



Mechanical and Enzymatic Digestion of Autologous Fat Grafting (A-FG): Fat Volume Maintenance and AD-SVFs Amount in Comparison



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Abstract

Background Currently, several techniques for autologous fat graft (A-FG) preparation aimed at obtaining purified tissue exist. Both mechanical digestions via centrifugation, filtration, and enzymatic digestion were considered the most effective with different impacts in terms of adult adipose-derived stromal vascular fraction cells (AD-SVFs) amount that volume maintenance.

Objectives This article aimed to report the in vivo and in vitro results, represented by fat volume maintenance and AD-SVFs amount, obtained by four different procedures of AD-SVFs isolation and A-FG purification based on centrifugation, filtration, centrifugation with filtration, and enzymatic digestion.

Methods A prospective, case-control study was conducted. In total, 80 patients affected by face and breast soft tissue defects were treated with A-FG and divided into four groups: $n=20$ were treated with A-FG enhanced with AD-SVFs obtained by enzymatic digestion (study group 1 [SG-1]); $n=20$ were treated with A-FG enhanced with AD-SVFs obtained by centrifugation with filtration (SG-2); $n=20$ were treated with A-FG enhanced with AD-SVFs obtained

by only filtration (SG-3); $n=20$ were treated with A-FG obtained by only centrifugation according to the Coleman technique (control group [CG]). Twelve months after the last A-FG session, the volume maintenance percentage was analyzed by magnetic resonance imaging (MRI). Isolated AD-SVF populations were counted using a hemocytometer, and cell yield was reported as cell number/mL of fat. **Results** Starting with the same amount of fat analyzed (20 mL), $50,000 \pm 6956$ AD-SVFs/mL were obtained in SG-1; $30,250 \pm 5100$ AD-SVFs/mL in SG-2; $33,333 \pm 5650$ AD-SVFs/mL in SG-3, while 500 AD-SVFs/mL were obtained in CG. In patients treated with A-FG enhanced with AD-SVFs obtained by automatic enzymatic digestion, a $63\% \pm 6.2\%$ maintenance of fat volume restoring after 1 year was observed compared with $52\% \pm 4.6\%$ using centrifugation with filtration, $39\% \pm 4.4\%$ using only centrifugation (Coleman), and $60\% \pm 5.0\%$ using only filtration.

Conclusions In vitro AD-SVFs cell analysis indicated that filtration was the most efficient system—between mechanical digestion procedures—thanks to the highest amount of cells obtained with fewer cell structure damage, producing in vivo, the most volume maintenance after 1 year. Enzymatic digestion produced the best number of AD-SVFs and the best fat volume maintenance.

Level of Evidence III This journal requires that authors assign a level of evidence to each article. For a full description of these Evidence-Based Medicine ratings, please refer to the Table of Contents or the online Instructions to Authors <http://www.springer.com/00266>.

Keywords Adipose-derived stromal vascular fraction cells · AD-SVFs · Adipose-derived mesenchymal stem cells · AD-MSCs · Centrifugation, filtration, enzymatic digestion · Regenerative plastic surgery, plastic surgery

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Introduction

In the last thirty years, several techniques for autologous fat graft (A-FG) preparation aimed at obtaining purified tissue have been described. Centrifugation and filtration of fat tissue, commonly identified as mechanical digestion procedures, and enzymatic digestion, all minimal manipulation procedures, were considered the most effective with different impacts in terms of adult adipose-derived stromal vascular fraction cells (AD-SVFs) amount that volume maintenance.

Several pathologies in the plastic surgery field like as hemifacial atrophy [1], Romberg syndrome [2], ulcers [3, 4], outcomes of breast cancer [5, 6] and breast hypoplasia [7, 8], scars [9], and face soft tissue effects [10] have been treated with greater benefits thanks to the A-FG, AD-SVFs, and new biotechnologies or biomaterials.

Such autologous procedures quickly make the patient's tissues and related cells available through safe methods and without side effects bypassing many limitations linked to allogeneic cell treatments. This is made possible by avoiding in vitro cell control and expansion, which requires good manufacturing practice (GMP)-approved facilities [9].

Recently, several studies tried to analyze the correlation between the procedure of A-FG purification and the enrichment of AD-SVFs with fat maintenance. On this point, the most interesting trials described a range of 58–61% maintenance of the volume after 3 years when A-FG was enriched with adipose-derived mesenchymal stem cells (AD-MSCs), compared with A-FG without AD-MSCs enrichment, which displayed only 29% maintenance [6–8, 10]. In the last years, procedures in AD-SVFs isolation and A-FG purification were deeply modified with a slow shift from enzymatic digestion based on collagenase use, to mechanical digestion based prevalently on centrifugation, filtration, and fragmentation. Additionally, the correlation between the AD-SVF amount and the fat volume maintenance has not been detailed described yet.

This article aimed to report the in vivo and in vitro results, represented by fat volume maintenance and AD-SVFs amount, obtained by four different procedures of AD-SVFs isolation and A-FG purification based on “Minimal Manipulation” such as centrifugation, filtration, centrifugation with filtration (all based on fat tissue' mechanical digestion), and enzymatic digestion.

