# Human *levator veli palatini* muscle: A novel source of mesenchymal stem cells for use in the rehabilitation of patients with congenital craniofacial malformations

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# Abstract

**Background.** Bone reconstruction in congenital craniofacial differences, which affect about 2-3% of newborns, has long been the focus of intensive research in the field of bone tissue engineering. The possibility of using mesenchymal stem cells in regenerative medicine protocols has opened a new field of investigation aimed at finding optimal sources of multipotent stem cells that can be isolated via non-invasive procedures. Here we analysed whether *levator veli palatini* muscle fragments, which can be readily obtained in non-invasive manner during surgical rehabilitation of cleft patients during palatoplasty, represent a novel source of MSCs with osteogenic potential.

**Methods**. We obtained *levator veli palatini* muscle fragments, in non-invasive procedure during surgical rehabilitation of 5 unrelated cleft palate patients (palatoplasty surgery). The *levator veli palatini muscle* fragments was used to obtain the mesenchymal cells using pre-plating technique in a clean rooms infrastructure and all procedures were performed at good practices of manipulation conditions. To prove that *levator veli palatini muscle* are mesenchymal stem cells they were induced to flow cytometry analysis and to differentiation into bone, cartilage, fat and muscle. To demonstrate the osteogenic potential of these cells *in vivo* a bilateral full thickness calvarial defect model was made in immunocompentent rats.

**Results.** Flow cytometry analysis showed that the cells were positive for mesenchymal stem cell antigens (CD29, CD73, CD90), while negative for hematopoietic (CD45) or endothelial cell markers (CD31). Moreover, these cells were capable of undergoing chondrogenic, adipogenic, osteogenic and skeletal muscle cell differentiation under appropriate cell culture conditions characterizing them as mesenchymal stem cell. Defects treated with CellCeram$^{TM}$ scaffolds seeded with *levator veli palatini* muscle cells showed significantly greater bone healing compared to defects treated with acellular scaffolds.

**Conclusion.** We have demonstrated that cells derived from *levator veli palatini* muscle have phenotypic characteristics similar to other mesenchymal stem cells, both *in vitro* and *in vivo*. Our findings suggest that these cells may have clinical relevance in the rehabilitation of patients with cleft palate and other craniofacial anomalies characterized by significant bone deficit.


# Introduction

The therapeutic potential of multilineage stem cells in bone tissue engineering is promising, as their use may confer minimal or no donor site morbidity. It is expected that bone tissue engineering will be used to treat a wide variety of conditions that present with bone deficit as a primary condition, including congenital malformations, or in the management of patients with secondary bone loss, as in the setting of trauma, oncologic resection, or osteoporosis.

Bone reconstruction in craniofacial diseases, which affect about 2–3% of newborns, has historically been the focus of intensive research (1). Due to its high incidence rate, estimated to occur of 1:2500 live births (2, 3), cleft palate (CP) stands out as one of the most intensively researched malformations.

In approximately 50% of cases, CP occurs as an isolated entity, whilst the remainder of cases are associated with various syndromes in which other structures are affected (4). In these syndromic cases patients may exhibit other facial bone malformations that need to be repaired, as in the case of Treacher-Collins syndrome (5) and Goldenhar syndrome (6). The gold standards in facial bone reconstruction in these patient populations include the use of autogenous bone grafts and distraction osteogenesis (5, 6). However, these surgical procedures may be subject to complications such as donor area morbidity, post-surgical reabsorption and infections (7, 8). To circumvent these problems, researchers have focused on the development of bone tissue engineering strategies and osteogenic materials that may offer alternative methods with comparatively minimal or no donor site morbidity (9, 10, 11, 12, 13). We previously reported that *orbicularis oris* muscle (OOM) fragments, obtained during cheiloplasty of cleft lip and palate (CL/P) patients, are a rich source of mesenchymal stem cells (MSCs) that may be useful for bone reconstruction when associated with a collagen scaffold (12). This observation prompted us to investigate whether it is possible to obtain MSCs from *levator veli palatini* muscle (LVPM) fragments. Like OOM during cleft lip repair, LVPM can be easily obtained during planned palatoplasty in CP patients.

Considering that 50% of CP cases are associated with craniofacial syndromes with significant bone defects in the absence of a cleft lip deformity, where OOM would otherwise not be accessible as part of a planned lip repair, LVPM could be an advantageous source of MSC. Here we describe the isolation and characterisation of stem cells from this new source, aiming, in the future, to use these cells in the rehabilitation of patients with craniofacial syndromes associated with CP.

## Material And Methods

Signed informed consent from all participants in this study was obtained from each patient or their legal guardian(s). Study approval was granted by the ethics committee of the Biosciences Institute of the University of São Paulo. The laboratory experiments were carried out at Hospital Sírio-Libanês, at Human Genome Research Center both in São Paulo, Brazil, and at Regenerative Bioengineering and Repair Laboratory, Department of Surgery, David Geffen School of Medicine at University of California, Los Angeles (UCLA).

# Levator veli palatini muscle (LVPM) collection and processing were performed at Good Practices of Manipulation(GMP)

LVPM fragments (n = 5) were obtained during palatoplasty of CL/P patients using a surgical technique (modified von Langenbeck repair) (14) with radical intravelar veloplasty (Fig. 1A, B). Surgical procedures were performed at Hospital Municipal Infantil Menino Jesus, São Paulo, Brazil and at Sobrapar hospital, Campinas Brazil. Harvesting of LVPM was executed at surgical operating room after antisepsis and asepsis and transported to Sírio-Libanês Hospital Laboratory. All procedures were performed at GMP conditions with maximum degree of decontamination and sterility.

According to regulatory local committee and under Brazilian Laws and resolution to regulate advanced cell therapies (National Sanitary Vigilance Agency – ANVISA – RDC n214, February 8th 2018) the Sírio-Libanês Hospital Laboratory facilities have recommended clean rooms infrastructure including air particulate control (HEPA filter) and airflow. There is also antechamber for individual protection vestment. Only human cells can be processes at laboratory site and all reagents from cell isolation to cryopreservation are certified, prion-free and apyrogenic following the guidelines for stem cell research and the development of new clinical therapies 2016, ISSCR (internet).

