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Author manuscript

*J Periodontol.* Author manuscript; available in PMC 2025 November 13.

## Upregulation of senescence-associated gene expression levels in human gingival tissue affected by periodontitis

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### Abstract

**Background:** Accumulation of senescent cells is increasingly recognized as a mechanism of aging and is considered an attractive therapeutic target for various age-associated diseases. The prevalence and severity of periodontitis increase with age, and preclinical studies have demonstrated that senescent cells could be a potential therapeutic target for age-associated periodontitis. However, clinical data linking cellular senescence and periodontitis is limited.

**Methods:** Gingival tissues affected with periodontitis and healthy controls were collected from patients with or without periodontitis in a cross-sectional study. RNA isolated from these samples was analyzed for senescence gene expression by qPCR assays. The associations of the gene expression levels with periodontal diagnosis, presence or absence of attachment loss or bleeding on probing, and age were examined. Additionally, we analyzed the expression of senescence genes in the gingival tissues of mice with or without ligature-induced periodontitis.

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#### Author Contribution

SJK, CB, YH, and MK have conceptualized and designed the study. SJK and CB have been involved in clinical sample collection. SJK, HY, YU, GJE, and MK have performed data acquisition and analysis. SJK, CB, and MK have drafted the manuscript. All the authors have been involved in data interpretation, revising manuscript critically, and have approved the manuscript submission.

#### Conflict of interest statement

The authors have no conflicts of interest, financial, or commercial relationships to declare.

Following the guideline of HUGO Gene Nomenclature Committee and Mouse Genome Informatics, gene names are italicized, while protein names are shown in non-italicized text.

**Results:** A total of 54 human gingival tissue samples were included in the study. Among the genes analyzed, *p16*, *TP53*, *MMP2*, and *MMP14* exhibited significantly higher expression in the periodontitis group compared to the control group. Furthermore, these genes were associated with clinical signs of periodontitis, such as bleeding score and attachment loss at diagnosis. There were no statistically significant positive correlations between gene expression levels and age. The ligature-induced periodontitis significantly increased expression levels of *p16* and other senescence genes in mouse gingival tissues.

**Conclusion:** Gene expression analysis indicates the accumulation of senescent cells in gingival tissue affected with periodontitis, supporting the concept that senotherapeutics could be effective in treating periodontitis.

## Summary

This study reports the upregulation of *p16*, *TP53*, *MMP2*, and *MMP14* in human gingival tissue affected with periodontitis, indicating the accumulation of senescent cells in the tissue.

## Plain language summary

Biological stressors cause cells to age, known as cellular senescence. These senescent cells play a significant role in age-related diseases and are considered a key target for treatment. Periodontitis, a gum disease, damages the tissues that support teeth and is a leading cause of tooth loss worldwide. When people get older, they have a higher chance of being affected by periodontitis. Studies using animal models have shown that the accumulation of senescent cells may be driving periodontitis in older individuals. Therefore, targeting senescent cells may be a potential treatment approach for periodontitis. However, clinical evidence showing the accumulation of senescent cells in gum tissue affected with periodontitis is limited. This study aimed to investigate whether periodontitis is accompanied by senescent cell accumulation in the gum tissue by examining gene expression in tissues with and without periodontitis. 27 healthy and 27 diseased, a total of 54 human gum tissues were collected during gum surgeries. Our analysis found that four genes related to cellular senescence (*p16*, *TP53*, *MMP2*, and *MMP14*) were significantly higher in samples affected with periodontitis. This indicates that senescent cells accumulate in diseased gum tissue. Additionally, results from an animal experiment suggested that being affected with gum disease may be a mechanism accelerating cellular aging in the gum tissue. The findings support the concept that targeting senescent cells would be an effective approach to treat gum disease.

## Keywords

Periodontitis; cellular senescence; gene expression; mouse ligature-induced periodontitis; gingival tissue

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## INTRODUCTION

Cellular senescence, stress-induced permanent proliferative arrest, is increasingly recognized as a mechanism that drives aging.<sup>1</sup> While cellular senescence plays a role in critical biological processes, such as embryonic development, tissue healing, and cancer defense, persistent senescent cells negatively impact our body by secreting tissue-damaging factors (senescence-associated secretory phenotype: SASP) and limiting stem cell proliferation.<sup>2</sup>

Studies have shown that senescent cells contribute to various pathological conditions, and senescent cells have been attracting researchers' attention as a promising therapeutic target.<sup>3</sup> To date, a variety of molecular targets to alleviate the negative impact of senescent cells have been identified, and anti-senescence approaches such as senomorphic (anti-SASP treatment) and senolysis (selective elimination of senescent cells) have demonstrated their efficacy in improving pathological conditions and extending lifespan in preclinical models.<sup>4</sup> Studies have demonstrated the diverse characteristics of senescent cells depending on biological contexts, such as tissue types and pathological conditions.<sup>5, 6</sup> Therefore, the selection of senotherapeutic agents will likely need to be tailored to the specific pathological condition in order to effectively eliminate the senescent cells responsible for the disease.<sup>7, 8</sup> Additionally, selecting appropriate cases based on the senescent characteristics appears crucial for achieving favorable outcomes with senotherapeutics.<sup>9</sup> Therefore, understanding the senescent characteristics of target diseases is vital for designing senotherapy.

Periodontitis is a bacterially induced inflammatory condition where tooth-supporting periodontal tissue is damaged. Prevalence of severe periodontitis steeply increases between the 3<sup>rd</sup> to 5<sup>th</sup> decade of life, indicating a strong association between age and the pathology of periodontitis.<sup>10, 11</sup> However, the underlying mechanism remains unclear. Preclinical studies using animal models have demonstrated that senescent cells accumulate in periodontal tissue, and anti-SASP treatment was effective in reducing periodontal inflammation and bone loss in aged mice.<sup>12–15</sup> While these studies suggest that the accumulation of senescent cells can disrupt periodontal tissue homeostasis, the clinical data linking periodontitis and cellular senescence is limited. Moreover, the senescent characteristics in human periodontal tissue have yet to be defined.

