PERIODONTAL THERAPY INFLUENCES DNA METHYLATION OF INFLAMMATORY GENES IN CHRONIC PERIODONTITIS

LA TERAPIA PARODONTALE INFLUENZA LA METILAZIONE DEL DNA DI GENI INFIAMMATORI NELLA MALATTIA PARODONTALE CRONICA

Farah Asa'ad [1] , Giulio Rasperini [1] , Valentina Bollati [2,3] ,Giorgio Pagni [1] , Rogerio M. Castilho [4,5], Eleonora Rossi [1], Francesca Pomingi [6], Letizia Tarantini [2], Dario Consonni [3], William V. Giannobile [5]

1 Department of Biomedical, Surgical and Dental Sciences, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, University of Milan, Milan, Italy

2 EPIGET-Epidemiology, Epigenetics and Toxicology Lab, Department of Clinical Sciences and Community Health, University of Milan, Milan, Italy

3 Epidemiology Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, University of Milan, Milan, Italy

4 Laboratory of Epithelial Biology, University of Michigan School of Dentistry, Ann Arbor, MI, USA

5 Department of Periodontics and Oral Medicine School of Dentistry & Department of Biomedical Engineering, College of Engineering, University of Michigan, Ann Arbor, MI, USA

6 Dental Hygienist, Private Dental Practice, Piacenza, Italy

Running title: Effect of Periodontal Therapy on Epigenetic Modifications

Abstract

Aim: To evaluate the influence of periodontal therapy on epigenetic modifications in chronic periodontitis patients compared to healthy individuals.

Methods: Ten patients with healthy periodontium and ten patients with chronic moderate periodontitis were enrolled. Gingival biopsies were collected at baseline for both groups and at 2 & 8 weeks post-periodontal therapy for the disease group (from normal and periodontitis sites). Random-intercept linear regression models were applied to evaluate methylation levels across groups at baseline and to assess changes in the disease group overtime, separately for normal and periodontitis sites.

Results: Periodontal therapy restored methylation levels of TNF-α, IFN-γ and COX-2 genes in periodontitis sites after 2 & 8 weeks to levels reported in normal sites. A DNA methylation gradient of COX-2 promoter region was observed at baseline for healthy group and periodontitis group (percentage mean in normal sites = 8.8 ± 5.7, p= 0.47 vs healthy; percentage mean in periodontitis sites = 13.2 ± 7.3, p = 0.03 vs healthy). Maintenance of high LINE-1 methylation on periodontitis sites was observed throughout all data points, suggesting up-regulation of methyltransferase in chronic disease.

Sessione Premio H.M. Goldman - H.M. Goldman Prize Session
XVIII Congresso Internazionale – 18th International Congress SIdP
Rimini (I), March 16th 2017

2

Conclusions: Periodontal therapy resets the DNA methylation of inflammatory genes to levels observed in normal sites. Maintenance of high levels of DNA methylation was higher in periodontitis sites, reflecting methyl-transferase up-regulation in chronic disease.

Keywords: Periodontal Diseases/therapy, Epigenetics, DNA Methylation, Inflammatory genes, Biomarkers

Abstract

Obiettivo dello studio: Valutare l'influenza della terapia parodontale sulle modifiche epigenetiche in pazienti con parodontite cronica rispetto a pazienti sani.

Metodi: Sono stati reclutati 10 pazienti parodontalmente sani e 10 affetti da parodontite cronica moderata. Lo studio si è articolato raccogliendo campioni di tessuto gengivale in tre momenti: baseline (per entrambi i gruppi), 2 settimane dopo la terapia parodontale (solo per il gruppo malato, eseguiti due prelievi per paziente: sito sano "**SS**" e sito malato "**SM**"), ad 8 settimane (solo per il gruppo test, rieseguiti i due prelievi). Sono stati usati "random-intercept linear regression models" per valutare le variazioni di metilazione tra gruppi al baseline ed eventuali cambiamenti nel gruppo malato, nei siti sia sani che malati, nel corso dello studio.

Risultati: La terapia parodontale ha riportato, dopo 2 e 8 settimane, i livelli di metilazione dei geni di TNF-α, IFN-γ e COX-2 dei SM ai livelli osservati nei SS.

Per COX-2, al baseline, è stato riscontrato una differenza di metilazione della regione promotrice del DNA tra pazienti sani e parodontopatici (percentuale media in SS= 8.8 ± 5.7, p= 0.47 rispetto ai pazienti sani. Percentuale media in SM= 13.2 ± 7.3, p = 0.03 rispetto ai pazienti sani).

