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Evaluation of DNA Methylation of Inflammatory Genes following Treatment of Chronic Periodontitis: a pilot case-control study

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ABSTRACT

OBJECTIVE: To evaluate the influence of periodontal therapy on DNA methylation in chronic periodontitis patients as compared to healthy individuals.

MATERIALS & METHODS: Twenty patients were enrolled into two groups: 1) 10 diagnosed as clinically healthy; and 2) 10 diagnosed with chronic periodontitis. Clinical measures were recorded and gingival biopsies were harvested at baseline (both patient groups) and at 2 and 8 weeks post-baseline for diseased individuals. Molecular DNA methylation analysis was performed by pyrosequencing for the putative inflammation-associated genes LINE-1, COX-2 IFN-γ, and TNF-α. Random-intercept linear regression models were applied to evaluate methylation levels across groups at baseline and the methylation changes over time in the diseased and normal tissues.

RESULTS: Periodontal therapy did not influence gene expression methylation of TNF-α, IFN-γ and LINE-1 levels at normal and periodontitis sites over time. However, it significantly reduced COX-2 methylation levels comparable to healthy individuals at both 2 and 8 weeks post-treatment ($P < 0.05$).

CONCLUSIONS: Periodontal therapy resets the DNA methylation status of inflammatory gene for COX-2 in periodontal disease patients. DNA methylation levels of TNF-α, IFN-γ and LINE-1 were sustained in periodontitis sites despite therapy. Future studies should consider an expanded panel of inflammatory genes over time. (ClinicalTrials.gov NCT02835898).

KEYWORDS: Periodontal Diseases/therapy, Epigenetics, DNA Methylation, Inflammatory genes, Biomarkers, Periodontal disease pathogenesis

CLINICAL RELEVANCE:

Scientific Rationale for Study: Little is known about the influence of periodontal therapy on DNA methylation levels in chronic periodontitis patients.

Principal Findings: Periodontal therapy is able to reset the levels of COX-2 methylation to

compatible levels observed in healthy patients on the short term. Methylation profile from normal tissues of periodontitis patients suggests they might be more prone to periodontal tissue breakdown.
Practical Implications: Local effects of the disease have an influence on the epigenetics of the tissues that may be modulated by the microbiota. Findings might help to better elucidate disease development in healthy sites of periodontitis patients.

INTRODUCTION

Periodontitis is a destructive disease of the tooth supporting tissues induced by bacterial biofilm **(Van Dyke & van Winkelhoff, 2013)** that eventually triggers an inflammatory host response influenced by environmental, genetic and epigenetic factors **(Borrell & Papapanou, 2005; Takashiba & Naruishi, 2006; Kornman, 2008)**. Of interest to this study, epigenetic modification can further regulate gene expression of an individual's immune response **(Schulz et al, 2016),** and despite the well-established influence of epigenetic modification in cancer and inflammatory diseases **(Fitzpatrick & Wilson, 2003; Adcock et al, 2007; El Gazzar et al, 2007; Ngollo et al, 2014)** little is known in the context of oral health **(Lod et al, 2014).** In general, epigenetics embodies the modifications of gene expression, without changing the DNA sequence **(Bird, 2002; Adcock et al, 2007)** through chemical alterations of DNA and associated proteins **(Barros & Offenbacher, 2014)** that evoke chromatin remodeling and a successive rapid inactivation or activation of genes **(Larsson et al, 2015).** Notably, such alterations occur through two major

mechanisms in human cells **(Shaw, 2006);** DNA methylation and histone modifications **(Wilson, 2008)** with the former offering a more stable form of gene regulation **(Bäckdahl et al, 2009).** Accordingly, most of the studies exploring the link between epigenetics and periodontal disease have investigated the changes in the DNA methylation of genes involved in the regulation of cytokine production, since these signaling molecules play a key role in periodontal tissue breakdown; for reviews see: **(Barros & Offenbacher, 2014; Lod et al, 2014; Larsson et al, 2015).** Recent studies in experimental periodontitis have also demonstrated the activation of inflammatory routes including the NFκB signaling pathway by histone modifications **(Martins et al, 2016).** Several studies demonstrated a lower level of DNA methylation of genes expressing pro-inflammatory cytokines in periodontitis patients, either chronic or aggressive, compared to those with a healthy periodontium **(Oliveira et al, 2009; Andia et al, 2010; Zhang et al, 2010 a, Ishida et al, 2012).** Nevertheless, hypermethylation has been also described for specific genes in the chronic state of periodontal disease, as a down-regulatory mechanism to prevent unrestricted tissue destruction **(Zhang et al, 2010 b).** Likewise, higher DNA methylation in chronic periodontitis has been also reported in recent studies **(Zhang et al, 2013, Kojima et al, 2016).** Although several epigenetic alterations have been described in periodontal disease, little is known about the efficacy of periodontal therapy in reestablishing normal DNA methylation levels in patients. The prospective positive effects of periodontal therapy on the methylation profile of DNA and specific genes came from the study of **Andia & colleagues (2015)** that found no change between healthy and periodontitis tissues after three months. These findings suggest that periodontal therapy can influence epigenetic modifications. However, variations in the methylation level between both groups were not assessed at baseline and samples of inflamed tissues were not investigated.

