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In vitro and *in vivo* investigations of osteogenic differentiation ability of dental pulp stem cells (DPSCs) and gingival mesenchymal stem cells (GMSCs) by use of nanostructured scaffolds

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Abstract

Thanks to the use of human mesenchymal stem cells (hMSCs), smart biomaterials and active biomolecules, Regenerative Medicine (RM) and Bone Tissue Engineering (BTE) can restore structure and function of injured tissues.

Among the different sources of hMSCs, the oro-facial hMSCs have promising *in vitro* and *in vivo* regeneration potential; in particular, dental pulp and gingiva are valuable sources of autologous hMSCs.

The aim of this PhD thesis is testing the *in vitro* and *in vivo* bone regeneration ability of hMSCs isolated from dental pulp and inflamed gingiva of periodontally-compromised teeth, up to now considered biological waste tissues and discarded during surgical procedures, on two commercial scaffolds, **FISIOGRAFT Bone Granular**®️ and **Matriderm**®️, in order to develop a low-cost and painless strategy of autologous bone tissue regeneration in patients affected by bone resorption.

This project is in line with the National Operational Program (PON) "Research and Innovation" (R&I) 2014-2020 and the National Strategy of Intelligent Specialization (SNSI), aiming to promote the research and the innovation of the country, with a particular interest on the Health Specialization Area.

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Summary

Periodontitis, affecting from 20 to 50 % of the global population, is probably one of the most common diseases of the human beings. It starts from a localized inflammation of gingiva, induced by the microorganisms of the dental plaque, and, if not properly treated, it progresses to periodontitis responsible for bone resorption and tooth loss. In these cases, the prosthetic rehabilitation is not possible and Guided Bone Regeneration (GBR) procedures have been widely used to regenerate the bone. The autologous bone graft is currently the "gold standard" of the GBR procedures to allow the placement of dental prosthesis; however, the surgical procedures, implicating additional surgical sites, lead to higher costs of interventions and risks of clinical complications. Tissue Engineering (TE) and Regenerative Medicine (RM) are doing many efforts to identify alternative treatments; and, among the hMSCs so far investigated, Dental Pulp Stem Cells (DPSCs) and Gingival Mesenchymal Stem Cells (GMSCs) demonstrated to have promising abilities of bone regeneration.

As integrating part of the PON R&I 2014-2020 and in line with the goals of the SNSI, the purpose of this PhD thesis is evaluating the *in vitro* and *in vivo* bone regeneration potential of DPSCs and GMSCs isolated from periodontally-compromised teeth, up to now considered biological waste tissues, on two commercial scaffolds, **FISIOGRAFT Bone Granular**® and **Matriderm**®, in order to develop a low-cost and painless strategy of autologous bone tissue regeneration in patients with bone resorption. Integrating the know-how of the University of Palermo and the expertise of the partner companies, this thesis aims to reach a Technology Readiness Level (TRL)-4 of the research, which constitutes the technological validation in laboratory, starting from the TRL-2, corresponding to the formulation of a technological concept.

DPSCs-the low cell yield and/or high rate of bacterial contamination of the dental pulp samples did not allow the isolation of DPSCs; for this reason, after one year of research this line was abandoned.

GMSCs-they were successfully isolated from gingiva of 18 healthy (Control group) and 20 periodontally-compromised teeth (Test group). After having confirmed the stem cell phenotype by doubling time assay, colony-forming unit assay, and the expression of

hMSC markers, the GMSCs both from Control and Test group were seeded on the **FISIOGRAFT Bone Granular**® and **Matriderm**® scaffolds to evaluate the *in vitro* and *in vivo* cell viability and bone differentiation ability.

*The results demonstrated that the GMSCs from inflamed gingiva of periodontally-compromised teeth, supported by the **Matriderm**® scaffold, may regenerate the bone in patients affected by bone resorption.*

CHAPTER **1**
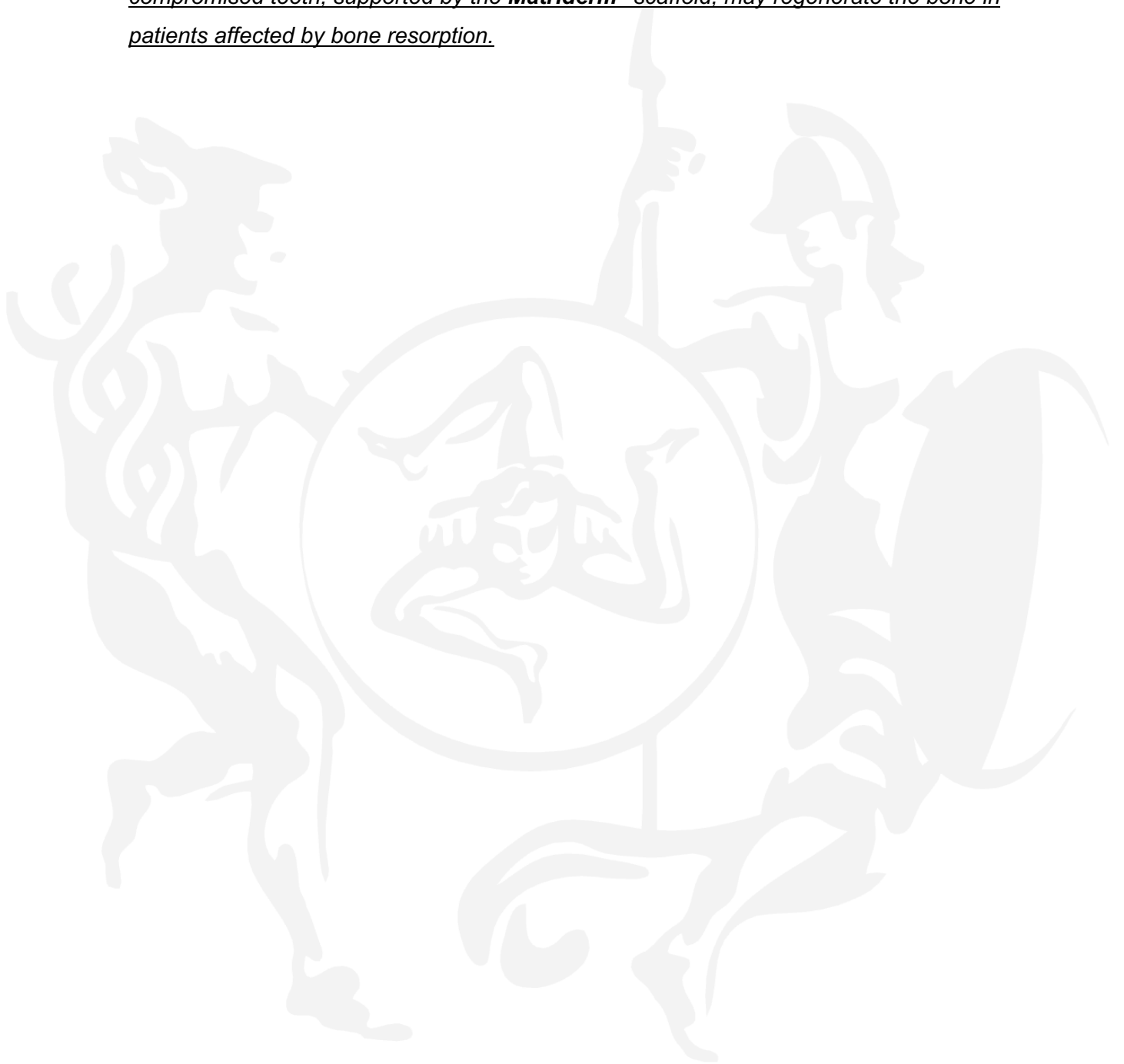
Background, Rationale and Objectives

1.1 Periodontal disease and bone resorption

Periodontitis is a multifactorial inflammatory disease affecting the tissues supporting the teeth: the gingiva, which is the soft tissue surrounding the teeth, the bone and the periodontal ligament, consisting of collagen fibers linking the tooth to the alveolar bone [1–3]. Periodontitis starts from a localized inflammation of the gingival tissue, named as gingivitis, which is induced by an imbalance on the microbial biofilm forming the dental plaque [4–6]. If not properly treated, the gingivitis progresses to periodontitis linked to bone resorption and tooth loss [7,8].

Affecting from 20 to 50 % of the global population, gingivitis and periodontitis are among the most common diseases of human beings, representing a serious problem for the global health. Even though it can affect children and adolescents, it particularly affects adults between 35 and 44 years old [9,10].

In case of teeth damage or loss, teeth are usually replaced by dental implants to restore the chewing, speech and aesthetic functions [11,12]; however, osteointegration, firstly described by Branemark in 1977 and defined as the correct structural and functional connection between bone and dental prosthesis, is possible only when a sufficient bone volume is available to place the dental implants and establish a strong connection [13,14]. It is widely known that patients affected by periodontitis suffer from bone resorption; in these patients, the restoring of the bone volume necessary to implant dental prosthesis is currently one of the main challenges of dentistry field [15–18] and alternative treatments are urgently needed [17,18].

1.2 Bone regeneration procedures

Guided Bone Regeneration (GBR), based on the application of grafting materials and membranes, represents the most commonly protocol to regenerate the bone and allow the placement of dental implants [19–21]. Noteworthy, thanks to the biocompatibility, osteoinductive and osteoconductive properties, the autologous bone graft is the "gold standard" of the GBR procedures, but its use is limited by the requirement of a second surgical site, resulting in increased possibility of clinical complications, morbidity, and higher costs of interventions. GBR procedures may also include the use allograft, xenograft or alloplastic materials, that can be used alone or in combination with autologous grafts [15,16,22]. Usually, membranes are employed in GBR procedures; they protect the defect from invasion of soft tissue cells, allowing the bone progenitor cells to reach the membrane and regenerate the bone, and can be resorbable (e.g. polylactic acid (PLA), collagen, polyglactin) or not-resorbable (e.g. titanium-reinforced expanded polytetrafluoroethylene)[20].

In this scenario, easier and low-cost dental surgical procedures are needed to regenerate the alveolar bone in patients with bone resorption, as periodontal patients.

1.3 Regenerative Medicine, Tissue Engineering and mesenchymal stem cells

Regenerative Medicine (RM) and Tissue Engineering (TE), also indicated as TERM, provide new strategies to treat diseases and regenerate injured tissues and organs [23,24].