Inoltre, nei siti con parodontite, è sempre risultato, un alto livello di metilazione di LINE-1, indice di iperespressione dell'enzima DNA metil-transferasi che si verifica in caso di malattia cronica.

Conclusioni: La terapia parodontale è in grado di riportare i livelli di metilazione dei geni analizzati nei SM ai livelli dei SS. Inoltre, l'ipermetilazione del genoma dei SM riflette l'upregulation dell'enzima DNAmetil-transferasi tipico della malattia cronica.

Keywords: Periodontal Diseases/therapy, Epigenetics, DNA Methylation, Inflammatory genes, Biomarkers

Introduction

Periodontitis is a multifactorial disease caused predominantly by gram-negative, anaerobic and microaerophilic bacteria that can colonize the subgingival regions **(Page, 1991; Page & Kornman, 1997),** and eventually trigger 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

Sessione Premio H.M. Goldman - H.M. Goldman Prize Session
XVIII Congresso Internazionale – 18th International Congress SIdP
Rimini (I), March 16th 2017

3

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 (turn off) or activation (turn on) 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)** that are reversible **(Lod et al, 2014),** and linked **(Jenuwein & Allis, 2001; Robertson & Wolffe, 2000).** Nevertheless, DNA methylation offers a more stable form of gene regulation **(Bäckdahl et al, 2009)** characterized by the addition of a methyl group, through DNA methyl-transferase enzymes, to the fifth carbon on the cytosine base within CpG islands, which are frequently found in the promoter region of the gene **(Sanders, 2006)**, consequently inhibiting gene expression.

Accordingly, most of the studies exploring the link between epigenetics and periodontal disease have investigated the changes in the *DNA methylation* of genes **(Baptista et al, 2014; Andia et al., 2015),** 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).** Emerging studies are also demonstrating the activation of inflammatory pathways like the NFkB signaling pathway by histone modifications **(Martins et al, 2016).**

Various 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, leading to over-expression of these mediators in inflamed tissues **(Oliveira et al, 2009; Andia et al, 2010; Zhang et al, 2010a; Ishida et al, 2012).** Nevertheless, hypermethylation has been interestingly described for certain genes in the chronic state of periodontal disease, as a down-regulatory mechanism to prevent unrestricted tissue destruction **(Zhang et al, 2010b).**

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 changes between healthy tissues and periodontitis after a three months follow-up. These findings suggest that periodontal therapy can influence epigenetic modifications. However, variations in the methylation level between both groups were not assessed at baseline (before therapy), 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 LINE-1, TNF-α, IFN-γ and COX-2 inflammatory genes in periodontitis patients following periodontal therapy in comparison to disease free subjects at both the site and patient levels.

Materials and Methods

This study was approved by the ethical committee of the University of Milan, Italy and was conducted in the period between October 2015 and June 2016.

- 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: "*disease free*" including ten patients with healthy

Sessione Premio H.M. Goldman - H.M. Goldman Prize Session
XVIII Congresso Internazionale – 18th International Congress SIdP
Rimini (I), March 16th 2017

4

periodontium and a "*periodontal disease group*" including ten chronic moderate periodontitis patients. All participants were voluntarily included in the study after explaining its objectives and obtaining their verbal and written informed consent.

Study participants fit the following inclusion criteria:

1. Patients older than 18 years of age.

2. Patients without any systemic diseases.

3. Non-smokers.

4. Patients of the same Caucasian origin (Italian descent).

5. **For the disease free group:** patients who showed no clinical signs of gingival inflammation and had no history of periodontitis were included.

6. **For the periodontal disease group:** patients diagnosed with chronic periodontitis, based on the American Academy of Periodontology (AAP) Workshop **(Armitage, 1999)** were included. Patients in this group must have had at least a normal site (PD < 4 mm with no signs of bleeding on probing, mobility and inflammation) and a periodontitis site (PD ≥ 5 mm) for later biopsy harvesting.

The exclusion criteria were the following:

1. Current pregnancy

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.

- **Study timeline & Periodontal Parameters:**

* *Baseline:* For all study participants, probing pocket 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. Next, gingival biopsies, using a 3 mm of diameter punch, were obtained from the disease free group during crown lengthening procedures or surgical removal of wisdom teeth. In the periodontal disease group, biopsies were harvested from two sites: a normal site and a periodontitis site with a purpose to compare methylation levels between both. Following biopsy collection, chronic periodontitis patients underwent conventional periodontal therapy; full mouth scaling and root planing with ultrasonic and manual instruments. Chlorhexidine mouthwash (0.2%) was prescribed for daily use (twice a day for twenty days).

