

*Original Article***Characterization of Adipose-Derived Mesenchymal Stem Cells from tissue harvested with the guided SEFFI technique and co-cultured with calcium hydroxyapatite**

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ABSTRACT

Increased interest in regenerative medicine in the last few years was registered. In parallel, a progressive increase in injectable calcium hydroxylapatite (CaHA) applications has been observed due to its applications in skin rejuvenation. This study combined micro fragmented fatty tissue (FAT) grafting with the CAHA filler procedure to observe the biological effect on FAT. Each sample of micro fragmented adipose tissue harvested using the guided Superficial Enhanced Fluid Fat Injection (guided SEFFI) technique were collected from 5 patients. The 5 samples were divided into aliquots to create two different experimental conditions: FAT and FAT combined with CaHA (FAT + R). Afterwards, the cellularity of mesenchymal phenotype and the in vitro differentiation capacity in mesenchymal lineages were assessed in both experimental conditions, FAT and FAT+R. Despite low cellularity observed for FAT+R compared to FAT, isolated cells could grow and expand in culture in both experimental conditions, thus proving their proliferative ability. Cells were proved to differentiate towards mesenchymal lineages, expressing mesenchymal markers by flow cytometry analysis. Combining emulsified harvested tissue prepared with guided SEFFI technique with CaHA products can be exploited to counteract the loss of volume and skin ageing of the human face and body. This approach to regenerative aesthetic treatment is a promising treatment for facial antiaging therapy.

INTRODUCTION

Skin rejuvenation treatments have been increasingly requested in the last few years(1-3). Nowadays, innovative procedures exploiting the effects of autologous stromal vascular fraction (SVF) and adipose-derived mesenchymal stem cells (AD-MSCs) led to the availability of methods meeting the current trend toward non-invasive and regenerative treatments for skin ageing (4-8).

Adipose tissue is a promising source of mesenchymal stem/stromal cells (MSCs), among which AD-MSCs, extracted from the SVF, possess antifibrotic and immunomodulatory abilities, and they have been shown to improve fat graft survival through angiogenesis induction (9-12).

Therefore, due to all these peculiarities, adipose tissue implantation has been used to enhance skin trophism, and this property has been applied to different fields of medicine, including plastic, reconstructive and aesthetic surgery and medicine (12-17). Interestingly, the role of fat injection in skin rejuvenation has been described (18).

Recently, in order to maximize the effect of fat, the combination of AD-MSCs with hyaluronic acid (HA) has been investigated, finding that materials were compatible and hypothesizing that the combination could be applied in clinical practice to enhance the skin rejuvenation potential of fat (19).

However, in the last few years, fillers such as calcium hydroxylapatite (CaHA) have become popular for their biostimulatory effects and versatility, leading to natural results and the possibility of being combined with other procedures (20, 21, 22).

This study aimed to investigate the biological effect of the CaHA filler (Radiesse®, Merz aesthetics, Frankfurt, Germany) on AD-MSCs prepared with the guided SEFFI technique (using SEFFILLER™ medical device produced by SEFFILINE srl Italy). In particular, the aim is to evaluate the impact of CaHA on mesenchymal SCs performance in terms of proliferation, immunophenotype and differentiation into adipose, bone and cartilage lineages in comparison with AD-MSCs derived from the lipoaspirates but cultured without filler.

MATERIALS AND METHODS

Biological Samples and Filler

This study was conducted in agreement with the Declaration of Helsinki-Ethical principle for medical research involving human subjects. One AA (FM) collected Lipoaspirates with a guided SEFFI technique (using SEFFILLER™ medical device produced by SEFFILINE srl Italy) from 5 healthy subjects. The procedure was performed under local anaesthesia. Lidocaine does not negatively impact the distribution, cell number, and viability of ASCs and preadipocytes (23).

Briefly, adipose tissue was harvested from the abdomen or the trochanteric region. Manual fat tissue aspiration was performed with a 10 mL syringe with a plunger lock mounted with the microperforated side-port cannula inserted into the patented guide (side port holes 800 microns). The adipose tissue's aspiration was performed by proceeding to a gentle back-and-forth fan movement throughout the sampling area. The guide guaranteed that the tunnelling was carried out in the subcutaneous tissue adjacent to the dermis, in the superficial adipose tissue (SAT) (Fig. 1).

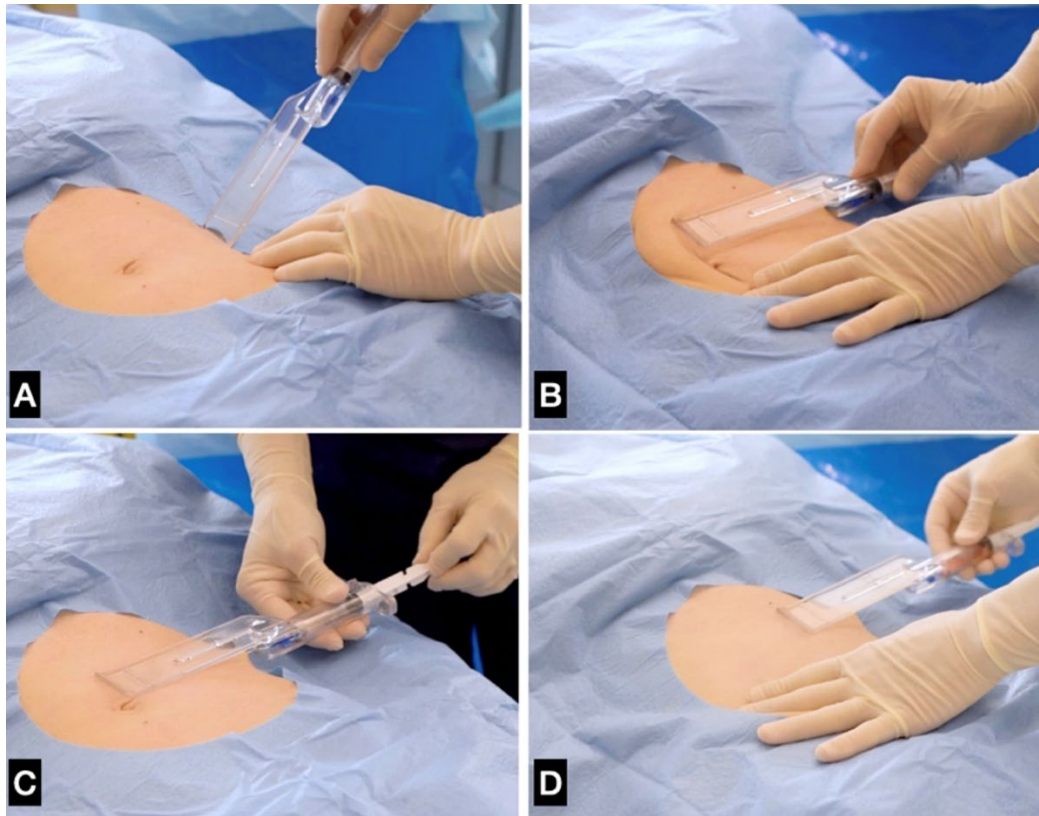


Fig. 1. *A): Introduction of the cannula; B): standardized harvesting procedure: 15mm under the skin; C): syringe plunger locked for tissue harvesting; D): harvested tissue.*

The aspiration ended after the collection of 5 ml of tissue. The harvested tissue was rinsed with saline solution and maintained vertically for a few minutes to let the content stratify by the force of gravity in two layers (Fig. 2).