Each sample was collected in HEPES-buffered Dulbecco Modified Eagle Medium/Hams F-12 1:1 (DMEM/F-12; Invitrogen, Carlsbad, CA) with 200 U/mL penicillin (Invitrogen, Carlsbad, CA) and 200 µg/mL streptomycin (Invitrogen, Carlsbad, CA), kept in 4 °C and processed within 24 hours. All LVPM samples were washed twice in phosphate-buffered saline (PBS, Gibco, Invitrogen, Carlsbad, CA), finely minced with a scalpel, put inside a 15 mL centrifuge tube, and incubated in 5 ml of TrypLE Express, (Invitrogen, Carlsbad, CA) for 30 minutes, at 37ºC. Subsequently, supernatant was removed with a sterile transfer pipette, washed once with 7 mL of DMEM/F-12 supplemented with 10% fetal bovine serum (FBS, HyClone, Hyclone Laboratories, Logan, UT), and pelleted by centrifugation at 400xg for 5 minutes at room temperature. The fragments were resuspended and transferred to 35-mm Petri dishes (Corning, NY) containing D-MEM/F12 culture medium with 15% FBS, 2 mM L-glutamine, 2 mM non-essential amino acids, 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA). After 2 weeks, cells were washed with PBS, then dissociated in trypsin solution and seeded at $1.0 \times 10^4$ cells per 25 $cm^2$ for the first passage. In order to prevent cell differentiation, cultures were maintained semi-confluent and they were subcultured every 4–5 days, with medium changes every 2–3 days.

To analyse the presence of aerobic and anaerobic bacteria and fungi in culture, it was used the automated microbial detection system Bact/Alert TM 3D (Bact/Alert- BioMérieux-Durham, NC) and for Micoplasm detection it was used MycoAlertTM (MycoAlert PLUS Mycoplasma detection Kit – Lonza). After these tests any positive results for them must be discarded and new harvesting is recommended according with the GMP laboratory rules.

# Flow cytometry

Flow cytometry analysis was performed by flow cytometry in a FACSCalibur flow cytometer (BD, Becton Dickinson Franklin Lakes, NJ) and analyzed in the CellQuest program (BD, Becton Dickinson Franklin Lakes, NJ). Cells were pelleted, resuspended in PBS (Gibco-Invitrogen, Carlsbad, CA) at a concentration of $1.0 \times 10^6$ cells/mL and stained with saturating concentration of antibodies. After a 45-minute incubation in the dark at room temperature, cells were washed three times with PBS and resuspended in 0.25 mL of cold PBS. In order to analyse expression of typical cell surface markers, cells were treated with the following anti-human conjugated antibodies: CD29-PE; CD31-FITC; CD45-PE; CD73-FITC; CD90-FITC, (Becton Dickinson, Franklin Lakes, NJ). Unstained cells were gated on forward scatter to eliminate particulate debris and clumped cells. A minimum of 5,000 events were acquired for each sample.

# Mesenchymal stem cell (MSC) differentiation

To evaluate the properties of mesenchymal stem cell differentiation, adherent cells (4th passage) underwent *in vitro* adipogenic, chondrogenic, osteogenic, and myogenic differentiation according to the following protocols:

# Adipogenic differentiation

Cells were seeded into 6-well plates (Corning, NY), at a density of $2.0 \times 10^5$ cells/well, in DMEM/High Glucose (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Hyclone), 1 µM dexamethasone, 100 µM indomethacin, 500 µM 3-isobutyl-1-methylxanthine, and 10 µg/mL insulin (all from Sigma-Aldrich, St. Louis, MO).

Fifteen days after induction, Oil Red-O (Sigma) staining was used to identify intracellular accumulation of lipid-rich vacuoles (12). Briefly, cells were fixed with 4% paraformaldehyde in PBS for 30 minutes, washed with PBS, and stained with a working solution of 0.16% Oil Red-O in PBS for 20 minutes (12).

# Chondrogenic differentiation

Approximately $2.5 \times 10^5$ cells were centrifuged in a 15 mL polystyrene tube at 400xg for 5 minutes, and the pellet was resuspended in 10 mL of basal medium. The basal medium consisted of DMEM/High Glucose (Invitrogen, Carlsbad, CA) supplemented with 1% insulin, transferrin, selenite (ITS Premix, Becton Dickinson, Franklin Lakes, NJ), 1% 100 nM dexamethasone (Sigma-Aldrich, St. Louis, MO), 1 mM sodium pyruvate (Gibco - Invitrogen, Carlsbad, CA), and 50 µM ascorbic acid-2 phosphate (Sigma-Aldrich, St. Louis, MO).

Without disrupting the pellet, cells were resuspended in 0.5 mL of chondrogenic medium, consisting of basal medium supplemented with 10 ng/mL transforming growth factor (TGF) β1 (R&D Systems, Minneapolis, MN) and 10% FBS, and maintained in a humidified atmosphere with 5% $CO_2$ at 37ºC.

On day one, tubes were gently turned over to acquire a single floating cell sphere. Medium was changed every four days. On day 21, samples were fixed in 10% formalin for 24 hours at 4ºC, and paraffin-embedded.

Cryosections (5 µm thick) were cut from the harvested micromasses and stained with toluidine blue to demonstrate extracellular matrix mucopolysaccharides (12).

# Myogenic differentiation

Cells were cultured in DMEM/High Glucose supplemented with 10% FBS (Hyclone), 5% horse serum (Sigma-Aldrich, St. Louis, MO), 0.1 µM dexamethasone, 50 µM hydrocortisone, and 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA) for 60 days (12). Differentiated LVPM cells were stained using immunofluorescence.

# Immunofluorescence

Immunofluorescence localisation of myosin and dystrophin was performed on muscle-differentiated cells of LVPM to confirm myogenic differentiation. Cells were washed twice with cold PBS, fixed with 4% paraformaldehyde/PBS for 20 minutes at 4ºC, and permeabilised with 0.05% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in PBS for five minutes. After blocking non-specific binding with 10% FBS/PBS for one hour at room temperature, incubations with the primary antibody (anti-dystrophin; Ab15277; Abcam, Cambridge, UK and monoclonal anti-myosin skeletal, Sigma) overnight at 4ºC and the secondary antibody (FITC IgG; Chemicon, Temecula, CA) for one hour at room temperature were performed. Nuclei were counter-stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO) for visualisation. As positive controls, we used normal human differentiated myotube cultures. As negative controls, we used non-differentiated LVPM-derived cells. The immunofluorescence slides were examined using an Axiovert 200 microscope (Axio Imager Z1, Carl Zeiss, Oberkochen, Germany).

