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ORIGINAL RESEARCH

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ABSTRACT

Objectives: The aim of this study was to evaluate the safety and efficacy of the use of FG-SVFs in face rejuvenation for esthetic improvement.

Methods: 33 female patients affected by face's soft-tissue defects with loss of volume, study group (SG), were treated with FG-SVFs, comparing results with a control group (CG) (n = 30) treated with fat graft not enhanced (FG). Clinical evaluation, a photographic assessment, magnetic resonance imaging (MRI), and ultrasound (US) were performed. Post-operative follow-up was performed at 1, 3, 7, 12, 24, 48, weeks, and then annually.

Results: SG patients showed 61% maintenance of the contour restoring and of volume after 3 years compared with the CG treated with FG, who showed 31% maintenance. 60.7% (n = 20) of SG patients, presented an increase of 6.6 mm in the soft tissue volume after 36 months, which was reported in only 33,3% (n = 10) of the CG. Volumetric persistence in the SG was higher than that in the CG (p <. 0001 vs. CG). MRI and US moreover confirmed the absence of important side effects, as fat necrosis, and cytosteatonecrotic areas.

Conclusions: The use of FG-SVFs was safe and effective in this series of a case treated.

1. Introduction

In the last century, several procedures, like the use of autologous grafts (e.g., bone grafts, cartilage grafts, and fat grafts), prosthesis and soft tissue fillers, have been suggested and tested for face remodeling [1–4].

The most used synthetic implants, like silicone prosthesis, polyethylene implants, and bone cement, showed acceptable results in selected cases. However, their long-term feasibilities have not been documented and potential complications, like infection, displacement, incompatibility, and rejection, reduced their use [1,2]. Although fillers like hyaluronic acid have recently become popular, these materials may not be used in all patients due to the necessity of repeat infiltrations and the possibility of an allergic reaction [3,4]. Autologous grafts, such as bone and cartilage grafts, dermal grafts and fat grafts, were considered preferable due to their biocompatibility and effectiveness in certain cases [1,2].

However, in traditional fat grafting prepared without the enrichment with Platelet-rich plasma (PRP) and/or stromal vascular fraction cells (SVFs), dissatisfaction in terms of unpredictable absorption rates, potential morbidities, and a lack of evidence regarding the long-term results, prompted the authors to describe the potential and efficacy of fat graft enhanced with adipose-derived stromal vascular fraction cells (FG-SVFs).

In the last 15 years, an increasing number of articles have been published on the use of FG-SVFs including breast augmentation [5–8], breast reconstruction [9–20], lower extremity ulcers [21,22], calvarial defects [23], craniofacial microsomia [24], and facial recontouring [25–30].

Adipose-derived stem cells (ASCs) can be identified in the mixed cell population referred to as SVFs [10]. These cells can be further isolated using minimal manipulation based on mechanical filtration and centrifugation or using enzymatic digestion.

Most recently, during the last year (2019) it was described the difference in terms of cell amount obtained, between the enzymatic digestion and mechanical procedures [31], evaluating the related impact in fat graft maintenance percentage in soft tissue defects [13,31]. Enzymatic digestion offered a better amount of nucleated SVFs cells compared to mechanical procedures, as previously reported [8,13,18,31] but with major costs and with more long-time procedures. Both, these procedures can be performed in a one-step surgery, where growth by cell culture is not performed. The EMA (i.e., European Medicines Agency), FDA (i.e., US Food and Drug Administration), and other similar bodies view adult cells, SVFs and ASCs included, as biological products that can be split into two classes: *'Minimally*

Manipulated' and *'Substantially Manipulated'*. Minimally manipulated products have undergone a small amount of manipulation through processes such as filtration, centrifugation, isolation, and more as used in this study.

Substantially manipulated products are those that have undergone a significant manipulation process like stem cells that have been expanded through cell cultures. In this last case, the rule's application of good manufacturing practice (GMP) for preparation was mandatory. Enzymatic digestion may be considered a minimal manipulation procedure only if some parameters were respected, as follows described.

In fact, in agreement with the reflection paper EMA/CAT/600280/2010 Rev 1, 20 June 2014, by the CAT, Line 10, according to which *'a similar basic capacity for a cell populace implies that the cells, when expelled from their unique condition in the human body are used to maintain the original capacity in a similar anatomical or histological condition'*, it is possible to presume that autologous use in a one-step medical procedure, minimal manipulation, omo-functional application *'used for an indistinguishable fundamental capacity in the beneficiary as in the donor,'*, and manipulation performed through devices in aseptic conditions (operatory room) would be conditions that do not require the rule's application of good manufacturing practice (GMP) for preparation, good clinical practices (GCP) for clinical use, or ethical committee underwriting.

SVFs may increase fat graft maintenance by improving vascularity, angiogenesis and the secretion of growth factors (GFs) that improve fat survival. Different researchers have published articles using fat graft enhanced with SVFs and ASCs, called in some case *"Cells Assisted Lipotransfert "*(CAL) [5,32] with favorable and unfavorable results employing different procedures of cells isolation [24,33,34].

Many studies have been published on the use of centrifuged fat graft with Coleman procedure [27,28], without any ASCs and/or SVFs addition.

Now, the authors feel the necessity to report the long-term follow up (3 years) of fat graft maintenance for esthetic purposes (face remodeling) in patients treated with FG-SVFs.

A multimodal imaging approach performed with Magnetic Resonance Imaging (MRI) and Ultrasound (US) was necessary for studying face tissue modification following fat graft injection.

The results reported suggest the efficacy of FG-SVFs and the satisfaction of the patients treated.

2. Materials and methods

2.1. Study overview and guidelines

This retrospective observational case-series study was performed following the principles reported in the Declaration of Helsinki and internationally consented ethics in clinical research [35]. A quality assessment was carried out based on the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) checklist [36]. All patients received, understood and signed detailed informed consent before any study procedure, about the protocol, including the risks,

benefits and alternative therapies. The study protocol was performed follows the European rules (1394/2007 EC) and EMA/CAT recommendations (20 June 2014 EMA/CAT/600280/2010 Rev 1). This article has been the object of a research contract with the University of Rome 'Tor Vergata', Italy, approved by rectoral degree with registration number D.R. 1467/2017.