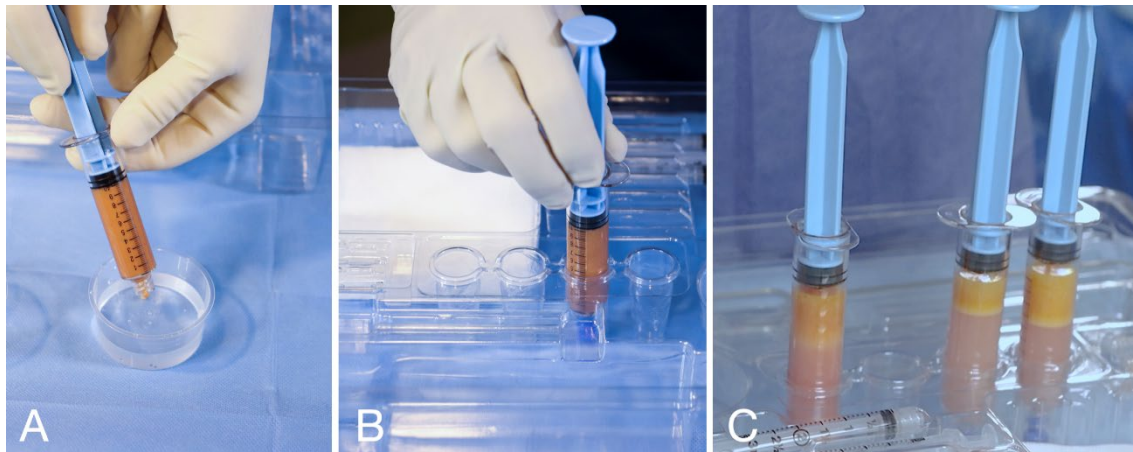


Fig. 2. *A): Harvesting tissue is rinsed with saline solution; B): Close the syringe with a lure-lock cap; C): Harvesting syringe is maintained in a vertical position.*

After a few minutes, the washing liquid was discharged, and the tissue was ready to be inserted in 5 disposable sterile plastic boxes without coding (Fig. 3) and promptly delivered to Tecnopolo “Mario Veronesi” by “Croce Blu” transfer in a dedicated refrigerated box. On arrival, each sample was marked by the attribution of an internal conventional number code; additionally, 10 vials (syringe) containing 1.5ml/each of the filler Radiesse® were delivered at room temperature (Fig. 3).

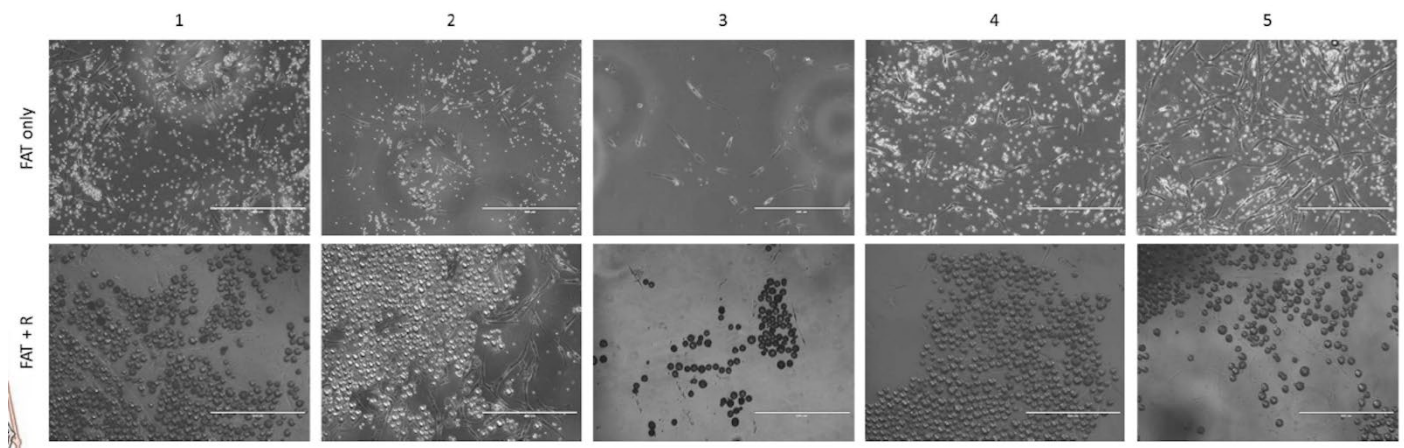


Fig. 3. *Photographs of adherent cells derived from both culture conditions, FAT and FAT+R. The pictures were collected from all donors after 8 days post seed by Evos Microscope at 10X magnification. Scale bar = 400µm.*

CaHA filler is a white material composed of HA suspended in a gel carrier that primarily consists of water, glycerine and sodium carboxymethylcellulose, as reported in the instruction for use (24). At the microscopical observation, the filler resulted in round-shaped micrometric spheres.

Cell isolation and combination with CaHA

The combination of lipoaspirates and Radiesse® were performed as defined and described in the previous study for lipoaspirates and HA (19).

Fat tissue was collected in a 30 ml syringe and washed 3 times with 15 ml of phosphate-buffered saline (PBS) to remove blood cells and used to perform two different culture conditions, following called “FAT” and “FAT + R”. In detail:

- FAT: 0.5 ml of adipose tissue (AT) were seeded on each well of MW6 (MW6 Cellstar Greiner Bio-one cat. 657 160) prefilled with 2ml of culture medium aMEM (MEM Alpha Medium (AMEM) Gibco ref. 22561-021; lot n. 2418642) enriched with 2.5% PLP, 0.2% Heparin, 1% Glutamine and 0.5% ciprofloxacin
- FAT + R: 0.6 ml of AT- Radiesse® mixture in a ratio of 1 (CaHA): 4 (lipoaspirate). Radiesse® was collected using a syringe connector and properly mixed until the fat was homogenously dispersed in the filler and then seeded. The two types of specimens were kept in an incubator at 37°C, 5%CO₂ for 4 days to allow the release and the adhesion of stromal cells to the plastic surface. The mixture was seeded on MW6, prefilled with 2ml of culture medium aMEM enriched with 2.5% PLP (Cell Culture-Human Platelet Lysate (PLP) Macopharma ref. BC0190030; lot.11410203DM), 0.2% Heparin, 1% Glutamine and 0.5% ciprofloxacin.

After the first 4 days, culture media were changed and refreshed every 2-3 days, being careful not to remove the AT floating fragment.