# Osteogenic differentiation

LVPM cells were cultured in osteogenic medium containing DMEM/Low Glucose (Invitrogen, Carlsbad, CA) with 0.1 μM dexamethasone and 50 μM ascorbic acid 2-phosphate. On day 9, β-glycerolphosphate (10 mM) was added to induce mineralisation, and on day 21, Von Kossa staining was performed in order to identify accumulation of mineralised calcium (12).

# Immunocompetent rat model used to test the in vivo osteogenic potential of LVPM cells

The Animal Research Ethics Committee at the University of São Paulo approved the use of Wistar immunocompetent 9-month-old male rats, body weight 320–420 g, in this experimental protocol (n = 5). The animals were kept in ventilated stands (Alesco, São Paulo, Brazil), in standardised air and light conditions, at a constant temperature of 22 °C with a 12-hour light/day cycle. They had free access to drinking water and standard laboratory food pellets.

The animals were anaesthetised with an intraperitoneal injection (0.3 mL/100 g of body weight) using a combination of ketamine hydrochloride (5%) and xylazine (2%). The heads of the rats were positioned in a cephalostat during the surgical procedure. A midline skin incision was performed from the nasofrontal area to the external occipital protuberance. The skin and underlying tissues, including the periosteum and the *temporalis* muscles were reflected laterally to expose the full extent of the calvaria.

# Fabrication of scaffold carriers

CellCeram™ (Scaffdex, Finland) was designed in a cylindrical shape with 4-mm diameter of a bioabsorbable 60% hydroxyapatite and 40% ß-tricalciumphosphate composite with a foam-type structure of 83% average porosity, and 200–400 μm of average pore size, with an overall range of 100–800 μm. The dimensions of the scaffolds were designed to match the planned calvarial rat defects in these experiments

# Cell preparation for transplantation procedure

We used CellCeram™ (Scaffdex, Finland) as a framework to seed $10^5$ undifferentiated LVPM stem cells and placed on a 35-mm plate (6-well plate; Corning, NY). The cells were supplemented with 2.5 mL of medium used for undifferentiated LVPM stem cells and incubated at 37 °C and 5% CO2 for 24 h prior to transplantation in order to adhere to the scaffold.

CellCeram™ scaffolds with adherent LVPM stem cells were transferred to the right cranial bone defect, and the cell-bearing CellCeram™ surface was positioned in direct contact with the dura mater.

# Creation and repair of calvarial defects

To evaluate the osteogenic potential of the LVPM cells, we performed two symmetric full-thickness cranial defects of 4 mm diameter in size on each parietal region of the animals. The cranial defect was created with a 4 mm diameter trephine drill, and constant irrigation with sterile physiological solution was used to prevent overheating of the bone.

The left sides (LS) of the skulls were arbitrarily selected as the control sides and were reconstructed with CellCeram™ scaffolds (Scaffdex, Finland). By comparison, the right-sided defects (RS) were reconstructed with CellCeram™ scaffolds that were seeded with $10^5$ undifferentiated LVPM stem cells. Scalps were repaired with 4 – 0 nylon sutures (Ethicon, São Paulo, Brazil), and the animals euthanised 30 days after cell transplantation. Calvaria were harvested for analysis at the time of euthanasia.

# Histological preparation

The calvaria of the animals were obtained for histological assessment following euthanasia at day thirty following surgery. Tissue samples were fixed in 10% formalin solution for 24 hours, decalcified in 5% formic acid for 48 hours, and paraffin-embedded. For the morphological study, 5-µm sections were stained with hematoxylin and eosin, and examined under a conventional light microscope.

## Results

After the first enzymatic dissociation, between 5–7 days of culture, adherent cells were characterized by homogeneous cell layers with a MSC-like phenotype. All cell strains were successfully expanded, frozen and thawed several times with no visible phenotypic alterations (Fig. 2).

# Flow cytometry analysis

None of the 5 LVPM cell populations expressed the CD45 hematopoietic lineage marker or the CD31 endothelial marker. The majority of cells expressed high levels of adhesion (CD29 and CD90) and MSC marker (CD73). (Fig. 3; Table I). These results indicate that the cells obtained from LVPM were mesenchymal in nature.

# Multilineage differentiation

Multilineage differentiation was performed for 5 independent samples of LVPM cells. No obvious qualitative differences in their differentiation potential were observed.

The plasticity of adherent cells obtained from LVPM was assessed 3 weeks after *in vitro* induction of osteogenic and chondrogenic differentiation. In addition, the potential of LVPM cells to differentiate into skeletal muscle cells was investigated after 40 days of *in vitro* treatment with induction medium and assessed by immunofluorescence. Induced LVPM cells reacted positively to myosin and dystrophin while undifferentiated cells showed negative reaction to these antibodies.

The LVPM cells from all 5 strains were able to undergo myogenic, adipogenic, chondrogenic, and osteogenic differentiation *in vitro* (Fig. 4). The adipogenic differentiation was observed after 15 days. Together, these results confirmed the mesenchymal nature of the isolated cells and their multipotency. Based on this observation, we renamed the cells isolated from LVPM as *Levator Veli Palatini* Muscle-Derived Stem Cells (LVPMDSC).

# In vivo osteogenic potential of LVPMDSC

The *in vivo* osteogenic potential of LVPMDSCs was assessed in a calvarial defect model in non-immunosuppressed Wistar rats. None of the experimental animals died of infection, nor any other complication as a result of surgery or the cell/scaffold transplantation process.

Histological examination of the cranial defect 30 days following surgery revealed greater new bone formation on the RS (scaffold + LVPMDSC) compared to the LS (acellular scaffold) (Fig. 5). The trabeculae of the newly formed bone (woven bone) observed in the RS defects were intermixed with granulation tissue and with remnants of the CellCeram™ biomaterial. By comparison, the LS defects healed with loose connective tissue exhibiting chronic inflammatory infiltrates, intermingled with larger amounts of scaffold remnant (Fig. 5).