* *Two weeks after periodontal therapy:* PPD, CAL & BOP, FMPS & FMBS were registered and gingival biopsies were harvested for the disease group only, from a normal and periodontitis site, to analyze the influence of periodontal therapy on epigenetic modifications, in comparison to baseline.

* *Eight weeks after periodontal therapy:* As at the 2 weeks time point, PPD, CAL, BOP, FMPS & FMBS were registered and gingival biopsies were harvested for the disease group only (from two sites), to analyze the influence of periodontal therapy on epigenetic changes, in comparison to those observed among disease free and periodontal disease groups at baseline and in the periodontal disease group at 2 weeks.

Sessione Premio H.M. Goldman - H.M. Goldman Prize Session
XVIII Congresso Internazionale – 18th International Congress SIdP
Rimini (I), March 16th 2017

5

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

Briefly, freshly harvested tissues were collected in vials and submerged in Allprotect Tissue Reagent (Qiagen, USA) to stabilize DNA immediately, and were stored at 2-8 °C for up to 6 months. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) following 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 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, 0.3 µl Pyrosequencing primer was annealed to the purified single-stranded PCR product, and Pyrosequencing was performed using the PyroMark MD System (Pyrosequencing, Inc.). The degree of methylation was expressed as a percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines (%5mC).

- Statistical Analysis:

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 normal and periodontitis sites. Statistical analyses were performed with Stata 13 (StataCorp. 2013) **(Rabe-Hesketh et al, 2008).**

Results

Twenty participants (8 males, 12 females) were included in this study, distributed into "disease free" (7 males, 3 females) and "periodontal disease" groups (3 males, 7 females). The age in the disease free 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 participants completed the study, as demonstrated in the CONSORT flow diagram **(figure 1).**

Success of periodontal therapy. We only reported the improvement of periodontal pocket depth in the disease group overtime, because this parameter provides an assessment of disease presence in respect to attachment loss/bone loss measurements which reflect past experience of periodontitis **(Tonetti & Claffey, 2005).** Periodontal therapy in the periodontal disease group was successful based on the changes in the mean of PPD over time. Mean PPD at baseline was 4.2 ± 0.4 mm indicating a moderate periodontal disease. After treatment mean PPD was 2.9 ± 0.4 mm (p < 0.001)

Sessione Premio H.M. Goldman - H.M. Goldman Prize Session
XVIII Congresso Internazionale – 18th International Congress SIdP
Rimini (I), March 16th 2017

6

at 2 weeks and 2.5 ± 0.3 (p < 0.001) at 8 weeks. In the disease free group PPD at baseline in all sites was ≤ 3mm. Demographic and clinical characteristics of the study sample are shown in **table (2).**

Periodontal disease patients present reduced methylation of TNF-α in normal and diseased sites compared with healthy population. The promoter region was more methylated in disease free 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.

IFN-γ present increased methylation within periodontitis tissues. The promoter region of IFN-γ gene was "*hypermethylated*" among all entities, with the disease free 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 disease free individuals (88.3% ± 2.1), while normal sites exhibited a slightly lower methylation (85.8% ± 6.5), implying a down-regulatory mechanism in periodontitis sites to cease further destruction of periodontal tissues. 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 disease free 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 highly "*hypomethylated*" in periodontium of disease free 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. High levels of COX-2 methylation in periodontitis sites can render an intrinsic protective mechanism capable of preventing the unrestrained breakdown of the periodontium. Such discrepancies in the mean of methylation between normal and periodontitis sites in comparison to free disease group at baseline were statistically significant (P=0.47 for normal sites vs disease free group, P=0.03 for periodontitis sites vs disease free 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 is still higher than the methylation level reported in the disease free 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). High levels of COX-2 methylation at baseline in the periodontal disease group can render an intrinsic protective mechanism capable of preventing the unrestrained breakdown of the periodontium

LINE-1 promoter methylation in periodontal disease. 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 disease free group (69.5% ± 3.5) and normal sites in diseased patients (69.4% ± 2.1), and remained fairly stable during the observation period. Nevertheless, periodontitis sites underwent increased methylation of the promoter region (70.2% ± 2.8), showing an increase in the methylation percentage at 8 weeks (71.7% ± 2.8). The

Sessione Premio H.M. Goldman - H.M. Goldman Prize Session
XVIII Congresso Internazionale – 18th International Congress SIdP
Rimini (I), March 16th 2017

7

variations in the methylation profile between different entities were not statistically significant (P value > 0.05).

