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ORIGINAL ARTICLE

Shared epigenetic alterations between oral cancer and periodontitis: A preliminary study

Andrea Gabusi[1] | Davide B. Gissi[1] | Sara Grillini[1] | Martina Stefanini[1] | Achille Tarsitano[1,2] | Claudio Marchetti[1,2] | Maria Pia Foschini[3] | Lucio Montebugnoli[1] | Luca Morandi[4,5]

[1]Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy

[2]Oral and Maxillo-facial Surgery Unit, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Bologna, Italy

[3]Section of Anatomic Pathology at Bellaria Hospital, Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy

[4]Functional MR Unit, IRCCS Istituto delle Scienze Neurologiche di Bologna, Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy

[5]IRCCS Istituto delle Scienze Neurologiche di Bologna, Bologna, Italy

Correspondence
Davide B. Gissi, Department of Biomedical and Neuromotor Sciences, Section of Oral Sciences, University of Bologna, Via San Vitale 59, 40159 Bologna, Italy.
Email: davide.gissi@unibo.it

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Abstract

Introduction: We recently developed a non-invasive sampling procedure for oral squamous cell carcinoma (OSCC) detection based on DNA methylation analysis of a panel of 13 genes. Oral cancer, as well as acute and chronic inflammatory diseases, may influence the methylation level of several genes in the oral cavity. In the present study, we evaluated the presence of periodontal disease (PD) and the methylation status using our 13-gene panel.

Methods: Oral brushing specimens were collected from three different patient groups: 23 gingival OSCC patients, 15 patients affected by PD, and 15 healthy volunteers lacking evidence of PD. DNA methylation analysis was performed and each sample was determined to be positive or negative based on a predefined cut-off value.

Results: Positive results were found for 23/23 OSCC patients, 3/15 PD patients, and 0/15 samples from healthy volunteers. The *GP1BB* and *MIR193* genes in the PD group exhibited mean methylation levels similar to OSCC patients. *ZAP70* showed different methylation levels among three groups.

Conclusion: Preliminary data identified shared epigenetic alterations between PD and OSCC patients in two inflammatory genes (*GP1BB* and *MIR193*). This study may help to identify potential links between the two diseases and serve as a starting point for the future research focused on pathogenesis.

KEYWORDS
13 gene panel, DNA methylation analysis, oral squamous cell carcinoma, periodontal disease

1 | INTRODUCTION

Chronic inflammation promotes carcinogenesis in many neoplastic diseases, including intestinal inflammation resulting in colon cancer, oesophageal inflammation resulting in oesophageal cancer, and liver inflammation increasing the prevalence of hepatocellular carcinoma (Coussens & Werb, 2002). Periodontal disease (PD) is a chronic inflammatory condition frequently encountered in the oral cavity that affects the tooth-supporting structure. It is caused by an exaggerated inflammatory response directed towards the supporting alveolar bone and results from interactions between periodontal pathogens and the immunological response of the host (Caton et al., 2018). Epidemiological research into PD has recently focused on the role of epigenetic modifications of inflammation-related

genes, including inflammatory cytokines and other inflammation-related genes (Li et al., 2018). Epigenetics is the study of inheritable changes in gene expression without changes in the underlying deoxyribonucleic acid (DNA) sequence. The main mechanisms of epigenetic regulation include DNA methylation, posttranslational modifications of histone proteins, and micro-ribonucleic acids (miRNAs). DNA methylation represents one of the most extensively studied epigenetic modifications: it refers to covalent addition of a methyl group to 5-carbon (C5) position of cytosine bases that are located 5′ to a guanosine base in a CpG dinucleotide. DNA hypermethylation at the promoter region usually results in transcriptional 'silencing' of a gene, whereas DNA hypomethylation results in a transcriptional 'activation' of a gene (Mascolo et al., 2012).

Barros and Offenbacher demonstrated that the epigenome differed between inflamed periodontal sites and non-inflamed sites in the same patient (Barros & Offenbacher, 2014). Zhang et al. found that TNF-α has a lower degree of methylation in patients with chronic periodontitis compared to healthy controls (Zhang et al., 2013). Similarly, Zhang et al. described IFN-γ hypomethylation in patients with chronic periodontitis vs. healthy controls (Zhang et al., 2010). These results describe a pro-inflammatory methylation profile for PD and confirm the status of chronic inflammation.