AD-MSCs Proliferation

Once the plastic culture was covered with “adherent” cells, cultures were extensively washed with PBS to remove oil, Evos Invert Microscope, objective 10X, acquired floating cells, debris and Radiesse® spheres and photographs of the cultures. A solution of 0.05%/0.02%trypsin/EDTA was added to each well and incubated at 37°C for 3 minutes to allow the detachment of adherent cells. Blocking media composed with DMEM (Dulbecco Modified Eagle Medium (DMEM) Gibco cod. 41965-039; lot. 2375262) supplemented with 10%FBS (Fetal Bovine Serum (FBS) Corning; lot. 01421001; ref 35-079-CV) were added to block the enzyme. Cells were then centrifuged at 1400 rpm for 5 minutes, counted using 0.4% trypan blue staining and plated at the density of 6000/cm² in culture media composed by aMEM supplemented with 2.5%PLP, 0.2%Heparin, 1% glutamine and 0.5% ciprofloxacin.

The medium was refreshed every 2-3 days until cells reached the confluence. The cultures were amplified for 4 passages (from P1 to P4). At Passage 4, adherent cells released from lipoaspirate were characterized by immunophenotypic analysis and differentiation assay.

During culture, cumulative Population Doubling (PD) and cumulative Population Doubling Time (PDT) were calculated from P1 to P4 to assess if Radiesse® could affect cell growth and proliferation.

The proliferation rate was analyzed by determining PD at the end of each passage for four passages using the following formula:

$$PD = \frac{\log(NH) - \log(N1)}{\log(2)}$$

Where “log” is the logarithm to base 10, NH denotes the number of cells retrieved at the end of the passage, and N1 denotes the number of cells seeded at the beginning of the passage. The number of PD at the end of each passage was added to the PD of previous passages to calculate cumulative PD. The PDT was calculated at the end of each passage for four passages using the following formula:

$$PDT = \frac{\text{Duration} (\log 2)}{\log(\text{final concentration}) - \log(\text{initial concentration})}$$

Where “log” is the logarithm to base 10 and the time unit indicated in the formula as “duration” is “day”. For calculating cumulative Doubling Time, the number of PDT at the end of each passage was added to the PDT of previous passages.

AD-MSCs Immunophenotype

Fluorescence-activated cell sorting (FACS) analyses for 7AAD, CD45, HLA-DR, CD31, CD90, CD73, CD105 and CD146 were performed on AD-MSCs after 4 culture passages. Before cell detachment for FACS analyses, images of cell cultures were acquired by Evos inverted microscope, with 10x magnification.

AD-MSCs were detached, counted and incubated in polypropylene tubes (0.3×10^6 /tube; VWR, Milan, Italy) in blocking buffer (100 μ L each 0.5×10^6 mesenchymal stem cells, MSCs) containing DMEM, 10% FBS, 0.1 M sodium azide and 66.6 mg/mL human immunoglobulin G (Sigma, Steinheim, Germany) for 20 min on ice. Cells were subsequently stained for 30 min on ice with primary antibodies (diluted in PBS with 0.1% bovine serum albumin (BSA; Sigma-Aldrich ref. B4287) and analyzed with a Cytotflex (Beckam culture). 7-Aminoactinomycin-D (7AAD)- staining (ViaProbe; BD Biosciences; 5 μ L/ 0.5×10^6 cells, according to the manufacturer's instructions) was evaluated by flow cytometry to detect apoptosis. Early apoptotic cells, taking up 7-AAD, become 7-AAD dim compared with living cells, which remain 7-AAD negative. Late apoptotic or necrotic cells, which have lost membrane integrity, appear 7-AAD bright.

An isotype control sample for each condition was used to exclude the autofluorescence background, and dead cells were excluded using 7-AAD staining. Analyses were performed using a Cytotflex by the same operator, who previously had shared measurement techniques with other operators. Data were analyzed using CytExpert software.

AD-MSCs differentiation assay

Bone Differentiation. Ten thousand cells/cm² were seeded, and bone induced by the addition to the culture media of 10 nM dexamethasone, 0.1 mM L-ADSCSorbic acid-2-phosphate, 10 mM beta-glycerol phosphate (all Sigma) and 100 ng/mL bone morphogenic protein BMP2 added only after 7 days of induction (PeproTech, Rocky Hill, NJ). After 2 weeks, differentiated AD-MSCs and controls were stained with 1.5% Alizarin Red-S (v/v; Sigma) in ddH₂O. red depots are observed by AxioObserver Z1 (Zeiss), objective 10x.

Adipogenic Differentiation. AD-MSCs were seeded at 10000 cells/cm² and induced towards the adipogenic lineage using DMEM with 1% P/S, 5% rabbit serum (Euroclone) and 2.5% horse serum (Hyclone) supplemented with 1 μ M dexamethasone, 60 μ M indomethacin, 10 μ M rh-insulin and 0.5 mM isobutylmethylxanthine (all from Sigma). AD-MSCs were kept in a differentiation medium for 5 days, and adipocyte differentiation was visualized with 1% Oil Red O (Sigma). After induction, differentiated cells and controls were microscopically visualized by Axio Observer Z1 (Zeiss) with 40x magnification.

Chondrogenic Differentiation. AD-MSCs were plated in 15 mL conical tubes (2×10^5 cells/mL) in culture media supplemented with 500 ng/mL bone morphogenic protein-6 (PeproTech, Rocky Hill, NJ), 10 ng/mL transforming growing factor- β (PeproTech, Rocky Hill, NJ), 50 mg/mL ITS+Premix (containing 6.25 μ g/mL insulin, 6.25 μ g/mL transferrin, 6.25 ng/mL selenous acid, 1.25 mg/mL BSA and 5.35 μ g/mL linoleic acid; BD Biosciences), dexamethasone (final concentration: 100 nM), 0.2 mM L-ADSCS ascorbic acid-2-phosphate, 100 μ g/mL sodium pyruvate (x 100) and 40 μ g/mL proline, all from Sigma). Cells were centrifuged to the bottom of the 15 mL conical tube and kept in an incubator with a controlled atmosphere (5% CO₂, 37°C). The medium was changed every 2 days while leaving the pellet undisturbed inside the tube. At 21 days of differentiation, the pellets were harvested, formalin-fix and paraffin-embedded. Four μ m-thick sections of induced samples and negative controls were stained explicitly with 1% Alcian blue solution (Sigma Aldrich) and counterstained with 0.2% nuclear fast red (Sigma-Aldrich). Pictures were acquired by optical direct Microscope AxioImager M2, 40x magnification.

Statistical analysis

Data were reported as average with a standard deviation error of the mean (SEM). T-test was applied, and the p-values obtained were considered statistically significant when $p < 0.05$.

RESULTS*Cellularity and proliferation*

A total of 5 consecutive patients with a mean age of 47.7 years were treated with the guided SEFFI (Superficial Enhanced Fluid Fat Injection) procedure to collect the fat tissue between February 2021 and March 2021.

