Journal of the International Academy of Periodontology (2023) **25/2**:69-80

DOI: https://doi.org/10.14436/1466-2094.25.2.069-080.oar

Salivary expression of novel inflammatory marker Plasminogen Activator Inhibitor-1 in health and disease: an interventional study

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Abstract

Objective: To assess levels of the biomarkers Plasminogen Activator Inhibitor-1 (PAI-1) and alpha 2-macroglobulin (2MG) in saliva and serum in healthy individuals and patients with and without type 2 diabetes mellitus (T2DM) before and after periodontal therapy.

Materials and Methods: The 90 recruited subjects were divided into the three following groups: Group 1, with 30 healthy subjects; Group 2, with 30 systemically healthy subjects with stage II and III periodontitis; and Group 3, with 30 patients with periodontitis and well controlled T2DM (PDM). Salivary and serum PAI-1 and α2MG levels were estimated by enzyme-linked immunosorbent assay, and correlated with clinical parameters before and three months after periodontal therapy, and the data obtained was statistically analyzed.

Results: There was an improvement in clinical parameters in both groups that underwent initial periodontal therapy. Salivary and serum PAI-1 and α2MG levels were upregulated in PDM group, compared to periodontitis alone at baseline. A significant reduction in the levels of the biomarkers was observed three months after periodontal therapy, and an improvement in glycaemic control was also seen PDM group.

Conclusion: PAI-1 was expressed in saliva and serum in healthy patients, and increased in periodontitis and diabetes patients, thus evidencing its possible role in periodontal inflammation and glucose regulation, and its levels decreased after periodontal therapy.

Keywords: Adipokines. Biomarkers. Periodontitis. Diabetes mellitus. Saliva.

Introduction

Periodontitis is a chronic inflammatory condition of the periodontium, which is brought on by pathogenic bacteria in conjunction with other risk factors. Diabetes and periodontitis are both inversely correlated and are significant global1 health problems (Preshaw and Bissett, 2019; Kim and Amar, 2006). Periodontal disease induces a gradual, irreversible inflammatory response that destroys tissue. In opposition to the bacterial flora present in dental plaque biofilm, local tissue and immune cells generate pro-inflammatory cytokines that cause periodontal tissue damage (Ebersole *et al*., 2013). The metabolic disorder diabetes mellitus (DM) is characterized by hyperglycemia brought on by insufficient insulin secretion, ineffective insulin, or both. Periodontal tissues are also among the many organs of the body affected by DM. The fact that both these conditions have a negative impact on each other has been amply confirmed by previous research (Kim and Amar, 2006). Chronic periodontal infections cause a chronic subclinical systemic inflammation in the body, which promotes insulin resistance (IR), and leads to poor glycemic control in people with diabetes (Simpson *et al*., 2015).

The plasminogen activator (PA) is a primary factor that catalyzes numerous processes of physiological and pathological nature, such as fibrinolysis, wound healing, extracellular matrix (ECM) degradation, and inflammation (Yarmolinsky *et al*., 2016). The conversion of dormant plasminogen into active non-specific plasmin, which facilitates fibrinolysis and wound healing, is made possible by the tissue type plasminogen activator (tPA). Adipocytes, endothelial cells, and hepatocytes secrete Plasminogen activator inhibitor-1 (PAI-1), which prevents activation of tPA (Schneider and Sobel, 2012).

The link between PAI-1 and insulin resistance (IR) and type 2 diabetes (T2DM) has been confirmed by observational and experimental studies on humans (Yarmolinsky

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et al., 2016; Ma *et al*., 2004; Al-Hamodi *et al*., 2012). Additionally, tPA and PAI-1 concentrations are higher in saliva and gingival crevicular fluid (GCF) than in serum plasma (Toyman *et al*., 2015; Yin *et al*., 2000; Kinnby et al., 1994). The deficiency in plasmin or PAI-1 has been linked to bleeding and periodontal damage (Wyganowska-Świątkowska *et al*., 2014).

Alpha 2-macroglobulin (α2MG) is a proteinase inhibitor that is a vital biomarker shown to be prominent in diabetes individuals (Yoshino *et al*., 2019). A number of diseases, including periodontal disease, can be diagnosed and prognosed using 2MG, according to previous studies (Chung *et al*., 2016; Rastogi *et al*., 2019). In patients with periodontitis, periodontal tissue secretes 2MG to reduce inflammation and prevent microbial growth (Ertugrul *et al*., 2013).

Eliminating the cause of localised chronic inflammation may help these individuals to improve their glycemic control. It is believed that scaling and root planing (SRP) is the most effective non-surgical periodontal therapy (NSPT). To our knowledge, few studies have compared salivary and serum levels of PAI-1 and 2MG in patients with periodontitis and T2DM, and only a small number of studies have examined the impact of NSPT on PAI-1 and 2MG levels. The hypothesis of the current investigation was that PAI-1 behaves as a pro-inflammatory adipokine in patients with periodontitis and T2DM, and thus NSPT may deplete its levels. Thus, the present study was done to assess levels of the biomarkers PAI-1 and 2MG in saliva and serum in healthy individuals and patients with and without type 2 diabetes mellitus (T2DM) before and after periodontal therapy.