Methods

Laws and Rules About Fat Tissue Manipulation

The EMA (i.e., European Medicines Agency) and FDA (i.e., US Food and Drug Administration) view adult cells as

biological products that can be split into two classes: “Minimal Manipulation” (a procedure that does not change biological characteristics and functions of cells, exclusively based on filtration, centrifugation, cutting decantation, fragmentation, cryopreservation, grinding, vitrification, shaping, freezing, lyophilization, soaking in antibiotic, cell separation/concentration/purification, irradiation, sterilization, without any cell expansion) and “Extensive Manipulation” (cell expansions and related cultures, cell differentiation, cell activation, genetic modification, and any processing that alters the biological, physiological, or structural characteristics of cells or tissues) [11]. As clearly reported, both extensive and minimal manipulation definitions do not mention the term enzymatic digestion, and for this reason, this procedure has been considered for several years a “gray zone,” between minimal and extensive manipulation, generating confusion. Enzymatic digestion, when used for the A-FG procedure, aims to isolate and expel the AD-SVFs from the fat tissue, and for this reason, it has been considered—not correctly—an extensive manipulation. In agreement with the reflection paper EMA/CAT/600280/2010 Rev 1, June 20, 2014, by the Committee for advanced therapies (CAT), Line 10, according to which “*a similar function and capacity for a cell population imply that the cells, when expelled from their specific condition in the tissue are used to maintain the original capacity in a similar histological or anatomical condition,*” it is possible to affirm that autologous use in a one-step surgery, based on minimal manipulation (without any cell expansion or culture), omo-functional application “*applied with the same capacity in the recipient as in the donor,*” and performed via medical devices in sterile condition (as in operatory room) would be situations that do not require the rule's application of GMP for preparation, good clinical practices (GCP) for clinical use, or ethical committee underwriting [11].

For these reasons, the enzymatic digestion of fat tissue when performed for autologous, and omo-functional use (collection from fat and re-injection to fat), for an intra-operative immediate re-grafting, may fall into the category of minimal manipulation.

Study Overview and Data definitions

A prospective case–control study, classified as evidence-based medicine (EBM) level 3, was performed fully respecting the Declaration of Helsinki and internationally consented ethics in clinical research [12]. The strengthening the reporting of observational studies in epidemiology (STROBE) checklist [13] was used to make a quality assessment. The protocols were applied fully respecting the European rules (EC 1394/2007) and EMA/CAT recommendations (June 20, 2014, EMA/CAT/600280/2010, Rev

1). This article was the object of a research contract between the first author (P.G.) and the University of Rome “Tor Vergata,” approved by Rectoral Decree R. D n. #1467/2017, continued in associate professor contract #13489/2021. All subjects enrolled signed an informed consent form, before any surgical treatment, in which detailed information about the study, including the side effects, benefits, and alternative procedures, has been reported.

Patient Population

Over 10 years (2011–2022), 80 patients affected by face and breast soft tissue defects were treated with A-FG and divided into four groups:

Twenty patients (12 females [F] and 8 males [M]) aged 18–60 years (average age 39), affected by breast soft tissue defects (outcomes of breast reconstruction $n=2$; Poland syndrome $n=1$ (Fig. 1A); outcomes of scars $n=3$; breast hypoplasia $n=4$) and face soft tissue defects (outcomes of scars $n=5$ [1-F and 4-M]; outcomes of burns $n=2$ [2-M]; Romberg syndrome $n=1$ [1-M]; signs of aging $n=2$ [1-F and 1-M]) were treated with A-FG enhanced with AD-SVFs obtained by enzymatic digestion using automatic extractor (study group 1 [SG-1]).

Twenty patients (11 females [F] and 9 males [M]) aged 18–60 years (average age 39), affected by breast soft tissue defects (outcomes of breast reconstruction $n=2$; tuberous breast $n=1$; outcomes of scars $n=2$; breast hypoplasia $n=4$) and face soft tissue defects (outcomes of scars $n=5$ [1-F and 4-M]; outcomes of burns $n=3$ [3-M]; Romberg syndrome $n=1$ [1-M]; signs of aging $n=2$ [1-F and 1-M]) were treated

with A-FG enhanced with AD-SVFs obtained by centrifugation with filtration (study group 2 [SG-2]).

Twenty patients (12 females [F] and 8 males [M]) aged 18–60 years (average age 39), affected by breast soft tissue defects (outcomes of breast reconstruction $n=1$; tuberous breast $n=1$; Poland syndrome $n=1$ (Fig. 2A–C); outcomes of scars $n=3$; breast hypoplasia $n=4$) and face soft tissue defects (outcomes of scars $n=5$ [1-F and 4-M]; outcomes of burns $n=2$ [2-M]; Romberg syndrome $n=1$ [1-M]; signs of aging $n=2$ [1-F and 1-M]) were treated with A-FG enhanced with AD-SVFs obtained by only filtration (study group 3 [SG-3]).

