Original Article

Relevance of single-nucleotide polymorphism to the expression of resistin gene affecting serum and gingival crevicular fluid resistin levels in chronic periodontitis and type 2 diabetes mellitus: A randomized control clinical trial

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Access this article online

Website: www.jisponline.com

DOI: 10.4103/jisp.jisp_361_18

Quick Response Code:

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Submission: 25-05-2018
Accepted: 25-10-2018

Abstract:

Aim: The present study is aimed to investigate whether single-nucleotide polymorphism (SNP) of resistin gene (RETN) at −420 and +299 sites, is associated with resistin levels in serum and gingival crevicular fluid (GCF) in periodontally healthy, chronic periodontitis (CP) with and without type 2 diabetes mellitus (T2DM) patients. **Materials and Methods:** Serum and GCF samples were procured from all the 60 patients (twenty in each group) of the three study groups i.e., periodontally healthy (Group I), CP (Group II) and CP with T2DM patients (Group III) to analyze resistin levels using enzyme-linked immunosorbent assay test and clinical parameters were assessed at baseline and at 3 months after scaling and root planing (SRP). RETN polymorphism at −420 and +299 was genotyped by polymerase chain reaction-restriction fragment length polymorphism technique. **Results:** Patients with SNP −420 and +299 were positively correlated with increased serum and GCF resistin levels in Group II and Group III patients. SRP led to substantial reduction in the serum and GCF resistin levels. **Conclusion:** These findings are suggestive of a biologic link between resistin, periodontal diseases, and periodontal diseases with T2DM and RETN SNP at −420 and +299 in imparting increased resistin levels in inflammatory and diabetic conditions.

Key words:

Chronic periodontitis, diabetes mellitus, genetics, resistin

INTRODUCTION

Periodontal disease results in attachment loss and alveolar bone destruction and has been linked with diabetes mellitus (DM).[1] Compromised wound healing, alteration in microbial flora and cellular and humoral functioning make DM a risk factor for periodontal disease.[2]

Recently, resistin an adipocytokine is reported to have a link between obesity and DM.[3] Resistin not only contributes to insulin resistance but also supplements in the inflammatory process through pro-inflammatory agents. There is a bidirectional relationship between resistin and periodontitis. Resistin acts as a pro-inflammatory molecule by secreting tumor necrosis factor alfa (TNF-α), interleukin (IL)-6 and IL-12, generating a positive feedback cycle inducing its own production. Elevation of resistin levels with periodontitis is attributed to the fact that resistin is expressed from polymorphonuclear leukocyte cells and macrophages, also there is resistin release from neutrophils by periodontopathogens due to lipopolysaccharide stimulation.[4] The

How to cite this article: Rode PA, Kolte RA, Kolte AP, Purohit HJ, Ahuja CR. Relevance of single-nucleotide polymorphism to the expression of resistin gene affecting serum and gingival crevicular fluid resistin levels in chronic periodontitis and type 2 diabetes mellitus: A randomized control clinical trial. J Indian Soc Periodontol 2019;23:131-6.

gene coding resistin is found on chromosome 19p13 with single-nucleotide polymorphisms (SNPs) reported at promoter as well as on coding sequences. C to G substitution at −420 position in the 5′ flanking region of the gene showed altered messenger ribonucleic acid (mRNA) levels in abdominal fat with increased serum and gingival crevicular fluid (GCF) resistin level[5] whereas SNP +299G>A with increased plasma resistin levels have been reported in Malaysian[6] and Japanese population.[7]

In the light of the above facts, we hypothesized in the present study that SNP of resistin gene (RETN) would influence the circulating levels of resistin and could be associated with inflammatory conditions such as DM and periodontal disease. The present randomized control trial was carried out to evaluate the resistin levels in serum and GCF at baseline and after 3 months of non surgical therapy and to find out its association with human RETN polymorphism in the promoter at −420 position and also in the coding sequence at +299 position in systemically healthy, chronic periodontitis (CP) and with and without type 2 DM (T2DM) patients.

In the present polymerase chain reaction (PCR)-based study, RETN −420C>G (rs1862513) and +299G>A (rs8745367) polymorphism were chosen as tags.