2.2. Patients

Between January 2007 and December 2019, 33 females patients (study group) (SG) diagnosed with loss of volume (hypoplasia) in zygomatic and cheek regions (15 patients with moderate grade of bilateral hypoplasia, 8 patients affected by high grade of bilateral hypoplasia, 5 patients with outcomes of hyaluronic acid filler, 5 patients with low grade of bilateral hypoplasia were treated with FG-SVFs for face rejuvenation. The SG was comprised of 33 females aged 19–68 years (average age 43.5). Pre-menopausal females were 25 (75.8%). To establish the long-term follow-up of fat graft maintenance, the authors compared the results obtained with a control group (CG) made up of 30 females patients treated with fat graft not enhanced with SVFs (FG) according to Coleman technique [27,28], (centrifuged fat graft alone). The CG comprised 30 females aged 20–61 years (average age 40.5), all affected by hypoplasia in zygomatic and cheek regions (15 patients with moderate grade of bilateral hypoplasia, 7 patients affected by high grade of bilateral hypoplasia, 5 patients with outcomes of hyaluronic acid filler, 3 patients with low grade of bilateral hypoplasia). Pre-menopausal females were 21 (70%). All enrolled patients (SG and CG were composed exclusively by females) were underwent a full pre-operative screening, including a complete clinical evaluation, photographic and instrumental assessment performed by MRI and US. The photographic assessment was done performing, for each patient, seven basic photographs, including the frontal view, quarter views (right and left), profile views (right and left), chin-up view (waters view), and chin-down view (helicopter view). Post-operative follow-up took place at 1, 3, 7, 12, 24, 48, weeks and then annually for five years.

2.2.1. Allocation sequence and quality assessment

The patient's allocation sequence was created the usage of an online randomization generator (https://www.randomizer.org) and turned into concealed by means of someone unrelated to the trial control group. The participants look at personnel, and outcome assessors were all blinded to remedy allocation, and blinding became maintained until all information had been analyzed. In detail, in all females treated, quality checks were performed based on the following criteria:

- SVFs preparation (in all patients of the study group, SVFs were obtained via a not-enzymatic method, based on mechanical procedures, represented by centrifugation and filtration).

- SVFs evaluation (in all patients the SVFs suspension had an average cells concentration to 33.250 ± 5.100 nucleated cells/mL of fat tissue processed).
- Fat harvest withdrew (within 150 mL for each patient).
- The quantity of FG-SVFs received (variable consistent with the size of the targeted area).
- Adverse response signaling (did not occur).

Quality evaluation was showed in a CONSORT flow diagram (Scheme 1).

2.2.2. Inclusion and exclusion criteria

Inclusion criteria had been the subsequent: age 19–68 years old, history of soft tissue defects with loss of volume and elasticity and signs of aging. Additional inclusion criteria in both groups were sufferers with BMI among 20 and 35 kg/m2, enough fat into the abdomen, thighs, flanks and inner knees regions (sites of fat harvest). Exclusion criteria were

divided into two types: local and general. The general criteria include pregnancy, anti-aggregating therapy, bone marrow aplasia, un-compensated diabetes, sepsis, and cancer. The local criteria encompass cancer, lack of substance and uncontrolled comorbidities. Tobacco use or genetic disorders were no longer considered as exclusion criteria.

2.2.3. Clinical information assessment

The following characteristics have been prospectively recorded within the dataset: demographic data, age, BMI, surgical management, surgical complications (Table 1). All the therapeutic options have been discussed and decided with the aid of a multidisciplinary team, consisting of a regenerative plastic surgeon and radiologist. During the first five years, patients were followed up every 6 months by clinical examination and every 12 months by surveillance with MRI and US. Abnormal scientific findings were in addition investigated as appropriate. Face soft tissue changes and
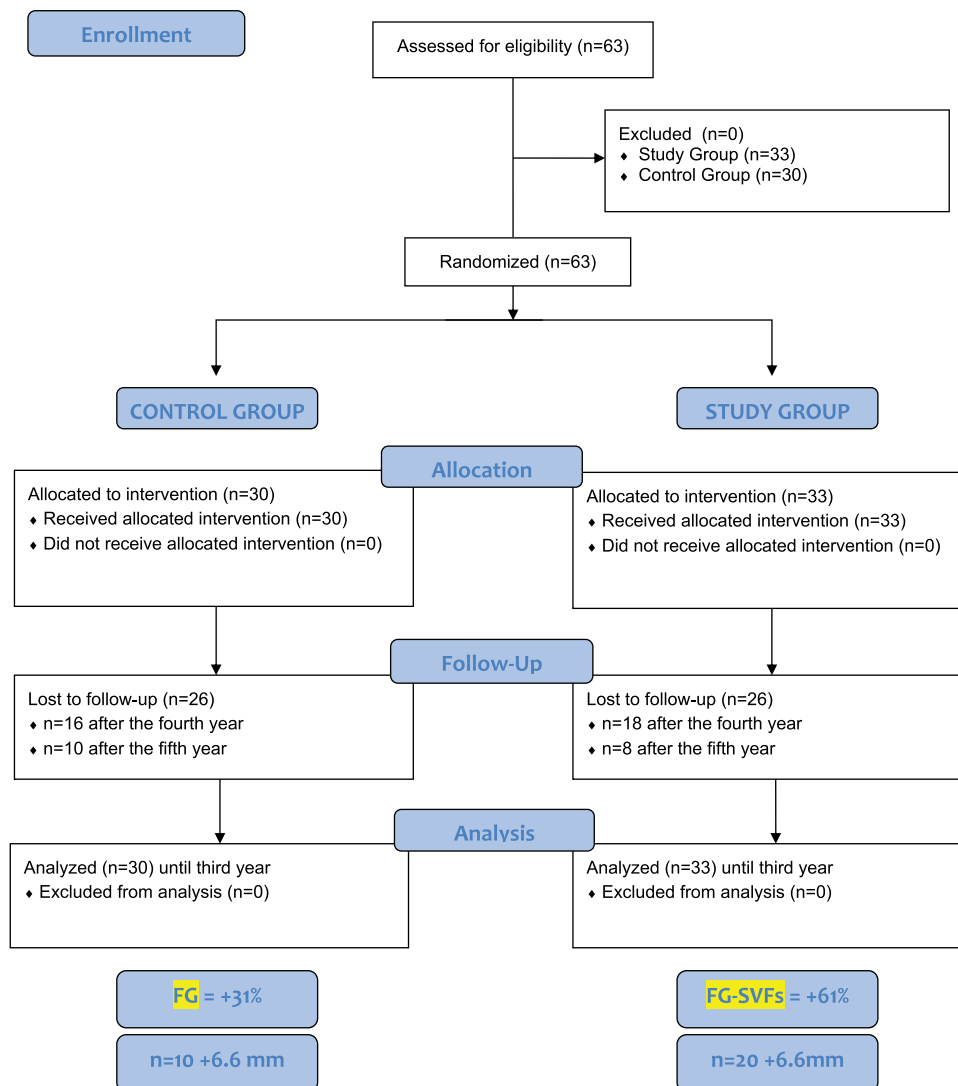
Scheme 1. CONSORT (Consolidated Standards of Reporting Trials) flow diagram.