Twenty patients (11 females [F] and 9 males [M]) aged 18–60 years (average age 39), affected by breast soft tissue defects (outcomes of breast reconstruction $n=2$; tuberous breast $n=1$; outcomes of scars $n=2$; breast hypoplasia $n=4$) and face soft tissue defects (outcomes of scars $n=5$ [1-F and 4-M]; outcomes of burns $n=3$ [3-M]; Romberg syndrome $n=1$ [1-M]; signs of aging $n=2$ [1-F and 1-M]) were treated with A-FG obtained by only centrifugation according to the Coleman technique (control group [CG]).

Patients' data and clinical assessment were detailed in a CONSORT flow diagram (Scheme 1) and Table 1.

An accurate pre-operative screening based on a full clinical evaluation and a photographic and instrumental assessment performed by magnetic resonance imaging (MRI) (Fig. 3A), and ultrasound (US), has been performed on all patients enrolled.

Inclusion and Exclusion Criteria

The following inclusion criteria were considered: age 18–60 years old, history of face and breast soft tissue



Fig. 1 Analysis of SG-1 patient, a 29-year-old male patient affected by Poland syndrome. **A** Frontal pre-operative view showing underdeveloped major and minor pectoralis muscles on the left side of chest; **B** post-operative in frontal view 6 months later the second fat graft

injection coinciding with T7. The second treatment was performed at T6. A great volume improving evenly distributed in the superior and inferior lateral and internal quadrant, peri-areolar region, and inferior and superior pole, obtaining excellent results

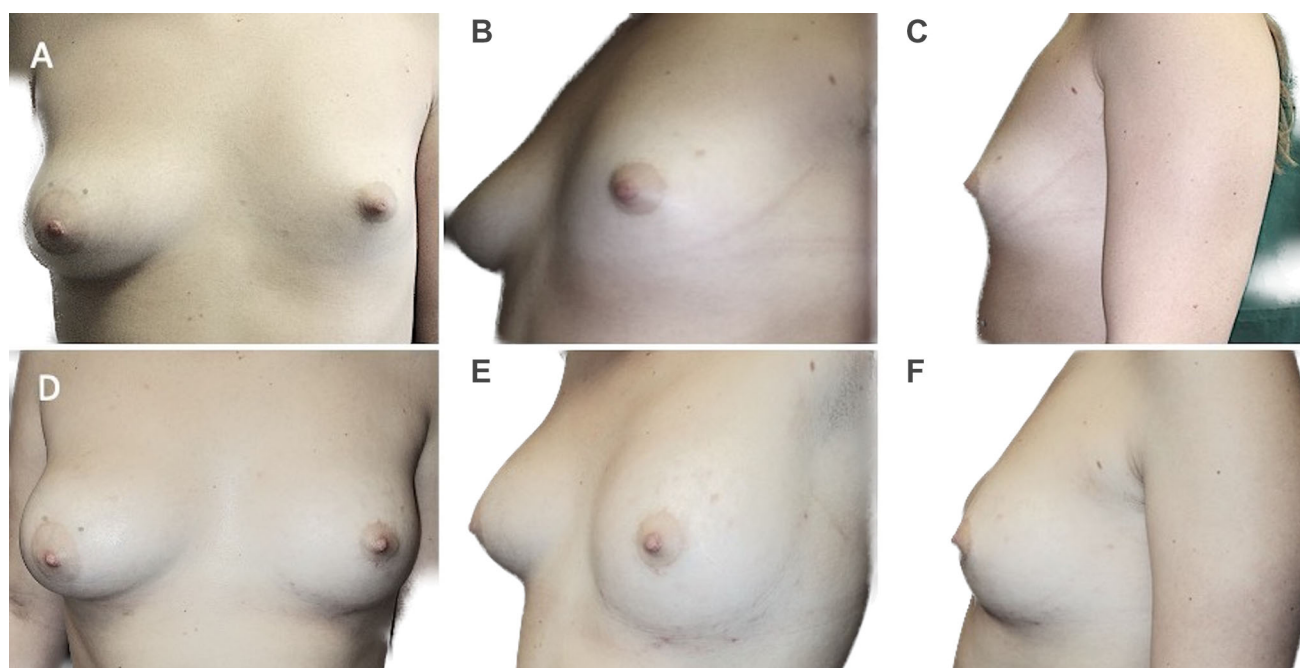


Fig. 2 Analysis of SG-3 patient, a 27-year-old female patient affected by Poland syndrome. **A** Frontal pre-operative view showing a high degree of hypoplasia in the left breast; **B** $\frac{3}{4}$ left pre-operative view showing underdeveloped major and minor pectoralis muscles with a high degree of breast hypoplasia; **C** lateral left pre-operative view showing absence projection of the breast; **D** frontal post-operative view 6 months later the second fat graft injection coinciding with T7 with significant volume improvement in the all quadrants

and upper and lower poles; **E** $\frac{3}{4}$ left post-operative view 6 months later the second fat graft injection coinciding with T7 with significant volume improvement; **F** lateral left post-operative view at T7 with great improvement of breast projection. The second treatment was performed at T6. A great volume improving evenly distributed in the superior and inferior lateral and internal quadrant, peri-areolar region, and inferior and superior pole, obtaining excellent results

defects, and a BMI between 18 and 35 kg/m². Additional inclusion criteria in SGs and CG were sufficient fat tissue in the sites of harvest as the abdomen, thighs, flanks, and inner knees regions. Following local and systemic exclusion criteria were considered: systemic exclusion criteria (bone marrow aplasia, anti-aggregating therapy, sepsis, cancer, and uncompensated diabetes), and local exclusion criteria (cancer loss of substance and uncontrolled comorbidities). Tobacco uses or genetic disorders were not considered exclusion criteria.