A final volume of lipoaspirates of 8.20 ± 1.20 ml was collected after blood cells and oil removal (Table I).

Table I. Amount of lipoaspirate collected.

ID SAMPLE	VOLUME FAT (ML)
#1	10
#2	12
#3	7
#4	4
#5	8
AVERAGE	8,2
SEM	1,36

Each biological specimen was used to set up both FAT and FAT + R experimental conditions (in the following table and paragraphs, we used the term “FAT” instead of adipose tissue prepared with guided SEFFI technique).

Lipoaspirates were maintained in culture for 4 days without medium replacement. On day 8, adipose tissue fragments were removed, and cell cultures were washed 3 times with PBS to remove oil floating debris and fibroblastoid elements in all specimens (Fig. 3). Only in the FAT+R samples were CaHA spheres observed. At day 13 after seeding, the vessel culture surface was covered with fibroblastoid cells, except for sample 3, where fewer adherent elements were visualized (Fig. 4). Cultures were treated with trypsin, and the number of cells counted was reported in Table II.

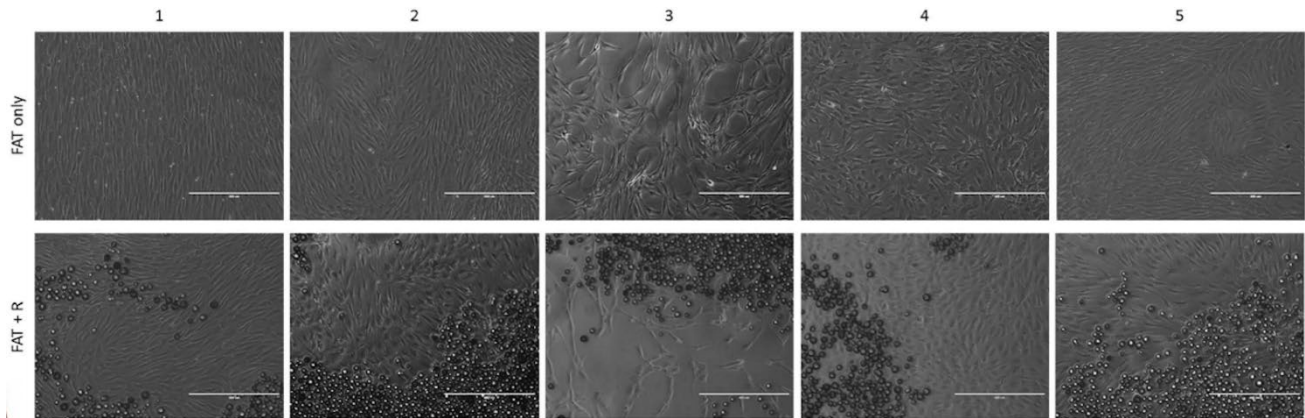


Fig. 4. Adherent cells derived from both culture conditions, FAT and FAT+R, had covered the plastic surface. The pictures were collected from all donors 13 days post-seed by Evos Microscope at 10X magnification. Scale bar = 400 μ m.

Table II. Adherent cells derived from FAT (A) and from FAT+R (B) were counted at 13 days post-seed and reported as row data (middle column) and for ml of lipoaspirate (right column).

ID SAMPLE	N OF ADHERENT CELLS	N OF ADHERENT CELLS/ML LIPOASPIRATE
FAT		
#1	1750000	583333
#2	2600000	866667
#3	1325000	441667
#4	1080000	540000
#5	2900000	966667
AVERAGE	1931000	679666,67
SEM	354310,6	100689,84
FAT+R		
#1+R	188888	62963
#2+R	1400000	466667
#3+R	155000	51667
#4+R	600000	300000
#5+R	333333	111111
AVERAGE	535444,2	198481,4
SEM	229925,22	80555,21

To better compare the number of cells released from FAT and FAT + R specimens, the row number of counted cells was reported to ml of lipoaspirate. Table II shows that an average value of 679.666 $\pm$ 100.680 cells/ml was counted for FAT samples, while the cells count was 198.481 $\pm$ 80.555 for FAT+R. The number of

cells counted resulted viable in all specimens. CaHA limited the number of cells released from FAT (Fig. 5), and the difference with FAT was statically significant (p -value <0.05). Adherent cells were cultured until 4 passages for FAT and FAT+R, sharing a similar cell morphology characterized by fibroblastoid shape (Fig. 6). In addition, in all samples, cells resembled preadipocytes, characterized by a squared shape with dark dots inside. These observations were expected considering the tissue sources from which cells were isolated. Both samples had a similar proliferation rate, as cumulative PD and PDT confirmed. No variability between tissue samples was detected, as shown by SEM of the graph (Fig. 7).

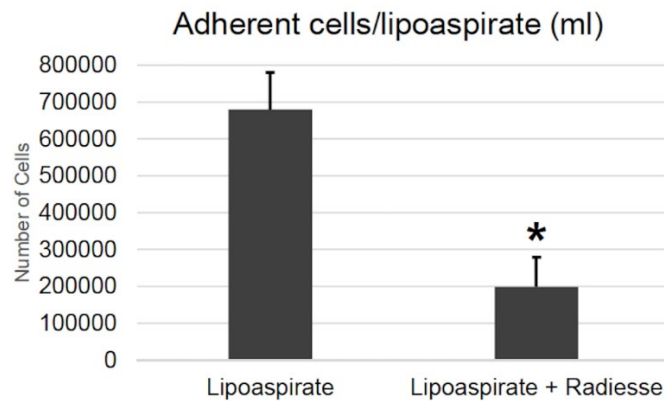


Fig. 5. Number of adherent cells derived from FAT (A) and FAT+R (B) were counted at 13 days post-seed. * P -value <0.05 .

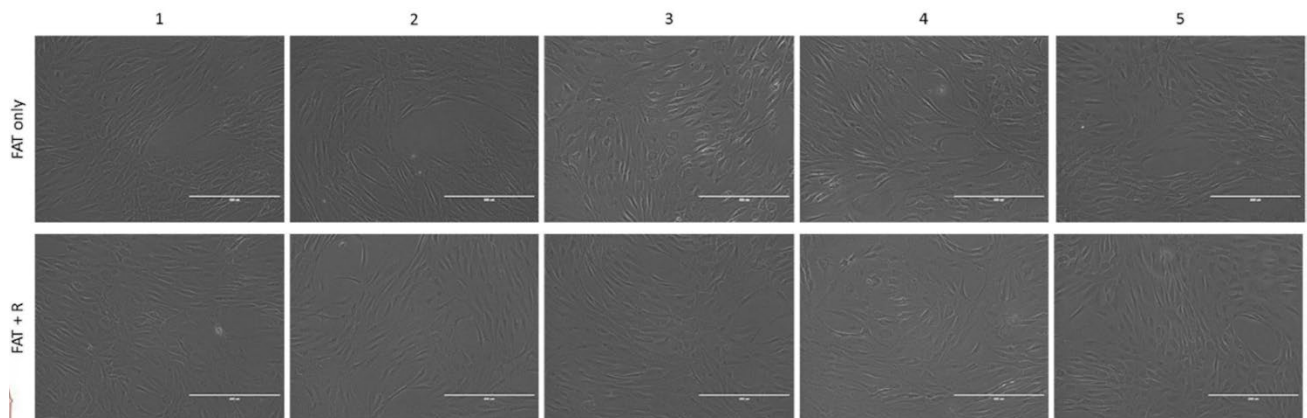


Fig. 6. Adherent cells (AD-MSCs) derived from both culture conditions, FAT and FAT+R cultured until passage 4 (P4). Pictures were acquired by Evos Microscope at 10X magnification. Scale bar = 400 μ m.