The role of DNA methylation in oral oncology and inflammation has been a topic of considerable interest in the last few years. Numerous authors recently identified hypermethylation and the subsequent silencing of several tumour suppressor genes and/or hypomethylation and the subsequent activation of proto-oncogenes in OSCC (Arantes et al., 2015; Demokan et al., 2010; Eljabo et al., 2018; Huang et al., 2014; Liyanage et al., 2019; Nagata et al., 2012; Pattani et al., 2010; Puttipanyalears et al., 2018; Schussel et al., 2013; Viet & Schmidt, 2008). Although PD is not considered a pre-neoplastic condition of the oral cavity, it has been identified as an independent risk factor for OSCC development in different epidemiological studies (Gopinath et al., 2020; Karmakar et al., 2020; Tezal et al., 2009). This has led some authors to identify a similar molecular pattern between PD and OSCC (Li et al., 2018; Planello et al., 2016).

Our group previously demonstrated the presence of epigenetic alterations and aberrant DNA methylation of the following genes associated with OSCC and its precursor lesions: *ZAP70*, *ITGA4*, *KIF1A*, *PARP15*, *EPHX3*, *NTM*, *LRRTM1*, *FLI1*, *MIR193*, *LINC00599*, *MIR296*, *TERT* and *GP1BB*. Linear discriminant analysis was used to develop an algorithm that clearly discriminated clinically healthy mucosa lesions from potentially malignant or malignant oral lesions (Morandi et al., 2017). A broad multicentre study confirmed the feasibility and diagnostic value of this minimally invasive procedure (Gissi et al., 2021).

In this study, we sought to investigate possible crosstalk between PD and OSCC in the epigenome using a set of 13 genes previously demonstrated to be altered in OSCC. Therefore, our 13-gene DNA methylation analysis was applied to brushing samples from patients with OSCC or PD and gingival brushing samples from healthy subjects.

2 | MATERIALS AND METHODS

All clinical investigations were conducted in accordance with the principles of the Declaration of Helsinki, and the study was approved by the local ethics committee (520/2018/Sper/AOUBo, 12 Dec 2018). All information pertaining to human material used in this study was managed using anonymous numerical codes.

We collected 53 oral brushing specimens from three different study groups of patients between January 2018 and January 2019 at the Department of Oral Sciences, University of Bologna.

GROUP 1 Patients (23) with a consecutive clinical and histological diagnosis of gingival OSCC. Oral brushing of these patients was performed on the entire surface of the suspected lesion before incisional biopsy, and samples were used in the study only after histological confirmation of OSCC.

GROUP 2 Patients (15) with a consecutive clinical and radiographical diagnosis of Stage III-IV localized or generalized PD according to the new classification proposed by the recent 2017 World Workshop on Periodontal and Peri-implant Diseases and Conditions (Caton et al., 2018). This group of patients was characterized by interproximal clinical attachment loss ≥5 mm at site of greatest loss, probing pockets depth >5 mm and bleeding on probing (BOP). In particular, in this group of patients, surfaces of attached gingiva correspondent to deepest pockets (either mandible or maxilla), with a pocket depth >5 mm with BOP, were gently brushed excluding areas of marginal and sulcular gingiva. The intention was to collect only keratinocytes avoiding presence of other cell types (i.e. inflammatory cells, eritrocytes) that may flaw methylation results.

GROUP 3 Healthy patients (15) studied as the control group. Cell sample collection via oral brushing was limited to gingival sites with signs of inflammation but in absence of bone resorption and a pocket depth <4–5 mm.

The study included only gingival brushing samples to minimize the confounding effect potentially derived from heterogeneous methylation levels in samples collected at different oral sites. Therefore, we only selected consecutive cases of gingival OSCC for GROUP 1. No patients in GROUP 2 or GROUP 3 had a history of OSCC or developed potentially malignant and/or neoplastic lesions during the study period.