Materials and Methods

A total sample of 90 patients were recruited from October 2019 to October 2021. Patients from outpatients visiting Department of Periodontics at D. A. Pandu Memorial R. V. Dental College and Hospital (Bangalore, India) were enrolled. Institutional Ethics Committee approval (IEC/ IRB NO: 318/VOL-2/2019) was obtained before the commencement of the study. According to the 2013 revision of the Helsinki Declaration, participants received a thorough explanation of the study protocol and treatment process in the language they were most familiar with, and a written informed consent was obtained. The study involved 90 subjects (25–60 years) with a minimum of 16 natural teeth, excluding third molars, divided into three groups based on the consensus report of World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions of 2017. Thirty healthy controls were included as Group 1, consisting of subjects with intact periodontium, bleeding on probing index (BOP) < 10%, probing pocket depth (PPD) ≤ 3mm and no clinical attachment loss (CAL) or radiographic signs of alveolar bone destruction. Group 2 included systemically healthy patients with periodontitis diagnosed, based on the Classification of 2017, as having Stages II or III, generalized, grade B periodontitis with CAL ≥ 3mm in 30% of the sites, and PPD ≥5 mm with at least six teeth. Group 3 included periodontitis patients as defined above, with well controlled T2DM (PDM) having HbA1C <7% with diabetes diagnosed at least one year prior to the study, as per the diagnostic criteria of the American Diabetes Association and taking oral hypo-glycaemic agents. Patients were excluded if they had type 1 DM, or any other systemic disease accompanying T2DM, pregnancy or lactation, or were current or former smokers. The exclusion criteria also included patients with history of periodontal therapy within the past six months, antibiotic or anti-inflammatory drug intake within the past three months, patients taking any others medications other than for DM, and patients with any intraoral lesions.

Intervention

All patients had saliva and serum samples taken at baseline and three months after NSPT. Clinical data was also recorded at the same time intervals. Plaque index (PI) and Gingival Index (GI) were assessed at four sites per tooth. Full-mouth PPD and CAL were documented at six sites per tooth, and the presence or absence of bleeding sites within 20 seconds of probing were dichotomously scored and the percentage of bleeding sites, recorded. All the clinical parameter recordings were performed using a UNC-15 probe, and recorded to the nearest millimeter by an experienced single calibrated examiner (SG) at baseline, and re-examined by the same clinician (SG) three months later. After baseline sample collection and clinical parameter recordings, all patients underwent initial periodontal therapy i.e. patient education, full mouth SRP, and oral hygiene instructions. Ultrasonic instruments (Acteon Satelec Suprasson, Merignac, France) were used for scaling and Gracey curettes (Hu-FriedyTM, Chicago, USA), for root planing. SRP was completed quarantine-wise in two consecutive visits over two days. Proper brushing technique and usage of interdental cleaning aids according to patient requirement was also demonstrated during oral hygiene instructions. Saliva and serum samples were once again taken at the three-month follow-up appointment, and oral hygiene recommendations were reiterated as deemed necessary. All patients with persistent pockets were treated according to the standard periodontitis treatment protocol after the collection of three month recall samples.

Sample collection

Collection of saliva

To reduce diurnal alterations in sample collection, samples were collected between 9:00 am to 10:00 am. Passive drooling was used to collect 5ml of whole, unstimulated saliva from each patient. The collected sample was centrifuged for 10 minutes at 5000 rpm (1975g), the

supernatant was transferred to a sterile plastic vial and kept at -80°C until use.

Collection of serum

A 5-ml syringe with 20-gauge needle was used for venipuncture in the antecubital fossa, to obtain 5ml of blood, which was then incubated upright at room temperature for 30-45 minutes (but no more than 60 minutes) to facilitate clotting. Centrifuging the blood for 10 minutes at 5000 rpm (1975g) separated the serum from the blood. The extracted serum was immediately put into a clean plastic container and kept there until the assay at -80°C.

Estimation of biomarkers

Commercially available kit was used to measure PAI-1 and α2MG levels by enzyme-linked immunosorbent assay (ELISA) method (Human PAI-1 ELISA Kit; Catlog nº PT-S11523, Progenbiolab Technologies, Hubei, China and Human α2MG ELISA Kit; Catlog nº PT-S11137, Progenbiolab Technologies, Hubei, China). The kit used a double-antibody sandwich ELISA for the sample analysis.

PAI-1 and α2MG analysis

All the reagents and samples were first thawed to room temperature. Standard reagent was diluted by pipetting 50µl standard dilution into each tube. Serial dilution was used to produce dilution series by pipetting 100µl standard (540pg/ml) in the first tube. 40µl of sample dilution was then pipetted into testing sample well, to which 10µl of saliva or serum sample was added and mixed gently. The wells were incubated for 30 minutes at 37°C after covering with an adhesive strip. The wash solution was diluted 30-folds using distilled water followed by addition of 50µl of Horseradish peroxidase conjugate reagent into each well, except the blank well. These wells were incubated at 37°C for 30 minutes and then washed. 50µl of Chromogen Solution A and Chromogen Solution B was added to each well and light avoided for 15 minutes at 37°C. This was followed by addition of 50µl of stop solution was into each well, so as to stop the reaction. The colour of solution changed from blue to yellow. Calculating the blank wells as zero, the absorbance was read at 450nm within 15 minutes of addition of stop solution.

Statistical analysis

The sample size was estimated using the software GPower v. 3.1.9.4. Considering the effect size to be measured (d) at 80%, power of the study at 80%, and the alpha error at 5%, the sample size needed was 86, which was rounded up to 90. Thus, each group comprised of 30 samples (30 samples x 3 groups = 90 samples).