MATERIALS AND METHODS

The study population comprised Caucasian population and included 60 patients equally divided into three groups; [Figure 1] within the age range of 35–65 years visiting the outpatient Department of Periodontics, VSPM Dental College and Research Centre and Nagpur. The study design was reviewed and approved by the Institutional Ethics Committee and was in accordance with the Helsinki Declaration. Before the initiation of the study, informed consent was obtained from the patients who agreed to participate voluntarily. The sample size to ensure adequate power for this study was calculated based on the results of the previous study,[5] where the data on mean concentration levels of serum and GCF resistin for the three groups were referred. The data for serum and GCF resistin resulted into an effect size of 0.92 and 0.46, respectively. A sample size of 20 per group was needed to achieve the stringent effect size of GCF resistin in the proposed study, with

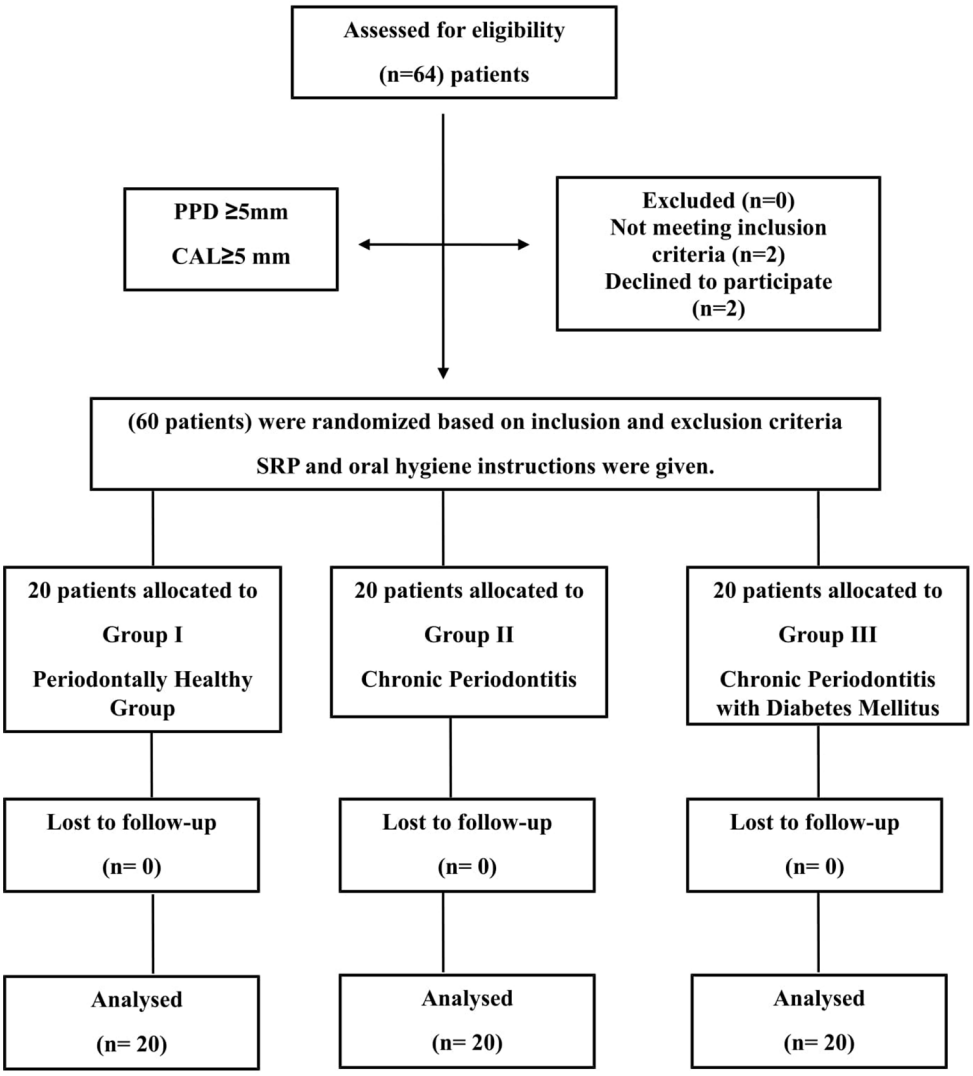

Figure 1: Consort flowchart of patients selection criteria. n – Number of patients

90% power and 95% confidence level. A dental and medical history was recorded for the selected patients, and an intraoral examination was conducted by a single examiner (RK). The intrarater reliability in the measurements of periodontal parameters was evaluated before treatment in the study groups. Intraclass correlation (ICC) coefficient with two-way mixed effects model was obtained for each periodontal parameter. The ICC ranged between 0.92 and 0.99 in the groups ($P < 0.0001$), indicating excellent intra-rater reliability.

