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Doctoral Dissertation
Doctoral Program in Bioengineering and Medical-Surgical Sciences (36th Cycle)

The impact of biological aging on periodontal ligament-derived stem cells and its implications for clinical periodontal regeneration: foundation for a translational approach.

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* * * * * *

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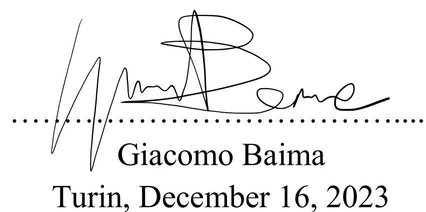

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Turin, December 16, 2023

Summary

Periodontal regeneration is the most desirable clinical outcome in the management of intraosseous and furcation periodontal defects. The aim is the complete restoration of the lost periodontal anatomy, i.e. the formation of new cementum, periodontal ligament (PDL) and alveolar proper bone. However, surgical techniques and biomaterials currently used limited the efficacy of periodontal regeneration. Cell therapy using autologous mesenchymal stem cells from the PDL (PDLSCs) may emerge as a solution to restore the architecture of the original periodontium due to their plasticity and ability to differentiate into osteo/cementoblast and periodontal ligament cell lines. However, a thorough characterization of these cell populations in patients affected by periodontitis is lacking, as well as understanding the impact of inflammatory-driven senescence and epigenetic aging on their regeneration potential.

This study aimed to conduct a comparative analysis between PDLSCs from healthy individuals (hPDLSCs) and those with periodontitis (pPDLSCs) to identify potential functional disparities attributed to their respective origins. The assessment encompassed colony-forming unit efficiency, multilineage differentiation capacity, immunophenotype, stemness, and senescence status, which were examined through flow cytometry, immunofluorescence, and β-galactosidase staining. Gene expression profiles were determined using RT-PCR. Moreover, a clinical transability of the findings was attempted, by assessing whether the presence of active inflammation and the expression of the senescence-associated secretory phenotype (SASP) in the gingival crevicular fluid (GCF) influenced the early wound healing (EHI) and the one-year outcomes of periodontal regeneration.

Both hPDLSCs and pPDLSCs exhibited similarities in their immunophenotype and their ability to differentiate into multiple lineages. However, pPDLSCs demonstrated a higher frequency of a senescent phenotype, expressing significantly

more p16 and p21 genes and, contemporary, more stemness genes such as OCT4. Moreover, pPDLSCs showed a higher expression pattern of P2X7R, a novel pro-inflammatory molecule involved in the purinergic signalling pathways. To investigate whether this senescent phenotype could be reverted, we tested the effect of an investigational small molecule inhibitor targeting DNA methyltransferase, known as RG108. RG108 application did not impact the proliferation and apoptosis of PDLSCs and had a non-significant effect on hPDLSCs. In contrast, a notable reduction in p16 and p21 expression was observed in pPDLSCs following treatment with 100μM RG108, together with an elevation in SOX2 and OCT4. Furthermore, the subset of PDLSCs co-expressing OCT4 and p21 diminished, and the adipogenic potential increased in pPDLSCs after RG108 treatment. Regarding the clinical findings, the presence of active inflammation (bleeding on probing) was a negative predictor for EHI at 2 weeks after surgery, as well as for the achievement of clinical success at one year. At the same time, a higher expression of SASP factors in the GCF (IL-1β, IL-6, MMP-8 and MMP-9) positively correlated with EHI, as well as with final probing pocket depth and clinical attachment level, suggesting a potential effect of inflammation and senescence on the attainment of periodontal regeneration.

In summary, pPDLSCs displayed a differential phenotypic and functional behavior compared to hPDLSCs, presenting a higher degree of cellular senescence. This phenotype was partially reversed through RG108. Clinically, among the other important parameters affecting the clinical outcomes of periodontal regeneration, SASP expression level represented a prognostic factor which deserves further investigation. The importance of addressing cellular senescence and biological aging to enhance the success of periodontal regeneration may broaden new horizons for future research and therapeutic applications.

Acknowledgment

And I would like to acknowledge Dr. Ilaria Roato for the important support she gave me in the laboratory analyses, together with all the scientists working in the group led by Prof. Federico Mussano. Moreover, I owe an acknowledgement to Dr. Federica Romano for her precious help with the statistics, as well as for being such a close collaborator during my entire PhD course, and hopefully beyond. Finally, I would like to thank my colleagues working in the Section of Periodontology for their help with clinical data collection and, most importantly, my mentor Prof. Mario Aimetti for being a constant source of inspiration and guidance throughout my scientific and clinical career.

Esto sin contar con que nuestra apreciación de lo importante y de lo accesorio, de lo grande y de lo pequeño, asiéntase en un falso juicio, en un verdadero error antropomórfico. En la Naturaleza no hay superior ni inferior, ni cosas accesorias y principales.

Santiago Ramon y Cajal,

Reglas y consejos sobre investigación científica.

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Introduction

1.1 Periodontitis and its Burden

Periodontitis is a chronic inflammatory, dysbiotic biofilm-initiated disease affecting the supporting anatomical structures of the teeth, which poses a significant global health challenge (Papapanou et al. 2018a; Peres 2019). Due to its high prevalence, periodontitis is recognized as one of the most frequent non-communicable diseases worldwide, with up to 10% of the global population affected by its most severe form (Aimetti et al. 2015; Trindade et al. 2023). Characterized by the pathological resorption of the periodontal tissues and potential tooth loss, periodontitis not only impacts oral health and overall quality of life, but also has implications for systemic health, including associations with cardiovascular diseases, diabetes, and other systemic conditions (Sanz, Marco Del Castillo, et al. 2020; Romandini et al. 2021; Romano et al. 2021; Baima et al. 2022). The chronic spill-out of pathogens and inflammatory mediators from the active periodontal pockets into the bloodstream is regarded as the causal link underlying the relationships between periodontitis and the other non-communicable diseases (Sanz et al. 2018; Hajishengallis and Chavakis 2021). More recently, it has been hypothesized that the culprit for these consistent associations may reside at a more fundamental level, in the form of an accelerated biological aging (Baima et al. 2021). Indeed, prolonged exposures to the proinflammatory environment associated with aging, commonly known as inflammaging and immunosenescence, can result in significant changes across various tissues, including the periodontium (Ebersole et al. 2016; Franceschi et al. 2018).

The multifactorial imbalance of the relationship between host and parasites occurring in destructive periodontal lesion and the resulting defective healing lead to heterogenous patterns of periodontal attachment and alveolar bone destruction, depending on patient-, tooth-, and site-level characteristics (D’Aiuto et al. 2005). A peculiar type of bone defect anatomy found in the most severe stages of

periodontitis is the infrabony defect, where the base of the pocket is located apical to the alveolar crest (Papapanou and Tonetti 2000). These defects are typically assessed through periodontal probing and radiographic imaging and play a crucial role in determining the severity of periodontitis, its risk of progression, and in planning the appropriate treatment strategies (Papapanou et al. 1991; Nibali et al. 2021).

1.2 Periodontal Regeneration

In order to tackle these common types of bone deformities, the treatment guideline recently approved by the European Federation of Periodontology (EFP) recommends periodontal regeneration (Nibali et al. 2020; Sanz, Herrera, et al. 2020). Periodontal regeneration techniques utilize various approaches to stimulate the body's natural healing mechanisms and encourage the reformation of damaged periodontal tissues (Nyman et al. 1982; Gottlow et al. 1986). The selection of the most appropriate regenerative strategy depends on patient and anatomical characteristics (Cortellini and Tonetti 2015), and it fundamentally lays on three key biological principles, that are an adequate supply of scaffolds, molecular mediators, and cells (Larsson et al. 2016). Moreover, an adequate blood supply and proper root surface decontamination of the site are fundamental corollaries for this finely tuned cascade of cellular and molecular events to occur (Cortellini and Tonetti 2015).

Historically, post-surgical periodontal healing has been characterized by the maturation of gingival connective tissue, partial regeneration of alveolar bone and cementum, and the epithelialization of the root surface (Caton and Zander 1979; Listgarten and Rosenberg 1979). This process, referred to as repair, typically involves the formation of a long junctional epithelium that offers protection against bacterial invasion and ankylosis. In the pursuit of enhancing the long-term prognosis of affected teeth, regenerative treatments were proposed in the early 1980s (Nyman et al. 1982). Unlike repair, periodontal regeneration aims for the creation of new cementum with attaching collagen fibers on previously affected root surfaces by periodontitis, along with the re-growth of alveolar bone. Melcher's 1976 proposition suggested that the cell types repopulating the root surface post-surgery play a crucial role in determining the nature of the attachment formed. Following flap surgery, the coagulum within the bone defect might be repopulated

by diverse cell types, including epithelial, gingival connective tissue, bone, and periodontal ligament cells—the latter believed to be responsible for genuine regeneration (Melcher 1976; Karring et al. 1980; Buser et al. 1990; Parlar et al. 2005). Notably, scientific evidence supporting the role of periodontal ligament-derived cells in selective cell repopulation and attachment restoration was provided by an early animal study (Karring et al. 1980). After reimplanting in the extraction socket roots that received different types of treatment, this study demonstrated that significant new connective tissue attachment was possible on roots affected by periodontitis, particularly in cases where the periodontal ligament remained intact. This insight led to early regenerative strategies aimed at preventing contact between the gingival epithelium and the root surface during the initial healing phase, accomplished by using a cell-occluding membrane (Gottlow et al. 1986).

Following these early concepts, decades of research have prompted a burgeoning evolution of surgical techniques and biologic materials (Ramseier et al. 2012; Cortellini and Tonetti 2015). Clinical protocols have been developed to enhance periodontal regeneration, including the use of various surgical techniques combined with bone grafts, guided tissue regeneration by the use of membranes (GTR), recombinant growth factors, enamel matrix derivative (EMD), hyaluronic acid, and combinations of the above (Sculean et al. 2015; Pilloni et al. 2021). Besides materials, soft tissue management is another key aspect to be considered when dealing with periodontal regeneration. First attempts were based on traditional flaps that were not able to provide full tissue closure in interproximal spaces, right above biomaterials were placed and exposing them to the risk of bacterial exposure (Ramfjord and Nissle 1974). A leap forward occurred when the first papilla preservation technique was introduced, placing a semilunar incision from the angular lines of two adjacent teeth and 3 mm apical to the edges of the defect (Takei et al. 1985). This concept was further advanced when modified and simplified papilla preservation techniques were introduced in order to allow a better flap design for papilla elevation (Cortellini et al. 1995; Cortellini et al. 1999). A further evolution toward minimal invasiveness was presented, in which a single flap procedures (Trombelli et al. 2007; 2009) or M-MIST (modified minimally invasive surgical technique) (Cortellini and Tonetti 2009) were suggested to access the defect area in order to provide less surgical trauma, more stabilization of the coagulum and to limit post-surgical recession.

