Original Article

Analysis of Plasma Homocysteine Levels in Patients with Chronic Periodontitis Before and After Nonsurgical Periodontal Therapy Using High-Performance Liquid Chromatography

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Abstract

Introduction: Homocysteine (Hcy), an inflammatory biomarker, is a sulfur-containing amino acid. Elevated levels of plasma Hcy are evident in various inflammatory conditions and have been described as an independent risk factor for cardiovascular disease. The literature has also stated that a similar association could exist between the chronic periodontitis and plasma-Hcy levels, in otherwise systemically healthy individuals. However, studies on Hcy levels in periodontitis are scarce. Hence, this study aimed to assess the levels of plasma Hcy in patients with chronic periodontitis before and after nonsurgical periodontal therapy. **Materials and Methods:** This longitudinal, case–control clinical study included a total of 60 patients who were divided into two groups. Periodontal parameters including Plaque Index, Gingival Index, Sulcus Bleeding Index, probing depth, and clinical attachment level were recorded at baseline and 12 weeks after periodontal therapy. A high-performance liquid chromatography analysis was performed to measure the Hcy levels. The results were evaluated statistically for intergroup pair-wise comparisons by Mann–Whitney U-test and intragroup comparison by Wilcoxon-matched pairs test. Correlation between the plasma-Hcy levels with other clinical parameters in all groups was done by Spearman's rank correlation method. **Results:** The Hcy was detectable in all the samples. At baseline, the mean levels of plasma Hcy were found to be low in the control group, whereas in the test group, it is found to be higher. These plasma-Hcy levels and all periodontal parameters were reduced significantly after nonsurgical periodontal therapy. **Conclusion:** The results demonstrated that plasma-Hcy levels are reduced after nonsurgical periodontal therapy but not to the levels comparable with those found in healthy individuals. Therefore, nonsurgical periodontal therapy may be used as an adjunctive Hcy-lowering therapy, contributing toward primary prevention against cardiovascular diseases.

Keywords: *Biological markers, cardiovascular disease, inflammation*

Introduction

Periodontal disease is a chronic inflammatory infectious disease affecting the supporting tissues of teeth including gingiva, periodontal ligament, cementum, and alveolar bone.[1] Periodontitis, which is a constant potential source of infection,[2] has been considered as a separate risk factor for diabetes, cardiovascular diseases, cerebrovascular diseases, respiratory diseases, and low birthweight rheumatoid arthritis through common pathophysiological pathways.[3,4] Cardiovascular disease is the single leading cause of morbidity and mortality globally as per the World Health Organization statistics,[5] wherein < 50% of the patients with atherosclerosis lack identifiable risk factors. As evidence documented that individuals with periodontitis have a greater risk of presenting endothelial dysfunction and cardiovascular diseases, the pathogenesis of periodontal disease and atherosclerotic disease can hence be related through common inflammatory cascades.[6]

Despite the various inflammatory mediators elevated in cardiovascular events, namely, intercellular adhesion molecule, IL-6, TNF-α, IL-8, and CRP;[7,8] homocysteine (Hcy), an inflammatory biomarker, was also evidenced in patients with cardiovascular diseases.[9]

Hcy, a sulfur-containing amino acid, which in the recent past has become a biomolecule of great importance for biochemists and clinicians alike. Hcy

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is exported into plasma where it circulates, mostly in its oxidized form, bound to plasma proteins as a protein-Hcy mixed disulfide with albumin (protein-SS-Hcy).[10] Under normal circumstances, most but not all of the Hcy formed in transmethylation reactions is remethylated back to methionine or is converted into cysteine in transsulfuration reactions. The B-complex vitamins play an essential role in the transformation and the excretion of the Hcy metabolism pathway,[11] the elevated levels of Hcy that is, hyper-Hcy (HHcy) has been associated with pathologic alteration in the vasculature, which is recognized as an independent cardiovascular disease risk factor.[11-13] The literature evidence also shows that a rise in plasma-Hcy concentration beyond 15 μmol (5–15 μmol) is a risk factor for cardiovascular disease.[14]

Not only this inflammatory biomarker associated with cardiovascular disease, but it has also been investigated in patients with rheumatoid arthritis, wherein the immunoinflammatory activation of rheumatoid arthritis contributes to the Hcy increase.[7]

It is evident from the literature that nonsurgical periodontal therapy decreases inflammatory cytokine levels in chronic periodontitis patients,[15] and hence it may be considered that by improving periodontal health, local and systemic inflammation can be reduced, which in turn reduces the complications associated with these conditions.