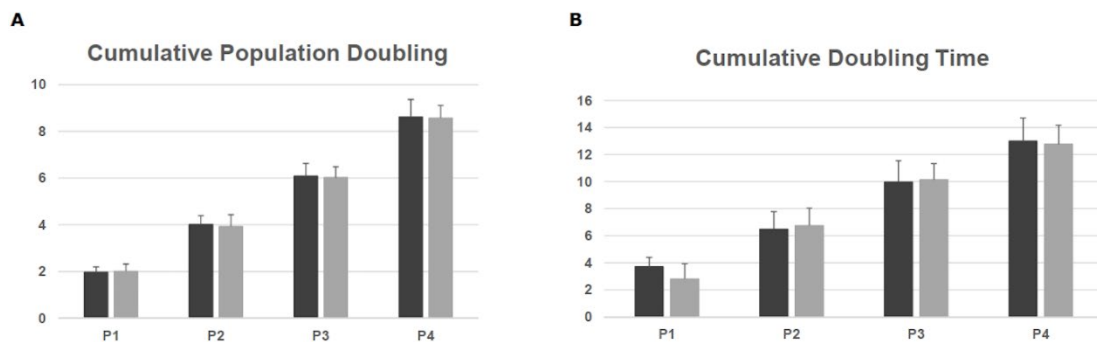


Fig. 7. Cumulative population doubling Cells (A) and Cumulative population doubling time (B) were calculated for adherent cells derived from both culture conditions, FAT (dark gray) and FAT+R (light gray) cultured until passage 4 (P4).

Immunophenotype analysis confirmed the MSC identity of the adherent fibroblastoid cells for both FAT and FAT+R. Accordingly, we detected an expression of over 95% for CD90, CD105, and CD73 and less than 5% for CD45, HLA-DR and CD31 (Table III, Fig. 8), while 7ADD resulted negative, confirming the viability of the cell population. However, CD31, a marker of endothelial cells, showed an 11.65% positivity for sample#1, suggesting contamination of the culture by endothelial cells, while the other samples reported an expression of less than 1%. Accordingly, CD146, a marker of pericytes, progenitors of endothelial cells, showed a 10% positivity for sample number 1 in both FAT and FAT+R, suggesting that sample number 1 resulted richer in angiogenic cells compared to the other samples (showing a positivity of less than 5%). This result could be related to other biological parameters (anatomic site, sample age) that should be further investigated.

Table III. Immunophenotype of adherent cells derived from FAT (A) and FAT+R (B) performed by Ctyoflex at passage 4.

ID SAMPLE	7ADD	HLADR	CD45	CD31	CD90	CD73	CD105	CD146
FAT								
#1	0,14	0,02	0,02	11,65	99,72	98,2	99,96	15,01
#2	0,25	0,8	0,17	0,02	na	na	81,12	6,16
#3	0,57	0,02	0,07	0,14	99,99	100	97,76	0,02
#4	0,31	0,02	0,05	0,03	100	100	99,43	0
#5	0,18	0,03	0,14	0	96,67	99,98	99,98	5,65
AVERAGE	0,29	0,18	0,09	2,37	99,1	99,55	95,65	5,37
SEM	0,08	0,16	0,03	2,32	0,73	0,4	3,66	2,75
FAT+R								
#1+R	0,01	0,13	0,05	0,02	99,24	99,94	98,73	10,32
#2+R	0,01	0	0,08	0,01	99,76	72,41	99,99	3,55
#3+R	0,05	0	0,04	0,01	na	99,9	99,96	2
#4+R	0,08	0,31	0,12	0,06	98,52	99,97	99,54	0,06
#5+R	0,01	0,38	0	0,2	100	100	100	0,78
AVERAGE	0,03	0,16	0,06	0,06	99,38	99,95	99,64	3,34
SEM	0,01	0,08	0,02	0,04	0,29	0,02	0,24	1,84

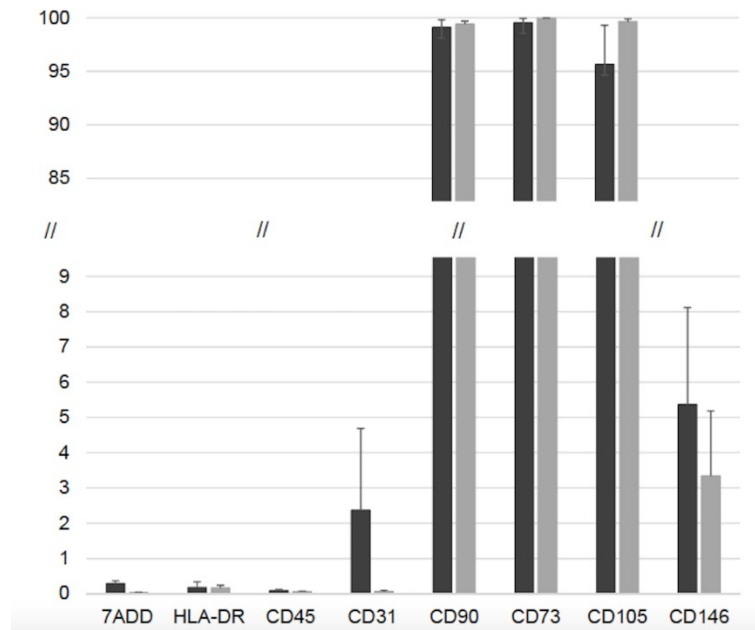


Fig. 8. Immunophenotype of adherent cells derived from both, FAT (dark gray) and FAT+R (light gray) at passage 4 (P4).

Adipogenic differentiation

Differentiation assays were performed to assess the multipotential differentiation. AD-MSCs derived from FAT were differentiated versus adipogenic lineage, as visualized by round red vacuole inside the cells positive to Oil Red Staining (Fig. 9a). When mixed with CaHA, although all samples preserved adipogenic potential, ADSCs appeared to have a reduced Oil Red signal (Fig. 9b). Additionally, sample #4, with or without CaHA, showed a reduced potential ability versus adipogenic lineage compared to other samples.