Thus, this study aimed to investigate senescence characteristics in human gingival tissue and its association with periodontitis by analyzing the expression levels of senescence-associated genes in gingival tissue samples with and without periodontitis. We also examined potential associations between senescence gene expression levels and other factors, such as age and smoking status. Additionally, to investigate the impact of periodontitis on the senescence burden in periodontal tissue, we induced an experimental periodontitis in mice and analyzed senescence-associated gene expression in gingival tissue.

## MATERIALS AND METHODS

### Clinical sample acquisition

This cross-sectional study was conducted on patients at the Graduate Periodontics Clinic at Indiana University, School of Dentistry. The protocol was reviewed and approved by the Institutional Review Board (#17678). The diagnosis of periodontitis was made as per the periodontal classification from the World Workshop 2017.<sup>16</sup> Patients receiving periodontal surgery (crown lengthening for non-periodontitis subjects, open flap debridement or osseous surgery for periodontitis subjects) who were over 18 years old with American Society of Anesthesiologist physical status Class I (a normal healthy patient) or II (a patient with mild systemic disease) were included in the study. Patients who did not meet inclusion criteria, pregnant or nursing women, or those presenting with active dental infections noted at the time of surgery were excluded. Samples from periodontitis subjects were collected from

sites affected by periodontitis with probing depths  $\geq$  4mm after non-surgical periodontal treatment, while samples from non-periodontitis subjects were from sites not affected by periodontitis and with probing depths  $<$  4mm. We obtained informed consent from all participants. All participants were recruited between February 6, 2023 and February 20, 2024. The following information was collected at the time of periodontal surgery:

- Age
- Sex
- Weight and height for BMI (body mass index) calculation
- The surgical site
- Periodontal diagnosis (A periodontal chart and a periapical radiograph taken within 6 months of recruitment was used, and the periodontal diagnosis was made according to the 2017 World Workshop on Periodontal and Periimplant diagnosis.<sup>16</sup>)
- Self-reported smoking status (current smoker, past smoker, and no history of smoking)
- Self-reported diabetes control status (diabetic: HbA1c $>$ 6.5%; pre-diabetic: HbA1c between 5.6 to 6.5%; and non-diabetic: HbA1c  $<$ 5.6%)
- Self-reported chronic kidney disease status (not diagnosed, diagnosed and on dialysis, diagnosed and not on dialysis)

During the periodontal surgery, gingival tissue, including epithelial tissue, subgingival connective tissue, and/or granulation tissue, from each subject was collected. To prevent RNA degradation in the samples, they were placed in 1.5 mL Eppendorf tubes containing 500  $\mu$ L of RNeasy lysis solution (Invitrogen™, Waltham, MA) and stored at 4°C until the subsequent RNA isolation procedure. The target sample size was set based on the sample sizes of previous publications that performed gene expression analysis of gingival tissue between periodontitis and non-periodontitis, which range from 11 to 30,<sup>17–21</sup> but not by power analysis because there were no publicly available dataset of senescent gingival gene expression studies at the time we designed the experiment.

### RNA isolation

50 to 100 mg of the tissue samples were transferred to 2.0 mm BashingBead™ lysis tubes (Zymo Research, Irvine, CA) with 500  $\mu$ L of TRIzol™ Reagent (Invitrogen™, Waltham, MA) and then homogenized. An additional 500  $\mu$ L of TRIzol reagent was added to the tubes to achieve an appropriate volume ratio of tissue and the reagent. Phase separation technique was used to isolate RNA, and the isolated RNA was reconstituted with 50 to 100  $\mu$ L of nuclease-free water. The quantity and quality of RNA was measured by absorbance.

### RT-qPCR analysis

0.5  $\mu$ g of RNA was used for reverse transcription to synthesize cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems®, Waltham, MA). The obtained cDNA was diluted 4- to 16-fold with nuclease-free water to serve as a cDNA template

for qPCR reactions. Due to the highly diverse characteristics of senescent cells, there is no single universal marker gene for senescent cells.<sup>22</sup> Therefore, an approach that analyzes multiple genes associated with key characteristics for senescent cells, such as cell cycle arrest, anti-apoptotic, and SASP, was employed. Targeted senescence genes for the analysis are as follows<sup>22</sup> (Primer sequences are listed in the Supplemental Table 1):

- Genes involved in cell cycle arrest: *p16*, *p21*, *TP53*, *p15*
- Anti-apoptotic genes: *BCL2*, *BCL2XL*
- SASP genes: *IL1B*, *IL6*, *IL17A*, *IL8*, *MMP2*, *MMP8*, *MMP12*, *MMP13*, *MMP14*

qPCR assay was performed using PowerUp SYBR Green master mix (Applied Biosystems®) on Quant Studio 3 Real-Time PCR system (Applied Biosystems®) by the personnel who are blind to the sample information. The relative standard curve method was used to calculate the relative gene expression levels. The results from qPCR reactions that did not yield an amplification curve due to low expression levels were considered an expression level of 0. Gene expression levels were normalized to those of *GAPDH*, a housekeeping gene. The average expression levels in the non-periodontitis control group were set to 1 for data visualization.

### Animal experiments

All animal experiments were conducted under the animal protocol approved by the IACUC of Indiana University School of Medicine and Augusta University. 10 week-old C57BL/6J male mice were challenged with ligature-induced periodontitis (LIP) as previously described.<sup>23</sup> Briefly, the maxillary left second molar of the animals was ligated with 5–0 silk sutures (Ethicon, Somerville, NJ) for 1, 3, 5, 7, 14, and 28 days. Sex-matched littermate mice were randomly assigned to either the group with or without the LIP challenge. Aged mice older than 24 months were obtained from the NIA Rodent colony. Gingival tissues with a 1 mm width from the palatal side were used for RNA isolation and subsequent gene expression analysis as described above. Based on the power analysis (type I error ( $\alpha$ ) = 0.05, type II error (Power) = 0.8, when SD is 20% of each mean value) of our previous datasets,<sup>23–25</sup> we initially predicted  $n=4$  to 8 will detect a difference in gene expression. Therefore, we first collected samples with a minimal sample size of 4, then performed power analysis again using actual data to determine the final sample size. Falling out of ligature or weight loss of more than 20% was used as exclusion criteria. No animals were excluded from the study.