Table 1. Patients data and fat graft assessment.

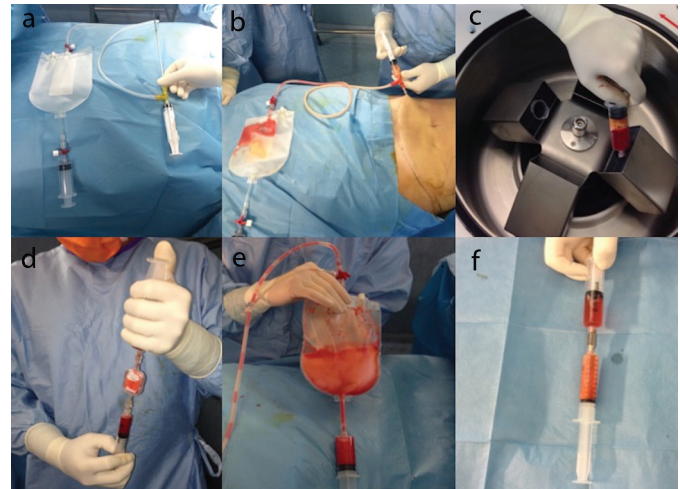|	Study Group (SG)	Control Group (CG)
Number of patients, no°	33	30
Age at surgery, yr	43,5 (min 19, max 68)	40,5 (min 20, max 61)
BMI at surgery, kg/m2	27 (min 21, max 33,16)	27 (min 21, max 33,16)
Bilateral HypoplasiaOutcomes of hyaluronic acid filler Pre-menopausalFat maintenance percentage (1 year later)Fat maintenance percentage (2 yearslater)Fat maintenance percentage (3 years later)Fat maintenance volume (3 years later)Cyst formation and Calcification Fat or Skin NecrosisSecond-Fat Injection (Re-treatment)Fat graft injected for patientFat Graft harvested volume	15 (moderate), 8 (high), 5 (low) 525 (75.8%)69% ± 5% (All patients) 64% ± 5% (All patients) 61% ± 5% (All patients) 6.6 mm ≥ (20 patients)30 022.5 mL (range 15–30 mL) 130 mL (range, 110 mL – 150 mL)	15 (moderate), 7 (high), 3 (low) 521 (70%)40% ± 5% (All patients) 34% ± 5% (All patients) 31% ± 5% (All patients) 6.6 mm ≥ (10 patients)40 222.5 mL (range 15–30 mL) 130 mL (range, 110 mL – 150 mL)

Figure 1. Preparation of Fat Graft Enhanced with Stromal Vascular Fraction Cells (FG-SVFs). (a) Aseptic closed circuit consisting of 3 mm cannulas connected with 20 mL vacuum syringe, tubes, and bag including filter. (b) Harvesting of fat tissue in the abdominal region. (c) Fat harvested (80 ml) centrifuged at 1700 rpm per 10 min. (d) 40 mL suspension extracted from the bag and filtered through a 120-?m filter obtaining 20 ml of the SVFs pellet. (e) The remaining part of a fat (30–60 mL) collected and centrifuged at 1700 rpm per 10 min obtaining 10 mL-25 mL of fat centrifuged. (f) Mixing of centrifuged fat with 5 mL of SVFs pellet resulting in approximately 22,5 mL (range 15 mL/30 mL) of FG-SVFs.

cysts, macro and micro-calcifications had been documented through a clinical exam, radiological assessments and/or pathological assessment.

2.3. Fat's donor area

The donor site area (abdomen and/or flanks and/or thighs and/or inner knees) was infiltrated with a cold solution containing 1 mL of adrenaline per 500 mL of saline solution (to reduce the bleeding during the treatment) and 3 mL of Naropin 7.5 mg/mL. The procedure becomes accomplished in sedation. Fat tissue was harvested after 6 minutes (min) using a 3-mm-diameter cannula and a 10-mL Luer-Look syringe.

