

Comparative Evaluation of Adipolin Expression in Gingival Crevicular Fluid and Serum of Healthy subjects and Periodontitis Patients with and without Type 2 Diabetes Mellitus

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Abstract: Background: Adipokine is a huge family of cytokines which are cell-signaling proteins secreted by adipose tissue released by White Adipose Tissue (WAT) and Adipolin (*FAM132A/CTRP12* gene) is the newest member added to the adipokine family. This research marks the first attempt to estimate and compare the Gingival crevicular fluid (GCF) and serum levels of Adipolin in healthy subjects and periodontitis patients, with and without type-2 diabetes mellitus (T2DM). **Methods:** The study population consisted of 10 patients each with healthy subjects and periodontitis with and without T2DM. GCF and serum samples were collected from each patient before non-surgical periodontal therapy. All the samples underwent enzyme linked immunosorbent assay (ELISA) test with an antibody specific to adipolin. **Results:** The mean GCF and serum adipolin levels were high in group I and III compared to group II and IV. Comparison of adipolin levels between the groups showed no statistically significant difference either in GCF ($P=0.68$) or serum ($P=0.85$). The comparison between GCF and serum adipolin levels in group IV showed statistically significant difference ($P<0.031$) The mean values showed a decrease in adipolin values as the disease rate progresses. Negative correlation was seen in serum and GCF adipolin with HbA1C. **Conclusion:** As the GCF and serum concentration of adipolin shows a gradual positive relation with the disease severity, within the limitations of the current study, it can be postulated adipolin could be possible an anti-inflammatory biomarker of periodontal disease.

Keywords: Adipolin; adipokines; periodontitis; type 2 diabetes mellitus

1. Introduction

Periodontitis results from the interaction of bacteria and the host response to bacterial challenge. Various environmental and acquired risk factors and genetic susceptibility can modify the disease. The clinical signs result from the host inflammatory response that develops to combat the biofilm.[1-3]

Microbial products trigger the release of cytokines, the dysregulation of which, in serum, saliva, and gingival crevicular fluid (GCF), lead to the disease. The role of cytokines as Interleukin-1, tumor Necrosis Factor-Alpha (TNF- α), Interferon γ (INF), adipocytokines and many others have been proven in the progression of periodontitis.[4-7] The increased level of cytokines seen in periodontitis can aggravate existing conditions, like atherosclerosis, diabetes, or rheumatoid arthritis.[8]

The two-way relationship between Periodontitis and Diabetes has long been established in year 1996 by Harald Loe and improvement in HbA_{1c} levels in Type 2 Diabetes mellitus (T2DM) patients after periodontal therapy has been observed.[9] Recent studies indicate that chronic low-grade inflammation is associated not only with the pathogenesis of T2DM but also with periodontal diseases where adipocytokines play an essential role in host's response to periodontal biofilm.[10] Thus, Adipolin is considered a candidate in linking inflammation to other metabolic disorders such as T2DM.[11]

Adipokine or adipocytokine is a huge family of cytokines which are cell-signaling proteins secreted by adipose tissue released by white adipose tissue. They are the modulator proteins derived from adipose tissue which regulate various metabolic functions as immune cell migration, adipocyte metabolism and inflammation etc. It includes leptin, adiponectin, omentin, chemerin, interleukin-6, visfatin and Adipolin. These adipokines affect insulin resistance, and may also have an impact on inflammation and immune responses.[12]

Adipolin (*FAM132A/CTRP12* gene) is the newest member added to the adipokine family in year 2011 by Takashi Enomoto which is involved in glycemic control and insulin sensitization. He found that like adiponectin, the circulating levels of adipolin decreased substantially in obese mice and administering adipolin resulted in an improvement in insulin sensitivity and glucose tolerance, and decreased adiposity and inflammation in obese and diabetic animal models.[13] It functions by acting as an anti-inflammatory by decreasing pro-inflammatory gene expression like TNF- α , IL- β which are potent mediators of periodontitis, and acts by decreasing the macrophage cell count [14].

An in-vitro study showed that adipolin acts as an insulin sensitizer role in diabetic patients by suppressing gluconeogenesis and promoting glucose uptake in hepatocytes and adipocytes.[15] Many theories explain the increased severity of periodontal disease in individuals with T2DM. Monitoring the persisting low-grade inflammation through the levels of cytokine and adipokine activity, may have a possible application for assessing the diabetic condition and development of periodontitis.[16] This research marks the first attempt to estimate and compare the GCF and serum levels of adipolin in healthy subjects and periodontitis patients, with and without T2DM.