### Statistical analysis

Chi-square tests were used to compare patient characteristics between the groups with or without periodontitis. Expression levels were log-transformed before analysis due to the non-normal distribution of the data. Comparisons between the groups for differences in senescence marker expression were analyzed using two-sample *t*-tests with heterogeneous variances. For genes that did not follow a normal distribution after log transformation, we additionally performed a non-parametric test and confirmed that the conclusion remained consistent between parametric and non-parametric tests. Analysis of covariance was used to

explore the effects of age, sex, BMI, smoking status, and diabetes status on the expression levels. The association between genes was analyzed using Pearson's correlation. A  $p$ -value less than 0.05 was used as a criterion for statistical significance. An extreme outlier that was more than 10 times larger than the next largest observation was omitted. Analyses and data visualizations were performed using SAS<sup>®</sup> version 9.4 (SAS Institute, Inc., Cary, NC, USA), GraphPad Prism version 10.3.1 (GraphPad Software, Boston, MA, USA), and R 4.2.0.

## RESULTS

### Demographic characteristics of the subjects

A total of 54 gingival tissue samples were examined in the study. Of the 54 samples, 27 (50%) were from sites affected with periodontitis, while the other 27 (50%) were from clinically healthy sites without periodontitis. This sample size was comparable to the senescence gene expression analysis of human gingival tissue in a previous study.<sup>26</sup> The mean probing depth of surgical sites were 2.426 mm with  $\pm 0.0988$  mm SE for non-periodontitis samples and 4.907 mm with  $\pm 0.1689$  mm SE for periodontitis samples. Age, gender, body mass, BMI, smoking status, diabetic status, and kidney disease status did not show significant differences between the groups, although the limited sample size in some subgroups does affect the ability to detect statistical significance (Table 1).

### Association between senescence-associated genes and periodontal disease status

Among the genes coding cell cycle inhibitors, *p16*, the most widely used senescence marker gene, and *TP53* were significantly higher in the periodontitis group than the group without periodontitis ( $p=0.010$  and  $0.017$ ), while *p15* and *p21* did not show differences between groups (Figure 1A and Supplemental Table 2). Among the genes for matrix metalloproteinases (MMPs), *MMP2* and *MMP14* were higher in the periodontitis group ( $p=0.045$  and  $<0.001$ , Figure 1B). No statistical differences were noted in inflammatory and anti-apoptotic genes between the groups (Figure 1C and D). After adjusting for patient characteristics, *BCLXL* and *p15* barely reached the significant  $p$  values ( $p=0.034$  and  $=0.040$ ). Because of the non-normality issue, we additionally performed non-parametric analysis for *p16* and *MMP13* and confirmed that the results were consistent with parametric analysis (Supplemental Table 2). Additionally, a network analysis among the genes upregulated in periodontitis patients was performed (Figure 1E). The strongest connectivity was between *MMP2* and *MMP14* ( $r=0.7281$ ,  $p<0.0001$ ). *MMP2* and *MMP14* expressions showed a stronger association with *p16* rather than with *TP53*. The association between *p16* and *TP53* was not strong enough to be highlighted in the analysis ( $r=0.1370$ ,  $p=0.3281$ ).

### Association of senescence gene expression with gingival inflammation and attachment loss

Additional analysis was performed on the expression levels of *p16*, *TP53*, *MMP2*, and *MMP14* to test if they were associated with the clinical parameters of periodontitis at the time of diagnosis. We used bleeding on probing (BOP) score 10% as the threshold for determining the healthy or inflamed.<sup>27, 28</sup> *p16* and *MMP14* expression levels were higher in the group with BOP score 10% than BOP <10% ( $p=0.008$  and  $=0.009$ , Figure 2),

while *TP53* and *MMP2* only showed increased tendency with no statistical significance ( $p=0.1369$  and  $0.1404$ ). *p16*, *MMP2*, and *MMP14* expressions were higher in the group with attachment loss caused by periodontitis (*p16*:  $p<0.001$ , *MMP2*:  $p=0.0484$ , *MMP14*:  $p=0.004$ ), while *TP53* was not ( $p=0.0645$ ). On the other hand, when analyzed for the association with attachment loss that is independent of periodontitis, none of the genes showed statistical significance ( $p=0.8681$ ,  $=0.1346$ ,  $=0.7983$ , and  $=0.8739$ ). Thus, *p16*, *MMP2*, and *MMP14* that showed connectivity in a network analysis (Figure 1E) exhibited high association with periodontal pathological conditions but not with tissue damage that was not associated with periodontitis.

### Association of senescence gene expression with age and other factors

In several organs, senescence marker levels increase with chronological age, and therefore chronological aging is considered a driver of cellular senescence.<sup>5, 6</sup> To examine if there is such an age effect on the expression levels of senescence genes in human gingival tissue, we performed Pearson's correlation and simple linear regression between age and senescence gene levels in gingival tissues. Initially, we analyzed the total population, including both periodontitis and non-periodontitis subjects. Unexpectedly, none of the tested genes showed a statistically significant positive correlation with age (Figure 3 and Supplemental Table 3). We still did not observe any significant positive correlation when we analyzed subjects with and without periodontitis separately (Supplemental Figures 1 and 2). Together with the results shown in Table 1, these data indicate that age is not a confounding factor in this study. Additionally, we explored potential associations between gene expression levels and patients' demographic information and found that *MMP12* expression decreased with a history of smoking in non-periodontitis subjects ( $p=0.019$ ) but not in periodontitis subjects ( $p=0.408$ ) (Supplemental Figure 3). Two-way ANOVA analysis showed a significant interaction between the diagnosis of periodontitis and the history of smoking on the *MMP12* expression levels ( $p=0.0256$ ). Analysis of covariance did not detect a significant effect of sex, BMI, or diabetic status on gene expression in the current dataset with a relatively small sample size for the analysis (Supplemental Table 4). Our cohort did not include enough chronic kidney disease patients for statistical analysis, therefore, a correlation analysis was not performed (Table 1).

