ORIGINAL RESEARCH

Gingival Crevicular fluid levels of Myeloid related proteins (MRP's) 8/14, MRP8 and MRP14 in clinical periodontal health and disease-A cross sectional study

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Abstract

Background: Myeloid-related protein complex (MRP)8/14, and its sub-units MRP8 and MRP14, are endogenous pro-inflammatory, damage associated molecular patterns (DAMP's). The GCF expression profile of these proteins in clinical periodontal health and disease may point out to their probable potential as proteomic biomarkers.

Aims and Objectives: The principal objective of our study was to estimate and compare the GCF levels of MRP8/14, MRP8 and MRP14 in clinical periodontal health and early to advanced stages of periodontal diseases. Specifically, we aimed to quantify and compare the GCF levels of these proteins in study groups of individuals with clinical periodontal health (Group 1), gingivitis or stage I periodontitis (Group 2), and stages III or IV periodontitis (Group 3).

Methodology: This cross-sectional study enrolled 60 participants into the three groups with 20 participants in each group. After periodontal examination and charting, GCF samples were collected using 2µL microcapillary pipettes, and samples were processed, stored, and assayed by enzyme-linked immunosorbent assay (ELISA). GCF levels of MRP8, MRP14, and MRP8/14 were expressed as mean and standard deviations. Kruskal Wallis test was used for between groups comparisons. Pairwise comparisons were done using Bonferroni correction. P-values <0.05 were considered statistically significant.

Results: This study observed significant differences in the groups' GCF levels of MRP8/14, MRP8 and MRP14.Pair wise comparisons showed that for MRP8/14, highly significant differences were observed between groups 1 and 2. Significant intergroup differences were also seen between groups 1 and 3. For the sub-units, MRP8 and MRP 14, significant intergroup differences were seen only between groups 1 and 2.

Conclusion: MRP8/14, MRP8 and MRP14 may play crucial roles in the early stages of periodontal diseases. Their GCF levels show promise as potential biomarkers for early periodontal disease.

Keywords: Periodontitis; Gingivitis; Gingival Crevicular Fluid; S100 Calcium Binding Protein A8; S100 Calcium Binding Protein A9; Myeloid-Related Protein-8; Myeloid-Related Protein-14

Introduction

Periodontal diseases involve microbe-initiated inflammation in the tooth supporting structures leading to connective tissue and alveolar bone loss, ultimately leading to tooth loss. This localized inflammation can also lead to dissemination of inflammatory mediators into circulation and contribute to the generation of low-grade systemic inflammation. Periodontal diseases are diagnosed and classified by clinical and radiographic evaluation. Currently used clinical measurements of periodontal tissue destruction such as pocket depth (PD), attachment loss (AL) and radiographic measures of alveolar bone loss are measures of past tissue damage and fall short in predicting disease susceptibility, current disease activity or response to therapy. Precision periodontics is focused on the incorporation of validated biomarkers which will enhance the screening and diagnosis of periodontal diseases.

Although periodontal diseases are initiated by dysbiotic microbial colonies in plaque biofilm, it is sustained by immunoinflammatory responses of the host. These responses involve recognition of pathogen or damage associated mo-

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lecular patterns (PAMP's or DAMP's) by pathogen recognition receptors (PRR's) and an ensuing cascade of complex innate and adaptive immune responses which are closely integrated and interdependent. Binding of exogenous PAMP's and endogenous 'alarmin'[1] molecules to receptors on immune cells are key events that initiate cell signaling events and cellular responses. Alarmins are DAMP's that are released from damaged or stimulated granulocytes, epithelial, and connective tissue cells. They signal tissue damage and can augment inflammation. Myeloid related proteins (MRP's) are 'alarmins'[1] which may have prominent role in both natural and acquired immune responses. Of them, MRP-8, MRP-14 and their heterodimer-MRP8/14 or calprotectin are being studied intensively for their potential utility as proteomic biomarkers for periodontal diseases. These proteins are also named as S100A8, S100A9 and S100A8/9 respectively and are principally expressed by neutrophils.[2] They are major constituents of the neutrophilic cytosol and are intracellularly involved in arachidonic acid metabolism, pathogen resistance and modulation of the cytoskeleton. Their extracellular release is upregulated during inflammation and these proteins facilitate neutrophilic chemotaxis and cytokine secretion.[2]