A systematic review with meta-analyses conducted by our group has evaluated the rate of pocket resolution after clinical periodontal regeneration (Aimetti et al. 2021). The resulting figure was the ability of minimally invasive flaps in achieving 92.1% of probing pocket depth (PPD) ≤ 4 mm. To date, regenerative treatment of intrabony defects in periodontal patients has proven to improve the prognosis of severely compromised elements with periodontal intrabony defects up to 10-15 years (Cortellini et al. 2020). Prognostic factors linked to the patient, the defect, and the site itself could significantly impact the outcomes of regenerative therapy. These factors may encompass aspects like plaque/inflammatory control, local anatomical characteristics, the surgical technique employed, as well as the inherent potential for wound healing (Tonetti et al. 1995; Nibali et al. 2021). Among the other factors driving the clinical success of periodontal regeneration, aging has been regarded to possess an effect, despite not significant (Mikami et al. 2022). However, traditional studies have only focused on chronological age, which might not be the most accurate marker of the cumulative damage to the biological systems and of the immune-inflammatory fitness of the patient (Kornman et al. 2017).

1.3 Periodontal Ligament-Derived Stem Cells

Contemporary regenerative strategies are primarily centered on promoting the intrinsic regenerative capabilities of periodontal tissues within the localized lesion (Cortellini and Tonetti 2007). However, surgical techniques and biomaterials currently used restrict the efficacy of periodontal regeneration to selected cases (i.e. two-three walls infra-bony defects), which only represent about 20% of the infrabony defects (Vrotsos et al. 1999). All the other infra-bony defects, together with the II-III degree furcation defects, the craters and the suprabony defects, still represent an unsolved challenge for predictable periodontal regeneration (Bartold et al. 2016). A significant limitation in addressing these issues may stem from the insufficient availability of stem/progenitor cells necessary to regenerate the lost tissues (Lin et al. 2015). In the last years, the emergence of bioengineered therapies has prompted extensive research into the application of cell-based treatments, particularly the utilization of undifferentiated mesenchymal cells in conjunction with various scaffold materials (Nuñez et al. 2019). Adult stem cells, recognized as somatic stem cells, are undifferentiated cells present in numerous adult tissues,

possessing the capacity to transform into specialized cells and sustain tissue well-being (Roato et al. 2021). The characterization of mesenchymal stem cells remains a complex task, and prevailing techniques yield diverse cell populations. Nevertheless, the International Society for Cellular Therapy has suggested minimal standards for identifying these cells based on their phenotypic characteristics, encompassing traits like plastic adherence, the expression of specific surface markers, and their capability to differentiate into multiple cell types in laboratory settings (Dominici et al. 2006).

The first characterization and isolation of Periodontal Ligament-Derived Stem Cells (PDLSCs) were reported in 2004, meeting criteria for identifiable mesenchymal stem cell (MSC)-like properties (Seo et al. 2004). Protocols for isolating human PDLSCs have since been published. Numerous studies have assessed the immunomodulatory properties and regenerative potential of these cells when combined with surgical strategies, using autogenous or allogeneic stem cells expanded in bioreactors (Qiang Li et al. 2020). Classical studies have confirmed their ability to give rise to fibroblasts, osteoblasts, and cementoblasts, whereas they were found to primarily originate from perivascular and paravascular regions (Roguljic et al. 2013). A systematic review on PDLSCs use in periodontal regeneration in animal models reported statistically significant improvements in 70.5% of the results (Bright et al. 2015). Some pilot clinical trials have already proven the efficacy of PDLSCs-based cell therapy for periodontal regeneration (Sánchez et al. 2020; Sun et al. 2023), despite their translation on practice routine being limited by economic and regulatory issues. Key issues also include determining whether PDLSCs constitute a single population or multiple populations with distinct functions, and whether the chronic exposure to a pro-inflammatory environment may affect their reservoir, skewing their fate to a more senescent pro-fibrotic phenotype (Roguljic et al. 2013).

1.4 Cellular Senescence

The periodontium relies on continuous remodeling, with cells and the extracellular matrix adapting to oxidative stressors and external forces. Postnatal and adult stem cells play crucial roles in maintaining tissue balance and aiding repair and regeneration (Bartold and Gronthos 2017). In adults, stem cells are

located in niches, microenvironments controlling tissue integrity (Mannino et al. 2022). As individuals age, the number of these niches decreases, limiting the potential for sample collection and obtaining multiple differentiation lines for tissue engineering. Indeed, age-related functional declines often result from stem cell exhaustion, leading to organ dysfunction (Lin et al. 2015).

The possibility that factors such as aging and inflammation may drive to the exhaustion of the regenerative potential of these stem cell populations, potentially impairing the outcomes of clinical techniques, has been proposed in the last years (Huang et al. 2016; Liu et al. 2022; Mikami et al. 2022). Indeed, both aging and chronic inflammation are known to induce cellular senescence, an evolutionary conserved mechanism which prevent cells from replicating when they get damaged (Muñoz-Espín and Serrano 2014). Cellular senescence embodies a multifaceted intrinsic process triggered in response to DNA damage, ultimately resulting in permanent cell cycle arrest and a decrease in the reserve of stem cells (Gorgoulis et al. 2019). Specifically, it is a stress-driven mechanism where telomere shortening initiates a p53-dependent pathway, leading to the activation of p21 and p16Ink4a (Campisi and d'Adda di Fagagna 2007). While mitosis primarily drives telomere shortening, various stress inducers like oxidative stress (von Zglinicki 2002), exposure to cigarette smoke (Nyunoya et al. 2006), and prolonged exposure to bacterial virulence factors like cytolethal distending toxins (Blazkova et al. 2010) can also induce replicative senescence. This stress-induced premature senescence is characterized by the presence of positive beta-galactosidase (SA-beta-Gal) staining and resistance to apoptosis (Toussaint et al. 2000). Beyond possessing a limited intrinsic potential for tissue healing, senescent cells exert a negative impact on the surrounding niche and on chronic systemic inflammation through the secretion of the so-called senescent-associated secretory phenotype (SASP) (Aquino-Martinez et al. 2021; Baima et al. 2021). SASP represents a cluster of proinflammatory cytokines, chemokines, and proteases released by these cells, which is known to bring about significant modifications to the surrounding microenvironment (Coppé et al. 2010; Nelson et al. 2012). Senescent cells typically cluster in regions associated with age-related conditions like cancer and osteoporosis, where they contribute to inflammation in a negative loop through the expression of SASP (Lee and Schmitt 2019). An excessive accumulation of senescent cells has been also linked to the development of atherosclerotic lesions

and osteoarthritis, while the targeted removal of such cells has shown promise in improving clinical outcomes in animal models (Coryell et al. 2021).

Following these pivotal discoveries, researchers have delved into the potential presence of senescent cells in the aged and diseased periodontium (Chen et al. 2022). Recent findings of senescent cells within periodontal tissues, attributed to the chronic gram-negative bacterial stimulation and related inflammation, may potentially provide key insights in the etiopathogenesis of periodontitis (Aquino-Martinez, Khosla, et al. 2020). Also, an animal study unveiled that young mice exposed to gram-negative lipopolysaccharide (LPS) demonstrated a premature accumulation of senescent-like osteocytes in the alveolar bone, indicated by increased expression of senescence-related markers like p53, p16ink4a, and p21 mRNA (Aquino-Martinez et al. 2021). Increased levels of DNA damage and an upsurge in SASP gene expression was also reported. In another murine study, a chronic hyperglycemic state led to a significant elevation in the detection of p16- and p21- cells in the gingival epithelium (Zhao et al. 2021). This also led to increased secretion of SASP factors (Coppé et al. 2010), including tumor necrosis factor-α and interleukin IL-1β, IL-6, IL-12 in the gingival crevicular fluid (GCF). As described, SASP did not only compromise the function of the gingival epithelial barrier, but transmitted senescence to neighboring epithelial cells through paracrine signaling (Zhao et al. 2021). Notably, even gingival MSCs from subjects without periodontitis could exhibit a senescent phenotype, characterized by reduced proliferation activity, increased p16 and p21 mRNA levels, and staining for SA-beta-Gal (Páez et al. 2020). Importantly, SASP secretion in these cells required an external inflammatory stimulus, such as LPS.

Regarding the potential mechanisms linking chronic inflammation and cellular senescence, increasing evidence has pointed towards the involvement of purinergic signaling in the activation of the intricate intracellular complexes known as inflammasomes. These molecular complexes play a pivotal role in regulating the release of potent inflammatory cytokines, including interleukin IL-1β, IL-6, and IL-18, contributing to SASP. Among the pathogens associated with periodontitis, *Porphyromonas gingivalis* has garnered particular attention for its ability to manipulate the immune response, specifically by dampening the activation of the NLRP3 inflammasome. A recent study shed light on the connection between this pathobiont and purinergic signaling through the purinergic P2X7 receptor (Ramos-

Junior et al. 2015). This receptor appears to play a crucial role in modulating IL-1β secretion induced by extracellular adenosine triphosphate (eATP), a common trigger for inflammation. The research revealed that *P. gingivalis*, utilizing its fimbriae, can impede eATP-induced IL-1β secretion, with the process being dependent on the purinergic P2X7 receptor. Importantly, the research also examined the modulation of P2X7 receptor and NLRP3 transcription in periodontitis, suggesting their relevance in its immunopathogenesis. However, a clear characterization of the patterns of expressions of P2X7R in the context of periodontal ligament cells is still lacking.

The notion of cellular senescence is intricately intertwined with stem cell depletion. The decline in adult stem cell function due to cellular senescence is considered a significant contributor to age-related reductions in tissue healing and regenerative potential (Sharpless and DePinho 2007). In a study conducted by Li et al. exploring the impact of aging on PDLSCs by comparing cells from young and elderly donors (X. Li et al. 2020), they observed diminishing proliferation, decreased multilineage (osteogenic, adipogenic, and chondrogenic) differentiation potential, and reduced immunosuppressive capacity of PDLSCs with advancing age, alongside an increase in apoptosis. Protein expression levels of Runx2, ALP, COL1A1, and PPARγ2 were notably reduced in PDLSCs from older subjects compared to younger individuals. Based on this evidence, it is possible to hypothesize that the continuous regeneration of impaired tissue within the periodontitis proinflammatory environment potentially results in an abnormal buildup of senescent cells, compromising the stem cells reservoir.