The inclusion criteria were patients aged more than 35 years with the presence of at least 20 natural teeth with moderate-to-severe clinical attachment loss (CAL) with >30% of sites involved.[8] The glycemic status of patients previously diagnosed with T2DM (having controlled T2DM since 5-10 year range) was confirmed by their glycated hemoglobin (HbA_1c)[9] and random blood sugar (RBS) level.

Pregnant women or lactating mothers, tobacco chewers, and cigarette smokers, patients who had undergone any periodontal therapy in the past 6 months, were on antibiotics, anti-inflammatory therapy, and with systemic diseases such as hypertension, renal disease, rheumatoid arthritis were excluded from the study. Patients were then categorized into three groups as:

1. Group I comprised 20 systemically and periodontally healthy individuals who presented with gingival index (GI) and plaque index (PI) score = 0, teeth with periodontal pocket depth (PPD) ≤3 mm, CAL = 0, with no evidence of radiographic bone loss and presenting with HbA_1c levels <6.5% and RBS levels <200 mg/dl
2. Group II comprised 20 systemically healthy patients with generalized moderate-to-severe CP and presented with GI and PI score of >1, PPD of ≥5 mm and CAL ≥5 mm that were positive for BOP and radiographic evidence of bone loss, HbA_1c levels <6.5%, and RBS levels <200 mg/dl
3. Group III comprised 20 generalized moderate-to-severe CP patients who were previously diagnosed with T2DM as obtained from their medical history and presented with GI and PI score of >1, PPD of ≥5 mm and CAL ≥5 mm that were positive for BOP and radiographic evidence of bone loss, HbA_1c levels 6.5–7% and RBS levels >200 mg/dl.

Serum sample collection

Serum was derived from 4 ml blood collected through venipuncture from antecubital fossa using a 20G needle and stored at −80°C until required; and 2 ml blood was used for extracting deoxyribonucleic acid (DNA).

Site selection and gingival crevicular fluid collection

Only one site per patient was selected on day 1 as a sampling site in CP groups (Group II and Group III), whereas in the healthy group to ensure adequate amount of GCF collection, multiple sites (3–5 sites per patients) were sampled with no signs of inflammation. In patient with CP, the site showing the greatest CAL and signs of inflammation was selected for sampling. GCF was collected by placing the microcapillary pipette at the entrance of gingival sulcus. To ensure atraumatic each sample collection was allotted a maximum of 10 min.[5]

Genotyping of resistin gene polymorphism

Genotyping was performed using PCR- restriction fragment length polymorphism (RFLP) technique.

Deoxyribonucleic acid extraction, polymerase chain reaction, and single-nucleotide polymorphism typing

25 µl of blood added to 500 µl phosphate-buffered solution and centrifuged at 10,000 rpm for 5 min and supernatant was discarded. To the remaining pellet, 0.5 M (mole) 50 µl NaoH was added and incubated at room temperature for 30 min. 50 µl of 1M Tris HCl was then added and vortexed to which 400 µl distilled water was added and stored at −20°C until further analysis. The details of amplification of DNA were obtained from National centre for biotechnology information-Primer Blast. Primers used were:

SNP −420

Forward – 5`TGTCATTCTCACCCAGAGACA-3`

Reverse – 5`TGGGCTCAGCTAACCAAATC-3`

SNP +299

Forward – 5`GAGAGGATCCAGGAGGTC – 3`

Reverse – 5`GTGAGACCAAACGGTCCCTG – 3`

The PCR amplification was done in PCR thermocycler machine. RFLP was carried out using 0.5 µl of Alu and Bpi I restriction endonuclease enzymes for SNP +299 and −420, respectively, using purified PCR products.

Evaluation of resistin from serum and gingival crevicular fluid

Samples were assayed for resistin levels using commercially available Enzyme-linked immunosorbent assay (RayBio® Human Resistin ELISA Kit, 3607 Parkway Lane, Suite. 200, Norcross, GA 30092 USA).

Clinical parameters were recorded along with the detection of serum and GCF resistin levels at baseline and after 3 months of scaling and root planning.

Statistical analysis

The presence or absence of SNPs was done using Fisher's exact test and z-test of proportion between independent samples, respectively. Anthropometric parameters such as RBS and HbA_1c levels were compared across groups using one-way analysis of variance (ANOVA). The statistical comparison of parameters within groups before and after treatment was performed using paired *t*-test. The comparison of resistin levels at pre- and post-stages across groups was performed using ANOVA. The *post hoc* analysis was performed using Tukey's HSD test. Statistical significance was tested at 5% level, and all the analyses were performed using computer software IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp, USA. programming language.