Centrifugation, Filtration, and Enzymatic Procedures

The enzymatic digestion (SG-1) was performed using Celution™ 800/CRS System (Cytori Therapeutics Inc., San Diego, CA, USA, <http://www.cytoritx.com>) obtaining a 365 mL average of A-FG enhanced with AD-SVFs as previously published [6, 7].

The centrifugation with filtration procedure (SG-2) was performed using Fatstkit (CORIOS Soc. Coop, San Giuliano Milanese, Italy, <https://www.cylex-italia.it/san-giuliano-milanese/corios-soc-coop-r-l-commercio-presidi-chirurgici-13161214.html>). In this case, an amount

of fat (480 mL average) was purified and enriched with AD-SVFs [8].

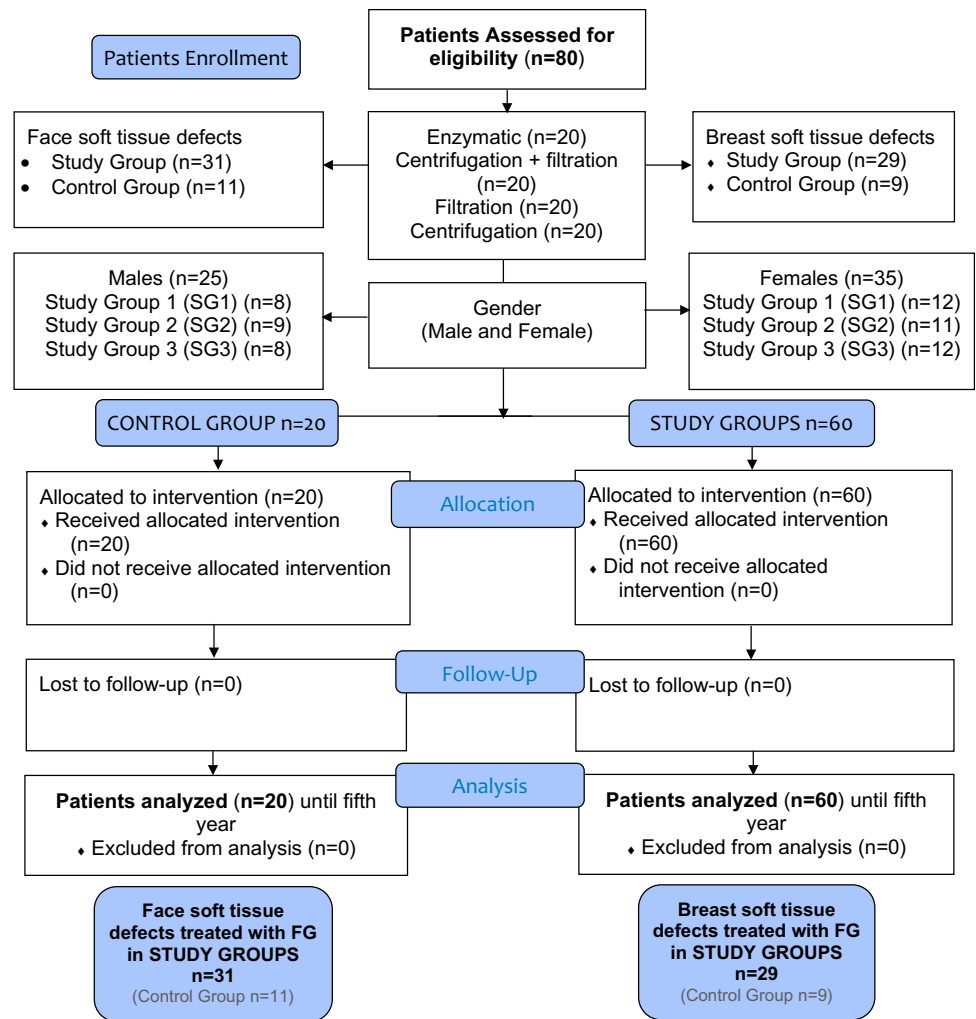
The filtration procedure (SG-3) was performed using Tissu-Trans Filtron®—Class IIa product—(Summit Medical, LLC 815 Vikings Parkway Suite 100 Saint Paul, MN 55121 USA, <https://shippertmedical.com/products/tissu-trans-filtron-250>). In this case, a 1000-mL closed inline filtration system was used, and fat (800 mL average in all patients—range 400/1200 mL) was collected and purified directly without any procedure of centrifugation, using a standard suction connected directly to the canister, permitting to filtering non-viable cells during harvest, saving valuable surgical time. At the end of the harvesting, an average of 400 mL of purified fat (200/600 mL) ready to be injected was obtained [14].