1.5 Cellular Rejuvenation for Clinical Translation

Senotherapy represents a promising frontier in medical research, involving the use of both synthetic and natural compounds meticulously designed to target and eliminate senescent cells or mitigate the expression of SASP factors (Campisi et al. 2019). Within this emerging field, senolytic agents such as rapamycin, MCC950, and various natural compounds have undergone experimental scrutiny as potential therapeutics aimed at tackling senescence (Power et al. 2023). However, it is important to acknowledge that clinical trials are still in their nascent stages, leaving questions about the safety and efficacy of these approaches unanswered.

Furthermore, the application of senolytic drugs in the context of periodontal medicine remains largely unexplored (Baima et al. 2021)

Considering these crucial factors, an innovative avenue to modulate the senescent cell phenotype in PDLSCs tackling telomere shortening has been hypothesized. This approach involves the inhibition of methylation at the telomerase reverse transcriptase (TERT) promoter region through the use of DNA methyltransferase inhibitor (DNMTi) drugs like RG108 (Assis et al. 2018). By employing this strategy, it becomes possible to induce the expression of TERT, thereby influencing the length of telomeres by the addition of the telomere repeat TTAGGG. This intervention holds the potential for exerting beneficial effects on cellular senescence, thereby expanding the scope of applications for stem cell therapy (Oh et al. 2015). Recently, RG108 was tested on bone marrow-derived MSCs from swine, resulting in positive effects on senescence, apoptosis, and the expression of pluripotency genes (Qi Li et al. 2020). Based on these premises, there is the rationale to test RG108 on PDLSCs to revert their acquired senescent phenotype.

1.6 Study Rationale

To date, few studies have preliminary showed that PDLSCs derived from inflamed periodontal tissues could be successfully isolated, retaining their proliferation and migration abilities (Park et al. 2011), although with impaired immunomodulatory capacities (Liu et al. 2012). Moreover, it seems to emerge the concept that PDLSCs are a quite heterogeneous cell population, with some of them already skewed toward a reparative phenotype (Roguljic et al. 2013). Therefore, there is the need to further explore how the biological mechanisms of aging and inflammation affect the intrinsic regenerative potential of the periodontium, both at the cellular and clinical level. Furthermore, while the emergence of senescent cells and the SASP in periodontal tissues has garnered recent attention, their precise role in modulating regenerative potential remains inadequately explored. By addressing these critical knowledge gaps, this research endeavors to provide a more holistic understanding of the intricate dynamics between aging, inflammation, and periodontal regeneration, with the ultimate goal of advancing therapeutic approaches for periodontal disease management.

Aims of the Study

Based on these premises, the present elaborate poses different specific objectives (SO):

- SO1: To investigate the phenotypic characteristics and multi-differentiating capabilities of PDLSCs derived from both periodontally healthy (hPDLSCs) and periodontitis patients (pPDLSCs).
- SO2: To assess the stemness and senescence characteristics of hPDLSCs and pPDLSCs, based on the single or double expression of stemness and/or senescent markers.
- SO3: To explore the underlying mechanisms by which inflammation affects the regenerative capacity of PDLSCs, particularly investigating the role of P2X7R pro-inflammatory molecule.
- SO4: To explore a new method to modulate the stemness/senescence status of PDLSCs through an epigenetic regulator drug RG108.
- SO5: To preliminary evaluate the clinical implications of age- and inflammation-related changes in the periodontal microenvironment (BoP and SASP expression in the gingival crevicular fluid) for periodontal tissue healing and regeneration.

Materials and Methods

3.1 *In vitro* Study

3.1.1 Participants and Sample Collection

Participants were consecutively recruited from among the inpatients of the C.I.R. Dental School, University of Turin. Severely compromised teeth of 12 patients suffering from stage III-IV periodontitis were collected; wisdom teeth of 5 healthy donors were used as controls. All patients were in good general health and provided written informed consent for sample collection. The study received the approval by the Ethical Committee of the A.O.U. Città della Salute e della Scienza of Turin (n° 0107683).

3.1.2 Cell Isolation and *in vitro* Culture

Immediately after extraction, the root was gently scraped, and the tissue fragments underwent rinsing with physiological saline buffer (PBS). Subsequently, they were subjected to digestion for 10 minutes using a solution comprising 1 mg/ml of dispase II and 3 mg/ml of collagenase I. Following this, the tissue particles were washed again and placed into a 10 cm diameter petri dish containing α-MEM and 15% fetal bovine serum (FBS), 5% penicillin/streptomycin, and 1% amphotericin B. These culture dishes were subsequently incubated in a humidified environment with 5% CO_2 at 37°C. The medium was renovated after 7 days, allowing the cells to proliferate over a period of 15-20 days until reaching confluence. Later, the cells were separated, counted, and cultured in a mesenchymal cell growth medium comprising α-MEM + Glutamax, along with 5% human platelet lysate (hpl), and 5% penicillin/streptomycin.

3.1.3 Determination of Colony-forming Unit (CFU)

Individual cell suspensions from both participant groups were plated into 6-multiwell plates at a density of 0.6×10^3 cells per well in basal medium and cultivated for a duration of 2 weeks. Following this, the cells were fixed with 4% paraformaldehyde and subsequently stained using crystal violet. Colonies were then observed and counted using a microscope.

3.1.4 Cell Culture Conditions for Multiple Differentiation

To explore the differentiation capacity of PDLSCs, the cells were cultured in different specialized media. The osteogenic medium (OM) comprised α-MEM supplemented with 10% FBS, 50μg/mL ascorbic acid, 10^{-8} M dexamethasone, and 10mM beta-glycerophosphate, sustaining the cultures for 30 days. Mineralized matrix formation was then examined using Von Kossa staining. In parallel, cells were kept in culture for 21 days in adipogenic and chondrogenic media. Oil red O staining was employed to visualize the presence of adipocytes containing lipid droplets, while chondrogenic differentiation was assessed by staining for Aggrecan (ACAN) in chondrocyte micromasses.

3.1.5 RNA Isolation and Real-Time

RNA extraction was carried out in accordance with the TRIzol protocol. Subsequently, 1 µg of RNA underwent retro-transcription to single-stranded cDNA utilizing the High-Capacity cDNA Reverse Transcription Kit.

The mRNA expression level of specific genes, including NANOG, SOX2, OCT4, p21, p16, BCL2, RUNX2, SOX9, PPARg, and P2X7R was assessed. The primer sequences are detailed in Table 1. The amplification protocol involved 40 cycles at an annealing temperature of 58° or 60°C. To normalize the gene expression data, β-actin was selected as the reference gene. Quantitative analysis was performed using the $2^{-\Delta\Delta Ct}$ method with CFX Manager software. The gene expression results are presented as mean ± SEM.

Table 1. Sequences of primers used for real time polymerase chain reaction.

gene	primer	5’-3’ sequence
PPARγ	FW	AGACAACCTGCTACAAGCCC
	REV	GGGCTTGTAGCAGGTTGTCT
CDKN2A, p16	FW	AGGTCATGATGATGGGCAGC
	REV	CACCAGCGTGTCCAGGAAG
CDKN1A, p21	FW	CAAGCTCTACCTTCCCACG
	REV	TCGACCCTGAGAGTCTCCAG
NANOG	FW	ACCCAGCTGTGTGTACTCAA
	REV	GGAAGAGTAAAGGCTGGGGT
OCT4	FW	CGAGAGGATTTTGAGGCTGC
	REV	CGAGGAGTACAGTGCAGTGA
SOX2	FW	CGAGAGGATTTTGAGGCTGC
	REV	CGAGGAGTACAGTGCAGTGA
BCL2	FW	ATGTGTGTGGAGAGCGTCAA
	REV	GGGCCGTACAGTTCCACAAA
SOX9	FW	AGCGAGCAGCAGCAGCAC
	REV	GAGTTCTGGTGGTCGGTGTAGTC
RUNX2	FW	CGAATGGCAGCACGCTATTA
	REV	TGGCTTCCATCAGCGTCAA
P2X7R	FW	GCTCTGGTGAGTGACAAGCT
	REV	GGTGTAGTCTGCGGTGTCAA
β-Actin	FW	CCCTGAAGTACCCCATCGA
	REV	AAGGTGTGGTGCCAGATTTTC

3.1.6 Flow Cytometry

Flow cytometry was employed to assess the expression of typical MSC markers in freshly isolated PDLSCs, utilizing a MSC phenotyping kit. Meanwhile, cells that were expanded and maintained in culture were identified as positive for CD105, CD73, CD90, and CD44, and negative for CD45 (Dominici et al. 2006). To achieve this, cultured cells were detached, and a labeling protocol for surface antigens was executed. Monoclonal antibodies that were fluorochrome-conjugated, along with isotypic controls, were used in the labeling process. Specifically, the following antibodies and controls were employed: human CD105 PE, CD73 FITC, CD44 FITC, CD45 PerCP, IgG1 PE, IgG1 FITC, IgG2a PerCP, and CD90 PerCP. Unstained cells were included as a control. For the detection of OCT4 and p21, a double intranuclear and cytoplasmic staining method was adopted.

As indicators of cell death, Annexin V, a phospholipid-binding protein, was employed, following the manufacturer's instructions. Data were acquired using a MACsQuant 10 flow cytometer and analyzed with MACsQuantify software. The results are presented as the percentage of cells which expressed specific markers, represented as mean ± SD.

3.1.7 Staining for β-galactosidase

Both cells underwent two passages in culture, and the degree of senescence was assessed using β-Galactosidase staining following the manufacturer's instructions provided in the senescence β-Galactosidase staining kit. In brief, PDLSCs were initially seeded in 6-multiwell plates and allowed to culture for 24 hours. Subsequently, the culture medium was aspirated, cells were washed, fixed, and then stained with the β-Galactosidase solution. This staining procedure was conducted overnight within a dry incubator. The resulting blue β-Galactosidase staining was observed and documented using a microscope.

3.1.8 Immunofluorescence

Immunofluorescent staining was utilized to examine the presence of OCT4 and p21 in PDLSCs. Initially, the PDLSCs were cultured in 8-well chamber slides. Following a 24-hour incubation at 37°C to facilitate cellular adhesion, the cells were fixed with 4% paraformaldehyde, permeabilized using TRIS-buffered saline (TBS) containing 0.5% Triton, and subsequently blocked with TBS containing 3% bovine serum albumin.