## Discussion

Initially defined as bone marrow precursors, new evidence suggests that MSCs are present in virtually all organs, possibly playing an important role in tissue maintenance and regeneration (15, 16, 17). The possibility of using these MSCs in regenerative medicine protocols has opened a new field of investigation aiming to find the best sources for obtaining multipotent stem cells, with a specific focus on sales that can be obtained in non- or minimally invasive ways.

In this study, we have demonstrated that LVPM fragments, which can easily be obtained in patients undergoing palatoplasty, are an additional source of multipotent MSCs, referred to herein as *levator veli palatini* muscle-derived stem cells (LVPMDSCs).

LVPMDSCs have similar characteristics to the *orbicularis oris* muscle-derived stem cells (OOMDSCs) obtained from cleft lip and palate patients, previously described by our group (12). The similarity between

them resides in their low level of commitment to the myogenic lineage, as observed by immunohistochemistry, in which undifferentiated LVPMDSCs and OOMDSCs did not display positive reaction to myosin and dystrophin markers. Furthermore, the immunophenotype of LVPMDSCs was comparable to that of OOMDSCs: they were strongly positive for mesenchymal and adhesion cell surface markers, but did not demonstrate the presence of endothelial or hematopoietic markers (12). This result shows that the pre-plating technique used here can be applied to isolate stem cells sharing similar properties from two distinct sources: *orbicularis oris* and *levator veli palatini* muscles. Whether this can be generalised to cells derived from other muscles remains to be investigated, since markers used for cell characterisation vary across studies (18, 19).

The mesenchymal/multipotential nature of LVPMDSCs was confirmed by the high expression of adhesion and MSC markers in these cells, together with their multilineage differentiation potential. These phenotypic hallmarks were similar to the ones seen in primary MSCs obtained from other sources, such as fat, dental pulp, bone marrow, fallopian tube, and umbilical cord vein (12, 13, 15–22).

Such features of LVPMDSCs, in conjunction with their capacity to increase bone formation *in vivo* when associated with the CellCeram™ scaffold, indicate that this cell type has the potential for clinical application, especially in bone tissue engineering protocols being developed to treat complex craniofacial malformations. Our data showed that LVPMDSCs seeded onto CellCeram™ scaffolds lead to significantly greater amounts of bone regeneration compared to acellular scaffolds, showing the osteogenic potential of these cells *in vivo*. Our results are consistent with other studies that have demonstrated how MSCs of alternate origin, when seeded onto biocompatible scaffolds, also lead to higher levels of new bone formation when compared to the use of acellular scaffolds (12, 20, 21).

Different types of biocompatible scaffolds have been used in tissue engineering research. These include collagen membranes (10, 12), hydroxyapatite (11, 23), calcium phosphate (24), and others. Here we suggest that CellCeram™, a biomaterial composed of hydroxyapatite and ß-tricalciumphosphate, is an effective alternative in this tissue engineering paradigm, as its size and three-dimensional shape can be custom-synthesized. This enables each CellCeram™ scaffold to be individually designed according to the precise anatomical requirements that define any specific bone defect.

We observed no post-surgical complications, such as wound infection or dehiscence, graft rejection, or any other overt sign of gross inflammation. The fact that immunocompetent animals were used in this study, and that these animals underwent xenotransplantation of human multipotent MSCs, suggests that LVPMDSCs helped mitigate an anticipated immunological response in such an experimental setting. This result is consistent with similar prior observations with other types of MSCs, in three previous works by our group (12, 20, 21). Moreover, it has been reported that MSCs possess immunomodulatory properties (23). Collectively, these findings suggest that heterologous LVPMDSCs may safely be used in clinical bone tissue engineering protocols without elevated risk of immunologic-mediated inflammatory responses.

Surgeons who treat congenital cream facial differences always try to decrease the number of operations in children requiring complex or staged bone reconstruction in the process of their craniofacial rehabilitation (10). The potential to minimize the numbers of required surgeries and, by extension, surgical morbidity by using MSCs seeded onto various scaffolds to generate new bone formation is an exciting prospect (10, 11, 13, 27).

# Conclusion

In summary, our study suggests that, in the future, LVPMDSCs associated with CellCeram$^{TM}$ scaffolds may be used to promote bone regeneration in craniofacial syndromes, specifically those that include cleft palate in their phenotype. For example, in patients with Treacher-Collins syndrome and Goldenhar syndrome that may require distraction or bone grafting procedures, autologous LVPMDSCs in association with CellCeram$^{TM}$ may provide a viable clinical alternative. The isolation and characterisation of LVPMDSCs opens new opportunities for the use of these cells in bone reconstruction.

To our knowledge, this is the first study to describe the isolation, *in vitro* expansion, and multilineage differentiation potential of mesenchymal stem cells derived from *levator veli palatini* muscle. These cells enhance bone regeneration *in vivo* when associated with CellCeram$^{TM}$. Thus, our results suggest that these cells are suitable for future applications in bone tissue engineering for craniofacial diseases.

# Abbreviations

OOM
Orbicularis oris muscle
CL/P
Cleft Lip and Palate
MSCs
Mesenchymal stem cells
LVPM
levator Veli Palatini Muscle
GMP
Good Practices of Manipulation
ANVISA
Agência Nacional de Vigilância Sanitária, Brazil (Brazilian National Sanitary Vigilance Agency)
DMEM/F12
Dulbecco Modified eagle medium/ HAMs F-12
FBS
Fetal Bovine Serum
PBS
Phosphate Buffered Saline
LVPMDSC

# Declarations

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### Data Availability

The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication. Please contact author for data requests.

### Ethical Approval

This project is approved by Animal Ethics Commit of Hospital Sírio-Libanês number (CEUA 2017-04) and Ethics Commit of Instituto de Biociências -USP

### Consent

Consent was received from the patients and/or legally responsible person.

### Conflicts of Interest

The authors declare that they have no competing interests.

### Authors' Contributions

Bueno D F contributed to the conceptualization, data curation, formal analysis, methodology, investigation, visualization, and wrote original draft preparation (all project); Kobayashi GS; Pinheiro CCG, Zuk, P.A contribution include resources, image editing, and data validation (laboratory processes). Resources and surgeries validation were also contributed by Tanikawa D Y S T; Raposo-Amaral CE; Rocha DL; Ferreira JRM and Jarrahy R. Bueno M R P did the conceptualization and supervision. The authors read and approved the final manuscript.