Mean methylation percentages of the selected genes and p values are summarized in **table 3.** DNA methylation patterns for each gene among different groups and in different points of time are demonstrated in **figure 2.**

Conclusions

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 presents 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 the tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and the cyclooxygenase-2, (COX-2, also known as prostaglandin-endoperoxide synthase 2 or PTGS2) as key genes associated with periodontal disease progression. We also carefully selected the long interspersed nuclear element-1 (LINE-1) gene as a genome-wide readout for methylation. Of note, our cohort of patients includes Caucasians of Italian descent, and careful interpretation of the results must take into consideration the potential ethnicity influence over epigenetic modifications **(Kwabi-Addo et al, 2010; Straughen et al, 2015).**

Based on the findings of the present study, we can conclude that; (i) periodontal treatment does influence the DNA methylation pattern of various inflammatory genes, (ii) in periodontitis sites, the promoter region of different inflammatory genes was either hypo- or hypermethylated, depending on the activation of a down-regulatory mechanism to limit ongoing destruction of periodontal tissues, (iii) periodontal therapy is not sufficient to reset the methylation levels of inflammatory genes in periodontitis sites to health levels of periodontitis-free patients, at least on the short term. However, DNA methylation in periodontitis sites was restored to that of normal sites. (iv) The methylation profile from normal tissues was either restored or not to healthy patient levels, depending on the inflammatory gene, which suggests that unaffected sites from periodontal disease patients are more prone to periodontal tissue breakdown with the presence of other factors and thus should frequently be monitored within a tailored maintenance phase of periodontal therapy. In fact, 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).**

On a global methylation perspective, periodontally-diseased sites showed a different DNA methylation profile in comparison to normal sites and disease free patients, even after periodontal treatment. 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. In this context, future investigations should further explore the identification of specific factors that affect the local epigenetics of periodontal soft and hard tissues.

Sessione Premio H.M. Goldman - H.M. Goldman Prize Session
XVIII Congresso Internazionale – 18th International Congress SIdP
Rimini (I), March 16th 2017

8

Despite these findings, the results of the study must 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 wasn't possible because 2 weeks after periodontal therapy soft tissues at the biopsy site were not completely healed. Also, findings of this study 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.

References

Adcock, I. M., Tsaprouni, L., Bhavsar, P. and Ito, K. (2007) Epigenetic regulation of airway inflammation. *Current Opinion in Immunology* **19**, 694–700

Andia, D. C., de Oliveira, N. F. P., Casarin, R. C. V, Casati, M. Z., Line, S. R. P. and de Souza, A. P. (2010) DNA methylation status of the IL8 gene promoter in aggressive periodontitis. *Journal of Periodontology* **81**,1336–1341

Andia, D. C., Planello, A. C., Portinho, D., da Silva, R. A., Salmon, C. R., Sallum, E. A., Junior, F. H. N. and de Souza, A. P. (2015) DNA methylation analysis of SOCS1, SOCS3, and LINE-1 in microdissected gingival tissue. *Clinical Oral Investigations* **19**, 2337–2344

Bäckdahl, L., Bushell, A. and Beck, S. (2009) Inflammatory signalling as mediator of epigenetic modulation in tissue-specific chronic inflammation. *International Journal of Biochemistry and Cell Biology*. **41**, 176–184

Baptista, N. B., Portinho, D., Casarin, R. C. V, Vale, H. F., Casati, M. Z., De Souza, A. P. and Andia, D. C. (2014) DNA methylation levels of SOCS1 and LINE-1 in oral epithelial cells from aggressive periodontitis patients. *Archives of Oral Biology*. **59**, 670–678

Barros, S. P. and Offenbacher, S. (2014) Modifiable risk factors in periodontal disease: epigenetic regulation of gene expression in the inflammatory response. *Periodontology 2000* **64**, 95–110

Bird, A. (2002) DNA methylation patterns and epigenetic memory. *Genes and Development* **16**, 6–21

Borrell, L. N. and Papapanou, P. N. (2005) Analytical epidemiology of periodontitis. *Journal of Clinical Periodontology* **32**, 132–158

Bollati, V., Baccarelli, A., Hou, L., Bonzini, M., Fustinoni, S., Cavallo, D., Byun, H. M., Jiang, J., Marinelli, B., Pesatori, A. C., Bertazzi, P. A. and Yang, A. S. (2007) Changes in DNA methylation patterns in subjects exposed to low-dose benzene. *Cancer Research* **67**, 876–880

El Gazzar, M., Yoza, B. K., Hu, J. Y.-Q., Cousart, S. L. and McCall, C. E. (2007) Epigenetic silencing of tumor necrosis factor alpha during endotoxin tolerance. *The Journal of biological chemistry* **282**, 26857–26864