The subsequent steps involved incubating the cells with primary antibodies, with p21 requiring a 1-hour incubation at room temperature, and OCT4 necessitating a 3-hour incubation period. Afterward, the cells were exposed to secondary antibodies linked to a fluorophore for 1 hour at room temperature. The antibodies used included unconjugated monoclonal antibodies specific to human OCT4 (9B7) and p21 (R.229.6), alongside their corresponding secondary antibodies (goat anti-Mouse IgG1 Alexa Fluor 568 and goat anti-rabbit IgG DyLight 488). Nuclei were counterstained using an antifade reagent with DAPI, and subsequent analysis was carried out using an inverted confocal microscope.

3.1.9 Statistical Analyses

Statistical analysis was conducted using Prism software (version 9.0). The data underwent Student's t-test to assess the differences between the hPDLSCs and pPDLSCs. Additionally, one-way ANOVA with the Bonferroni post hoc test was employed to assess the gene expression data across various groups (CTRL, DMSO,

and RG108 treated). A significance level of $p < 0.05$ was employed to determine statistical significance.

3.2 Clinical Study

The protocol for the clinical study was approved by the Institutional Ethical Committee (protocol number 00309/2021). The study complies with the ethical standards of the Declaration of Helsinki and it has been reported following the CONSORT guideline. Again, all patients enrolled provided signed informed consent.

3.2.1 Study Design and Population

The research was designed as a prospective study, where consecutive patients who had undergone steps I-II of periodontal therapy were considered for inclusion between January and November 2022. Two calibrated examiners evaluated the eligibility based on specific criteria. Inclusion criteria comprised: 1) diagnosis of stage III or IV periodontitis (Papapanou et al. 2018b); 2) full-mouth plaque score (FMPS) and full-mouth bleeding score (FMBS) of <15%; 3) completion of steps I-II of periodontal treatment at least 2 months priorly; 4) tooth with residual PPD ≥ 6 mm, BoP+, and a radiographic intrabony component ≥ 3 mm, lacking furcation involvement (Aimetti et al. 2023), considered suitable for a minimally invasive procedure (Cortellini and Tonetti 2007) (Fig. 1A).

Exclusion criteria encompassed: 1) age < 18 years; 2) current smokers; 3) contraindications to surgery; 4) systemic diseases that could impact periodontal healing; 5) pregnancy and lactation; 6) a history of periodontal surgery at the experimental teeth.

3.3.2 Surgical Procedure

All the experimental sites underwent a MIST procedure under magnification (Cortellini and Tonetti 2007). The full-thickness flap was minimally raised both on the buccal and oral side, avoiding vertical releasing incisions. Granulation tissue was scraped from the bony surfaces of the defect, and the root was debrided using

minicurettes/ultrasonic device and chemically treated by EDTA. Regenerative procedure was carried out using a combination of enamel matrix derivatives and bone xenograft (Nibali et al. 2020; Sanz, Herrera, et al. 2020; Aimetti et al. 2021) (Fig. 1B-C). The flaps were then repositioned and sutured in order to obtain passive primary closure (Fig. 1D).

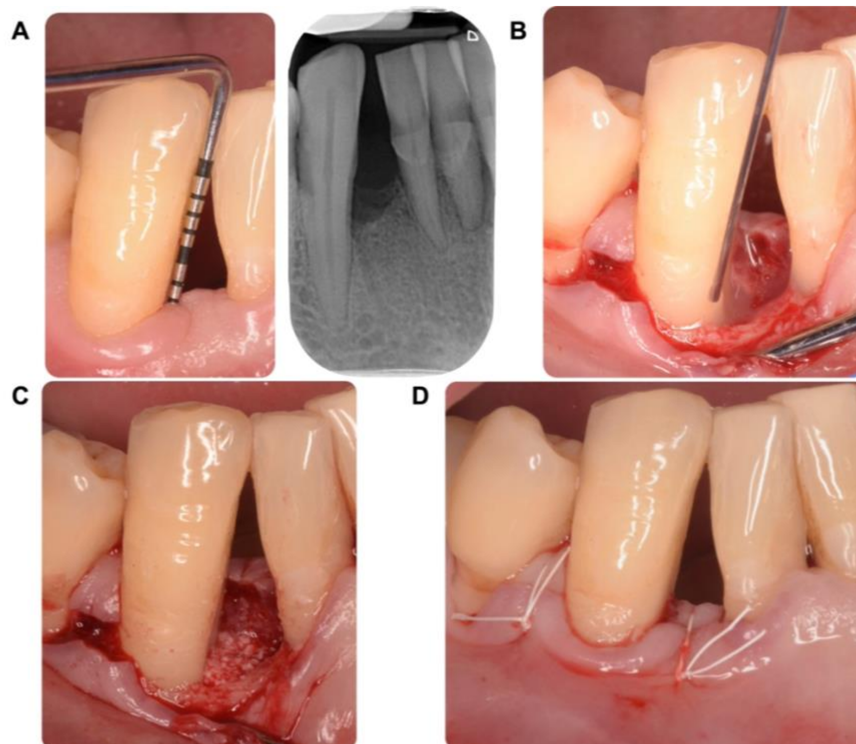

Figure 1. Description of the clinical procedure. Clinical and radiographic images showing the presence of a deep probing pocket depth associated to the radiographic aspect of an intrabony defect (A). After flap elevation, the defect is treated with a combination of enamel matrix derivatives (B) and bone xenograft (C). Eventually, the minimally invasive flap was sutured to obtain primary intention healing (D).

3.3.3 Post-operative and Maintenance Care

Patients were prescribed analgesic medication (ibuprofen 600 mg, every 8 hours for 3 days). Clinical control and wound medication were performed one week later. Sutures were removed 2 weeks after surgery. During the postoperative period, patients were advised to avoid toothbrushing and flossing in the operated area.

3.3.4 Clinical Measurements

Clinical measurements were performed at the selected defects by using a manual 1-mm graduated periodontal probe (PCP-UNC 15) at the day of surgery (T1) and 1 year after periodontal regenerative surgery (T3) by the same blinded

examiner. The following clinical parameters were assessed: presence/absence of bacterial plaque (PI), presence/absence of BoP, PPD, gingival recession (REC), clinical attachment level (CAL) and the width of keratinized tissue. Since the first two of weeks after treatment are the most significant in determining the stability of the wound, the EHI index (Wachtel et al. 2003) was evaluated at 14 days (T2) by the same blinded examiner.

3.3.5 Sampling of GCF

The GCF was collected from the experimental sites before conducting any clinical examination, ensuring prevention of blood contamination. The process involved isolating the sites with cotton rolls and meticulously removing any supragingival plaque. After gently drying the areas using an air syringe, GCF samples were obtained using paper strips. These strips were carefully inserted into the pockets until encountering slight resistance and were left in place for 30 seconds. Any strip contaminated with blood was excluded from the study. The volume of collected GCF was measured, and each GCF-containing strip was placed into separate coded sealed Eppendorf microcentrifuge tubes, each containing 100 μL of sterile phosphate-buffered saline (PBS). These samples were stored at -80°C until further processing.

3.3.6 Multiplex Bead Immunoassay

Levels of the markers IL-1β, IL-6, IL-12, MMP-8, and MMP-9 were assessed using the highly sensitive Bio-Plex Suspension Array System, according to the manufacturer's provided protocols. In brief, specialized anti-cytokine antibody-conjugated beads were loaded into individual wells of a 96-well plate. Following a washing step, standards and undiluted GCF samples were added to their respective wells and allowed to incubate for 30 minutes. Subsequently, the plates were washed, and biotin-conjugated detection antibodies were introduced. After an additional 30-minute incubation and subsequent washing, streptavidin-conjugated PE was added for a 10-minute interval. The resulting complexes were then solubilized by introducing Bio-Plex assay buffer to each well and subjected to analysis by the Bio-Plex Suspension Array System to determine the total quantities of each marker.

3.3.7 Statistical Analysis

Normality of the data distribution was assessed using the Shapiro-Wilk test. Differences in continuous variables were evaluated through paired t-tests or Wilcoxon-test, depending on data distribution. Categorical variables were compared through the chi-square test or Fisher's exact test as appropriate. The EHI values were categorized into two groups: one denoting favorable wound healing (EHI 1, 2, and 3) and the other indicating unfavorable healing characterized by incomplete flap closure and necrosis (EHI degrees 4 and 5). A multiple stepwise backward logistic regression model was constructed to identify predictors of favorable EHI, and the results were presented as odds ratios (OR) with 95% confidence intervals (CI). Spearman's and Pearson's correlation analyses were performed to investigate associations between GCF cytokine values and EHI scores as well as clinical outcomes, respectively. Statistical significance was defined as p-values less than 0.05. All statistical analyses were carried out using commercially available software (IBM SPSS Statistics, version 27).

Results

4.1 *In vitro* Study

Part of the results described within the present chapter has been previously published in Roato, Baima, et al. 2023.

4.1.1 Characteristics of PDLSCs

PDL tissues obtained from individuals with periodontitis and healthy individuals resulted in a non-homogeneous cell population (Fig. 2A). This cohort consisted of a minor subset of MSCs, marked by CD105, CD73, CD90, and lacking CD45 expression, alongside non-MSCs expressing CD14, CD19, CD34, CD45, and epithelial cells characterized by EpCam1 (specific data not presented). An immunophenotype analysis was conducted on PDLSCs from initial passage (p0) to passage 3 (p3), demonstrating a comparable proportion of MSCs between patients and healthy controls across passages, with the exception of p3, where pPDLSCs exhibited a higher proportion, $p < 0.05$ (Fig. 2B). A consistently high and stable MSC proportion between the two groups was maintained throughout the subsequent passages (specific data not provided). Crystal violet staining evidenced how both hPDLSCs and pPDLSCs initially formed small colonies when cultured in mesenchymal growth medium (Fig. 2C). After reaching confluence (p0), they were further expanded for additional 8 passages.

