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Gamma Glutamyl Transferase- A Link between Oxidative Stress and Periodontitis in Smokeless Tobacco Users and Non Users with Chronic Periodontitis

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Authors' contributions

This work was carried out in collaboration between all authors. Author SIP designed the study, wrote the protocol, managed the literature searches and wrote the first draft of the manuscript. Authors VJ, PS and PD monitored the entire procedure of the study. Author NG managed the analyses of the study. Author NS helped to performing the procedure. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Long-term usage of tobacco related products are the potential generators of free radicals which alter the cellular antioxidant defense system. Changing the balance towards an increase in the pro-oxidants over the capacity of the antioxidants is defined as oxidative stress (OS), which leads to oxidative damage. Gamma-glutamyl transpeptidase/transferase (GGT), a liver function marker plays a key role in regulating cellular levels of the antioxidant molecule glutathione, hence is a critical enzyme in maintaining cellular redox homeostasis.
Aim: To investigate and compare the level of GGT in smokeless tobacco users (STU) and non tobacco users (NTU) with chronic periodontitis (CP).
Materials and Methods: The study comprises of 50 subjects with the age group 20-64 years categorized as smokeless STU with CP (n=25) and non tobacco users NTU with CP (n=25). 2 ml of

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blood from antecubital vein and 2 ml of unstimulated saliva was obtained and evaluated for GGT. Clinical parameters such as Plaque index (PI), Gingival Index (GI), Pocket probing depth (PD), Clinical attachment levels (CAL) were recorded. The statistical analysis was carried out using Chi-square test / Fisher's exact probability test for the inter-group comparison of categorical variable. The statistical significance of inter-group difference in the continuous variables is tested using independent sample t test after confirming the underlying normality assumption.

Results: Within the reference range, level of GGT was significantly higher in STU with CP than NTU with CP.

Conclusion: Estimation of GGT levels being an easy, reliable and inexpensive test, can become an emerging tool for the diagnostic assessment of periodontal disease for early detection of OS suggesting its high scope to be recognized as a biomarker for OS damage and thereby establishing both oral and systemic health.

Keywords: Gamma-glutamyl transferase; smokeless tobacco users; non tobacco users; saliva; serum; oxidative stress.

1. INTRODUCTION

Periodontitis is a chronic inflammatory disease that affects the supporting tissues of the teeth, the gingiva, the periodontal ligament, the cementum and the alveolar bone [1]. This disease occurs as a result of excessive accumulation of dental plaque around the gingival margins. Virulent micro-organisms that colonize the plaque biofilm produce toxins that arouse the host immune response characterised by influx of immune and inflammatory cells into the periodontal tissues resulting in production of cytokines, inflammatory mediators, free radicals, and reactive oxygen species (ROS), by these cells, that mediate tissue destruction characterized clinically as pocket formation, pus discharge, tooth mobility, and eventually tooth loss [2].

Periodontitis is a multifactorial disease associated with several risk factors, among which tobacco has been found to be associated with severe periodontal destruction [3].

The term smokeless tobacco (SLT) is used to describe tobacco that is consumed without burning or heating at the time of consumption [4]. Long-term usage of tobacco related products are the potential generators of free radicals that alter the cellular antioxidant defence system [5].

ROS collectively describes oxygen free radicals and other non-radical oxygen derivatives involved in the oxygen radical production. These include superoxide, hydroxyl (OH), hydroperoxyl, nitric oxide (NO), alkoxy, singlet oxygen, ozone, hypochlorous acid and hydrogen peroxide [6]. Under normal physiologic conditions the reactive oxygen species and antioxidants are in an equilibrium. Whenever there is a shift in this delicate equilibrium either by increased ROS release or by its increased activity or by a reduced antioxidant defense mechanism, oxidative stress (OS) results. Oxidative stress was defined by *Sies* [7] as a disturbance in the pro-oxidant–antioxidant balance in favour of the former, leading to potential damage. Therefore if the homeostasis is interrupted in favour of ROS, an oxidative stress situation is created.

