

Regenerative potential of the Bichat fat pad determined by the quantification of multilineage differentiating stress enduring cells

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allow to classify them as multilineage differentiating stress enduring cells. This data set allows to collect novel information about regenerative potential of Bichat fat pad that could explain the success of its employment in reconstructive and regenerative medicine.

Introduction

As described by Sbarbati *et al.*,¹ adipose tissue is not characterized by the same morphological aspects in all depots and, until now, it was possible to classify the adipose tissue primarily into three different types (namely, structural, fibrous and deposit), based on the morphological and ultrastructural features.¹ Basing on this classification, it was possible to describe others fat depots such as the trochanteric² and the facial fat pads.³

More recently, in addition to the description of the collagen compartment, the adipocytes organization, the blood vessels network and the presence of fibroblasts, the attention of researchers has been focusing also on the regenerative potential of the adipose tissue.⁴⁻⁶ The subcutaneous adipose tissue is one of the sources of mesenchymal stem cells (MSCs), characterized by high regenerative potential, as described in the literature.⁷ Isolation of stem cell from the adipose tissue is not mandatory to obtain tissue regeneration. In fact, the lipofilling technique allows to inject purified fat in the area of interest: injected fat provides a large amount of stem niches, in which stem cells are included, capable to cause a *restitutio ad integrum* of the damaged tissues.⁸⁻¹¹ Isolated and purified adipose derived MSCs, were studied and characterized by numerous authors that identified in these cells the regenerative potential of the adipose tissue. So, today, surgeons employ for regenerative surgery both purified adipose tissue and adipose derived MSCs.^{9,10} It is possible to isolate adipose MSCs from fat depots harvested from all body districts, but the best characterized adipose MSCs were purified and isolated from the abdominal fat depot which represents the largest fat pad in humans.^{11,12} Very recently, surgeons and researchers investigated also the regenerative potential, defined as the percentage of stem niches or MSCs, also of the trochanter, knee, and facial fat pads, with a growing attention for Bichat fat pad.^{13,14} In addition of a different architecture for each fat pad, the concept has been emerging of a different content of regenerative units, among the different fat depots.¹³

The Bichat, or buccal, fat pad is a fat structure located in the masticatory space, that divides the chewing muscles from each

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other, from the zygomatic arch and from the ramus of the mandible; in this manner the intermuscular motion is enhanced, in both mastication and speech. Anatomically, the Bichat fat pad consists of a central encapsulated depot with four extensions: buccal, pterygoid, superficial and deep temporal. The fat pad proceeds along the anterior wall of the masseter muscle and the periosteum of the posterior wall of the maxilla. The buccal extension lies superficially within the cheek (responsible for cheek fullness); the pterygoid extension occupies the deep medial aspect of the mandibular ramus and the lateral surface of the medial and lateral pterygoid muscles. The size of Bichat fat pad is structurally the same, among different people, regardless of the overall body weight and fat distribution. Its volume is about 10 mL with a size of about 60 mm long, 50 mm wide, 6 mm thick. Another characteristic is the blood vessels distribution represented by vessels from the maxillary (buccal and deep temporal), superficial temporal artery (transverse facial branch) and facial arteries (small branches).¹⁴⁻¹⁷

In the scientific literature, a paucity of ultrastructural and morphological knowledge about Bichat fat pad exists, even if this depot is currently used, in particular in maxillo-facial and oral surgery, for the correction of peri-implant defects and in lip or cheek volumes restoration.¹⁸⁻²¹ Important features of the Bichat fat pad have never been deeply analyzed, and the presence and the characteristics of its regenerative units have not been described yet. Wakao *et al.*^{22,23} recently isolated a peculiar sub-population of MSCs, defined as multilineage differentiating stress enduring cells (MUSE cells), and demonstrated their very promising regenerative potential. In the present investigation, we provide a detailed ultra-

Abstract

Published studies regarding Bichat fat pad focused, quite exclusively, on the implant of this adipose depot for different facial portions reconstruction. The regenerative components of Bichat fat pad were poorly investigated. The present study aimed to describe by an ultrastructural approach the Bichat fat pad, providing novel data at the ultrastructural and cellular level. This data sets improve the knowledge about the usefulness of the Bichat fat pad in regenerative and reconstructive surgery.

Bichat fat pads were harvested from eight patients subjected to maxillofacial, dental and aesthetic surgeries. Biopsies were used for the isolation of mesenchymal cell compartment and for ultrastructural analysis. Respectively, Bichat fat pads were either digested and placed in culture for the characterization of mesenchymal stem cells (MSCs) or were fixed in 2% glutaraldehyde and processed for transmission or scanning electron microscopy. Collected data showed very interesting features regarding the cellular composition of the Bichat fat pad and, in particular, experiments aimed to characterized the MSCs showed the presence of a sub-population of MSCs characterized by the expression of specific markers that

structural description of the Bichat fat pad associated with a characterization of its multilineage MSCs, in order to support the use of this adipose depot in reconstructive surgery.