TE was defined for the first time in 1993 by Langer and Vacanti as "*an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ*". It is able to regenerate tissues by relying on three main factors: stem cells, biomaterials and bioactive molecules. On the other side, RM has been defined as "*the process of replacing or regenerating human cells, tissues or organs to restore or establish normal*

function" by Mason & Dunnill in 2008 and takes advantage of stem cell ability to differentiate towards different cell types, being immunomodulatory and regenerate tissues in combination to nanomedicine, biomedicine and TE itself [25–28].

With their ability of clonogenicity, self-renewal and multi-differentiation ability, stem cells are the key to the regenerative process [29,30]. The autologous hMSCs isolated from human adult tissues represent the ideal stem cell population to employ for autografts [30–34].

Several hMSC sources have been identified as valuable sources for cellular therapy in TERM. Bone marrow (BM), umbilical cord blood and adipose tissue are currently among the most investigated tissues as source of hMSCs; however, the harvesting methods can be invasive and painful and, especially in case of BM, the number, the differentiation potential and the maximal life cycle of hMSCs decrease with the age of the subject [35,36]. More recently, the efforts to identify other available sources of hMSCs, have led to characterize the oro-facial hMSCs, with a demonstrated potential of *in vitro* and *in vivo* tissue regeneration.

1.4 Dental pulp stem cells

Among the different stem cell "niches" found in the mouth, the pulp cavity of the tooth demonstrated to be a promising source of autologous hMSCs. The DPSCs were discovered by Gronthos et al in 2000, that found the presence of neural crest-derived stem cells in the dental pulp [37]. In detail, the ectoderm-mesoderm interaction leads to the development of the tooth germ, where the neural crest cells differentiate in dental pulp, papilla and follicle.

The hMSCs isolated from dental pulp of permanent teeth are called DPSCs; the hMSCs isolated from deciduous teeth are defined as SHEDs (Stem Cells from Human Exfoliated Deciduous Teeth) [38–40].

DPSCs and SHEDs demonstrated to have clonogenic activity, multi-differentiation potential and immunomodulation ability [39–41], and, if compared to BM-MSCs, they are easier to be harvested and demonstrated to have higher proliferation rate and osteo-differentiation ability [37,41–43]. Noteworthy, it was

recently demonstrated that DPSCs, isolated from inflamed tissues, have enhanced stem cell properties and multi-lineage differentiation capability [44]. These properties make them promising candidates for TERM applications [41,45,46].

1.5 Gingival mesenchymal stem cells

In the oral cavity, also gingiva [37,41,47–52], which is the soft tissue separating the periodontium from outer space, is considered a promising source of autologous hMSCs. The GMSCs, isolated for the first time in 2009 by Zhang et al. [50], represent a subpopulation of gingival fibroblasts, with well-demonstrated *in vitro* and *in vivo* abilities of self-renewal, multi-lineage differentiation, and immunomodulatory properties [53–55].

Many biological characteristics make GMSCs ideal for TERM procedures: (i) they are easy to isolate and the patient can be submitted to the surgical biopsy without worrying about delayed healing; in addition, the vast majority of dissected gingival tissue is usually discarded during routine surgical procedures; (ii) in presence of specific conditions, they are able to differentiate towards mature osteoblasts, chondrocytes and adipocytes, expressing the relative cell lineage markers, phenotype and activity; (iii) they have a higher proliferation rate and multi-differentiation ability than bone marrow mesenchymal stem cells (BM-MSCs); (iv) they display a stable phenotype, karyotype and normal telomerase activity in long-term cultures [54,56–61].

Furthermore, as described for the DPSCs, the inflammatory microenvironment, characterizing the periodontally-affected periodontium, has demonstrated to positively affect the stem cell properties of GMSCs, showing higher proliferation rate, expression of hMSC markers, and ability of multi-lineage differentiation [44].

1.6 Biomaterials

Bone TERM is an interdisciplinary field that integrates principles of life sciences, medicine, chemistry and engineering, and investigates the relationship between structure and function of human tissues. Along with hMSCs, biomaterials are one of the main pillars of bone TERM and, as widely demonstrated, their composition,

structure and properties influence the cell attachment, growth and multi-differentiation [62,63].

The biomaterials can be synthetically produced or naturally derived. The advantage of using synthetic biomaterials is that they can be artificially modified (e.g. pore sizes, interconnection degree) as much as they need; however, their biocompatibility is lower than natural biomaterials. On the other hand, even if the natural biomaterials are weaker and softer than synthetic biomaterials, they are much more flexible and can adapt their shape to the required forms. Furthermore, the natural biomaterials usually contain specific natural domains that support and guide the cells in their development, thus improving the biological interactions with the tissue [39,62].

All the biomaterials should have optimal mechanical properties and a functional micro-architecture with well-distributed and interconnected pores along the surface, to ensure the *in vivo* neovascularization [64,65].

The integration between the expertise of the partner companies in smart biomaterials and the know-how of the University of Palermo on hMSCs contributed to carry on a research for the development of a quality biomedical experimental product with a TRL-4 and in line with the goals of one of the most currently investigated topics in the Health Specialization Area, the Regenerative and the Personalized Medicine.

FISIOGRAFT Bone Granular®, from GHIMAS Spa (Bologna, Italy) and **MatriDerm®** from Medskin Solution, Dr. Otto Suwelack Skin and Health Care GmbH (Billerbeck, Germany) were used in this thesis project.

1.7 FISIOGRAFT Bone Granular®

Many types of scaffolds have been developed for bone TERM. The bone is a living tissue composed by an organic phase (i.e. collagen) and inorganic phase, mainly constituted by inorganic-based compounds as hydroxyapatite [66]; for this reason, hydroxyapatite (HA) and calcium phosphate derivates, mimicking natural bone inorganic phase, have been mostly used for bone regenerative purposes

[67,68]. They can be used alone or in combination with additives such as polymers and bioactive molecules to repair bone defects [69].

FISIOGRAFT Bone Granular® (GHIMAS Spa, Bologna, Italy) is a synthetic scaffold consisting of granules derived from a HA sponge of nanometric dimensions, with a morphological structure that mimics trabecular bone with very thin trabeculae, allowing the new bone to occupy a volume greater than that of xenografts. From the morphological point of view the synthetic sponge, based on nanometric hydroxyapatite, is homogeneous; it has a pore size from 500 to 1000 µm and an interconnected porosity, which is optimal for cell proliferation [70]. Noteworthy, a clinical study from Stacchi et al. [71] recently demonstrated that after 6 months from **FISIOGRAFT Bone Granular®** implant in patients with maxillary sinus bone defect, the vital bone percentage was approximately 35 %, with a bone marrow space percentage of approximately 45 % and a residual graft percentage of roughly 21 %; in addition, after 12 months the implant survival rate was 96.4%, demonstrating its *in vivo* bone regeneration ability.

1.8 MatriDerm®

Type I collagen is the most represented organic polymer of bone matrix and plays an important role in the complex process of bone formation and remodeling. For these reasons, thanks to the excellent biocompatibility, biodegradability and weak antigenicity, and the ability of collagen fibrils to serve as a template for bone mineralization, collagen is a biomaterial widely used for tissue regeneration [72,73].

MatriDerm® scaffold (Medskin Solution, Dr. Otto Suwelack Skin and Health Care GmbH, Billerbeck, Germany) is a three-dimensional matrix consisting of collagen type I (bovine collagen) and elastin (extracted from bovine ligamentum nuchae), possessing a porosity approximately of 100 µm and obtained by the Advanced CryoSafe™ Method, that preserves and refines the natural features and properties of biomaterials. Many studies recently demonstrated that **MatriDerm®** is able to support the crucial steps of tissue regeneration: cell migration and ingrowth, proliferation and neo-angiogenesis, essential for the regeneration process, successfully regenerating skin and cartilage tissues [74–76].

1.9 Bioactive molecules

In bone regeneration techniques, besides to biomaterials with osteoinductive and osteoconductive properties, vascularization, growth factors, and mechanical environment are other crucial factors to reach the therapeutic goals [77,78]. Growth factors and bioactive molecules are frequently used in association with biomaterials to improve the regeneration process; it was demonstrated that pure collagen materials don't have enough osteoinductive activity to stimulate bone formation and many strategies, based on scaffold incorporation or hMSC treatment with bioactive molecules, have been developed [72,73,79].

Biochanin A (5,7-dihydroxy-4'-methoxy-isoflavone), an isoflavone most commonly found in legumes as red clover (Trifolium pratense), is commercially available as a nutraceutical, a natural supplement used alone or in association to drugs to treat many diseases involving the bone health [80,81]. Acting as a natural modulator of the α and β estrogen receptors, Biochanin A induces the transcriptional pathways physiologically activated by estrogens and inhibited during human pathological conditions as osteoporosis in post-menopausal women [82–86]. In particular, Biochanin A showed to enhance the osteoblastic differentiation pathway and inhibit the osteoclastic differentiation pathway [87,88], contributing to maintain the bone health [80,81].

In vitro and *in vivo* studies successfully showed that Biochanin A prevents the bone loss derived by ovariectomy, stimulating the osteoblastic activity and inhibiting the osteoclastic activity [87,88].

Aim of the PhD thesis

The project of this PhD thesis is well-integrated in the National Operational Program (PON) "Research and Innovation" (R&I) 2014-2020 and the National Strategy of Intelligent Specialization (SNSI), with a particular interest on the Health Specialization Area for the application of the scientific knowledge and technology of biomedical and biotechnological fields; the project is based on the promotion and development of the Regenerative and Personalized Medicine to improve the life quality of patients affected by chronic-degenerative diseases as periodontitis.

As part of this program, the aim of this thesis project is the assessment of the *in vitro* and *in vivo* bone regeneration ability of GMSCs and DPSCs isolated from periodontally-compromised teeth, up to now considered waste tissues, on two commercial nano-structured scaffolds, **FISIOGRAFT Bone Granular®** and **Matriderm®**, in order to develop a low-cost and painless strategy of autologous bone tissue regeneration for patients with bone resorption.

The low cell yield and/or high bacterial contamination of dental pulp samples did not allow the DPSC isolation and evaluation of bone regeneration ability; instead, the GMSCs were successfully isolated from healthy (Control group) and inflamed gingiva (Test group). Stem cell phenotype was confirmed by doubling time assay, colony-forming unit assay, and expression of hMSC markers, while the *in vitro* and *in vivo* cell viability and bone differentiation ability of GMSCs from Control and Test group were evaluated on the **FISIOGRAFT Bone Granular®** and **Matriderm®** scaffolds, in presence or not of the isoflavone Biochanin A with pro-osteoblastic activity.

The hMSC know-how of the University of Palermo and the biomaterial expertise of the companies worked together to reach a biomedical experimental product characterized by a TRL-4, i.e. the technological validation in the laboratory, starting from the TRL-2, corresponding to the formulation of a technological concept.