To mitigate the effect of oxidative stress the body has evolved potent antioxidant mechanisms. In this regard, the role of Gamma-glutamyl transferase (GGT) is important. GGT was first described in 1950 by Hanes et al. [8] as an enzyme of cellular membranes as it is present in the cell membranes of many tissues including the kidneys, bile duct, pancreas, gall bladder, spleen, heart, brain and seminal vesicles and serves the potential role as a marker in the inflammatory condition of these tissues [9]. GGT was also found on the outer cell envelope of Treponema denticola, the periodontal pathogen, thus predicting a probable role of GGT in propagation of the organism within inflamed periodontal tissues [10]. GGT is present normally in blood in low levels of 5-50 U/L of serum.

Cellular GGT also plays an important role in antioxidant defense systems. GGT induction increases intracellular glutathione, which is protective against OS and thereby GGT can be considered as an early and sensitive enzyme related to OS [11].

Saliva has become an emerging tool for the diagnostic assessment of various oral and systemic diseases, particularly periodontal disease as it can easily be collected in large volume by non-invasive methods and does not need skilled technicians and special equipments for its collection.

There are few studies on the analysis of the salivary chemical constituents in general, and the evaluation of GGT by saliva analysis in particular, from India. Hence the aim of the present study was to investigate and compare the level of GGT in smokeless tobacco chewers (STU) and non- tobacco users (NTU) with chronic periodontitis (CP).

2. MATERIALS AND METHODS

In order to improve the power of study and to reduce bias 50 subjects aged 19-64 years were selected from the outpatient department of Department of Periodontics, Yogita Dental College and Hospital, Khed, India. The study subjects were divided into two groups Group I comprised of smokeless tobacco users (STU) with chronic periodontitis (CP) (n=25), Group II comprised of non tobacco users (NTU) with chronic periodontitis (CP) (n=25). The protocol for this cross-sectional study was approved by the Ethical Committee of the Institution. Subjects that fulfilled the inclusion criteria were included in the study.

Inclusion Criteria were:

1) Individuals having chronic periodontitis with pocket probing depth $\geq$ 4 mm and clinical attachment level of 3 mm or more at more than 30% of all sites in the mouth [12].
2) Subjects having not less than 20 permanent teeth
3) In case of smokeless tobacco users, who used only tobacco in the form of chewing with chewing habit $\geq$1 year and frequency of consumption of atleast once a day.

Exclusion Criteria were:

1) Current and former smokers, alcohol consumers.
2) Former tobacco users or current users of tobacco product other than smokeless tobacco chewer such as snuff users.
3) Individuals on any medications such as cholesterol lowering drugs, antibiotics cardiovascular, anti depressant drugs within the past 3 months.
4) Individuals with underlying systemic disease.
5) Individuals who had undergone periodontal treatment within the past 6 months.
6) Pregnant and lactating mothers.

A written informed consent was obtained from all the participants who were willing to participate in the study. During clinical examination information such as duration of use and frequency of consumption of smokeless tobacco was obtained from every patient and clinical parameters such as Plaque index (PI) by Silness and Loe, [13] Gingival Index (GI) by Loe and Silness, [13] Pocket probing depth (PD), Clinical attachment level (CAL) were recorded on six sites per tooth (mesiobuccal, distobuccal, midbuccal, mesiolingual, distolingual and midlingual) by the same examiner using a University of North Carolina 15 probe (UNC 15- Hu-Friedy, Chicago, IL, USA), calibrated in millimetres.

2.1 Collection of Samples

An unstimulated saliva of 2 ml was collected according to the method given by Navazesh [14]. The sample was collected between 9 am–12 noon. The participants were asked to rinse their mouths thoroughly with water to remove any food debris and then were asked not to chew, talk or perform any activity with their oral activity and saliva was allowed to accumulate in the floor of the mouth for approximately two minutes, then after the two minutes, they were asked to spit the saliva into sterile plastic containers and were instructed to avoiding forcible spitting. The salivary samples obtained were transported to the laboratory where they were analysed by semi automated biochemistry analyser (Erba Manheim CHEM-5 Plus V_2) for GGT levels in saliva.

From every subject, under aseptic precautions, 2 ml of venous blood was withdrawn in sterile test tubes coated with clot accelerator. The test tubes were centrifuged at 2500 rpm for 15 minutes. The serum that was formed was transferred into plain test tube using a dropper and was transported to the laboratory where they were analysed by semi automated biochemistry analyser (Erba Manheim CHEM-5 Plus V_2) for GGT levels in the serum.