All the data analysis was accomplished using the software Statistical Package for Social Sciences for Windows version 22.0 released 2013 (Armonk, New York: IBM Corp).

Descriptive analysis of all the explanatory and outcome parameters was done using frequency and proportions for categorical variables, and mean and SD for continuous variables.

Mean values of various clinical parameters among the three groups at baseline were compared using one-way ANOVA test followed by Tukey's *post-hoc* test and Kruskal-Wallis test followed by Dunn's *post-hoc* test for the salivary and serum PAI-1 and α2MG levels. Independent Student *t*-test was used to compare the mean values of clinical parameters between Groups 2 and 3, and Mann-Whitney test was used to compare mean PAI-1 and α2MG levels in saliva and serum samples between Groups 2 and 3 at baseline. Student paired *t*-test was used to compare the mean values of clinical parameters between baseline and three months period in Groups 2 and 3, and Wilcoxon signed-rank test was used to compare the mean PAI-1 and α2MG levels in saliva and serum samples between baseline and three months period in Groups 2 and 3.

The relationship between salivary and serum PAI-1 and 2MG levels and the clinical parameters was evaluated using the Spearman's correlation test at baseline in all the groups and Groups 2 and 3 after three months. Using clinical parameters from Groups 2 and 3 at baseline and after three months, stepwise multiple linear regression analysis was used to predict the levels of PAI-1 and 2MG in saliva and serum samples. The level of significance was set at $p<0.05$.

Results

The study included 90 participants, with 30 participants in each group. The demographic characteristics were analyzed using Kruskal-Wallis test and chi-square test. In Group 1, both males and females were equally distributed. In both Groups 2 and 3, 56.7% (n=17) were males while 43.3% (n=13) were females. The mean age in Group 1 was 44.47± 6.87 years; in Group 2, was 41.60 ± 8.17 years; and in Group 3, was 45.20 ± 7.45 years. There was no significant difference in the mean age between the groups.

Figure 1A compares the mean values of various clinical parameters among the three groups at baseline, using one-way ANOVA test followed by Tukey's *post-hoc* test and Kruskal-Wallis test followed by Dunn's *post-hoc* test for the salivary (Figure 1B) and serum (Figure 1C) PAI-1 and α2MG levels. At baseline, statistically significant difference was seen in GI, PI, BOP, PPD and CAL between the groups, with Group 1 having the lowest value and Group 3, the highest value. Salivary and serum PAI-1 and α2MG values were significantly higher in Group 3, as compared to Groups 1 and 2.

Figure 1D compares the mean values of various clinical parameters at the three months period using Student *t*-test, and salivary (Figure 1E) and serum (Figure 1F) PAI-1 and α2MG levels using Mann-Whitney test.

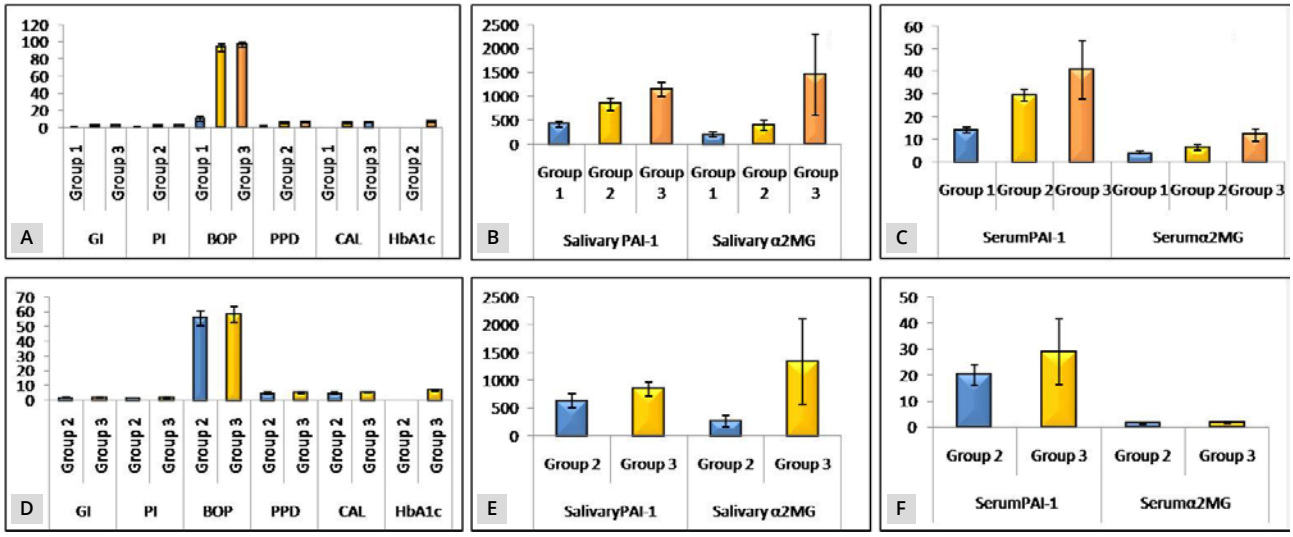

Figure 1. Comparison of mean values of various parameters. A) Comparison of mean values of various clinical parameters between the three groups at Baseline. B) Comparison of mean values of salivary PAI-1 and α2MG levels between the three groups at Baseline. C) Comparison of mean values of serum PAI-1 and α2MG levels between the three groups at Baseline. D) Comparison of mean values of various clinical parameters between Groups 2 and 3 after three months. E) Comparison of mean values of salivary PAI-1 and α2MG levels between Groups 2 and 3 after three months. F) Comparison of mean values of serum PAI-1 and α2MG levels between Groups 2 and 3 after three months.