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CHAPTER **2**

Materials and Methods

2.1 Ethics

The protocol was approved by the Internal Ethical Committee of the University Hospital A.O.U.P "P. Giaccone" of Palermo (Internal registry: 5/2014). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

2.2 Patient identification and tissue extraction

22 Healthy adult patients (ages 18-75) who needed extraction of wisdom teeth for orthodontic reasons (Control group) and 26 adult patients (ages 18-75) who needed extraction of molars suffering from periodontitis (mobility grade III) (Test group), without suspected or visible pregnancy in females, were recruited for the study.

Before the extraction, each patient made a mouth rinse with 0,2% chlorhexidine for one minute (Meridol®, Gaba Vebas S.r.l., Rome, Italy) to decontaminate the oral cavity. Gingival tissues were resected from gingiva flaps during oral surgery procedures.

2.3 Sample collection and establishment of primary cell cultures

The samples were processed in the Laboratory of Regenerative Medicine (Department of Health Promotion, Mother and Child Care, Internal Medicine and Medical Specialties-ProMISE) headed by the Professor Carla Giordano.

After surgery, the pulpal and gingival tissues were collected in a 50-ml tube with cold, sterile Dulbecco's Phosphate Buffer Saline Solution w/o Calcium w/o Magnesium (DPBS w/o Ca^{2+}/Mg^{2+}) (Euroclone, Milan, Italy), containing 0,25 mg/ml Levofloxacin, 0,40 mg/ml Gentamicin, 5 mg/ml Meropenem, and 0,25 mg/ml Fluconazole, transported to the laboratory within 30 minutes and digested within 3 hours.

The tissues were firstly mechanically digested using sterile scalpels and secondly enzymatically digested using a solution of Collagenase Type II (Gibco, Milan, Italy) 1 mg/ml for 2 hours at 37°C under agitation. After centrifugation of digests containing pulpal or gingival primary cells at 1200 rpm for 6', the supernatant was removed, the pellet was re-suspended in fresh Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM F-12) (Thermo Fisher Scientific, Milan, Italy), containing 10% of fetal bovine serum (FBS) (Euroclone, Milan, Italy), 100 μg/ml Levofloxacin, 50 μg/ml Gentamicin, 50 μg/ml Meropenem, and 1,5 μg/ml Fluconazole, transferred in T25 culture flask (EuroClone, Milan, Italy), referred to as passage 0 (P0) and incubated at 37 °C and 5% CO2. The primary cells started to adhere to the flask in 4–5 days, and when they reached 80% of confluence (approximately 2 weeks), they were sub-cultured referred to as P1. By subculture P3, the antibiotic and antifungal cover was decreased and by subculture P4 it was completely abolished. Primary cells between P1 and P6 were used for the experiments in this study.

hMSCs of dental pulp and gingiva from healthy patients are referred to as respectively H-DPSCs and H-GMSCs; hMSCs of dental pulp and gingiva from patients with periodontitis are referred to as respectively P-DPSCs and P-GMSCs.

2.4 Colony-Forming Unit Fibroblast Assay (CFU-F)

H-GMSCs and P-GMSCs (P1) were seeded in 10-cm dishes at a density of 300 cells/dish and cultured under conventional conditions, replacing old medium every 3 days. After 14 days, the cells were washed twice with DPBS, fixed in 4% paraformaldehyde and stained with 0.1% crystal violet. Only cellular groups

containing more than 50 cells were considered as colonies. Three sets of experiments for each sample were performed for calculations.

2.5 Population doubling (DT) and cell proliferation curve

The proliferation rate of H-GMSC and P-GMSCs was evaluated by trypan blue assay (Sigma-Aldrich, Milan, Italy) following the manufacturer's instructions. H-GMSCs and P-GMSCs (P2) were seeded at a density of 4×10^3 cells/cm^2 in a 24-well plate and grown up to 120 h. The cells were counted every 24 hours by observation under the optical microscope, after being stained with trypan blue. The DT was calculated in according to the literature data (Roth V. 2006 on the website http://www.doublingtime. com/compute.php). Three sets of experiments for each sample were performed for calculations.

2.6 Flow cytometric immunophenotyping

H-GMSCs and P-GMSCs (P5) were harvested and the cell pellet was re-suspended in DPBS w/o Ca^{2+}/Mg^{2+}, at a concentration of 1x10^{6}cells/ml; then, 5x10^5 cells/100 µl of cell suspension was used for every cytofluorimetric test.

Briefly, the H-GMSCs and P-GMSCs were tested for expression of hematopoietic stem cell surface markers using FITC human anti-HLA-DR and anti-CD45 monoclonal antibodies, and MSC surface markers using FITC human anti-CD29, CD90 and CD105, and PE human anti-CD73 (Table 1). Table 1 describes the conditions of antibody dilution, incubation and detection, following the manufacturer's instructions.

All reactions were then acquired using the FACS Calibur flow cytometer (Becton-Dickinson, New Jersey, USA) and analyzed by the CellQuest Pro software. Specific IgG isotype antibodies were used as internal negative control. Unstained cells were used as negative control and BM-MSCs as a positive control (not shown).

2.7 Isolation of total RNA and Real Time quantitative PCR (qRT-PCR)

Isolation and purification of total RNA was performed using the RNeasy Mini Kit (Qiagen, California, USA), according to the manufacturer's instructions. RNA quantity and quality were evaluated by NanoDrop 2000 (Thermo Fisher Scientific, Milan, Italy); 2µg of hMSC total RNA were reverse-transcribed to cDNA in a volume of 20µl with Oligo dT primers using the QuantiTect Reverse Transcription Kit (Qiagen, California, USA). To evaluate the stem cell gene profile and *in vivo* bone differentiation, quantitative PCR (qPCR) was performed using the QuantiNova SYBR Green PCR Kit and the RotorGene Q Instrument (Qiagen, California, USA). Briefly, the cDNA samples were mixed with the SYBR Green PCR master mix and specific pair of primers presented in Table 2. The qPCR conditions were as follows: denaturation at 95 °C for 3 min for 1 cycle, followed by 44 cycles of denaturation at 95 °C for 20 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 60 s. Three technical replicates were performed for every sample. The specificity of the amplified products was determined by melting peak analysis. The relative expression of target genes was calculated using the ΔΔCt method according to the guidelines [89]. β-actin was used as housekeeping gene to normalize the expression of target genes and BM-MSCs, used as a positive cell control, were used to compare gene expression. The results were presented in histograms using GraphPad Software and setting at 1 the gene expression of the positive cell control. hMSCs at P3 were used for the RT-qPCR analysis.

2.8 Biomaterials

The biomaterials used in the study were: **<u>FISIOGRAFT Bone Granular</u>®**, from GHIMAS Spa (Bologna, Italy) and constituted by sintered nanohydroxyapatite (NHA) microgranules, with a diameter between 250 and 500 µm, a pore size from 500 to 1000 µm and obtained by crashing HA porous blocks; **<u>MatriDerm</u>®**, from Medskin Solution (Dr. Otto Suwelack Skin and Health Care GmbH, Billerbeck, Germany) and constituted by a three-dimensional matrix consisting of collagen (bovine collagen) and elastin (extracted from bovine ligamentum nuchae), with a porosity approximately of 100 µm and obtained by the Advanced CryoSafe™

Method, able to preserve and refine the natural features and properties of biomaterials.

2.9 Cell seeding

Both types of biomaterials were provided by the companies in sterile conditions. They were incubated in culture media for 30 minutes at 37°C and 5% CO_2, prior to cell seeding.

For the viability test, 7400 cells/cm^2 were seeded in 5 mg of the **FISIOGRAFT Bone Granular®** scaffold [90], in low-adhesion 96 well plate in order to inhibit the attachment of the cells to the bottom of the well and avoid false positive. After seeding, they were incubated at 37°C and 5% CO_2 and the viability of H-GMSCs and P-GMSCs was evaluated after 24, 48, 72 hours by Water Soluble Tetrazolium Salt 1 (WST1).

To perform the viability test on the **MatriDerm®** scaffold, 10000 cells/ cm^2 were seeded in the scaffolds, using a 24 well plate. After cell seeding, the scaffolds were incubated at 37°C and 5% CO_2 for 5 minutes without culture medium to promote the cell attachment; then, 1 ml of complete fresh medium was added to each scaffold and kept at 37°C and 5% CO_2. After 24, 48, 72 hours 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate the viability of the cells.

2.10 WST1 viability assay

WST1 viability assay was performed to evaluate the viability of H-GMSCs and P-GMSCs (P3) seeded on the **FISIOGRAFT Bone Granular®**. H-GMSCs and P-GMSCs without scaffolds were used as controls.

Briefly, after 3 hours of incubation with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt at 37°C and 5% CO_2, the absorbance of the supernatant was read at 450 nm, using a microplate reader.

2.11 MTT viability assay

MTT viability assay was performed to evaluate the viability of H-GMSCs and P-GMSCs (P3) seeded on the **MatriDerm®** scaffold. H-GMSCs and P-GMSCs without scaffolds were used as controls.

Briefly, after 4 hours of incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt at 37°C and 5% CO^2, the absorbance of the supernatant was read at 570 nm, using a microplate reader.

2.12 Live/Dead assay

Live/Dead assay was performed to evaluate the survival of H-GMSCs and P-GMSCs (P5) seeded on the **MatriDerm®** scaffold. Briefly, a dye mix of Ethidium Bromide (100 µg/ml) and Acridine Orange (100 µg/ml) in DPBS was used for the staining. At 24, 48 and 72 hours the scaffolds were washed twice with DPBS (100 µl), every wash was run for 5 minutes by slight agitation.

Live/Dead dye mix (30 µl) was added to each scaffold for 5 minutes and images were acquired using a Nikon fluorescence microscope (10 X) by FITC (green) and TRITC (red) filters. All images were overlaid with FITC and TRITC channels to indicate respectively live and dead cells.

2.13 DAPI/Actin Green assay

The confocal microscopy analysis was performed to evaluate the colonization rate of the **MatriDerm®** scaffold by H-GMSCs and P-GMSCs (P5) and the distribution of the cells.