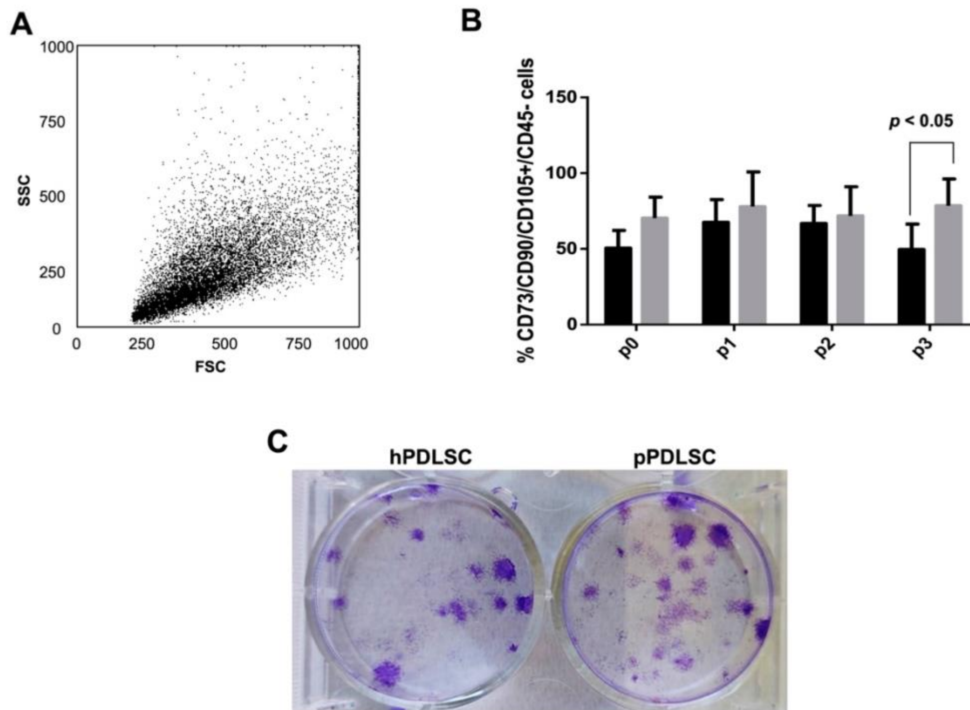

Figure 2. Examination of PDLSCs following their isolation. In panel A, the dot plot illustrates the diverse cellular morphology observed shortly after PDL isolation. As indicated in panel B, the proportion of PDLSCs co-expressing CD73, CD90, and CD105, while lacking CD45 expression, remained consistent and similar between hPDLSCs and pPDLSCs up to passage 2. However, at passage 3, this proportion was notably higher in pPDLSCs, with a significance level of $p < 0.05$. Furthermore, both hPDLSCs and pPDLSCs exhibited the ability to generate small colonies, which were visualized through crystal violet staining (panel C).

The master gene expression related to chondrocytes, adipocytes, and osteoblasts was examined in both cell groups. The analysis revealed comparable levels of SOX9 and RUNX2 expression (Fig. 3A, C), while the expression of PPARγ was increased in pPDLSCs than in hPDLSCs, with a significance of $p < 0.05$ (Fig. 3B). Related to osteogenic genes, we observed an upregulation of ALP and COLL1 in pPDLSCs, which is typically associated with inflammatory conditions. Interestingly, we also detected an increased expression of P2X7R in pPDLSCs, a purinergic signaling marker recently implicated in the development of inflammatory reactions.

Later, both hPDLSCs and pPDLSCs were cultured in conventional mesenchymal growth medium and specific differentiation media. The cells exhibited the formation of chondromasses containing ACAN+ chondrocytes when subjected to chondrogenic differentiation medium (depicted in Fig. 3E-G), while they lacked the presence of chondrocytes when cultured in the standard medium (illustrated in Fig. 3D-F). Adipogenic differentiation medium prompted the

generation of Oil red O-positive adipocytes (as shown in Fig. 3I-M), a feature not observed in the standard medium (as seen in Fig. 3H-L). Finally, the osteogenic medium induced the differentiation of osteoblasts, characterized by the formation of mineralized nodules (Fig. 2O-Q), whereas such nodules were not observed in the standard medium (Fig. 2N-P).

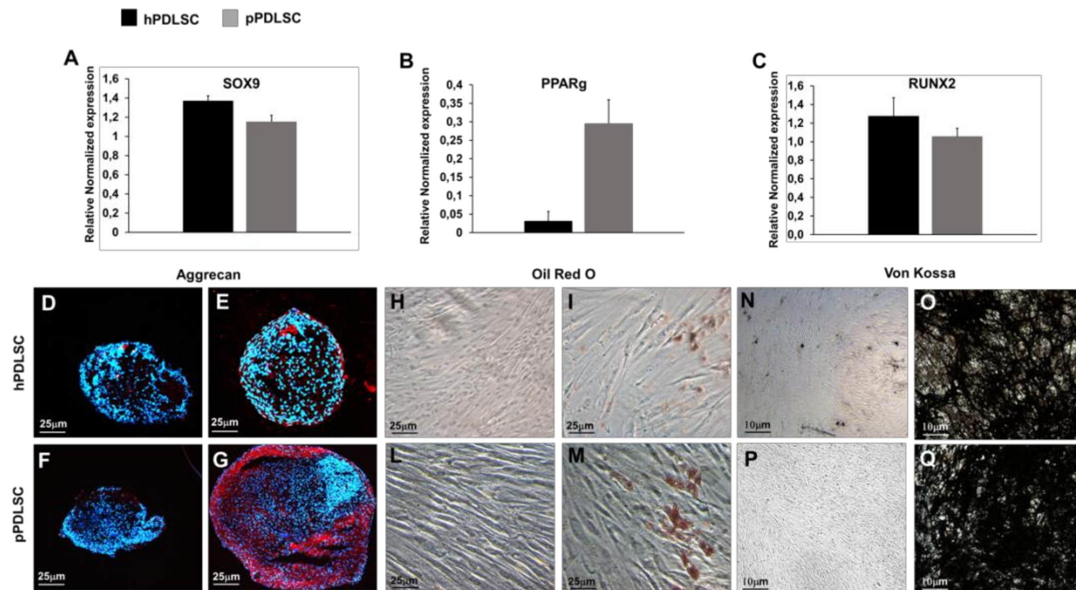

Figure 3. Multi-differentiating capabilities of PDLSCs. The expression of the chondrocyte master gene SOX9 (indicated in A) and the osteoblast marker RUNX2 (depicted in C) exhibited similarities between hPDLSCs and pPDLSCs. However, concerning adipocytes, PPARγ expression was notably higher in pPDLSCs compared to hPDLSCs, with statistical significance ($p < 0.05$) (highlighted in B). Upon exposure to chondro-differentiation medium, both hPDLSCs and pPDLSCs differentiated into ACAN+ chondrocytes, forming micromasses (demonstrated in E-G), whereas they did not exhibit differentiation in the basal medium (as shown in D-F). When subjected to adipo-differentiation medium, they gave rise to Oil red O-stained adipocytes (illustrated in I-M), a feature absent in the standard medium (as visualized in H-L). In the presence of osteogenic medium, osteoblasts formed mineralized nodules (presented in O-Q), whereas no such formation was observed in the standard medium (depicted in N-P). The graphs represent the mean value of gene expression ± SEM.

4.1.2 Impact of Aging and Inflammation on PDLSCs

A distinctive characteristic of MSCs is the stemness-related gene expression. Therefore, we assessed the expression levels of SOX2, OCT4, and NANOG, revealing that these genes were more prominently expressed in pPDLSCs compared to hPDLSCs (Fig. 4A). Notably, as we observed that pPDLSCs tended to grow slowly and exhibited a shift from their characteristic spindle shape to a more square-like shape with increasing passages (data not shown), we examined the expression of two senescence markers, p16 and p21. Our findings indicated a significant elevation of these markers in pPDLSCs relative to hPDLSCs (Fig. 4B).

Consequently, we postulated that pPDLSCs might experience a form of replicative senescence, and we tested this hypothesis by conducting β-Gal staining at an early passage. While hPDLSCs displayed negative β-Gal staining, many pPDLSCs exhibited positive staining, indicative of a senescent state (Fig. 4C-D). Furthermore, our flow cytometry analysis revealed that a subset of PDLSCs concurrently expressed stemness and senescence markers, specifically OCT4 and p21. Notably, this specific pattern was more prevalent in pPDLSCs than in hPDLSCs (38 ± 16.3 vs. 14.7 ± 10.9, mean ± SD) (Fig. 4E). Immunofluorescence staining further confirmed the expression of OCT4 in both hPDLSCs and pPDLSCs, with a higher number of cells simultaneously expressing OCT4 and p21 in pPDLSCs (Fig. 4F-G). Eventually, our findings also indicated a significantly more elevated expression of the inflammatory marker P2X7R in pPDLSCs with respect to hPDLSCs (Fig. 5).

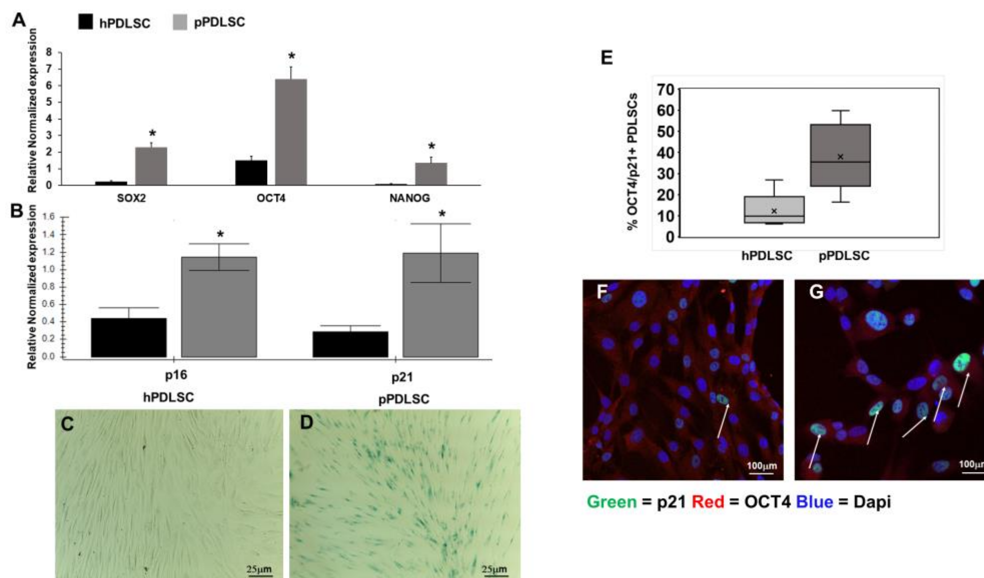

Figure 4. Identification of a subset of senescent cells in pPDLSCs. In comparison to hPDLSCs, pPDLSCs exhibited significantly higher expression levels of stemness genes, including SOX2, OCT4, and NANOG (indicated by * $p < 0.05$) (as illustrated in A). Furthermore, senescence-associated genes, p16 and p21, were notably upregulated in pPDLSCs, denoted by *$p < 0.01$ (as depicted in B). While β-Gal staining was negative for hPDLSCs, many cells in pPDLSCs were β-Gal+, confirming their senescent state (as presented in C-D). Intracellular staining revealed a subset of PDLSCs concurrently expressing both stemness (OCT4) and senescent (p21) markers (shown in E), where 'x' signifies the mean value of the percentage of PDLSCs positive for both OCT4 and p21. Immunofluorescence staining demonstrated a higher number of cells co-expressing OCT4 and p21 (indicated by white arrows) in pPDLSCs compared to hPDLSCs (illustrated in F-G).