Based on these observations, we conducted a clinical study to monitor the changes in DNA methylation of long interspersed nuclear element-1 (LINE-1), cyclooxygenase-2 (COX-2), interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α) inflammatory genes in periodontitis patients following periodontal therapy in comparison to healthy subjects at both the site and patient levels.

MATERIALS & METHODS

This investigation was approved by the ethical committee of the University of Milan, Italy and was conducted during the period between October 2015 and June 2016. This short-term, prospective cohort study is registered at ClinicalTrials.gov (Identifier: NCT02835898).

- Study Participants & Inclusion Criteria:

From the pool of patients attending a private dental practice in Piacenza- Italy, twenty individuals were enrolled equally in two groups: 10 healthy patients displaying a disease-free periodontium and 10 moderate, chronic periodontitis patients, since the present clinical investigation is considered a pilot investigation. All participants were voluntarily enrolled into the study after explaining its objectives and obtaining their verbal and written informed consent.

Study participants fit the following inclusion criteria:

1. Patients 18 years of age or older.
2. Patients without any reported systemic diseases.
3. Non-smokers or ex-smokers who had quit smoking for at least one or more years prior to enrollment in the study.
4. Patients of Caucasian origin.
5. **For the healthy group:** patients who showed no clinical signs of gingival inflammation or history of periodontitis were included. In this group, periodontal probing depths (PPD) in all sites were ≤ 3 mm, without any signs of tooth mobility, bleeding on probing (BOP) or clinical attachment loss (CAL).
6. **For the periodontal disease group:** patients diagnosed with chronic periodontitis according to the American Academy of Periodontology (AAP) Workshop definition **(Armitage, 1999)** were included. Patients in this group displayed a minimum of a single "healthy site" (PPD < 4 mm without signs of bleeding on probing, mobility or inflammation) and a periodontitis site (PPD ≥ 5mm with bleeding on probing at baseline) for subsequent biopsy harvesting.

The exclusion criteria were the following:

1. Pregnant patients

2. Patients who reported the use of antibiotics and/or non-steroidal anti-inflammatory drugs (NSAIDs), for at least one month before enrolment in the study.

3. Patients who received periodontal therapy within the last three months prior to enrollment in the study.

- **Study timeline & Periodontal Parameters:**

* ***Baseline:*** For periodontitis participants, periodontal probing depth (PPD), clinical attachment level (CAL), bleeding on probing (BOP), full mouth plaque score (FMPS) and full-mouth bleeding score (FMBS) were recorded at six sites around each tooth. As for healthy patients, only periodontal screening and recording (PSR) was registered, in accordance with the principles of good clinical practice; the periodontal charting is completed when the patient presents with two or more sites of a PD ≥ 5mm **(Tonetti & Claffey, 2005).**

  Next, gingival biopsies were obtained from the healthy patients during surgical removal of wisdom teeth, using a 3 mm diameter punch. In the periodontal disease group, biopsies were harvested from two sites: a normal site and a periodontitis site with the purpose to compare methylation levels between both clinically distinct sites. Following biopsy harvest, chronic periodontitis patients underwent conventional periodontal therapy consisting of full mouth scaling and root planing with ultrasonic and manual instruments. Chlorhexidine mouthwash (0.2%) was prescribed for daily use (twice daily for 20 days).

* ***Two and eight weeks after periodontal therapy:*** PPD, CAL & BOP, FMPS & FMBS were measured and gingival biopsies were harvested for the disease group only, from both a normal and a periodontitis site, to analyze the influence of periodontal therapy on epigenetic modifications as comparison to each time point. Two weeks point of time was selected to evaluate the early response to periodontal treatment, while eight weeks was chosen because the re-evaluation of periodontal parameters after periodontal therapy varies between 4 and 8 weeks (ideally 4 to 6 weeks). However, 4 weeks and 6 weeks points of time might be a little bit short for adequate stability of periodontal parameters, therefore, 8 weeks was chosen as a reasonable point of time as a first re-evaluation following periodontal therapy **(Segelnick & Weinberg, 2006).**

In the periodontitis group, it was not possible to harvest biopsies from the same site at different points of time, therefore, tissue sampling was done in a manner to match the clinical parameters at all points of time as the following: for healthy sites; PPD < 4 mm without bleeding on probing or

signs of inflammation at all points of time. For periodontitis sites; PPD ≥ 5mm with bleeding on probing at baseline. For 2 weeks and 8 weeks point of time, biopsies were harvested from sites of PPD ≥ 5mm without bleeding on probing but already showed BOP at baseline, as documented in the periodontal chart.