Briefly, after 2, 7 and 10 days, the scaffolds were fixed with 4% paraformaldehyde in DPBS (300 µl) at room temperature for 15 minutes. They were washed with DPBS and incubated with 0,1% Triton-X 100 in DPBS (300 µl) at room temperature for 4 minutes. Finally, they were incubated with 1:1000 DAPI (Sigma Aldrich, Milan, Italy) in distilled H_2O (300 µl) at room temperature for 30 minutes to stain the nuclei, and 2 drops/ml ActinGreen™ 488 ReadyProbes™ Reagent (Thermo Fisher Scientific, Milan, Italy) in DPBS (300 µl) at room temperature for 1 hour to stain cellular cytoskeleton.

The scaffolds were analyzed by a Nikon A1 confocal microscope and the software ImageJ (http://imagej.nih.gov/ij/). The volumetric analysis has been performed by NIS Elements AR software (Nikon).

2.14 *In vitro* GMSC bone differentiation on the <u>MatriDerm®</u> scaffold

To test the bone differentiation ability of H-GMSCs and P-GMSCs (P3) grown in the <u>MatriDerm®</u> scaffold, the cells were grown in 24 well plates to confluence under standard culture conditions and then maintained in home-made osteogenic differentiation medium (ODM) consisting of: DMEM F-12 supplemented with 10% FBS, 10 nM dexamethasone (Sigma Aldrich, Milan, Italy), 10 mM glycerophosphate (Sigma Aldrich, Milan, Italy), and 0.05 mM ascorbic acid (Sigma Aldrich, Milan, Italy), with or without the isoflavone Biochanin A at two different concentrations, 300 nM and 1 µM. H-GMSCs and P-GMSCs cultured without scaffolds were used as control. After 21 days of culture in the ODM, H-GMSCs and P-GMSCs with or without the scaffolds were stained with Alizarin Red S (Sigma Aldrich, Milan, Italy) to detect the calcium deposits. Briefly, scaffolds were transferred in a new 24 well plate and H-GMSCs and P-GMSCs with or without the scaffolds were gently washed with DPBS, fixed with 4% paraformaldehyde solution for 15 minutes at room temperature and rinsed twice with distilled H_2O. Cells were stained with 40 mM Alizarin Red S (pH 4.1) for 30 minutes at room temperature with gentle shaking, washed with DPBS and observed under a light optical microscope. The images were acquired with a Nikon DS-fi1. Due to the thickness of the scaffolds, only images of control H-GMSCs and P-GMSCs were acquired. The quantification of the calcium deposits in H-GMSCs and P-GMSCs with or without the scaffolds was then evaluated by measurement of Alizarin Red S optical density (OD) at 550 nm. Three sets of experiments for each sample were performed for calculations.

2.15 *In vivo* GMSC bone differentiation on the <u>MatriDerm®</u> scaffold

All experimental procedures and protocols were approved by the "OPBA – Organismo Preposto al Benessere degli Animali" of the "A.Mirri" Experimental

Zooprophylactic Institute of Sicily (approval No. 1061/2015) and by the Italian Minister of Health (approval No.383/2018).

All the surgical procedures will be performed at the "A.Mirri" Experimental Zooprophylactic Institute of Sicily.

Twenty-four (24) 4 week-old athimic immunodeficient nude rats will be used for the study. All animals will be housed in the facility of the "A.Mirri" Experimental Zooprophylactic Institute of Sicily, and maintained on a 12-h light/12-h dark cycle with free access to rodent feeding and water. After two weeks of housing in well-controlled conditions of temperature, light and humidity, the animals will be randomly divided into groups for the experiment as follows (see Fig.1):

1) time-point 1 (4 weeks) including 8 animals; 4 animals receiving **MatriDerm**® scaffold/H-GMSCs, 4 animals receiving MatriDerm® scaffold/P-GMSCs;

2) time-point 2 (6 weeks) including 8 animals; 4 animals receiving **MatriDerm**® scaffold/H-GMSCs, 4 animals receiving MatriDerm® scaffold/P-GMSCs;

3) time-point 3 (8 weeks) including 8 animals; 4 animals receiving **MatriDerm**® scaffold/H-GMSCs, 4 animals receiving MatriDerm® scaffold/P-GMSCs.

In detail, 200.000 cells will be seeded on each scaffold under standard culture conditions; after having reached the confluence, they will be maintained in ODM for 1 week to induce bone differentiation; for each group, a suspension of H-GMSCs and P-GMSCs without the scaffold will be prepared as a control. Next, the scaffolds with pre-conditioned H-GMSCs/P-GMSCs and the cell suspension of pre-conditioned H-GMSCs/P-GMSCs will be respectively implanted and injected subcutaneously on the right side and the left side of the dorsal surface of each group of animals.

After each time-point, the animals will be sacrificed and dissected to explant the scaffolds and the control tissues afterward used to perform histological analysis and RT-qPCR analysis of bone differentiation markers: RUNX2; Osteopontin (OPN); Osteocalcin (OCN); Alkaline Phosphatase (ALP) and Collagen 1 A1 (COL1A1).

2.16 Statistical analysis

All the experiments of the study were performed in triplicate, and results are reported as means ± SD and compared by the Student's unpaired two-sample T-test. $P \leq 0.05$ was considered statistically significant.

CHAPTER **3**

Results

3.1 Proliferation rate of adherent H-GMSCs and P-GMSCs

22 Healthy patients (Control group) and 26 periodontally-affected patients (Test group) were used in the study to isolate DPSCs and GMSCs. For each patient, a gingival flap was used to extract gingival tissue. None of the harvested dental pulp tissues allowed the isolation of DPSCs, because of low cell yield and/or high rate of bacterial contamination. Ten of the harvested gingival tissues, respectively 4 of the Control group and 6 of the Test group were removed from the study, because of high bacterial contamination. For all the 38 gingival samples, after a sequential mechanic and enzymatic digestion, it was generated a cell suspension as it is shown in the Figure 2 A, B. Primary cells (P0), derived from both Control and Test group, started to adhere to the flask approximately between the 4° and the 5° day from digestion. All gingival primary cells from both Control and Test group cultures showed a typical fibroblast-like morphology, an homogeneous shape and size (Fig. 2 C, D) and reached 80% of confluence between 12 and 18 days; both populations initially showed the same rate of cell growth. After having reached the confluence, they were trypsinized and sub-cultured referring to them as P1 and showed a modification in behavior: as it is highlighted in cell growth curve (Fig. 3 A), 24 h after seeding, P-GMSCs started to proliferate faster than H-GMSCs, showing a higher proliferation rate. The doubling time (DT) was calculated as 26.4 ± 2 h vs. 30.2 ± 1 h ($p \leq 0.05$) respectively for P-GMSCs and H-GMSCs (Fig. 3 B).

3.2 Clonogenic potential of H-GMSCs and P-GMSCs

To analyze the clonogenic potential of H-GMSCs and P-GMSCs, the CFU assay was performed. GMSCs from both Control and Test group were able to form adherent colony-forming units on plastic dish after 14 days of incubation under standard conditions (Fig. 3 C), even if an increase in the number of CFU colonies was observed in P-GMSCs compared to the healthy counterpart, thus showing a higher clonogenic activity. The counting performed by software ImageJ showed 156.8 ± 9.3 and 116.7 ± 5.9 ($p \leq 0.01$) CFU colonies after 14 days of culture (Fig. 3 D) respectively for P-GMSCs and H-GMSCs.

3.3 Immunophenotyping of H-GMSCs and P-GMSCs

Both population of GMSCs analyzed resulted negative for the hematopoietic surface markers CD45 and HLA-DR (Fig. 4 A) and positive for putative adult MSC surface markers CD73, CD29, CD90 and CD105 (Fig. 4 B). CD73 and CD29 were highly expressed in all samples (approximately 100 %); however, a slightly increased expression of CD90 and CD105 was detected in P-GMSCs compared to H-GMSCs ($p \leq 0.05$) (Table 3).

The expression of adult MSC nuclear markers Oct4, SOX2 and NANOG was positive in both populations, even if it was higher in P-GMSCs than H-GMSCs ($p \leq 0.05$) (Fig. 4 C). The adult MSC profile was more highly expressed in P-GMSCs than H-GMSCs.

3.4 WST1 cell viability assay on the FISIOGRAFT Bone Granular®

H-GMSCs and P-GMSCs were seeded on the **FISIOGRAFT Bone Granular®** in presence of low-adhesion conditions and the viability evaluated at 24, 48 and 72 hours by WST1 assay (Fig. 5 A). The histogram showed that the viability of H-GMSCs and P-GMSCs, grown in presence of the scaffold, results decreased (approximately 50 %) compared to H-GMSCs and P-GMSCs, grown without the scaffold, both in standard and low-adhesion conditions, demonstrating that the properties of the scaffold are not suitable for *in vitro* experimental purposes.

3.5 MTT cell viability assay on the __MatriDerm__® scaffold

H-GMSCs and P-GMSCs were seeded on the __MatriDerm__® scaffold for 24, 48 and 72 hours and the viability was then evaluated by MTT assay (Fig. 5 B). The data displayed in the histogram demonstrated the continuous cell growth in presence of the scaffold and a higher proliferation rate of both H-GMSCs and P-GMSCs in presence of the scaffold compared to control cells, grown without the scaffold. As expected, the proliferation rate of P-GMSCs was higher than H-GMSCs.

3.6 Live/Dead assay on the __MatriDerm__® scaffold

The viability and the distribution of GMSCs from healthy and periodontally-affected tissues in the __MatriDerm__® scaffold was also evidenced by the Live/Dead assay (Fig. 6 A). Approximately 100% of both H-GMSCs and P-GMSCs, seeded in the scaffold for 24, 48 and 72 hours, were viable; the density of the cells increased in every time-point and was higher for P-GMSCs than H-GMSCs. We also observed that both H-GMSCs and P-GMSCs tended to align along the direction of collagen fibrils.

3.7 DAPI/Actin Green assay on the __MatriDerm__® scaffold

After nuclear and cytoskeleton staining, a confocal microscopy analysis was performed to evaluate the colonization rate of the __MatriDerm__® scaffold by H-GMSCs and P-GMSCs after 2, 7 and 10 days of culture under standard conditions. Different areas of the scaffold were taken into consideration and images were acquired. Considering the thickness of the scaffold used (1 mm), the results in Fig. 6 B showed that both H-GMSCs and P-GMSCs were able to colonize roughly 200 μm of the scaffold and were homogeneously distributed in the axis x and y. In particular, H-GMSCs colonized 190,336 μm of the scaffold in depth, P-GMSCs colonized 182,80 μm of the scaffold in depth. In addition, we observed an increased density of cells up to 10 days, with a higher increase for P-GMSCs compared to H-GMSCs, demonstrating the ability of the cells to colonize the scaffold and grow homogeneously.