# References


1. Gorlin RJ, Cohen MM, Jr, Hennekam RCM. Orofacial clefting syndrome: general aspects. In: Gorlin RJ, Cohen MM, Jr, Hennekam RCM, editors. Syndromes of the Head and Neck. 4th ed. *New York*: *Oxford University Press*; 2001. pp. 850–76.
2. Vanderas AP. Incidence of cleft lip, cleft palate, and cleft lip and palate among races: a review. Cleft Palate J. 1987;24:216.
3. Murray JC, Daack-Hirsch S, Buetow KH, Munger R, Espina L, Paglinawan N, Villanueva E, Rary J, Magee K, Magee W. Clinical and epidemiologic studies of cleft lip and palate in the Philippines. Cleft Palate Craniofac J. 1997;34:7.
4. Jones MC. Etiology of facial clefts: prospective evaluation of 428 patients. Cleft Palate J. 1988;25:16.
5. Thompson JT, Anderson PJ, David DJ Treacher Collins syndrome: protocol management from birth to maturity. *J Craniofac Surg* **20**, 2028, 2009.
6. Canter HI, Kayikcioglu A, Saglam-Aydinatay B, Kiratli PO, Benli K, Taner T, Erk Y. Mandibular reconstruction in Goldenhar syndrome using temporalis muscle osteofascial flap. J Craniofac Surg. 2008;19:165.
7. Younger EM, Chapman MW. Morbidity at bone graft donor sites. J Orthop Trauma. 1989;3:192.
8. Esparza J, Hinojosa J. Complications in the surgical treatment of craniosynostosis and craniofacial syndromes: apropos of 306 transcranial procedures. Childs Nerv Syst. 2008;24:1421.
9. Alonso N, Tanikawa DY, Freitas RS, Canan L, Jr, Ozawa TO, Rocha DL. Evaluation of maxillary alveolar reconstruction using a resorbable collagen sponge with recombinant human bone morphogenetic protein-2 in cleft lip and palate patients. Tissue Eng Part C Methods. 2011;16:1183.
10. Gimbel M, Ashley RK, Sisodia M, Gabbay JS, Wasson KL, Heller J, Wilson L, Kawamoto HK, Bradley JP. Repair of alveolar cleft defects: reduced morbidity with bone marrow stem cells in a reabsorbable matrix. J Craniofac Surg. 2007;18:895.
11. Tanikawa DYS, Pinheiro CCG, Almeida MCA, Oliveira CRGCM, Coudry RA, Rocha DL, Bueno DF Deciduous Dental Pulp Stem Cells for Maxillary Alveolar Reconstruction in Cleft Lip and Palate Patients. *Stem Cells Int*. 2020 Mar 12;2020:6234167.
12. Bueno DF, Kerkis I, Costa AM, Martins MT, Kobayashi GS, Zucconi E, Fanganiello RD, Salles FT, Almeida AB, do Amaral CE, Alonso N, Passos-Bueno MR. New source of muscle-derived stem cells

with potential for alveolar bone reconstruction in cleft lip and/or palate patients. Tissue Eng Part A. 2009;15:427.

13. Pinheiro CCG, Leyendecker Junior A, Tanikawa DYS, Ferreira JRM, Jarrahy R, Bueno DF. Is There a Noninvasive Source of MSCs Isolated with GMP Methods with Better Osteogenic Potential? Stem Cells Int. 2019 Nov;6;2019:7951696.
14. Trier WC, Dreyer TM. Primary Von Langenbeck palatoplasty with levator reconstruction: rationale and technique. The cleft palate journal. 1984;21:254–62.
15. Caplan AI. Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. Tissue Eng. 2005;11:1198.
16. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang J, Mizuno H, Afonso ZC, Fraser JK, Benhaim P, Hedrick MH. Human Adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002;13:4279.
17. Da Silva Meireles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci. 2006;119:2204.
18. Sun JS, Wu SY, Lin FH. The role of muscle-derived stem cells in bone tissue engineering. Biomaterials. 2005;26:3953.
19. Sinanan AC, Buxton PG, Lewis MP. Muscling in on stem cells. Biol Cell. 2006;98:203.
20. de Mendonça Costa A, Bueno DF, Martins MT, Kerkis I, Kerkis A, Fanganiello RD, Cerruti H, Alonso N, Passos-Bueno MR. Reconstruction of large cranial defects in nonimmunosuppressed experimental design with human dental pulp stem cells. J Craniofac Surg. 2008;19:204.
21. Jazedje T, Perin PM, Czeresnia CE, Maluf M, Halpern S, Secco M, Bueno DF, Vieira NM, Zucconi E, Zatz M. Human fallopian tube: a new source of multipotent adult mesenchymal stem cells discarded in surgical procedures. J Transl Med. 2009;18:7.
22. Secco M, Zucconi E, Vieira NM, Fogaça LL, Cerqueira A, Carvalho MD, Jazedje T, Okamoto OK, Muotri AR, Zatz M. Mesenchymal stem cells from umbilical cord: do not discard the cord! Neuromuscul Disord. 2008;18:17.
23. Mankani MH, Afghani S, Franco J, Launey M, Marshall S, Marshall GW, Nissenson R, Lee J, Tomsia AP, Saiz E. Lamellar spacing in cuboid hydroxyapatite scaffolds regulates bone formation by human bone marrow stromal cells. Tissue Eng Part A. 2011;17:1615.
24. Goshima J, Goldberg VM, Caplan A. The origin of bone formed in composite grafts of porous calcium phosphate ceramic loaded with marrow cells. Clin Orthop. 1991;269:274.
25. Jazedje T, Bueno DF, Almada BV, Caetano H, Czeresnia CE, Perin PM, Halpern S, Maluf M, Evangelista LP, Nisenbaum MG, Martins MT, Passos-Bueno MR, Zatz M. Human fallopian tube mesenchymal stromal cells enhance bone regeneration in a xenotransplanted model. Stem Cell Rev. 2012;8:355.
26. Ren G, Zhang L, Hao X, Xu G, Zhang Y, Roberts A, Zhao RC, Shi Y. Mesenchymal stem cells mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell. 2008;2:141.