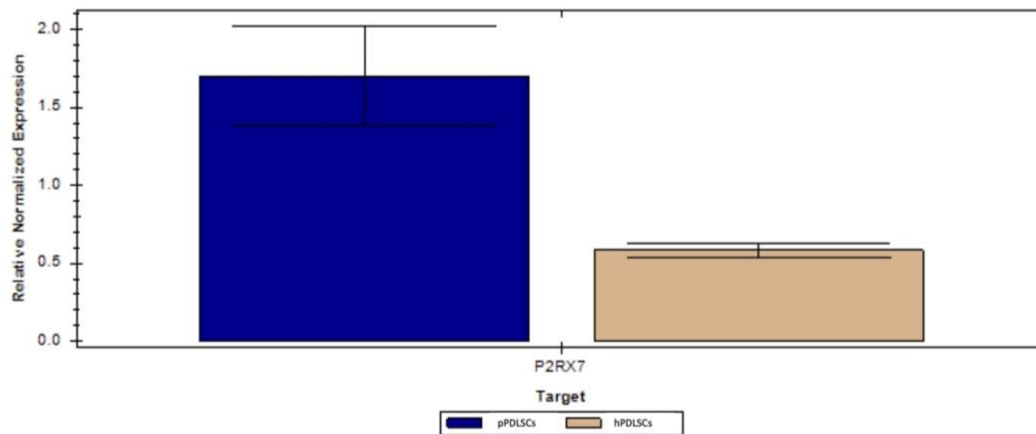

Figure 5. P2X7R expression in hPDLSCs and pPDLSCs. The inflammatory gene P2X7R was significantly more expressed in pPDLSCs than in hPDLSC * $p < 0.05$.

4.1.3 RG108 Modulates the Status of PDLSCs

We examined the influence of two different concentrations of RG108 (50 μM and 100 μM) on PDT and cell viability. A 5-day culture period did not reveal any substantial differences in the growth rates between hPDLSCs and pPDLSCs under various culture conditions. In terms of BCL2 anti-apoptotic gene, it was observed that pPDLSCs showed a significantly reduced expression than hPDLSCs (as shown in Fig. 6A). Notably, treatment with RG108 at 100 μM led to an increase in the expression of BCL2 in pPDLSCs, bringing it to levels similar to those observed in hPDLSCs (as depicted in Fig. 6B). Annexin V, the apoptotic marker, exhibited higher expression in pPDLSCs in comparison to hPDLSCs, although the difference was not statistically significant.

Furthermore, we explored the impact of RG108 treatment on the expression of p16 and p21, and found no significant variations in hPDLSCs. However, when we analyzed pPDLSCs treated with 100 μM RG108, a significant reduction in the expression of both p16 and p21 was evident (as illustrated in Fig. 7A-B). Additionally, we investigated whether RG108 treatment could influence the expression of stemness genes, and in hPDLSCs, we observed no significant alterations (Fig. 7C), but in pPDLSCs, treatment with 100μM RG108 led to an increase in SOX2 and OCT4 expression (Fig. 7D). Additionally, we examined whether RG108 might modulate the PDLSCs subset which co-expressed OCT4 and p21. This experiment revealed a decrease in pPDLSCs treated with 100μM RG108, while no significant modulation was detected in hPDLSCs (Fig. 7E).

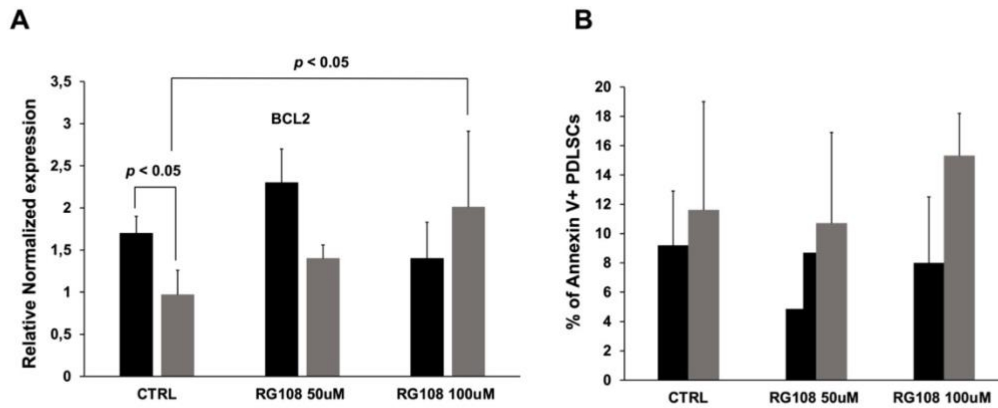

Figure 6. Effect of RG108 on BCL2 and annexin. The expression of the anti-apoptotic gene BCL2 was notably lower in pPDLSCs than in hPDLSCs, showing statistical significance ($p < 0.05$) (as seen in Fig. A). However, upon treatment with 100 μM RG108, there was a significant increase in BCL2 expression in pPDLSCs ($p < 0.05$) (as shown in Fig. B). Moreover, we observed a tendency toward higher levels of Annexin V expression in pPDLSCs in comparison to hPDLSCs.

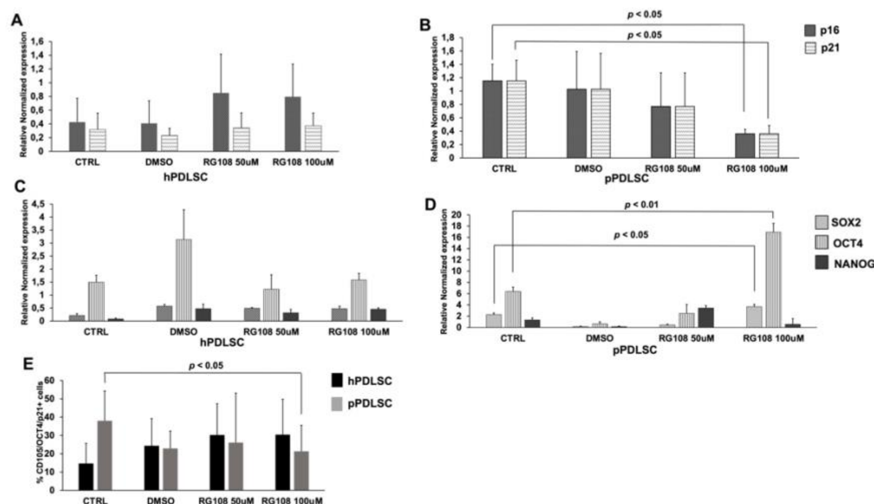

Figure 7. RG108 affected the senescent phenotype of pPDLSCs. In hPDLSCs, RG108 showed no significant variations in p16 and p21 expression under any conditions (as illustrated in Fig. A). However, in pPDLSCs, the application of RG108 at 100μM led to a notable reduction in both p16 and p21 expression, demonstrating statistical significance ($p < 0.05$) (as demonstrated in Fig. B). While the expression of stemness genes, including SOX2, OCT4, and NANOG, remained largely unaffected in hPDLSCs (Fig. C), treatment with RG108 at 100μM induced a noteworthy increase in SOX2 ($p < 0.05$) and OCT4 ($p < 0.01$) in these cells (Fig. D). Furthermore, RG108 contributed to a decrease in the subset of pPDLSCs co-expressing OCT4 and p21 ($p < 0.05$), without significant modulation observed in hPDLSCs (Fig. E).

We also explored whether RG108 had an impact on the multi-differentiating abilities of PDLSCs. In both hPDLSCs and pPDLSCs, osteogenic and chondrogenic abilities remained unaltered (as depicted in Fig. 8A-B). It is worth noting that PPARγ expression was initially very low in hPDLSCs and remained unaffected by RG108. Conversely, a significant up-regulation was observed in pPDLSCs (as shown in Fig. 8E-F). Additionally, when cultured under adipogenic

conditions, a higher number of cells harboring lipid droplets was observed in pPDLSCs treated with 100µM RG108 (as indicated in Fig. 8C-D).

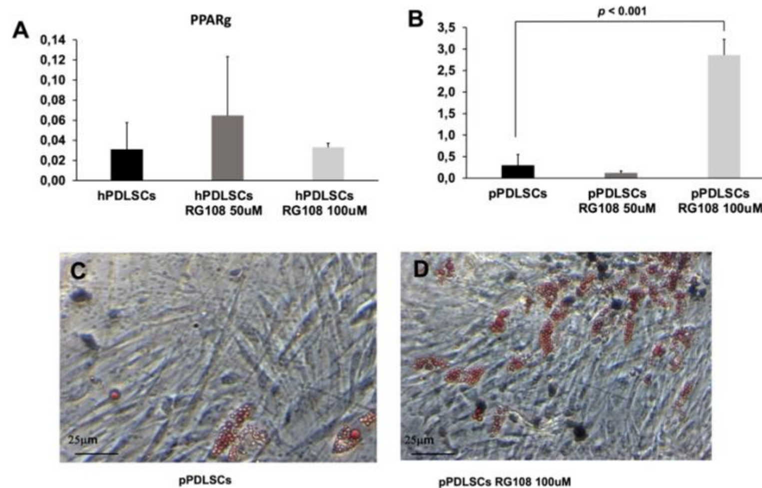

Figure 8. RG108 enhanced the adipogenic potential in pPDLSCs. The chondrogenic and osteogenic capabilities in both hPDLSCs and pPDLSCs remained unaffected by RG108, while a noteworthy up-regulation of PPARγ was observed in pPDLSCs ($p < 0.01$) (as illustrated in A-B). In pPDLSCs treated with RG108, there was a notable increase in their adipogenic potential, indicated by the increased formation of cells containing lipid droplets (as shown in C-D).

4.2 Clinical Findings

4.2.1 Patient Characteristics and Clinical Outcomes

Forty-four patients (26 females; mean age: 57.3 ± 8.5 years) providing one experimental site each were consecutively enrolled. Their characteristics both at patient- and tooth-level are summarized in Table 2.

Table 2. Characteristics of the experimental sample at the baseline.

Variables	Values
Patient level	
Age (years; mean ± sd)	57.3 ± 9.1
Females/Males (n)	26/18
FMPS (%; mean ± sd)	10.6 ± 1.8
FMBS (%; mean ± sd)	8.1 ± 1.7
Tooth level	
Tooth type (anterior/premolar/molar; %)	34.1/38.6/27.3
Dental arch (maxilla, mandible; %)	61.4/38.6
KT width at buccal site (mean ± sd)	4.1 ± 1.2

FMPS, full-mouth plaque score; FMBS, full-mouth bleeding score; KT, keratinized tissue; sd, standard deviation.