3.8 *In vitro* H-GMSC and P-GMSC bone differentiation on the <u>MatriDerm</u>® scaffold

To test the bone differentiation ability of H-GMSCs and P-GMSCs seeded on the <u>MatriDerm</u>® scaffold, the cells, with or without the scaffold, were grown to confluence in 24 well plates under standard culture conditions and then maintained in home-made ODM, in presence or not of 300 nM and 1 µM Biochanin A; the bone differentiation rate was assessed by Red S Alizarin assay (Fig.7). After 21 days, the <u>MatriDerm</u>® scaffold seems to support the bone differentiation of H-GMSCs and P-GMSCs with an increase in osteoblastic differentiation ability of GMSCs grown in the scaffold ($p \leq 0.05$); moreover, the presence of Biochanin A at the concentration of 1 µM seems to induce a slight increase in bone differentiation with respect to the standard ODM (Fig. 7 A, B).

3.9 *In vivo* H-GMSC and P-GMSC bone differentiation on the <u>MatriDerm</u>® scaffold

After evaluation of experimental *in vitro* results, it is planned to use twenty-four (24) 4 week-old athymic immunodeficient nude rats for the *in vivo* study.

CHAPTER **4**

Discussion

Periodontitis is prevalent both in developed and developing countries and affects roughly the 20-50% of the global population. The high prevalence of periodontitis in young and old people makes it a serious public health concern. It occurs when the inflammation on gingival tissue, mainly induced by the microorganisms of the dental plaque, is not properly treated and the gingiva, along with periodontal ligament and alveolar bone, progressively resorbs creating a pocket responsible for tooth loss [7,8]. In these patients, no dental implant can be placed and the physiological oral functions are seriously compromised [11,12,91].

Integrated in the PON R&I 2014-2020, this PhD thesis is in line with the SNSI that promotes the smart and inclusive development of the country. With a particular interest on the Health Specialization Area, this PhD project aims to improve the life quality of patients with chronic-degenerative diseases as periodontitis.

Nowadays, GBR represents the most used protocol to regenerate the bone and allow the placement of dental implants [19]. In particular thanks to the high biocompatibility, osteoinductive and osteoconductive properties, the autologous bone graft represents the "gold standard" of GBR; however, it requires a second surgical site, which means higher costs of interventions and increased probability of clinical complications [19–21]; thus, alternative treatments are urgently needed.

In the past few years, TERM is one of the most discussed topics in medicine as alternative strategy to treat tissue defects; thanks to the well-known properties of hMSCs, adult multipotent cells characterized by clonogenicity, self-renewal and multi-lineage differentiation, many smart procedures have been developed to regenerate tissues as bone [39]. In the light of the currently used source of hMSCs (i.e. bone marrow, umbilical cord blood and adipose tissue) [35,36], a more accessible and low-cost hMSC source is necessary and the oral cavity seems to be a valuable candidate [37,41,47–52]. Among the different sources of hMSCs identified in the mouth [41,47–51], dental pulp and gingiva demonstrated to be very promising; DPSCs and GMSCs have high regenerative potential, with even increased proliferation and multi-lineage differentiation abilities compared to BM-MSCs [54,56,61]. In addition, it was recently demonstrated that inflammation conditions positively influence their stem cell and multi-lineage differentiation abilities [44].

Therefore, based on the current national priorities of the technological development in the Health Specialization Area (i.e. Regenerative and Personalized Medicine), harvesting hMSCs from oral tissues undergone inflammation, as dental pulp and gingiva from periodontally-compromised teeth up to now considered waste tissues, constitutes an encouraging, easy and low-cost alternative to the GBR traditional strategies.

In vitro study

Isolation and promising hMSC properties of P-GMSCs- GMSCs were successfully isolated from gingiva of healthy and periodontally-compromised teeth, and as previously demonstrated [44], they confirmed to have increased clonogenic activity, expression of hMSC markers and increased ability to differentiate towards osteoblastic cells than H-GMSCs, confirming that the inflamed microenvironment positively affects the regeneration potential of

GMSCs. The waste tissue, as gingiva from periodontally-compromised teeth, can be successfully employed as a source of autologous hMSCs.

***In vitro* bone differentiation of P-GMSCs in the Matriderm® scaffold** - to evaluate if GMSCs from waste gingiva could be successfully employed to regenerate the bone, and contribute to the development of a biomedical experimental product with a TRL-4, we tested the ability of P-GMSCs and H-GMSCs to *in vitro* grow and bone differentiate in two different types of scaffolds provided by the companies involved in the study: **FISIOGRAFT Bone Granular®** and **Matriderm®**.

HA and calcium phosphate derivates, that mimic the inorganic phase of the natural bone, are widely used for bone regenerative purposes [67,68]. The synthetic **FISIOGRAFT Bone Granular®**, provided by the partner company Ghimas Spa, consists of HA granules with pores from 500 to 1000 µm and interconnected porosity optimal for cell proliferation. The morphological structure of **FISIOGRAFT Bone Granular®** mimics the trabecular bone, allowing the new bone to greatly occupy the volume of the scaffold. The **FISIOGRAFT** could be a promising scaffold to support the growth and the bone differentiation of GMSCs. A recent clinical study on patients with maxillary sinus bone defect showed that it successfully regenerates the bone defect [71]. However, as widely accepted, one of the main challenges in the *in vitro* systems is re-producing the *in vivo* cell microenvironment, which is a very complex task. This is constituted by factors able to influence the environment of a cell or a group of cells, with direct or indirect effects on cell behavior and phenotype. A single cell is affected by the composition and structure of the extracellular matrix surrounding cells, growth factors, cytokines, hormones and other bioactive molecules with autocrine, endocrine and paracrine effects; besides, physical and mechanical factors, due to the movement of the organism or the physiological fluids as blood, have to be taken in consideration [92]. From our results, deriving from the WST1 viability assay, the **FISIOGRAFT Bone Granular®** doesn't support the growth of the GMSCs *in vitro*, since approximately 50 % of both P-GMSCs and H-GMSCs

showed lower viability in presence of the scaffold. It might be caused by the marked difference between the *in vitro* and the *in vivo* cellular microenvironment; thanks to the *in vivo* blood supply, many factors can coordinate the biomaterial remodeling and degradation along with the attraction of hMSCs to the target site, supporting the tissue regeneration. It was demonstrated that the cells have different behaviors in 2D and 3D cultures; they start to behave differently when explanted from native three-dimensional (3D) tissues and grow as an *in vitro* monolayer [93]. Therefore, depending on the *in vitro* or the *in vivo* microenvironment, GMSCs could differently react to the presence of the scaffold. Type I collagen is the most represented organic polymer of bone matrix and plays an important role in the process of bone formation and remodeling. For these reasons, nowadays, also thanks to the excellent biocompatibility, biodegradability and weak antigenicity, and the ability of the fibrils to guide the bone mineralization, the collagen is widely used for tissue regeneration [72,73]. We tested the *in vitro* growth and bone differentiation properties of P-GMSCs and H-GMSCs on the **MatriDerm®** scaffold, a three-dimensional matrix consisting of collagen type I (bovine collagen) and elastin with a porosity approximately of 100 μm, from the company Medskin Solution (Dr. Otto Suwelack Skin and Health Care GmbH, Billerbeck, Germany). Our study demonstrated that **MatriDerm®** can support the growth of H-GMSCs and P-GMSCs. The viability results showed a progressively increasing rate of cell growth in presence of the scaffold. These data were also confirmed by the Live/Dead and DAPI/Actin Green assays, demonstrating that the H-GMSCs and P-GMSCs, showing the typical fibroblast-like shape, homogeneously colonized the scaffold guided by the collagen fibrils. In addition, we observed an increased cell density up to 10 days of culture. These data suggested that the **MatriDerm®** scaffold may promote the adhesion and growth of GMSCs both from Control and Test group.

To assess the bone differentiation ability of H-GMSCs and P-GMSCs in the **MatriDerm®** scaffold, they were grown on the scaffold under osteoblastic differentiation conditions. The results derived from Alizarin S Red assay showed an increased mineralization rate of both H-GMSCs and P-GMSCs grown on the

scaffold compared to the control cells. However, it was demonstrated that pure collagen materials don't have enough osteoinductive activity to stimulate bone formation, and many strategies, based on scaffold incorporation or MSC treatment with bioactive molecules, have been developed [79]. Biochanin A (5,7-dihydroxy-4'-methoxy-isoflavone), an isoflavone most commonly found in legumes as red clover (Trifolium pratense), acts as a natural modulator of the α and β estrogen receptors [82–86], enhancing the osteoblastic differentiation and inhibiting the osteoclastic differentiation [87,88], thus contributing to maintain the bone health [80,81]. Su et al. recently demonstrated that Biochanin A, with the concentration of 300 nM, supports the osteoblastic differentiation [88]. A slight increase in the mineralization rate of H-GMSCs and P-GMSCs, grown in presence of Biochanin A, was observed; however, these results need to be confirmed and more deeply investigated in further studies to identify the optimal concentration of Biochanin A supporting the bone differentiation, and to evaluate the molecular mechanism potentially involved.

In vivo study

The results of the *in vitro* study lead to evaluate the H-GMSCs and P-GMSC *in vivo* bone regeneration potential in the **MatriDerm®** scaffold.

The *in vitro* results and the successful *in vivo* application of the **MatriDerm®** for dermal and cartilage regeneration[75,76,94], lead us to conceive that the H-GMSCs and P-GMSCs, supported by a biocompatible scaffold as **MatriDerm®**, may regenerate the bone *in vivo*.

Conclusions

Patients suffering from jaw bone loss, as periodontal patients, urgently need a low-cost strategy for alveolar bone defect regeneration to place dental implants and restore the oral functions. Bone TE along with RM reproduces tissues and organs by use of adult hMSCs, smart biomaterials and bioactive molecules.