Materials and Methods

Adipose tissue harvesting

Bichat fat pads were harvested from eight 23/25-year-old patients (5 men and 3 women), during bi-maxillary surgery, from the circumvestibular incision during the Le Fort I osteotomy.

All patients were informed about the surgery protocol and about the harvesting of Bichat fat pad for scientific purposes, and they had signed an informed consent. Samples were split into three portions: two portions were employed for ultrastructural evaluation performed by transmission and scanning electron microscopy (TEM and SEM, respectively), the third one for the MSCs isolation and characterization.

Moreover, abdominal adipose tissue was harvested by liposuction performed on women subjected to breast reconstruction. Also in this case all the enrolled patients had signed an informed consent for the harvesting of adipose tissue both for breast reconstruction and for research aims. Liposuction was based on the infiltration with a cold saline solution with the addition of 1:400,000 of epinephrine and 20 mL of lidocaine 0.5% every 500 mL. Adipose tissue was removed using Coleman's instrumentation. Lipoaspirate purification was obtained by centrifugation of 3 min at 3000 rpm to allow the separation of fat from its water content and from the oil produced by the destruction of damaged adipocytes.

TEM

Samples of adipose tissue were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer for 4 h, post fixed in 1% osmium tetroxide in the same buffer for 2 h, dehydrated in gradient acetone, and finally embedded in Epon-Araldite mixture (Electron Microscopy Sciences, Fort Washington, PA, USA). The semi-thin sections were examined by light microscopy and stained with toluidine blue in order to select the region of interest for the ultra-thin sections. Seventy-nm-thick ultra-thin sections were cut with an Ultracut E ultramicrotome (Reichert, Wien, Austria), placed on Cu/Rh grids, stained with lead citrate and observed using a FEI Morgagni 268D electron microscope (FEI, Eindhoven, The Netherlands).

SEM

Samples of adipose tissue were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer for 4 h, post fixed in 1% osmium tetroxide in the same buffer for 1 h, dehydrated in gradient ethanol, critical point dried (CPD 030, Balzers, Vaduz, Liechtenstein), fixed to stubs with colloidal silver, sputtered with gold by a MED 010 coater (Balzers), and examined with a FEI XL30 scanning electron microscope (FEI).

Isolation of adipose MSCs

Adipose tissue specimens were digested with type I collagenase in 2% bovine serum albumin (BSA) to obtain the stromal vascular fraction (SVF) ready for culture. For the digestion procedure, 1 mL of the adipose tissue was incubated at 37°C with 5 mL of collagenase type I, 2% v/v, for 40–45 min. After digestion, the sample was centrifuged at 100 g for 5 min, and the supernatant was discarded. The SVF pellet in the 15 mL vial was re-suspended with NH_4Cl to lyse blood cells, filtered and then centrifuged at 100 g for 10 min. The resulted SVF was re-suspended in growth medium, DMEM (Dulbecco's Modified Eagle Medium) containing 10% Fetal Bovine Serum (FBS) and 1% of a mix of penicillin and streptomycin (Life Technologies, Carlsbad, CA, USA), in a 25 cm² culture flask (Falcon™, BD Biosciences, Franklin Lakes, NJ, USA) for 3 days. Culture vessels were kept in the incubator (Sanyo, Osaka, Japan) at 37°C, in a 5% CO₂ atmosphere and 100% humidity. After 3 days, the cells were detached by a 10 min exposure to trypsin/EDTA (Life Technologies) followed by inactivation of the trypsin through addition of DMEM containing 15% FBS (Life Technologies). After centrifugation for 10 min at 150 g, the cell pellets were re-suspended in the same culture medium. The number of vital cells was determined using a Bürker chamber under a conventional light microscope, excluding dead cells by the Trypan blue exclusion test. The cells were cultured till passage 4, corresponding to 14 days of culture, and the expansion rate was determined by calculating the population doubling level over the different passages: $\log(N/\text{No}) \times 3.33$ (N = number of cells in the next passage; No = number of cells in the previous passage).

Characterization of MSCs

As positive MSCs membrane markers, CD73-APC and CD105-APC (eBiosciences, San Diego, CA, USA), at the dilution respectively of 1:200 and 1:1000, were used, whereas CD45-FITC and CD34-FITC (BD biosciences, Italy) were taken as negative MSC markers, and were used at the dilution respectively of 1:200 and 1:500. Isotype control monoclonal antibodies were

used to exclude the non-specific binding (mouse IgG1-APC, IgG1-PE and IgG2a-FITC; BD biosciences, used at the dilution respectively of 1:22, 1:1000, 1:200 and 1:500). Briefly, cells were stained for 10 min at 4°C with the different antibodies and then centrifuged for 6 min at 100 g and re-suspended in sterile PBS. The detection of fluorescence was performed using a BD FACS Canto II (BD Biosciences).