Demographic and clinical variables such as age, oral site, sex and smoke were recorded in each group. In OSCC group, TNM status and periodontal stage were also recorded.

Table 1 summarizes the clinical features of the study population.

2.1 | Oral brushing method

Brushing specimens were collected from three different study groups of patients according to a previously described protocol (Morandi et al., 2015, 2017; Gissi et al., 2018, 2019; Gissi, Gabusi, et al., 2020; Gissi, Fabbri, et al., 2020). After brushing, each cytobrush sample was placed in a 2-ml tube containing DNAshield (Zymo Research) and stored at −20°C for DNA/RNA preservation.

TABLE 1 Demographic and clinical features of the studied population

	n.	Sex	Median age	Smoking status	Sampling site	TNM status	Periodontal stage
OSCC group	23	11 males 12 females	70.35 ± 11.95	4 smokers 19 no-smokers	14 superior gum 9 inferior gum	5 T1N0M0 5 T2N0M0 1 T4N1M0 10 T4N0M0 1 T4N3M0 1 TIS	2 stage I-II 21 stage III-IV
Periodontal disease group	15	7 males 8 females	58.67 ± 16.92	3 smokers 12 no-smokers	7 superior gum 8 inferior gum		
Healthy control group	15	6 males 9 females	49.6 ± 19.13	6 smokers 9 no-smokers	9 superior gum 6 inferior gum		

2.2 | DNA methylation analysis

Thirteen-gene DNA methylation analysis was performed as described by Morandi et al. (Morandi et al., 2017). Briefly, DNA from exfoliated cells was purified using the MasterPure Complete DNA Purification Kit™ (MC85200; Lucigen). 100–500 ng of DNA were treated with sodium bisulphite using the EZ DNA Methylation-Lightning Kit™ (D5031; Zymo Research) according to the manufacturers' instructions. The quantitative DNA methylation analysis included next-generation sequencing for the following genes: *ZAP70*, *ITGA4*, *KIF1A*, *PARP15*, *EPHX3*, *NTM*, *LRRTM1*, *FLI1*, *MIR193*, *LINC00599*, *MIR296*, *TERT* and *GP1BB*. Libraries were prepared using the Nextera™ Index Kit following a locus-specific bisulphite amplicon approach (Morandi et al., 2017) and loaded into MiSEQ (15,027,617, Illumina). The FASTQ output files underwent quality control processing (>Q30) and were converted into FASTA format in a Galaxy Project environment (Afgan et al., 2018). The methylation ratio of each CpG was calculated in parallel using various tools: BSPAT (http://cbc. Case.edu/BSPAT/index.jsp) (Hu et al., 2015) and BWAmeth in a Galaxy Project environment (Europe), followed by the MethylDackel tool (https://github.com/dpryan79/MethylDackel), EPIC-TABSAT (Krainer et al., 2019) and Kismeth (Gruntman et al., 2008). Once having catalogued methylation levels for all CpGs in an excel file, the evaluation of differential DNA methylation with group comparison was performed by Methylation plotter (Mallona et al., 2014).

In our previous study (Morandi et al., 2017), the best CpGs identified by ROC analysis were used to generate an optimal algorithm based on multiclass linear discriminant analysis. This allowed us to accurately identify OSCC with a threshold of 1.06 as the best value for sensitivity and specificity (AUC = 0.981). Values exceeding the threshold of 1.06 were considered to indicate positive results.

2.3 | Statistical analysis

Based on our generated algorithm score, each sample was analysed as a numeric variable in the statistical analysis. The standardized skewness and kurtosis values were not within the data range for a normal distribution. Therefore, a multilevel mixed-effects ordered logistic regression with stepwise selection was fitted to evaluate the relationships between algorithmic scores and the following variables: group (OSCC/PD/control), sex (male/female), age (<65/>65 years), smoking status (no/yes), periodontal stage (stage I-II/III-IV) and brushing site (maxilla/mandible). The same analysis was performed for the methylation level of the 13 genes (*ZAP70*, *ITGA4*, *KIF1A*, *PARP15*, *EPHX3*, *NTM*, *LRRTM1*, *FLI1*, *MIR193*, *LINC00599*, *MIR296*, *TERT*, *GP1BB*). Multiple comparison tests with a Bonferroni correction were used to evaluate whether differences between groups were statistically significant. *p*-values <0.05 were considered to indicate statistical significance for all analyses.