### Experimental periodontitis increased senescence marker gene expressions in mouse gingival tissue

We observed an association between periodontitis and senescence gene expression in Figure 1, but their causal relationship remains unknown. To test whether periodontal inflammation can induce senescence gene expression in gingival tissue, we challenged young mice (10-week-old) with mouse ligature-induced periodontitis (LIP) and analyzed gene expressions in their gingival tissues. We also included aged mice older than 24 months to assess the age effects on gene expression. We first analyzed inflammatory characteristics of LIP by analyzing expression levels of inflammatory genes such as *Il6*, *Il1b*, *Il17a*. LIP strongly induced *Il6* and *Il1b* expression 1 day after the induction, indicating the strong acute inflammatory response in the early stage of LIP. After the initial peak on day 1, *Il1b* expression maintained slightly increased levels until day 14, and then showed a second peak at day 28 (Figure 4A). On the other hand, *Il17a*, a marker for adaptive immune

response that is more relevant to chronic inflammation, did not increase until day 14 and then increased at day 28. These results indicate that the inflammatory characteristics in LIP differ significantly between timepoints, consistent with previous studies that characterize LIP inflammation.<sup>23, 29, 30</sup> The inflammatory gene expression in aged mice without LIP was comparable with young control mice. The LIP induced *p16* expression peaking at day 28 (Figure 4B). Aged mice also showed elevated *p16* levels, however, it was significantly lower than day 28 of LIP. *Mmp2* was increased by LIP but not in aged mice. We did not observe an increase in *Trp53* or *Mmp14* in either mice challenged by LIP or aged mice. We then analyzed additional senescence marker genes, *p19*, a gene coding another cell cycle inhibitory protein,<sup>31</sup> and *Cd274*, a gene coding PD-L1 that plays a role in immune evasion of senescent cells,<sup>32</sup> and found that both LIP and aging significantly increased the expression levels of these genes (Figure 4C). The induction of *Cd274* by LIP was more significant compared to aging.

## DISCUSSION

We found higher expression levels of senescence genes in the gingival tissues affected with periodontitis compared to the healthy control group (Figure 1). The expression levels of those senescence genes were highly associated with the BOP status and attachment loss caused by periodontitis (Figure 2). Furthermore, none of the gene expression levels showed a significant correlation with age (Figure 3). On the other hand, our animal experiment found LIP challenge increased senescence marker expression (Figure 4). Together with a recent study that reported *p14ARF* gene expression and p16 protein expression as well as senescence-associated  $\beta$ -galactosidase activity in human gingival tissues of periodontitis patients,<sup>26</sup> our data indicate that senescent cells accumulate in periodontal tissue in association with periodontal inflammation and damage, and periodontitis would be an inducer of cellular senescence in periodontal tissues. Previous animal studies have suggested that the accumulation of senescent cells exacerbates inflammation and tissue damage in periodontitis.<sup>12, 13, 33</sup> Accordingly, it is likely that there is a feedback loop between periodontitis and senescent cell accumulation in periodontal tissue, in which periodontal inflammation induces cellular senescence in periodontal tissues, and accumulated senescent cells exacerbate periodontitis. These results support that senotherapeutics targeting senescent cells would be an effective approach to treating periodontitis as previously proposed based on animal studies.<sup>14, 15, 33, 34</sup>

In this study, we found that *MMP2* and *MMP14* levels increased in the gingival tissue affected with periodontitis. Increased levels of MMPs in periodontal tissue affected with periodontitis has been described,<sup>26, 35</sup> and cellular senescence might be an induction mechanism of MMPs in periodontitis. MMPs have been considered as drivers of inflammatory tissue damage in periodontitis due to their capability to degrade extracellular matrix. Although further functional studies are needed to clarify the biological impact of senescence-associated MMPs on the pathology of periodontitis, it is likely that senotherapeutics would help normalize tissue MMP levels.

Several biological stressors, such as inflammation, bacterial burden, aging, obesity, and diabetes, have been proposed to trigger the generation of senescent cells.<sup>22, 36</sup> Previous *in*

*vitro* studies have shown that stimulation with *Fusobacterium nucleatum* or *Porphyromonas gingivalis* induces senescent phenotypes in human immortalized gingival epithelial cell line and mouse primary alveolar bone cells, indicating bacterial burden can be a driver of cellular senescence in periodontal tissue.<sup>13, 37</sup> In the present study, we showed that the LIP significantly induced senescence gene expression *in vivo* for the first time (Figure 4). Considering that LIP inflammation is bacterially induced,<sup>23, 38</sup> bacterial stimulation would play a role in the induction of senescence gene expression in the mouse gingival tissues as well, and this could be a potential mechanism for inducing senescence gene expression in human gingival tissue affected with periodontitis. On the other hand, chronological aging may not be a significant driver of cellular senescence in gingival tissue. It is reported that senescent burden increases with chronological aging in various tissues.<sup>5, 6</sup> However, none of the tested genes positively correlated with age in human gingival tissue in the present study (Figure 3), suggesting that chronological aging alone is insufficient to increase senescent burden in human gingival tissue. Supporting the concept, LIP increased expression levels of some senescence marker genes, such as *p16*, *Mmp2*, and *Cd274*, in gingival tissues to significantly higher levels than that of aged mice (Figure 4).