Multilineage differentiating stress enduring (MUSE) cells characterization by flow cytometry

MSCs isolated from Bichat fat pads of different patients, were analyzed by flow cytometry for the expression of two markers that characterized this sub-population of stem cells defined as pluripotent. The antibodies employed to perform analysis were an anti-SEEA3-AF488 (eBiosciences; dilution used 1:1000) and an anti-CD105-APC (eBiosciences; dilution used 1:1000). Cells were incubated at 4°C for 30 min in PBS solution with each antibody and then washed by centrifugation and resuspension in fresh PBS. Isotype controls (Rat IgM Isotype Control Alexa Fluor 488 and Mouse Ig1K Isotype Control APC) were used to exclude the not specific binding and were diluted 1:1000.

Results

Ultrastructural evaluation of Bichat fat pad compared with the abdominal adipose tissue

The morphological evaluation showed that the Bichat fat pad and the abdominal adipose tissue were not substantially different. At SEM, both in the Bichat and the abdominal fat pads, unilocular mature adipocytes, of 60 µm, were organized in large clusters (Figure 1) well defined by thick collagen septa that divided the Bichat fat pad in lobules (Figure 2A). In the abdominal adipose tissue, the adipocyte clusters were more well-defined and homogeneously organized in the whole biopsy (Figure 1G). Bichat fat pad appeared entirely surrounded by a capsule characterized by thick collagen fibers (Figure 1 B,C,E,F), but in some SEM images also mature elements were covered by very thin basket-like collagen structure, as much as it occurs also in the abdominal adipose tissue (Figure 1 E,F); this is especially evident at higher magnification both in Bichat and in abdominal fat pads (Figure 1 C,F,I). At SEM, the abdominal adipose tissue is characterized by a denser collagen scaffold surrounding the adipocytes (Figure 1 G,H).

TEM images help to clarify these pecu-

liarities of the two different adipose tissues. In the Bichat fat pad, a scarcely electron-dense material was observed among the adipocytes, with axially-oriented spacer collagen fibers (Figure 3 A,B). Moreover, blood arterioles were detectable (Figure 3C) and associated to the presence of fibroblasts (Figure 3C) and by elements characterized by elevated nucleus/cytoplasm ratio, by the paucity of organelles in the cytoplasm and with a basal membrane in common with blood vessels (Figure 3D). Numerous lipid droplets were found inside mature adipocytes (Figure 3 A). In Bichat fat pad it is possible to detect stem cells and fibroblasts close to arterioles (Figure 3 B,C,D). It is possible to recognize stem cells basing on the observation of nuclei and by the presence of rare mitochondria and organelles. In the abdominal adipose tissue, TEM micrographs showed unilocular mature adipocytes more closed to each other, in comparison to the Bichat adipocytes. Some blood capillaries and macrophages were detectable (Figure 3 F,G), but the stem cells

were not easily visible in the extracellular space (Figure 3 E-H).

In the adipocytes from the Bichat fat pad, numerous long and thin cytoplasmic protrusions were observed (Figure 4A), whereas the abdominal adipocytes were more spherical in shape (Figure 4B).

MSCs isolation from the Bichat fat pad and the abdominal adipose tissue

Irrespective of the donor's gender, immediately after isolation (0 h), no substantial differences were observed between the abdominal and the Bichat fat pads (Figure 5 A,B), even if in some cases the number of isolated cells is higher from the abdominal depots (Figure 5B). During the following passages of culture (3, 7, 14 days after isolation), Bichat fat pad was characterized by evident homogeneity in the mitotic rate (Figure 5A), that results similar in all examined samples, while the samples of abdominal fat were characterized different doubling rate (Figure 5B). The One-Way ANOVA test was employed to ascer-

tain statistically significant differences between the population doubling of Bichat fat pads and abdominal adipose tissue ($P=0.00001$).

At SEM, the MSCs isolated from the Bichat depots had the elongated shape typical of stem cells (Figure 5C), with large cytoplasm and long protrusions. At TEM, it was possible to detect the presence of numerous lipid droplets in the cytoplasm. Mitochondria and other organelles were relatively scarce. Also this aspect is typical of non-differentiated cells (Figure 5D).

Identification of MUSE cells in the Bichat fat pad and the abdominal adipose tissue

Flow cytometry analysis confirmed that the cells isolated from Bichat and abdominal adipose tissue specimens, examined at each time point, were classifiable as MSCs, due to the expression of CD105 ($99\% \pm 1$ for abdominal and $97\% \pm 2$ for Bichat), CD73 (100% for abdominal and $99\% \pm 1$ for Bichat). These cells were