3 | RESULTS

3.1 | Thirteen-gene DNA methylation algorithm score analysis

GROUP 1 OSCC patients had a mean score of 3.06 ± 1.4 (range 1.11–8.1), and all 23 (100%) specimens scored higher than the threshold value of 1.06. A mean score of 0.73 ± 0.7 (range −0.6 – 2.4) was calculated from brushing specimens collected from GROUP 2 PD patients. Three of 15 (20%) specimens exceeded the threshold value of 1.06 previously validated for OSCC discrimination (Figure 1) (Morandi et al., 2017). The control group had a mean score of −0.89 ± 1.02 (range − 2.77 − 0.87), and all specimens collected scored below 1.06.

Multilevel mixed-effects ordered logistic regression revealed significant differences between the groups ($p < 0.01$; Figure 1). No other patient variables analysed (age, sex, smoking status, periodontal stage and site) had a significant effect on the calculated score. Post-hoc analysis with the Bonferroni correction comparing the effect size in each group indicated that samples from PD patients had significantly higher scores than brushing specimens from healthy controls. As expected, higher scores were found for OSCC lesions compared to the other two groups (Figure 1).

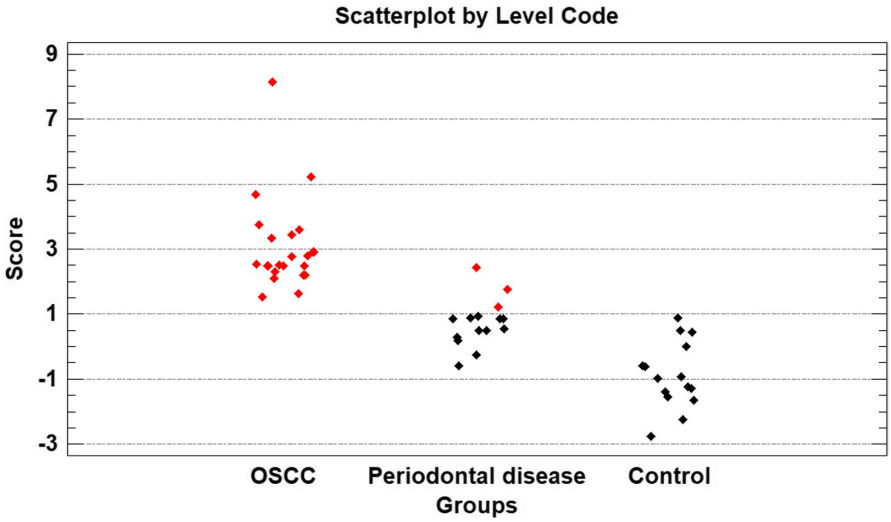

FIGURE 1 Scatterplot showing the scores calculated using the algorithm for the OSCC, PD, and control groups. Red dots correspond to brushing samples with scores that exceeded the threshold value (>1.06)

3.2 | Analysis of individual CpGs

The methylation pattern of OSCC clearly differed from normal samples as previously reported (Morandi et al., 2017). Ten genes were found to be hypermethylated and three hypomethylated in OSCC.

Multilevel mixed-effects ordered logistic regression showed that the only statistically significant variable for methylation pattern differences was group divison for all but one gene (*MIR296*). Multiple comparison analyses identified differences in the methylation pattern between groups. *GP1BB* had similar methylation level in GROUP 1 and GROUP 2 (hypomethyled), whereas a distinct methylation level was found in the group of healthy controls (GROUP 3). *MIR193* had similar methylation level in GROUP 1 and GROUP 2 (hypermethyled) and significantly different with respect to the group of healthy controls (GROUP 3). The methylation profile of *ZAP70* differed across the three groups (Figure 2).