The p16 pathway and the p21/p53 pathway play an essential role in the induction and maintenance of cellular senescence, making *p16* and *p21* vital markers for senescent cells.<sup>39, 40</sup> The contribution of each pathway on senescent phenotypes seems to vary depending on tissue types or biological stressor causing cellular senescence.<sup>41</sup> In the p21/p53 pathway, p53 serves as an activator for the cyclin-dependent kinase inhibitor p21, while p16 directly inhibits G1 cyclin-dependent kinases in the p16 pathway. In the present study, we found *p16* levels were increased in the gingival tissues affected with periodontitis, indicating the activation of p16 pathway. On the other hand, *p21* levels were not increased in the periodontitis group, although we saw an increase in *TP53* levels. A recent study suggested that p53 is a mediator of periodontal inflammation and bone loss.<sup>42</sup> The upregulated *TP53* in gingival tissues from periodontitis patients may be involved in host immune responses rather than contributing to cellular senescence. Additionally, our network analysis showed that *MMP2* and *MMP14* levels had a stronger association with *p16* compared to *TP53*. These results suggest that the p16 pathway plays a more dominant role in regulating the senescent phenotypes in the gingival tissues than the p21/p53 pathway. p16 would be a more suitable marker for senescent cells than p21 when characterizing senescent cells in gingival tissues affected with periodontitis.

Previous studies reported that non-surgical periodontal treatments effectively reduced gingival inflammation, even to lower levels than healthy control for some markers.<sup>20, 43</sup> In alignment with those reports, our results showed that inflammatory gene expression was comparable between the periodontitis group and the control group, indicating that non-surgical treatments effectively reduced gingival inflammation. Upregulation of inflammatory gene expression is one of the common characteristics of senescent cells, however, inflammatory genes we analyzed in this study, *IL1B*, *IL6*, *IL8*, and *IL17A*, might not be a major characteristic of cellular senescence in gingival tissues affected by periodontitis. On the other hand, other senescence-associated genes remained upregulated after non-surgical treatment, suggesting the persistence of senescent cells, most likely due to their anti-apoptotic characteristics and ability to evade immune surveillance.<sup>32, 44–46</sup> Meanwhile,

studies using animal models have shown transient senescent cell populations that play a role in the tissue healing process.<sup>47, 48</sup> Considering the dynamic course of the pathology of periodontitis, there might be such transient senescent cells involved in the pathological process. Further studies are needed to comprehensively understand the role of senescent cells in periodontitis.

A history of smoking is a well-recognized significant risk factor for developing periodontitis.<sup>49</sup> In the present study, although we did not see increased senescence gene expressions in smokers, we found that *MMP12* levels were decreased with a history of smoking in non-periodontitis patients (Supplemental Figure 3). Furthermore, two-way ANOVA identified a significant interaction between a history of smoking and periodontitis on *MMP12* levels. Similarly, a previous study reported that *MMP12* levels in saliva decreased in smokers.<sup>50</sup> In contrast to the observations in oral cavity, it has been shown that cigarette smoke exposure significantly increased *MMP12* levels in mouse lung tissue.<sup>51</sup> Thus, the effect of smoking on tissue *MMP12* levels can be varied depending on the tissue type. The role of *MMP12* in the pathophysiology of periodontal tissue is not well understood, and the biological impact of reducing *MMP12* levels is unknown. *MMP12* is highly expressed by macrophages and mediates their functions such as chemotaxis and phagocytosis.<sup>51–54</sup> Declined *MMP12* levels in the gingival tissue may be involved in the mechanism by which smoking increases the risk of periodontitis by altering macrophage functions.

### Limitations of the study

In the present study, we used crude samples composed of different tissue types, including epithelium, gingival connective tissue, blood vessels, and immune cells. Therefore, we were unable to identify the cell types that were responsible for the senescent characteristics in the gingival tissue affected by periodontitis. According to a recent publication investigating senescent characteristics in similar subjects<sup>26</sup>, cellular senescence in gingival tissue could likely coincide in multiple cell types, including epithelial cells, gingival fibroblasts, and immune cells. Furthermore, our data are limited to gene expression and cannot assess the functional impact of cellular senescence on the pathology of periodontitis. To clarify the role of cellular senescence in periodontitis and the molecular mechanisms of cellular senescence induction in periodontal tissue, further research using different approaches, such as cell culture or genetic animal models, will be necessary. A larger clinical study will be needed to assess the association between patient factors (age, sex, BMI, smoking status, diabetes status, CKD) and expression levels.

### CONCLUSION

In conclusion, the expression levels of *p16*, *TP53*, *MMP2*, and *MMP14* were significantly higher in periodontally diseased human gingival tissues compared to the controls, indicating the accumulation of senescent cells in gingival tissue affected with periodontitis. Furthermore, our data suggest that periodontitis is a driver of cellular senescence in gingival tissue. These findings provide a rationale for the application of senotherapeutics in the treatment of periodontitis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

This study was supported by NIDCR (R03DE032101, R03DE034787, and R01DE034757), the American Academy of Implant Dentistry Foundation (AAIDF), Indiana University School of Dentistry, and Augusta University Dental College of Georgia.

## Data availability

All the data are available through the corresponding authors upon reasonable request.