27. Hibi H, Yamada Y, Ueda M, Endo Y. Alveolar cleft osteoplasty using tissue-engineered osteogenic material. Int J Oral Maxillofac Surg. 2006;35:551.

# Figures

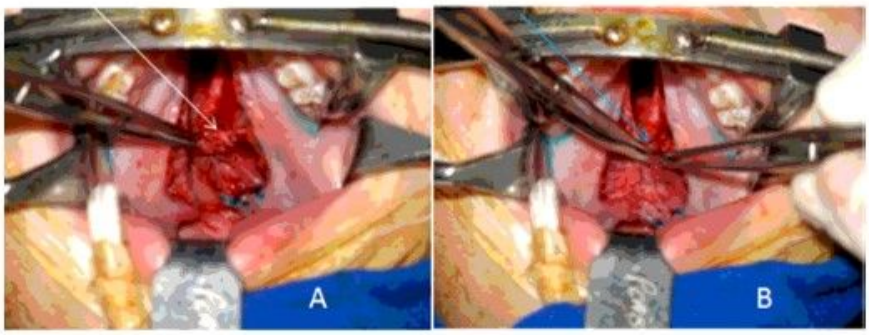
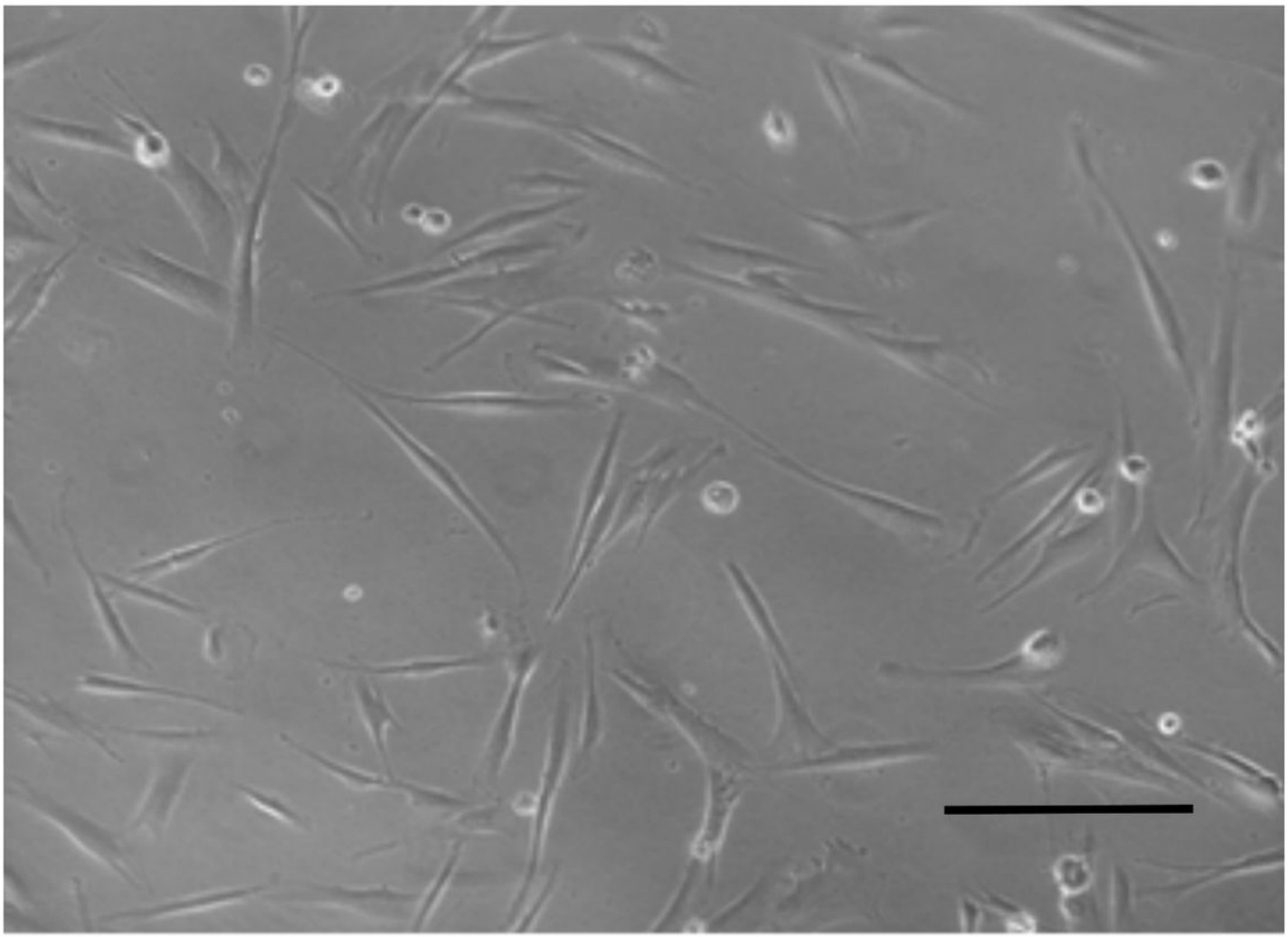