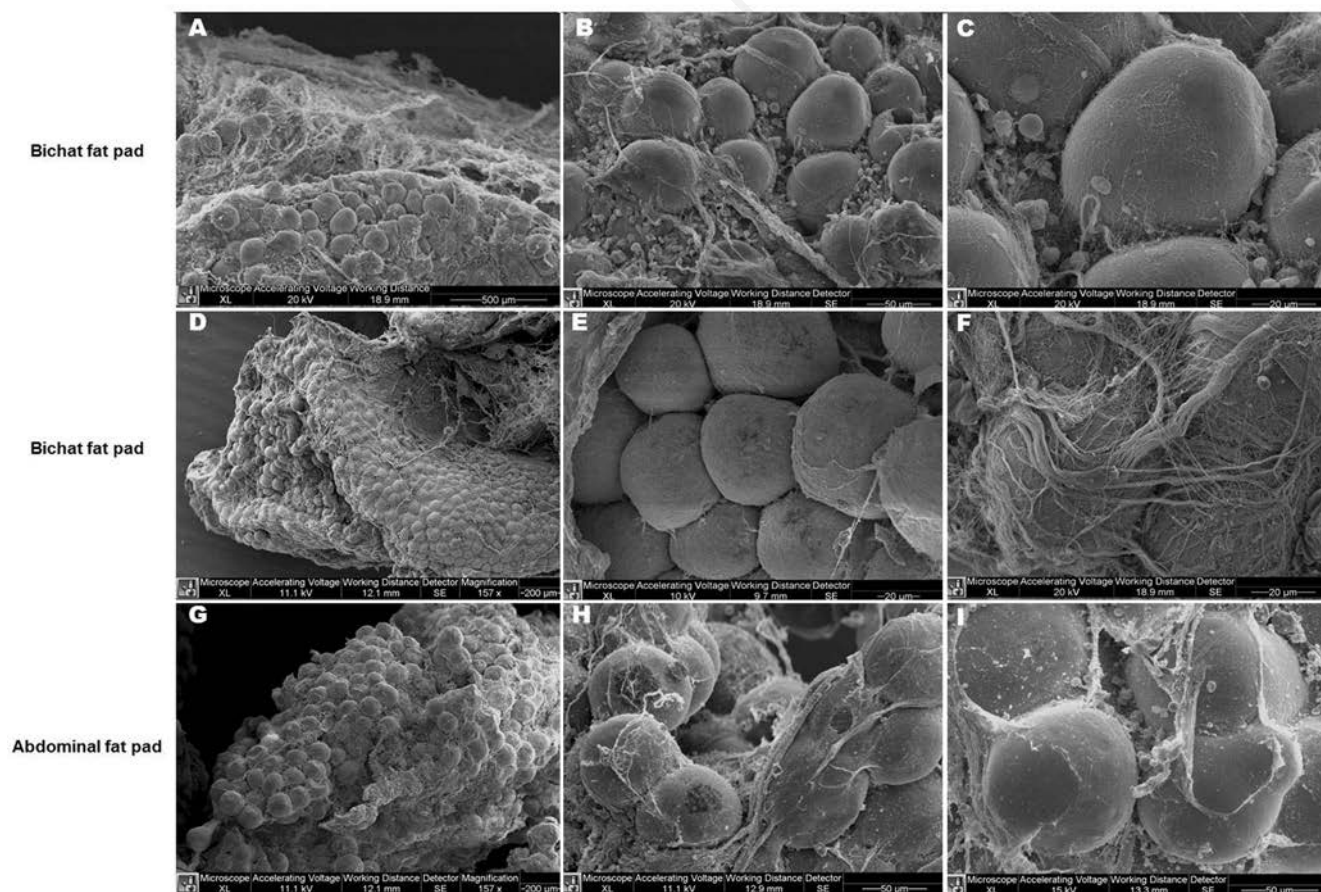


Figure 1. SEM micrographs of abdominal and Bichat fat pad. Panels A-F show the organization of Bichat fat pad while panels G-I show abdominal fat pad structure. Tissues appeared not dramatically different even if Bichat fat pad is characterized by thinner collagen fibers.

negative for CD34 and CD45 (*data not shown*). Labeling of these cells with specific antibodies allowed to identify the percentage of MUSE cells in the population of MSCs, due to the simultaneous expression

of SEEA3 and CD105 markers. The flow cytometric analysis showed three groups of patients with low, medium or high percentage of MUSE cells among the isolated MSCs from the Bichat fat pad (Figure 6A).

The patients were classified based on the median fluorescence intensity (SEEA3-AF488 /isotype ratio) and on the percentage of cells expressing both CD105 and SEEA3 (Figure 6B).