RESULTS

HbA_1c was considered to be confirmatory test to validate the diabetic condition of the patient along with RBS test and was used to classify patients into different groups. All the clinical parameters were assessed at baseline and post nonsurgical periodontal therapy. Results indicated a

significant reduction in the means of PI, GI, PPD, and CAL after a comprehensive nonsurgical periodontal therapy using one-way ANOVA [Table 1].

At pretreatment as well as posttreatment phase, the difference in the mean serum resistin levels across study groups were highly significantly with $P < 0.0001$, using one-way ANOVA. Further, the mean GCF resistin levels, before as well as after treatment across groups showed highly significant difference with $P < 0.0001$.

As per the mechanism of RFLP, in Group II, 13 patients were found to have SNP at −420 site and 11 patients showed to have SNP at +299 site. While in Group III, 13 patients were found to have SNP at −420 site and 15 patients were found to have SNP at +299 site [Table 1 and Figures 2a, b, 3a, b].

When comparison of pre- and post-levels of resistin in three groups was done, in Group I, the serum as well as GCF resistin levels before and after treatment showed significant difference with *P* value of 0.0002 using paired *t*-test [Table 2] In Group II and Group III, the difference was highly significant with $P < 0.0001$.

Significantly higher resistin levels were found only in serum and not in GCF samples with SNPs at −420 and +299 as compared to the samples showing absence of SNPs. When influence of presence of SNP at −420 over resistin levels was analyzed statistically significant difference was found in serum as well as GCF. When observed for influence of presence or absence of SNP at +299, a statistically significant difference was found in serum resistin levels of Group III patients [Table 3].

Table 1: Descriptive statistics for various parameters according to groups (*n*=20)

Parameters	Group I - Healthy	Group II - CP	Group III - CP with T2DM	*P*
PI				
Pretreatment	0.41±0.31[a]	1.43±0.35[b]	1.40±0.20[b]	<0.0001[§]
Posttreatment	0[a]	0.19±0.11[b]	0.21±0.17[b]	<0.0001[§]
GI				
Pretreatment	0.07±0.04[a]	1.51±0.51[b]	1.86±0.39[c]	<0.0001[§]
Posttreatment	0[a]	0.14±0.09[b]	0.28±0.27[c]	<0.0001[§]
PPD				
Pretreatment	1.26±0.22[a]	4.62±0.78[b]	6.28±1.52[c]	<0.0001[§]
Posttreatment	0.43±0.35[a]	2.58±0.60[b]	4.01±1.38[c]	<0.0001[§]
CAL				
Pretreatment	0[a]	5.28±0.80[b]	7.14±1.59[c]	<0.0001[§]
Posttreatment	0[a]	3.12±0.77[b]	4.51±1.38[c]	<0.0001[§]
Serum resistin				
Pretreatment	0.09±0.08[a]	0.74±0.34[b]	0.87±0.42[b]	<0.0001[§]
Posttreatment	0.004±0.01[a]	0.08±0.08[b]	0.12±0.07[b]	<0.0001[§]
GCF resistin				
Pretreatment	0.15±0.15[a]	1.55±0.75[b]	2.43±0.62[c]	<0.0001[§]
Posttreatment	0.003±0.001[a]	0.12±0.14[b]	0.22±0.15[c]	<0.0001[§]
SNP: −420				
Present		13	13	0.9999[k]
Absent		7	7	
SNP: +299				
Present		11	15	0.3203[k]
Absent		9	5	

[§]Statistically highly significant difference at *P*<0.001; *P* values obtained using one-way ANOVA for continuous variables, [k]Statistically nonsignificant difference at *P*>0.05; obtained using Fisher's exact test, [a,b,c]Statistically nonsignificant difference using Tukey's *post hoc* test. GI – Gingival index; PI – Plaque index; PPD – Probing pocket depth; CAL – Clinical attachment level; GCF – Gingival crevicular fluid; SNP – Single nucleotide polymorphism; CP – Chronic periodontitis; T2DM – Type 2 Diabetes mellitus; *n* – Number of sites treated for each group; ANOVA – Analysis of variance; *P* – *P*- value

Table 2: Intragroup comparison of change in means of serum and gingival crevicular fluid resistin levels