Nine genes (*KIF1A*, *PARP15*, *ITGA4*, *NTM*, *EPHX3*, *LINC00599*, *FLI1*, *LRRTM1*, and *TERT*) were found to have unique methylation levels in GROUP 1 OSCC patients (only *TERT* resulting hypomethylated), whereas no differences between GROUP 2 PD patients and GROUP 3 control subjects were noted. Table 2 presents quantitative mean methylation levels of most informative CpGs of each gene and the results of multiple comparative analyses.

Figure S1 shows the methylation profile plots for each CpG investigated (total number: 231) of all 13 genes obtained using the Methylation Plotter tool (Mallona et al., 2014). Figure S2 shows box plots for each single gene. File S1 summarized all aggregated data and Kruskal–Wallis calculation. Among the 231 CpG analyzed, only 5 out of 20 for *LINC00599*, 2 out of 14 for *EPHX3*, and 3 out of 4 for *MIR296* were found not statistically significant (see Figure S1 for details).

4 | DISCUSSION

In this preliminary study, we applied 13-gene DNA methylation analysis to gingival brushing specimens collected from patients with PD and compared the results to those from brushing specimens from gingival OSCC lesions and gingiva specimens from healthy volunteers.

The aim of this study was to investigate possible epigenetic resemblances between PD patients and OSCC patients. Some authors reported a significantly higher OSCC risk in periodontally compromised patients (Gopinath et al., 2020; Karmakar et al., 2020; Tezal et al., 2009). Present results are in agreement with previous reports since 21/23 (91.3%) of consecutive gingival OSCC patients enrolled in the present study also exhibited signs of stage III-IV periodontal disease.

Because chronic periodontitis only affects gingival mucosa, brushing samples from patients with PD were collected from the most representative gingival area. We minimized the hypothetical bias of heterogeneous methylation in GROUP 1 by only including oral carcinomas that developed in the gingiva. Likewise, we limited the collection of oral brushing samples to the gingival area for GROUP 3 (control group of healthy volunteers).

As expected, the scores of 100% of the OSCC brushing specimens exceeded the threshold value of 1.06, whereas all 15 patients from the control group (GROUP 3) and 12 of 15 PD patients had scores indicating negative results. Three of the 15 patients affected by PD (20%) had scores that exceeded the threshold value. Multiple comparisons showed that the mean methylation levels in PD patients (GROUP 2) was significantly lower than those in GROUP 1 OSCC patients ($p < 0.05$) and significantly higher ($p < 0.05$) than those in the control group (GROUP 3).

The mean methylation levels of 9 out of the 13 genes (*KIF1A*, *PARP15*, *ITGA4*, *NTM*, *EPHX3*, *LINC00599*, *FLI1*, *LRRTM1 and TERT)* were similar between the PD and control group but differed for the OSCC patients. Two genes (*MIR193* and *GP1BB*) had the same methylation levels in the OSCC and PD groups. *ZAP70* had a distinct mean methylation level in each group, with the PD group having a mean score below that of the OSCC group and above that of the control group.

GP1BB encodes a transmembrane protein that constitutes the receptor for the von Willebrand factor. It mediates platelet