MRP8/14 and its homodimers has been investigated in both GCF and saliva for its potential as a biomarker substance and these studies have yielded mixed results.[3] The 2017 world workshop jointly organized American Academy of Periodontology (AAP) and European Federation of Periodontology (EFP) on the classification of periodontal and peri-implant diseases and conditions underlined the need for integrating biomarkers for more precise case definitions of periodontal health and diseases. Combining a biomarker or panel of biomarkers into the diagnostic algorithm will improve the staging and grading of periodontal and peri-implant diseases.[4] Gingival crevicular fluid (GCF) is housed in the gingival crevice/periodontal pocket, and its volume, nature, and composition are dictated by periodontal inflammation. GCF is composed of locally present and recruited immune cells, microbe- and host-derived mediators, electrolytes, and organic molecules. Although investigators have quantified GCF levels of MRP8/14,[5,6&7] MRP8 and MRP14[8] their distinct, simultaneous and differential expression patterns in different stages of periodontal disease need further clarification. We hypothesized that the expression of these proteins in GCF would be altered in periodontal diseases than in health. The objective of our study was to estimate and compare the GCF levels of MRP8/14, MRP8 and MRP14 in clinical periodontal health, gingivitis or stage I periodontitis and in stages III or IV periodontitis. In this study, we clinically assessed the periodontal parameters and quantified the GCF levels of MRP8/14, MRP8 and MRP14 by ELISA in periodontal health and disease.

Methodology

This cross-sectional analytical study was carried out after approval from the Institutional Ethics Committee and Scientific Review Board of Saveetha Dental College (IHEC/PhD/PERIO-1621/21/230). The present study was done in strict adherence with the Helsinki Declaration of 1975, as revised in 2000 and was reported following the strengthening the reporting of observational studies in epidemiology (STROBE) guidelines. Consecutive samples were drawn from patients in the 25 to 55-year-old age group visiting the out-patient clinics of the Saveetha Dental College over a period of four months from December 2022 up to March 2023.

The sample size was calculated using G Power and the minimum sample size was fixed at 18 per group using inputs of 0.9 for anticipated effect size, a type 1 error of 5%, and with a power of 80%. Subjects with self-reported systemic diseases, obesity, smoking habits, those who had received periodontal therapy six months before sample collection, and those who received antibiotic or anti-inflammatory medications in the preceding three months were excluded from the study. A body mass index (BMI)>30 kg/m^2 was the standard used to define obesity. Participants who consented to the study were categorized and enrolled into Group 1 - 20 participants with clinically healthy periodontium, Group 2 - 20 patients with either gingivitis or stage 1 periodontitis, and Group 3 - 20 patients with stages III or IV periodontitis. The Gingival Index(GI) 9 was scored in a scale of 0-3 in all study participants, and detailed periodontal charting was performed for all patients with stages III or IV periodontitis. A University of North Carolina (UNC) 15 probe was used to record probing pocket depth (PPD), and the gingival margin level (GML) in relation to the cemento-enamel junction (CEJ) (+/- from CEJ) at six sites per tooth. Clinical attachment loss (CAL) was calculated and recorded. Mean PPD and mean CAL were calculated for each patient. Percentage of sites with PPD> 5mm and CAL> 5mm were recorded. Clinical periodontal and gingival health was defined according to the consensus reported by workgroup 1 of the World Workshop in 2017 on the Classification of Periodontal and Peri-Implant Diseases and Conditions, as a periodontium with less than 10% bleeding on probing (BOP) sites and with gingival sulcus depth measures <3mm.10A BOP scoring(dichotomous yes/no) for the presence of >10% bleeding sites and gingival sulcus depth measures <3mm defined cases of gingivitis on an intact periodontium.11 Stage 1 periodontitis was defined based on a clinical finding of interdental CAL of 1-2 mm on two non-adjacent teeth, and stages III and IV periodontitis were defined as the presence of interdental CAL >5mm on two non-adjacent teeth as per the criteria proposed for staging of periodontitis by the 2017 AAP and EFP world workshop.[4] All participants were informed about the diagnosis of their periodontal status and professional oral hygiene instructions were given.[12]