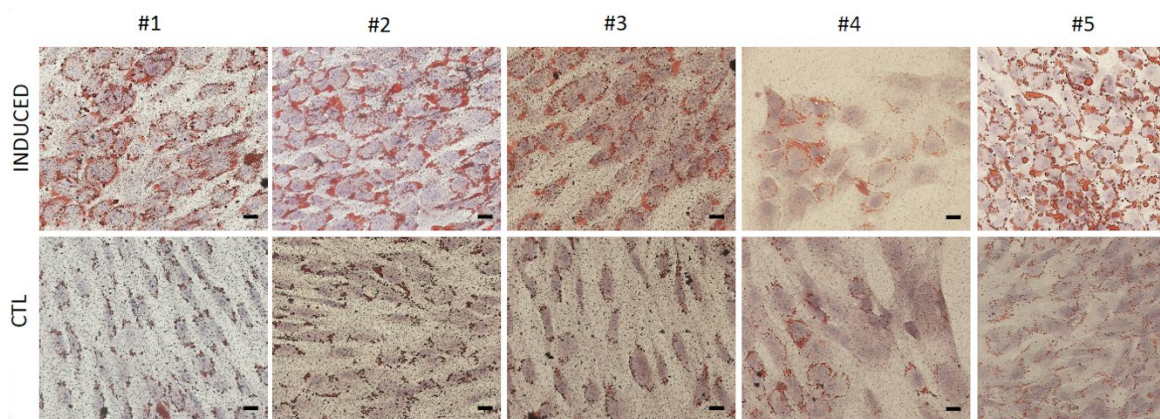


Fig. 9a. Adipogenic differentiation assay of lipoaspirate samples. Round red vacuole inside the cells resulted positive to Oil Red Staining in induced specimens (upper panel) and was not detected in the control culture (bottom panel). Images were acquired with Observer Z1 (Zeiss) at a magnification of 40x. Scale bar = 20µm.

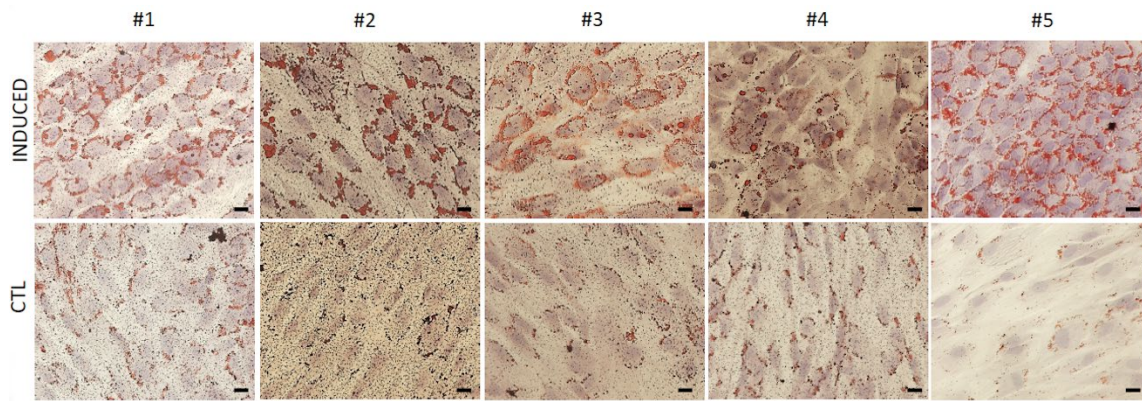


Fig. 9b. Adipogenic differentiation assay of lipoaspirate combined with Radiesse samples. Round red vacuole inside the cells resulted positive to Oil Red Staining in induced specimens (upper panel) and was not detected in the control culture (bottom panel). Images were acquired with Observer Z1 (Zeiss) at a magnification of 40x. Scale bar = 20 μ m.

Osteogenic differentiation

We also investigated bone differentiation potential. AD-MSCs bone differentiation developed in culture mineralized matrix with calcium depots detected by cytochemical staining such as Alizarin Red used in this study. Only samples #1 and #5, derived from FAT, developed Alizarin Red positive depots, suggesting a bone stimulus, while no signal was observed in the other samples (Fig. 10a). However when Radiesse® was added, we observed an increase in red signal for samples #1 and #5 and the presence of red depots also in sample #2 and sample #3, suggesting that the CaHA spheres in Radiesse® could lead to an increased bone differentiation (Fig. 10b).

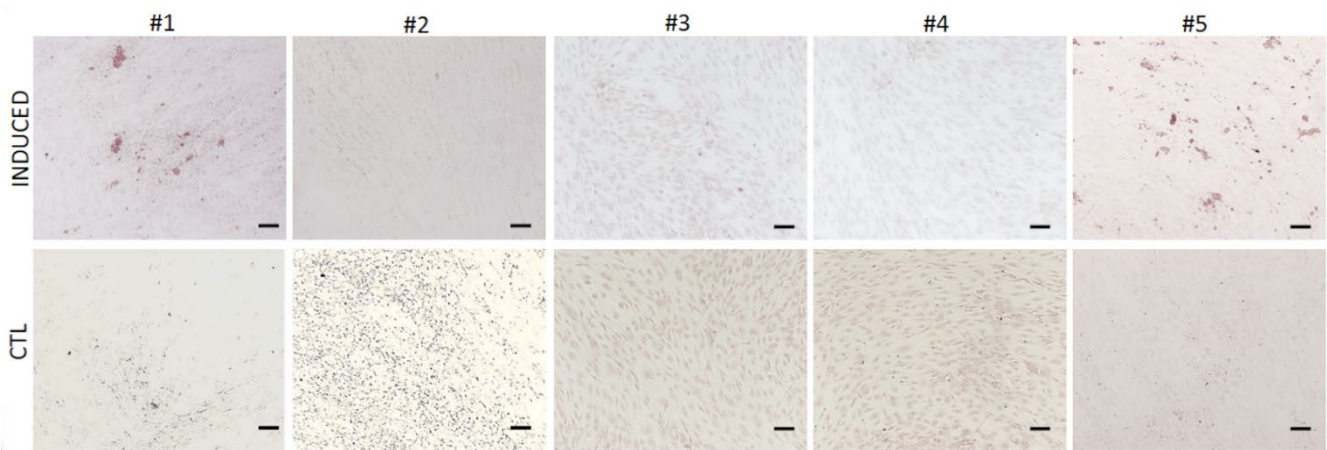


Fig. 10a. Osteogenic differentiation assay of lipoaspirates samples. Alizarin Red positive depots resulted positive to Alizarin Red Staining in donors #1 and #5 in induced specimens (upper panel) and were not detected in the control culture (bottom panel). Images were acquired with Observer Z1 (Zeiss) at a magnification of 10x. Scale bar = 100 μ m.

