Original Research

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Expression of High Mobility Group Box 1 and Receptor for Advanced Glycation End Products in the Gingival Tissues of Periodontitis Patients with Type 2 Diabetes Mellitus

Ramya Raj, Devapriya Appukuttan, Sangeetha Subramanian, Prakash PSG, Jasmine Crena

Department of Periodontics and Oral Implantology, SRM Dental College, Chennai, India

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Corresponding Author: Devapriya Appukuttan, Department of Periodontics and Oral Implantology, SRM Dental College, Chennai, India
Phone: +91 9840197121 **E-mail:** devapriyamds@gmail.com **ORCID:** orcid.org/0000-0003-2109-1135

Abstract

Objective: Diabetes mellitus (DM) and periodontitis (PD) share similar pathophysiology including altered immune-inflammatory responses, and evidence implicates high mobility group box 1 (HMGB1)-receptor for advanced glycation end products (RAGE) axis in amplifying inflammation. Therefore, we aimed to investigate their expression in gingival tissues from individuals with Stage III/IV PD with and without type 2DM (T2DM).

Materials and Methods: Thirty-three gingival tissue samples were taken from Group I: Systemically and periodontally healthy, Group II: Systemically healthy with PD and Group III: PD with T2DM. Tissue and gene expression were evaluated by immunohistochemistry and reverse transcription quantitative-polymerase chain reaction. The staining intensity distribution (SID) and the mean percentage of positive stained cells (MPPC) scores were reported.

Results: When compared to group I, the gene expression of HMGB1 and RAGE were 2.85 and 2.50 times higher in PD+T2DM, respectively ($p<0.001$). MPPC and SID for HMGB1 and RAGE expression were high in Groups II and III, with increased expression in PD+T2DM. Furthermore, when compared to Group I, the expression was significantly higher ($p<0.001$). In the 33 samples, MPPC and SID scores for HMGB1 and RAGE in the epithelium and connective tissue significantly correlated with clinical parameters ($p<0.001$). With a diagnostic accuracy of 87.88% [95% confidence interval (CI): 72.67-95.18] and a positive and negative predictive value of 95% and 76.92%, respectively, HMGB1 was able to distinguish between periodontal health and disease. RAGE had a diagnostic accuracy of 93.94% (95% CI: 80.39-98.32) with a positive and negative predictive values of 91.67% and 100%, respectively.

Conclusion: Increased HMGB1 and RAGE expression underscores their significance in amplifying inflammation in PD associated with T2DM. Furthermore, both can be used as diagnostic biomarkers.

Keywords: High mobility group box 1, receptor for advanced glycated end products, periodontitis, diabetes mellitus, reverse transcription quantitative-polymerase chain reaction, immunohistochemistry

Introduction

Inflammation is a crucial physiological response that the host mounts in response to a range of unpleasant stimuli, and while it is ideally protective, if it is not treated properly, it can be harmful (1). Periodontitis is one such chronic "immune-inflammatory disease" associated with a dysbiotic oral microbiota in a susceptible host (2). The principal mediators of inflammation in periodontitis are pathogen associated molecular patterns and damage associated molecular patterns (DAMP), as well as their receptors such as toll-like receptors (TLR), nucleotide oligomerization domain like receptors, retinoic acid inducible gene like receptors, C type lectin receptors, an absent in melanoma-2 like receptor, scavenger receptors, receptor for advanced glycation end products (RAGE), triggering receptor expressed on myeloid cells-1, G protein coupled receptors, and ion channels. Furthermore, cytokines, chemokines, kinins, complements, leukotrienes, prostaglandins, vasoactive amines, and their target cells all contribute to periodontal damage (3).

ORCID: R. Raj 0000-0003-1071-7745, D. Appukuttan 0000-0003-2109-1135, S. Subramanian 0000-0002-9352-6081, P. PSG 0000-0003-4243-5865, J. Crena 0000-0001-9788-3898

The groundwork for DAMPs' involvement in the activation of innate immune responses was put forth in 1994 by Polly Matzinger's danger theory (4). These molecules serve as "danger signals" or "alarmins" and are endogenous host-derived extracellular and intracellular elements that are released in the context of cell stress, injury, infection, or programmed cell death (5). Many DAMPs have been identified so far, including high mobility group box 1 (HMGB1), heat shock proteins, histones, F-Actin, cyclophilin A, uric acid, S100 proteins, and others, and these biomolecules mediate sterile inflammation and are implicated in the pathogenesis of several diseases including periodontitis (6).

The first DAMP to be recognized was the non-histone DNA binding protein, HMGB1. It is normally localized in the nucleus but, in response to certain stimuli, it translocates into the cytoplasm and is secreted extracellularly, where it serves as both a cytokine that promotes inflammation and one that aids in tissue repair (7). *In vitro* studies have shown increased secretion of HMGB1 from gingival epithelial cells, gingival fibroblast, periodontal ligament cells and macrophages. Furthermore, similar increased expression has been demonstrated in the gingival crevicular fluid and serum of periodontitis patients (8-14). Extracellular HMGB1 binds to RAGE, TLR-4, TLR-2, and TLR-9 more frequently than to Macrophage Antigen-1, Syndecan-3, CD 24-Siglec10, CXCR4, Integrin, and a variety of other receptors (7). HMGB1-RAGE and HMGB1-TLR4 binding are the key mechanisms through which HMGB1 drives inflammation (15). The discovery of HMGB1-RAGE binding was first made by Hori et al. (16) in 1995 and since then, the axis has become recognized as a key participant in the pathogenesis of a number of disorders, including acute lung damage, preeclampsia, rheumatoid arthritis, diabetes, cancer, autoimmune diseases, and periodontal disease (7,15).

The binding of HMGB1 to RAGE activates the two canonical mitogen-activated protein kinases (MAPKs), extracellular regulated protein kinases (ERK1/2) and p38 MAPK, resulting in the nuclear translocation of NF-kB and the release of proinflammatory cytokines, which further upregulate RAGE expression and HMGB1 secretion, thereby maintaining the inflammatory phenotype. RAGE is also involved in endocytosis of HMGB1-LPS complex and activation of caspase-1 mediated pyroptosis (17,18). Therefore, RAGE is imperative for HMGB1-induced cell migration, proliferation, regeneration, inflammation, autophagy, injury, neurite growth, metabolism and immunity.

RAGE is a multi-ligand receptor that is expressed on the surface of different types of cells, including cancer cells, immune cells, neurons, smooth muscle cells and the accumulation of RAGE ligands in an inflammatory milieu further upregulates RAGE expression in a positive feedback loop. RAGE binding activates several pathways and transcription factors including NF-kB, STAT3, AP-1, and so on resulting in cell proliferation, migration, survival, cell homeostasis, repair and regeneration, secretion of inflammatory cytokines and tumor formation (19,20). Hence, it may be inferred that RAGE is essential for many physiological processes and, when dysregulated, contributes to the pathophysiology of a multitude of diseases. In periodontitis and diabetes-related periodontitis, RAGE is upregulated and is associated with increased ROS production, upregulated adhesion molecules on endothelial cells, and accelerated NF-kB translocation (21). The levels significantly correlate with the severity of periodontal destruction. Additionally, in individuals with and without type 2 diabetic mellitus (T2DM), the RAGE gene polymorphism is linked to chronic periodontitis (22). The above findings underscore the significance of RAGE in mediating inflammatory periodontal destruction.

Ito et al. (23) demonstrated the collaborative function of HMGB1-RAGE after interleukin (IL)-1β stimulation in human gingival fibroblast and epithelial cells, indicating their synergistic role in periodontal inflammation. Comparable outcomes were shown by Pei et al. (24), Akutagawa et al. (25), and He et al. (26) in diabetic mouse models with and without periodontitis. Until recently, the expression of HMGB1 and HMGB1-RAGE in periodontal disease associated with T2DM from human gingival tissue samples has been barely studied. Hence, it is crucial to comprehend this axis in order to clarify its function in the pathophysiology of periodontitis and in T2DM-associated periodontitis. According to our hypothesis, human gingival tissue samples from cases of periodontal disease associated with type 2 diabetes will express higher levels of HMGB1 and RAGE than periodontally healthy control samples. Therefore, the purpose of this research is to analyze the expression of HMGB1 and RAGE in the gingival tissues of individuals with and without T2DM and Stage III/IV periodontitis.

Materials and Methods

Study Design, Ethics and Informed Consent

This cross-sectional case-control study was designed, planned and executed in the outpatient clinics of the Department of Periodontics and Oral Implantology, SRM Dental College, Ramapuram between August 2020 and May 2022. The research proposal was submitted to the institutional review board of SRM University and approval was received (SRMDC/IRB/2020/MDS/No.506, date: 05.12.2022). The study was registered in the clinical trial registry of India and the allotted CTRI number

was CTRI/2022/02/040169. Autonomy of the research participants was ensured by obtaining written and verbal informed consent after the research objectives, protocol, risk and benefits were clearly and completely explained.

Sample Size Calculation

Sample size was calculated with G*Power software (version 3.1.9.4) using an effect size of 0.75, with 95% power and 5% alpha error, based on the study by Abbass et al. in 2012 (27). To reach significant difference between the groups, 33 gingival tissue samples were required. Based on the research objectives, 3 groups of study population were planned and 11 gingival tissue samples were collected from each group.

Subject Recruitment and Diagnosis

Thirty-three gingival tissue samples were collected from patients categorized into three study groups - Group I: Systemically and periodontally healthy (n=11), Group II: Systemically healthy subjects with localized or generalized stage III/IV periodontitis (n=11) and Group III: Localized or generalized Grade B, Stage III/IV periodontitis with T2DM (n=11). The subjects attending the clinics were screened, HbA1c for DM and radiographs were requested, followed by final diagnosis. They were enrolled in the study if they met the inclusion and exclusion guidelines laid down for the research. Once the participants agreed to participate, informed consent was obtained.