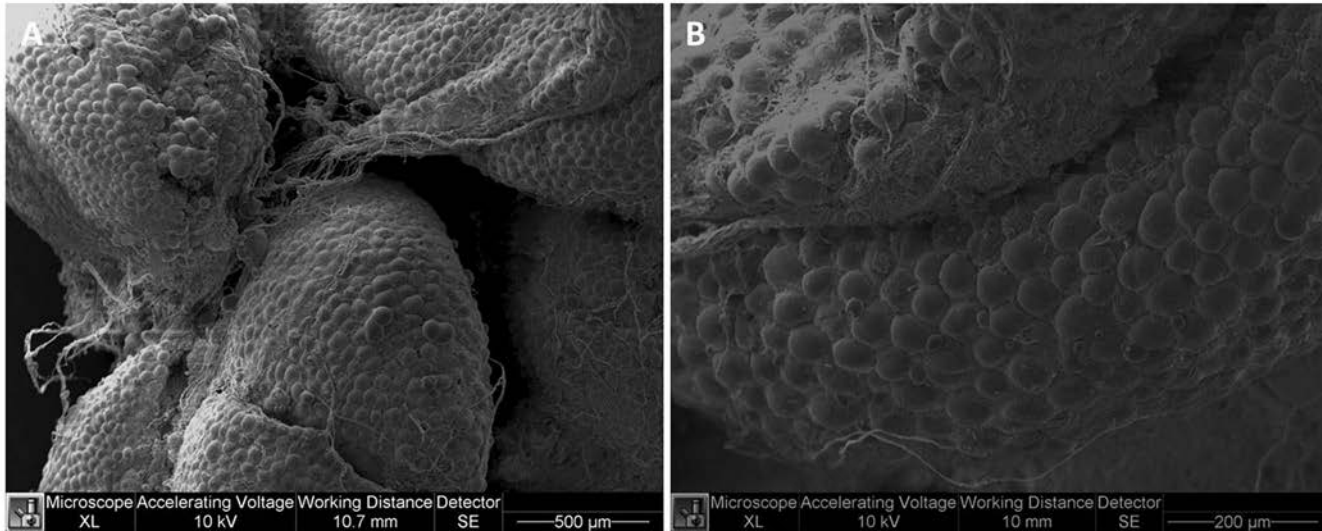


Figure 2. SEM micrographs of fat pad. Panels show the organization of Bichat fat pad in lobules containing mature adipocytes and surrounding by very thin collagen envelop. On the contrary, lobules are separated from each other by thick collagen septa.

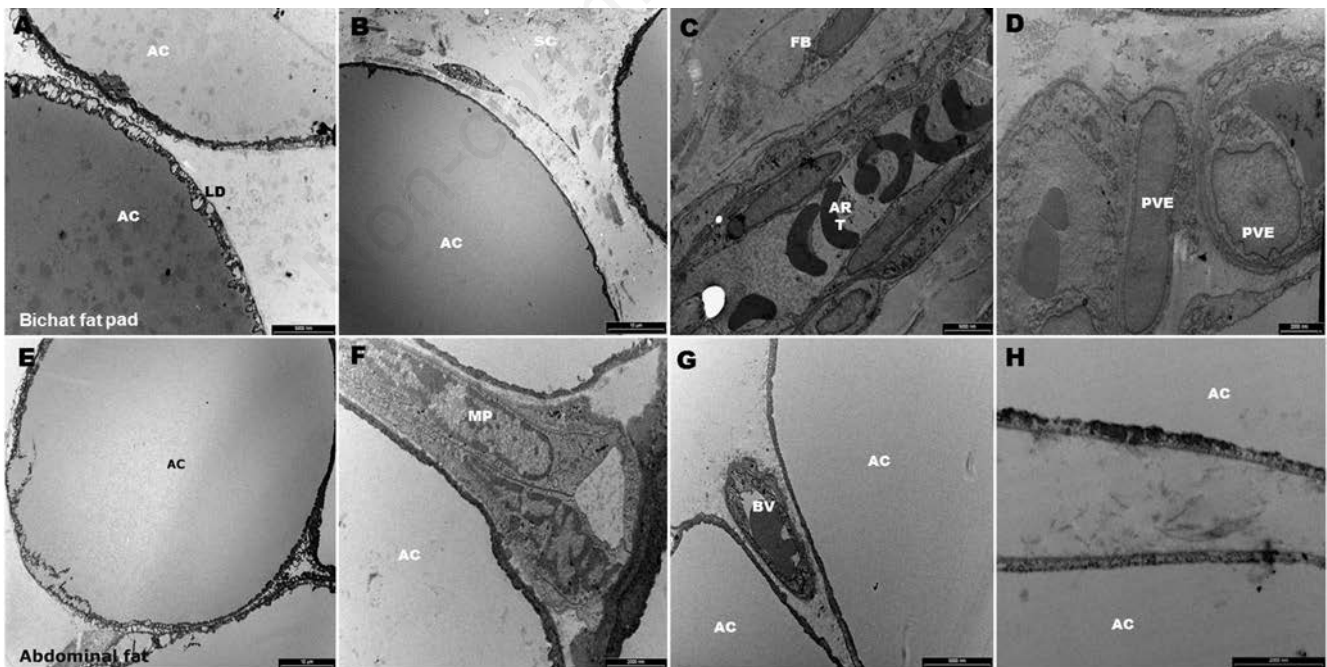


Figure 3. TEM micrographs of Bichat and abdominal fat pads. The ultrastructural analysis of two different fat pads evidences the presence of unilocular adipocytes (AC) surrounded by scarcely electron-dense material and in Bichat fat pad by spacer collagen (SC). In Bichat fat pad adipocytes are characterized by a membrane rich in lipid droplets (LD) and the presence of arterioles (ART) is relevant. Moreover fibroblasts (FB) and perivascular elements (PVE) are detectable in Bichat fat pad. Abdominal adipose tissue is characterized by the presence of mature unilocular adipocytes (AC) and macrophages (MP). Small blood vessels (BV) are visible.