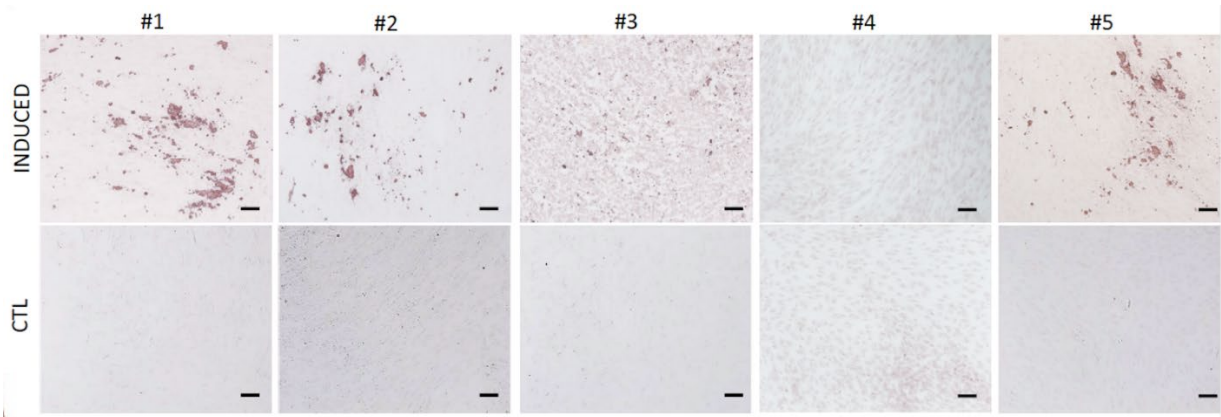


Fig. 10b. Osteogenic differentiation assay of lipoaspirates combined with Radiesse samples. Alizarin Red positive depots resulted positive to Alizarin Red Staining in donors #1, #2, #3 and #5 in induced specimens (upper panel) and were not detected in the control culture (bottom panel). Images were acquired with Observer Z1 (Zeiss) at a magnification of 10x. Scale bar = 100 μ m.

Chondrogenic differentiation

In addition, all samples from both FAT and FAT+R showed positivity for chondrogenic differentiation. Blue signal-marked cartilage matrix elements, such as glycosaminoglycan, were detected in all samples (Fig. 11a, b).

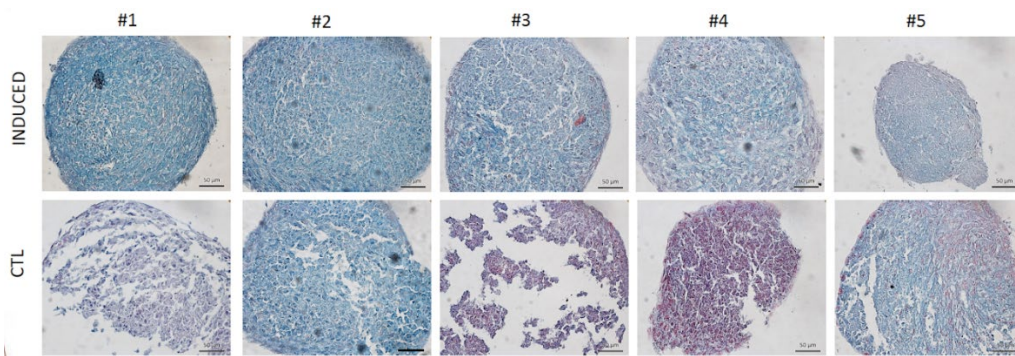


Fig. 11a. Chondrogenic differentiation assay of lipoaspirate samples. Cartilage matrix elements, such as glycosaminoglycan, resulted positive to Alcian Blu staining in induced specimens (upper panel); a red violet signal of nuclear fast red staining was observed in the control culture (bottom panel). Images were acquired with Imager M2 (Zeiss) at a magnification of 40x. Scale bar = 50 μ m.

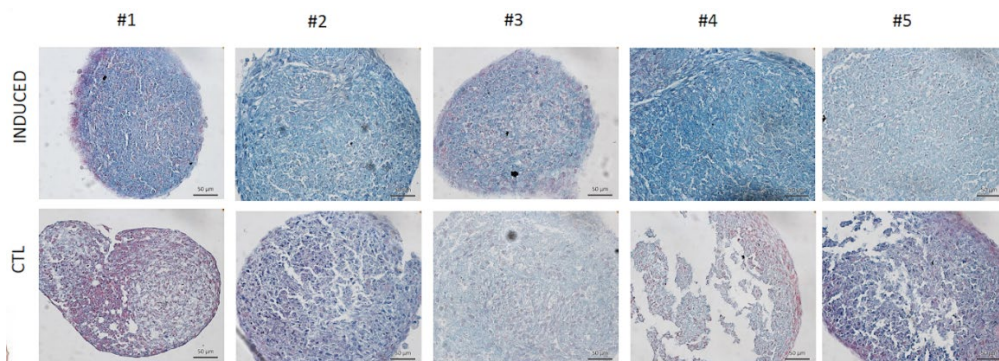


Fig. 11b. Chondrogenic differentiation assay of lipoaspirate combined with Radiesse samples. Cartilage matrix elements, such as glycosaminoglycan, resulted positive to Alcian Blu staining in induced specimens (upper panel); a red violet signal of nuclear fast red staining was observed in the control culture (bottom panel). Images were acquired with Imager M2 (Zeiss) at a magnification of 40x. Scale bar = 50 μ m.

DISCUSSION

However, the effects of the combination of adipose tissue prepared with guided SEFFI technique with HA have been recently explored, highlighting that this combination can be exploited to counteract the loss of volume and skin ageing and can be employed as a promising treatment for facial antiaging therapy (19).

An increasing interest in both adipose tissue prepared with guided SEFFI technique and CaHA has been recently observed in clinical practice, although no studies explored this combination. In the current study, we aim to investigate the biological effect of CaHA filler on AD-MSCs, highlighting its biocompatibility with adipose tissue prepared with guided SEFFI technique, together with its ability to promote the differentiation into adipose, bone and cartilage lineages in comparison with AD-MSCs derived from the lipoaspirates but cultured without CaHA filler.

AD-MSCs were successfully isolated from all samples and in both culture conditions, FAT and FAT + R, respectively, with or without CaHA. Interestingly, CaHA did not exert any cytotoxic impact on isolated AD-MSCs, as observed during the first count with trypan blue, supporting the biocompatibility of the combination of FAT and CaHA.

Interestingly, the isolated cells preserved the same proliferation rate, with PDT and doubling cells similar for both groups (FAT and FAT+R). Immunophenotype results obtained by FACS analysis revealed that the adherent cells from both the culture conditions (FAT and FAT+R) showed MSC-specific markers, as previously reported in literature where, in culture, AD-MSCs retain markers in common with other MSCs (25).

Regarding differentiation ability, the adipogenic and chondrogenic potential is well preserved in both conditions, FAT and FAT+R and an increased marking with Alizarin red is observed when adding CaHA. This last result may be associated with a stimulus toward osteogenic differentiation, as reported under certain circumstances (26, 27), or it can be related to the fact that Alizarin red is a marker for calcium-rich deposits; therefore, not surprisingly more cells are marked in the presence of a calcium and hydroxylapatite-based filler (28). Of note, increased osteogenic differentiation of AD-MSCs has also been observed when combined with a specific HA-based scaffold (19).