Inclusion Criteria

Subjects more than 18 years (both men and women) fulfilling the criteria for the study were categorized into 3 groups. Group I- subjects with good systemic health, with at least 20 natural teeth excluding third molars, full mouth plaque score (FMPS) ≤20%, full mouth bleeding score (FMBS) ≤10%, probing pocket depth (PPD) ≤3 mm with no clinical attachment loss (CAL). Group II-Subjects with FMPS >20%, FMBS >10%, PPD ≥6 mm and CAL ≥5 mm, bone loss observed in the IOPA, involving ½ or more on the root surface determined either as localized or generalized stage III/IV periodontitis (28). Group III-Subjects diagnosed with localized or generalized stage III/IV periodontitis (based on the Group II eligibility criteria) and DM with HbA1c <7%.

Subjects were excluded if they were under therapy with immunosuppressants and/or antibiotics and/or anti-inflammatory medications and/or any drugs during the last 6 months, that could influence the periodontium. Expectant or nurturing women, both current and past smokers and those treated for periodontitis or orthodontic treatment in the past were excluded. Subjects with a history of any other systemic disease other than T2DM and those diabetic individuals giving a history of diabetic complications, taking insulin injections, HbA1c level more than 7% were not eligible.

Periodontal Examination

The following periodontal clinical variables were recorded by a single investigator (RR) using an UNC-15 periodontal probe: Site specific plaque index (SSPI), site specific bleeding index (SSBI), site specific probing pocket depth (SS-PPD), site specific clinical attachment loss (SS-CAL), FMPS, and FMBS.

Collection of Gingival Tissue Samples

During crown lengthening or during tooth extraction for orthodontic purposes, Group I patients had samples of their gingival tissue collected. For Groups II and III, tissue samples were taken from teeth with a poor prognosis that were recommended for extraction from participants with stage III/IV periodontitis with and without T2DM. Local anesthetic agent was administered and using BP blade no: 15 c, internal bevel and crevicular incisions were made on the buccal/lingual/interproximal area and two sections of tissue samples were excised. For RT-qPCR analysis, the tissue was placed in RNA later solution and for immunohistochemistry (IHC), it was placed in 10% neutral buffered formalin.

RT-qPCR

Total RNA was extracted from the homogenized tissues using a TRIR kit (total RNA isolation reagent) (TRIzol-C Reagent, Sisco Research Laboratories Pvt. Ltd., India, Cat No. #41066) after standardizing the weight of the tissue sample (20-25 mg). The purity of the RNA pellets were quantified using Thermo Scientific TMNanoDrop™ Lite Spectrophotometer (Cat No. #: ND-LITE-PR). The samples were stored on ice and subjected to the reverse transcription procedure using a high capacity cDNA reverse transcription kit (SYBR Premix Ex Taq; Tli RNase H Plus,TaKaRa, Cat. #RR420A, Japan). Real time PCR was carried out to quantify the gene expression (T100 Thermal Cycler, Bio-Rad Laboratories, Inc. USA, Cat No. #1861096).

The following primers were used for analysis: HMGB1 (sense - 5′-ACTACCGAGTCCGAGTCTACC-3′, anti-sense- 5′-CCACCTTATTAGGGACACTGG-3′), RAGE (sense- 5′-TATGGCAAAAGCGGACAAGG-3’, anti-sense- 5′-CTTCGCAACATCACCAATGGA-3’), GAPDH (sense- 5′-GAGTCAACGGATTTGGTCGT-3′, anti-sense-5′-GACAA GCTTCCCGTTCTCAG-3′) (Eurofins Genomics India Pvt Ltd, Bengaluru). As an internal control, the GAPDH gene was used. The reaction volume was set to 20 µL with lid temperature 105°C. The polymerase chain reaction (PCR) protocol was followed, initial denaturation at 95°C for 3 min was followed by 40 cycles of PCR, each lasting 10 seconds, with annealing at 60°C for 30

seconds and extension at 72°C for 20 seconds. The no template control, which lacked any template DNA, was used in all reactions, which were carried out in triplicate. To identify the presence of numerous amplicons, non-specific products, and impurities, melt curve analysis was carried out using thermal cycling programmed at 50-95°C for each sample. CFX 96 real time system software was used to analyze the results (Bio-Rad Laboratories, Inc. USA, Cat No. # 1845096). Relative gene expression of the target gene was derived based on the endogenous control and the fold quantification was carried out using the comparative CT method given by Livak and Schmittgen (29).

Immunohistochemistry

The samples were dehydrated in a series of graded alcohols, cleared through two changes of xylene, and then embedded in a paraffin block after the tissues were fixed in 10% formalin for 24-48 hours. On a microtome, a tissue block was sectioned at 5-8 μm thickness. The slides were then processed for examination using both haematoxylin and eosin and IHC to analyze the expression of HMGB1 and RAGE. Tris-EDTA (pH 6.0) was used for antigen retrieval and TRIS wash buffer was used. The primary antibody was made into 1: 20 and 1:50 dilutions for HMGB1(Invitrogen, Thermo Fisher Scientific Inc. NYSE: TMO Waltham, MA, USA) and RAGE (Invitrogen, Thermo Fisher Scientific Inc. NYSE: TMO Waltham, MA, USA) respectively. 25 μL of primary antibody solution was applied to the slides covering the tissue sections and incubated overnight and treated with secondary antibody (PolyExcel HRP/DAB Detection System kit, PathnSitu co. USA). The slides were counterstained for hematoxylin and cleared in xylene and mounted using DPX (Di-sterene dibutyl Phthalate Xylene) and coverslip. Each sample had a positive control (HMGB1-liver tissue and RAGE- lung tissue). The percentage of positively stained cells (MPPC), the proportion of positively stained cells (MPI) and the intensity of staining (SI) were determined based on the study of Tobón-Arroyave et al. in 2005 (30). A staining-intensity-distribution (SID) score was calculated by multiplying the MPI by SI in each field. Two experienced oral pathologists manually scored each sample using a grid from four separate fields, and the average was calculated for each sample. The kappa statistics for inter-examiner agreement was 0.81.

Statistical Analysis

For statistical analysis, IBM SPSS Statistics for Windows, Version 26.0, Armonk, NY: IBM Corp., released in 2019, was used. The significance limit was set at 0.05 (α=0.05). To determine if the raw data were distributed normally or not, the Kolmogorov-Smirnov and Shapiro-Wilks tests were used. Except for age, all other variables did not exhibit normal distribution, according to the normality test. Therefore, both parametric and non-parametric tests were used to statistically analyze the data. One-Way ANOVA was used for comparing the mean age between the groups and the Pearson chi-square test was applied to compare the gender distribution. The Kruskal-Wallis analysis and Bonferroni correction test were used for intergroup comparison of other parameters. The Spearman Rank correlation was utilized to evaluate the linear relationship between clinical and RT-qPCR/IHC variables. Receiver operating characteristic (ROC) curve analysis was carried out to find the optimal cut-off values to predict the diagnostic ability of HMGB1 and RAGE to distinguish between periodontal health vs GCP and GCP+DM. Sensitivity and specificity of the test, positive predictive and negative predictive values were also reported.

Results

Age and Gender Description

In groups I, II, and III of the study, the mean ages were 45.1 ± 5.9, 47.3 ± 9.9 and 46.9 ± 7.8 years, respectively. One-Way ANOVA showed no significant difference in the mean age between the three groups ($p \geq 0.05$). Group I, II, and III included 7 males (63.6%) and 6 females (36.4%), 5 males (45.5%) and 6 females (54.5%) and 5 males (45.5%) and 6 females (54.5%), respectively. There were no differences in the gender distribution across the groups based on the Pearson's chi-squared analysis ($p \geq 0.05$).

Clinical Characteristics with Intergroup Comparison

The mean and median values of the periodontal parameters recorded in all the three groups have been shown in Table 1. Increased site specific and full mouth plaque and bleeding scores were observed in the periodontally diseased groups (II and III). Likewise, PPD and CAL indicating periodontal destruction were also higher in the periodontally diseased groups (II and III). Intergroup comparison showed a statistically significant difference in all the clinical parameters recorded between the groups ($p<0.001$). The Bonferroni correction test for intergroup pairwise comparison of clinical parameters showed a statistically significant difference between Group I and II and between Group I and III ($p<0.001$). No significant difference was observed between Group II and Group III ($p>0.05$) in terms of clinical parameters (Table 1 given in the Supplemental File).

Relative Quantification Data from RT-qPCR Analysis

Lower CT values indicating increased mRNA transcripts of HMGB1 and RAGE were observed in the diseased periodontium when compared to periodontal health and

comparison between the groups was significant statistically ($p<0.001$). ΔCT and ΔΔCT values of both HMGB1 and RAGE gene expression were also statistically significant ($p<0.001$) between the groups. Increased fold change in the gene expression of both the biomarkers was observed in Group II and III (Table 2).