2. MATERIALS AND METHODS:

2.1. Participant Selection

Forty individuals (20 females and 20 males) aged 20 to 50 years were selected and recruited from the outpatient section, Department of Periodontics in an institution. The patients were divided in 4 groups viz. 10 healthy subjects (Group I), 10 healthy subjects with T2DM (Group II), 10 periodontitis patients without T2DM (Group III) and 10 Periodontitis patients with T2DM (Group IV).

Individuals with aggressive periodontitis, type1diabetes mellitus, pregnancy, human immunodeficiency virus (HIV) infection, smoking and alcoholism, hematological and immune system disorders, bone disorders, Patients on contraceptives or antibiotic therapy in the last 6 months, and those receiving any periodontal treatment were excluded.

The periodontitis patients who were showing radiographic evidence of bone loss and clinical attachment loss (CAL) \geq 5mm (severe), with a minimum of 6 teeth with a pocket probing depth (PD) of \geq 5 mm, bleeding on probing (BOP) in at least 2 separate quadrants, and gingival index (GI) $>$ 1, plaque index (PI) \geq 1 and Individuals with a minimum of 14 teeth, and age of $>$ 20 years were included. Healthy subjects were approved if the full-mouth probing pocket depth (PPD) was \leq 3 mm, clinical attachment loss (CAL)=0, GI \leq 1 and PI \leq 1 (absence of clinical inflammation). Radiographic bone loss was recorded dichotomously to differentiate between periodontitis patients and healthy subjects.

The diabetic patients diagnosed with T2DM were screened and their glycemic condition was evaluated by the glycated hemoglobin A1c (HbA_{1c}) levels. Patients with HbA_{1c} levels between 7% -8% (moderately controlled) were recruited.[17] It was

determined that none of the participants changed their prescription over the past 3 months preceding the study.

Ethical clearance for the study was obtained from the institutional ethical review board. The study procedure was explained to the patients and an informed consent was obtained from those who agreed to participate voluntarily in the study. Two examiners were trained to check for clinical parameters. Calibration was done and reliability was checked with a kappa value of 0.8 agreement.

2.2. Selection of Site and Sample Collection:

On the first day, clinical and radiological examinations, group allocation, and sampling-site selections were done, and samples were collected on the following day. This was done to prevent the contamination of GCF with blood from probing of the inflamed sites. In Group I and II, multiple sites with absence of inflammation were sampled by pooling GCF to ensure adequate amount for the study. In Group III and IV, sites with the highest clinical signs of inflammation, attachment loss and >5mm probing depth were selected. The area was dried with a blast of air, and then the supragingival plaque was removed without touching the marginal gingiva. The GCF samples were collected in 30 seconds using the intracrevicular method. The GCF obtained was immediately transferred into plastic vials and stored at -70°C until the time of assay.

For serum collection, the skin over the antecubital fossa was disinfected and two ml of blood was collected by venipuncture using 20-gauge needle with 2ml syringe and immediately transferred to the laboratory. Samples were allowed to clot at room temperature and after one hour, the samples were centrifuged at 3000 rpm for 15 minutes to separate serum component. Serum was extracted from the blood and stored at -70°C till the assay procedure.

2.3. Adipolin Assay

The supernatants were then collected, and Adipolin concentration was evaluated by sandwich enzyme linked immunosorbent assay (ELISA) employing standard commercial equipment (Bioassay Technology, Shanghai Biotech Ltd). The recommended detection parameters for the Adipolin by manufacturers were 0.05 - 20ng/ml. The measurement of absorbance of each well was obtained under 450nm wavelength, which was carried out within the 10 minutes after adding stop solution. The concentration of adipolin in the samples were then determined by comparing the Optical density (OD) values for each well was calculated with standard curve by using My Assay software.

3. Statistical Analysis

The data was entered and analyzed using the Statistical Package for Social Sciences (SPSS) for Windows, Version 28.0. (Armonk, NY: IBM Corp) Confidence intervals were set at 95%, and a p-value \leq of 0.05 was considered statistically significant. One way Analysis of Variance (ANOVA), Pearson's correlation coefficient test, Chi-square tests were carried out to compare adipolin levels among groups. Spearman's correlation coefficient test was used to correlate adipolin levels to clinical parameters

4. Results

There were no statistical differences in age, gender distribution between the groups. The full mouth parameters and sample sites PI, GI, PPD and CAL showed notable increase as the disease severity increases viz. from Group I to Group IV. (Table 1)

The comparison of mean PI, GI, PPD and CAL between group I & III, group I & IV, group II & III and group II & IV respectively showed statistically significant difference $P < 0.0001$. There was no statistically significant difference ($P > 0.05$) in group I & II and group III & IV. (Table 2)

The mean GCF Adipolin values in group I, II, III and IV were 10.71 ± 6.76 ng/ml, 8.27 ± 5.09 ng/ml, 9.02 ± 3.38 ng/ml and 8.75 ± 2.7 ng/ml. The mean GCF adipolin levels were high in group I and III compared to group II and IV. Comparison of adipolin levels between the groups showed no statistically significant difference ($P=0.68$).