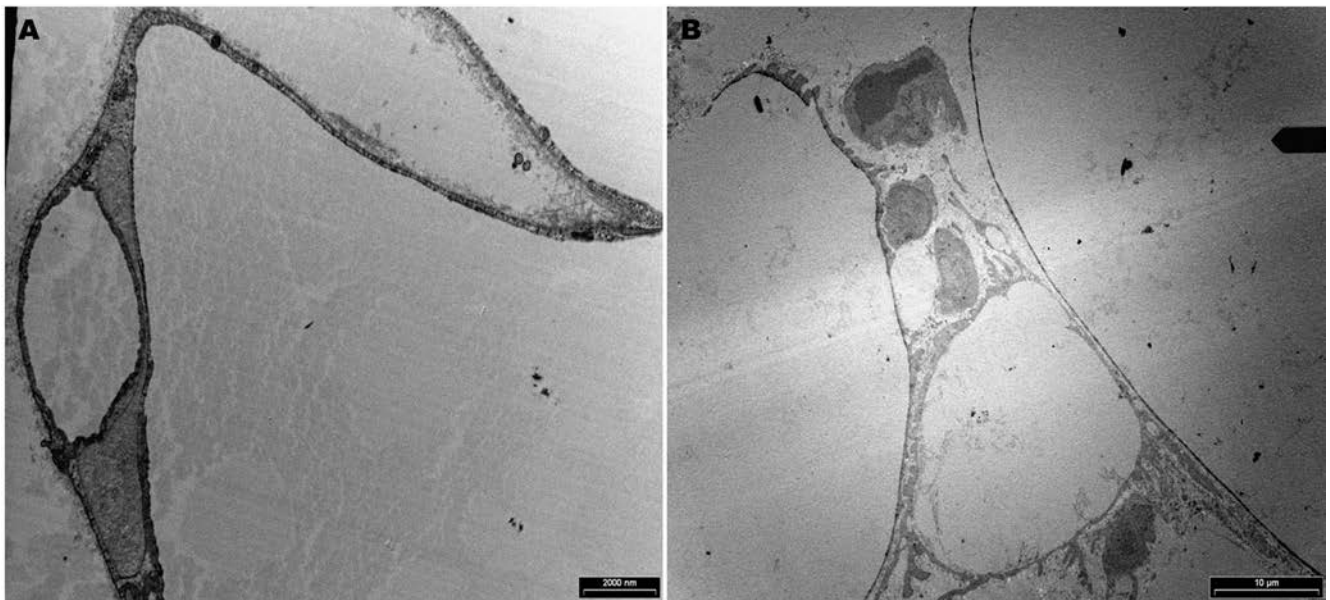


Figure 4. TEM micrographs of Bichat and abdominal adipocytes. Bichat adipocytes are characterized by long cytoplasm protrusions presenting numerous lipid droplets (A), while abdominal adipocytes are spherical and their cytoplasm is occupied by unique lipid