Table 1. Result of the Kruskal-Wallis test for intergroup comparison of clinical parameters between the Groups I, II and III

Clinical Parameter	Central Tendency	Groups			p-value
		Group I	Group II	Group III	
SSPI	Mean ± SD	0.1 ± 0.1	2.9 ± 0.2	2.9 ± 0.3	p<0.001*
	Median (Q1-Q3)	0.00 (0.00-0.3)	3.00 (2.8-3.0)	3.00 (2.8-3.0)	
SSBI	Mean ± SD	0.0 ± 0.1	2.9 ± 0.2	2.8 ± 0.3	p<0.001*
	Median (Q1-Q3)	-	3.0 (3.0-3.0)	3.0 (2.8-3.0)	
SS-PPD (mm)	Mean ± SD	1.9 ± 0.3	7.5 ± 1.4	7.7 ± 1.2	p<0.001*
	Median (Q1-Q3)	2.0 (2.0-2.0)	8.0 (6.0-9.0)	7.0 (7.0-9.0)	
SS-CAL (mm)	Mean ± SD	-	9.6 ± 1.8	9.8 ± 1.2	p<0.001*
	Median (Q1-Q3)	-	9.0 (9.0-11.0)	10.0 (9.0-11.0)	
FMPS (%)	Mean ± SD	9.7 ± 7.1	99.3 ± 2.4	99.8 ± 0.6	p<0.001*
	Median (Q1-Q3)	10.0 (6.3-13.0)	100.0 (100.0-100.0)	100.0 (100.0-100.0)	
FMBS (%)	Mean ± SD	2.3 ± 7.5	99.3 ± 2.4	99.8 ± 0.6	p<0.001*
	Median (Q1-Q3)	-	100.0 (100.0-100.0)	100.0 (100.0-100.0)	

*P<0.05, statistically significant, SS-PI: Site-specific plaque index score, SS-BI: Site-specific bleeding index score, SS-PPD: Site-specific mean probing pocket depth, SS-CAL: Site-specific mean clinical attachment loss, FMPS: Full mouth plaque score, FMBS: Full mouth bleeding score, Q1-Q3 lower quartile to upper quartile

Table 2. The relative quantification data from RT-qPCR analysis with the Kruskal-Wallis test for intergroup comparison between Group I, II and III

RT-qPCR data	Central Tendency	Groups			p-value
		Group I	Group II	Group III	
HMGB1 CT	Mean ± SD	33.9 ± 0.6	32.8 ± 0.8	31.4 ± 1.0	p<0.001*
	Median (Q1-Q3)	33.7 (33.45-34.23)	32.98 (32.56-33.34)	31.1 (30.99-31.98)	
HMGB1 ΔCT	Mean ± SD	7.1 ± 0.6	6.5 ± 0.73	5.6 ± 0.5	p<0.001*
	Median (Q1-Q3)	7.00 (6.67-7.54)	6.22 (5.89-7.03)	5.6 (5.5-5.7)	
HMGB1 ΔΔCT	Mean ± SD	.	-0.59 ± 0.73	-1.4 ± 0.5	0.007*
	Median(Q1-Q3)	.	-0.84 (-1.17 to-0.03)	-1.5 (-1.6 to -1.4)	
HMGB1 fold change	Mean ± SD	1.00	1.7 ± 0.73	2.8 ± 1.1	p<0.001*
	Median (Q1-Q3)	1.00	1.8	2.8	
		(1.0-1.0)	(1.0-2.3)	(2.7-3.0)	
RAGE CT	Mean ± SD	34.4 ± 1.0	32.6 ± 0.7	31.7 ± 0.6	p<0.001*
	Median (Q1-Q3)	34.66	32.980	31.8	
		34.1-35.2	32.0-33.3	31.2-32.1	
RAGE ΔCT	Mean ± SD	7.3 ± 1.0	7.0 ± 1.0	6.4 ± 0.9	0.014*
	Median (Q1-Q3)	7.6	7.1	6.5	
		6.9-8.1	6.4-7.9	6.1-7.0	
RAGE ΔΔCT	Mean ± SD	.	-0.3 ± 1.0	-1.0 ± 0.9	0.036*
	Median (Q1-Q3)	.	-0.2	-0.8	
		.	-0.9- 0.5	-1.2 to -0.3	
RAGE fold change	Mean ± SD	1.0	1.6 ± 1.6	2.5 ± 2.5	p<0.001*
	Median (Q1-Q3)	1.0	1.2	1.8	
		1.0-1.0	0.7-1.9	1.2-2.3	

*P-value <0.05, statistically significant, NS: Not significant, CT: Cyclic threshold, Q1-Q3: Lower quartile to upper quartile

Table 3. Spearman Rank Correlation results for the clinical parameters and the data from RT-qPCR for the total study sample

Parameters		HMGB1 CT	HMGB1 ΔCT	HMGB1 ΔΔCT	HMGB1 fold change	RAGE CT	RAGE ΔCT	RAGE ΔΔCT	RAGE fold change
SSPI	r-value	-0.63	-0.54	-0.09	0.63	-0.75	-0.42	-0.22	0.43
	p-value	0.00*	0.001*	0.66	0.00*	0.00*	0.01*	0.32	0.01*
SSBI	r-value	-0.67	-0.52	-0.06	0.64	-0.73	-0.31	0.04	0.37
	p-value	0.00*	0.00*	0.78	0.00*	0.00*	0.07*	0.82	0.03*
SS-PPD	r-value	-0.67	-0.51	-0.09	0.54	-0.71	-0.38	-0.14	0.43
	p-value	0.00*	0.00*	0.68	0.001*	0.00*	0.02*	0.52	0.01*
SS-CAL	r-value	-0.62	-0.50	-0.09	0.55	-0.68	-0.33	-0.08	0.36
	p-value	0.00*	0.00*	0.69	0.001*	0.00*	0.05*	0.72	0.03*
FMPS	r-value	-0.70	-0.56	-0.16	0.65	-0.73	-0.37	-0.02	0.40
	p-value	0.00*	0.00*	0.45	0.00*	0.00*	0.03*	0.90	0.02*
FMBS	r-value	-0.72	-0.57	-0.16	0.66	-0.72	-0.34	-0.02	0.41
	p-value	0.00*	0.00*	0.45	0.00*	0.00*	0.04*	0.90	0.01*

SS-PI: Site-specific plaque index score, SS-BI: Site-specific bleeding index score, SS-PPD: Site-specific mean probing pocket depth, SS-CAL: Site-specific mean clinical attachment loss Q1-Q3 lower quartile to upper quartile, CT: Cyclic threshold

Table 4. Area under the curve

Variable	AUC (95% CI: LB-UB), P-value	Sensitivity (95% CI: LB-UB)	Specificity (95% CI: LB-UB)	PPV (95% CI: LB-UB)	NPV (95% CI: LB-UB)	Diagnostic accuracy (%)
HMGB1 CT	0.963 (0.90, 1.00), <0.05*	86.36% (66.66, 95.25)	90.91% (62.26, 98.38)	95.00% (76.39, 99.11)	76.92% (49.74, 91.82)	87.88%
RAGE CT	0.963 (0.88, 1.00), <0.05*	100.00% (85.13, 100.00)	81.82% (52.3, 94.86)	91.67% (74.15, 97.68)	100.00% (70.08, 100.00)	93.94%

LB: Lower bound, UB: Upper bound, PPV: Positive predictive value, NPV: Negative predictive value, AUC: Area under the ROC Curve, CI: Confidence interval

Table 5. Descriptive statistics and Kruskal-Wallis test for intergroup comparison of HMBG1 in epithelium and connective tissue for Group I, II, III

	IHC Parameter	Group I	Group II	Group III	p-value
HMGB1					
EPITHELIUM	MPPC	25.1 ± 10.2	59.4 ± 11.3	68.2 ± 7.5	p<0.001*
	SI	0.8 ± 0.5	2.6 ± 0.39	2.9 ± 0.1	p<0.001*
	MPI	1.7 ± 0.5	2.7 ± 0.3	8.8 ± 0.4	p<0.001*
	SID	1.6 ± 0.9	7.3 ± 1.7	8.8 ± 0.4	p<0.001*
CONNECTIVE TISSUE	MPPC	16.2 ± 9.9	41.7 ± 9.9	49.5 ± 9.4	p<0.001*
	SI	0.4 ± 0.5	1.7 ± 0.5	2.1 ± 0.5	p<0.001*
	MPI	1.1 ± 0.4	2.3 ± 0.4	2.5 ± 0.4	p<0.001*
	SID	0.8 ± 2.0	4.2 ± 2.0	5.6 ± 1.7	p<0.001*
RAGE					
EPITHELIUM	MPPC	23.9 ± 9.3	60.7 ± 8.5	65.7 ± 9.7	p<0.001*
	SI	0.9 ± 0.4	2.6 ± 0.3	2.7 ± 0.3	p<0.001*
	MPI	1.5 ± 0.5	2.9 ± 3	2.9 ± 0.2	p<0.001*
	SID	1.4 ± 0.7	7.7 ± 8	8.1 ± 1.2	p<0.001*
CONNECTIVE TISSUE	MPPC	20.7 ± 7.2	40.1 ± 13.6	51.8 ± 12.7	p<0.001*
	SI	0.78 ± 0.4	1.7 ± 0.6	2.1 ± 0.6	p<0.001*
	MPI	1.3 ± 0.3	2.2 ± 0.6	2.6 ± 0.4	p<0.001*
	SID	1.2 ± 0.67	4.2 ± 2.5	5.9 ± 2.4	p<0.001*

*P<0.05, statistically significant, MPPC: Mean percentage of positively stained cells, SI: Staining intensity, MPI: Mean proportion of positively stained cells, SID: Staining intensity distribution

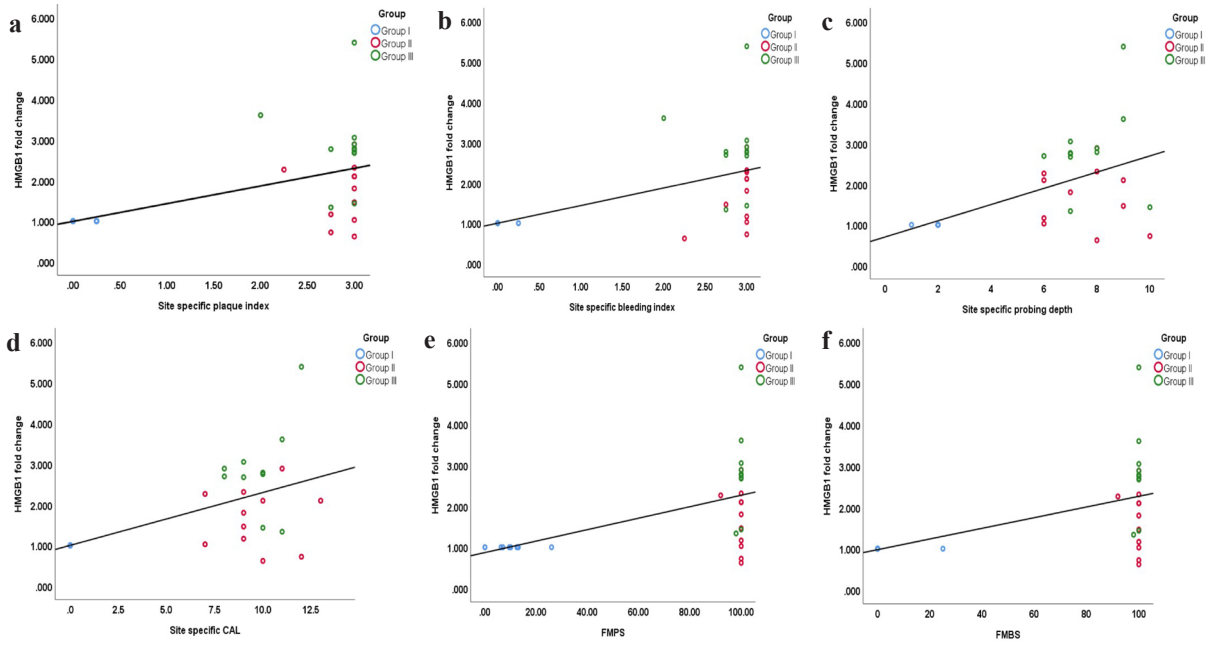

Figure 1. Scatter plot showing the correlation between fold change values of HMGB1 gene expression with site specific plaque index, site specific bleeding index, site specific probing depth, site specific CAL, FMPS and FMBS.