After three months, differences in the various clinical parameters between the two experimental groups were not statistically significant, except for GI. However salivary and serum PAI-1 and α2MG values were significantly higher in Group 3, as compared to Group 2. Table 1 shows comparison of mean values of different parameters between baseline and three months period in Groups 2 and 3 using Student paired *t*-test for clinical parameters and Wilcoxon signed-rank test for salivary and serum PAI-1 and α2MG levels. In both Groups 2 and 3 there was a statistically significant difference in all the clinical parameters, as well as salivary and serum PAI-1 and α2MG levels between baseline and three months post-therapy. At three months post-intervention, the mean values were significantly decreased in all clinical parameters (p<0.001), and for serum PAI-1 (p= 0.01) and α2MG (p= 0.001) levels. Comparison of mean HbA1c levels (%) between baseline and three months in Group 3 showed a statistically significant decrease in HbA1c levels (p<0.001) three months post-SRP.

Table 2 assessed the relationship between salivary and serum PAI-1 and α2MG levels and various clinical parameters, using Spearman's correlation test in all the groups at baseline. At baseline the salivary and serum PAI-1 and α2MG levels showed a weak to moderate positive correlation with the various parameters in Group 1, which did not reach statistical significance, except between salivary PAI-1 and serum PAI-1, GI and PI. In both Groups 2 and 3 salivary and serum PAI-1 and α2MG levels showed a significant weak to moderate positive correlation with the various parameters. Despite salivary α2MG levels, serum PAI-1 and serum α2MG levels demonstrated a weak positive correlation, it did not reach statistical significance. Table 3 demonstrates Spearman's correlation test between salivary and serum PAI-1 and α2MG levels and the various parameters in Groups 2 and 3 at three months, which showed a significant weak to moderate positive correlation. However, serum PAI-1 and serum α2MG levels also demonstrated a weak positive correlation, but it did not reach statistical significance.

Table 4 analyzed stepwise multiple linear regression for predicting salivary and serum PAI-1 and α2MG levels by clinical parameters in Group 2 and Group 3 at baseline. In Group 2, for every 1-mm increase in PPD, there was 92.82 ng/ml increase in salivary PAI-1 levels, with a variability of 31%, which was statistically significant (p = 0.001). For every 1-mm increase in CAL, there was an increase in serum PAI-1 levels by 1.34 ng/ml (p < 0.001, variability 20%), in salivary α2MG levels by 43.16 ng/ml (p=0.04, variability 14%) and an increase in serum α2MG levels by 0.70 ng/ml (p=0.03, variability 15%), which were statistically significant. In Group 3, for every 1-mm increase in CAL, there was an increase in salivary PAI-1 levels by 158.51 ng/ml (p < 0.001, variability 41%), in serum PAI-1 levels by 13.19 ng/ml (p < 0.001, variability 37%), in salivary α2MG levels by 758.88 ng/ml (p=0.002, variability 29%) and an increase in serum α2MG levels by 2.51 ng/ml (p=0.001, variability 33%) which were statistically significant.

Table 1. Comparison of mean values of various parameters between baseline and 3 months period in Group 2 and Group 3.

Group	Parameters	Time	n	Mean	SD	Mean diff.	P-Value
Group 2	GI	Baseline	30	2.09	0.36	0.63	<0.001*[a]
		Three months	30	1.46	0.31		
	PI	Baseline	30	2.07	0.34	0.64	<0.001*[a]
		Three months	30	1.43	0.26		
	BOP	Baseline	30	92.84	4.01	37.01	<0.001*[a]
		Three months	35	55.83	5.08		
	PPD	Baseline	35	5.44	0.75	1.03	<0.001*[a]
		Three months	35	4.41	0.84		
	CAL	Baseline	35	5.16	0.88	0.46	<0.001*[a]
			35	4.70	0.93		
	HbA1c	-	-	-	-	-	-
	Salivary PAI-1 (ng/ml)	Baseline	30	832.67	124.33	201.30	<0.001*[b]
		Three months	30	631.36	128.98		
	Salivary α2MG (ng/ml)	Baseline	30	389.22	102.79	120.55	0.001*[b]
		Three months	30	268.67	102.22		
	Serum PAI-1 (ng/ml)	Baseline	30	29.43	2.63	9.28	<0.001*[b]
		Three months	30	20.15	3.96		
	Serum α2MG (ng/ml)	Baseline	30	6.27	1.36	2.16	<0.001*[b]
		Three months	30	4.11	1.31		
Group 3	GI	Baseline	30	2.40	0.34	0.76	<0.001*[a]
		Three months	30	1.64	0.40		
	PI	Baseline	30	2.40	0.38	0.87	<0.001*[a]
		Three months	30	1.52	0.65		
	BOP	Baseline	30	96.11	2.68	38.10	<0.001*[a]
		Three months	30	58.01	5.41		
	PPD	Baseline	30	6.02	0.67	1.42	<0.001*[a]
		Three months	30	4.59	0.58		
	CAL	Baseline	30	5.76	0.60	0.75	<0.001*[a]
			30	5.01	0.42		
	HbA1c	-	30	6.72	0.38	0.32	<0.001*[a]
			30	6.40	0.40		
	Salivary PAI-1 (ng/ml)	Baseline	30	1137.43	146.99	296.19	<0.001*[b]
		Three months	30	841.24	130.85		
	Salivary α2MG (ng/ml)	Baseline	30	1451.27	846.59	118.86	<0.001*[b]
		Three months	30	1332.41	773.04		
	Serum PAI-1 (ng/ml)	Baseline	30	40.73	12.95	11.88	0.01*[b]
		Three months	30	28.85	12.60		
	Serum α2MG (ng/ml)	Baseline	30	11.89	2.61	2.21	0.001*[b]
		Three months	30	9.67	2.71		