Table 3 reports the comparison between baseline and post-treatment clinical outcomes. Periodontal regeneration yielded to significant changes at 1 year in term of BoP and PPD reduction, as well as CAL gain, whereas it did not significantly affect recession.

Table 3. Characteristics of treated sites at baseline and at 1 year after periodontal regeneration.

Variables	Baseline	1-year	p-value
Presence of plaque (%)	16.7	12.7	<0.05
BoP (%)	50.0	35.0	<0.05
PPD (mm; mean ± sd)	7.6 ± 2.2	4.1 ± 1.4	<0.01
REC (mm; mean ± sd)	2.0 ± 1.6	2.2 ± 1.7	0.93
CAL (mm; mean ± sd)	9.6 ± 2.6	6.3 ± 2.2	<0.01

BoP, bleeding on probing; PPD, probing pocket depth; CAL, clinical attachment level; REC, gingival recession; sd, standard deviation.

4.2.3 Impact of Clinical Inflammation and SASP on EHI

To investigate potential predictors of enhanced or diminished EHI at the 2-week post-surgery, a logistic regression model was employed. The analysis revealed that the presence of a non-contained defect (OR = 0.128; 95%CI: 0.021– 0.768) and the presence of BoP at T1 (OR = 0.110; 95%CI: 0.019–0.654) were associated with a reduced likelihood of achieving an improved EHI (detailed in Table 4).

Table 4. Logistic regression analysis was performed to identify factors that predict favorable wound healing outcomes (defined as EHI scores of 1, 2, or 3) following periodontal regenerative surgery.

Variables	OR	95% (CI)	P-value
Type of bony defects			
Contained	1		
Non-contained	0.128	0.021-0.768	0.024
Presence of BoP at T1			
Yes	1		
No	0.110	0.019-0.654	0.015

BoP, bleeding on probing; CI, confidence interval; OR, odds ratio; T1, day of the surgery.

Regarding SASP factors, the amount of IL-1β ($r = .73$, $p < .001$), IL-6 ($r = .76$, $p < .001$), MMP-8 ($r = .32$, $p < .05$) and MMP-9 ($r = .60$, $p < .001$) in the GCF at T1 and EHI scores showed a statistically significantly positive correlation (Table 5).

Table 5. Correlation between SASP factors and EHI at 2 weeks after surgery (Spearman Rho coefficient).

SASP factor	r	P value
IL-1β	0.73**	0.000
IL-6	0.76**	0.000
IL-12	-0.21	0.154
MMP-8	0.32*	0.049
MMP-9	0.60**	0.000

IL, interleukin; MMP, matrix metalloproteinase; SASP, senescence-associated secretory phenotype.

4.2.5 Impact of SASP on Periodontal Regeneration

In relation to the influence of an increased inflammatory expression profile on the outcomes of periodontal regeneration, a statistically positive relationship was noted between the levels of IL-6 and MMP-9 detected in the GCF and the PPD observed at the 12-month post-surgery. Also, the same positive correlation was found between IL-1β, IL-6, and MMP-9 and CAL at 12 months (Table 6), indicating how higher SASP expression predicted worse healing outcomes.

Table 6. Correlation between SASP factors and clinical outcomes (PPD and CAL) at 12 months after surgery (Spearman Rho coefficient).

SASP factor	PPD at 12 months	P value	CAL at 12 months	P value
IL-1β	0.30	0.148	0.51*	0.010
IL-6	0.56**	0.008	0.58**	0.006
IL-12	-0.2	0.209	-0.06	0.772
MMP-8	0.09	0.663	0.07	0.775
MMP-9	0.45*	0.028	0.44*	0.030

CAL, clinical attachment level; IL, interleukin; MMP, matrix metalloproteinase; PPD, probing pocket depth; SASP, senescence-associated secretory phenotype.

Discussion

5.1 Interpretation of *In vitro* Results

In light of the increasing significance of PDLSCs for periodontal regeneration purposes, the present study aimed to conduct a comparative analysis between hPDLSCs and pPDLSCs. Additionally, it sought to investigate the presence of senescent cells and the potential of RG-108 in mitigating the adverse consequences of the inflamed microenvironment on pPDLSCs. To comprehensively characterize PDLSCs, we initiated immunophenotypic profiling of these cells immediately following their isolation through mechanical harvesting and subsequent enzymatic digestion of the periodontal ligament, an assessment conducted before passage zero (p0). The original cell population displayed significant heterogeneity, consisting of a small subset of MSCs characterized by the criteria outlined by the International Society for Cellular Therapy (Dominici et al. 2006). Concurrently, this sample included non-MSC elements and epithelial cells, typically removed during in vitro passages as the culture medium favors the growth of MSCs. Analysis across three passages revealed a notable variation at passage three (p3), signifying a greater presence of MSC markers in cells obtained from teeth affected by periodontitis (Fig. 1D). Intriguingly, CD73 and CD90 consistently maintained levels exceeding 95%, whereas CD105 exhibited variable expression and appeared more influenced by the cellular milieu, particularly in the context of healthy versus inflamed environments. Indeed, recent attention has been directed toward understanding the role of CD105 in MSCs, particularly its potential association with immunomodulatory functions (Lyamina et al. 2023). It is also worth noting that variations in CD105 expression can depend on cell culture conditions and passages (Mark et al. 2013). Studies have shown that CD105-negative MSCs possess significant inhibitory effects on lymphocyte expression when compared to CD105+ cells (Mousavi Niri et al. 2009). In this investigation, we observed that pPDLSCs displayed significantly upregulated levels of CD105 than hPDLSCs at p3, aligning

with findings illustrating a decrease in MSC immunosuppressive properties in subsequent passages (Klinker et al. 2017).

In order to uncover potential functional disparities between hPDLSCs and pPDLSCs, an investigation into their capacity for multi-differentiation, specifically towards cartilage, adipose tissue, and bone, was further conducted. This evaluation was founded on the examination of mRNA expression levels of master genes associated with these respective tissues, as well as the generation of tissue-like structures in vitro. While SOX9 and RUNX2 expression levels were comparable between the two groups, consistent with the differentiation into ACAN+ chondrocytes and the formation of mineralized nodules, a notable distinction was observed in the expression of PPARγ. The observed difference between pPDLSCs and hPDLSCs was statistically significant, and this contrast was also evident in the accumulation of lipid droplets, as confirmed by Oil red O staining. PPARγ, a well-recognized transcription factor, becomes activated by ligands that bind to PPAR response elements within the nucleus. Its regulatory function extends to the expression of multiple genes, influencing diverse cellular processes such as differentiation, metabolism, and pathways associated with inflammation (Rogue et al. 2010). This transcription factor exhibits high expression in a range of cell types, including endothelial cells, smooth muscle cells, and monocytes/macrophages. Moreover, previous research has demonstrated its involvement in ameliorating the effects of inflammation, often mediated by PPARγ agonists (Han et al. 2017). As reported in this study, the observed upregulation of PPARγ in pPDLSCs is an unprecedented finding. However, it aligns with the hypothesis that this heightened PPARγ expression may represent a defensive response to the persistent inflammatory milieu from which these cells were obtained.

Periodontitis is a chronic inflammatory condition stemming from a gradual transition of oral microbiota, evolving from a symbiotic to a dysbiotic state at the subgingival level (Hajishengallis 2015). Periodontal pathobionts such as *Aggregatibacter actinomycetemcomitans* and *P. gingivalis* are known to release toxins, inducing genotoxic effects (Blazkova et al. 2010). *P. gingivalis*, for instance, releases LPS and gingipains (Hajishengallis and Chavakis 2021), thereby perpetuating a state of continuous cellular stress. This prolonged stress may contribute to the onset of replicative senescence in PDLSCs and other oral MSCs (Feng et al. 2014). Notably, LPS has also been shown to promote the accumulation

of senescent osteocytes in young alveolar bone (Aquino-Martinez, Rowsey, et al. 2020), eventually leading to tooth loss. Sustained exposure to LPS has been associated with genetic and oxidative harm, leading to the eventual manifestation of a cellular senescence phenotype, as previously noted (Martin and Frisan 2020). Furthermore, periodontitis contributes to the establishment of a persistent state of low-grade inflammation characterized by increased concentrations of cytokines, acute-phase mediators (C-reactive protein, fibrinogen), and alterations in metabolic markers (Baima et al. 2022; Botelho et al. 2022). Our hypothesis posits that the chronic inflammatory conditions stemming from periodontitis, whether local or systemic, may lead to an increase in the subset of PDLSCs expressing both stemness and senescence-related genes (Baima et al. 2021). This phenomenon is perceived as an attempt to confront the bacterial insult and initiate the restoration of damaged periodontal ligament tissue. In normal physiological conditions, senescent cells play a role as initiators of tissue remodeling, a complex multistep process. In this context, senescent cells typically self-eliminate, as they are damaged and aim to promote tissue renewal (Muñoz-Espín and Serrano 2014). However, in pathological conditions like periodontitis, this regenerative process is initiated but remains incomplete, resulting in the accumulation of senescent cells (Cugini et al. 2013; Aquino-Martinez et al. 2020 Dec 20). Moreover, at the initial phases, the release of SASP is meant to promote plasticity and regeneration of the lost tissues (Campisi and d'Adda di Fagagna 2007). Also in this case, prolonged exposure to SASP leads to a vicious cycle of accumulation of senescent cells (Coppé et al. 2010). Our results corroborate this disrupted regenerative process, as we observed an elevation in the expression of stemness genes in pPDLSCs in comparison to hPDLSCs. Concomitantly, these cells exhibited increased expression of senescent markers, including p16, p21, and β-gal, resulting in a phenotype that more closely resembles replicative senescence. Notably, our discovery of a diverse PDLSC population expressing varying levels of stemness and senescent markers is of particular significance. This is especially noteworthy because while an increase in p21 and p16 expression is expected in aging PDLSCs (Fan et al. 2019; Baima et al. 2021), the simultaneous expression of OCT4 and p21 in a subset of PDLSCs is a novel finding. Additionally, we investigated potential alterations in molecules associated with the apoptotic process in pPDLSCs. We observed a notable decrease in BCL2 expression in pPDLSCs compared to

hPDLSCs, suggesting a downregulation of apoptosis inhibition. On the contrary, there was a marginally higher expression of Annexin V observed in pPDLSCs compared to hPDLSCs. BCL-2 is recognized for its role as a suppressor of apoptosis. (Granville and Gottlieb 2002). Our hypothesis centered around the decreased expression of BCL2 in pPDLSCs, potentially linked to the challenging conditions within the periodontitis microenvironment, characterized by an abundance of free radicals. Previous studies have indicated that the application of H2O2 to PDLSCs results in the inhibition of BCL2 (Jia et al. 2018). However, the reduced expression of BCL2 in pPDLSCs suggests that these cells might not have completely shifted to a replicative senescent state (Seluanov et al. 2001), thereby potentially remaining amenable to rescue and rejuvenation. Furthermore, pPDLSCs showed a higher expression pattern of P2X7R, a novel pro-inflammatory molecule involved in the purinergic signalling pathways (Giannuzzo et al. 2015). This receptor appears to play a crucial role in modulating IL-1β secretion induced by extracellular eATP, a common trigger for inflammation, making itself a potential molecular link between inflammation, cellular senescence and SASP secretion at the periodontal level.