2.4. Fat graft enhanced with stromal vascular fraction cells (FG-SVFs)

The cells isolation and fat tissue purification procedure mainly exhibit two steps. Step one starts with a closed and not-invasive liposuction (130 mL average in all patients – range 110 mL/150 mL -) performed with a 3 mm cannula connected with an aseptic closed circuit consisting of 20 mL vacuum syringe, connection tubes and bag including filter (Figure 1a). Adipose tissue was harvested in in the abdominal region (Figure 1b) and/or flank and thighs. Maintaining aseptic technique, the plunger of the 20 mL-syringes Luer-Look was unscrewed (Figure 1c) and the tip was closed with a sterile cap. The first half of the fat harvested (80 ml) underwent filtration and centrifugation cycles at 1700 right per minute (rpm) per 10 min (Figure 1c), after which 40 mL of the suspension was extracted from the bag. The suspension was further filtered through 120-μm filter (Figure 1d), and 20 ml of the SVFs suspension was obtained. In

the second step, the remaining part of fat tissue harvested (30–60 mL) was centrifuged at 1700 rpm per 10 min. Once completed, the 10 mL-25 mL of fat centrifuged (Figure 1e) was added and mixed with 5 mL of SVFs suspension (Figure 1f) resulting in approximately 22,5 mL (range 15 mL/30 mL) of SVFs-enhanced fat tissue for grafting that the authors called Fat Graft Enhanced with Stromal Vascular Fraction Cells (FG-SVFs). Using specific 1.5-mm-diameter micro-cannulas for implantation, the FG-SVFs was transferred into 1-ml syringes and aseptically re-injected into the soft-tissue defects of the face.

2.4.1. Analysis of the SVFs vascularization potential and growth factors secretion

The procedure of fat graft enhancement was based on two steps and two related fat harvesting during the same surgical procedure, as previously described. The first step was aimed to isolate a SVFs pellet from a first amount of fat tissue. The second step was aimed to obtain a purified fat graft ready to be enhanced with SVFs pellet previously obtained (Figure 2). The major concentration of adipose-derived stromal vascular nucleated cells in FG-SVFs aimed to improve:

- Cells capacity to secrete several GFs as Vascular Endothelial Growth Factor (VEGF);
- The neo-angiogenesis and fat vascularization;
- The Extracellular Matrix (ECM) guidance cues promoting endothelial sprouting;
- The new micro-capillary networks, which deliver the proper nutrients and oxygen to the fat implant.
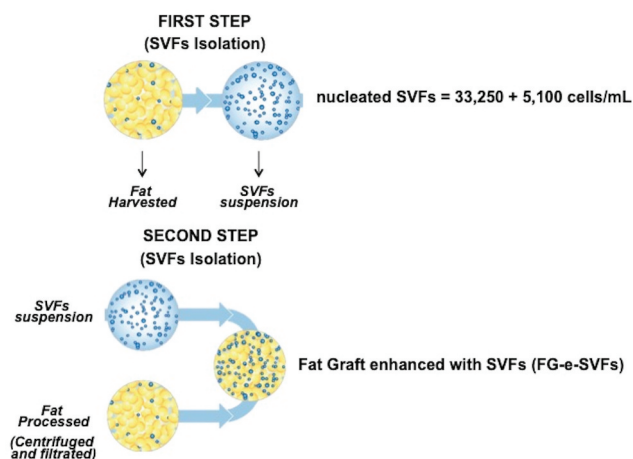
Figure 2. The rationale of SVFs isolation and enrichment of fat graft. Analysis of the procedure of fat graft enhancement, based on two steps: The first step aimed to isolate an SVFs pellet from the first amount of fat tissue; The second step aimed to obtain a purified centrifuged fat graft ready to be enhanced with SVFs pellet previously obtained.
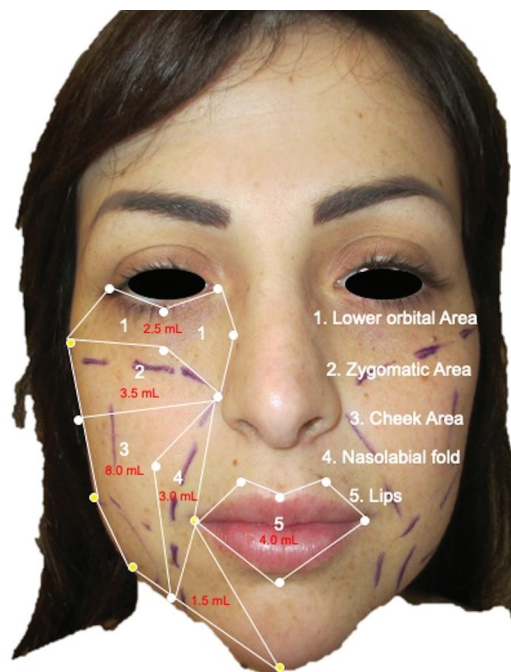

Figure 3. Analysis of the study design of fat injection, identifying the targeted areas, to perform face rejuvenation, represented by 5 regions: zygomatic region, cheek region, lower orbital area, nasolabial fold, and lips. The amount of fat injected (FG-SVFs) in each treated area was reported.

2.5. Fat graft not-enhanced with stromal vascular fraction Cells (FG)

Fat Graft not Enhanced with Stromal Vascular Fraction Cells (FG) was performed according to Coleman technique. Adipose tissue was collected from the abdomen and/or flanks and/or thighs and/or inner knees using the same cannula used in FG-SVFs. Maintaining asepsis, we took the plungers off the syringes; after remaining them with a cap and we positioned them within the sterile centrifuge. The syringes containing fat tissue had been processed for 3 min at 3.000 rpm. This technique acquired purified adipose tissue, keeping the integrity of the however isolating the fluid fat portion from the serous bloody part. The purified and centrifuged fat become inserted in 1-mL Luer-Look syringes and aseptically re-injected using 1.5-mm-diameter micro-cannulas for implanting. None of SVFs or ASCs addition become performed.