* Statistically significant.
[a] Student paired *t*-test.
[b] Wilcoxon signed-rank test.
GI = gingival index.
PI = plaque index.
BOP = bleeding on probing.
PPD = probing pocket depth.
CAL = clinical attachment level.
HbA1c = glycosylated hemoglobin.
PAI-1 = plasminogen activator inhibitor-1.
α2MG = alpha 2-macroglobulin.

Table 2. Spearman's correlation test to assess the relationship between salivary and serum PAI-1 and α2MG levels and clinical parameters at baseline.

Group	Parameters	Values	Salivary PAI-1	GI	PI	BOP	PPD	CAL	HbA1c	Serum PAI-1	Salivary α2MG	Serum α2MG
Group 1	Salivary PAI-1	rho	1	0.44	0.593	0.181	0.133	-	-	0.074	0.327	0.091
		P-value	-	0.015*	<0.001*	0.339	0.49	-	-	0.697	0.078	0.631
	Serum PAI-1	rho	-0.383	0.097	-0.354	0.204	0.092	-	-	1	0.001	0.141
		P-value	0.037*	0.611	0.055	0.281	0.634	-	-	-	0.998	0.458
	Salivary α2MG	rho	0.327	0.481**	0.125	0.354	0.217	-	-	0.001	1	-0.097
		P-value	0.078	0.007	0.511	0.055	0.259	-	-	0.998	-	0.611
	Serum α2MG	rho	-0.24	-0.116	-0.104	0.383*	0.248	-	-	0.141	-0.097	1
		P-value	0.202	0.54	0.584	0.037	0.195	-	-	0.458	0.611	-
Group 2	Salivary PAI-1	rho	1	0.51	0.48	0.44	0.54	0.55	-	0.60	0.58	0.58
		P-value	-	0.004*	0.007*	0.02*	0.002*	0.002*	-	0.001*	0.001*	0.001*
	Serum PAI-1	rho	0.60	0.39	0.39	0.31	0.38	0.42	-	1	0.55	0.53
		P-value	0.001*	0.03*	0.03*	0.07	0.04*	0.02*	-	-	0.002*	0.003*
	Salivary α2MG	rho	0.58	0.39	0.40	0.32	0.39	0.43	-	0.55	1	0.55
		P-value	0.001*	0.03*	0.03*	0.07	0.03*	0.02*	-	0.002*	-	0.002*
	Serum α2MG	rho	0.58	0.39	0.39	0.31	0.38	0.42	-	0.53	0.55	1
		P-value	0.001*	0.03*	0.03*	0.07	0.04*	0.02*	-	0.003*	0.002*	-
Group 3	Salivary PAI-1	rho	1	0.54	0.46	0.42	0.56	0.56	0.57	0.58	0.56	0.56
		P-value	-	0.002*	0.01*	0.02*	0.001*	0.001*	0.001*	0.001*	0.001*	0.001*
	Serum PAI-1	rho	0.58	0.54	0.46	0.42	0.56	0.56	0.57	1	0.57	0.55
		P-value	0.001*	0.002*	0.01*	0.02*	0.001*	0.001*	0.001*	-	0.001*	0.002*
	Salivary α2MG	rho	0.56	0.54	0.46	0.42	0.56	0.56	0.57	0.57	1	0.54
		P-value	0.001*	0.002*	0.01*	0.02*	0.001*	0.001*	0.001*	0.001*	-	0.003*
	Serum α2MG	rho	0.56	0.55	0.46	0.42	0.57	0.57	0.57	0.56	0.54	1
		P-value	0.001*	0.002*	0.01*	0.02*	0.001*	0.001*	0.001*	0.001*	0.003*	-

* Statistically significant.
The correlation coefficients are denoted by 'rho'.
Minus sign denotes negative correlation.
Correlation coefficient range:
0.0 = No Correlation
0.01 - 0.20 = very weak correlation.
0.21 - 0.40 = weak correlation.
0.41 - 0.60 = moderate correlation.
0.61 - 0.80 = strong correlation.
0.81 - 1.00 = very strong correlation.

GI = gingival index.
PI = plaque index.
BOP = bleeding on probing.
PPD = probing pocket depth.
CAL = clinical attachment level.
HbA1c = glycosylated hemoglobin.
PAI-1 = plasminogen activator inhibitor-1.
α2MG = alpha 2-macroglobulin.

Table 3. Spearman's correlation test to assess the relationship between salivary and serum PAI-1 and α2MG levels and clinical parameters in Group 2 and Group 3 at three months period.