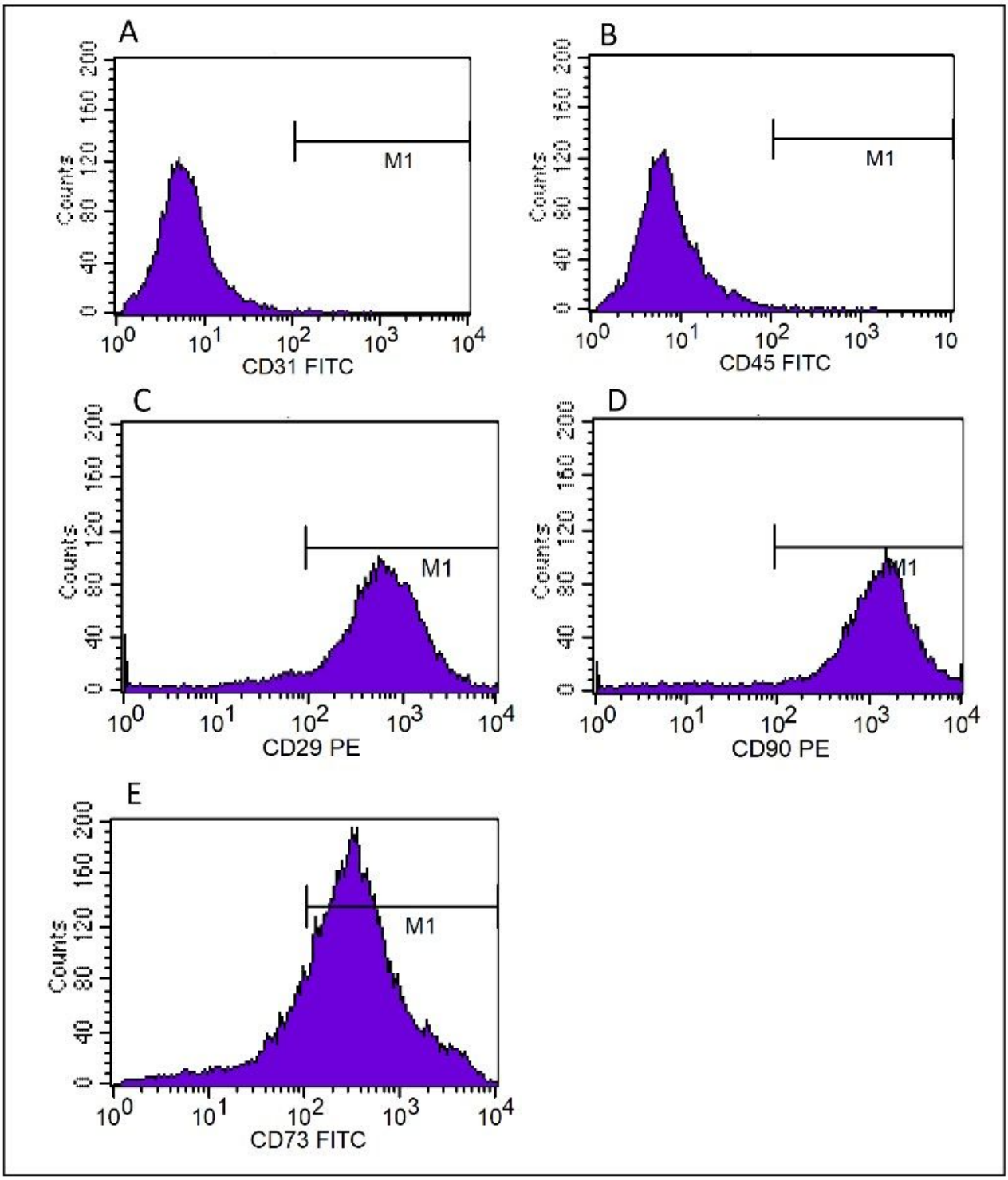

**Figure 1**

Levator veli palatini muscle (1A, white arrow) and small piece levator veli palatini muscle harvested to obtain the cell cultures after palatoplasty (1B, blue arrow)



## Figure 2

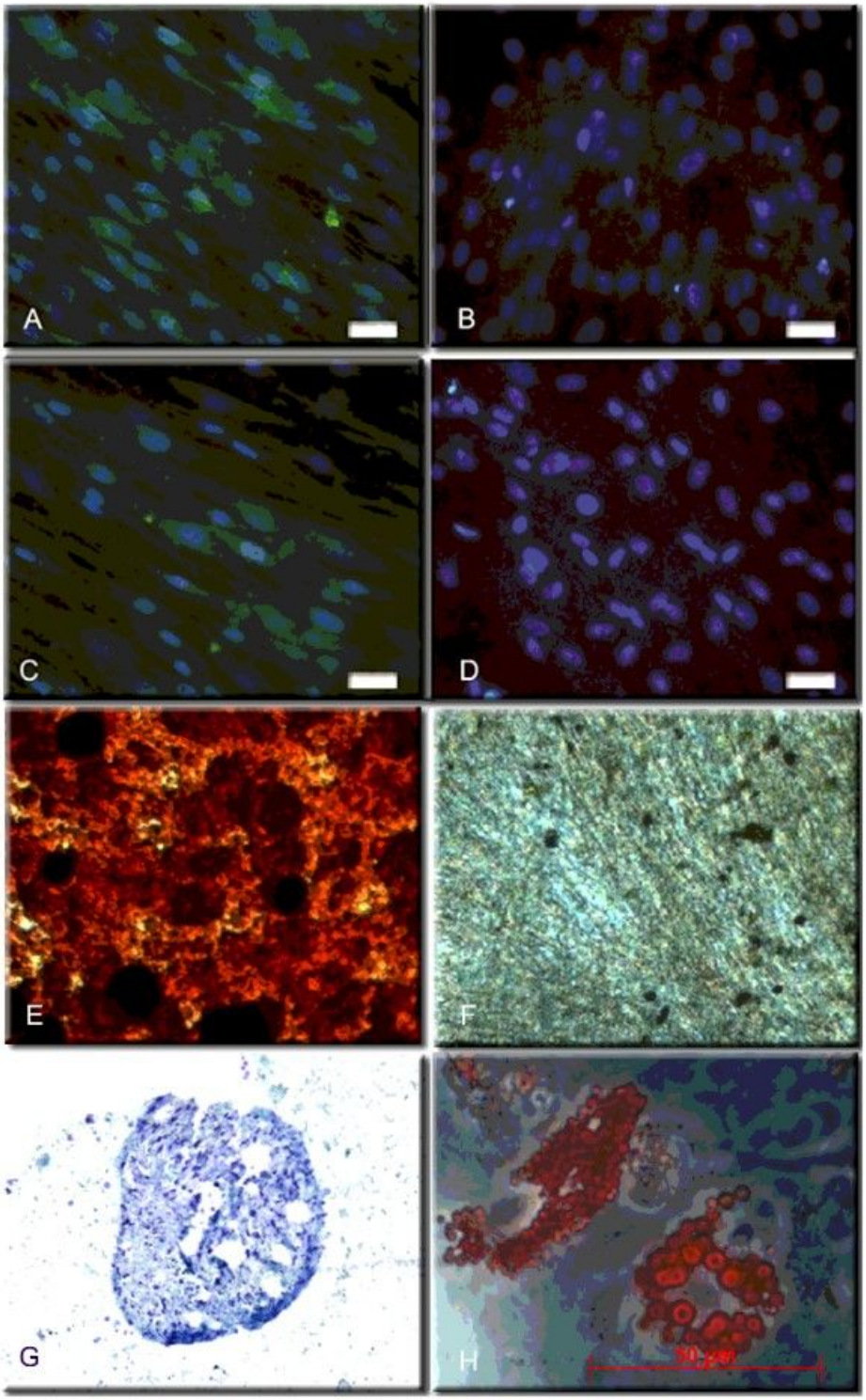
Levator Veli Palatini Muscle-Derived Stem Cell (LVPMDSC) fibroblast-like morphology. Scale bars: 50 μm.