A single calibrated examiner made the measurements and harvested the gingival biopsies (GR), while the same experienced dental hygienist (FP) performed the conventional periodontal therapy on all patients of the periodontitis group.

- **Sample Collection, DNA Extraction, and Bisulfite Treatment:**

Briefly, freshly harvested tissues were collected and submerged in Allprotect Tissue Reagent (Qiagen, USA) to stabilize DNA immediately, and were stored in vials at 2-8 °C for up to 6 months. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, USA) according to the manufacturer's recommendations.

EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA) was used to treat 500 ng DNA (concentration 25 ng/µl) according to the manufacturer's protocol. Bisulfite-treated DNA was eluted in 300 µl of M-Elution Buffer.

- Analysis of DNA Methylation:

Analysis of DNA methylation was performed using previously published methods, **(Bollati et al, 2007; Tarantini et al, 2013)** with minor modifications. Briefly, a 50 µl PCR was carried out in 25 µl of GoTaq Hot Start Green Master Mix (Promega, Madison, WI, USA), 1 pmol of the forward primer, 1 pmol of the biotinylated reverse primer, 25 ng of bisulfite-treated genomic DNA and water. Primers used for DNA methylation analysis and PCR cycling conditions are shown in **table 1.** The biotin-labeled primers were used to purify the final PCR product using Sepharose beads. The PCR product was bound to Streptavidin Sepharose HP (Amersham Biosciences, Uppsala, Sweden) and the Sepharose beads containing the immobilized PCR product were purified, washed, denatured using a 0.2 M NaOH solution, and washed again using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Inc., Westborough, MA), as recommended by the manufacturer. Then, 0.3µl pyrosequencing primer was annealed to the purified single-stranded PCR product, and pyrosequencing was performed using the PyroMark MD System (Pyrosequencing, Inc., Westborough, MA). The degree of methylation was expressed as a percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines (%5mC).

The CpG positions were selected according to data published in literature for the same assessed genes **(Dawsey et al, 2008; Madrigano et al, 2012; Cantone et al, 2016),** which ensures

consistency in terms of repeatability of the assay.

- Statistical Analysis:

To compare gender and age distribution between the two groups we use the chi-square and the Wilcoxon-Mann-Whitney test, respectively. In order to take into account correlations within subjects, random-intercept linear regression models were applied to evaluate methylation levels across groups at baseline and to evaluate methylation changes over time in the periodontal disease group, separately for the disease-free and periodontitis sites. Statistical analyses were performed with Stata 13 (StataCorp. 2013) **(Rabe-Hesketh & Skrondal, 2008).** Statistical significance was set at p value < 0.05.

RESULTS

Twenty participants (8 males, 12 females) were included in this study, distributed into "healthy" (5 males, 5 females) and "periodontal disease" groups (3 males, 7 females). The age in the healthy group was between 25 and 69 years old (mean = 53.3 ± 12.3 years), while the range was 26-60 years old in the periodontal disease group (mean = 46.6 ± 10 years). Nineteen out of the initial 20 participants completed the study with one dropout at the 8th week **(Figure 1).**

Periodontal therapy in the periodontal disease group was successful based on the changes in the mean of PPD, CAL & BOP over time. Mean PPD at baseline was 4.2 ± 0.4 mm, which became 2.5 ± 0.3 mm on 8th week ($p < 0.001$). In the healthy group, PPD at baseline in all sites was ≤ 3mm. Clinical attachment level in the periodontitis group was 4.2 ± 0.9 at baseline, indicating a moderate periodontal disease. Eight weeks post-therapy, mean CAL became 3.6 ± 0.9 mm ($p = 0.01$). As for bleeding on probing, mean percentage at baseline was 63% in periodontally-diseased individuals and decreased up to 5% at the end of the evaluation period ($p < 0.001$).

Demographic and clinical characteristics of the study sample are shown in **Table (2),** while **table (3)** reports clinical characteristics of the harvested sites in the periodontitis group at the time of biopsy. A marked improvement in PPD of periodontitis sites from which biopsies were harvested can be noted at 2 and 8 weeks ($p < 0.001$). Due to site selection characteristics, sites affected by periodontal disease must have had BOP at baseline while healthy sites in periodontitis patients must not have had BOP at baseline. For 2 weeks and 8 weeks point of time, BOP greatly reduced in all sites of the mouth following periodontal therapy. Therefore it was possible to harvest biopsies from sites that showed no BOP at these points of time, but had BOP at baseline as documented in the periodontal chart.