2.2 Principle

GGT levels were estimated by Carboxy Substrate Method wherein the reagent for bilirubin assay consists of L-γ-glutamyl-3-carboxy-4 nitroanilide and glycylglycine. 0.1ml of sample and 1 ml of reagent were pipetted and mixed together in a cuvette in a swirling motion and incubated for 1 min at room temperature (15℃ to 30℃). After 1 minute the cuvette was fed into the semi automated biochemistry

analyser. GGT in the sample catalyses the transfer of amino group between L-γ-glutamyl-3-carboxy-4 nitroanilide and glycylglycine to form L-γ-glutamyl glycylglycine and 5-amino-2-nitrobenzoate. The rate of formation of 5-amino-2-nitrobenzoate was measured as an increase in absorbance in accordance with the principles of spectrophotometry and the intensity of light at 405 nm was converted into a digital readout correlating with the levels of GGT in the sample.

2.3 Statistical Analysis

The sample size calculation was based on the effect sizes through previously published studies. The minimum sample size 25 in each study group (total 50) provides 80% power of study (type II error 20% - beta error) with type I error 5% - alpha error to detect the clinically significant difference in the average GGT (serum as well as salivary) between two study groups and the statistical values less than 0.05 were considered to be statistically significant.

The data was analysed using Statistical Package for Social Sciences (SPSS version 16.0, Incorporated Chicago, USA) for MS Windows. The data on categorical variables is shown as n (% of cases) and data on continuous variable is shown as mean and standard deviation (SD) across two study groups. The inter-group comparison of categorical variables is performed using Chi-square test / Fisher's exact probability test. The statistical significance of inter-group difference in the continuous variables is tested using independent sample t test after confirming the underlying normality assumption.

3. RESULTS

The mean ± standard deviation of age of the subjects from Group I and Group II were 44.36 ± 9.50 years and 42.64 ± 10.66 years respectively and in both the study groups, the majority of subjects were in the age group 25.0 to 44.0 years (Group I – 14 subjects and Group II– 14 subjects). The age distribution did not differ significantly between two study groups (P-value>0.05) [Table 1, Fig. 1].

The distribution of mean Serum GGT is significantly higher in Group I compared to the Group II (P-value<0.001). The distribution of mean Saliva GGT is significantly higher among the STU with CP compared to the NTU with CP (P-value<0.001) [Table 2, Fig. 2].

Pearson's correlation coefficient test was used to observe the correlation between periodontal parameters i.e. PI, GI, PD, CAL and GGT in serum and saliva [Table 3].

Serum GGT did not show significant correlation with all periodontal parameters (PI, GI, PD and CAL) in both the study groups (P-value>0.05 for all) [Fig. 3].

Salivary GGT showed negative and significant correlation with CAL in STU with CP (P-value<0.01) and it showed positive and significant correlation with PD in NTU with CP (P-value<0.05). Salivary GGT did not show significant correlation with PI, GI, PD in STU with CP and PI, GI, CAL in NTU with CP (P-value>0.05 for all) [Fig. 3].

Table 1. The distribution of age

Age group (years)	Group I (n=25)		Group II (n=25)		P-value (Group I vs Group II)
	N	%	N	%	
25.0 – 34.0	3	12.0	7	28.0	0.445^{NS}
35.0 – 44.0	11	44.0	7	28.0	
45.0 – 54.0	5	20.0	6	24.0	
55.0 – 64.0	6	24.0	5	20.0	
Total	**25**	**100.0**	**25**	**100.0**	

*Values are n (% of cases). P-value by Chi-Square test. P-value <0.05 is considered to be statistically significant. *P-value<0.05, **P-value<0.01, ***P-value<0.001, NS: Statistically Non-Significant*

Table 2. The distribution of GGT levels in serum and saliva

GGT levels	Group I (n=25)		Group II (n=25)		P-value (Group I vs Group II)
	Mean	SD	Mean	SD	
Serum GGT (U/L)	75.38	10.18	43.35	6.61	0.001^{***}
Saliva GGT (U/L)	9.00	0.84	6.69	0.69	0.001^{***}

*Values are Mean and SD. P-value by independent sample t test. P-value<0.05 is considered to be statistically significant. *P-value<0.05, **P-value<0.01, ***P-value<0.001, NS: Statistically Non-Significant*