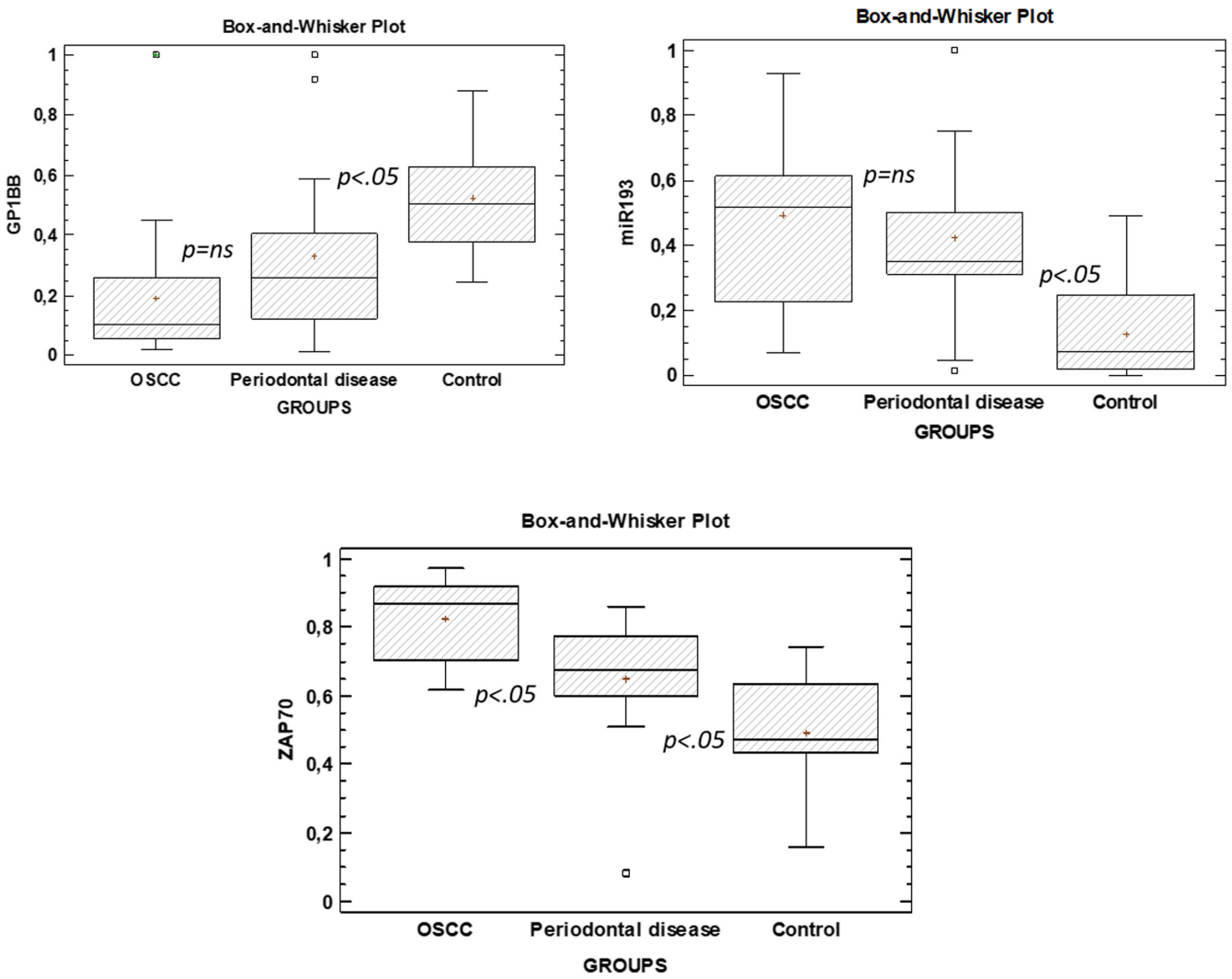

FIGURE 2 Box plot showing the between-group scores for *GP1BB*, *MIR193*, and *ZAP70* based on the CpG positions used in the choice algorithm. *MIR193* (hypermethyled) and *GP1BB* (hypomethyled) were methylated at similar levels in the OSCC and PD groups, whereas different methylation levels were found in the control group. The methylation profile of ZAP70 differed among the three groups