Parameters	Groups	Pretreatment	Posttreatment	*P*
Serum resistin (ng/ml)	Group I	0.09±0.08	0.004±0.01	0.0002[¶]
	Group II	0.74±0.34	0.08±0.08	<0.0001[§]
	Group III	0.87±0.42	0.12±0.07	<0.0001[§]
GCF resistin (ng/µl)	Group I	0.15±0.15	0.003±0.004	<0.0002[¶]
	Group II	1.55±0.75	0.12±0.14	<0.0001[§]
	Group III	2.43±0.62	0.22±0.15	<0.0001[§]

[§]Statistically highly significant difference at *P*<0.001, [¶]Statistically significant difference at *P*<0.0002. GCF – Gingival crevicular fluid; *P* – *P*- value

DISCUSSION

The study evaluates the impact of inflammation and increased insulin concentration on inflammatory biomarker resistin along with RETN SNPs at −420 and +299 position and in turn analyze the effective resolution of inflammatory state after nonsurgical periodontal therapy of 3 months through decreased levels of resistin in serum and GCF. Here, influence of age and sex on resistin levels is minimized by recruiting equal number of males and females in each group and confining age within a range of 35–65 years.

There was a significant improvement in clinical parameter such as PPD, CAL, PI, and GI after nonsurgical periodontal therapy in both the groups but the Group II showed better improvement as compared to Group III. A positive correlation of both serum and GCF resistin levels with all the clinical parameters were observed in the present study. It is reported in many studies that DM patients propose a risk for the initiation of inflammation. Hence, it was probable to find lesser improvement in clinical parameter after therapy as compared to absence of DM.[10]

Taking an account of resistin to be found in abundance in peripheral blood mononuclear cells and macrophages,[11] in our study higher resistin levels in serum and GCF were found in Group II and Group III at baseline which substantiates the previous findings[11] and considers resistin as a potential marker of inflammation.[12] Since *in vitro* expression of resistin is seen to be affected by release of inflammatory cytokines such as IL-6, TNF-α, and IL-1β[13] as well as by maturation of monocytes into macrophages, it accentuates the possibility of periodontal inflammation influencing resistin expression.[14]

In our study, reduction in serum and GCF resistin levels from baseline to 3 months posttherapy was observed in all the groups. In contrast to our study, Gonçalves *et al*.[15] did not observe any significant reduction in serum and GCF levels of inflammatory markers including resistin. It might be because; several residual sites might have remained unaltered for both groups despite the maintenance and periodontal therapies, which sustained the bacterial load higher enough maintaining systemic inflammation even after therapy. In our study, although significant reduction in resistin levels was observed posttherapy, the mean reduction was lesser in Group III as

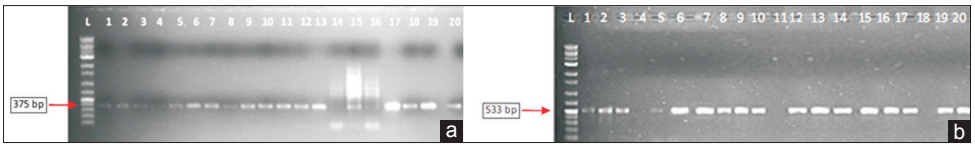

Figure 2: (a and b) Polymerase chain reaction analysis for the detection of resistin gene at +299 loci and at −420 loci, respectively. Polymerase chain reaction analysis of blood samples from 20 patients. L denotes Ladder for deoxyribonucleic acid marker of 1 Kb. Lane 1–20 represents 20 samples. Lane 1–10 from Group II, lane 11–20 from Group III. Amplification of resistin gene at +299 loci and −420 loci is indicated by the polymerase chain reaction product of 375 bp (a) and 533 bp, respectively (b)

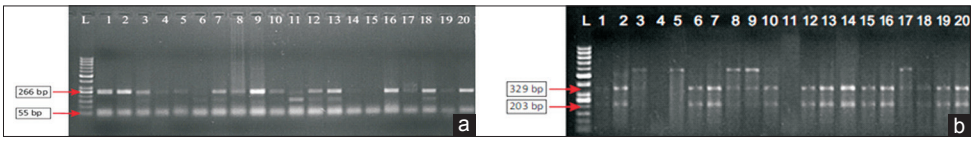

Figure 3: (a and b) Restriction fragment length polymorphism detection of resistin gene at +299 loci and at −420 loci, respectively. Restriction fragment length polymorphism analysis of blood samples from 20 patients. L denotes Deoxyribonucleic acid marker of 50 bp. Lane 1–20 represents 20 samples. Lane 1–10 from Group II, lane 11–20 from Group III. Amplification of resistin gene with restriction fragment length polymorphism product of 266 bp and 55 bp indicates polymorphism at +299 loci (a); amplification of the resistin gene with restriction fragment length polymorphism product of 329 bp and 203 bp indicates polymorphism at −420 loci (b)