The mean serum Adipolin values in group I, II, III and IV were 7.68 ± 6.5 ng/ml, 5.89 ± 5.7 ng/ml, 6.37 ± 5.25 ng/ml and 5.93 ± 2.76 ng/ml. The mean serum adipolin levels were high in group I and III compared to group II and IV. Comparison of adipolin levels between the groups showed no statistically significant difference ($P=0.85$). The comparison between GCF and serum adipolin levels in group I, II and III showed no statistically significant difference ($P=0.32$, $P=0.33$ and $P=0.97$) respectively. Whereas in group IV showed statistically significant difference ($P<0.031$) (Table 3)

The Spearman's correlation test was used to observe correlation in the adipolin levels of GCF and serum and clinical parameter i.e., PPD and CAL in group III and IV. In group III, correlation between adipolin (GCF) versus PPD and CAL showed no statistically significant difference with $P=0.86$ and $P=0.46$. Correlation between adipolin (serum) versus PPD and CAL also showed no statistically significant difference with $P=0.93$ and $P=0.72$. In group IV, correlation between adipolin (GCF) versus PPD and CAL showed no statistically significant with $P=0.52$ and $P=0.35$ and correlation between adipolin (serum) versus PPD and CAL also showed no statistical significant difference with $P=0.46$ and $P=0.32$. (Table 4 and 5). The HbA1c results showed negative correlation with serum and GCF adipolin levels in all the groups. However, there is statistically significant difference ($p<0.05$) seen in group IV.

Table 1. Demographic data of the study population

	Gender		Mean Age (in years)
	Male	Female	
Group I	5	5	32.3
Group II	5	5	40.3
Group III	5	5	41.2
Group IV	6	4	37.1

Table 2. Clinical parameters in the study groups

	PI	GI	PPD (in mm)	CAL (in mm)
Group I	0.66 ± 0.26	0.61 ± 0.24	1.6 ± 0.96	0
Group II	0.57 ± 0.26	0.50 ± 0.27	1.2 ± 0.69	0
Group III	1.90 ± 0.53	1.74 ± 0.52	5.3 ± 0.74	5.85 ± 0.84
Group IV	1.91 ± 0.42	1.94 ± 0.55	5.5 ± 0.35	6.04 ± 0.43

**indicates statistically significant difference ($p<0.05$) between group I and III, I and IV, II and III and II and IV ($P < 0.001$); † No statistical significant difference between group I and II, group III and IV ($P>1, P>1$). PI: Plaque index; GI: Gingival index; PPD: Probing pocket depth; CAL: Clinical attachment loss

Table 3. Comparison of Serum and GCF Adipolin levels in all the groups

Group	Serum Adipolin (ng/ml)	GCF Adipolin (ng/ml)	P-value
Group I	7.68	10.71	0.32
Group II	5.89	8.27	0.33
Group III	6.37	9.02	0.97
Group IV	5.93	8.75	0.031*

*indicates statistically significant difference ($p<0.05$)

Table 4. Correlation between change in levels of adipolin (GCF) and adipolin (serum) with change in clinical parameters of periodontitis severity (PPD, CAL) in group III.

Adipolin	P value for PPD	rho coefficient	P value for CAL	rho coefficient
GCF	0.86	0.06	0.46	0.26
Serum	0.93	0.03	0.72	-0.13

Table 5. Correlation between change in levels of adipolin (GCF) and adipolin (Serum) with change in clinical parameters of periodontitis severity (PPD, CAL) in group IV.

Adipolin	P value for PPD	rho coefficient	P value for CAL	rho coefficient
GCF	0.52	-0.23	0.35	-0.33
Serum	0.46	-0.26	0.32	-0.35

PPD: Probing pocket depth; CAL: Clinical attachment level.