The only centrifugation procedure (CG) was performed using the Coleman procedure [15].

A-FG Injecting Technique

The A-FG injection in all groups (SG-1, SG-2, SG-3, and CG) was performed using the “Gentle-technique” [16] based on slow and controlled movements, implanting linear deposits of A-FG in the subcutaneous tissue for soft tissue

Scheme 1 CONSORT
(consolidated standards of reporting trials) flow diagram on patients' enrollment, including soft tissue defects and treatment assessment



defects of the face, and in the breast, supra-fascial, retro-glandular, and intra-glandular (not into pectoralis major muscle), through multiple tunnels and seven different entrances (infra-mammary fold [located at 130°, 180°, 220°], higher external quadrant [290°], lower-external quadrant [240°], higher internal quadrant [65°], and lower-internal quadrant [110°]) using 1.5-mm cannulas [16].

In Vivo Evaluation: Fat Maintenance and Clinical Evaluation

Fat maintenance evaluation has been performed using MRI and US, while clinical assessment and quality checks were performed as the following protocol reported. Each subject underwent 3 clinical analyses performed by the first author (P.G.). During the first visit (lasting not less than 60 min), every patient was clinically analyzed by performing a strict anamnesis and placing several questions regarding her expectations.

A complete clinical evaluation of the soft tissue defects was performed by analyzing:

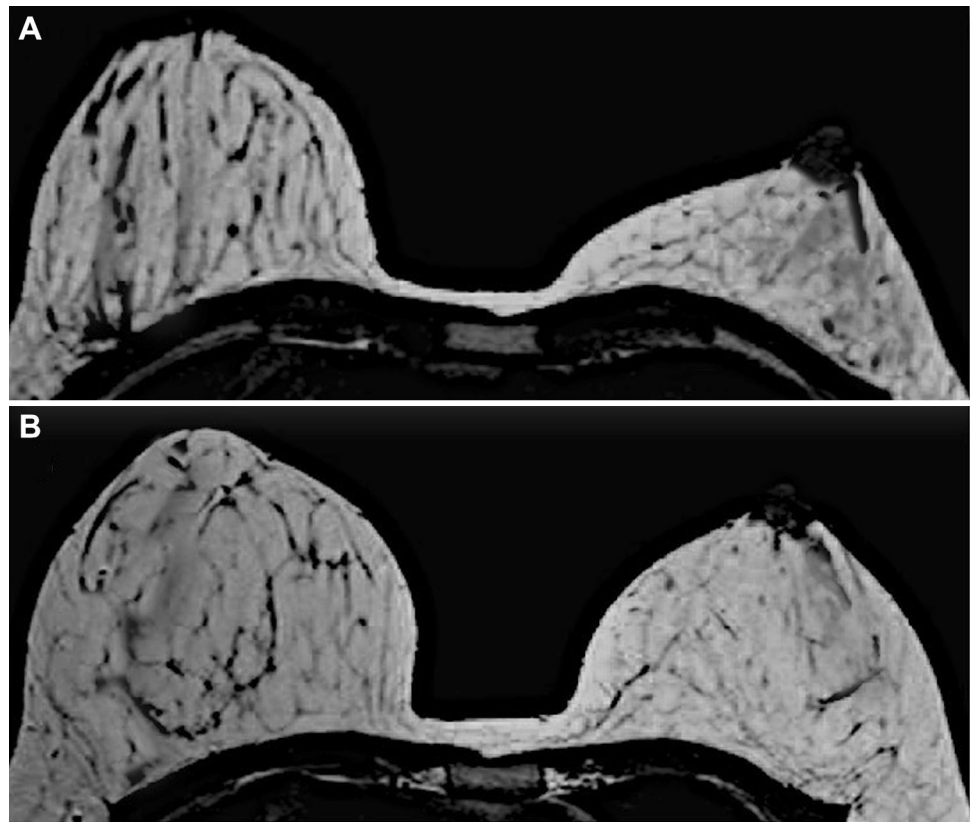
- The anatomical region of soft tissue defects (face or breast)
- The degree of soft tissue defects (high, moderate, low),
- The presence of scars,
- If the defect was unilateral or bilateral,
- The degree of asymmetry (high, moderate, low),

The distribution of body fats has been analyzed. The surgical procedure to adopt was established based on the patient's expectations, clinical evaluation, and inclusion and exclusion criteria. Risks and side effects related to the surgical strategy identified were discussed accurately. The second clinical evaluation was aimed to evaluate the patient's suitability based on blood and instrumental analysis results. In particular, the radiologist evaluated the instrumental analysis outcomes, and the psychologist the eventual presence of body image perception disorder, dysmorphophobia, personality, and/or mood disorders. The