2.6. Fat injection technique

The processed fat tissue has been injected for face rejuvenation, prevalently, into 5 regions: zygomatic region, cheek region, lower orbital area, naso-labial fold and lips (Figure 3) The selected regions to treat were decided, in all patients, on the necessary corrections analyzed via MRI scans and clinical assessment. The processed fat tissue injections were performed using the *"Gentle technique"* [7] based on a gradual and gentle injection implanting linear deposits of fat graft from the deepest soft tissue to the most superficial [7,13]. For this reason, the FG-SVFs and FG have been implanted, only in subcutaneous space (not into epidermis), in multiple tunnels with gradual and controlled movements through different entrances (naso-labial fold and temporal area) to underline the

importance of a non-traumatic procedure to maximize the integrity of the grafted tissue and to maximize the contact surface between the fat injected and the host's capillaries [7,13]. The diffusion of nutrients from neighboring capillaries is vital for adipocyte survival and favors their integration with the surrounding tissue [7,13]. According to the patient's needs, 15 mL/30 mL (average, 22,5 mL) of fat grafting was injected for each procedure. The incisions have been closed with 6–0 Monocryl sutures.

2.7. In Vivo assessment

2.7.1. Instrumental face volume assessment

The MRI scansion was performed in all patients before the first treatment, again at 6 and 12 months later the FG-SVFs and FG injections and then annually. In fact, in the post-operative follow-up, the US, and MRI scansions were performed annually, after the first year, with the aim of determining the face volume and macro and micro-calcifications. A 1.5 Tesla scanner (Hitachi, MS, Echelon Oval, Tokyo, Japan) was employed with 3 mm thick slices. OsiriX software, (Pixmeo, CA, USA) has been used to calculate face volume. Two calculations were done per exam and the average determined was taken as the final face volume. Based on MRI scans acquired, volumetric fat site assessments into the face were also calculated and assessed using a three-dimensional reconstruction on a separate workstation (ADW 4.0; GE Medical Systems, Milwaukee, Wis.).

2.7.2. Clinical face volume assessment

Clinical outcomes were evaluated with objective- and subjective-evaluation. The objective evaluation has been performed by the surgical team, while subjective-evaluation has been performed by patients. The surgical team-evaluation was based on clinical analysis, applying a scale of six degrees (excellent, good, discreet, enough, poor, inadequate). The patient self-evaluation has been performed applying the same six degrees previously reported. The factors/variables that have been considered were pigmentation, vascularization, pliability, thickness, itching, and pain.

2.8. Characterization, isolation and expansion of ASCs and SVFs derived from fat harvested tissue

Fat tissue harvested has been washed 3 times with phosphate-buffered saline (PBS) and suspended in an equal volume of PBS and 0.1% collagenase type I (C130; Sigma- Aldrich, Milan, Italy, EU, http://www.sigmaaldrich.com) pre-warmed to 37°C. The fat graft has been located in a shaking water bath at 37°C with agitation for sixty min and centrifuged for ten min at 600 g. The supernatant with mature adipocytes was collected. The SVFs pellet, containing ASCs, has been re-suspended in erythrocyte lysis buffer (155 mM NH4Cl, 10 mM KHCO3, and 0.1 mM ethylenediaminetetraacetic acid -EDTA-) and incubated for five min at room temperature. Later centrifugation for five min, the pellet has been re-suspended in few microliters of growth medium and passed through a 100-µm Falcon strainer (Becton and Dickinson, Sunnyvale, CA, USA, http://www.bd.com), and the cellular population was counted using a hemocytometer. Then digestion has been plated in Dulbecco's Modified Eagle's Medium (DMEM) (Euroclone, Pavia, Italy, EU, https://www.euroclonegroup.it) added with 10% (v = v) fetal bovine serum (FBS; Euroclone, Pavia, Italy, EU, https://www.euroclonegroup.it), 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Fungizone, Invitrogen, Milan, Italy, EU, http://www.invitrogen.com), at a density of 2500–5000 cells/cm^2 of surface area. This initial passage of primary cell culture was referred to as passage 0 (P0). Later 48 hours of incubation at 37°C at 5% CO2, the cultures have been washed with PBS and maintained in the stromal medium until they achieved 75–90% confluence. ASCs were passaged by trypsin (0.05%) digestion and plated at a density of 5000 cells/cm^2 (P1). The medium was changed every 3 days, as previously reported [25].

2.9. Statistical analysis

Comparison among SG and CG was carried out with Student's t-test or Mann-Whitney for face volumetry, fat graft volume, cellular counting, the question of the self-assessment questionnaire, and surface markers expression. The data have been expressed by mean (range and standard deviation), median (range), and percentages. For histological parameters assessment, data have been expressed as mean values ± standard error of the mean (SEM). A two-tailed p-value of less than 0.05 was considered significant.

3. Results

3.1. In Vivo evaluation

The injections of FG-SVFs and FG were successfully performed in all patients (SG and CG). The follow-up was performed after baseline (T0) at 1 week (T1), 3 weeks (T2), 7 weeks (T3), 3 months (T4), 6 months (T5), 12 months (T6) and then annually. Really, the follow-up was performed in all patients (SG and CG) until the third year later the last fat graft injection. Many patients were not available to come back at control the two years later. In fact, after the third year, 15 patients (46%) of the SG and 14 patients (47%) of the CG were controlled at the fourth year, whereas 7 patients (21%) of the SG and only 4 patients (13%) of the CG were controlled at the fifth year. Mean follow-up was 36 months (range 12–60 months). The mean age of females was 41 (range 20–62).

3.1.1. Instrumental face volume evaluation

Injected fat tissue survival was evaluated with instrumental MRI and ultrasound. The patients treated with FG-SVFs showed 61% maintenance of the contour restoring and of three-dimensional volume after 3 years compared with the patients of the CG treated with FG, who showed 31% maintenance. In 60.7% (n = 20) of patients treated with FG-SVFs, we observed a restoration of the face contour and an increase of 6.6 millimeters in the 3-dimensional volume after 36 months, which was reported in only 33,3% (n = 10) of patients in the CG. Volumetric persistence in the SG was higher than that in the CG ($p < .$ 0001 vs. CG). MRI has detected cyst formation, micro calcifications, macro calcifications, and cytosteatonecrotic areas. Cyst formation and calcifications were identified in 3 patients in the SG and in 4 patients in the CG (p = 0.053). Fat necrosis was not identified. 2 patients in the CG underwent a second treatment. In the long-term follow-up, side effects like infections and skin necrosis were not observed in either group.