GCF sampling for ELISA: GCF was collected using 2µL microcapillary pipettes (Drummond Microcaps). GCF collection was done from each patient's most inflamed/deepest sulcus/pockets. A colored marking was given at exactly half the length of these pipettes. After obtaining consent, study participants were comfortably seated on the dental chair. The head was placed 45 degrees to the floor for sample collection from the maxillary teeth, and the mandible was placed parallel to the floor for mandibular sites. Under illumination from the dental chair, the sample collection site was determined. Supragingival debridement was done using a universal scaler, sites of collection were dried using gauze packs, rolls of cotton were placed into the buccal vestibule and the microcapillary pipette was

carefully slid into the sulcus/pocket, ensuring minimal contact with the lateral wall of the sulcus. The microcapillary pipette was placed at the sites of collection for 7 minutes until GCF rose till the color-coded mark on the pipette. Sites from where GCF did not rise in the pipette up to the marked line at the end of 7 minutes, were abandoned and alternate sites were chosen. The GCF obtained in the pipette was placed in a 2ml Eppendorf tube and was immediately transferred to the biochemistry laboratory. 200µL of phosphate-buffered saline (PBS) at pH 7 was added into each Eppendorf tube, and the pipette contents were washed out into the PBS. The GCF samples were then stored at -80 degrees centigrade (°C). On the day of the assay, all samples and ELISA kit components were brought to room temperature, and quantification of MRP8/14, MRP8 and MRP14 were done by ELISA using ELISA kits (R&D Systems, Minneapolis, MN, USA) following the guidelines provided by the manufacturer. Standard curves were generated for both proteins. The proteins were quantified according to the values of optical density (OD) read by a microplate reader at 450nm. Protein concentrations were expressed in picograms/milliliter (pg/ml).

Table 1: Comparison of GCF levels of MRP8, MRP14 & MRP 8/14 among the study groups

Groups	GCF Protein level in pg/ml mean (SD)		
	GCF MRP8*	GCF MRP14*	GCF MRP8/14*
Clinical periodontal health	135.47 (52.65)	153.28 (59.57)	1558.82 (99.24)
Gingivitis or Stage 1 Periodontitis	81.23 (52.67)	91.91 (59.60)	1691.15 (214.90)
Stages III or IV Periodontitis	98.3 (59.92)	111.22 (67.80)	1632.05 (67.32)

* Kruskal Wallis test, the p-value is statistically significant.

Table 2: Pair wise comparison of GCF levels of MRP8, MRP14 & MRP8/14 among the study groups.

Pair Wise Comparison	GCF Protein level in pg/ml mean (SD)		
	GCF MRP8 p value	GCF MRP14 p value	GCF MRP8/14
Clinical periodontal health- Gingivitis or Stage 1 Periodontitis	0.003	0.003	<0.001
Clinical periodontal health- Stages III or IV Periodontitis	0.05	0.05	0.021
Gingivitis or Stage 1 Periodontitis- Stages III or IV Periodontitis	0.813	0.813	0.087

Statistical analysis

Study data were entered into spreadsheets. Analysis was done by SPSS. The GCF levels of MRP8/14, MRP8 and MRP14 were expressed as their mean and standard deviation values. Shapiro- Wilk test was used to test if the data followed normal distribution. As the data did not follow normal distribution, the Kruskal Wallis test was used for between group comparisons. Pairwise comparisons were done using Bonferroni correction. P-values <0.05 were considered to be statistically significant.

Results

GCF samples were drawn from 60 subjects. Group 1 comprised 20 participants with clinical gingival and periodontal health, Group 2 had 20 subjects diagnosed with gingivitis or stage 1 periodontitis, and Group 3 comprised of 20 participants with either stages III or IV periodontitis. Of the 60 participants, 38 (63%) were males, and the rest were females. The average GI index values were 0.53±0.38, 2.29±0.45, and 2.57±0.36 in groups 1, 2, and 3, respectively. Mean PPD and CAL in group 3 were 5.15±0.53 and 3.87±0.61 respectively. In group 3, the percentage of sites with CAL >5mm was 49.24 ± 13.10, and percentage of sites with PPD >5mm was 47.14 ± 11.8.