adhesion in arterial circulation and its mutations are associated with Bernard-Soulier syndrome, a rare inherited bleeding disorder (Savoia et al., 2011). A recent paper demonstrated that some pathogenic bacteria, such as *Porphiromonas gingivalis*, may interact with platelets to trigger their activation and aggregation (Chen et al., 2020). *MIR193* has been found to be suppressed in various tumours, including OSCC, melanoma, and breast and lung cancers (Khordadmehr et al., 2019). It is also associated with numerous immune diseases, including inflammatory bowel disease, lupus erythematosus, and rheumatoid arthritis (Khordadmehr et al., 2019). A recent paper found low *MIR193* expression in inflamed gingiva (Ogata et al., 2014). *ZAP70* encodes a 70-kDa ζ-chain-associated protein kinase, a tyrosine kinase normally expressed by natural killer cells and T cells. Its hypermethylation has only been associated with an unfavourable disease course for chronic lymphocytic leukaemia (Claus et al., 2014) and OSCC (Marsit et al., 2009; Morandi et al., 2017), including disease progression and lower overall survival. It is well known that natural killer cells and T cells play prominent roles in not only carcinogenesis but also chronic inflammatory diseases (Seidel et al., 2020). These three genes may have contributed to the elevated scores of patients with PD. In a previous study, a cytological examination applied to the same procedure of oral brushing, revealed only the presence of keratinocytes from the upper and medium layers (Gissi et al., 2018). In the present study, gingival brushing cell collection protocol excluded inflamed areas of marginal and sulcular gingiva in group 2 and 3 with the aim to collect only keratinocytes. However, the absence of cytological examination in the design of the present study cannot exclude the presence of inflammatory cells in brushing cell collection and future investigations should be designed to evaluate this confounding factor in the interpretation of DNA methylation profiles.

There are no known studies in the literature that demonstrate the presence of an altered methylation pattern for any of the 13 genes with respect to specific inflammatory conditions of the oral cavity, such as PD. These reported data seem to support common

TABLE 2 Multiple range test with Bonferroni correction comparing quantitative methylation levels of most informative CpGs of each gene in three different groups (OSCC group, periodontal disease group and control group)

Gene	Position	OSCC (23 cases)	Periodontal disease (15 cases)	Control (15 cases)
ZAP70	*Chr2: 98340854*	0.82 ± 0.12^{a}	0.65 ± 0.19^{b}	0.49 ± 0.4^{c}
*GP1BB**	*Chr22: 19710956*	0.19 ± 0.22^{a}	0.33 ± 0.3^{a}	0.52 ± 0.19^{b}
KIF1A	*Chr2: 241759621*	0.31 ± 0.29^{a}	0.02 ± 0.06^{b}	0.01 ± 0.01^{b}
PARP15	*Chr3: 122296586*	0.18 ± 0.21^{a}	0.04 ± 0.07^{b}	0.03 ± 0.02^{b}
ITGA4	*Chr2: 1823229.02*	0.15 ± 0.19^{a}	0.02 ± 0.02^{b}	0.02 ± 0.01^{b}
NTM	*Chr11:131781167*	0.21 ± 0.26^{a}	0.01 ± 0.03^{b}	0.02 ± 0.03^{b}
*miR193A**	*Chr17: 29886944*	0.49 ± 0.26^{a}	0.42 ± 0.3^{a}	0.12 ± 0.14^{b}
EPHX3	*Chr19: 15342885*	0.38 ± 0.34^{a}	0.01 ± 0.04^{b}	0.01 ± 0.01^{b}
LINC00599	*Chr8: 9760888*	0.09 ± 0.14^{a}	0.01 ± 0.02^{b}	0.01 ± 0.02^{b}
FLI1	*Chr11:128564158*	0.13 ± 0.22^{a}	0.01 ± 0.02^{b}	0.01 ± 0.01^{b}
miR296	*Chr20: 57392374*	0.92 ± 0.19^{a}	0.98 ± 0.03^{a}	0.98 ± 0.03^{a}
LRRTM1	*Chr2: 80531799*	0.24 ± 0.26^{a}	0.02 ± 0.03^{b}	0.06 ± 0.07^{b}
TERT	*Chr5: 1279758*	0.92 ± 0.06^{a}	0.97 ± 0.03^{b}	0.98 ± 0.02^{b}

Note: The means within a row with unlike superscript letters (^{abc}) were significantly different at $p < 0.05$.

(*) indicates CpGs with superimposable methylation profiles between OSCC group and Periodontal Disease group but distinct with respect to Control group.

epigenetic pathways between PD and OSCC, which is in agreement with previous reports. Li et al. used bioinformatics to identify seven differentially expressed genes (*MPPED1*, *PROC*, *TUBA4B*, *PLD6*, *PSHPH4*, *RSPH9* and *CSPG4*) with similar methylation levels in both OSCC and chronic periodontitis (Li et al., 2018). Planello et al. found that 21% of the hypermethylated CpG sites in chronic periodontitis were hypermethylated in a dataset of 301 OSCC patients (Planello et al., 2016).