Table 6. Correlation between change in levels of adipolin (GCF) and adipolin (Serum) with change in HbA1c levels

Groups		Mean	SD	rho coefficient	P value
Group I	GCF Adipolin	10.71	6.76	-0.39	0.26
	Serum Adipolin	7.68	6.50	-0.11	0.76
	HbA1c	4.56	.38		
Group II	GCF Adipolin	8.27	5.09	-0.57	0.08
	Serum Adipolin	5.89	5.70	-0.32	0.35
	HbA1c	6.18	.26		
Group III	GCF Adipolin	9.02	3.38	-0.12	0.72
	Serum Adipolin	6.37	5.25	-0.01	0.96
	HbA1c	5.03	.26		
Group IV	GCF Adipolin	8.75	2.70	-0.79	0.006*
	Serum Adipolin	5.90	2.76	-0.28	0.42
	HbA1c	7.66	.40		

*indicates statistically significant difference (p<0.05)

5. Discussion

Adipolin (FAM132A/CTRP12/Clq/TNF-related protein gene) is a novel adipokine, influenced by a decrease in Kruppel-like factor-15(KLF-15) and in T2DM patients the insulin resistance disrupts the insulin–adipolin homeostatic response, which could lead to the lower adipolin levels.[18,19] Predominantly macrophages and almost all major type of immune cells are involved in the linkage of endocrine function of adipose tissue in systemic metabolic regulation.[20]

The metabolic disarrangement found in diabetic condition is related to alterations in adipocyte metabolism. Loss of periodontal attachment and alveolar bone started early in the diabetic population. Along with being a passive reservoir of triglycerides, the adipose tissue, produces high levels of adipokines that can result in the initiation and progression of disease through dysregulated immune responses. These adipocytokines play an

important role in periodontal disease activity by stimulating monocytes which increases the production of inflammatory cytokines by altering the host immune response that results in a higher susceptibility to bacterial infections.[14] Thus, the present study was undertaken to study the GCF and serum levels of adipolin in healthy subjects and periodontitis patients, with and without T2DM.

As the inclusion of an equal number of males and females in each group and the selection of individuals within the age range of 20-50 years, the gender and age of the subjects had no impact on the Adipolin concentration in the present study. It's possible that the varying concentrations seen within each group are the results of the disease being in a different stage when the GCF and serum samples were taken, which would explain the wide range of values.

Our study found that the subjects included in Group II and IV had moderate levels of HbA_{1c} ranging from 7-8%. This criterion was used to avoid the bias which could have occurred in case of extreme values. This range of HbA_{1c} is in accordance with the Diabetes diagnosis criteria as sorted by American Diabetes Association.[17] Values measuring HbA_{1c}< 7% are good control subjects and the detection of biomarker may not be feasible. Studies have shown a link between high HbA_{1c} and periodontitis, where it is stated that HbA_{1c} was significantly associated with pocket depth, clinical attachment level and plaque index and insulin resistance.[21-23] To avoid shift in biomarker values resulting from fluctuation in HbA_{1c} all patients with moderate HbA_{1c} levels were included. The prevalence of advanced periodontal disease was significantly higher among T2DM persons as compared to non-diabetic persons of the Pima Indian community and the rate of periodontal disease progression in T2DM is 3 times that in non-diabetic persons.[24]

The PI and GI scores show a gradual rise from Group I to Group IV. The evaluation of PPD and CAL values are important indicators of periodontal diseases. The inter-group comparison of mean PPD and CAL values showed statistically significant difference indicating higher periodontal destruction in Group III and IV than Group I and II. This suggested that Group III and IV had "severe" periodontitis according to American Academy of Periodontology's classification. [25]

Adipolin, though, is predominantly seen in adipose tissue, their mRNA profiles are also expressed in various tissues by quantitative real time PCR analysis. Minor quantities are also found in liver, kidney, leukocyte and cardiac tissues. The source of cytokine has an impact on the regulation of marker and interaction with the glycemic index.[15]

In current study, the presence of Adipolin in serum of diseased and healthy conditions was seen. The mean serum Adipolin levels ranged from 5.89ng/ml to 7.68ng/ml; concluding that the severity of periodontitis in T2DM patients is associated with the decreased levels of adipolin.