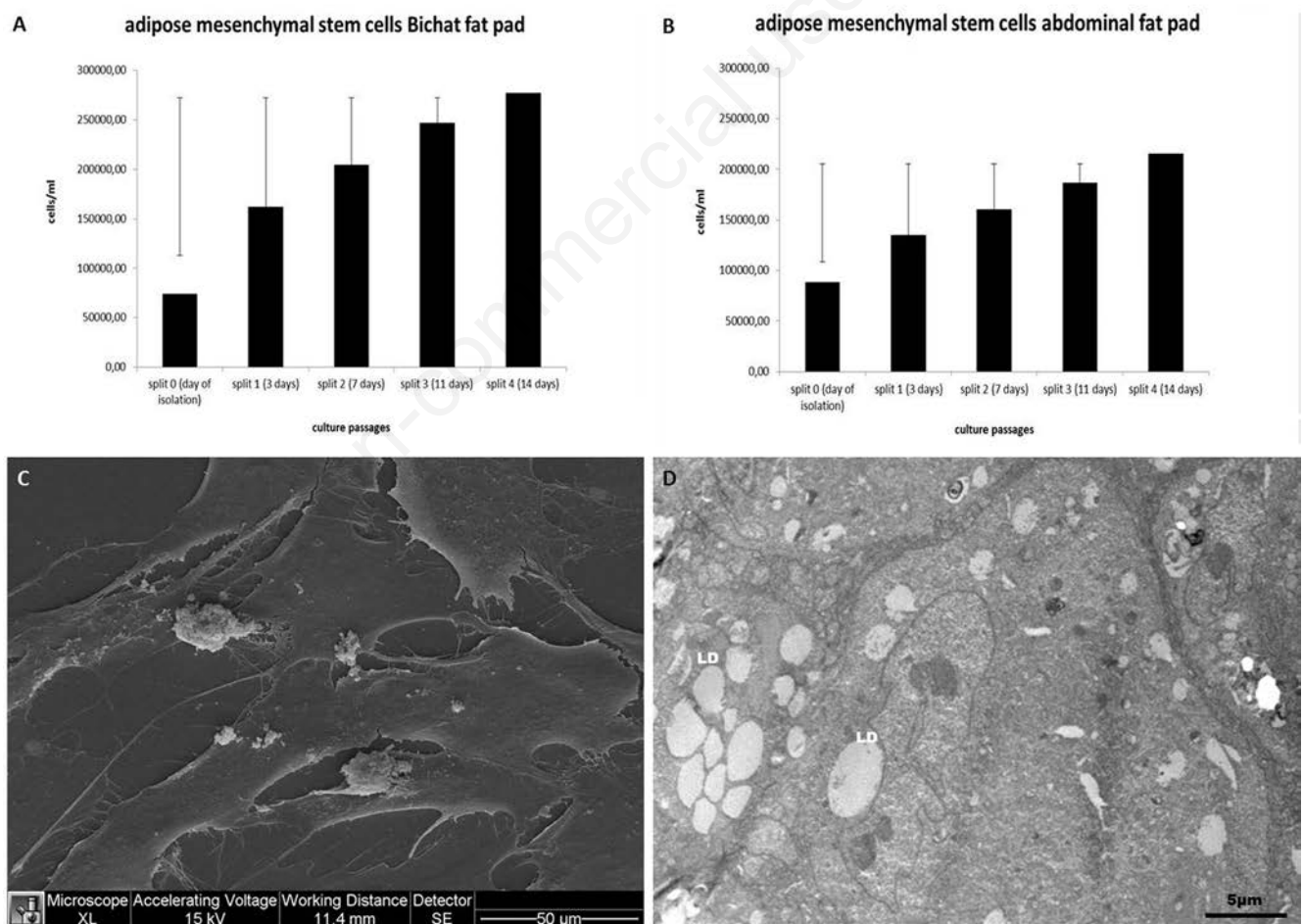


Figure 5. Mesenchymal stem cell isolation from Bichat and abdominal fat pad. A,B) number of isolated cells/mL at each culture passage. Cell population doubling was monitored for 14 days both for Bichat and abdominal fat pad. C) SEM micrograph of MSCs isolated from Bichat fat pad. D) TEM of the pellet of MSCs isolate from Bichat fat pad. Using a cell pellet the mesenchymal stem cells appeared to be closer to each other. However, they are characterized by cytoplasm poor in organelles and by the presence of small lipid droplets.

Discussion

In the literature, several papers described the histology of Bichat fat pad and its employment in regenerative plastic surgery. The first to use Bichat fat pad for fat graft was Egyedy in 1977.²⁰ During the period 1977-2017, numerous manuscripts described the use of Bichat fat pad in regenerative medicine. Bichat fat pad was proposed for fat graft and described for numerous application such as treatment of congenital oro-antral and/or oro-nasal diseases, congenital cleft palate repair, oral submucosa fibrosis, intraoral malignant defect. Recent studies showed that human adipose stem cells isolated from the Bichat fat pad possess all the suitable characteristics for bone tissue engineering, both *in vitro* and *in vivo*.^{15,16,21,24} These last studies were focused on animal MSCs implanted in animal model of diseases, while in the present investigation we performed an ultrastructural and cytometric analysis on the human Bichat fat pad, and found differences with the abdominal fat pad which is largely used for reconstructive surgery.

Harvesting of the abdominal fat pad requires liposuction, but in a patient with facial deformity or with mandibular or oral defects, that require a surgical procedure to correct them, it could definitely be preferable to harvest the adipose tissue from more easily accessible areas. After harvesting,

Bichat fat pad could be processed, easily, to obtain an emulsion injectable using a 27G needle, as referred by the most of surgeons.¹⁷ In abdominal fat pad, the collagen basket like the structure that surrounds adipocyte appeared denser than that observed around Bichat adipocytes and its emulsification requires the employment of specific instrumentation, in order to obtain an injectable preparation similar to that obtained manually when Bichat fat pad was processed. Moreover, this deficiency of thick collagen fibers of Bichat promotes its classification as visceral fat. These aspects evidence that Bichat fat pad may represent an important reservoir of adipose tissue to be exploited for maxillofacial or oral reconstructive surgery. This is especially true if we consider also the presence of perivascular elements (PVE) (Figure 3), characterized by the same aspect of cells previously described by Tran *et al.* in 2012.¹⁸ The PVE seems to be more frequently present in Bichat fat pad than in abdominal adipose tissue, and this aspect suggests the high presence of regenerative units.¹⁸ After the digestion of Bichat fat pad and abdominal adipose tissue, performed in order to determine the regenerative capability of these two adipose depots, isolated cells were observed at TEM. Their cytoplasm was characterized by some lipid droplets and scarce mitochondria and other organelles, suggesting that they were not-differentiated