Fitzpatrick, D. R. and Wilson, C. B. (2003) Methylation and demethylation in the regulation of genes, cells, and responses in the immune system, *Clinical Immunology* **109**, 37–45

Ishida, K., Kobayashi, T., Ito, S., Komatsu, Y., Yokoyama, T., Okada, M., Abe, A., Murasawa, A. and Yoshie, H. (2012) Interleukin-6 Gene Promoter Methylation in Rheumatoid Arthritis and Chronic Periodontitis. *Journal of Periodontology* **83**, 917–925

Jenuwein, T. and Allis, C. D. (2001) Translating the histone code. *Science (New York, N.Y.)* **293**, 1074–1080

Kornman, K. S. (2008) Mapping the pathogenesis of periodontitis: a new look. *The Journal of periodontology* **79**, 1560–1568

Kwabi-Addo, B., Wang, S., Chung, W., Jelinek, J., Patierno, S. R., Wang, B. D., Andrawis, R., Lee, N. H., Apprey, V., Issa, J. P. and Ittmann, M. (2010) Identification of differentially methylated genes in normal prostate tissues from African American and Caucasian men. *Clinical Cancer Research* **16**, 3539–3547

Sessione Premio H.M. Goldman - H.M. Goldman Prize Session
XVIII Congresso Internazionale – 18th International Congress SIdP
Rimini (I), March 16th 2017

9

Larsson, L., Castilho, R. M. and Giannobile, W. V. (2015) Epigenetics and its role in periodontal diseases: a state-of-the-art review. *Journal of periodontology* **86**, 556–568

Lod, S., Johansson, T., Abrahamsson, K. H. and Larsson, L. (2014) The influence of epigenetics in relation to oral health. *International Journal of Dental Hygiene* **12**, 48–54

Martins, M. D., Jiao, Y., Larsson, L., Almeida, L. O., Garaicoa-Pazmino, C., Le, J. M., Squarize, C. H., Inohara, N., Giannobile, W. V. and Castilho, R. M. (2016) Epigenetic Modifications of Histones in Periodontal Disease. *Journal of Dental Research* **95**, 215–222

Mdala, I., Olsen, I., Haffajee, A. D., Socransky, S. S., Thoresen, M. and De Blasio, B. F. (2014) Comparing clinical attachment level and pocket depth for predicting periodontal disease progression in healthy sites of patients with chronic periodontitis using multi-state Markov models. *Journal of Clinical Periodontology* **41**, 837–845

Ngollo, M., Dagdemir, A., Karsli-Ceppioglu, S., Judes, G., Pajon, A., Penault-Llorca, F., Boiteux, J.-P., Bignon, Y.-J., Guy, L. and Bernard-Gallon, D. J. (2014) Epigenetic modifications in prostate cancer. *Epigenomics* **6**, 415–426

Oliveira, N. F. P., Damm, G. R., Andia, D. C., Salmon, C., Nociti, F. H., Line, S. R. P. and De Souza, A. P. (2009) DNA methylation status of the IL8 gene promoter in oral cells of smokers and non-smokers with chronic periodontitis. *Journal of Clinical Periodontology* **36**, 719–725

Page, R. C. (1991) The role of inflammatory mediators in the pathogenesis of periodontal disease. *Journal of Periodontal Research* **26**, 230–242

Page, R. C. and Kornman, K. S. (1997) The pathogenesis of human periodontitis: an introduction. *Periodontology 2000* **14**, 9–11

Robertson, K. D. and Wolffe, A. P. (2000) DNA methylation in health and disease. *Nature reviews. Genetics* **1**, 11–19

Sanders, V. M. (2006) Epigenetic regulation of Th1 and Th2 cell development. *Brain, Behavior, and Immunity* **20**, 317–324

Schulz, S., Immel, U. D., Just, L., Schaller, H. G., Gläser, C. and Reichert, S. (2016) Epigenetic characteristics in inflammatory candidate genes in aggressive periodontitis. *Human Immunology* **77**, 71–75

Shaw, R. (2006) The epigenetics of oral cancer. *International Journal of Oral and Maxillofacial Surgery* **35**, 101–108

Straughen, J. K., Sipahi, L., Uddin, M., Misra, D. P. and Misra, V. K. (2015) Racial differences in IGF1 methylation and birth weight. *Clinical Epigenetics* **7**, 47

Takashiba, S. and Naruishi K. (2006) Gene polymorphisms in periodontal health and disease. *Periodontology 2000* **40**, 94–106