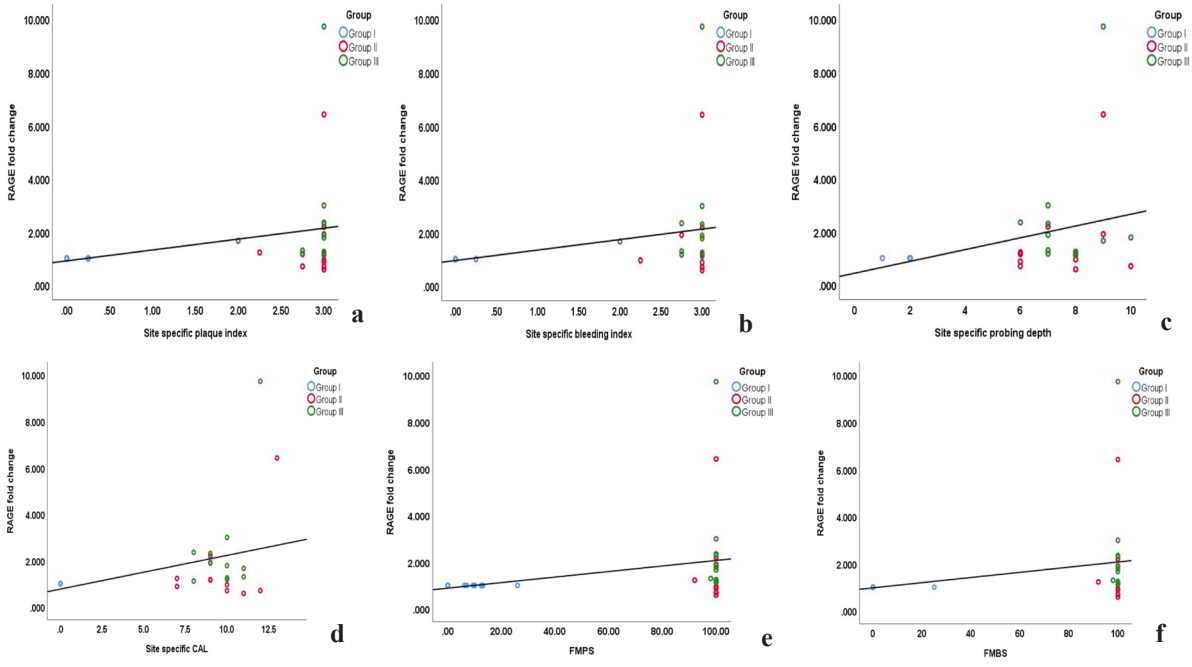

Figure 2. Scatter plot showing the correlation between fold change values of RAGE gene expression with site specific plaque index, site specific bleeding index, site specific probing depth, site specific CAL, FMPS and FMBS.

Pairwise comparison of fold change values indicated a statistically significant difference in the HMGB1 gene expression between Group I and III ($p<0.001$) and no statistically significant difference was observed between Group II and III and between Group I and II ($p<0.001$). Similarly, significant difference in the RAGE gene expression was observed between Group I and III and between Group II and III ($p<0.001$). A statistically significant increase in the RAGE gene expression was observed in the diabetes associated periodontitis group when compared to that the periodontitis group. No significant difference in the fold change was observed between Group I and II ($p>0.05$) (Table 2 given in the Supplemental File).

Correlation Between the Clinical Parameters and the RT-qPCR Data

There was no statistically significant correlation between the RT-qPCR data of HMGB1 and RAGE and

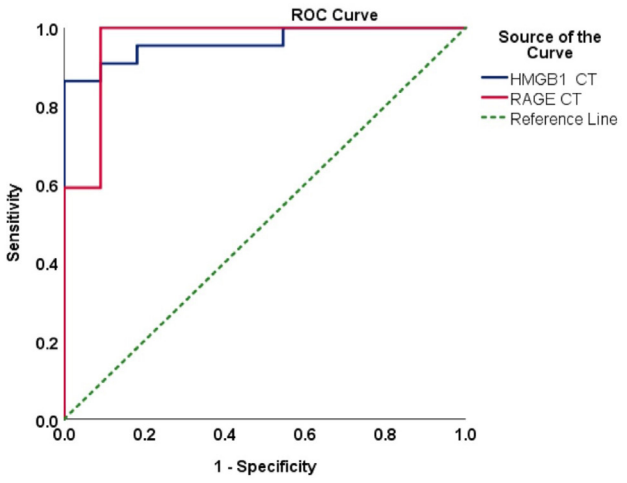

Figure 3. Shows the ROC analysis to evaluate the utility of HMGB1 and RAGE to distinguish between periodontal health and disease.

the clinical parameters in Group I and II (Tables 3 and 4 given in the Supplemental File). In Group III, the RAGE CT value inversely correlated with the SSPI and SSBI (p=0.012 and p=0.008, respectively) (Table 5 given in the Supplemental File). Overall, a statistically significant correlation was observed between the CT, ΔCT and fold change values of HMGB1 and RAGE gene expression in terms of clinical parameters in all the 33 subjects (p<0.05). There was a statistically significant inverse correlation (p<0.05) between CT and ΔCT values of HMGB1 and RAGE gene expression and all clinical parameters. A statistically significant positive correlation (p<0.05) was observed between the fold change values of HMGB1 and RAGE gene expression with all the clinical parameters (Table 3) (Figures 1 and 2).

ROC Analysis

The AUC for both markers was 0.96 with statistical significance (p<0.001). The diagnostic accuracy was 87.9% [95% confidence interval (CI): 72.7-95.2%], the positive and negative predictive values were 95.0% and 76.9% respectively for HMGB1. The diagnostic accuracy was 93.9% (95% CI: 80.4-98.3%), positive and negative predictive values were 91.7% and 100.0%, respectively, for RAGE (Table 4) (Figure 3).

IHC Analysis

HMGB1 and RAGE Expression

Table 5 shows a statistically significant difference when comparing the IHC parameter for HMGB1 and RAGE expression between the three groups in both the epithelium and the CT. MPPC, SI and SID scores were highest in the T2DM periodontitis patients (Group III) for both HMGB1 and RAGE, when compared to Group I and II (p<0.001).