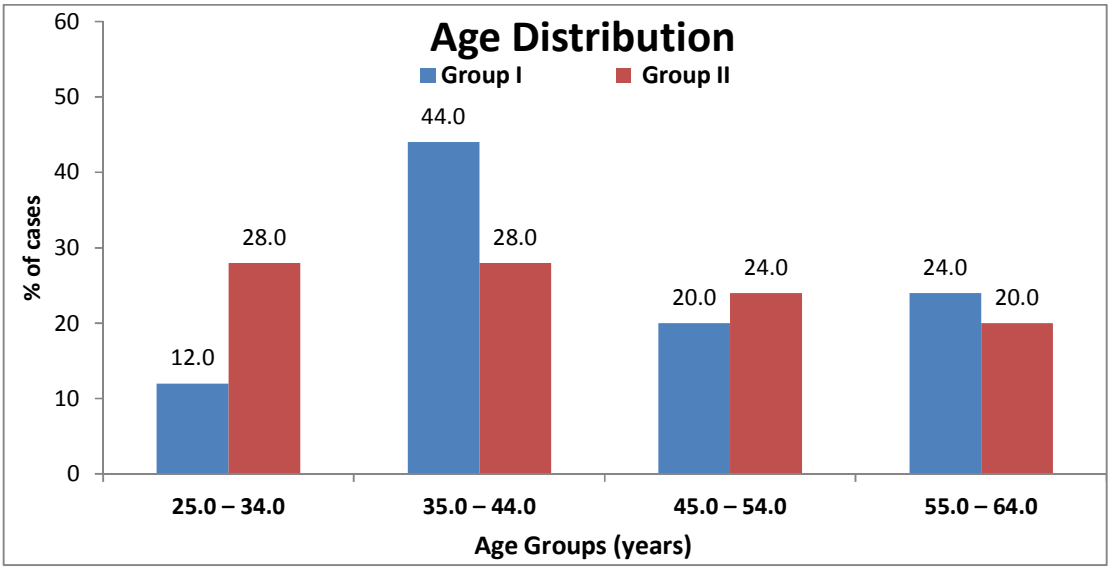

Fig. 1. The distribution of age among the two groups

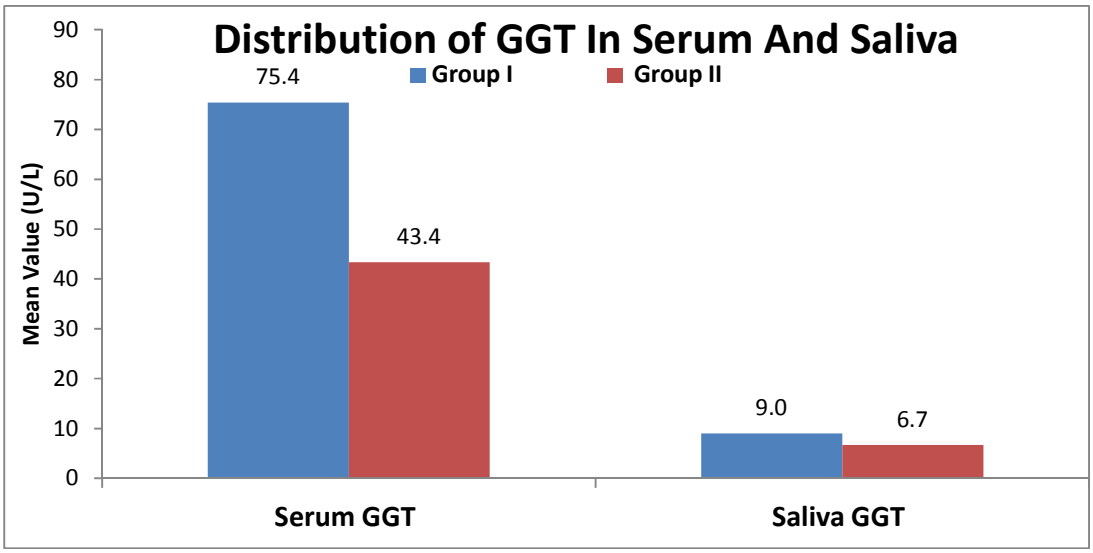

Fig. 2. The distribution of GGT levels in serum and saliva among the two groups

Table 3. The correlation of GGT with periodontal parameters

Sample type	Correlation between	Group I (n=25)		Group II (n=25)	
		r-value	P-value	r-value	P-value
Serum	GGT with PI	-0.003	0.989^{NS}	0.075	0.720^{NS}
	GGT with GI	0.163	0.435^{NS}	-0.008	0.970^{NS}
	GGT with PD	0.057	0.787^{NS}	-0.024	0.911^{NS}
	GGT with CAL	0.301	0.143^{NS}	-0.055	0.793^{NS}
Saliva	GGT with PI	0.114	0.586^{NS}	-0.199	0.340^{NS}
	GGT with GI	-0.338	0.098^{NS}	-0.210	0.314^{NS}
	GGT with PD	-0.222	0.287^{NS}	0.458	0.021^{*}
	GGT with CAL	-0.506	0.010^{**}	0.069	0.743^{NS}