Table 1 Patients' data and clinical assessment

	Enzymatic digestion (SG-1)	Centrifugation with filtration (SG-2)	Filtration alone (SG-3)	Centrifugation alone (CG)
Number of patients, no	20	20	20	20
Age at surgery, yr	39,0 (min 18, max 60)	39,0 (min 18, max 60)	39,0 (min 18, max 60)	39,0 (min 18, max 60)
BMI at surgery, kg/m ²	26,5 (min 18, max 35)	26,5 (min 18, max 35)	26,5 (min 18, max 35)	26,5 (min 18, max 35)
Sex	12 Females 8 Males	11 Females 9 Males	12 Females 8 Males	11 Females 9 Males
Breast soft tissue defects	(Outcomes of breast reconstruction $n=2$; Poland Syndrome $n=1$; outcomes of scars $n=3$; breast hypoplasia $n=4$)	(Outcomes of breast reconstruction $n=2$; tuberos breast $n=1$; outcomes of scars $n=2$; breast hypoplasia $n=4$)	(Outcomes of breast reconstruction $n=1$; tuberos breast $n=1$; Poland Syndrome $n=1$; outcomes of scars $n=3$; breast hypoplasia $n=4$)	(Outcomes of breast reconstruction $n=2$; tuberos breast $n=1$; outcomes of scars $n=2$; breast hypoplasia $n=4$)
Face soft tissue defects	(Outcomes of scars $n=5$ [1-F and 4-M]; outcomes of burns $n=2$ [2-M]; Romberg syndrome $n=1$ [1-M]; signs of aging $n=2$ [1-F and 1-M])	(Outcomes of scars $n=5$ [1-F and 4-M]; outcomes of burns $n=3$ [3-M]; Romberg syndrome $n=1$ [1-M]; signs of aging $n=2$ [1-F and 1-M])	(Outcomes of scars $n=5$ [1-F and 4-M]; outcomes of burns $n=2$ [2-M]; Romberg syndrome $n=1$ [1-M]; signs of aging $n=2$ [1-F and 1-M])	(Outcomes of scars $n=5$ [1-F and 4-M]; outcomes of burns $n=3$ [3-M]; Romberg syndrome $n=1$ [1-M]; signs of aging $n=2$ [1-F and 1-M])
Pre-menopausal	14 (70%)	11 (55%)	12 (60%)	15 (75%)
Only one session	15 (75%)	12 (60%)	13 (65%)	4 (20%)
Mean A-FG enhanced with AD-SVFs obtained for each treatment	365 mL (range 180–550 mL)	480 mL (range 190–770 mL)	800 mL (range 400–1200 mL)	480 mL (range 190–770 mL)
Second session	5 (25%) at T6 insufficient final volume re-treated	8 (40%) at T6 insufficient final volume re-treated	7 (35%) at T6 insufficient final volume re-treated	12 (60%) at T6 insufficient final volume re-treated
AD-SVFs amount obtained starting by 20 mL	254,500 $\pm$ 35,124/mL (biologist) 50,000 $\pm$ 6956 (automatic extractor)	30,250 $\pm$ 5100/mL	33,333 $\pm$ 5650/mL	500 $\pm$ 23/mL
Volume maintenance percentage after one treatment	63% $\pm$ 6.2% at T12 (All patients)	52% $\pm$ 4.6% at T12 (All patients)	60% $\pm$ 5.0% at T12 (All patients)	39% $\pm$ 4.4% at T12 (All patients)
Cyst formation and calcification	4 (20%)	3 (15%)	2 (10%)	3 (15%)
Fat necrosis	0	0	0	0

Fig. 3 T2-weighted MRI scans of the patient shown in Fig. 2. **A** Pre-operative situation of the patient with a high degree of hypoplasia in the left breast. **B** Post-operative obtained 6 months later the second fat graft injection coinciding with T7 with significative volume improvement in the left breast



third clinical meeting was useful to undersign the informed consent (P.G. and patient) and to clarify any doubts.

During the first two years, patients were analyzed at 1, 2, 4, 8, 12, 24, 48 weeks and then annually for 2 years by clinical examination while at 1 month (T3), 6 months (T6), 12 months (T7), and then annually until the second year later the last procedure by MRI and US. The MRI images were collected on axial and sagittal planes. Based on MRI scans acquired, volumetric assessments of the soft tissue defects were also calculated. They were evaluated by adopting a 3-D reconstruction on a separate workstation (ADW 4.0; GE Medical Systems, Milwaukee, Wis.). Twelve months (T7) after the last A-FG session, the volume maintenance percentage was analyzed. All examinations were performed and analyzed in a blinded fashion by two radiologists experienced in interpreting MRI imaging.

In Vitro Evaluation: AD-SVFs Analysis Obtained from Centrifugation, Filtration, and Enzymatic Digestion

For manual enzymatic digestion, fat was suspended in an equal volume of PBS and 0.1% collagenase type I (C130; Sigma-Aldrich, Milan, Italy) prewarmed to 37 °C [17]. Fat tissue was centrifuged for 10 minutes at 600 g after being continuously shaken in a water bath at 37 °C for 60 min.

The supernatant, containing mature adipocytes, was aspirated. The SVF pellet has been resuspended in erythrocyte lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA) and incubated for five minutes (min) at room temperature. After centrifugation at 1100 rpm for 5 min, the pellet was resuspended in a few microliters of growth medium and passed through a 100-μm Falcon strainer (Becton and Dickinson, Sunnyvale, CA). Isolated AD-SVF populations (from manual and automatic enzymatic digestion, filtration, and only centrifugation) were counted using a hemocytometer with trypan blue staining exclusion. Cell viability by trypan blue exclusion was consistently more than 98% [18]. Cell yield was reported as cell number/mL of fat. Results represented the mean of five samples/groups.