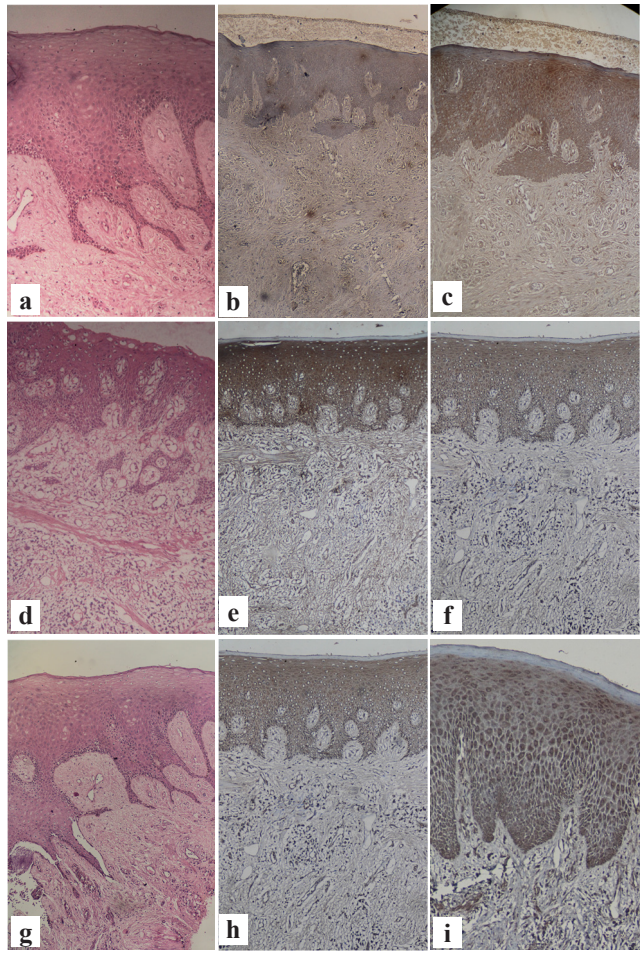

Figure 4. (100x magnification) (a) The H&E section of Group I exhibits keratinized stratified squamous epithelium with long rete pegs with proliferative nature. The underlying connective tissue shows moderately fibrous st roma with scattered inflammatory cells and moderate vascularity, (b) the IHC section of HMGB1 in Group I shows keratinized epithelium with underlying connective tissue where the epithelium does not show any positivity for the marker. There is a scattered expression of HMGB1 within the inflammatory cells of the connective tissue, (c) shows diffuse positivity of RAGE within the epithelium of moderate intensity in both the nucleus and cytoplasm of the cells. The underlying connective tissue exhibits a mild expression of the markers within the inflammatory cells and the stroma, (d) shows H&E section in Group II of keratinized stratified squamous epithelium which is hyperplastic and highly proliferative in nature. The underlying connective tissue exhibits diffuse infiltration of inflammatory cells and numerous capillaries indicating high vascularity within the lesion, (e) the IHC section of HMGB1 in Group II shows strong positivity for the HMGB1 marker in both the nucleus and cytoplasm of the epithelial cells. The underlying connective tissue shows a diffused expression of HMGB1 of moderate intensity in the inflammatory cells and fibroblasts, (f) RAGE in Group II exhibits diffuse positivity in the epithelium with both membranous and nuclear patterns of moderate intensity. The underlying connective tissue shows scarce positivity in the capillaries, (g) the H&E section of Group III exhibits hyperplastic keratinized epithelium with elongated rete pegs and prominent intercellular bridges. The epithelium is proliferative in nature with an active basal layer. The underlying connective tissue exhibits moderate fibrous stroma with diffuse chronic inflammatory cell infiltration and vascularization, (h) exhibits a strong positive expression of HMGB1 in both the epithelium and connective tissue in Group III. The epithelium shows both nuclear and cytoplasmic expression of the marker with dense expression in the basal and spinous layers. The underlying connective tissue shows intense positivity within the inflammatory cells and the fibrous stoma, (i) exhibits the expression of RAGE in both the epithelium and connective tissue. Within the epithelium, there is dense positivity of the marker within the basal, parabasal and the lower half of the spinous layers while the other layers show positivity of moderate intensity. The underlying connective tissue exhibits a diffuse expression of the marker with localized dense areas of positivity.

The H&E and IHC stainings for tissue expression of HMGB1 and RAGE are shown in Figure 4.

There was a statistically significant difference between Group I and II, and Group I and III in the epithelium and CT in terms of MPPC and SID scores of HMGB1 and RAGE expression. However, such a relationship was not observed between Group II and III, except for the SID score in the epithelium for HMGB1 expression (Table 6 shown in the Supplemental File). No statistically significant correlation was observed between the clinical parameters and the HMGB1, RAGE IHC parameters in Group I, II, and III but a statistically significant correlation was found with the total study population ($p<0.05$) (Tables 7 and 8 shown in the Supplemental File). Similar MPPC and SID scores were observed in the expression of both HMGB1 and RAGE in the epithelium and the CT with no significant difference between the groups (Table 9 shown in the Supplemental File).

Discussion

The scientific literature is replete with research papers suggesting that systemic disorders influence oral health, and vice-versa (31). When periodontal pathogens, their toxins, harmful metabolic by products, host immune-inflammatory response mediators, DAMPs, acute phase reactants, and a variety of other pathogenic factors enter the systemic circulation from the oral cavity, they may act alone or in conjunction with other risk factors to contribute to the pathogenesis of non-oral diseases (5). Many pathways and mechanisms have been suggested and investigated until now, yet there remain a lot of unexplored domains that demand in-depth exploration.

Danger molecules have recently been linked to the pathogenesis of a number of systemic diseases, and HMGB1 has been linked to the pathogenesis of both T2DM and periodontitis (6). In cultured periodontal ligament cells, Hasegnawa (32) found that HMGB1 secretion was associated with elevated levels of RAGE, TLR-2, and TLR-4 mRNA as well as IL-6 and IL-11 production. Both HMGB1-RAGE and TLR ligation accelerated and exacerbated chronic inflammation via NF-kB signal transduction (33). The binding stimulates the migration of leukocytes and monocytes into the inflammatory milieu, endothelial dysfunction, autophagy, neutrophil NETosis, dendritic cell differentiation and maturation, activation of Th17 cells, regulation of B cell activation, and induction of inducible nitric oxide (32). Moreover, HMGB1, RAGE expression are increased in T2DM and associated with diabetes complications like diabetic retinopathy, diabetic neuropathy, diabetic cardiomyopathy, and diabetic liver injury, among others, by encouraging M1 macrophage polarization, establishing insulin resistance by activating NF-kB, inducing pancreatic β cell death and inflammation (34). The HMGB1-RAGE complex and the RAGE-AGE axis have thus been proposed to have a role in the etiology of periodontal disease and T2DM (35). Therefore, we sought to compare the expression of HMGB1 and RAGE in periodontitis patients with and without type 2 diabetes mellitus. Furthermore, the relationship with periodontal disease severity was investigated.

To investigate the expression of HMGB1 and RAGE in gingival tissues, we used RT-PCR and IHC techniques. When compared to periodontally healthy individuals, it was shown that the gene expression of HMGB1 was 2.8 times higher in patients with periodontitis and T2DM which is in line with published data (9-11,23,36-39). This could be attributed to the inflammatory milieu within the periodontium stimulated with LPS and pro-inflammatory cytokines which leads to stimulate the transcription of HMGB1 gene from the nucleus into the cytoplasm and its consequent active secretion. Moreover, the periodontal ligament cells undergo pyroptosis, necrosis, apoptosis, necroptosis, ferroptosis, NETosis, and other forms of cell death all of which cause HMGB1 to be passively secreted (40).

Jiang et al. (10) demonstrated upregulated HMGB1 levels that positively correlated with pro-inflammatory cytokines in periodontitis. Further, it was both sensitive (89.71%) and specific (95%) in diagnosing periodontitis with potential utility as a biomarker. In the current study, ROC analysis similarly revealed that HMGB1 could distinguish between periodontal health and disease with a diagnostic accuracy of 87.88% and positive and negative predictive values of 95% and 76.92%, respectively. On the contrary, Lin et al. (41) observed that smokers with chronic periodontitis had reduced HMGB1 expression in their GCF. They attributed their findings to nicotine, which might inhibit NF-kB activation and reduce HMGB1 secretion via specific pathways (41).

In accordance with the RT-qPCR results, IHC showed increased HMGB1 expression in both the epithelium and the connective tissue of gingival tissues from periodontitis with and without T2DM, with the latter group showing the highest expression in this study. Significantly, increased HMGB1 positive cells with strong staining intensity was seen in both the periodontally diseased groups when compared to periodontal health. Similar findings in periodontitis have been described in previous studies (21,23,24,27,33,38,42-44). Listyarifah et al. (45) also found elevated HMGB1 expression in the basal layer of the sulcular epithelium and in every layer of the junctional epithelium with cytoplasmic localization in periodontitis. Additionally, in periodontitis, there were considerably more HMGB1-positive cells with cytoplasmic localization in the lamina propria than in gingivitis. The intracellular localization of HMGB1 was not investigated in this work.

RAGE gene expression was found to be 2.5 times higher in patients with periodontitis and T2DM it had a diagnostic accuracy of 93.94%, with positive and negative predictive values of 91.67% and 100%. Similar findings were reported previously (23,25,31,44,46,47). When compared to healthy periodontium, IHC labelling revealed more positive cells with strong staining intensity for RAGE in the epithelium and connective tissue of periodontitis and diabetes associated periodontitis groups. This is consistent with the data from Abbass et al. (27), Rajeev et al. (43) and Katz et al. (44). In this study, however, no statistically significant difference was seen between the diabetes and non-diabetic periodontitis groups. Chang et al. (21) and Katz et al. (44) reported similar findings. On the contrary, Schmidt et al. (19), Rajeev et al. (43) and Lalla et al. (48) reported significant differences in RAGE expression in patients with DM associated periodontitis. Majority of the evidence emphasized RAGE overexpression in periodontal tissues in patients with or without T2DM. Increased RAGE expression could be attributable to the accumulation of AGE and non-AGE RAGE ligands in the inflammatory periodontium, such as HMGB1, S100/calgranulin, and Mac-1. These molecules may upregulate RAGE and chronic inflammation. Katz et al. (44) observed 50% higher RAGE expression in the gingival tissues of patients periodontitis and diabetes than that in systemically healthy periodontitis patients. Similarly, Li et al. (46) found that in the presence of AGEs, periodontal ligament fibroblasts exhibited higher levels of RAGE and showed enhanced apoptosis. In line with this study, Akutagawa et al. (25) discovered an increase in RAGE mRNA and protein levels in diabetic mouse models.

Although the HMGB1-RAGE axis has been implicated in the pathogenesis of several chronic inflammatory diseases, evidence is scarce to show their role in patients with periodontitis and diabetes. The combined expression of HMGB1 and RAGE in periodontitis has been studied *in vitro* cell culture and in diabetic murine models (23-26). However, the HMGB1-RAGE axis in human gingival tissue from diabetes associated periodontitis has received little attention. The increased HMGB1 and RAGE expression were observed in this study (24). This study the authors found increased HMGB1 and RAGE expression in the epithelial and connective tissue layers in diabetes-associated periodontitis compared to that of the periodontally healthy and periodontitis group, possibly due to the increased accumulation of AGE and other RAGE ligands in the gingival tissues of diabetic individuals. Increased HMGB1 expression in diabetes may also be associated with increased activation of inflammasomes.

In this study, we found a significant positive correlation between HMGB1 and RAGE tissue expression and fold change values with all clinical parameters in the entire sample. This indicates that the expression of these markers increased with the severity of the disease. Such a finding would be expected since HMGB1 and RAGE are associated with chronic inflammation such as T2DM and periodontitis. Yu et al. (13), Lin et al. (41), Jiang et al. (10), and Paknejad et al. (8) have also reported that HMGB1 expression was associated with the severity of periodontal disease and this is consistent with our findings. Rajeev et al. (43), Chang et al. (21), and Lalla et al. (48) have reported a comparable positive correlation of RAGE expression with clinical parameters. No significant correlation was observed between markers and clinical parameters across groups in this study, which may be due to the small sample size.