Regarding DNA methylation of TNF-α gene, the promoter region was more methylated in healthy individuals (36.6% ± 9.2), compared to normal & periodontitis sites of the periodontal disease group (34.1% ± 5.2 and 31.4% ± 7.8 respectively), indicating a relatively more active gene expression of TNF-α in the state of disease. Methylation status remained almost stable in normal sites throughout the evaluation period. In the periodontitis sites, however, methylation level was almost stable up to 2 weeks. Afterward, it started to rise reaching 33.7% ± 7.4 at 8 weeks, which is quite close to that reported in normal sites at baseline. Nonetheless, these results were not statistically significant (P value > 0.05), neither among groups nor among sites.

As for the promoter region of IFN-γ gene, it was hypermethylated among all entities, with the healthy group displaying the highest methylation percentage among all (88.6% ± 1.4) reflecting a

decreased gene expression of IFN-γ in the state of periodontal health. Regarding the periodontal disease group, the level of methylation in periodontitis sites was almost comparable to healthy individuals (88.3% ± 2.1), while normal sites exhibited a slightly lower methylation (85.8% ± 6.5). Through time, DNA methylation in periodontitis sites started to decline, reaching at 8 weeks a percentage almost equivalent to that reported in normal sites at baseline (86.2% ± 3.8). On the other hand, methylation levels started to increase in normal sites, approaching a level at 8 weeks a comparable to the healthy group (88.8% ± 2.8). None of these findings was statistically significant (P value > 0.05).

Periodontal therapy reduces the methylation of COX-2 promoter. Unlike TNF-γ and IFN-γ, COX-2 promoter region was hypomethylated in periodontium of healthy patients (6.7% ± 7.6). DNA methylation was nearly as twice as high in the periodontitis sites (13.2% ± 7.3), while on normal tissues from periodontal disease patients it was 8.8% ± 5.7. The discrepancy in the mean of methylation between periodontitis sites in comparison to healthy group at baseline was statistically significant (p = 0.03 for periodontitis sites vs healthy group). Following periodontal treatment, methylation percentage decreased dramatically in periodontitis sites, at 2 weeks, almost to half (5.8% ± 3.2, p <0.001 vs baseline), with a very slight rise from 2 weeks to 8 weeks (7.2% ± 4.3, p = 0.004 vs baseline), which was comparable to the methylation level reported in the healthy group. On the contrary, the mean percentage of methylation steadily declined in normal sites, reaching 5% ± 1.9 at 8 weeks, P=0.03 vs baseline).

To evaluate the DNA methylation at a broader level, we explored the methylation profile of LINE-1 promoter region. We observed that the mean percentage of methylation was similar between the healthy group (69.5% ± 3.5) and normal sites in diseased patients (69.4% ± 2.1), and remained fairly stable during the observation period. As for periodontitis sites, a slight increase in the methylation of the promoter region at 8 weeks was observed (71.7% ± 2.8 vs 70.2% ± 2.8 at baseline). These findings were not statistically significant (p value > 0.05).

Mean methylation percentages of the selected genes and p values are summarized in **Table 4.** Time pattern of DNA Methylation among different groups for TNF-α, IFN- γ, COX-2 and LINE-1 are demonstrated in **figure 2.**

DISCUSSION

Here we provide for the first time evidence of epigenetic modifications mediated by periodontal therapy. Due to the well-established association of methylation with stable gene regulation, we chose to evaluate the status of DNA methylation instead of the acetylation of genes related to inflammation **(Bäckdahl et al, 2009).** Our study shows the expression pattern of methylated genes before periodontal therapy and during the two months follow-up. Since alterations in the methylation patterns might vary from site to site within the same individual **(Barros & Offenbacher, 2014),** we assessed the epigenetic modifications in normal and periodontitis sites within the same chronic periodontitis patient. We also compared our data to tissue samples from healthy periodontium. Our study gave emphasis to the methylation status of TNF-α, IFN-γ, and COX-2, as key genes associated with periodontal disease progression. We also carefully selected LINE-1 gene as a genome-wide readout for methylation. Of note, our cohort of patients includes Caucasians, and careful interpretation of the results must take into consideration the potential ethnic influence over epigenetic modifications **(Kwabi-Addo et al, 2010; Straughen et al, 2015).**