Tarantini, L., Bonzini, M., Tripodi, A., Angelici, L., Nordio, F., Cantone, L., Apostoli, P., Bertazzi, P. A. and Baccarelli, A. A. (2013) Blood hypomethylation of inflammatory genes mediates the effects of metal-rich airborne pollutants on blood coagulation. *Occupational and Environmental Medicine* **70**, 418–25

Tonetti, M.S. and Claffey, N. (2005) European Workshop in Periodontology group C. Advances in the progression of periodontitis and proposal of definitions of a periodontitis case and disease progression for use in risk factor research. Group C consensus report of the 5th European Workshop in Periodontology. *Journal of Clinical Periodontology* **32**, 210-213

Wilson, A. G. (2008) Epigenetic regulation of gene expression in the inflammatory response and relevance to common diseases. *Journal of Periodontol*ogy **79**, 1514–1519

Zhang, S., Crivello, A., Offenbacher, S., Moretti, A., Paquette, D. W. and Barros, S. P. (2010 a) Interferon-gamma promoter hypomethylation and increased expression in chronic periodontitis. *Journal of Clinical Periodontology* **37**, 953–961

Zhang, S., Barros, S. P., Niculescu, M. D., Moretti, A. J., Preisser, J. S. and Offenbacher, S. (2010 b) Alteration of PTGS2 promoter methylation in chronic periodontitis. *Journal of Dental Research* **89**, 133-137

10

Sessione Premio H.M. Goldman - H.M. Goldman Prize Session
XVIII Congresso Internazionale – 18th International Congress SIdP
Rimini (I), March 16th 2017

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/AAAAAAATTAAACC	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	Chr 6 Pos1:31651172; Pos2:31651157; Pos3:31651155; Pos4: 31651149

* Nucleotides at which DNA methylation was measured are underlined

* Chr = Chromosome, Pos= Position

Sessione Premio H.M. Goldman - H.M. Goldman Prize Session
XVIII Congresso Internazionale – 18th International Congress SIdP
Rimini (I), March 16th 2017

11

Table 2. Demographic and clinical characteristics of the study sample

Demographic/Clinical characteristics	Disease Free Group (n =10)	Periodontal Disease Group (n =10)		
Males/Females	7/3	3/7		
Age (years) (Mean ± SD)	53.3 ± 12.3	46.6 ± 10.0		
Probing Depth (mm) (Mean ± SD)	*At baseline*	*At baseline*	*Two weeks after periodontal treatment*	*Eight weeks after periodontal treatment*
	≤ 3	4.2 ± 0.4	2.9 ± 0.4	2.5 ± 0.4
		Reference	P <0.001	P <0.001

*P-values from random intercept linear regression model.

Table 3. Methylation of selected genes across groups and over time. Results of random-intercept linear regression models

Genes	Group	Methylation percentage (Mean ± SD) At baseline	Two weeks after periodontal treatment	Eight weeks after Periodontal treatment
TNF-α	Disease Free	36.6 ± 9.2	-	-
	Periodontal Disease/Normal Sites	34.1 ± 5.2	33.9 ± 6.5	34.0 ± 9.3
		P=0.44 vs Disease free	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 Disease free	P=0.97 vs baseline	P=0.50 vs baseline
IFN-γ	Disease Free	88.6 ± 1.4	-	-
	Periodontal Disease/Normal Sites	85.8 ± 6.5	87.7 ± 3.5	88.8 ± 2.8
		P=0.10 vs Disease free	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 Disease free	P=0.89 vs baseline	P=0.08 vs baseline
COX-2	Disease Free	6.7 ± 7.6	-	-
	Periodontal Disease/Normal Sites	8.8 ± 5.7	6.6 ± 3.6	5.0 ± 1.9
		P=0.47 vs Disease free	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 Disease free	P <0.001 vs baseline	P=0.004 vs baseline
LINE-1	Disease Free	69.5 ± 3.5	-	-
	Periodontal Disease/ Normal Sites	69.4 ± 2.1	70.0 ± 2.3	69.5 ± 1.4
		P=0.92 vs Disease free	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 Disease free	P=0.61 vs baseline	P=0.22 vs baseline

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

Figure 1. CONSORT Flow Diagram of the Study Sample

```
            ┌─────────────────────────────────┐
            │  Total number of eligible patients│
            │            (n =20)               │
            └─────────────────────────────────┘
                           │
              ┌────────────┴────────────┐
              ▼                         ▼
      ┌───────────────┐         ┌───────────────┐
      │  Disease free │         │    Chronic    │
      │ group (n = 10)│         │   Moderate    │
      │               │         │ Periodontitis │
      │               │         │ group (n = 10)│
      └───────────────┘         └───────────────┘
              │                         │
              ▼                         ▼
```