The results of this study highlighted the importance of upregulated inflammatory biomolecules, such as HMGB1 and RAGE, and the associated exaggerated inflammatory response in the pathogenesis of T2DM with periodontitis. Furthermore, this study suggests that these biomolecules can be used as diagnostic markers for periodontitis. Targeting these biomolecules and blocking their activation and subsequent downstream signaling pathways can help to reduce inflammation in the treatment of periodontitis. The current work advances our understanding of the disease's pathophysiology and highlights the critical function of the HMGB1-RAGE axis in diabetes-related periodontitis.

Study Limitations

Small sample size, cross-sectional study design, lack of protein estimation are some of the limitations of our study, and *in vitro* studies are warranted to confirm clinical findings. Only subjects with controlled T2DM receiving medication with oral hypoglycemics were recruited in this study. This may have influenced the results as metformin, the most common drug, has been shown to result in decreased HMGB1 and RAGE expression (49). Future studies analyzing patients with uncontrolled type 2 diabetes may expand our knowledge of the role of these biomarkers in periodontitis associated with type 2 diabetes.

Conclusion

Type 2 diabetes mellitus patients with stage III/IV periodontitis have significantly higher levels of HMGB1 and RAGE expression than those who are systemically healthy with periodontitis and periodontally healthy controls. Patients with type 2 diabetes may be more prone to periodontitis due to higher HMGB1-RAGE levels. Therapeutic targeting of the HMGB1-RAGE axis and associated signaling pathways may thus pave the way for the development of innovative periodontitis treatment options in type 2 diabetes mellitus patients.

Ethics

Ethics Committee Approval: The research proposal was submitted to the institutional review board of SRM University and approval was received (SRMDC/IRB/2020/MDS/No.506, date: 05.12.2022). The study was registered in the clinical trial registry of India and the allotted CTRI number was CTRI/2022/02/040169, REF/2022/02/051245.

Informed Consent: Autonomy of the research participants was ensured by obtaining written and verbal informed consent.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: R.R., D.A., S.S., P.P., Concept: R.R., D.A., S.S., P.P., J.C., Design: R.R., D.A., S.S., P.P., J.C., Data Collection or Processing: R.R., D.A., S.S., P.P., Analysis or Interpretation: R.R., D.A., S.S., P.P., Literature Search: R.R., D.A., S.S., J.C., Writing: R.R., D.A., S.S., J.C.

Conflict of Interest: No conflict of interest was declared by the authors.

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Supplemental Table 1. Results of the Bonferroni correction test for Intergroup pairwise comparison of clinical parameters

Clinical Variable	Pairwise group comparison	p-value
SSPI	Group I – Group II	<0.05*
	Group I – Group III	<0.05*
	Group II – Group III	0.99, NS
SSBI	Group I – Group II	<0.05*
	Group I – Group III	<0.05*
	Group II – Group III	0.99, NS
SS-PPD (in mm)	Group I – Group II	<0.05*
	Group I – Group III	<0.05*
	Group II – Group III	0.99, NS
SS-CAL (in mm)	Group I – Group II	<0.05*
	Group I – Group III	<0.05*
	Group II – Group III	0.99, NS
FMPS (%)	Group I – Group II	<0.05*
	Group I – Group III	<0.05*
	Group II – Group III	0.99, NS
FMBS (%)	Group I – Group II	<0.05*
	Group I – Group III	<0.05*
	Group II – Group III	0.99, NS

*P<0.05, statistically significant, NS: Not significant, SS-PI: Site-specific plaque index score, SS-BI: Site-specific bleeding index score, SS-PPD: Site-specific mean probing pocket depth, SS-CAL: Site-specific mean clinical attachment loss, FMPS: Full mouth plaque score, FMBS: Full mouth bleeding score, Q1-Q3: Lower quartile to upper quartile

Supplemental Table 2. Bonferroni corrected test results for pairwise comparisons of data from RT-qPCR analysis

Variable	Pairs	p-value
HMGB1 CT	Group I – Group II	0.024*
	Group I – Group III	<0.05*
	Group II – Group III	0.108, NS
HMGB1 ΔCT	Group I – Group II	0.315, NS
	Group I – Group III	<0.05*
	Group II – Group III	0.04*
HMGB1 ΔΔCT	Group III – Group II	0.007*
HMGB1 FOLD CHANGE	Group I – Group II	0.090, NS
	Group I – Group III	<0.05*
	Group II – Group III	0.058, NS
RAGE CT	Group I – Group II	0.018*
	Group I – Group III	<0.05*
	Group II – Group III	0.174, NS
RAGE ΔCT	Group I – Group II	0.999, NS
	Group I – Group III	0.012*
	Group II – Group III	0.149, NS
RAGE ΔΔCT	Group II – Group III	0.036*
RAGE FOLD CHANGE	Group I – Group II	0.999, NS
	Group I – Group III	<0.05*
	Group II – Group III	0.023*

*P-value<0.05, statistically significant, NS: Not significant, CT: Cyclic threshold, Q1-Q3: Lower quartile to upper quartile

Supplemental Table 3. Spearman Rank Correlation between the clinical parameters and the data from RT-qPCR for group I

Parameters		HMGB1 CT	HMGB1 ΔCT	HMGB1 ΔΔCT	HMGB1 fold change	RAGE CT	RAGE ΔCT	RAGE ΔΔCT	RAGE fold change
SSPI	r-value	-0.34	0.05	-	-	-0.02	-0.11	-	-
	P-value	0.29 NS	0.86 NS	-	-	0.93 NS	0.73 NS	-	-
SSBI	r-value	0.26	0.37	-	-	0.52	-0.07	-	-
	P-value	0.43 NS	0.25 NS	-	-	0.09 NS	0.82 NS	-	-
SS-PPD	r-value	-0.50	-0.50	-	-	-0.15	-0.10	-	-
	P-value	0.11 NS	0.11 NS	-	-	0.65 NS	0.77 NS	-	-
SS-CAL	r-value	-	-	-	-	.	.	-	-
	P-value	-	-	-	-	.	.	-	-
FMPS	r-value	0.19	-0.20	-	-	0.05	0.03	-	-
	P-value	0.56 NS	0.55 NS	-	-	0.87 NS	0.91 NS	-	-
FMBS	r-value	0.15	-0.30	-	-	0.50	0.40	-	-
	P-value	0.65 NS	0.37 NS	-	-	0.11 NS	0.22 NS	-	-

*P-value <0.05, statistically significant, NS: Not significant, SS-PI: Site-specific plaque index score, SS-BI: Site-specific bleeding index score, SS-PPD: Site-specific mean probing pocket depth, SS-CAL: Site-specific mean clinical attachment loss, Q1-Q3: Lower quartile to upper quartile, CT: Cyclic threshold

Supplemental Table 4. Spearman Rank Correlation between the clinical parameters and the data from RT-qPCR for group II

Parameters		HMGB1 CT	HMGB1 ΔCT	HMGB1 ΔΔCT	HMGB1 fold change	RAGE CT	RAGE ΔCT	RAGE ΔΔCT	RAGE fold change
SSPI	r-value	0.14	-0.12	-0.12	0.12	-0.15	-0.02	-0.02	0.02
	P-value	0.68 NS	0.72 NS	0.72 NS	0.72 NS	0.65 NS	0.94 NS	0.94 NS	0.94 NS
SSBI	r-value	-0.30	-0.47	-0.47	0.47	-0.41	0.12	0.12	-0.12
	P-value	0.36 NS	0.14 NS	0.14 NS	0.14 NS	0.20 NS	0.72 NS	0.72 NS	0.72 NS
SS-PPD	r-value	-0.18	0.11	0.11	-0.11	-0.19	-0.17	-0.17	0.17
	P-value	0.59 NS	0.73 NS	0.73 NS	0.73 NS	0.56 NS	0.61 NS	0.61 NS	0.61 NS
SS-CAL	r-value	0.27	0.00	0.00	-0.00	-0.20	0.20	0.20	-0.20
	P-value	0.41 NS	0.99 NS	0.99 NS	0.99 NS	0.55 NS	0.55 NS	0.55 NS	0.55 NS
FMPS	r-value	-0.20	0.30	0.30	-0.30	-0.20	0.20	0.20	-0.20
	P-value	0.55 NS	0.36 NS	0.36 NS	0.36 NS	0.55 NS	0.55 NS	0.55 NS	0.55 NS
FMBS	r-value	-0.20	0.30	0.30	-0.30	-0.20	0.20	0.20	-0.20
	P-value	0.55 NS	0.36 NS	0.36 NS	0.36 NS	0.55 NS	0.55 NS	0.55 NS	0.55 NS

*P-value <0.05, statistically significant, NS: Not significant, SS-PI: Site-specific plaque index score, SS-BI: Site-specific bleeding index score, SS-PPD: Site-specific mean probing pocket depth, SS-CAL: Site-specific mean clinical attachment loss, Q1-Q3: Lower quartile to upper quartile, CT: Cyclic threshold

Supplemental Table 5. Spearman Rank correlation between the clinical parameters and the data from RT-qPCR for Group III

Parameters		HMGB1 CT	HMGB1 ΔCT	HMGB1 ΔΔCT	HMGB1 fold change	RAGE CT	RAGE ΔCT	RAGE ΔΔCT	RAGE fold change
SSPI	r-value	-0.07	0.01	0.01	-0.01	-0.72	-0.42	-0.42	0.42
	P-value	0.82 NS	0.97 NS	0.97 NS	0.97 NS	0.01*	0.19 NS	0.19 NS	0.19 NS
SSBS	r-value	-0.23	-0.07	-0.07	0.07	-0.74	-0.23	-0.23	0.23
	P-value	0.48 NS	0.82 NS	0.82 NS	0.82 NS	0.01*	0.48 NS	0.48 NS	0.48 NS
SS-PPD	r-value	0.02	-0.36	-0.36	0.36	-0.16	0.14	0.14	-0.14
	P-value	0.94 NS	0.27 NS	0.27 NS	0.27 NS	0.63 NS	0.67 NS	0.67 NS	0.67 NS
SS-CAL	r-value	0.09	-0.18	-0.18	0.18	0.10	-0.13	-0.13	0.13
	P-value	0.78 NS	0.59 NS	0.59 NS	0.59 NS	0.75 NS	0.69 NS	0.69 NS	0.69 NS
FMPS	r-value	-0.20	-0.50	-0.50	0.50	-0.20	-0.20	-0.20	0.20
	P-value	0.55 NS	0.11 NS	0.11 NS	0.11 NS	0.55 NS	0.55 NS	0.55 NS	0.55 NS
FMBS	r-value	-0.20	-0.50	-0.50	0.50	-0.20	-0.20	-0.20	0.20
	P-value	0.55 NS	0.11 NS	0.11 NS	0.11 NS	0.55 NS	0.55 NS	0.55 NS	0.55 NS