Taking these aspects into consideration, it was hypothesized that periodontal intervention therapy might reduce the systemic inflammatory burden, which in turn could act as a Hcy -lowering therapy and serve a dual purpose in not only managing the periodontal disease but also in the management of other chronic inflammatory conditions. Hence, an attempt has been made in this study to analyze the Hcy levels in chronic periodontitis patients before and after nonsurgical periodontal therapy.

Materials and Methods

This 3-month follow-up case–control study included a total of 60 patients, of which 30 patients who were diagnosed with chronic periodontitis[16] were allocated to test group and 30 patients with healthy periodontium were allocated to the control group. The study protocol was reviewed and approved by the Institutional Review Board and Ethical Committee of Vishnu Dental College, Andhra Pradesh. Written informed consent was obtained from all the participants after thorough explanation of the nature of risks and benefits of the clinical investigation and associated procedures. The sample size was calculated based on a pilot study with a standard deviation value of Hcy levels 8.27 μmol/l, and the difference between the two groups with 6 μmol/l with a confidence level of 95% and a statistical power of 80%. Patients diagnosed with systemic and infectious diseases, patients on any medications from the past 6 months, smokers, and pregnant women were excluded from the study.

Criteria for inclusion of periodontally healthy individuals (Group A):

Patients with uniform probing depths of 3 mm or below and with no evidence of loss of attachment but might be showing some signs of gingival inflammation.

Criteria for inclusion of chronic periodontitis patients (Group B):

- Age range of 25–65 years
- A minimum of 20 teeth in the mouth
- Patients diagnosed with moderate-to-severe periodontitis according to American Academy of Periodontology guidelines of 1999
- The presence of at least four sites in different teeth with clinical attachment loss ≥3 mm (moderate-to-severe periodontitis)
- Patients who had not received periodontal treatment in the past 1 year.

The primary end-point of the study was to assess plasma-Hcy levels and the secondary end-point was to assess periodontal parameters, namely, Plaque Index,[17] Gingival Index,[18] Sulcular Bleeding Index,[19] probing depth, and clinical attachment level before and after the nonsurgical periodontal therapy (NSPT). The parameters were assessed at baseline and at 3 months from the baseline visit. At the initial visit, blood sample was collected and periodontal parameters were assessed, followed by NSPT and at 3 months from the baseline visit again periodontal parameters were assessed and the blood sample collection was performed to assess Hcy levels. After NSPT, oral hygiene instructions were given to each patient.

Methodology

Patients were advised to come after an overnight fasting for the blood sample collection before scaling and root planning, wherein a 5 ml of venous blood was collected through venipuncture from the median cubital vein and at 3 months from the baseline visit. These blood samples were transferred to a test tube containing ethylenediaminetetraacetic acid anticoagulant. The blood samples were centrifuged at 3500 rpm for 10 min to extract the plasma, and this plasma was immediately transferred to a plastic veil and stored at −20°C until Hcy analysis was carried out with high-performance liquid chromatography method HPLC equipment, waters india pvt Ltd. bangtore, India, and the column used for HPLC, Inertsil ODS C18 of size 4.6 mm × 250 mm, 5 μm, detector used was dual λ absorption spectrometer.

Sample preparation

All the samples were assayed for Hcy using HPLC with dual λ absorption spectrometer, and the assay protocol as proposed by Shaik.[20]

Hcy and 1-heptane sulfonic acid were purchased from tokyo chemical industry (India) Pvt. Ltd. The internal standard (chlorogenic acid) was purchased from Sigma-Aldrich, Co., India. The organic solvents (methanol and acetonitrile) were of HPLC grade and were purchased from Sigma-Aldrich (India).