Findings of this study showed that periodontal therapy significantly reduced COX-2 methylation levels comparable to healthy individuals at both 2 and 8 weeks after treatment. The methylation status of the COX-2 gene (also known as prostaglandin-endoperoxide synthase 2 or PTGS2) was inspected because it is the enzyme that synthesizes prostaglandin E2 (PGE2) and governs its production. As is known, PGE2 is a key inflammatory mediator in periodontal disease and is well correlated with periodontal inflammation and alveolar bone loss **(Goodson et al, 1974; Offenbacher et al, 1992; Tipton et al, 2003; Noguchi & Ishikawa, 2007; Reynolds et al, 2007).** We found that COX-2 gene promoter was hypomethylated in biopsies retrieved from the healthy group (6.7%), while DNA methylation was as twice higher in the periodontitis sites of the periodontal disease group (13.2%). Similarly**, Loo & colleagues** described a hypermethylated

COX-2 in 19% of patients with chronic periodontitis. In concordance, **Zhang et al (2010 b)** identified a hypermethylated PTGS2 promoter in periodontal disease tissues, associated with lower levels of PTGS2 transcription. Therefore, high levels of COX-2 methylation can render an intrinsic protective mechanism capable of preventing the unrestrained breakdown of the periodontium **(Zhang et al, 2010 b).** However, it must be noted that the methylation frequency of the COX-2 gene promoter was much higher in our study than that reported by **Zhang & Colleagues (2010 b);** 6.7% vs. 0.85% in the healthy sites and 13.2% vs. 4.3% in the periodontitis-affected sites, as they measured DNA methylation of the COX-2 gene promoter using a different technique i.e., bisulfite specific PCR, cloning, and sequencing.

Other findings in literature might further confirm the down-regulation of COX-2 in the state of chronic disease; in a previously published cross-sectional epidemiological study, the attachment level was negatively associated with the levels of PGE_2 in the gingival crevicular fluid (GCF) **(Zhong et al, 2007).** Hence, it was suggested that chronic inflammation might serve as a new "set-point" in which certain inflammatory mediators are down regulated to prevent unrestricted destruction of periodontal tissues **(Zhang et al, 2010 b)**.

Regarding other biomarkers, findings of the present clinical investigation revealed that periodontal therapy did not influence DNA methylation of TNF-α, IFN-γ and LINE-1 levels at normal and periodontitis sites over time ($P > 0.05$), indicating that epigenetic alterations might be locally sustained for some mediators even after the elimination of periodontal inflammation. Nonetheless, certain observations can still be underlined; DNA methylation status of TNF-α gene promoter was almost stable in normal sites throughout the evaluation period and wasn't restored to that of the healthy group. This might indicate a different methylation profile in healthy tissues of individuals with and without history of periodontitis. Such information might help to better elucidate disease development in healthy sites in susceptible periodontitis patients, which needs to be further investigated for implementing appropriate intervention plans **(Mdala et al, 2014**). However, this hypothesis needs to be confirmed in future studies exploring a wide panel of inflammatory cytokines. TNF-α gene was selected for DNA methylation analysis due to its important role in the pathogenesis of periodontal disease and tissue destruction **(Page, 1991).**

With respect to IFN- γ gene promoter, hypermethylation was evident among all entities even after periodontal therapy, which might be a usual feature in healthy and inflamed periodontal tissues. In fact, hypermethylation of IFN- γ gene promoter has already been reported in another study **(Viana et al, 2011)** probably due to the presence of distinctly active mixed cell population in the sample as previously suggested **(Winders et al, 2004; Stefani et al, 2013).** IFN- γ gene was evaluated in the present study because of its association with the severity and progression of periodontal disease

(Garlet et al, 2003).

For each gene, CpG positions were selected based on previously published data **(Dawsey et al, 2008; Madrigano et al, 2012; Cantone et al, 2016),** as they are more consistent in terms of repeatability of the assay. Four CpG islands were analyzed for COX-2 and TNF- α genes while two were assessed for IFN- γ gene. Although higher number of CpG islands was reported in literature for these genes **(Zhang et al, 2010 a; Zhang et al, 2010 b; Zhang et al, 2013),** this can be attributed to the different technique utilized for DNA methylation analysis in the noted studies. Our clinical investigation is a pilot study, and with the limited amount of available tissues, we were more interested in evaluating DNA methylation of more genes than covering the entire promoter of one single gene. Furthermore, we were not able to cover a larger sequence due to the limited amount of harvested tissues.

LINE-1 was selected to evaluate the DNA methylation more comprehensively, as it encloses a high CpG density and comprises about 17% of the genome **(Kitkumthorn & Mutirangura, 2011; Newman et al, 2012)** with the total genomic methylation content being consistent with the methylation of such repetitive elements **(Lange et al, 2012).** Despite the lack of statistically significant findings, LINE-1 hypermethylation was maintained in normal and periodontitis sites, overtime, reflecting an overall genome stability.