Group	Parameters	Values	Salivary PAI-1	GI	PI	BOP	PPD	CAL	HbA1c	Serum PAI-1	Salivary α2MG	Serum α2MG
Group 2	Salivary PAI-1	rho	1	0.38	0.40	0.35	0.45	0.44	-	0.58	0.56	0.55
		P-value	-	0.04*	0.03*	0.04*	0.01*	0.02*	-	0.001*	0.001*	0.002*
	Serum PAI-1	rho	0.58	0.39	0.41	0.34	0.45	0.44	-	1	0.53	0.51
		P-value	0.001*	0.04*	0.03*	0.05	0.01*	0.02*	-	-	0.003*	0.004*
	Salivary α2MG	rho	0.56	0.39	0.41	0.35	0.45	0.43	-	0.53	1	0.55
		P-value	0.001*	0.03*	0.02*	0.04*	0.01*	0.02*	-	0.003*	-	0.002*
	Serum α2MG	rho	0.55	0.388	0.410	0.351	0.45	0.43	-	0.51	0.55	1
		P-value	0.002*	0.034*	0.024*	0.057	0.01*	0.02*	-	0.004*	0.002*	-
Group 3	Salivary PAI-1	rho	1	0.44	0.38	0.37	0.48	0.44	0.46	0.54	0.56	0.53
		P-value	.	0.02*	0.04*	0.04*	0.008*	0.01*	0.01*	0.003*	0.001*	0.003*
	Serum PAI-1	rho	0.54	0.43	0.37	0.37	0.47	0.43	0.45	1	0.53	0.55
		P-value	0.003*	0.02*	0.04*	0.04*	0.009*	0.02*	0.01*	-	0.003*	0.002*
	Salivary α2MG	rho	0.56	0.41	0.40	0.36	0.42	0.46	0.44	0.53	1	0.57
		P-value	0.001*	0.02*	0.03*	0.04*	0.02*	0.01*	0.02*	0.003*	-	0.001*
	Serum α2MG	rho	0.53	0.43	0.37	0.37	0.43	0.47	0.45	0.55	0.57	1
		P-value	0.003*	0.02*	0.04*	0.04*	0.02*	0.009*	0.01*	0.002*	0.001*	-

* Statistically significant.
The correlation coefficients are denoted by 'rho'.
Minus sign denotes negative correlation.
Correlation coefficient range:
0.0 = no correlation.
0.01 - 0.20 = very weak correlation.
0.21 - 0.40 = weak correlation.
0.41 - 0.60 = moderate correlation.
0.61 - 0.80 = strong correlation.
0.81 - 1.00 = very strong correlation.
GI = gingival index.
PI = plaque index.
BOP = bleeding on probing.
PPD = probing pocket depth.
CAL = clinical attachment level.
HbA1c = glycosylated hemoglobin.
PAI-1 = plasminogen activator inhibitor-1.
α22MG = alpha 2-macroglobulin.

Table 4. Stepwise multiple linear regression analysis for predicting salivary and serum PAI-1 and α2MG levels by clinical parameters in Group 2 and Group 3 at Baseline.

Group	Parameter	IV	b	SE	t	P-value	R^2
Group 2	Salivary PAI-1	Constant	327.43	142.33	2.301	0.03*	0.31
		PPD	92.82	25.91	3.582	0.001*	
	Serum PAI-1	Constant	22.54	2.66	8.483	<0.001*	0.20
		CAL	1.34	0.51	2.628	0.01*	
	Salivary α2MG	Constant	166.54	107.99	1.542	0.13	0.14
		CAL	43.16	20.64	2.091	0.04*	
	Serum α2MG	Constant	2.44	1.73	1.414	0.17	0.15
		CAL	0.70	0.31	2.240	0.03*	
Group 3	Salivary PAI-1	Constant	224.95	206.08	1.092	0.28	0.41
		CAL	158.51	35.61	4.451	<0.001*	
	Serum PAI-1	Constant	-35.23	18.83	-1.870	0.07	0.37
		CAL	13.19	3.26	4.054	<0.001*	
	Salivary α2MG	Constant	-2917.36	1310.19	-2.227	0.03*	0.29
		CAL	758.88	226.42	3.352	0.002*	
	Serum α2MG	Constant	-2.54	3.91	-0.649	0.52	0.33
		CAL	2.51	0.68	3.707	0.001*	

* Statistically significant.
PAI-1 = plasminogen activator inhibitor-1
α2MG = alpha 2-macroglobulin.
PPD = probing pocket depth.
CAL = clinical attachment level.

Table 5 presents the stepwise multiple linear regression for predicting salivary and serum PAI-1 and α2MG levels by clinical parameters in Groups 2 and 2 at three months period. In Group 2, for every 1-mm increase in CAL, there was an increase in salivary PAI-1 levels by 65.01 ng/ml ($p = 0.009$, variability 22%), serum PAI-1 levels by 2.50 ng/ml ($p = 0.001$, variability 34%), salivary α2MG levels by 56.72 ng/ml ($p = 0.004$, variability 27%) and an increase in serum α2MG levels by 0.63 ng/ml ($p = 0.01$, variability 21%), which were statistically significant. In Group 3, for every 1-mm increase in PPD, there was 121.00 ng/ml increase in salivary PAI-1 levels ($p = 0.002$, variability 29%), which was statistically significant. For every 1-mm increase in CAL, there was an increase in serum PAI-1 levels by 16.58 ng/ml ($p = 0.002$, variability 30%), salivary α2MG levels by 658.79 ng/ml ($p = 0.006$, variability 24%) and an increase in serum α2MG levels by 3.07 ng/ml ($p = 0.009$, variability 22%), which were statistically significant.