Table 3: Comparison between pretreatment parametric levels corresponding to single-nucleotide polymorphism –420 and +299 for Groups II and III

SNPs	Groups	Serum resistin Pretreatment (ng/ml)	*P*	GCF resistin Pretreatment (ng/µl)	*P*
SNP –420					
Present	Group II	0.85±0.31	0.0592[k]	1.69±0.72	0.2649[k]
Absent	Group II	0.55±0.31		1.27±0.78	
Present	Group III	1.01±0.44	0.0177[¶]	2.28±0.67	<0.0001[§]
Absent	Group III	0.61±0.25		2.55±0.74	
SNP +299					
Present	Group II	0.84±0.38	0.1297[k]	1.81±0.74	0.0769[k]
Absent	Group II	0.62±0.24		1.23±0.65	
Present	Group III	1.02±0.38	<0.0001[§]	2.34±0.66	0.2024[k]
Absent	Group III	0.42±0.10		2.69±0.44	

[§]Statistically highly significant difference at $P<0.001$, [¶]Statistically significant difference at $P<0.05$, [k]Statistically nonsignificant difference at $P>0.05$. GCF – Gingival crevicular fluid; SNP – Single-nucleotide polymorphism; *P* – *P*- value

compared to Group II and Group I, and the results were similar to those obtained by Youn *et al*.[16]

Our results showed that SNP –420 and SNP +299 at the RETN locus were strongly associated with increased serum resistin levels. However, when this same evaluation was done in relation to GCF, we did not find a significant association of –420 and +299 SNPs with increased resistin levels. This shows that resistin values might be linked with gene polymorphism of +299 site but not associated with metabolic disorders or CP state always.

In the meta-analysis[17] a significant association was observed between SNP –420C>G and resistin levels in Asian populations, thus suggesting some ethnic-specific genetic effects related to heterogeneity. The results of our study are in accordance with the previous studies in which several SNPs have been identified in the human RETN.[18,19] One of these (a C to G substitution at position –420 in the 5′ flanking region of the gene) was found to be associated with increased resistin mRNA levels in abdominal fat by altering the transcriptional activity and serum resistin levels were also elevated. The GG genotype of resistin SNP at –420 was seen to be associated with T2DM.[20] Association of –420C>G polymorphism with obesity was reported in a meta-analysis with G allele as a risk factor.[21] In our study, we found an association of both SNPs with increased resistin levels in serum which is similar to that reported by Suriyaprom *et al*.[22] However, study conducted by Hivert *et al*.[23] did not show an association with resistin levels possibly because +299 G>A polymorphism occurring in an intron, generally has not been considered to have regulatory functions. However, the variation of results among populations about the RETN variation, –420C>G and +299G>A, and resistin levels may contribute appreciably to differences in gene expression phenotypes by ethnicity.

The correlations drawn from our study suggests that CP and T2DM play an important role in elevating the levels of resistin and elevated resistin levels can thereby worsen the periodontal status of the diabetic patient. In T2DM patients with periodontitis, the pro-inflammatory cytokines enhance insulin resistance by inhibiting insulin signaling and thus inflammatory burden is increased as compared to nondiabetic condition and so reduction in resistin levels after therapy is comparatively lower than in patients of CP without T2DM. Few limitations of the study are selection of the patients was made on the basis of clinical parameters and a larger sample size with longitudinal evaluation is desired.

CONCLUSION

Within limits of the study, it can be concluded that in the presence of polymorphism of RETN at most frequently found

loci such as −420 and +299, the resistin levels of GCF and serum are almost always seen to be elevated, which reduced after a comprehensive 3 months of nonsurgical periodontal therapy in CP with and without T2DM. This SNP might not be the sole reason for enhancing the inflammatory burden in immune-compromised patients, but definitely is a major factor for its causation. Hence, SNP's of resistin if identified in patients with DM and obesity, it would help in establishing a therapeutic protocol at an early stage, thereby preventing the patients getting prone to periodontitis.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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