*P-value <0.05, statistically significant, NS: Not significant, SS-PI: Site-specific plaque index score, SS-BI: Site-specific bleeding index score, SS-PPD: Site-specific mean probing pocket depth, SS-CAL: Site-specific mean clinical attachment loss, Q1-Q3: Lower quartile to upper quartile, CT: Cyclic threshold

Supplemental Table 6. Bonferroni corrected pairwise comparisons between the groups to compare the expression of HMGB1 AND RAGE in the epithelium and the connective tissue in Group I, II and III

			GROUP I and GROUP II (p-value)	GROUP I and GROUP III (p-value)	GROUP II and GROUP III (p-value)
HMGB1	EPITHELIUM	MPPC	0.00*	0.00*	0.13, NS
		SID	0.00*	0.00*	0.01*
	CONNECTIVE TISSUE	MPPC	0.00*	0.00*	0.16, NS
		SID	0.00*	0.00*	0.11, NS
RAGE	EPITHELIUM	MPPC	0.00*	0.00*	0.63, NS
		SID	0.00*	0.00*	1.0, NS
	CONNECTIVE TISSUE	MPPC	0.001*	0.00*	0.07, NS
		SID	0.005*	0.00*	0.15, NS

*P-value <0.05, statistically significant, NS: Not significant, MPPC: Mean percentage of positively stained cells, SID: Staining intensity distribution

Supplemental Table 7. Non-parametric Spearman's rank correlation between the clinical parameters and MPPC and SID of HMGB1 expression in Group I, II, III and total samples (33)

Groups		HMGB1	r/p-value	SSPI	SSBI	SS-PPD	SS-CAL
Group I	Epithelium	MPPC	r-value	-	-	0.40	-
			P-value	-	-	0.21	-
		SID	r-value	-	-	0.47	-
			P-value	-	-	0.14	-
	Connective tissue	MPPC	r-value	-	-	-0.30	-
			P-value	-	-	0.36	-
		SID	r-value	-	-	-0.31	-
			P-value	-	-	0.34	-
Group II	Epithelium	MPPC	r-value	0.00	0.5	-0.08	-0.07
			P-value	1.00	0.11	0.81	0.82
		SID	r-value	0.10	0.51	0.06	0.15
			P-value	0.76	0.1	0.85	0.65
	Connective tissue	MPPC	r-value	-0.45	-0.45	-0.19	-0.27
			P-value	0.15	0.15	0.56	0.41
		SID	r-value	-0.5	-0.4	-0.09	-0.07
			P-value	0.11	0.21	0.78	0.82
Group III	Epithelium	MPPC	r-value	0.05	0.05	0.08	-0.1
			P-value	0.88	0.88	0.80	0.76
		SID	r-value	-0.14	-0.14	-0.23	0.30
			P-value	0.66	0.66	0.48	0.35
	Connective tissue	MPPC	r-value	0.00	0.00	-0.34	0.29
			P-value	1.00	1.00	0.29	0.37
		SID	r-value	0.15	0.15	-0.18	0.61
			P-value	0.64	0.64	0.59	0.04
Total Samples (33)	Epithelium	MPPC	r-value	0.79	0.81	0.70	0.69
			P-value	0.00*	0.00*	0.00*	0.00*
		SID	r-value	0.81	0.81	0.82	0.72
			P-value	0.00*	0.00*	0.00*	0.00*
	Connective tissue	MPPC	r-value	0.72	0.73	0.65	0.74
			P-value	0.00*	0.00*	0.00*	0.00*
		SID	r-value	0.72	0.73	0.65	0.74
			P-value	0.00*	0.00*	0.00*	0.00*

*P-value <0.05, statistically significant, NS: Not significant, SS-PI: Site-specific plaque index score, SS-BI: Site-specific bleeding index score, SS-PPD: Site-specific mean probing pocket depth, SS-CAL: Site-specific mean clinical attachment loss, Q1-Q3: Lower quartile to upper quartile, MPPC: Mean percentage of positively stained cells, SI: Staining intensity, MPI: Mean proportion of positively stained cells, SID: Staining intensity distribution

Supplemental Table 8. Non-parametric Spearman's rank correlation between the clinical parameters and MPPC and SID of RAGE expression in Group I, II, III and total samples (33)

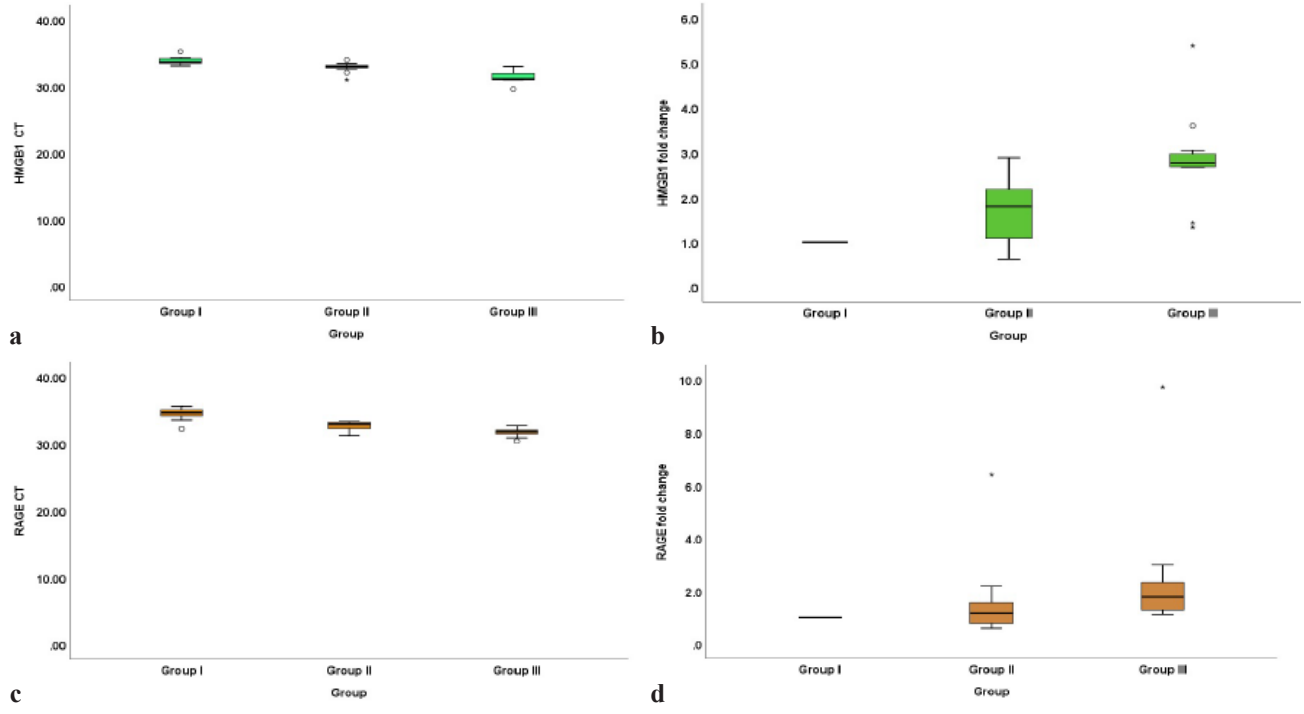
GROUPS		RAGE	r/P-value	SSPI	SSBI	SS-PD	SS-CAL
Group I	Epithelium	MPPC	r-value	-	-	-0.50	-
			P-value	-	-	0.11	-
		SID	r-value	-	-	-0.51	-
			P-value	-	-	0.10	-
	Connective tissue	MPPC	r-value	.	.	0.30	.
			P-value	.	.	0.36	.
		SID	r-value	.	.	0.30	.
			P-value	.	.	0.36	.
Group II	Epithelium	MPPC	r-value	0.40	0.30	0.51	0.33
			P-value	0.22	0.36	0.10	0.31
		SID	r-value	0.36	0.36	0.38	0.25
			P-value	0.26	0.26	0.23	0.45
	Connective tissue	MPPC	r-value	-0.40	0.05	-0.20	-0.27
			P-value	0.22	0.88	0.54	0.42
		SID	r-value	-0.42	0.10	-0.35	-0.38
			P-value	0.19	0.75	0.29	0.24
Group III	Epithelium	MPPC	r-value	0.10	0.10	-0.10	0.43
			P-value	0.76	0.76	0.75	0.18
		SID	r-value	0.22	0.22	-0.18	0.44
			P-value	0.51	0.51	0.59	0.17
	Connective tissue	MPPC	r-value	0.45	0.45	-0.25	-0.45
			P-value	0.16	0.16	0.45	0.89
		SID	r-value	0.45	0.45	-0.32	0.00
			P-value	0.16	0.16	0.32	0.97
Total (33)	Epithelium	MPPC SID	P-value	0.80	0.79	0.78	0.80
			r-value	0.00*	0.00*	0.00*	0.00*
		SID	P-value	0.83	0.83	0.72	0.80
			P-value	0.00*	0.00*	0.00*	0.00*
	Connective tissue	MPPC	r-value	0.71	0.76	0.59	0.59
			P-value	0.00*	0.00*	0.00*	0.00*
		SID	r-value	0.76	0.81	0.61	0.61
			P-value	0.00*	0.00*	0.00*	0.00*