The low cell yield and/or high rate of bacterial contamination of the dental pulp samples did not allow the isolation and evaluation of bone regeneration potential of DPSCs; instead, *GMSCs were successfully isolated from healthy and inflamed gingiva of periodontally-compromised teeth. Compared to dental pulp, gingiva is a more accessible source of hMSCs, and the procedure to isolate the GMSCs is quicker and much easier than that one used for DPSCs, resulting a more convenient source of autologous hMSCs for clinical routine in line with the principles of the SNSI.*

Probably due to the marked difference between the *in vitro* and the *in vivo* cellular microenvironment and behavior, *the H-GMSCs and P-GMSCs* were not able to grow in the **FISIOGRAFT Bone Granular**®, but they *showed to progressively grow, homogeneously distribute and bone differentiate in the **MatriDerm**® scaffold, with a slight increase in the osteodifferentiation rate after treatment with the Biochanin A. The results, derived from the integration of the hMSC know-how of the University of Palermo and the biomaterial expertise of the partner companies, contributed developing a biomedical experimental product that may successfully improve the quality of life of the periodontal patients and was validated in the laboratory (TRL-4).*

These data need to be further investigated in future studies; however, the results suggest that *the P-GMSCs from discarded tissues, supported by a biocompatible scaffold as **MatriDerm**®, may really support the development of a low-cost and painless strategy of autologous bone tissue regeneration suitable for clinical routine*; in line with the goals of the PON R&I 2014-2020 and SNSI, this represents an easier and 100 % biocompatible alternative to the traditional GBR procedures to treat not only bone defects caused by periodontitis but also any other type of bone defect.

CHAPTER **5**

Figures and Tables

5.2 Tables

Table 1: Human anti-monoclonal antibodies list used in flow cytometry analysis for mesenchymal stem cell marker detection.

Fluorescently-conjugated antibody/localization marker	Brand/code number	Dilution	Incubation
CD-105/FITC, surface	Milteny Biotec, 130-098-774	1:11	30', +4°C
CD-29/FITC, surface	Milteny Biotec, 130-101-256	1:11	30', +4°C
CD-90/FITC, surface	Milteny Biotec, 130-114-859	1:50	30', +4°C
CD-73/PE, surface PE	Milteny Biotec, 130-111-908	1:50	30', +4°C
CD-45/FITC, surface	Milteny Biotec, 130-110-631	1:50	30', +4°C
HLA-DR/FITC, surface	BD Pharmingen, 555811	1:5	30', +4°C

Table 2: Real-Time qPCR primer sequence list for amplification of mesenchymal stem cell cDNA.

Gene	Primer sequence	Brand/code number
β-actin	F:5'CCACACTGTGCCCATCTACG3' R:5'AGGATCTTCATGAGGTAGTCAGTCAG3'	Eurofins Genomics
Nanog		QT01844808
Oct3/4		QT00210840
SOX2	F:5'GGAGACGGAGCTGAAGCCGC3' R:5'GACGCGGTCCGGGCTTGTTTT3'	MWG
RUNX2	F:5'TACGACTGGACGCTGGTGC3' R:5'TTCATGGGTCGCTTGACGT3'	MWG
OPN (Osteopontin)	F:5'TGTGGGTTTCAGCACTCTGGTCA3' R:5'AAGCGAGTTGAATGGTGC3'	MWG
OCN (Osteocalcin)	F:5'CTGACCTCACAGATGCCAAG3' R:5'GTAGCGCCGGAGTCTGTTC3'	MWG
ALP (Alkaline Phosphatase)	F:5'GCTTCAAACCGAGATACAAGCA3' R:5'GCTCGAAGAGACCCAATAGGTAGT3'	Eurofins Genomics
COL1A1 (Collagen 1 A1)	F:5'TCTGCGACAACGGCAAGGTG3' R:5'GACGCCGGTGGTTTCTTGGT3'	Eurofins Genomics

F=forward; R=reverse

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Table 3: Expression levels of MSC markers in healthy and periodontally-affected GMSCs.

MSCs	CD-105	CD-29	CD-90	CD-73
H-GMSCs	34.3 ±1.2	98±0.94	82.01±0.81	99.1±0.14
P-GMSCs	57±2.1	100±0.05	97.05±0.8	99.2±0.5

5.3 Figures

TIMEPOINT 1	TIMEPOINT 2	TIMEPOINT 3
Day 30 (4° week)	Day 45 (6° week)	Day 60 (8° week)

Day 0 (implantation)

GROUP 1
macroscopic (*in vivo* imaging) and **microscopic** (histological and RT-qPCR analyses of osteoblastic differentiation markers) **investigations**

GROUP 2
macroscopic (*in vivo* imaging) and **microscopic** (histological and RT-qPCR analyses of osteoblastic differentiation markers) **investigations**

GROUP 3
macroscopic (*in vivo* imaging) and **microscopic** (histological and RT-qPCR analyses of osteoblastic differentiation markers) **investigations**