## Figure 3

Immunophenotype analysis of LVPMDSCs. Related graphs, where it is possible to compare, for each of the 5 analysed markers, the negative control (unlabelled LVPMDSCs, in grey) and the experimental population of LVPMDSCs labelled with specific antibodies (in black). Negative reaction (< 5%) for endothelial marker CD31: 0.96% (A) and hematopoietic marker CD45: 1.14% (B). Positive reaction was

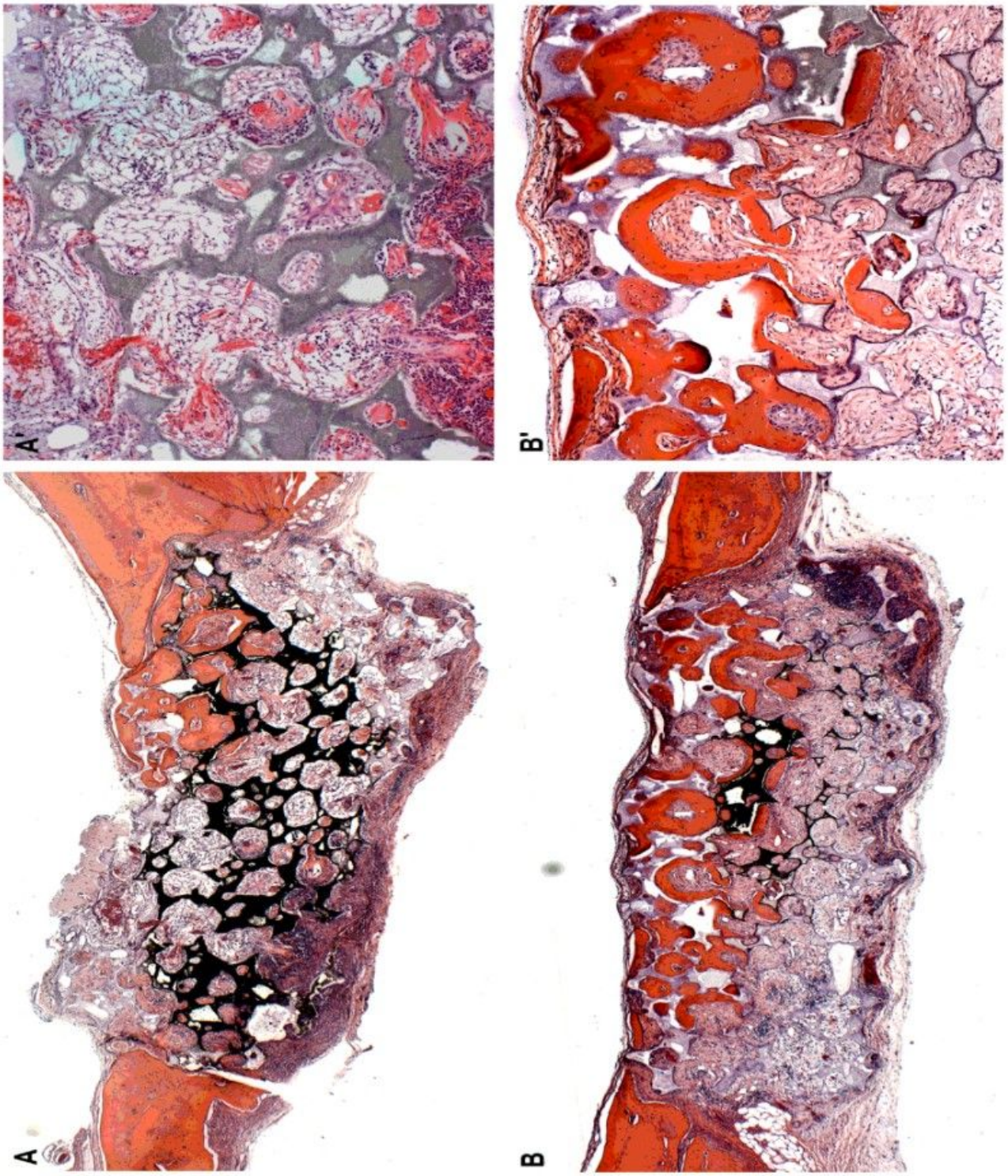
observed for the adhesion markers CD29: 99.82% (C); CD 90: 98.06% (D) and for the mesenchymal marker CD73: 99.66% (E). Abbreviations: CD, Cluster of Differentiation.




## Figure 4

In vitro staining of LVPMDSCs. Myogenic differentiation labelled with: anti-myosin (A) and anti-dystrophin (C). Undifferentiated cells labelled with anti-myosin (B) and anti-dystrophin (D). Osteogenic differentiation: Von Kossa staining revealing calcified extracellular matrix 21 days after osteogenic

induction (E) and its negative control (F); Adipogenic differentiation: Oil Red-O staining (G); Chondrogenic differentiation: toluidine blue staining (H). Scale bars: 50 μm.




## Figure 5

Histological analysis of bone neoformation at 30 days post-surgery. Rat defect (right side) seeded with LVPMDSC associated with CellCeramTM, revealing higher bone neoformation in different magnifications (B, B') when compared with the defect (left side) where only CellCeramTM was applied (A, A'). In B and B'

we observe greater bone neoformation intermixed with granulation tissue with few remnants of CellCeramTM (in black/gray), whilst and in A and A' the defect is filled with loose connective tissue exhibiting chronic inflammatory infiltrate, intermingled with remnants of CellCeramTM and only a small number of trabeculae of recently formed bone.

## Supplementary Files

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- CoverLetterLVPM1.docx