PDLSCs possess the capacity for multi-differentiation, as indicated by the equivalent expression patterns of both osteogenic and chondrogenic master genes observed in both hPDLSCs and pPDLSCs within our series. Interestingly, we noted that the expression of the adipogenic master regulator, PPARγ, was higher in pPDLSCs in comparison to hPDLSCs. This observation led us to focus on potential regulators of gene expression that might govern these transformations in cell phenotype. Particularly, we delved into epigenetic mechanisms, particularly DNA methylation, recognized as key players in controlling the differentiation of MSCs (Pittenger et al. 2019). In this context, RG108 is a DNA methylation inhibitor, acting by specifically inhibiting the enzymatic activity of DNMTs. RG108 is well-known for its utility in experimentally modulating epigenetic gene regulation (Brueckner et al. 2005). We hypothesized that RG108 could induce a reversal of the senescent state observed in pPDLSCs. This hypothesis was rooted in previous findings that demonstrated RG108 capability to induce a global reduction in DNA methylation levels (Assis et al. 2018). Additionally, it was shown to locally reduce methylation levels at the promoters of key pluripotency genes, namely OCT4 and NANOG. The consequent decrease caused an elevation in the RNA and protein

levels within bone marrow MSCs. OCT4, SOX-2, and NANOG function as crucial regulators maintaining cellular pluripotency (Boyer et al. 2005). The increased expression of DNMT1, governed by OCT4 and NANOG, is essential in preserving the methylation status during DNA replication. This involves DNMT1 binding to the promoter region, subsequently restraining the expression of p16 and p21 genes. Ultimately, this molecular cascade promotes the undifferentiated state of MSCs (Tsai et al. 2012). Our research findings confirmed this hypothesis, marking the first time that RG108 induced a similar response in pPDLSCs, akin to its impact on bone marrow MSCs. These effects were not only observed at the genetic level but were also reflected in changes in protein expression, notably the reduction of the subset of PDLSCs that co-expressed OCT4 and p21 in teeth affected by periodontal compromise.

Moreover, we delved into whether this "rejuvenation" effect induced by RG108 could extend to the multi-differentiation potential of pPDLSCs. Our research demonstrated a significant enhancement in the adipogenic potential of pPDLSCs following RG108 treatment, while the expression patterns of other key master genes, SOX9 and RUNX2, remained unaltered. These findings point to the potential utility of RG108 as a valuable tool for future applications of in situ periodontitis treatment.

5.2 Interpretation of Clinical Results

Another key aim of the present elaborate was to evaluate how clinical inflammation and the expression of SASP in the GCF influence the outcomes of periodontal regeneration. Prior studies suggest that minimizing both the bacterial biofilm load and the site-specific inflammatory burden is crucial before undergoing regenerative periodontal treatment (Heitz-Mayfield et al. 2006; Aimetti et al. 2023). Indeed, higher plaque indexes and lack of inflammation control prior to surgical therapy have been associated to worse clinical outcomes in previous investigations (Tonetti et al. 1995; Aljateeli et al. 2014). However, scarce evidence was related to their impact on short-term healing outcomes. In our study, sites which underwent the intervention without BoP achieved better wound healing outcomes (flaps closed for primary intention with a small amount of fibrin). On the other hand, pre-surgical local inflammation was linked to poorer outcomes concerning EHI, marked by a considerable fibrin clot presence in the interproximal area and occasionally

resulting in partial necrosis of the interproximal tissue (Wachtel et al. 2003). These short-term findings highlight that minimizing local inflammation before surgical intervention can enhance site healing, particularly crucial for sustaining coagulum stability and safeguarding the biomaterial used for regenerating the intrabony defect from contamination (Heitz-Mayfield et al. 2006, Nibali et al. 2019). We have also observed a significant impact for the morphology of the bony defect in determining the healing pattern. Indeed, the presence of a containing intrabony defect was associated to improved EHI, as expected from the strong evidence that a more supportive defect anatomy leads to more favorable outcomes in periodontal regeneration (Nibali et al. 2021).

When dealing with molecular markers of cellular senescence, we examined the role of SASP factors in influencing clinical outcomes. Positive correlations were observed between IL-1β, IL-6, MMP-8, and MMP-9 in the GCF and EHI scores, underlining the potential impact of inflammation and senescence on early wound healing. Furthermore, the study investigated how SASP expression levels affected the long-term results of periodontal regeneration. Notably, statistically significant positive associations were established between pre-surgical levels of IL-1β, IL-6, and MMP-9 in the GCF and the parameters of PPD and CAL at the 12-month follow-up. Conversely, there was no notable impact observed for MMP-8 and IL-12. Comparison of the present data with the existing literature is difficult, since very few studies have investigated the impact of the cytokine expression pattern in the GCF on the long-term outcomes of periodontal regeneration. Indeed, most of the authors have analyzed the effect of periodontal therapy on the initial variation of interleukins and MMPs, confirming a significant decrease for IL-1β, IL-6, and MMP-8/9 (Dolińska et al. 2022; Aimetti et al. 2023). From a more comprehensive perspective, SASP can be broadly categorized into several groups, including pro-inflammatory cytokines like IL-1α/β, IL-6, and IL-8; chemokines; proteases encompassing MMPs and activators of plasminogen; growth factors like VEGF, TGF-β, and GM-CSF; and extracellular vesicles (Yue et al. 2022). Among these markers, we have employed the array of molecules which had a stronger scientific validation as diagnostic and prognostic markers of periodontitis (Arias-Bujanda et al. 2019; Koidou et al. 2020). To the best of our knowledge, this is the first study in which the GCF markers were hypothesized as independent variables at the baseline, capable of influencing the long-term clinical results of periodontal

therapy. The novelty also consisted in considering this array of well-known molecules as proxies for the senescent state of the subgingival environment prior to periodontal regeneration.

5.3 Limitations and Future Directions

While this study provides valuable insights into the phenotypic variations between hPDLSCs and pPDLSCs, there are some limitations that should be acknowledged. Firstly, the first part of the elaborate primarily focused on *in vitro* analyses, and future investigations could benefit from *in vivo* experiments to better elucidate the practical implications of these findings. Second, the potential impact of other (epi)genetic factors on PDLSC behavior was not explored extensively, and future research might delve into the molecular underpinnings of the observed disparities. Distinguishing between young and elderly patients could uncover age-related variations in PDLSC behavior. Third, our data on the role of P2X7R is still preliminary and warrants further investigation. Fourth, there are currently no validated clinical formulations for the use of RG108 in humans, emphasizing the need for future studies to explore its clinical transability.

When dealing with the second part of the dissertation, this is the first study to assess the impact of SASP/inflammatory molecular factors in the GCF on the outcomes of periodontal regeneration in a relatively large sample size. Needless to say, the clinical assessment of the real senescent state of a tissue/organ is far from being comprehensively reflected by an array of expressed cytokines. However, despite the entire medical literature has acknowledged the significance of cellular senescence in the pathogenesis of chronic inflammatory diseases, accurate chair/bed-side biomarkers are currently lacking. Furthermore, the generalizability of these findings is restricted due to the exclusion of patients with risk factors linked to less favorable healing outcomes, including individuals who smoke and those with diabetes. Overall, the clinical applications of these findings in the context of periodontal regeneration and other regenerative medicine approaches remain largely unexplored. Regulating the inflammatory response and managing the release of cytokines, especially by controlling their activation or inhibition in a precise time and location, could present an important avenue for periodontal tissue

engineering (Morand et al. 2017). In this regard, further research should aim to bridge the gap between the laboratory and clinical settings.

As the global population ages, the prevalence of periodontal diseases and the need for effective treatment options are increasing. This study can contribute to the advancement of periodontal regeneration techniques by shedding light on the role of biological aging and inflammation in the behavior of PDLSCs. This study somehow underlines how, among the other well-known factors affecting the outcomes of periodontal regeneration, there might be a significant role played by an inner biological potential of the patient at the site-level, which we hypothesize being influenced by its background senescent state. Ultimately, this study paves the way for exciting future research directions, such as harnessing epigenetic modulation for the rejuvenation of PDLSCs and expanding our knowledge of their role in tissue regeneration and personalized medicine. Understanding these factors is important for developing more effective and tailored regenerative strategies based on the patient's inner regenerative potential.

Conclusions

The findings from this research highlighted notable differences in thephenotypic and functional characteristics of hPDLSCs compared to pPDLSCs. Specifically, pPDLSCs displayed a higher expression of PPARγ and P2X7R genes, along with an increased presence of cells expressing markers linked to both stemness and cellular senescence. Crucially, these pPDLSCs were not in a state of replicative senescence, not displaying resistance to apoptosis. Furthermore, the DNMT inhibitor RG108 led to a notable reversal of these characteristics in pPDLSCs, alleviating the senescent phenotype.

Regarding the clinical findings, the presence of active inflammation (BoP) was a negative predictor for early wound healing at 2 weeks after surgery, as well as for the achievement of clinical success at one year. At the same time, a higher expression of SASP factors in the GCF (IL-1β, IL-6, and MMP-9) positively correlated with final PPD and CAL. These novel human findings suggest that higher SASP expression levels may predict less favorable healing outcomes and emphasize the need to: 1) consider these factors when planning periodontal regenerative procedures; 2) explore novel therapeutic approaches to modulate inflammation/senescence within the periodontal microenvironment.

In general, the content of the present dissertation has the potential to inform more personalized treatment approaches to enhance patient care. By identifying the impact of chronic inflammation and biological aging on periodontal regeneration, clinicians can anticipate treatment challenges and modify treatment plans accordingly. This may lead to higher success rates and improved long-term outcomes for patients undergoing periodontal regeneration procedures, especially in sites which have already undergone advanced destruction. Finally, the insights gained from our endeavor may pave the way for the development of targeted therapeutic interventions that mitigate the negative effects of aging and inflammation on PDLSCs.

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