3.1.2. Clinical face volume evaluation

30 patients of the SG (91%) (Figures 4a and 5a and Supplementary Figure 1a) underwent the FG-ASCs referred full satisfaction about texture, softness and volume contours (Figure 4b and Supplementary Figure 1b) versus only 11 patients of the CG (37%). In SG the major part of females was satisfied with the results of fat grafting, recommending this procedure to a friend, and 23 patients (70%) would available, to undergo the fat grafting procedure again (Table 2).

Regarding the self-evaluation of cosmetic results, scores ranged from 3 to 6 in CG and from 1 to 4 in SG (p = 0.031). The results reported show a hard trend in patients of the SG to be more satisfied (Figures 4b and 5b) than patients in the CG. Satisfaction grade assessment questionnaire analysis showed that all people in both groups (SG and CG) would choose to undergo face rejuvenation/recontouring with fat injections, and they were sufficiently informed about risks and complications of this treatment (included the risk of reabsorption of fat graft and the high possibility to repeat the treatment more times) (Table 2).

Figure 4. 27 years old female patient affected by moderate bilateral zygomatic hypoplasia treated with FG-SVFs. (a) The pre-operative situation of the face in frontal view. (b) Post-operative situation after 36 months displaying maintenance of fat graft injected in zygomatic, temporal and lower orbital area with very satisfying esthetic outcomes.

Figure 5. 27 years old female patient (the same patient showed in Figure 1) with focusing on adipose tissue in the thighs and inner knees. (a) The pre-operative situation in the frontal view of the fat's donor site represented by thighs and inner knees. (b) Post-operative situation after 36 months without localized adiposity in thighs and inner knees with good esthetic outcomes.

3.2. In Vitro evaluation

In 12 selected patients with simple randomization (randomly), the authors calculated nucleated SVFs that were 33,250 ± 5,100 cells/mL of fat tissue processed (FTP). The percentage of ASCs was 1–3% of the total amount of nucleated SVFs for each mL of FTP. The allocation sequence has been created using an online randomization generator (https://www.randomizer.org) and has been concealed by a person unrelated to the trial management group. The female patients, surgical and radiologic teams, and outcome assessors have been all blinded to treatment allocation, and blinding has been maintained until all data had been analyzed. Histological analysis of fat graft before transplantation, comparing FG-SVFs with FG, was performed by hematoxylin and eosin staining (Figure 6a–d). The FG showed normal-shaped adipocytes at 10× magnification (Figure 6a) and 40× magnification (Figure 6c). The FG-SVFs composed of stromal scaffolding of adipose tissue showed cell clusters (small group >15 cells of round-shaped cells within the fat context) at 10× magnification (Figure 6b) and 40× magnification (Figure 6d).

4. Discussion

Fat grafting is an important clinical application in esthetic regenerative plastic surgery. The simplicity of the procedures, the absence of prostheses and of subsequent visible scar prompted an increasing interest in this procedure.

Fat graft procedure has been used since many years by surgeons-scientists with documented experience in fat's manipulation, in different fields as breast augmentation [5–8], breast reconstruction [9–20], lower extremity ulcers [21,22], calvarial defects [23], craniofacial microsomia [24], facial recontouring [25–30]

On this basis, the authors feel the necessity to better explain the correct approach to have the more natural results and the less fat resorption, represented by face volume analysis, the technique of fat injection and methods of fat enhancement.

Regarding the technique of fat injection, it is fundamental to choose the correct method. The authors used the Coleman procedure for many years, both for the fat preparation method (trough 3000 rpm centrifugation per 3 min) and

Table 2. Patient's satisfaction data.

|	Study Group (SG)	Control Group (CG)
Patients no°	33	30
Self evaluation of cosmetic results (score range 1–6/excellent-very poor)	30 (Fully Satisfied): 11 (Excellent/1) 9 (Very good/2) 10 (Good/3) 3(Not/Fully/ Satisfied): 3 (Sufficient/4)	11 (Fully Satisfied): 11 (Good/3)10(Not/Fully/ Satisfied): 10 (Sufficient/4) 9(Not/Satisfied): 7 (Poor/5) 2 (Very poor/6)
Satisfaction of final result	3023	114
Available to next fat graftingRecommend the fat injection to a friend	30	11
Available to face recontouring with fat injectionsSufficiently informed about risks and complications	3033	1730
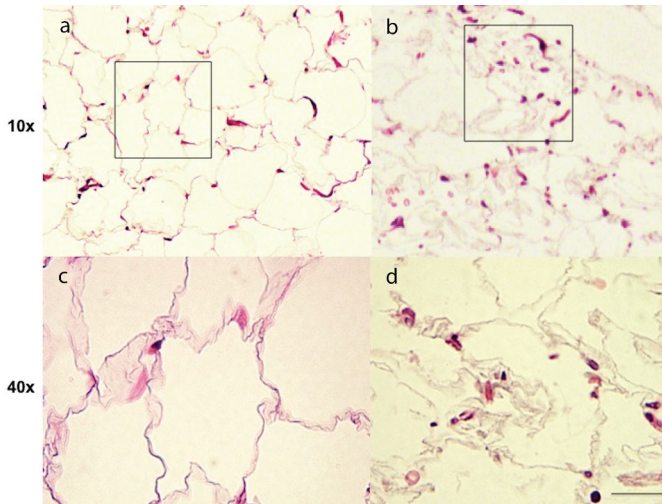
Figure 6. Histological analysis of fat graft before transplantation, in hematoxylin and eosin staining, comparing FG-SVFs with FG, (a) Normal-shaped adipocytes in FG at 10× magnification. (b) Stromal scaffolding of adipose tissue in FG-SVFs showed cell clusters represented by small group >15 cells of round-shaped cells within the fat context at 10× magnification. (c) Normal-shaped adipocytes in FG at 40× magnification. (d) Stromal scaffolding of adipose tissue in FG-SVFs showed cell clusters represented by small group >15 cells of round-shaped cells within the fat context at 40× magnification.