Statistical Analysis

A comparison between SGs (SG-1, SG-2, SG-3) and CG was made with the Student's *t* test or Mann–Whitney for the self-assessment questionnaire. The data obtained are expressed by mean (range), median (range), and percentages. A two-tailed *p* value less than 0.05 was identified as significant, while a *p* value less than 0.0001 was identified as extremely significant.

Results

In Vivo Fat Maintenance and Clinical Outcomes

Regarding the in vivo results (Fig. 4A), after the A-FG, the volume retention rates were not similar in the four groups. In patients treated with A-FG enhanced with AD-SVFs obtained by enzymatic digestion, using an automatic extractor (SG-1) a $63\% \pm 6.2\%$ maintenance of fat volume restoring after 1 year was observed (Fig. 1B) compared with $52\% \pm 4.6\%$ using centrifugation with filtration (SG-2) and $60\% \pm 5.0\%$ using only filtration (SG-3) (Fig. 2D–F) as shown from MRI (Fig. 3B). On the other way, only a $39\% \pm 4.4\%$ maintenance of fat volume after 1 year using centrifugation (Coleman technique) (CG) was observed.

The in vivo results showed that the volume fat maintenance obtained by enzymatic digestion using an automatic extractor (SG-1) was higher with $63\% \pm 6.2\%$ compared to $52\% \pm 4.6\%$ (SG-2) ($p < 0.0001$), to $60\% \pm 5.0\%$ (SG-3) ($p < 0.0305$), and to $39\% \pm 4.4\%$ (CG) ($p < 0.0001$).

The difference between (SG-1) and (SG-3) ($p < 0.0305$) was statistically significant, while the difference between (SG-1), (SG-2) (SG-3), and (CG) was extremely statistically significant ($p < 0.0001$).

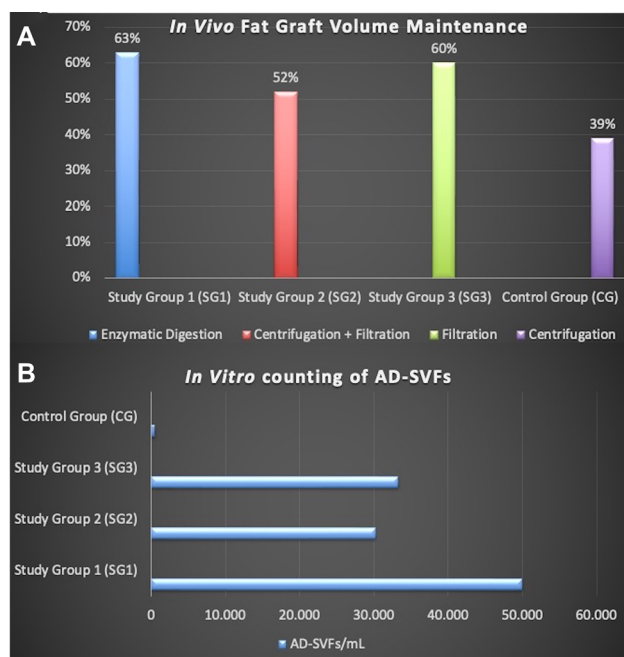


Fig. 4 In vivo and in vitro results analysis. **A** Graph bar of volume fat graft maintenance obtained by different procedures: enzymatic digestion (SG1), centrifugation+filtration (SG2), filtration (SG3), and only centrifugation with Coleman technique (CG). **B** Graph bar showing AD-SVF cell yield obtained by the different procedures: enzymatic digestion (SG1), centrifugation+filtration (SG2), filtration (SG3), and only centrifugation with Coleman technique (CG)

US showed oily cysts in 60.5% of soft tissue analyzed average at T3, 56.5% at T6, and 33.9% at T7, whereas MRI detected oily cysts only in 5.5% and 4.5% at T3 and T6, respectively, while 3.5% at T7. US resulted in extremely specific to identify oil cysts compared to MRI.

At T3 and T6, the cytosteatonecrotic areas identified on both US and MRI were unchanged (7.5%), whereas at T7 those cytosteatonecrotic areas were increased on ultrasound (11.2%) and even more on the magnetic resonance imaging scans (15.5%). MRI and US both resulted in specific to identify cytosteatonecrotic area until T6, except at T7 where MRI resulted more specific than the US.

The fat volume maintenance was analyzed using 3-D reconstruction of MRI images collected on axial and sagittal planes resulting in the only instrumental test able to perform the volume analysis.