GCF levels of MRP8, MRP14 and MRP8/14 in groups 1, 2, and 3 are shown in Table 1. GCF MRP8/14 was significantly different between the groups (p<0.05). GCF MRP8/14 was higher in groups 2 and 3 than in group 1 and was highest in group 2. Pair-wise comparisons showed significant difference between groups 1 and 2 (p<0.001) and between groups 1 and 3 (p=0.021). There was no significant difference between groups 2 and 3 (p=0.087). (Table 2).

Mean values of MRP8 were significantly different between the three groups (p<0.05). GCF MRP8 was highest in group 1 and lowest in group 2. Inter-group comparison using post hoc analysis showed that mean MRP8 was significantly different in group 2 with gingivitis or stage 1 periodontitis compared to the group 1 with clinically healthy periodontium(p=0.003). However, the inter group differences of MRP8 was not significantly different between groups 3 and 1(p=0.05) or between groups 3 and 2 (p=0.813). (Table 2).

Similar to MRP8, a comparison of the means of MRP14 among the three groups showed a statistically significant difference (p<0.05). GCF MRP 14 was highest in periodontal health and lowest in the gingivitis or stage 1 periodontitis group. Post hoc analysis showed that mean MRP14 was significantly different in the gingivitis or stage 1 periodontitis group compared to the group with clinically healthy periodontium (p=0.003). However, the differences observed for GCF-MRP14 between groups 3 and 1 (p=0.05) or between groups 3 and 2 were not significantly different (p=0.813). (Table 2). GCF levels of MRP8, MRP 14 and MRP8/14 are depicted graphically in Graphs 1,2 and 3 respectively.

Discussion

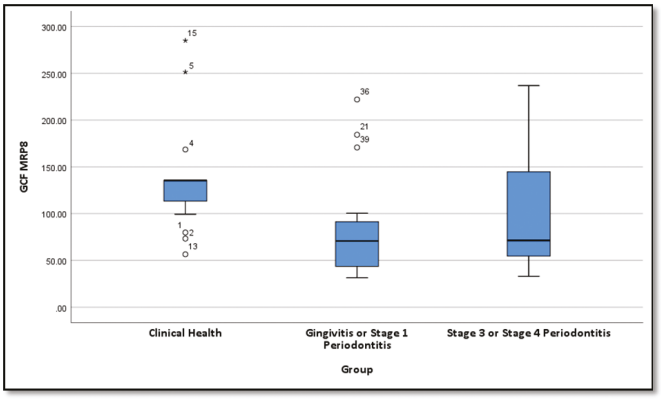
This observational study estimated and compared GCF levels of MRP8/14 and its sub units-MRP8 and MRP14 among three study groups categorized based on their clinical peri-

odontal status. We estimated GCF levels of MRP's to identify their probable involvement in the etiopathogenesis of periodontal inflammation and tissue destruction and to specifically study the changes in their GCF expression profiles in clinical periodontal health and diseases.

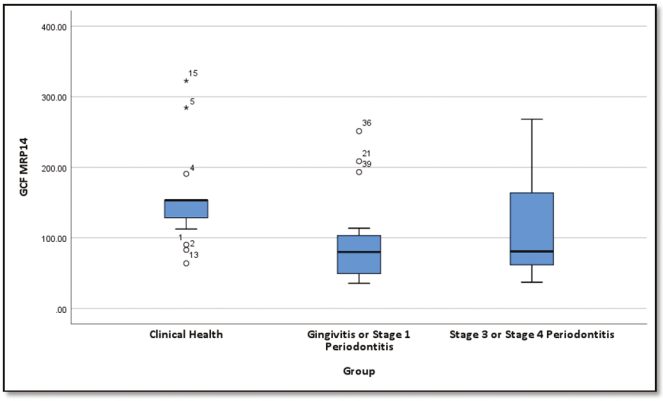
MRP's are endogenous DAMP's or alarmins that mediate inflammation. The MRP's are three calcium-binding proteins belonging to the S100 family: MRP8 (S100A8/Calgranulin A), MRP14 (S100A9/Calgranulin B), and MRP6 (S100A12). MRP8/14(Calprotectin), the heterodimer of MRP8 and 14 is thought to be the more physiologically relevant and stable form.[2] However, the subunits MRP8 and MRP14 are of recent relevant interest in periodontal inflammation. Although saliva and GCF are diagnostic vehicles that can be non-invasively collected for proteomics, we studied GCF for the expression of MRP's as it may be more reflective and sensitive to alterations in the gingival sulcus or periodontal pockets. GCF is considered both as a physiologic oral fluid with defensive functions and as an exudate in inflammation, that originates from the vascular plexus of the connective tissue of gingiva. The permeability of the gingival epithelium facilitates the release of several low molecular weight molecules from the serum into the GCF. In addition to serum excipients, GCF contains biological mediators and products of subjacent tissue remodeling. In our study, GCF collection was done using microcapillary pipettes, and we observed that we could collect GCF with greater ease from sites of deeper pockets and active clinical inflammation.