Fig.1 Overview of the experimental protocol for the evaluation of the *in vivo* bone differentiation ability of H-GMSCs and P-GMSCs on **MatriDerm**® scaffold. *In vivo* imaging, and histological and RT-qPCR analyses of osteoblastic differentiation markers analysis are performed at 4, 6 and 8 weeks from day of implantation.
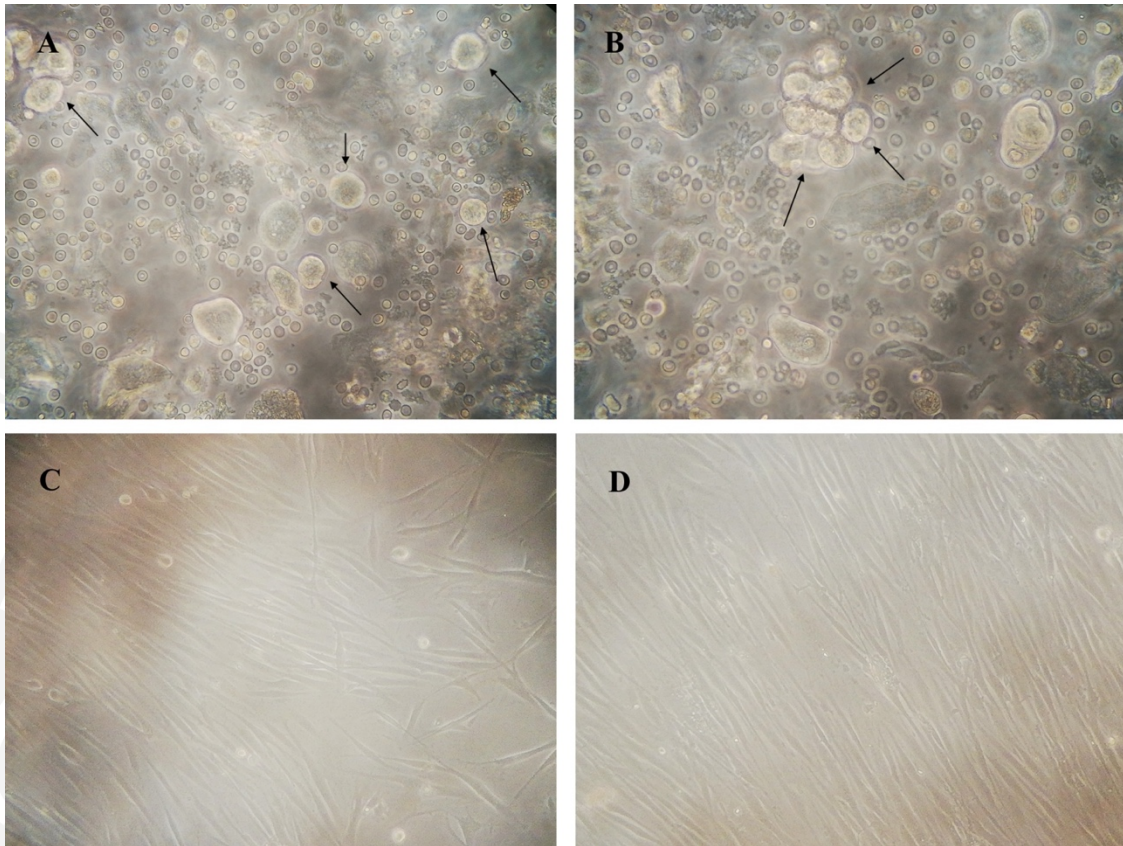
Fig.2 GMSC cultures (P0). Representative image of (A) healthy and (B) periodontally-compromised GMSCs immediately after mechanical and enzymatic digestion, showing a rounded morphology (10X); representative image of (C) healthy and (D) periodontally-compromised GMSCs at 7° day from digestion, with the typical fibroblast-like morphology (10X).
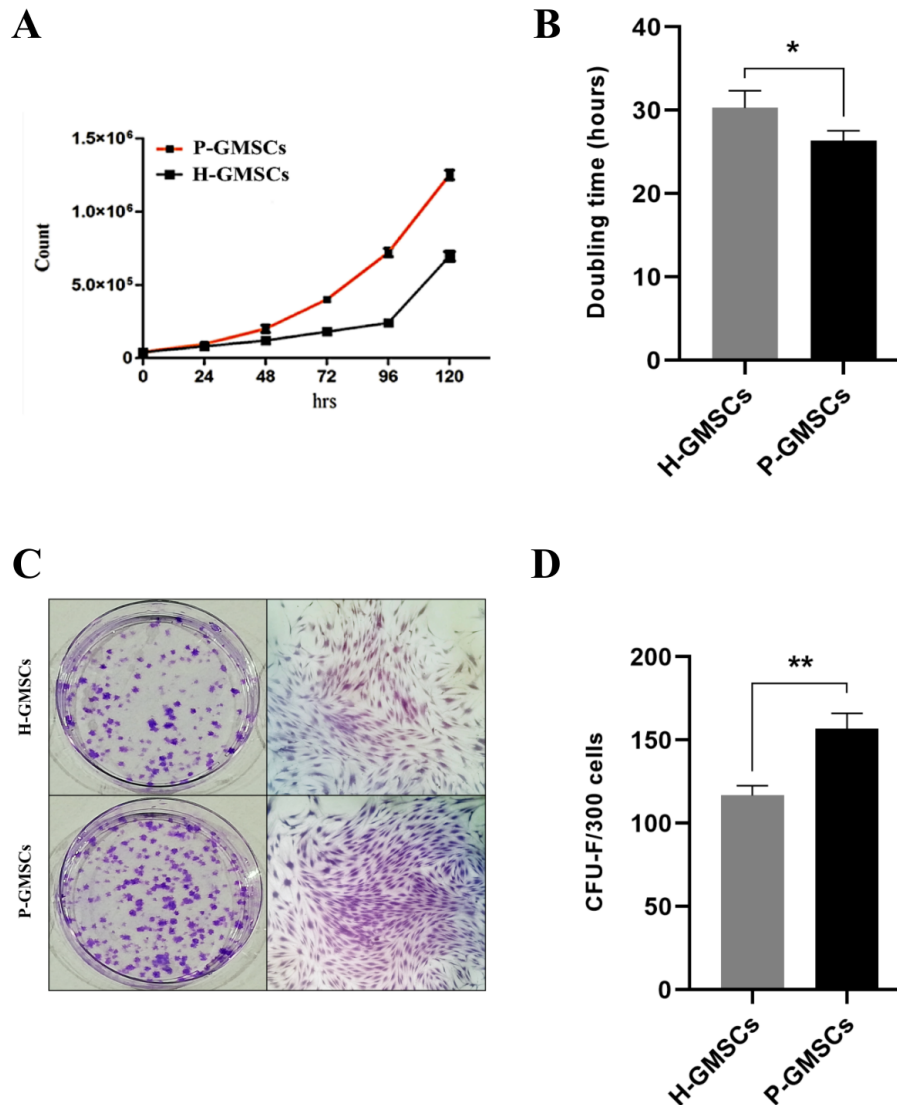
Fig. 3 Cell growth analysis and Colony-Forming Unit Fibroblast Assay (CFU-F). Figures (A) and (B) respectively show the cell growth curve of H-GMSCs and P-GMSCs (P2) evaluated by Trypan blue viability assay and the doubling time of H-GMSCs and P-GMSCs calculated according to the literature data (http://www.doublingtime. com/compute.php); figures (C) and (D) respectively show the colonies (<50 cells) (left) and the monolayer subculture (right) of H-GMSCs and P-GMSCs (P1) stained with Crystal Violet, and the quantification histogram of the CFU-F; data are reported as mean values ± SD of three independent experiments. *P-value *P≤0.05; **P≤0.01.*
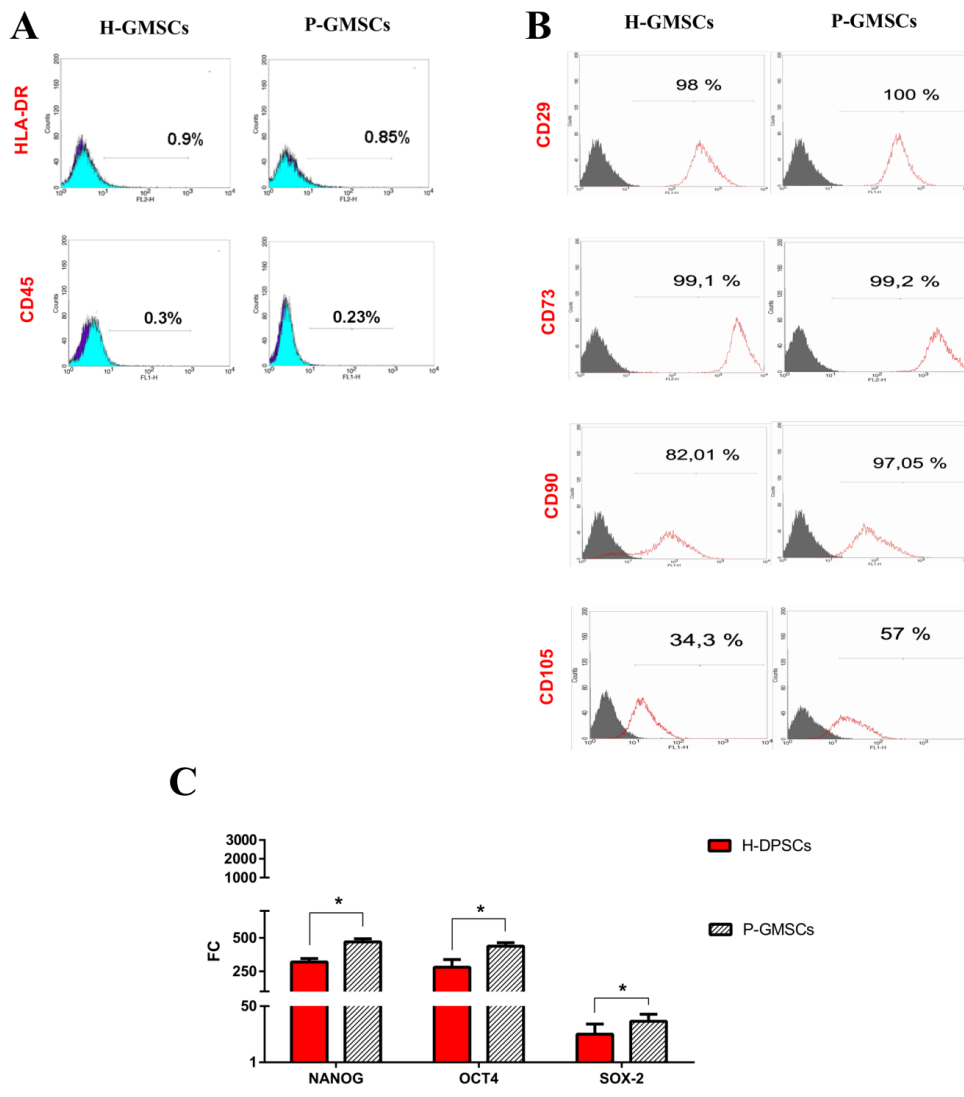
Fig.4 Mesenchymal stem cell feature analysis. Representative fields of flow-cytometric analysis of (A) hematopoietic stem cell markers CD45 and HLA-DR and (B) MSC markers CD29, CD73, CD90 and CD105 in H-GMSCs and P-GMSCs (P5) (control: isotype anti-IgG1 for CD45, CD29, CD90, CD73 and CD105; isotype anti-IgG2 for HLA-DR); (C) the histogram shows the expression of nuclear MSC markers NANOG; Oct4 and SOX-2 in H-GMSCs and P-GMSCs (P3). Data are reported as mean values ± SD of three independent experiments. Actin-β was used as housekeeping gene; FC= fold change; the mRNA expression of analyzed genes was normalized against BM-MSCs (positive control); *p-value *P≤0.05*.
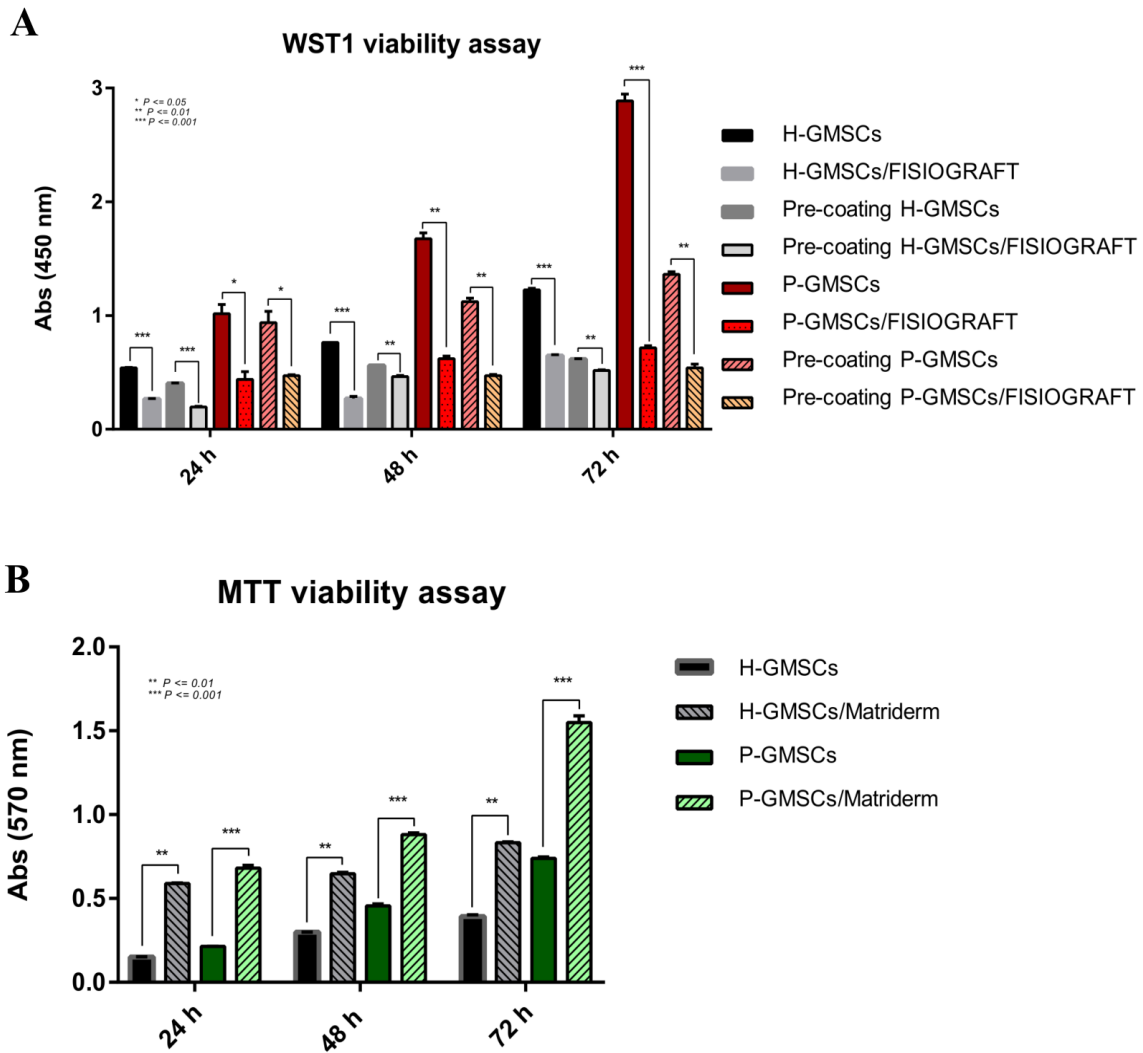
A