Based on the findings of the present study, we can conclude that: (i), periodontal therapy is not sufficient to reset the methylation levels of TNF-α, and IFN-γ inflammatory genes to levels of healthy patients; (ii) periodontal therapy is sufficient to reset the levels of COX-2 methylation to compatible levels observed in healthy patients; (iii) The methylation profile from normal tissues suggests that unaffected sites from periodontal disease patients might be more prone to periodontal tissue breakdown; (iv) Epigenetic modifications result in the activation of down-regulatory mechanisms to prevent unrestricted periodontal tissue destruction; and finally (v) On a global methylation perspective, hypermethylation of LINE-1 was maintained in periodontitis and normal sites, overtime.

The results of the study should be interpreted with caution as it has certain limitations. Gingival biopsies of normal and inflamed tissues were harvested from different sites and not the same one during different points of time. Sample collection from the same site is preferable when evaluating the influence of periodontal therapy over time, however, that was not possible because 2 weeks after periodontal therapy, soft tissues at the biopsy site were not completely healed. Another study limitation is the lack of longitudinal data on the healthy subjects as it only captured the temporal changes in the chronic periodontitis group; it was not possible to harvest multiple biopsies for

healthy participants from an ethical standpoint. Simultaneous analysis of DNA methylation and mRNA expression could have strengthened the consistency of our findings. However, the limited amount of tissue recovered from tissue biopsies, and the difficulty of collecting larger samples especially in control subjects did not allow for DNA and RNA analysis in the same subject. Also, limited numbers of CpG sites were analyzed in our pilot study, despite being the same positions previously reported in literature. Hence, future studies are needed to allow us cover all the CpG sites within the promoter region.

Findings of this investigation represent data on the short-term; thus, epigenetic changes still need to be monitored on the long-term to understand if the methylation status in chronic periodontitis could turn out similar to that in healthy individuals with no history of periodontitis, following an effective periodontal therapy, maintenance, and compliance.

CONCLUSIONS

Periodontal therapy was able to reset the DNA methylation status of COX-2 gene in chronic periodontitis patients, while DNA methylation levels of TNF-α, IFN-γ and LINE-1 were sustained in the periodontal disease group even after periodontal therapy. These findings suggest that the local effects of the disease have an influence on the epigenetics of the tissues that may be modulated by environmental factors, including the microbiota. Future investigations should further explore the identification of specific factors that affect the local epigenetics of periodontal soft and hard tissues. Moreover, future studies need to consider an expanded panel of inflammation associated epigenetic changes over time in periodontal diseases.

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Table 1. Primers Used for DNA methylation analysis and PCR cycling conditions

Sequence ID	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Sequencing Primer (5' to 3')	Sequence analyzed* (5' to 3')	Annealing conditions	Fragment size (bp)	Chr and CpG positions
Repetitive element methylation analysis							
LINE-1	TTTTGAGTTAGGTGTGGGATATA	Biotin-AAAATCAAAAAATTCCCTTTC	AGTTAGGTGTGGGATATAGT	TTC/TGTGGTGC/TGTC/TG	50°C for 30 seconds	146	
Gene-specific methylation analysis							
COX-2	GGAGATTAGTTTAGAATTGGTTTT	Biotin-AATCCCCACTCTCCTATCTAATCC	AAGAAGAAAAGATATTTGG	C/TGGAAATTTGTGC/TGTTTGGGGC/TGGTGGAATTC/TGGGG	59°C for 60 seconds	139	Chr1 Pos1: 186649540 Pos2: 186649552 Pos3: 186649561 Pos4: 186649570
IFN-γ	Biotin-GTTTTTTGGATTTGATTAGTTTGA	CAATAACAACCAAAAAAACCCA	TATAACTTATATATTTCATC	G/ATTTCCG/AAAAAATTAAACC	54°C for 60 seconds	143	Chr12 Pos1: 66840192 Pos2: 66840186
TNF-α	Biotin-TGAGGGGTATTTTTGATGTTTGT	CCAACAACTACCTTTATATATCCC	ATAAACCCTACACCTTCTAT-	CTCA/GATTTCTTCTCCATCA/GCA/GAAAACA/GAAAA	57°C for 60 seconds	208	Chr6 Pos1:31651172 Pos2:31651157 Pos3:31651155