MRP 8/14 (Calprotectin or S100A8/9) is chiefly expressed by granulocytes and is a modulator of inflammation. It is also an important antimicrobial peptide. It has been extensively studied as a biomarker for many conditions such as cystic fibrosis, inflammatory bowel disease, rheumatoid arthritis, psoriasis, glomerulonephritis and periodontitis which are all underlined by chronic inflammation.[2] Our study results show that GCF levels of MRP8/14 were significantly different in the three groups. We observed that GCF MRP8/14 was higher in periodontal diseases than in health. It was highest in our study group 2 comprising of subjects with either gingivitis or stage 1 periodontitis and lowest in study participants of group 1 with clinical periodontal health, underlining its active role in innate immune responses and early periodontal inflammation. Our study results agree with studies that reported higher values for GCF MRP8/14 in individuals with periodontal disease than in subjects with periodontal health.[5,6] However, our results are not in concordance with studies that have reported lower calprotectin concentrations in GCF of chronic periodontitis patients.[7] In this study, GCF MRP8/14 was highest in the gingivitis group pointing out to its role as an antimicrobial peptide as discussed previously by Afacan B et al in their study on the effects of full mouth disinfection on GCF MRP8/14.[13] Our findings point out that GCF MRP8/14 can distinguish between states of periodontal health and disease. Evidence suggests that GCF MRP8/14 levels may reflect crevicular neutrophilic count.14 GCF levels of MRP8/14 was reported to be significantly corelated with myeloperoxidase which is a neutrophil azurophilic granular constituent.[14] A study report of GCF calprotectin in experimentally induced gingivitis observed considerable inter individual variability in the protein levels and identified two response patterns, a pattern with higher level of MRP8/14 and less of plaque, gingival inflammation and pocket depth than the other pattern with higher plaque levels, GI and PD suggestive of a protective role of this protein.[15] The authors reported that GCF levels of MRP8/14 at baseline was corelated to level of gingival inflammation at 10 days of experimental abstinence of oral hygiene procedures. They also suggested that GCF levels of MRP8/14 may reflect an individual susceptibility to periodontal diseases. In our study, significant pair wise group differences were observed for MRP8/14 between study groups 1 and 2 and groups 1 and 3. (Table 2). The Intergroup differences observed between study groups 1 and 2 was highly significant. GCF MRP8/14 may be an early screening agent for gingival inflammation.

GCF levels of MRP8/14 were reported to be higher in sites with periodontal tissue destruction than in control sites.[16] The concentration and content/site of this leukocytic protein complex was reported to be higher in sites which deeper pockets. Higher concentrations of this protein was also reported in sites with higher GI17 which bled on probing18 than sites which did not bleed. GCF MRP8/14 levels were observed to corelate with biochemical markers such as collagenase and aspartate amino transferase.[17] However other research groups have reported that GCF calprotectin cannot differentiate periodontally