*P-value <0.05, statistically significant, NS: Not significant, SS-PI: Site-specific plaque index score, SS-BI: Site-specific bleeding index score, SS-PPD: Site-specific mean probing pocket depth, SS-CAL: Site-specific mean clinical attachment loss, Q1-Q3: Lower quartile to upper quartile, MPPC: Mean percentage of positively stained cells, SI: Staining intensity, MPI: Mean proportion of positively stained cells, SID: Staining intensity distribution

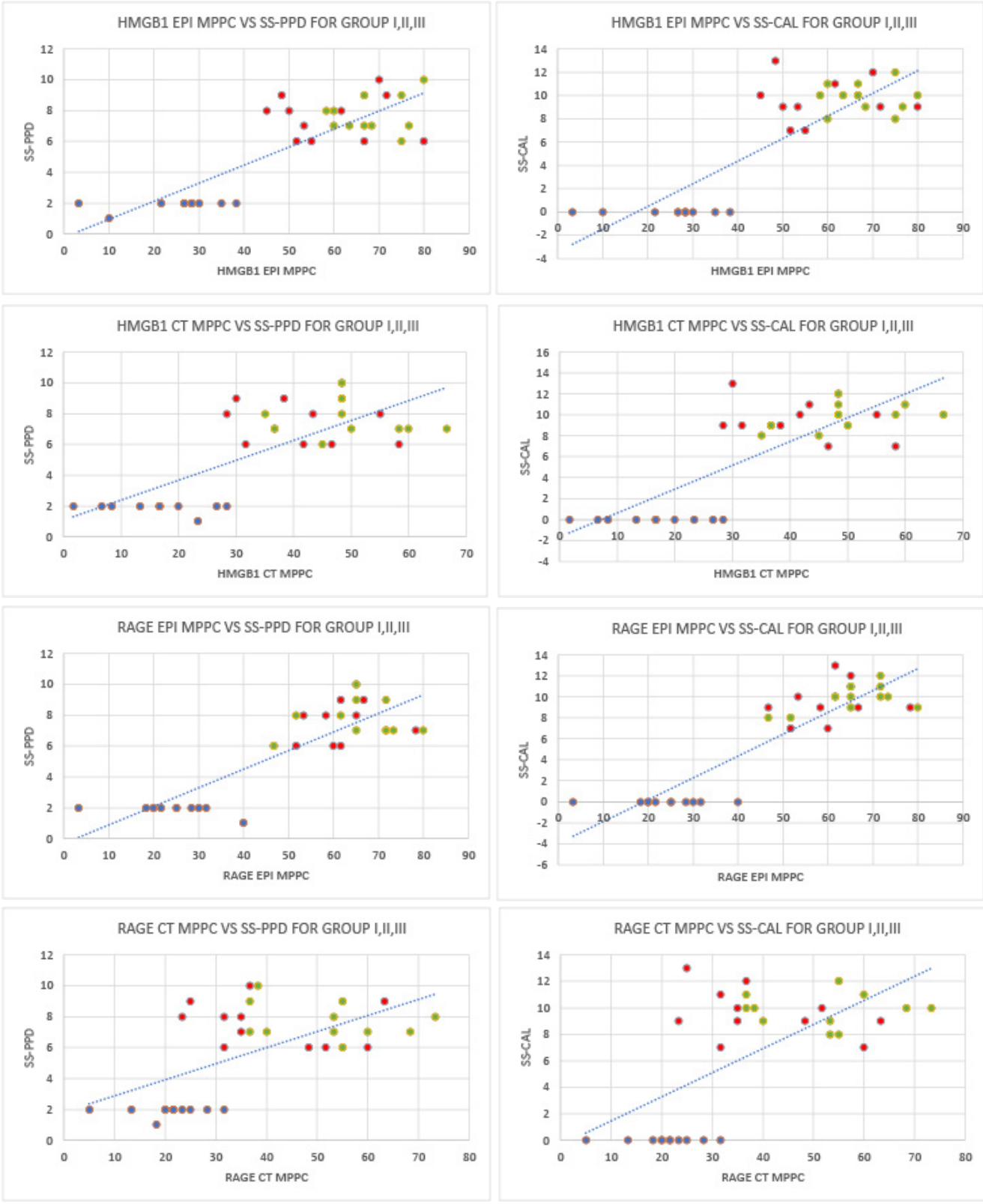
Supplemental Table 9. Related-Samples Wilcoxon Signed-Rank test comparison between the MPPC and SID values of HMGB1 and RAGE in in the epithelium and the connective tissue for Group I, II, III

Groups			HMGB1	RAGE	p-value
		MEAN			
Group I	Epithelium	MPPC	25.15	23.93	0.68, NS
		SID	1.63	1.45	0.63, NS
	Connective tissue	MPPC	16.66	21.66	0.16, NS
		SID	0.33	1.33	0.16, NS
Group II	Epithelium	MPPC	59.39	60.75	0.44, NS
		SID	7.33	7.75	0.35, NS
	Connective tissue	MPPC	41.66	40.15	0.56, NS
		SID	4.21	4.18	09, NS
Group III	Epithelium	MPPC	68.18	65.75	0.75, NS
		SID	8.82	8.18	0.06, NS
	Connective tissue	MPPC	49.54	51.81	0.64, NS
		SID	5.69	5.93	0.64, NS

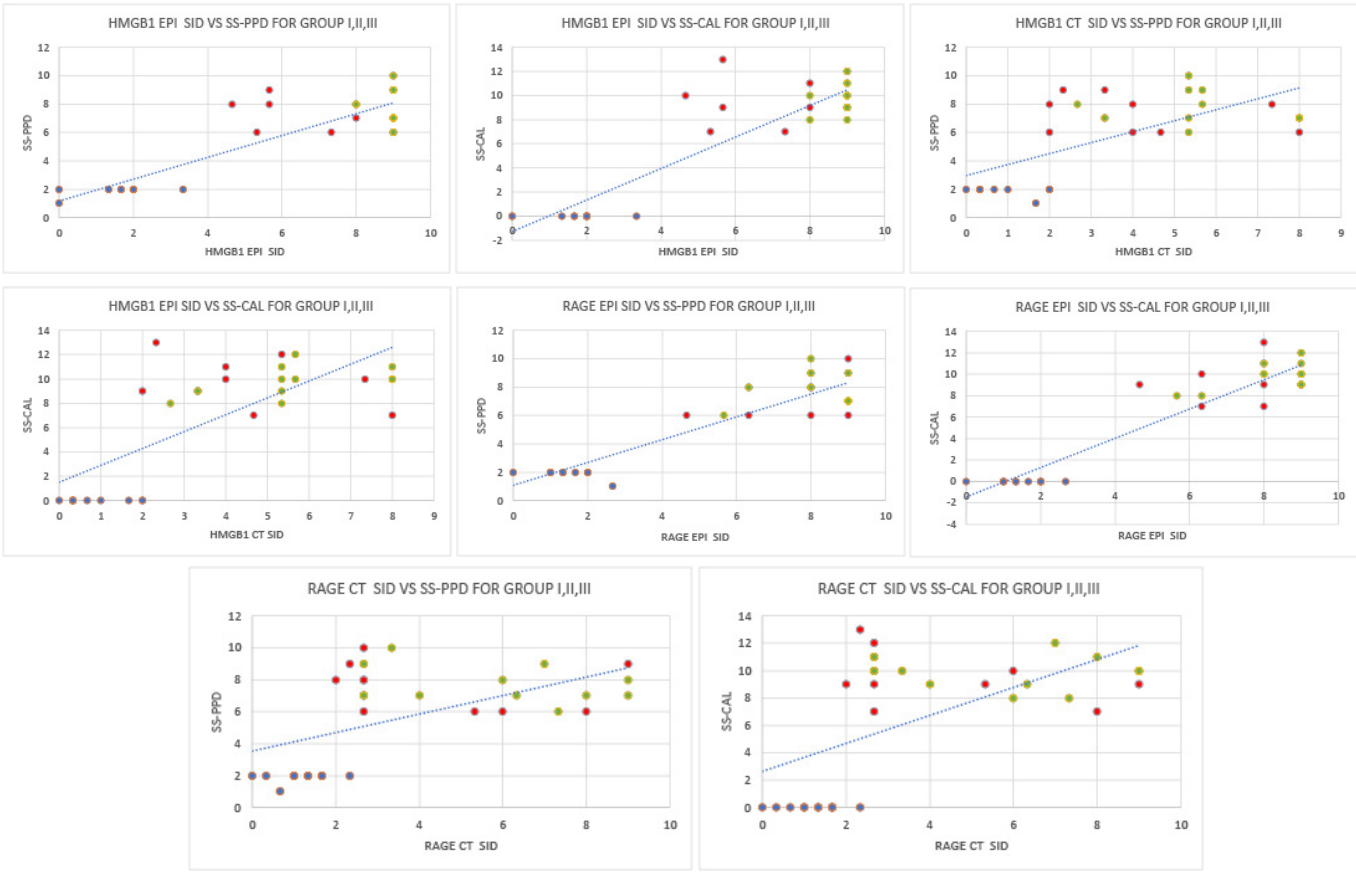
*P-value <0.05, statistically significant, NS: Not significant, MPPC: Mean percentage of positively stained cells, SID: Staining intensity distribution



Supplemental Figure 1. Box and Whisker plot demonstrating the CT and fold change values of HMGB1 and RAGE gene expression in Group I, II and III subjects.



Supplemental Figure 2. Scatter plot depicting correlation between HMGB1 & RAGE MPPC VS SS-PPD, SS-CAL in epithelium and connective tissue.



Supplemental Figure 3. Scatter plot depicting correlation between HMGB1 & RAGE SID VS SS-PPD, SS-CAL in epithelium and connective tissue.