B

Fig.5 Cell viability analysis. (A) WST1 viability values of H-GMSCs and P-GMSCs (P3) grown in the **FISIOGRAFT Bone Granular**® scaffold for 24, 48 and 72 hours; (B) MTT viability values of H-GMSCs and P-GMSCs (P3) grown in the **MatriDerm**® collagen scaffold for 24, 48 and 72 hours; data are reported as mean values ± SD of three independent experiments; *p-values *P≤0.05, **P≤0.01, ***P≤0.001.*
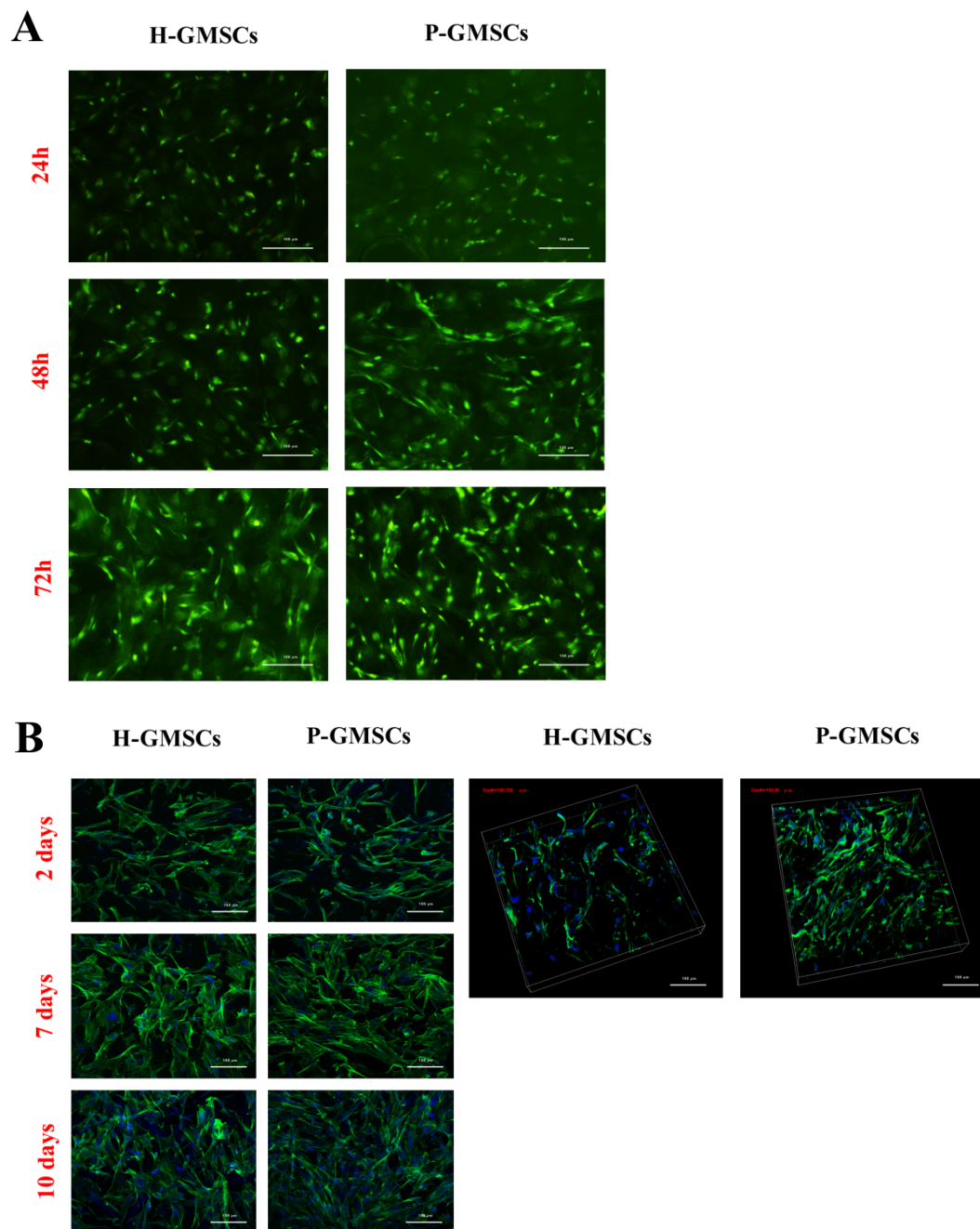
Fig. 6 Cell distribution analysis. (A) Fluorescent representative images of a Live/Dead assay of H-GMSCs and P-GMSCs (P5) grown for 24, 48 and 72 hours in the **MatriDerm**® collagen scaffold (4X); (B) (left) MaxI P and (right) volumetric images of DAPI/Actin Green confocal microscopy assay of H-GMSCs and P-GMSCs (P5) grown for 2, 7 and 10 days in the **MatriDerm**® collagen scaffold (4X); scale bars = 100 μm; depth=190,336 μm for H-GMSCs; depth=182,80 μm for P-GMSCs.
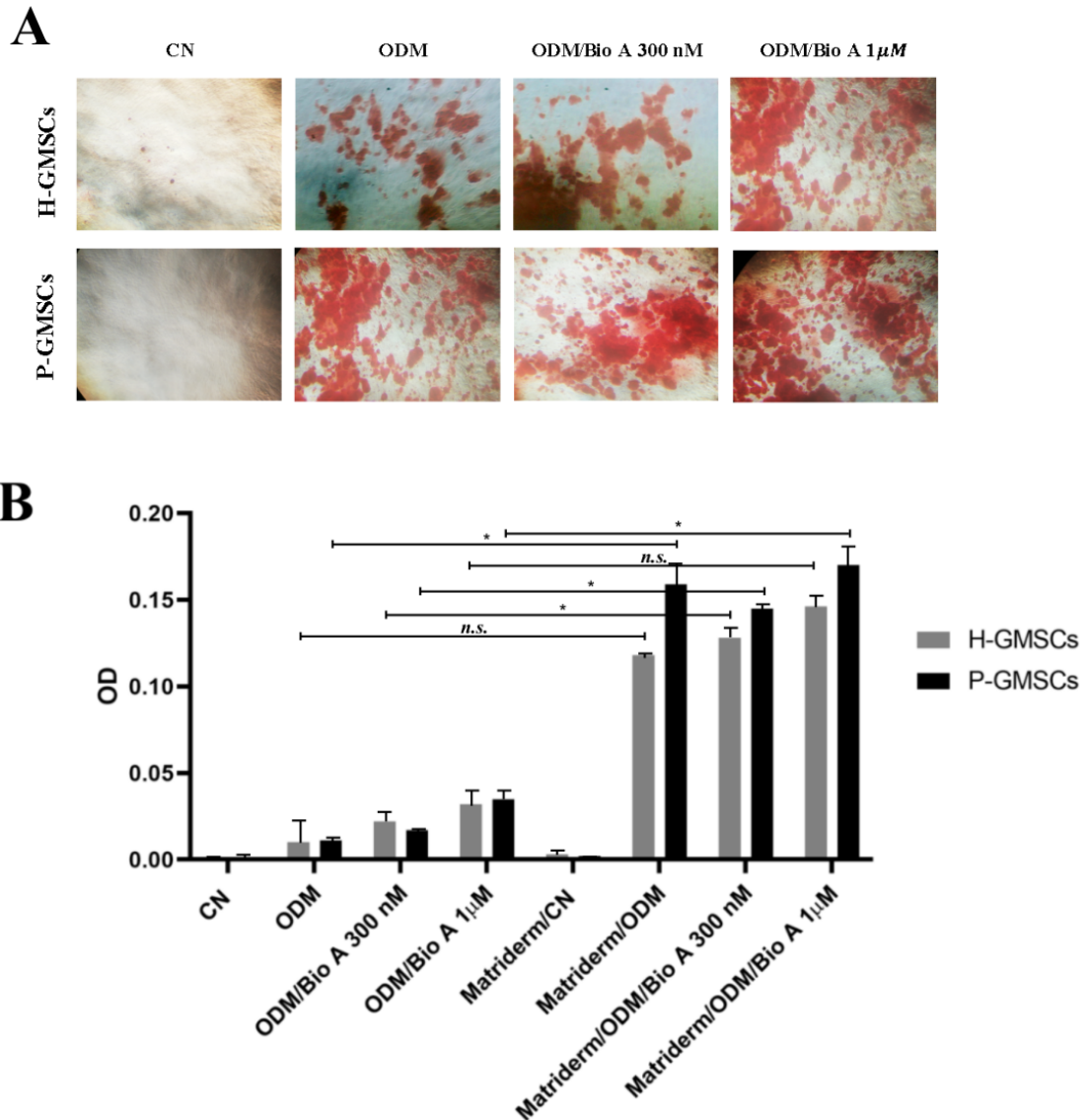
Fig. 7 Osteoblastic differentiation assay. (A) Representative images of control H-GMSCs and P-GMSCs grown in osteogenic differentiation medium (ODM), with or without Biochanin A 300 nM and 1 µM, and stained with Red S Alizarin (4X); (B) histogram representing the quantitative analysis of Red S Alizarin by spectrophotometry (550 nm OD), of H-GMSCs and P-GMSCs grown in ODM, in presence or not of the **MatriDerm**® collagen scaffold, with or without Biochanin A 300 nM and 1 µM. Data are reported as mean values ± SD of three independent experiments; *p-values *P≤0.05.*

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Scientific products (attached)

Publications discussed in the PhD thesis:

<ins>**2017**</ins>

- **Cristaldi M**, Mauceri R, Seidita F, Lo Muzio L, Campisi G. *Mesenchymal stem cells derived from inflamed gingival tissue for in vivo bone tissue engineering: preliminary results*. Poster presentation at XIV Congresso Nazionale della Società Italiana di Patologia e Medicina Orale (SIPMO), Rome (Italy), 2017.

<ins>**2018**</ins>

- **Cristaldi M**, Mauceri R, Tomasello L, Pizzolanti G, Zito G, Alessandro R, Giordano C, Campisi G *Human exfoliated deciduous teeth and oral mucosa: promising applications in tissue regeneration*. Journal of Pediatrics and Pediatric Medicine (2018).

- **Cristaldi M**, Mauceri R, Tomasello L, Pizzo G, Pizzolanti G, Giordano C, Campisi G. *Dental pulp stem cells for bone tissue engineering: a review of the current literature and a look to the future.* Future Medicine (IF 2.4) (2018).

<ins>**2019**</ins>

- **Marta Cristaldi**, Rodolfo Mauceri, Giuseppina Campisi, Giuseppe Pizzo, Riccardo Alessandro, Laura Tomasello, Maria Pitrone, Giuseppe Pizzolanti, Carla Giordano. *Growth and bone differentiation of waste gingiva-derived mesenchymal stem cells on a commercial scaffold*. Frontiers in Cell and Developmental Biology (IF 5.2) (submitted-2019, under review).

Other publications in collaboration with the departments involved in the PhD thesis project (University of Palermo):

2017

- Raimondo S, **Cristaldi M**, Fontana S, Saieva L, Monteleone F, Conigliaro A, Zito G, Alessandro R. *The phospholipase DDHD1 is a new target for the development of antitumor therapies in colorectal cancer*. Abstract presentation at ISCaM2017 - 4th Annual Meeting - Cancer Metabolism, Bertinoro (Italy), October 19-21 (2017).

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<u>**2019**</u>

Marta Cristaldi, Rodolfo Mauceri, Olga Di Fede, Giovanna Giuliana, Giuseppina Campisi, Vera Panzarella. *Salivary biomarkers for Oral Squamous Cell Carcinoma diagnosis and follow-up: current status and perspectives*. Frontiers in Physiology (IF 3.2) (2019).