for the fast and dynamic way of infiltration. For many years, Gentile et al. [7,13] have begun to compare the different methods of centrifugation, filtration, and fat enhancement, for various uses in regenerative plastic surgery, like to breast augmentation/reconstruction or face recontouring, verifying that a more delicate and three-dimensional infiltration distributed in the different compartments of the breast or the face, was able to reduce the percentage of fat resorption over time, and also to have more natural results compared with a prosthesis [37]. Starting with this concept, in the present article, the processed fat tissue has been injected for face recontouring into 5 regions: zygomatic region, cheek region, lower orbital area, nasolabial fold and lips. The area destined to receive the fat injection was decided, in all patients, based on the necessary corrections analyzed through MRI scans and clinical assessment. The processed fat tissue injection was performed using the *"Gentle technique"* previously described for breast augmentation [7], based on a slow and gentle injection implanting linear deposits of fat graft from the deepest soft tissue to the most superficial [7,13]. For this reason, the FG-SVFs and FG were implanted, only in subcutaneous space (not into the epidermis or not under/into the muscle), in multiple tunnels with slow and controlled movements through different entrances (nasolabial fold and temporal area) to underline the importance of a non-traumatic procedure to maximize the integrity of the grafted tissue and to maximize the contact surface between the fat processed and the host's capillaries [7,13]. Applying this technique also for the face, the authors favoring the diffusion of nutrients from neighboring capillaries, that as known, essential for adipocyte survival improving their integration with the surrounding tissue [7,13].

Regarding the methods of fat enhancement, in this article, supplementation of autologous fat injection using FG-SVFs improves the soft tissue volume of the face, compared to FG.

FG-SVFs was obtained by mechanical centrifugation and filtration of adipose tissue, according to the minimal manipulation rules, presenting less reabsorption compared with a not enhanced fat graft. To prevent resorption, it is fundamental to do each step of the procedure carefully, having attention to each detail.

As previously published [8,13,17–19,25,26,29,31], several different concentrations of SVFs nucleated cells were obtained by the use of minimal manipulation procedures, enzymatic digestion, and substantial manipulation. In fact, by manual extraction, using cell cultures in the laboratory (considered substantial manipulation) it was obtained approximately $250,000 \pm 34,782$ SVFs nucleated cells per milliliter of FTP [8,13,17–19,25,26,29,31]. When enzymatic digestion was used, via automatic extractor, $50,000 \pm 6,956$ nucleated cells/ml of FTP was obtained [8,13,17–19,25,26,29,31]. Using an automatic extractor based on mechanical filtration and centrifugation (as used in the present study), the cell yield was about $33,250 \pm 5,100$ nucleated cells/ml of FTP. The related impact in terms of fat graft maintenance was previously reported [8,13,17–19,25,26,29,31].

The positive outcomes derived from SVFs adding could be explained by the cell's capacity to secrete several GFs, as Vascular Endothelial Growth Factor (VEGF) – a potent pro-angiogenic factor, that improves neo-angiogenesis and fat vascularization, and provide physical Extracellular Matrix (ECM) guidance cues promoting endothelial sprouting [38–40]. This SVFs addition may increase fat survival through improved vascularization, leading to reduced resorption of the graft, as observed in the present work. This concept, related to the activity of SVFs that improve the fat graft survival and maintenance, is supported by observations from other surgical treatments, such as that for a calvarial defect and breast reconstruction after partial mastectomy with radiotherapy damage, as previously reported.

In fact, injected fat graft must survive through a diffusion mechanism until active blood supply is reestablished. Thus, survival of the graft must be balanced between this mechanism and hypoxia-induced cell death. GFs released by SVFs may therefore pro-survival of the fat injected through increased blood vessel density within the same graft with a significant improvement in graft retention as also reported in an animal study, using gene therapy to deliver VEGF to the graft [41]. In fact, in this animal study, a significant improvement in graft retention at 15 weeks was reported. Also, the early establishment of new micro-capillary networks, which deliver the proper nutrients and oxygen to the implant, might contribute to the improved outcomes observed [41].

Herly M, et al. [42] in an interesting retrospective, longitudinal cohort study, reported a long-term evaluation of fat graft maintenance, using CT and MRI in 108 patients affected by removal outcomes of vestibular schwannoma. The average baseline fat graft volume was 18.1 ± 4.8 ml. They reported that the average time to reach a steady-state was 806 days

(2.2 years average) after transplantation, showing at this time, average fat graft retention of 50.6%. Additionally, fat graft retention over time was significantly higher in men than in women (57.7% versus 44.5%; $p < 0.001$). They concluded that fat grafts continue to shrink long after the initial hypoxia-induced tissue necrosis has been cleared, thus indicating that factors other than blood supply may be more influential for fat graft retention.

Comparing these results with those obtained in the present study, several analogies appear to be reported. The same instrumental analysis was performed using MRI and CT, a strategical follow-up was 2.2 years (Herly M et al) and 3.0 years (Gentile et al) respectively and finally, a similar fat volume was injected 18.1 mL (Herly M et al) versus 22.5 mL (Gentile et al). However, fat retention was different, respectively 61% (Gentile et al) versus 50.6 (Herly M. et al). In agreement with the concept of Herly M et al. [42], above mentioned, several factors may contribute to fat retention both before and after the hypoxia-induced tissue necrosis. A three-dimensional and kindly fat injection like "Gentle technique" and enrichment with SVFs appears to be factors improving the fat maintenance. Additionally, appears to be fundamental to evaluate the correct ratio between the volume of the recipient area and the amount of injected fat [8] and the presence of fibrosis and scars in the area to treat.