The standard solution was prepared using DL-Hcy and the solutions were run in the HPLC unit to note the absorption spectrum and generate Hcy retention time, linearity, and precision graphs on the attached computer monitor screen

The test and control samples were thawed, and prepared for analysis by the addition of 2 ml acetonitrile and 10 µl of methanol, and centrifuged to obtain a supernatant solution to which 1 ml of chlorogenic acid was added as an internal standard. The mobile phase was prepared using methanol and 1-heptane sulfonic acid with a pH adjusted to 2.3 using orthophosphoric acid.[20] An injection volume of 10 µl was required for sampling into HPLC. The retention time for Hcy was 2.051 min at an excitation wavelength of 210 nm, and for internal standard, the retention time was about 5 min. Hcy was thus identified based on the retention time as confirmed by the standards. The concentrations of Hcy obtained were initially expressed as parts per million and later converted to the SI unit for Hcy, that is, µmol using the AMA manual of style online convertor. At the initial visit, periodontal parameters were assessed, followed by blood sample collection and NSPT and was done and after 3 months again blood sample collection and other parameters were assessed.

Statistical analysis

Data analysis was performed using statistical software SPSS 20 0 IBM corporation. Chicago Stata 8 0. statacorp Medcalc 9.0.1. medcalc software Systat 12 0. syslat software Inc, CA, USA. The data were described using mean and standard deviation. Mann–Whitney U-test was used for intergroup comparisons and Wilcoxon-matched pairs test for intragroup comparison. The correlation between the plasma-Hcy levels with other clinical parameters in all groups was done by Spearman's rank correlation method.

$P < 0.05$ was considered to be statistically significant for all analyses.

Results

The study included a total of 30 chronic periodontitis patients comprising of 12 male and 18 female patients and 30 patients who are periodontally healthy individuals comprising of 19 males and 11 female patients were recruited in the study [Table 1] The mean age standard deviation in test and control groups are SD 45.53(7.64). SD 41.57(6 63) years respectively which is depicted in Table 2. Intragroup comparisons of PI, Gl, Bl, PD and mean CAL are depicted in Tables 3-7 which are statistically significant Inter group comparisons between test and control with mean preoperative mean PI, mean Gl, mean PD, and mean CAL are depicted in Tables 8-12 respectively wherein, there is a statistically significant difference between test and control groups. There is statistically significant decrease in PD before and after treatment [Tables 13 and 14] On the other hand, Hcy levels for test group (26.39±9.01µ mol/l) and control group (14.05±1.98µmol/l) showed significant difference which is depicted in Table 15. Also a significant statistical difference in decrease in probing depths before and after treatment were depicted in Table 16. The preoperative and post-operative correlations amongst the different parameters of PI, Gl, Bl, PD, CAL and Hcy levels are presented in Tables 17 and 18.

Discussion

Periodontal disease is initiated by bacterial-plaque biofilm attached to tooth surfaces, resulting in damage to the supporting structures comprising of gingiva, periodontal ligament, cementum, and alveolar bone. Along with the biofilm, products of periodontal pathogens consisting of enzymes, toxins, and lipopolysaccharides induce an inflammatory response in the host. Furthermore, an uncontrolled, overexuberant inflammatory response in chronic periodontitis associated with an oxidative stress-induced inflammatory pathogenesis, has been linked with systemic diseases such as cardiovascular disease, diabetes mellitus, and rheumatoid arthritis.[2,3] Considering the above findings associated with periodontal disease, recent research has focused on the objective measures such as biomarkers to assess the current disease status and also to predict any association between the periodontitis and other systemic diseases. Among the innumerable biomarkers that have been evidenced in the periodontal literature, Hcy is one among them.

Hcy is an inflammatory biomarker which is considered as a potent risk factor for cardiovascular disease. As hyperhomocysteinuria IE elevated levels of Hcy can induce damage to vascular walls either directly through the protein homocysteinylation (Hcyn) or through an oxidative

Table 1: Distribution of male and females in the study groups (test and control)

Sex	Test group (%)	Control group (%)	Total
Male	12 (40.00)	19 (63.33)	31
Female	18 (60.00)	11 (36.67)	29
Total	30 (100.00)	30 (100.00)	60

Table 2: Comparison of two study groups (test and control) with mean age by *t*-test

Groups	Mean±SD
Test group	45.53±7.64
Control group	41.57±6.93
Total	42.89±7.34
t	0.7472
P	0.4579

SD: Standard deviation

Table 3: Comparison of pre- and post-operative Plaque Index scores in test group by Wilcoxon matched pairs test

Time	Mean±SD	Mean difference	SD difference	Percentage of change	*Z*	*P*
Preoperative	1.64±0.43	0.92	0.38	56.32	4.6225	0.0001*
Postoperative	0.72±0.32					