This marker showed an anti-inflammatory curve in serum. Similar observation was done by Enomoto (2011) [13] where adipolin administration in diet-induced obese mice ameliorated insulin resistance and increased insulin sensitivity. Its supplementation further reduced accumulation of macrophages and pro-inflammatory gene expression in the adipose tissue. It had an inhibitory impact on the production of cytokines that contribute to inflammation in response to lipopolysaccharides and TNF-. This points to the fact that insulin-resistant states are characterised by decreased amounts of the adipokine that suppresses inflammation.[26]

Our study is the pioneer to discover the presence of adipolin in GCF. The mean GCF adipolin values in Group I, II, III and IV were 10.715± 6.76 ng/ml, 8.27± 5.09 ng/ml, 9.02± 3.38 ng/ml and 8.75± 2.7 ng/ml respectively. The mean values showed a steady downward inclination representing the decrease in adipolin values as disease rate progresses. As a result, the anti-inflammatory property of Adipolin is proven. No statistically significant difference was observed in the adipolin levels in 4 groups. This speculation might be explained by the study undertaken by Mehrdadi (2016) [27] which hypothesised that variations in adipolin levels, as well as the consequences that are associated with them, are dependent on the metabolic and genetic make-up of the participants.

This study recorded a peculiar characteristic of adipolin levels between the diabetic and non-diabetic groups. Adipolin levels were found decreased in Group II and Group IV as compared to Group I and Group III; the Serum Adipolin and GCF Adipolin levels were lower in patients with T2DM than patients without T2DM. This can suggest the systemic effect of diabetes on the periodontal status and thus affects the adipolin levels and diabetic condition aggravates the periodontal disease.

Wei et al (2012) [15] showed that after administration of anti-diabetic drug rosiglitazone on mice models, Adipolin exerted its insulin sensitizing action by suppression of gluconeogenesis and thereby increasing glucose uptake in hepatocytes. A similar action was reported by Tan et al (2013) [28] documenting that concentration of serum adipolin in polycystic ovary syndrome (PCOS) was lower compared to healthy group. This action might be due to metabolic disorders that accompany PCOS like obesity, insulin resistance and inflammation. Additionally, adipolin levels were increased in PCOS patients after administration of Metformin.[29]

Another interesting fact observed was in comparison of Adipolin concentration in GCF and serum in Group IV showed statistically significant difference. Group IV included periodontitis with T2DM and the status of one condition is influenced by another i.e., presence of periodontitis enhanced T2DM condition or vice versa. This suggested that the combined dual effect of Periodontitis as well as T2DM on the level of Adipolin is significant and thus shows the resultant difference in comparison. A similar study conducted by Zimmerman G. (2013) [30] checked the levels of adiponectin obese and non-obese subjects with and without periodontitis in serum and GCF and concluded that adiponectin levels were lower in periodontitis than in non-periodontitis groups.

Levels of adipolin observed in GCF were higher than the serum levels. This elevated levels of the adipokine in GCF might be contributed by the systemic "spill" of cytokine via the circulation. Offenbacher (1996) was the first person to propose this theory. He stated that cytokines produced within the tissues work locally to trigger specific cellular targets and are destroyed within a localised zone. However, if the stressor continues, the cellular cytokine receptors may become overloaded, which will result in decreased cytokine clearance and a "spill" of systemic cytokines through the circulation.[31,32]

The study also tried to show correlation between PPD, CAL and levels of Adipolin. This association shows a constant relation. The mean values of PPD and CAL increase with rising disease intensity. Congruently, the mean levels of Adipolin in Serum and GCF show a rise. Though there was no statistically significant correlation found, it gave similar results to the study conducted by P Bharti (2013) showed that with decrease in PPD, the levels of adiponectin increased. The negative correlation observed in GCF adipolin levels with HbA1c in periodontitis and T2DM patients could be due to increase local and systemic inflammatory burden.[33]

This study is first of its kind to have detected Adipolin in the periodontium. The study showed that decreased concentrations of adipolin is detected in serum and GCF of T2DM patients with and without periodontitis than healthy subjects and periodontitis patients.

Alongside, the change in trend of expression of marker is also studied and a steady correlation is obtained i.e., with increasing disease severity, a decrease in biomarker level was observed. It showed that adipolin levels change according to the level of inflammatory burden present in different stages of disease. Thus, suggesting adipolin as potential marker of inflammation in periodontitis and T2DM.

Further longitudinal studies are recommended to be carried out with larger subject population. Later, evaluation of the levels of adipolin in GCF and serum before and after periodontal therapy should be carried out to confirm the role of adipolin as an anti-inflammatory marker in periodontal disease.

6. Conclusion

Adipolin as an anti-inflammatory biomarker could be a possible connecting link between periodontitis and other inflammatory conditions like obesity, atherosclerosis and hypertension. Adipolin levels in periodontitis as a predictive parameter in progression of systemic conditions like obesity, T2DM and metabolic syndrome. Futuristic chair side diagnostic kits can be devised to assess the adipolin levels as possible biomarkers for periodontitis and obesity related conditions

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