The data from this limited study with a small population raise a clinical interesting issue: the interpretation of the three patients affected by PD with methylation scores exceeding the threshold value, which was previously validated for OSCC patients. Altered expressions of DNA methylation, miRNAs, and signalling pathways have been reported to play crucial roles in the onset and progression of both PD and OSCC, but knowledge in this domain is still limited (Li et al., 2018; Planello et al., 2016). Strict follow-up of these patients is ongoing and none of the patients developed OSCC upon completion of this manuscript. Nevertheless, it is important to verify whether the three positive PD patients have an increased risk of developing OSCC or if they are 'simply' false-positive cases.

In a seminal study, 13-gene DNA methylation analysis revealed extremely high levels of accuracy (96.7% of sensitivity and 100% of specificity); indeed 28/29 OSCC were detected as positive whereas 65/65 healthy donors were detected as negative (Morandi et al., 2017). Successively, a recent multicentre study confirmed the high sensitivity of our procedure (103/110 OSCC patients detected as positive) but revealed the 15.1% of false positive cases (16/110 healthy volunteers detected as positive) (Gissi et al., 2021). The present study identified a potential clinical variable (periodontal disease) that may influence the methylation profile of our 13-gene panel. However, in a good first-line screening method, we can accept high false positive cases, who will be further verify by histological examination, but not false negative cases causing dangerous implications in the management of the high-risk patients. A differential diagnosis between a suspected gingival malignant lesion and a gingival site of a PD patient characterized by interproximal clinical attachment loss is rarely a clinical problem. Instead, further investigations on methylation profile of gingival reactive lesions (i.e. epulis), sometimes similar in clinical aspect to a neoplastic lesion, may be helpful to test the specificity of our procedure.

A potential limit of this preliminary study, in addition to the small population, is a significant median age difference among groups of patients (OSCC group 70.35 ± 11.95, PD group 58.67 ± 16.92 and healthy control group 49.6 ± 19.13). It is well known that aging can affect global genome methylation (Bollati et al., 2009); however, in the present study, none of the clinical variables analysed (smoking habits, sex, age) influenced the data. Further investigation is needed to analyse for other local factors (i.e. the examination of microflora and their associations with the inflammatory micro-environment) and systemic variables that may influence the methylation status in a larger and homogeneous cohort of patients.

5 | CONCLUSION

The data from this preliminary study indicate that PD patients may exhibit methylation levels comparable to those of patients with OSCC for some inflammatory genes. In addition, our results highlight how distinct epigenetic profiles differentiate PD patients from healthy controls and suggest distinct pathogenic

mechanisms. This pilot study may provide the theoretical basis for future research that focuses on pathogenesis and potential links between OSCC and PD.

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AUTHOR CONTRIBUTIONS

Andrea Gabusi: Investigation; writing – original draft. **DAVIDE B. GISSI:** Conceptualization; writing – original draft. **Sara Grillini:** Data curation; investigation. **Martina Stefanini:** Data curation; methodology. **Achille Tarsitano:** Conceptualization; data curation. **Claudio Marchetti:** Project administration; resources; supervision. **Maria P Foschini:** Project administration; supervision; writing – review and editing. **Lucio Montebugnoli:** Methodology; project administration; supervision; validation. **Luca Morandi:** Formal analysis; investigation; resources; software; writing – review and editing.

CONFLICT OF INTEREST

As a possible conflict of interest, Luca Morandi, Davide B Gissi, and Achille Tarsitano submitted a patent (the applicant is the University of Bologna) in November 2016 to the National Institute of Industrial Property; however, we believe that this is a natural step of translational research (bench-to-bedside) and guarantee that the scientific results are true. The remaining authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/odi.14251.

ORCID

Andrea Gabusi https://orcid.org/0000-0002-7247-8615
Davide B. Gissi https://orcid.org/0000-0002-0195-7694

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