*P<0.05. SD: Standard deviation

Table 4: Comparison of pre- and post-operative Gingival Index scores in test group by Wilcoxon matched pairs test

Time	Mean±SD	Mean difference	SD difference	Percentage of change	*Z*	*P*
Preoperative	1.48±0.40	0.95	0.42	64.44	4.5771	0.0001*
Postoperative	0.53±0.43					

*P<0.05. SD: Standard deviation

Table 5: Comparison of pre- and post-operative Bleeding Index scores in test group by paired *t*-test

Time	Mean±SD	Mean difference	SD difference	Percentage of change	*Z*	*P*
Preoperative	67.28±17.20	39.66	18.15	58.95	11.5615	0.0001*
Postoperative	27.62±15.84					

*P<0.05. SD: Standard deviation

Table 6: Comparison of pre- and post-operative probing depth scores in test group by paired *t*-test

Time	Mean±SD	Mean difference	SD difference	Percentage of change	*Z*	*P*
Preoperative	4.09±0.88	1.22	0.90	29.87	7.1909	0.0001*
Postoperative	2.87±0.61					

*P<0.05. SD: Standard deviation

Table 7: Comparison of pre- and post-operative clinical attachment level scores in test group by Paired *t*-test

Time	Mean±SD	Mean difference	SD difference	Percentage of change	*Z*	*P*
Preoperative	4.65±1.27	1.04	1.13	22.42	4.8589	0.0001*
Postoperative	3.60±0.92					

*P<0.05. SD: Standard deviation

Table 8: Comparison of study groups (test and control) with preoperative mean Plaque Index scores by Mann-Whitney U-test

Group	*n*	Mean±SD	Mean rank	*U*	*Z*	*P*
Test group	30	1.65±0.43	45.50	0.00	−6.6530	0.0001*
Control group	30	0.26±0.10	15.50			

*P<0.05. SD: Standard deviation

stress response Hcyn may cause protein functional damage by inactivation of the enzyme lysyl oxidase,[21] which is required for collagen modification and this HHcy has been recognized as a risk factor for cardiovascular diseases. In HHcy, Hcyn proteins accumulate in the vascular wall surfaces which are recognized by the macrophages, leading to the phagocytosis and the destruction of the endothelial cells. If the responsible agent (homocysteination proteins) is present continually on endothelial cells, it would eventually lead to atherosclerotic plaque formation. This atherosclerotic plaque formation also enhances the pro-oxidant activity and nitric oxide expression, ultimately changing the endothelial cell phenotype from anticoagulant to procoagulant;[22] other probable mechanisms that promote atherosclerotic action of Hcy are molecular target hypothesis and endoplasmic stress hypothesis, and it may also be assumed that periodontal tissue damage may accelerate specific remethylation reactions of DNA, RNA, and various proteins during tissue repair, with consequent generation of S-adenosyl Hcy and release of Hcy, a mechanism which is similar to that seen in autoimmune conditions, such as rheumatoid arthritis. These mechanisms suggest the pathways through which inflammatory processes in chronic periodontitis influence the plasma-Hcy concentrations. It was also hypothesized that the periodontal inflammatory cascade can affect the methionine metabolism leading to HHcy.

Hcy can be detected in a variety of different laboratory techniques, which include chromatography, electrophoresis, and immunoassay. In immunoassay laboratory technique, the commonly used are enzyme-linked immunosorbent assay (ELISA), chemiluminescent, and fluorescent detection. However, the use of HPLC with dual λ detector was opted over ELISA for the detection and estimation of Hcy, as the former shows higher sensitivity and wider range over the latter. The HPLC protocol validated in a study[23] has been employed in the present study to evaluate the plasma-Hcy concentration in chronic periodontitis before and after NSPT.