	Pos4: 31651149

* Nucleotides at which DNA methylation was measured are underlined

* Chr = Chromosome, Pos= Position

Table 2. Patient Demographic and Clinical Characteristics

Demographic/Clinical characteristics	**Healthy (n =10)**	**Periodontal Disease (n =10)**	**P –Value ***
Males/Females	5/5	3/7	0.36
Age (years) (Mean ± SD)	53.3 ± 12.3	46.6 ± 10.0	0.11
Periodontal Probing Depth (mm) (Mean ± SD)	≤ 3 (*at baseline)*	4.2 ± 0.4 *(at baseline)*	ND
		2.9 ± 0.4 *(2 weeks after periodontal treatment)*	P < 0.001
		2.5 ± 0.3 *(8 weeks after periodontal treatment)*	P < 0.001
Clinical Attachment Level (mm) (Mean ± SD)	≤ 3	4.2 ± 0.9 *(at baseline)*	ND
		3.9 ± 1.2 *(2 weeks after periodontal treatment)*	P = 0.21
		3.6 ± 0.9 *(8 weeks after periodontal treatment)*	P = 0.01
Bleeding on Probing (%) (Mean ± SD)	0%	63 ± 25% *(at baseline)*	ND

	7 ± 6% *(2 weeks after periodontal treatment)*	P < 0.001
	5 ± 6% *(8 weeks after periodontal treatment)*	P < 0.001

*For gender and age, p-values were calculated by chi-squared and Wilcoxon-Mann-Whitney test, respectively. For periodontal variables, p-values were obtained from random intercept linear regression model.

* ND= Not determined

Table 3. Clinical Characteristics of Biopsied Sites in the Periodontitis Group, at baseline, 2 weeks and 8 weeks following Periodontal Therapy

	PPD (mean ± SD)		
Biopsy Site	***At baseline***	***Two weeks after periodontal treatment***	***Eight weeks after Periodontal treatment***
Periodontal Disease/Normal Sites	*3.0 ± 0.0*	2.8 ± *0.4*	2.7 ± *0.5*
		P = 0.18 vs baseline	***P= 0.03 vs baseline***
Periodontal Disease/ Periodontitis Sites	7.1 ± *1.7*	5.5 ± *0.8*	4.4 ± *0.9*
	P<0.001 vs normal sites	***P<0.001 vs baseline***	***P<0.001 vs baseline***

	CAL (mean± SD)		
Biopsy Site	***At Baseline***	***Two weeks after Periodontal treatment***	***Eight weeks after Periodontal treatment***
Periodontal Disease/Normal Sites	3.6 ± *0.8*	3.6 ± *1.0*	3.4 ± *1.9*
		P= 1.00 vs baseline	*P= 0.72 vs baseline*
Periodontal Disease/ Periodontitis Sites	8.4 ± *2.6*	7.7 ± *2.5*	5.6 ± *2.5*
	P<0.001 vs normal sites	*P= 0.23 vs baseline*	***P<0.001 vs baseline***

*P-values calculated with random-intercept linear regression models.

Table 4. Methylation of LINE-1, COX-2 IFN-γ, and TNF-α. genes across groups and over time*. Results of random-intercept linear regression models.

		Methylation percentage (Mean ± SD)		
Genes	***Group***	***At baseline***	***Two weeks after periodontal treatment***	***Eight weeks after Periodontal treatment***
TNF-α	**Healthy**	36.6 ± 9.2	-	-
	Periodontal Disease/Normal Sites	34.1 ± 5.2	33.9 ± 6.5	34.0 ± 9.3
		P=0.44 vs healthy	*P=0.92 vs baseline*	*P=0.90 vs baseline*
	Periodontal Disease/ Periodontitis Sites	31.4 ± 7.8	31.5 ± 8.5	33.7 ± 7.4

		P=0.11 vs healthy	*P=0.97 vs baseline*	*P=0.50 vs baseline*
IFN-γ	**Healthy**	88.6 ± 1.4	-	-
	Periodontal Disease/Normal Sites	85.8 ± 6.5	87.7 ± 3.5	88.8 ± 2.8
		P=0.10 vs healthy	*P=0.33 vs baseline*	*P=0.14 vs baseline*
	Periodontal Disease/ Periodontitis Sites	88.3 ± 2.1	88.4 ± 2.2	86.2 ± 3.8
		P=0.85 vs healthy	*P=0.89 vs baseline*	*P=0.08 vs baseline*
COX-2	**Healthy**	6.7 ± 7.6	-	-
	Periodontal Disease/Normal Sites	8.8 ± 5.7	6.6 ± 3.6	5.0 ± 1.9
		P=0.47 vs healthy	*P=0.18 vs baseline*	***P=0.03 vs baseline***
	Periodontal Disease/ Periodontitis Sites	13.2 ± 7.3	5.8 ± 3.2	7.2 ± 4.3
		P=0.03 vs healthy	***P<0.001 vs baseline***	***P=0.004 vs baseline***
LINE-1	**Healthy**	69.5 ± 3.5	-	-
	Periodontal Disease/ Normal Sites	69.4 ± 2.1	70.0 ± 2.3	69.5 ± 1.4
		P=0.92 vs healthy	*P=0.36 vs baseline*	*P=0.91 vs baseline*
	Periodontal Disease/ Periodontitis Sites	70.2 ± 2.8	70.7 ± 2.8	71.7 ± 2.8
		P=0.58 vs healthy	*P=0.61 vs baseline*	*P=0.22 vs baseline*