┌─────────────────────────────────┐   ┌─────────────────────────────────┐
│ - Clinical parameters were   │   │ - Clinical parameters were   │
│   registered at baseline   │   │   registered at baseline, then 2 │
│   │   │   weeks and 8 weeks post-   │
│ - Gingival biopsies were   │   │   periodontal therapy   │
│   obtained only at baseline   │   │   │
│   during crown lengthening   │   │ - Gingival biopsies were   │
│   procedures or surgical   │   │   harvested from two sites at   │
│   removal of wisdom teeth   │   │   all points of time: a normal   │
│   │   │   site and a periodontitis site │
└─────────────────────────────────┘   └─────────────────────────────────┘

```
                           ▼
            ┌─────────────────────────────────┐
            │  19 Patients completed the study │
            └─────────────────────────────────┘
```
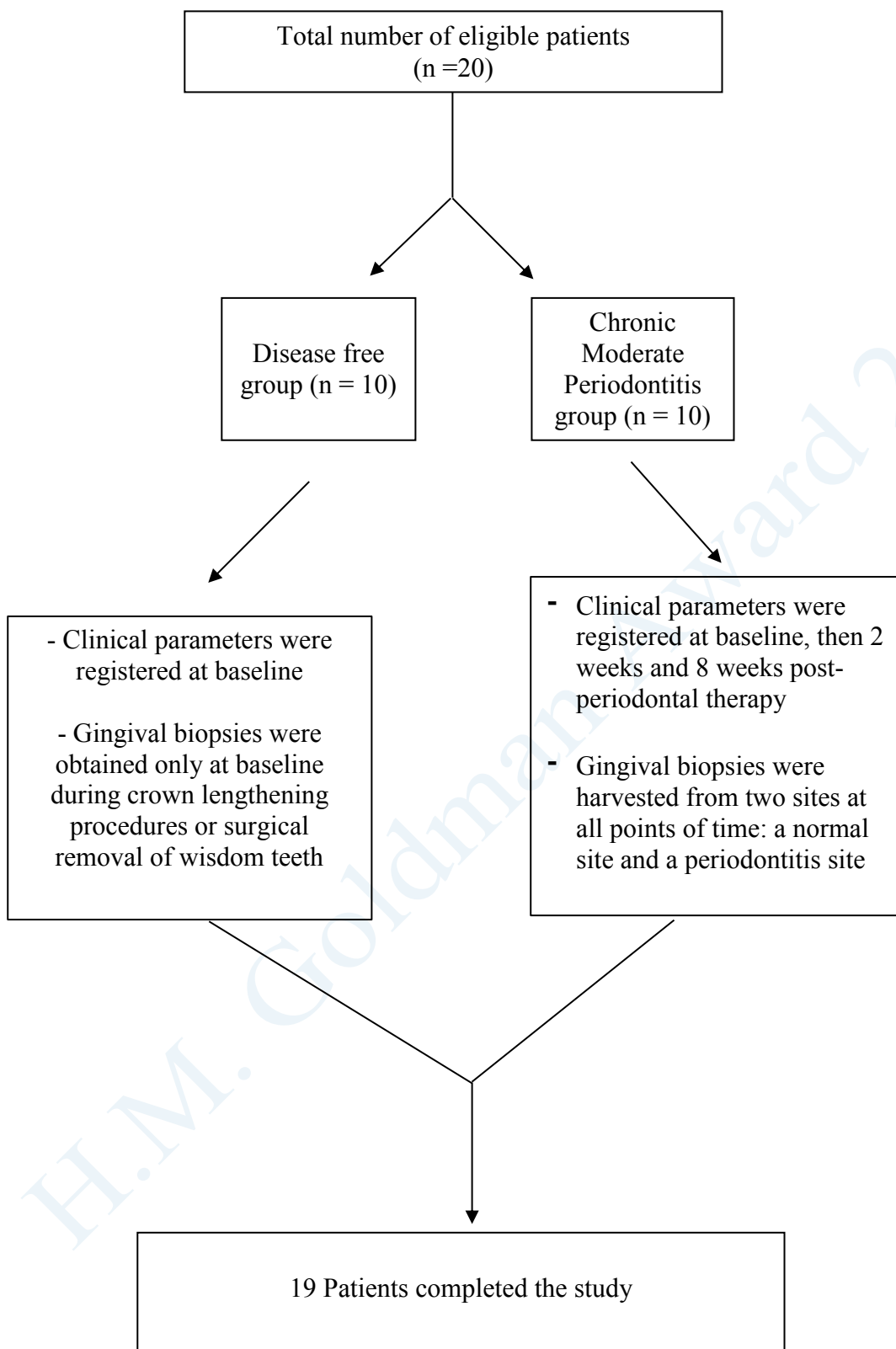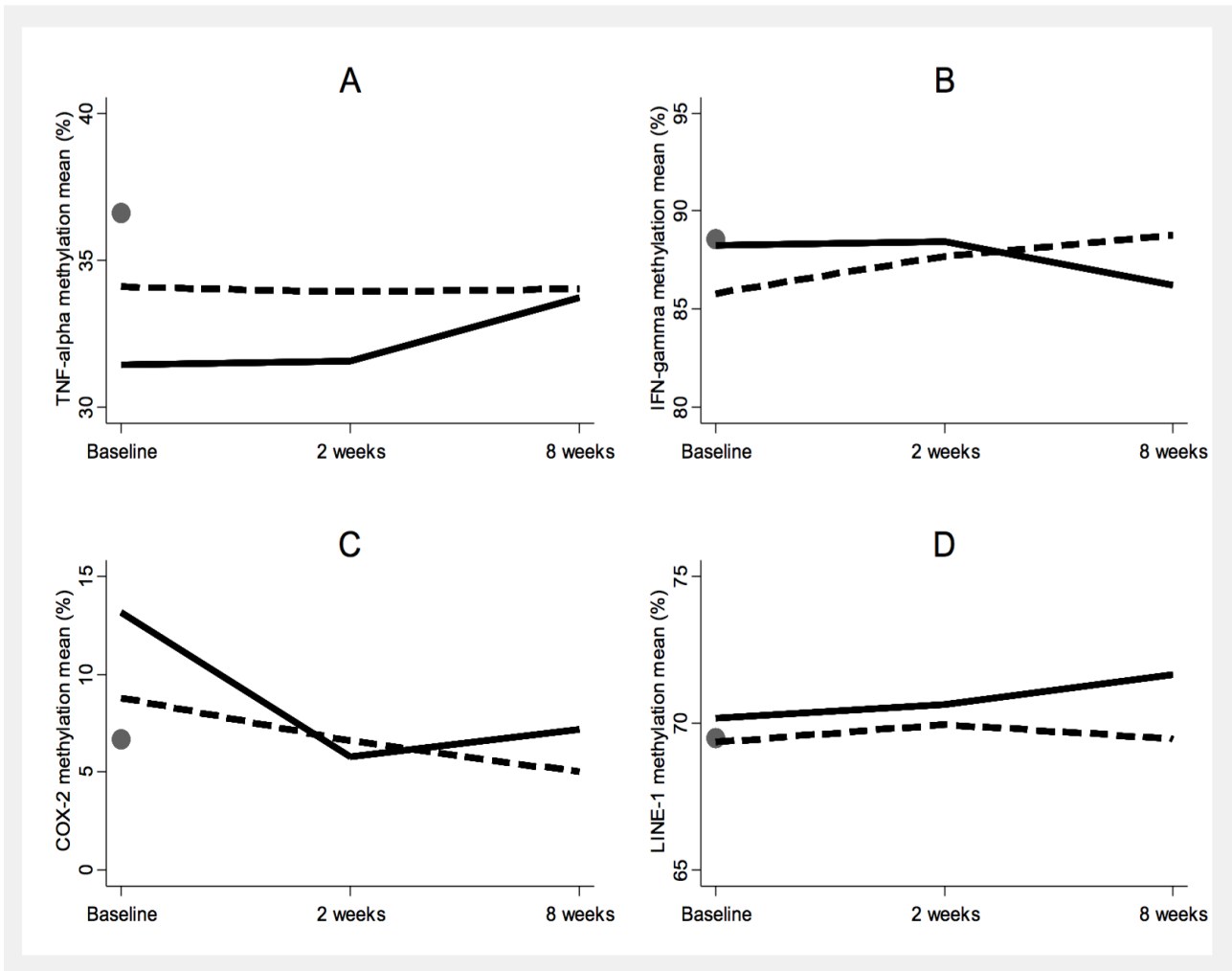
Figure 2. DNA methylation patterns among different groups and at different points of time, for TNF-α, IFN- γ, COX-2 and LINE-1

Circle: Disease-free group

Dashed line: Periodontal disease group, normal sites

Continuous line: Periodontal disease group, periodontitis sites