Table 9: Comparison of study groups (test and control) with preoperative mean Gingival Index scores by Mann-Whitney U-test

Group	*n*	Mean±SD	Mean rank	*U*	*Z*	*P*
Test group	30	1.48±0.40	45.50	0.00	−6.6530	0.0001*
Control group	30	0.09±0.04	15.50			

*P<0.05. SD: Standard deviation

Table 10: Comparison of study groups (test and control) with preoperative mean Bleeding Index scores by independent *t*-test

Group	*n*	Mean±SD	SE	*t*	*P*
Test group	30	68.49±17.30	3.16	15.8316	0.0001*
Control group	30	17.29±3.82	0.70		

*P<0.05. SD: Standard deviation; SE: Standard error

Table 11: Comparison of study groups (test and control) with preoperative mean probing depth scores by independent *t*-test

Group	*n*	Mean±SD	SE	*t*	*P*
Test group	30	4.05±0.87	0.16	11.3235	0.0001*
Control group	30	2.23±0.17	0.03		

*P<0.05. SD: Standard deviation; SE: Standard error

Table 12: Comparison of study groups (test and control) with preoperative mean clinical attachment level scores by Independent *t*-test

Group	*n*	Mean±SD	SE	*t*	*P*
Test group	30	4.59±1.25	0.23	10.1201	0.0001*
Control group	30	2.17±0.40	0.07		

*P<0.05. SD: Standard deviation; SE: Standard error

As per the literature available, it can be considered that if the inflammatory states were really implicated in the genesis of hyperhomocysteinuria, a decrease in plasma-Hcy concentration could be expected following periodontal therapy, owing to the reduction of systemic inflammatory load.[15] Moreover, a study suggested that plasma-Hcy levels above 14 μmol/l[24] warrant a treatment to decelerate the growth of carotid plaque formation. Nonsurgical periodontal therapy belongs to the first phase of periodontal treatment, wherein the primary objective is to completely remove the gingival inflammation provoking elements (i.e., plaque, calculus, and endotoxins) from the tooth surface, thereby helping in improving the host defense by restoring the disturbed oxidant: antioxidant balance. Restoration of this oxidant: antioxidant balance may help to delay or control the onset of various systemic inflammatory diseases, at least those initiated by periodontitis.[25] Hence, an attempt was made to evaluate the efficacy of nonsurgical periodontal therapy on plasma-Hcy levels in chronic periodontitis patients.

The results of the present study indicated a rise in the plasma Hcy concentrations in test group (preoperative 26.67 ± 8.77 μmol/L) when compared to the control group (14.05 ± 1.98 μmol/L). The results obtained are in conjunction with the previous studies which demonstrated elevated plasma Hcy concentrations using ELISA in similarly defined chronic periodontitis subjects over the controls.[6,26] On the contrary, Hcy concentrations in chronic periodontitis patients have been reported in the studies using chemiluminescent immunoassay.[27,28]

The elevated plasma Hcy in our test group may be a consequence of the persistent immunoinflammatory activation by periodontal pathogens. There could be several possible mechanisms underlying the link between chronic periodontitis and plasma-Hcy. Pro-inflammatory cytokines, such as IL-6, which may be released from inflamed periodontal pockets and may give rise to acute-phase reactants in the systemic circulation. McCarty[29] has reported that IL-6 may interact with Vitamin B6 metabolism and compromise cystathionine β-synthase activity, thereby elevating plasma-Hcy concentrations. This suggests that hyperhomocysteinuria could be expected by the release of IL-6 from the inflamed pocket walls of the periodontal tissues.[29] On the other hand, a significant reduction of plasma-Hcy levels in postoperative test group to 19.87 ± 6.44 μmol/l) was evident after 12 weeks of NSPT. This reduction of plasma-Hcy levels connotes the reinstitution of the Hcy–methionine homeostasis which may be attributable to the presumed reduction in the serum IL-6 and reactive oxygen metabolites.

This finding is in an agreement with a previous study demonstrating elevation in Hcy concentrations with incrementing severity of periodontal disease.[6] These observations reinforce the hypothesis that plasma-Hcy concentration is positively correlated with increasing periodontal destruction and that it is lowering partly depends on the residual pocket depth and clinical attachment level after periodontal therapy.