## ABBREVIATIONS

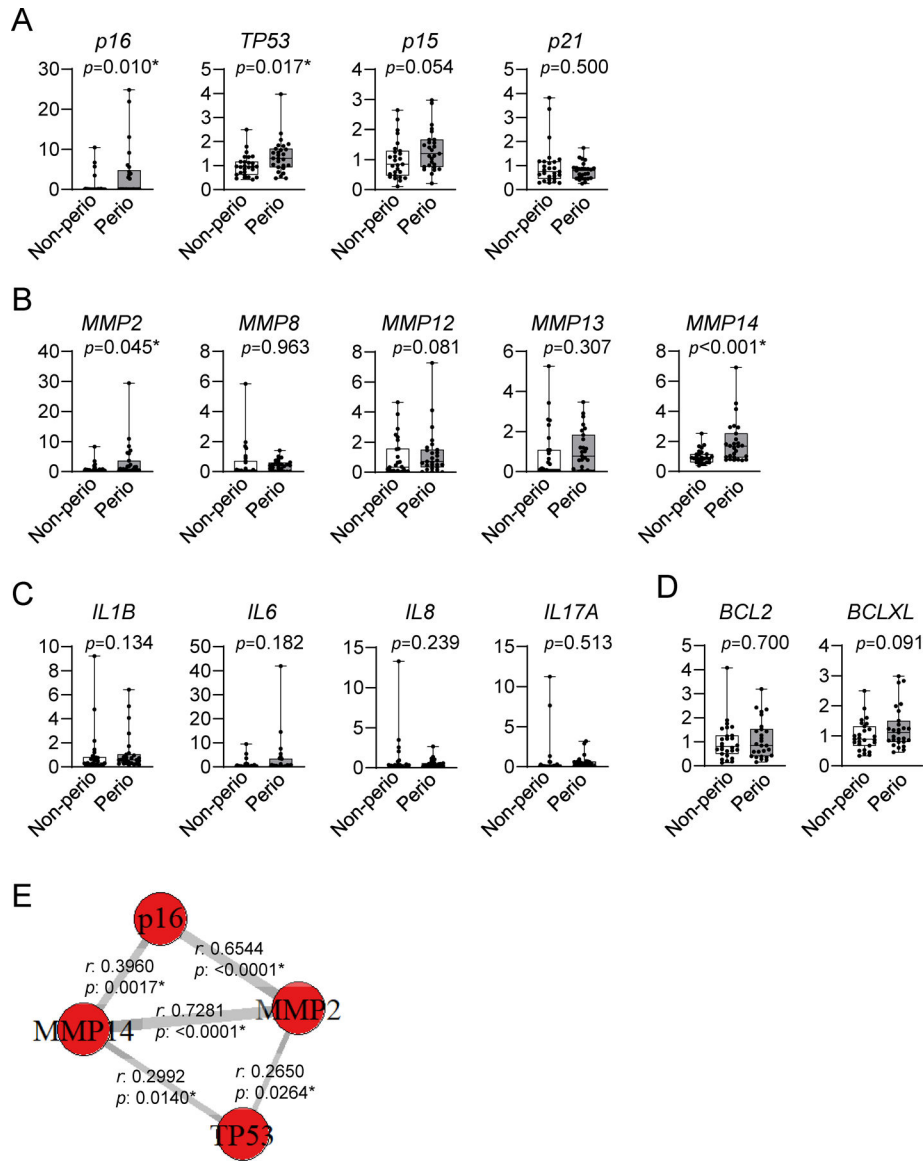
<b>ASA</b>	American Society of Anesthesiologists
<b>BCL2</b>	B-cell leukemia/lymphoma 2 protein
<b>BCLXL</b>	B-cell lymphoma extra-large protein
<b>BMI</b>	body mass index
<b>CDKN</b>	cyclin-dependent kinase inhibitor
<b>cDNA</b>	complementary deoxyribonucleic acid
<b>GAPDH</b>	glyceraldehyde-3-phosphate dehydrogenase
<b>HbA1c</b>	hemoglobin A1c
<b>IFN</b>	interferon
<b>IL</b>	interleukin
<b>LIP</b>	ligature-induced periodontitis
<b>MMP</b>	matrix metalloproteinase
<b>p16</b>	a CDKN coded by CDKN2A gene. CDKN2A gene encodes two CDKNs, p16 and p14ARF for human or p19ARF for mouse
<b>qPCR</b>	quantitative/real-time polymerase chain reaction
<b>RNA</b>	ribonucleic acid
<b>SASP</b>	senescence-associated secretory phenotype
<b>TP53</b>	tumor protein P53 (human)
<b>Trp53</b>	transformation related protein 53 (mouse equivalent for human TP53)