*Values are Pearson's r-values. P-value<0.05 is considered to be statistically significant. *P-value<0.05, **P-value<0.01, ***P-value<0.001. NS: Statistically Non-Significant*

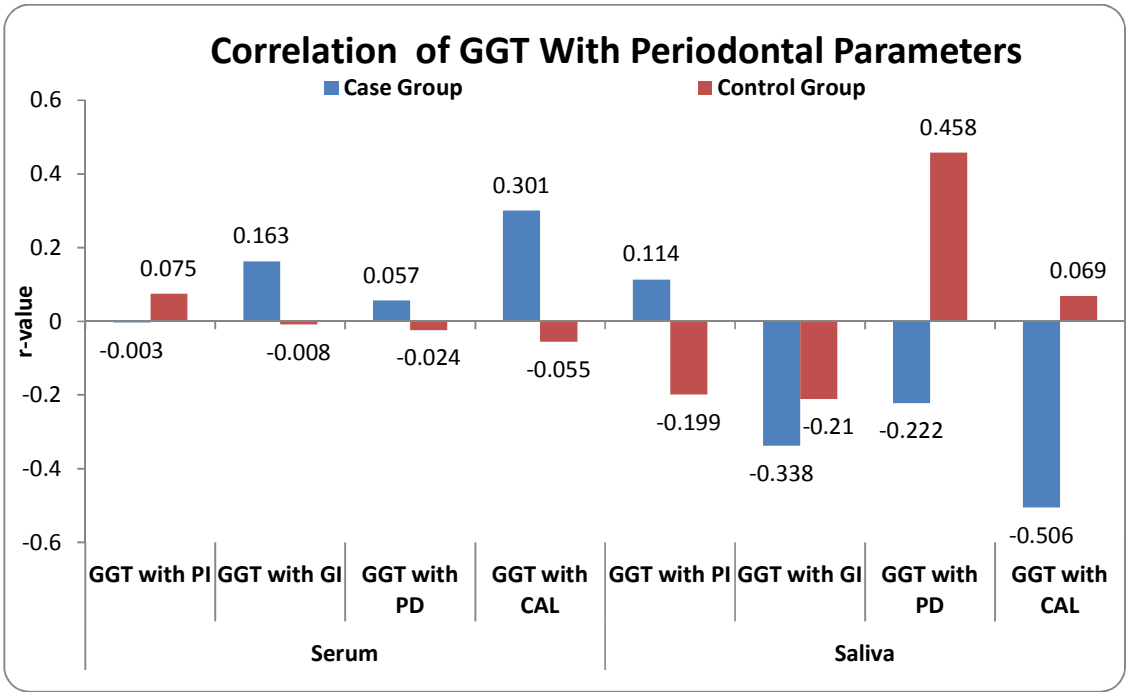

Fig. 3. The correlation of GGT levels in serum and saliva with periodontal parameters

4. DISCUSSION

Chronic periodontitis is usually seen affecting higher age groups. This fact is seen in the present study (Table 1, Fig. 1). Periodontitis is characterised by the inflammatory and immune reactions induced by the bacterial plaque. However OS in periodontitis is independent of gender and age [15].

During periodontal disease the infiltration of neutrophils represents the main event in the host's response to bacterial invasion. Neutrophils have both intracellular and extracellular oxidative and non-oxidative mechanisms for controlling bacterial invasion. The oxidative mechanism of neutrophils and other phagocytes results in the formation of reactive oxygen species (ROS) for killing the bacteria [2]. Lipopolysaccharides of plaque bacteria activate the toll like receptor-4 which in turn causes the activation of osteoclasts and increases the concentration of matrixmetalloproteinases (MMPs) which ultimately causes periodontal tissue damage [16]. Recruitment and activation of polymorphonuclear leukocytes and the inflammatory cytokines hastens the ROS production [17]. ROS have extremely short half-life yet they can cause substantial tissue damage by initiating free radical chain reactions. Therefore the body contains a number of protective anti-oxidant mechanisms, whose specific role is to remove ROS, as soon as they are formed, or to repair the damage caused by ROS. Glutathione is one such antioxidant that plays a major role in maintaining the intracellular redox balance and thus regulating signalling pathways which are affected by OS [18].