Graph 1: Comparison of GCF MRP8 between the groups



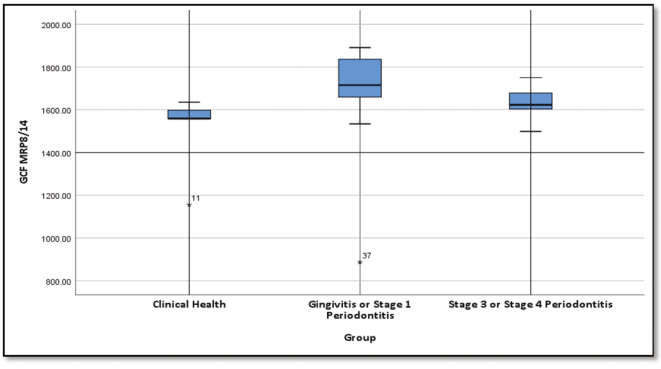
Graph 2: Comparison of GCF MRP14 between the groups

diseased from non-diseased sites in patients with chronic periodontitis.[6]

In our study GCF MRP8 and MRP14 was seen to be significantly different between the groups with highest values observed in group with periodontal health and lowest values seen in group in gingivitis or stage 1 periodontitis group. Post hoc comparisons showed that intergroup differences were statistically significant only between study groups 1 and 2. Earliest reports on the presence of the homodimer MRP8 in GCF were from Lundy[19] and Kojiama et al.[20] The latter research group reported the presence of both MRP8 and 14 in GCF of periodontitis patients. Lundy et al quantified MRP8 in periodontally inflamed sites by microbore high pressure liquid chromatography (HPLC) and reported highest amounts in sites of periodontal inflammation. They reported that MRP8 is a major response protein in chronic periodontitis.[21] At the patient level, we observed a decrease in levels of these proteins in gingivitis and periodontitis when compared to the group with periodontal health. Investigation of Kim et al[8] also reported the same trend we observed in our study. They also reported that MRP8 and MRP14 levels were lower in GCF of patients with periodontal diseases, with lowest values observed in the gingivitis group. Compared to health, the lowered levels of these proteins in periodontal diseases could point to a decreased or inadequate immune response.

Nishii K et al.[1] reported the different and inconsistent expression levels of MRP8 & MRP14 in the gingival epithelial cells of experimental germ-free rats. They reported that gingival epithelium of axenic mice expressed only MRP8, while neutrophils in the junctional epithelium expressed both MRP8 and MRP14. MRP8 expression was seen in the gingival epithelial cells of conventional mice. and it was increased in the presence of bacteria. The authors reported that gingival epithelial cells expressed MRP14 in the presence of bacteria, suggesting that MRP14 expression was induced by infection. This report also points out that the expression and role of these proteins in gingival inflammation may be distinct and diverse.[1]

Within our literature search and to our understanding of this specific domain, this is the first study where the heterocomplex MRP8/14 and its constituents have been explored simultaneously in clinical periodontal health, gingivitis and periodontitis. A previous investigation had examined GCF MRP8/14 and its homodimers in health and experimentally induced gingivitis[15] and another study investigated GCF MRP8/14 and MRP14 in periodontal health, disease and after therapy.[6] We have observed that the expression of the homodimers is much lesser than the heterocomplex and this has been supported by previous evidence.[15] Another merit of our study was that case definitions of stages I, III and IV periodontitis, gingivitis and clinical periodontal health were according to the new international classification laid down by the AAP-EFP world work shop on classification scheme for periodontal and peri-implant diseases and conditions. Meticulous attention to sampling sites and collection method of GCF by trained periodontists is also a definite merit of this study. Sites with deeper pockets and clinical inflammation were identified for GCF collection. Although samples visibly contaminated by blood were discarded, very slight contamination with blood or saliva could have occurred in sample collection. Our study is limited by its cross-sectional design wherein a causal relationship could not be drawn. It is also limited by its small sample size. A comparison of healthy and diseased sites in periodontitis patients could not be made because GCF could not be collected in the micro capillary pipettes from healthy sites with shallow sulci.



Graph 3: Comparison of GCF MRP8/14 between the groups

Conclusion

GCF expression of MRP8/14, MRP8 and MRP14 is significantly different in study groups comprising of subjects with stages III or IV periodontitis, gingivitis or stage I periodontitis and clinical periodontal health. Inter group comparisons showed consistent and significant differences between groups with periodontal health and gingivitis. The potential of these molecules as probable early markers of periodontal inflammation and disease activity needs to be explored further.

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