Table 5. Stepwise multiple linear regression analysis for predicting salivary and serum PAI-1 and α2MG levels by clinical parameters in Group 2 and Group 3 at three months period.

Group	Parameter	IV	b	SE	t	P-value	R^2
Group 2	Salivary PAI-1	Constant	325.59	111.01	2.933	0.007*	0.22
		CAL	65.01	23.17	2.806	0.009*	
	Serum PAI-1	Constant	8.38	3.13	2.682	0.01*	0.34
		CAL	2.50	0.65	3.835	0.001*	
	Salivary α2MG	Constant	1.91	85.32	0.022	0.98	0.27
		CAL	56.72	17.81	3.185	0.004*	
	Serum α2MG	Constant	1.14	1.14	1.003	0.33	0.21
		CAL	0.63	0.24	2.649	0.01*	
Group 3	Salivary PAI-1	Constant	285.47	167.71	1.702	0.10	0.29
		PPD	121.00	36.24	3.339	0.002*	
	Serum PAI-1	Constant	-54.21	23.99	-2.260	0.03*	0.30
		CAL	16.58	4.77	3.474	0.002*	
	Salivary α2MG	Constant	-1693.63	1020.11	-1.660	0.11	0.24
		CAL	658.79	220.41	2.989	0.006*	
	Serum α2MG	Constant	-5.70	5.45	-1.046	0.31	0.22
		CAL	3.07	1.08	2.830	0.009*	

* Statistically significant. PAI-1 = Plasminogen activator inhibitor-1. α2MG = alpha 2-macroglobulin. PPD = probing pocket depth. CAL = clinical attachment level.

Discussion

Many physiological functions, as well as pathological events including acute and chronic inflammatory reactions, depend on the plasminogen activator system. The pathogenesis and development of T2DM have been linked to procoagulant and fibrinolytic markers. The main tPA inhibitor is PAI-1, which plays a crucial role in fibrinolysis and downregulates it. Earlier studies have found associations between elevated PAI-1 levels and obesity, insulin resistance (IR), impaired glucose tolerance (IGT), and type 2 diabetes (T2DM) (Yarmolinsky *et al*., 2016; Schneider and Sobel, 2012; Ma *et al*., 2004). In people with type 2 diabetes, there has been evidence of increased PAI-1 concentration and activity (Pannacciulli *et al*., 2002). To explain the connection between PAI-1 and T2DM, various theories have been formulated. The activities of the plasminogen-activating (PA) system are maintained in balance by urokinase, tPA, and PAIs such PAI-1 and P2 (Schneider and Sobel, 2012; Wyganowska-Świątkowska *et al*., 2014). Plasmin plays a key role in the regulation of connective tissue breakdown, which is necessary for the progression of inflammatory lesions, by acting directly on the components of connective tissue, as well as indirectly by activating the proforms of metalloproteinases (Birkedal-Hansen H, 1995). PAI-1 influences cell migration, which is connected to the inflammatory response (McMahon *et al*., 2001; Czekay *et al*., 2003). Studies have suggested that PAI-1 may be crucial to the emergence and interaction of obesity and IR (Ma *et al*., 2004; Festa *et al*., 2002). In two prospective studies, elevated PAI-1 levels were found to be a predictor of the onset of the metabolic syndrome (Alessi *et al*., 2011; Ingelsson *et al*., 2007).

The onset and development of periodontal diseases may be influenced by the fibrinolytic system's destructive potential, suggesting that gingival inflammatory disease may be influenced by the plasminogen activation cascade. These PA system components are involved in the development and progression of periodontal disease, as well as the healing of periodontal wounds, cell migration, and tissue remodelling in the periodontal region (Yarmolinsky *et al*., 2016; Wyganowska-Świątkowska *et al*., 2014; Kinnby, 2002). The complex biological processes that control the proteolytic events that take place in the ECM of periodontal tissue are carefully controlled by interactions between cells and growth factors. These interactions set off a series of intracellular events that lead to the formation of new tissue (Ebersole *et al*., 2013). Enzymes like tPA and PAI-1 orchestrate the local inflammatory responses, as well as the synthesis of distinct ECM molecules (Ebersole *et al*., 2013; Wyganowska-Świątkowska *et al*., 2014; Ghosh and Vaughan, 2012). The tissue destruction observed in periodontal disease may be caused by the PA system. PAs and PAIs have been previously studied in GCF (Toyman *et al*., 2015; Yin *et al*., 2000; Kinnby *et al*., 1994). It was hypothesized that PAs and inhibitors might be produced locally in gingival tissue and GCF in patients with periodontitis, as elevated levels of tPA were found in gingival tissue and GCF samples (Yin *et al*., 2000; Kinnby *et al*., 1994; Kinnby *et al*., 1999). In periodontal tissue, PAI-1 is associated with an inflammatory response. Inflamed gingiva significantly increased the expression of the mRNA-binding protein of PAI-1 (Na *et al*., 2012).

In the present study, we assessed levels of the biomarkers PAI-1 and 2MG in saliva and serum in healthy individuals and patients with and without type 2 diabetes mellitus (T2DM) before and after periodontal therapy. Intergroup comparison did not show any statistically significant differences related to age and sex among the study groups.