Table 13: Comparison of pre- and post-operative probing depth with 3-5 mm scores in test group by paired *t*-test

Time	Mean±SD	Mean difference	SD difference	Percentage of change	*Z*	*P*
Preoperative	51.89±16.04	28.00	19.07	53.96	7.7698	0.0001*
Postoperative	23.89±14.83					

*P<0.05. SD: Standard deviation

Table 14: Comparison of pre- and post-operative probing depth with >5 mm scores in test group by paired *t*-test

Time	Mean±SD	Mean difference	SD difference	Percentage of change	*Z*	*P*
Preoperative	11.54±13.71	7.43	9.68	64.40	4.0617	0.0004*
Postoperative	4.11±5.96					

*P<0.05. SD: Standard deviation

Table 15: Comparison of study groups (test and control) with preoperative mean homocysteine level scores by Independent *t*-test

Group	*n*	Mean±SD	SE	*t*	*P*
Test group	30	26.67±8.77	1.60	7.6891	0.0001*
Control group	30	14.05±1.98	0.36		

*P<0.05. SD: Standard deviation; SE: Standard error

Table 16: Comparison of pre- and post-operative homocysteine level scores in test group by Paired *t*-test

Time	Mean±SD	Mean difference	SD difference	Percentage of change	*Z*	*P*
Preoperative	26.39±9.01	6.52	4.61	24.69	7.4735	0.0001*
Postoperative	19.87±6.44					

*P<0.05. SD: Standard deviation

Table 17: Correlations among different preoperative parameters by Karl Pearson's correlation coefficient

Variables	Summary	PI	GI	BI	PD	CAL	Homocysteine level
PI	*r*	-					
	P	-					
GI	*r*	0.749**	-				
	P	0.0000	-				
BI	*r*	0.777**	0.834**	-			
	P	0.0001	0.0001	-			
PD	*r*	0.2740	0.2770	0.3660	-		
	P	0.1590	0.1540	0.0550	-		
CAL	*r*	0.391*	0.3250	0.432*	0.913**	-	
	P	0.0400	0.0910	0.0220	0.0001	-	
Homocysteine level	*r*	0.0520	0.1350	0.2190	0.1460	0.0860	-
	P	0.7950	0.4920	0.2630	0.4600	0.6640	-

**Correlation is significant at the 0.01 level (two-tailed), *Correlation is significant at the 0.05 level (two-tailed). PI: Plaque Index; GI: Gingival Index; BI: Bleeding Index; PD: Probing depth; CAL: Clinical attachment level

Hence, within the limitations of the study, it can be summarized that with an increase in periodontal inflammation, there is a substantial rise in the concentration of Hcy in plasma. The interventional therapy, including scaling and root planning reduces the periodontal inflammation and Hcy levels, which in turn could reduce the risk of cardiovascular diseases.

Limitations of the study

As the main enzyme involved in the metabolism of Hcy requires the presence of cofactors such as Vitamin B_6 and B_{12}, the deficiencies of these vitamins are also associated with elevated levels of Hcy in various pathological conditions. Therefore, it is important that the levels of these B vitamins need to be elucidated to provide a better representation of the inflammatory conditions.

Hence, further studies can be performed with a large sample size and an assay of Vitamin B_6, B_{12}, and IL-6 need to be included to provide a holistic picture of role of Hcy in periodontal disease.

Table 18: Correlations among different postoperative parameters by Karl Pearson's correlation coefficient

Variables	Summary	PI	GI	BI	PD	CAL	Homocysteine level
PI	*r*	-					
	P	-					
GI	*r*	0.875**	-				
	P	0.0001	-				
BI	*r*	0.528**	0.605**	-			
	P	0.0040	0.0010	-			
PD	*r*	0.1460	0.1980	0.474*	-		
	P	0.4580	0.3130	0.0110	-		
CAL	*r*	0.1010	0.0310	0.0570	0.609**	-	
	P	0.6080	0.8770	0.7730	0.0010	-	
Homocysteine level	*r*	0.0700	0.0450	0.437*	0.3630	0.3460	-
	P	0.7220	0.8200	0.0200	0.0570	0.0710	-

**Correlation is significant at the 0.01 level (two-tailed), *Correlation is significant at the 0.05 level (two-tailed). PI: Plaque Index; GI: Gingival Index; BI: Bleeding Index; PD: Probing depth; CAL: Clinical attachment level

Conclusion

The results of the present study showed that the plasma-Hcy levels were found to be elevated in patients with chronic periodontitis as compared to patients with healthy periodontal status. These levels reduced significantly following nonsurgical periodontal therapy, but not to the levels evident in periodontally healthy individuals. One of the various benefits of nonsurgical periodontal therapy is the reduction of Hcy levels which may contribute toward prevention of cardiovascular diseases. However, this needs to be evaluated further in longitudinal studies with larger sample size.

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Conflicts of interest

There are no conflicts of interest.

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