elements. When isolated from tissue and better characterized by immune phenotyping, these cellular elements had shown pluripotent stem cell properties, because of the simultaneous expression of CD105 and SSEA3 markers, as reported by Wakao *et al.* in 2011-2012^{22,23} and by Kitada *et al.* in 2012.²⁵ These pluripotent mesenchymal stem cells, isolated from Bichat fat pad, were characterized also by higher mitosis rate than the pluripotent cells isolated by abdominal adipose tissue. Of course, there are different protocols for adipose tissue harvesting from each fat depot, but there are no differences, in term of number of isolated regenerative units, among the numerous adipose tissue harvesting methodologies. In fact, each device employed for lipoaspiration is equipped with filtrating systems that do not affect the regenerative capabilities during tissue processing. The percentage of pluripotent stem cells, that could be considered as a biomarker for the regenerative capability of adipose tissue, determined with immune sorting technique, showed that Bichat fat pads of young patients frequently were characterized by high population of pluripotent elements. Thus, for this category, Bichat fat pad could represent the alternative to the liposuction and to injection of abdominal adipose tissue, in the case of facial reconstructive surgeries.

Despite the relatively small number of subjects examined, our preliminary study

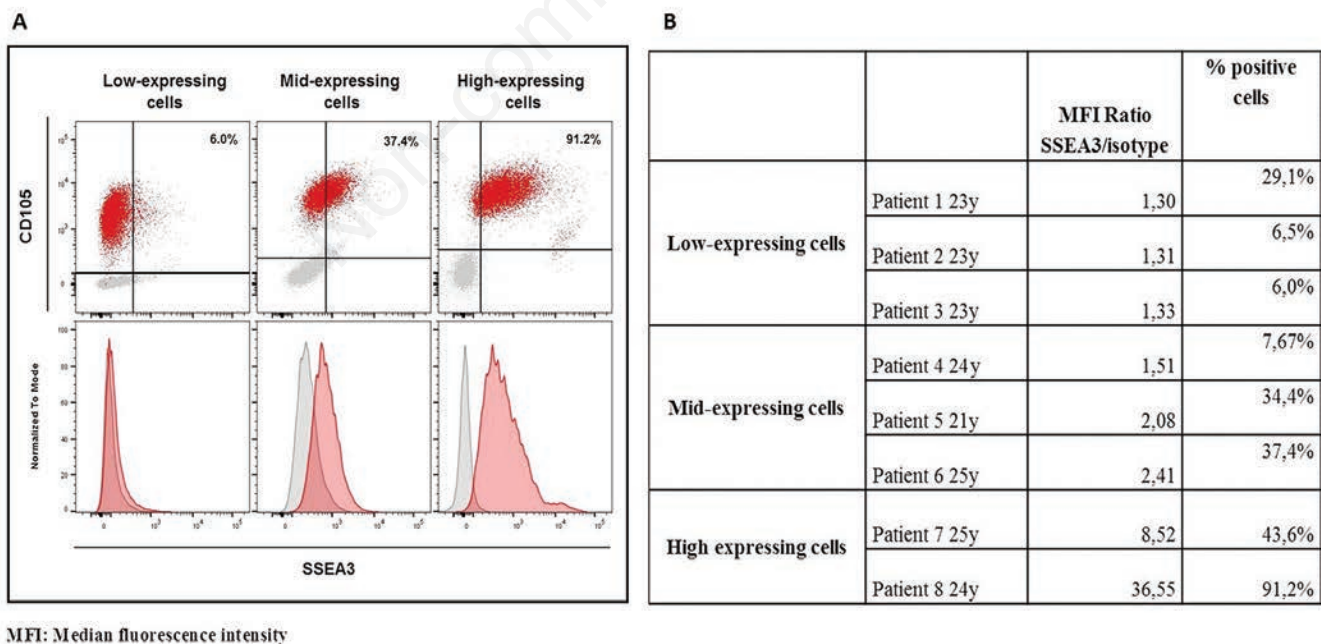


Figure 6. Flow cytometry evaluation of MUSE cells into isolated mesenchymal stem cells. A) Plots and histograms of MSCs incubated with anti-SSEA3 and anti-CD105 showed the three different median fluorescence intensity (MFI) values of three representative patients classified for low, medium and high level of MUSE cells presence. B) The table shows the MFI and the percentage of SSEA3 and CD105 expressing cells in each patient.

indicates the high regenerative potential of this fat pad that combined with the harvesting easiness in the case of facial reconstructive surgery, makes, this fat pad an especially appropriate source of regenerative units to obtain optimal results, especially in young patients.

So far, the regenerative potential of Bichat fat pad was evaluated only after surgery and described in few published papers.^{14-16,18-24,26,27} The results in the present paper provide a novel description of the regenerative potential of Bichat fat pad, based on the presence of numerous cellular elements characterized by pluripotency, scarce in the abdominal adipose tissue. Studying the regenerative potential of Bichat fat pad may represent a milestone in reconstructive and aesthetic facial surgery, in particular for young patients suffering from traumatic facial events. The use of the Bichat fat pad, opportunely processed in order to inject with a small needle the fat in the site of damage, will allow to implant a tissue, especially rich in regenerative units. In the next future, this practice could be preferable to the injection of abdominal fat pad that must be harvested by liposuction which is, anyway, a traumatic procedure.

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