At baseline, the PDM group had significantly greater baseline levels of all clinical measures (GI, PI, BOP, PPD, and CAL) and biomarkers (PAI-1 and 2MG) in serum and saliva than the periodontitis and healthy groups. PAI-1 functions as the principal inhibitor of tPA and urokinase plasminogen activator (uPA), the activators of plasminogen and hence fibrinolysis. Hence, it is present in minor quantities in healthy individuals, and a balance is maintained between the activators and inhibitors. The production is upregulated in inflammatory conditions and also obesity and IR. This could explain the increased levels in periodontitis and PDM groups. Given the bidirectional relationship between diabetes and periodontitis (Preshaw and Bissett, 2019; Simpson *et al*., 2015), these findings show that diabetes has a deleterious impact on the periodontium, as well as on the regulation of cytokines in inflammation. Hyperglycemia-induced systemic inflammation and the local inflammatory response in the periodontium in periodontitis upregulate inflammatory cytokines in periodontal tissues, exacerbating periodontal disease (Preshaw and Bissett, 2019; Simpson *et al*., 2015). Because most adipokines affect insulin sensitivity and inflammation, there is an increase in pro-inflammatory adipokines in T2DM patients. Research has shown that glucose increases the expression of the PAI-1 gene in adipose tissue, vascular smooth muscle cells, and endothelial cells (Sjoholm and Nystrom, 2005; Suzuki *et al*., 2002). Therefore, it is reasonable to assume that as blood glucose levels rise, PAI-1 gene expression will be raised, leading to higher levels of PAI-1 in the blood. According to the literature (Yin *et al*., 2000; Kinnby *et al*., 1994; Kinnby *et al*., 1999), the plasminogen activators and their inhibitors are generated locally in inflamed gingival tissue and GCF. This may help to explain why the salivary PAI-1 levels were higher in the present study. According to Na *et al*. (2014), the signaling pathways for extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase, and c-Jun N-terminal kinase (JNK) are required for PAI-1 activation. These pathways can be activated by many different kinds of cells in response to various extracellular inputs. In periodontal disease, the host's inflammatory responses may therefore trigger these signaling pathways and raise PAI-1 levels.

All clinical indicators significantly improved in the periodontitis and PDM groups from baseline to three months after the NSPT. This suggests that the NSPT had a positive effect on both groups, as reported in the study by Kardesler *et al*. (2011). This finding was also in accordance with a meta-analysis (Teshome and Yitayeh, 2016) that showed substantial improvement after NSPT among type 2 diabetic patients with periodontitis. Kiran *et al*. (2005) reported a significant decrease in CAL three months after NSPT, which is in agreement with the present study. The study also found that biomarkers decreased significantly from baseline to three months after NSPT, a finding that is similar to previous studies (Tüter *et al*., 2013; Chung *et al*., 2016) showing that NSPT was effective in reducing serum and salivary markers. Other studies (Bizzarro *et al*., 2007; Akman *et al*., 2012) have demonstrated the significance of serum PAI-1 as a marker in periodontal disease, with PAI-1 levels increasing with inflammation and disease progression.

Behle *et al*. (2009) conducted a study that estimated serum PAI-1 levels in patients with periodontitis receiving comprehensive periodontal treatment, and found significant reduction in six weeks, which was similar to the present study.

Taylor *et al*. (2010) also observed decrease in serum PAI-1 levels within three months of beginning periodontal treatment. Periodontal treatment reduces the intraoral bacterial bioburden, which consequently

diminishes periodontal inflammation and furthermore influences systemic inflammation. In the present study, the reduction of salivary PAI-1 reflected the decrease in tissue inflammation caused by periodontal treatment.

Another notable finding in the present study was the significant reduction in HbA1c levels from baseline to three months after treatment in Group 3. Following NSPT, there was a 0.3% drop in HbA1c levels in Group 3, which, as reported by other studies (Chen *et al*., 2012; Katagiri *et al*., 2009; Simpson *et al*., 2015; Baeza *et al*., 2020), implicates in better glycemic control. Thus, in accordance with previous literature, periodontal treatment brings down HbA1c levels in patients with T2DM. Consistent with this, a study by Yarmolinsky *et al*. (2016) stated that decrease in HbA1c levels by non-surgical periodontal treatment lowers diabetes associated microvascular problems by 35%, thus improving overall health.

Therefore, pro-inflammatory adipokines, such as PAI-1, may be important mediators of inflammation in periodontal disease and a risk factor for diabetes, according to the findings of the present study. However, studies with a more extended follow-up and varying severities of periodontal disease may provide a more significant knowledge of the plausible connection between PAI-1 and periodontal disease.

Conclusion

The findings of the present study allow for the following deductions. The biomarkers PAI-1 and 2MG were expressed in saliva and serum of healthy individuals and patients with and without type 2 diabetes mellitus (T2DM). Healthy individuals had lower levels of the biomarkers, while patients with periodontitis and T2DM had significantly higher levels of PAI-1 and 2MG in serum and saliva than individuals with periodontitis alone. There was statistically significant reduction in the levels of PAI-1 and 2MG in the periodontitis patients following treatment. The results of the current investigation indicate that salivary and serum levels of PAI-1 and 2MG are strongly correlated with periodontitis and periodontitis with T2DM. As a result, they may contribute to the pathophysiology of periodontitis and have potential application with regard to the same.

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