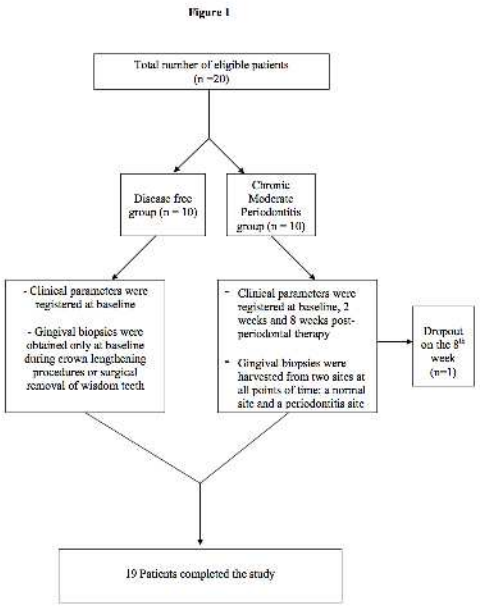
*The sample size was 10 in each group-moment combinations, except for eight weeks (one subject lost to follow-up)

**P-values calculated with random-intercept linear regression models.

FIGURE LEGENDS

Figure 1. CONSORT Flow Diagram of the Study Population

Figure 2. Time pattern of DNA Methylation among different groups for TNF-α (A), IFN- γ (B), COX-2 (C) and LINE-1 (D). Circle represents healthy groups, intermittent line represents healthy sites in periodontitis group and solid line represents periodontitis sites in periodontitis group.



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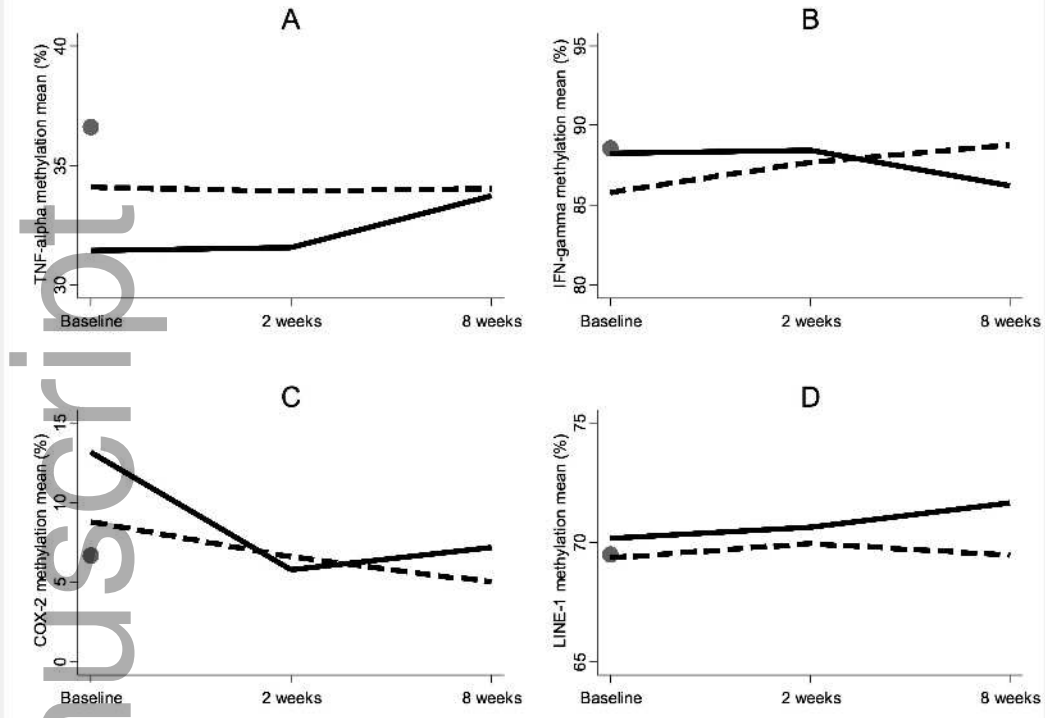

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