GGT being an intracellular enzyme plays an important role in antioxidant defense systems. The primary role of GGT is to catalyse the extracellular reduced glutathione (GSH), allowing for precursor amino acids to be assimilated and reutilized for intracellular GSH synthesis, thereby maintaining, a continuous "GSH cycling" across the plasma membrane [19]. Thus GGT promotes the cellular supply of GSH, the most important antioxidant of the cell involved in free radical scavenging catalysed by glutathione peroxidase. Therefore GGT is a good indicator to assess cellular damage as it reflects the pathological changes occurring in the periodontal tissues [20].

In the present study subjects with chronic periodontitis showed high levels of GGT in both serum and saliva which can be attributed to periodontal tissue degradation and release of GGT from the damaged cells of the periodontal

tissues into the gingival crevicular fluid and saliva, as well as in the surrounding fluids. This was in accordance to a study done by Arati C et al. [21] Other studies showing similar results in serum and saliva include those by Todorovic T et al. [20] Sreeram M [22] and Agnihotram G et al. [23].

Tobacco plays a major role in the prevalence and severity of periodontal diseases [24]. In the present study smokeless tobacco users with chronic periodontitis showed higher levels of GGT in both serum and saliva as compared to non tobacco users with chronic periodontitis. This can be attributed to the presence of nicotine in tobacco that produces reactive oxygen species which induces hepatic mitochondria and microsomal cellular degeneration, dose dependent increase in lipid peroxidation of hepatic mitochondria and microsomes as well as hepatic DNA damage leading to oxidative tissue damage and apoptosis [25]. In accordance with the reports of Bagchi et al. [26] and Teitz [27], subtle membrane changes due to oxidative stress allows passage of intracellular enzymes like GGT to the extracellular space [28]. This strongly suggests that smokeless tobacco induces OS. In the present study the smokeless tobacco users also exhibited OS.

Currently in the field of OS, biomarkers have been gaining a lot of importance. There is emphasis on development of functional biomarkers of OS status [28]. Although clinical parameters like bleeding on probing, probing depth, clinical attachment level and radiographic assessment of alveolar bone loss provide information on the severity of periodontitis, they do not measure the disease activity [29].

There is a need of rapid, reliable and simple diagnostic test that can evaluate periodontal disease and identify the patients at risk. Diagnostic tests based on host salivary and immune factors facilitate early detection of patients at risk for periodontal diseases, measure the periodontal disease activity and thereby allows appropriate intervention, decreases the need for more aggressive treatment and improves the response to periodontal therapy [30].

Lim JS et al. [31] repeatedly found that the activity of serum GGT was unchanged, during the 40 week, when frozen stored samples were thawed and then frozen again after each testing. The activity of GGT can correlate with the amount of damage and can indicate the prognosis of the course of this disease [20].

From the present study, it is evident that by estimation of GGT in chronic periodontitis patients, the degree of oxidative damage can be assessed. Estimation of GGT would have important clinical significance to prevent further periodontal damage and thereby maintain a healthy periodontium in the high risk cases. By correcting the underlying deficiency of antioxidants, periodontal health can be improved. This can help in the management of periodontitis, by arresting it in early stages and avoiding the possible consequences. The limitations of the present study include a small sample size. So, further elaborate studies on the estimation of GGT before and after scaling and root planing levels are needed to depict the actual role of these parameters in the initiation and promotion of periodontitis. Also, further longitudinal studies are needed to determine if elevated levels of GGT alone can be a predictor for the development of systemic conditions.

5. CONCLUSION

There are no gold standard methods yet for measuring OS generated during periodontal tissue destruction. Estimation of GGT levels being an easy, reliable and inexpensive test, can become an emerging tool for the diagnostic assessment of periodontal disease for early detection of OS suggesting its high scope to be recognized as a biomarker for OS damage. GGT can be a promising option to establish both oral and systemic health. Long term studies are needed to justify the role of GGT as a biomarker.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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