In addition, SVFs may improve the fibrogenic activity of fibroblasts that favor, like vascularization, fat tissue survival, and three-dimensional organization. Thus, the fat graft survival is more probable when SVFs addition is performed and fat necrosis is reduced potentially due to improved vascular development in the treated area. This research, suggests an in vivo tissue-engineering approach based on an optimized microenvironment, supporting the correct architectural adipocyte distribution, and on better cell-to-cell interaction that favors fat tissue survival; this approach could offer early protection from surrounding inflammatory events.

Regarding the face volume evaluation, it is fundamental, in the pre-operative phase, to identify the regions needing correction, performing face volume analysis, shape and symmetry evaluation. Clinical evaluation is very fast and simple mainly based on experience and skin references. Three-dimensional (3D) evaluation with MRI proved to be a method, accurate and effective for volume estimation depicting in vivo face shape and symmetry.

Volume can be obtained with automatic or manual contouring on T1-weighted images and thanks to the uncompressed prone position shape and symmetry in vivo can be depicted with volume rendering 3D images.

Using MRI any deformity, asymmetry and post-operative changes can be correctly located, estimated and evaluated as well as a loss of fat volume. Face volume modification and shape changes can be compared during the follow-up thanks to the reproducibility of the assessment.

Despite the appeal of fat grafting technique, and the advantages reported, some problems still remain concerning the final face volume, the application of a standardized method for injection technique aimed to improve the fat survival reducing also the cyst formation, the controversial role of SVFs, and the necessity to repeat the treatment in some cases.

Post-operative sequelae of fat grafting may be represented by cyst formation, micro, macro-calcifications, and cytosteato-necrotic areas, as detected by MRI scan in the present work. In fact, cyst formation and calcifications were identified in 3 patients in the SG and in 4 patients in the CG ($p = 0.053$)). Instead, fat necrosis and skin necrosis were not reported. 2 patients of the CG underwent a second treatment. In the long-term follow-up, adverse events like infections and skin necrosis were not observed in both groups.

In the light of this concept, the use of a *"Gentle technique"*, the injection of fat only in subcutaneous space, the identification of an optimal volume of fat to inject into the face, reduced but not prevented the calcification and cyst formation.

As reported in previous studies [17,18], the enhancement of SVFs to fat graft not seems to improve the carcinogenesis. On the other hand, fat tissue's secretions, i.e. adipokines that are modulated during obesity, could have *'remote'* effects on mammary carcinogenesis [17–20]. For this reason, the FG-SVFs may be considered safe in esthetic regenerative surgery and in particular for breast augmentation [8] and for face recontouring [29,30]. Additionally, combined treatment with PRP and insulin favors chondrogenic and osteogenic differentiation of human ASCs in three-dimensional collagen scaffolds [43] promoting an application in cartilage and bone regeneration.

The use of autologous SVFs aims to regenerate damaged tissues through their use in isolated suspensions or in combination with the fat tissue from which they have been derived (enrichment procedures). This stems from the necessity to move from *'substitutive surgery'*, born with transplants, and represented in plastic surgery by the use of osseointegrated implant techniques [44–48] to *'regenerative surgery'* with the regeneration of organs and tissues induced through autologous cells or where this is not yet possible, the use of autologous tissue grafts [49–51].

Recently, there has been a sensible increase in the clinical trials analyzing the SVFs enhanced fat graft. A strong point of the presented study, compared with others, was the randomization and the blinding evaluation as Evidence-Based Medicine (EBM) level 1.

On the other side, an initial limitation was the unclear description of the procedure performed in the study group successively better explained. Additional initial limits appear to be the necessity to choose a technique versus another (SVFs enrichment vs. centrifugation alone) but the results showed in terms of fat graft maintenance clarify this concept.

A current limit is the absence of an SVFs enrichment standardized procedure, and a protocol largely shared.

5. Current and future challenges

The authors demonstrated that the gentle injection of FG-SVFs results in an increased fat graft survival in patients

affected by face hypoplasia. The authors concluded that the approach based on a correct face evaluation performed by MRI, SVFs-enhanced fat graft as FG-SVFs (based on minimal manipulation), and a gentle injection is a more effective procedure compared with traditional fat grafting identified as "Lipofilling" based on centrifugation alone and without three-dimensional fat injection. Additionally, it may be considered a reliable alternative to fillers or implants. The results obtained, suggested that FG-SVFs is effective and safe and that, SVFs favor fat tissue survival. In both cases, patients of SG and CG were treated with procedures based on purification of fat tissue (washing/centrifugation/filtration) and for this reason, in this work, the authors confirmed, with the results reported, the necessity to use fat tissue underwent to purification procedure

6. Expert opinion

The standardization of the FG-SVFs use in Regenerative Plastic Surgery presents a challenge for the scientific community. The absence of a protocol widely shared, related to the SVFs isolation (enzymatic digestion vs mechanical centrifugation/filtration), the kind of injection, and the fact that fat graft maintenance is not well evaluated can lead to the mistake interpretation for the soft tissue corrections. Since the FG-SVFs use may be considered a minimal manipulation procedure, fully respecting the institutional guidelines and European Rules, its standardized use, as the first choice for the plastic surgeon, appears to be essential to treat the soft tissue defects of the face.

Acknowledgments

Authors' contributions

P Gentile designed the studies, performed the procedures, analyzed the results, wrote the paper, wrote editing review, dealt with methodology and data analysis and conducted the study as the leader; C Calabrese dealt with methodology and software; D De Fazio dealt with methodology and data curation; B De Angelis dealt with project administration and software; S Garcovich dealt with methodology, software, and visualization; A Sterodimas dealt with project administration and software; A Trivisonno dealt with methodology and visualization; J Pizzicannella dealt with methodology; L Dionisi dealt with rules. All authors made substantial contributions to concept and design of the study, revised the manuscript, and gave their approval to the final version of the manuscript.

Declaration of interest

Reviewer disclosures

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