A potential limitation of the study is that an apparent reduction in the number of adherent cells in samples when lipoaspirates were combined with CaHA was observed under experimental conditions, despite the absence of a cytotoxic effect. We hypothesize that there is a reduction of available plastic surfaces covered by the filler and not available for cells. Therefore, to better clarify whether the filler could limit the release of ADSCs from lipoaspirate, a further study using the Transwell approach is encouraged, where lipoaspirates are seeded in the bottom of the well, and Radiesse® added in the Transwell; in any case, a similar result was observed when AD-MSCs were combined with HA (19).

Further studies are necessary to understand the real mechanisms involved in the differentiation of adipose tissue prepared with guided SEFFI technique when CaHA has been added and to address in vitro limitations of the current study.

REFERENCES

1. Guida S, Pellacani G, Bencini PL. Picosecond laser treatment of atrophic and hypertrophic surgical scars: In vivo monitoring of results by means of 3D imaging and reflectance confocal microscopy. *Skin Res Technol* 2019; 25(6):896-902. doi: 10.1111/srt.12755. Epub 2019

2. Galimberti MG, Guida S, Pellacani G, Bencini PL. Hyaluronic acid filler for skin rejuvenation: The role of diet on outcomes. A pilot study. *Dermatol Ther* 2018; 31(4):e12646. doi: 10.1111/dth.12646. Epub 2018 Jul 17. PMID: 30019474
3. Guida S, Nisticò SP, Farnetani F, et al. Resurfacing with Ablation of Periorbital Skin Technique: Indications, Efficacy, Safety, and 3D Assessment from a Pilot Study. *Photomed Laser Surg* 2018; 36(10):541-547. doi: 10.1089/pho.2018.4479
4. Zuk PA, Zhu M, Mizuno H, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 2001; 7:211-228.
5. Zuk PA, Zhu M, Ashjian P, et al. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 2002; 13:4279-4295.
6. Zuk PA (2010) The adipose-derived stem cell: looking back and looking ahead. *Mol Biol Cell* 2010; 21:1783-1787.
7. Crisan M, Yap S, Casteilla L, et al. A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 2008; 3:301-313.
8. Tallone T, Realini C, Böhmeler A, et al. Adult human adipose tissue contains several types of multipotent cells. *J Cardiovasc Transl Res* 2011; 4:200-210.
9. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J Cell Physiol* 2007; 213:341-347.
10. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. *J Cell Biochem* 2006; 98:1076-1084.
11. Blaber SP, Webster RA, Hill CJ, et al. Analysis of in vitro secretion profiles from adipose-derived cell populations. *J Transl Med* 2012; 10:172.
12. Coleman SR Structural fat grafts: the ideal filler? *Clin Plast Surg* 2001; 28:111-119.
13. Li K, Li F, Li J, et al. Increased survival of human free fat grafts with varying densities of human adipose-derived stem cells and platelet-rich plasma. *J Tissue Eng Regen Med* 2017; 11:209-219.
14. Sultan SM, Barr JS, Butala P, et al. Fat grafting accelerates revascularization and decreases fibrosis following thermal injury. *J Plast Reconstr Aesthet Surg* 2012; 65:219-227.
15. Del Papa N, Di Luca G, Sambataro D, et al. Regional implantation of autologous adipose tissue-derived cells induces a prompt healing of long-lasting indolent digital ulcers in patients with systemic sclerosis. *Cell Transplant* 2015; 24:2297-2305.
16. Charles de Sá L, Gontijo de Amorim NF, Takiya CM, et al. Antiaging treatment of the facial skin by fat graft and adipose-derived stem cells. *Plast Reconstr Surg* 2015; 135:999-1009.
17. Rigotti G, Charles de Sá L, Gontijo de Amorim NF, et al. Expanded Stem Cells, Stromal-Vascular Fraction, and Platelet-Rich Plasma Enriched Fat: Comparing Results of Different Facial Rejuvenation Approaches in a Clinical Trial. *Aesthet Surg J* 2016; 36:261-270.
18. Crowley JS, Kream E, Fabi S, Cohen SR. Facial Rejuvenation With Fat Grafting and Fillers. *Aesthet Surg J* 2021; 41(Suppl 1):S31-S38. doi: 10.1093/asj/sjab014
19. Gennai A, Bovani B, Colli M, Melfa F, Piccolo D, Russo R, Clementoni MT, Zia S, Roda B, Zattoni A. Evaluation of the Number, Biophysical and Multipotent Characteristics of Adipose Derived Stem Cells Harvested by SEFFI Procedure and Interaction with Different Type of Hyaluronic Acids *Int J Regenr Med* 2021; 4(2):2-10. doi: 10.31487/j.RGM.2021.02.02
20. Rovatti PP, Pellacani G, Guida S. Hyperdiluted Calcium Hydroxylapatite 1: 2 for Mid and Lower Facial Skin Rejuvenation: Efficacy and Safety. *Dermatol Surg* 2020; 46(12):e112-e117. doi: 10.1097/DSS.0000000000002375
21. Casabona G. Combined Calcium Hydroxylapatite Plus Microfocused Ultrasound for Treating Skin Laxity of the Chest and Buttocks. *J Drugs Dermatol* 2022; 21(1):27-30. doi: 10.36849/JDD.2022.6368
22. Bartoletti E, Melfa F, Renzi M, Rovatti P, Systematic review of the literature on the proprieties, quality and reliability of Calcium hydroxyapatite: results of an Italian expert meeting. *Aesthetic Medicine* 2022; 8(1).
23. Grambow F, Rutkowski R, Podmelle F, Schmoeckel K, Siegerist F, Domanski G, Schuster MW, Domanska G. The Impact of Lidocaine on Adipose-Derived Stem Cells in Human Adipose Tissue Harvested by Liposuction and Used for Lipotransfer. *Int J Mol Sci* 2020; 21(8):2869.

24. <https://radiesse.com/app/uploads/EM00543-04.pdf>.
25. Bourin P, Bunnell BA, Casteilla L, et al. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). *Cytotherapy* 2013; 15(6):641-8. doi: 10.1016/j.jcyt.2013.02.006
26. Amann E, Amirall A, Franco AR, et al. A Graded, Porous Composite of Natural Biopolymers and Octacalcium Phosphate Guides Osteochondral Differentiation of Stem Cells. *Adv Healthc Mater* 2021; 10(6):e2001692. doi: 10.1002/adhm.202001692
27. Ebrahimi Z, Irani S, Ardeshtyrlajimi A, Seyedjafari E. Enhanced osteogenic differentiation of stem cells by 3D printed PCL scaffolds coated with collagen and hydroxyapatite. *Sci Rep* 2022; 12(1):12359. doi: 10.1038/s41598-022-15602-y
28. Gregory CA, Gunn WG, Peister A, Prockop DJ. An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. *Anal Biochem* 2004; 329(1):77-84. doi: 10.1016/j.ab.2004.02.002