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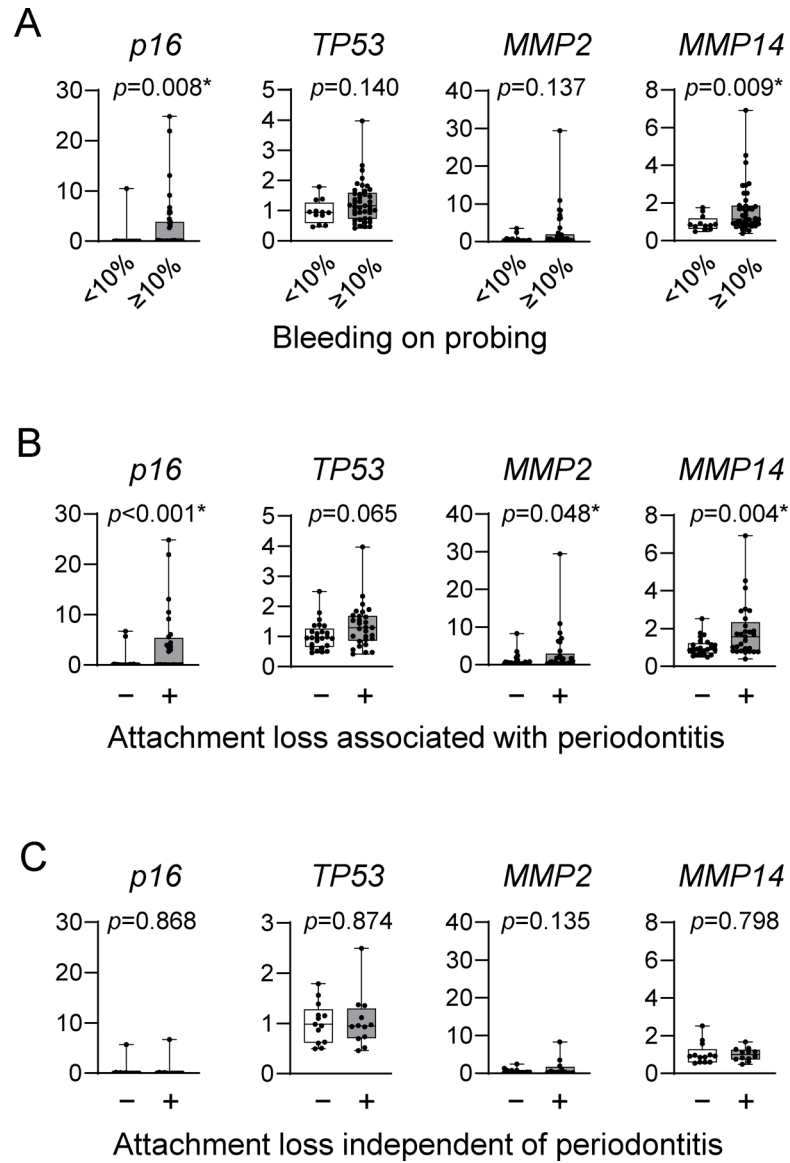
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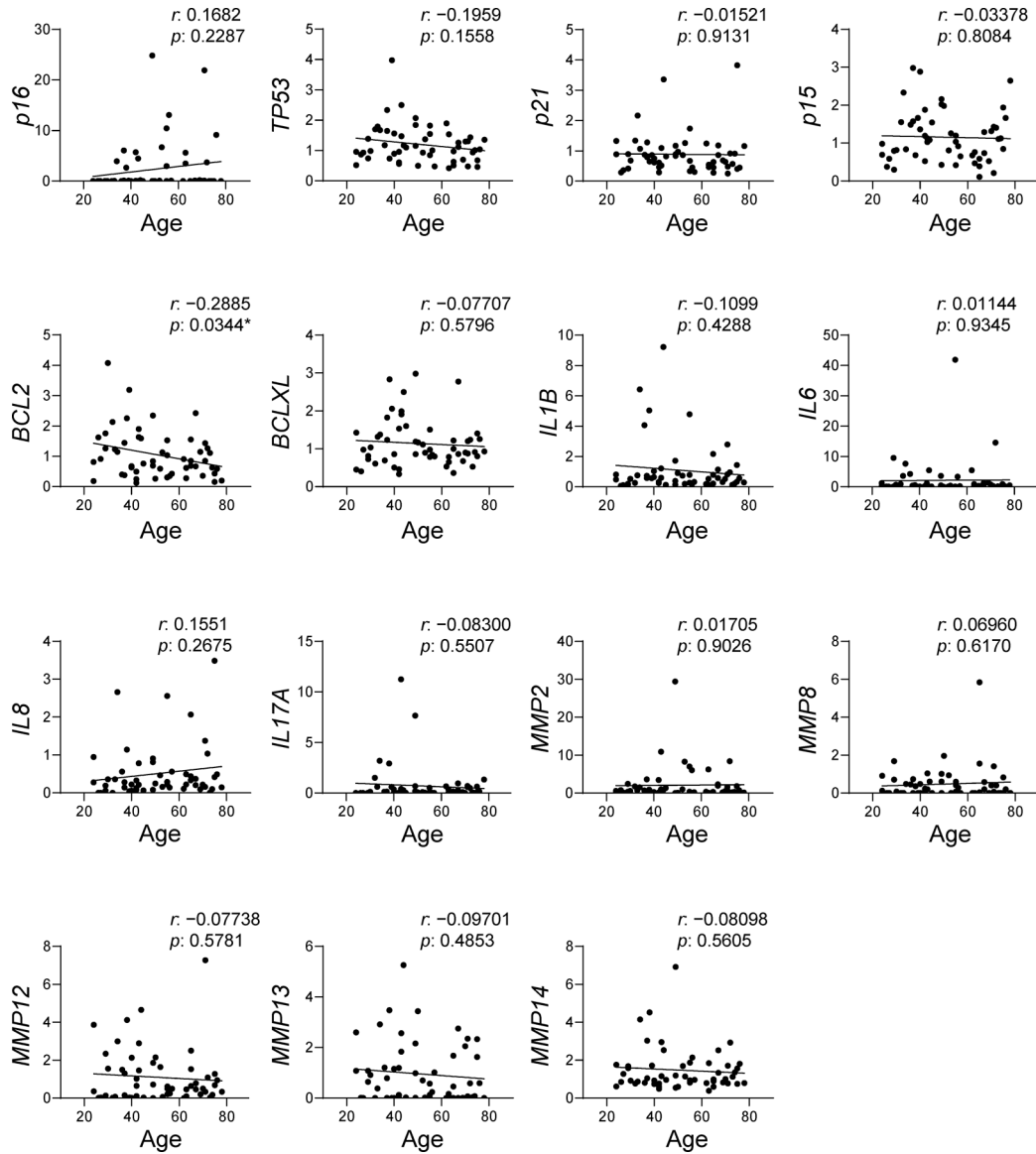
**Figure 1. Senescence-associated gene expression in gingival tissue.**

Gene expression analysis was performed by RT-qPCR assay. Data are presented with box and whisker plots. Each dot represents an individual sample. Relative gene expression levels were normalized with the expression levels of *GAPDH*, and average expression levels in non-periodontitis control group were set as 1.  $p$  values were determined by the Student's  $t$ -test on log-transformed data.  $p$  values less than 0.05 are highlighted with a star symbol (\*). **A**) Genes involved in cell cycle arrest. **B**) MMP genes. **C**) Genes for inflammatory cytokines. **D**) Anti-apoptotic genes. **E**) Network analysis visualizing the correlation between the genes upregulated in the gingival tissues of periodontitis patients.  $p < 0.05$  was the cutoff value to draw edges. The thickness of edges between nodes reflect the magnitude of correlation coefficient ( $r$ ). Results of the statistical analysis for A-D are summarized in Supplemental Table 2.



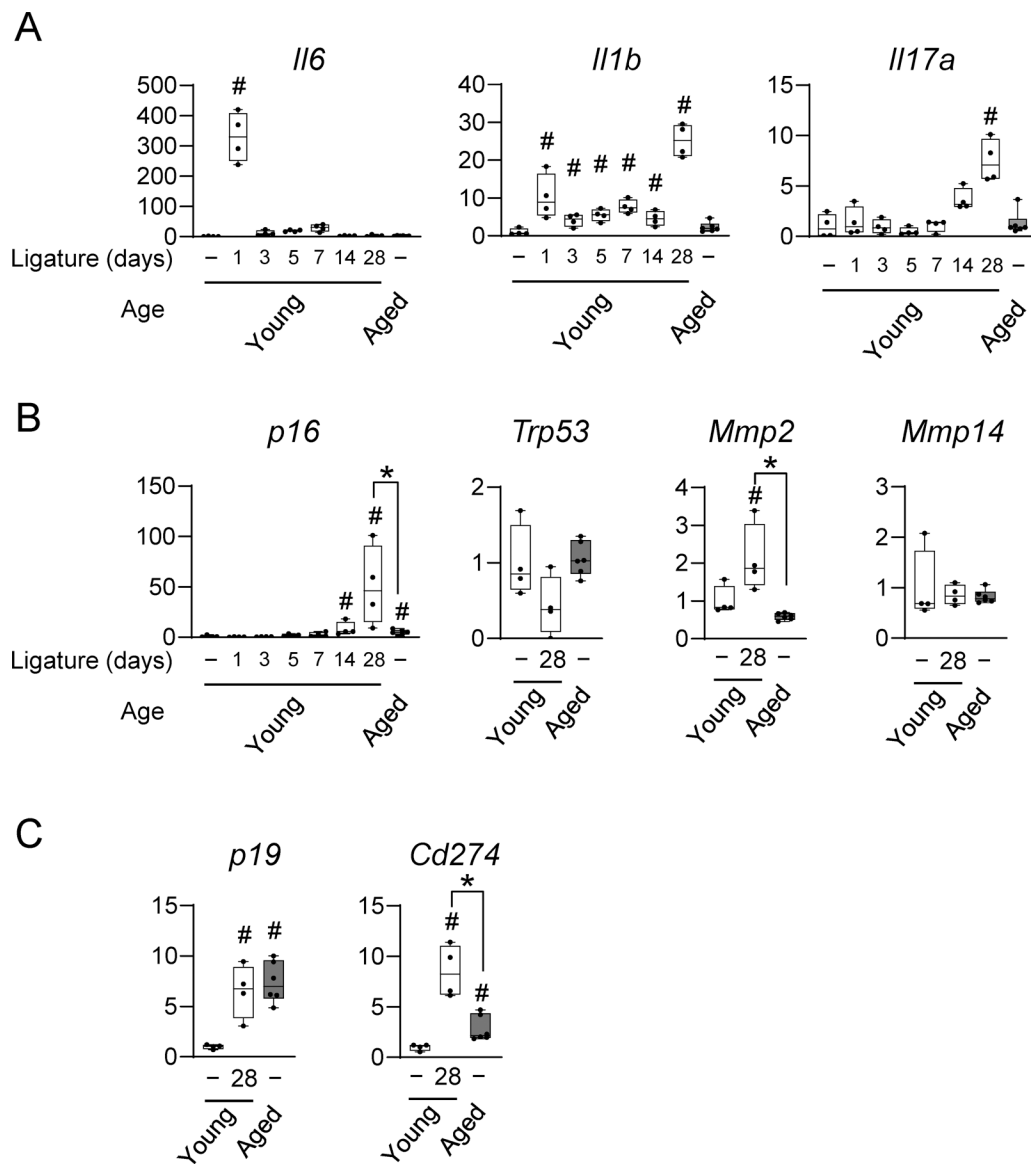
**Figure 2. Association between clinical features and gene expression.**

**A)** Gene expression comparison between BOP score <10% and ≥10% at the time of diagnosis. **B)** Gene expression comparison between with and without attachment loss associated with periodontitis. **C)** Gene expression comparison between no attachment loss and attachment loss that is not associated with periodontitis. *p* values were analyzed by the Student's *t*-test. *p* values less than 0.05 are highlighted with a star symbol (\*).



**Figure 3. Correlation between senescence-associated genes and age.**

The correlation between age and gene expressions was analyzed using Pearson's correlation and linear regression. Samples include both non-periodontitis and periodontitis groups. The results of analysis in which samples were separated based on the presence or absence of periodontitis are shown in Supplemental Figures 1 and 2. None of the tested genes showed statistically significant positive correlations with age.



**Figure 4. Mouse ligature-induced periodontitis induced senescence gene expression in gingival tissues.**

RT-qPCR analysis of gene expression in gingival tissues from young mice with or without LIP and aged (>24 months old) mice without LIP. A) Inflammatory genes. B) Gene expression of *p16*, *Trp53*, *Mmp2*, and *Mmp14*. C) Additional senescence genes. Gene expression levels were normalized with the expression levels of *Gapdh*, and average expression levels in young mice without LIP were set as 1. *p* values were analyzed by One-way ANOVA followed by Tukey-Kramer post hoc test. #:  $p < 0.05$  compared to young baseline control; \*:  $p < 0.05$  between groups.

**Table 1.**

Patient characteristics collected at the time of the periodontal surgery.

Characteristic	Level	Without periodontitis	Periodontitis	<i>p</i> -value
Age	<40	8 (30%)	7 (26%)	0.942
	40–60	10 (37%)	10 (37%)	
	>60	9 (33%)	10 (37%)	
Sex	F	16 (59%)	12 (44%)	0.276
	M	11 (41%)	15 (56%)	
BMI	Healthy	3 (11%)	8 (30%)	0.06
	Overweight	9 (33%)	12 (44%)	
	Obese	15 (56%)	7 (26%)	
Smoker	Never Smoker	16 (59%)	16 (59%)	0.901
	Past Smoker	8 (30%)	7 (26%)	
	Smoker	3 (11%)	4 (15%)	
Diabetes	Non-diabetic	23 (85%)	23 (85%)	not available
	Pre-diabetic	1 (4%)	3 (11%)	
	Diabetic	3(11%)	1 (4%)	
CKD	Not diagnosed	25 (93%)	26 (96%)	not available
	Diagnosed and not on dialysis	2 (7%)	1 (4%)	