In Vitro Counting of AD-SVFs

Regarding in vitro results (Fig. 4B), starting with the same amount of fat analyzed (20 mL) in every patient for each group (a total of 80 samples), respectively, $254,500 \pm 35,124$ AD-SVFs/mL and $50,000 \pm 6956$ AD-SVFs/mL were obtained using enzymatic digestion performed by a biologist (only for test—not applied in surgical re-injection) and an automatic extractor (used for re-injection, SG-1); when centrifugation with filtration was used, the cell yield was about $30,250 \pm 5100$ AD-SVFs/mL (SG-2); only filtration showed $33,333 \pm 5650$ AD-SVFs/mL (SG-3), while only centrifugation according to Coleman technique showed 500 ± 52 AD-SVFs/mL (CG).

The in vitro results showed that the number of AD-SVFs obtained by enzymatic digestion using an automatic extractor (SG-1) was higher with $50,000 \pm 6956$ AD-SVFs/mL compared to $30,250 \pm 5100$ AD-SVFs/mL (SG-2) ($p < 0.0001$), to $33,333 \pm 5650$ AD-SVFs/mL (SG-3) ($p < 0.0001$), and to 500 ± 52 AD-SVFs/mL (CG) ($p < 0.0001$). The two-tailed p value was less than 0.0001.

By conventional criteria, this difference is extremely statistically significant.

Study Limitations

Several limitations led to the presence of bias in the present work. Firstly, regarding the SGs, a lack of a single standardized protocol for the isolation methods of AD-SVFs must be highlighted, as well as standardized enrichment procedures. A total of four procedures, three based on enrichment of A-FG performed by AD-SVFs isolation of which one was enzymatic and two were mechanical (centrifugation with filtration and filtration only), while one based on purification (without enrichment) have been analyzed. These differences made the groups non-

homogeneous and influenced the fat maintenance percentage as shown. Secondly, the bias has been represented by several differences in terms of anatomical areas treated (breast and face).

Thirdly, in each case, the bias was limited by the “custom-made approach” for every patient, in which the choice of the amount of A-FG to inject and the related amount of fat to collect was made based on the kind of defect and of fat tissues available. Additional perspectives studies and controlled trials will be necessary.

Discussion

Several procedures, kits, and devices permitting enzymatic and mechanical digestions of fat tissue, with different outputs in terms of AD-SVF counting and fat maintenance, have been described in the literature.

Regarding enzymatic digestion, using the Celution® system, a study by Lin et al. [19] showed a cell number of 2.95×10^5 cells/mL with viability of 86.6%. In another research where different devices were compared, the Celution® system demonstrated the highest cell yield (2.41×10^5 cells/g) when compared to multi-station (1.07×10^5 cells/g), Lipo-Kit GT (0.35×10^5 cells/g), and CHA STATION™ (0.05×10^5 cells/g) as reported by Aronowitz et al. [20]. A-FG enhanced with AD-SVFs obtained by the Celution® system improved breast contour in a clinical trial performed by Perez-Cano et al. [21] for breast conservation therapy [21]. Using other kits and devices, AD-SVFs obtained by GID SVF-1™ were $7.19 \pm 2.11 \times 10^5$ nucleated cells/mL of dry fat tissue [22]. Khan et al. [23] described a procedure that resulted in a $\sim 6 \times 10^5$ cells/mL (66% viability) for donor 1 and $\sim 1 \times 10^6$ cells/mL (51% viability) for donor 2, while on the same way, Stubbers and Coleman [24] described a procedure for cell isolation yielding 4.9×10^6 – 24.7×10^6 total nucleated cells/100 g of fat harvested. Domenis et al. [25] analyzed three different kits (Lipo-Kit®, Celution®, and Fastem®), where A-FG enhanced with AD-SVFs, obtained by these procedures, increased thickness, and long-term volume maintenance of A-FG in outcomes of breast reconstruction when compared to not-enhanced A-FG.

Regarding the mechanical digestion based on filtration, centrifugation, washing, and purification of fat tissue, two different studies performed by Schafer et al. [26] and Zhu et al. [27] in which Puregraft® was used, displayed augmented tissue viability, and reduced quantity of red blood cells, free lipids, and contaminants when compared to other A-FG. Dos-Anjos Vilaboa et al. [28] reported the results obtained by fat tissue washed and purified via GID 700™ displaying significantly reduced amounts of lactate

dehydrogenase, triglycerides, and hematocrit maintaining the adipose graft osmolarity. AD-SVFs obtained by the filtration device LipiVage™ displayed endothelial and mesenchymal progenitor cells, maintaining their differentiation capacity when used as fibrin spray [29]. Ferguson et al. [30] additionally reported that LipiVage™ produced a higher number of adipocytes and sustained a higher level of intracellular enzyme (glycerol-3-phosphatase dehydrogenase (G3PDH)) activity within fat grafts. Research published by Bianchi et al. [31] reported that cells obtained from Lipogems® showed a significantly higher concentration of mature pericytes, AD-MSCs, exosomes, and a lower quantity of hematopoietic cells when compared to isolated cells with enzymatic digestion. Victor [32] created a procedure that aimed to isolate AD-SVFs by applying ultrasonic cavitation and yielding 1.67 – 2.24×10^7 cells with a viability of 97.1–98.9% while Bright et al. [33] promoted the dissociation of fat tissue by lysing mature adipocytes by applying ultrasonic cavitation with a yield of about 2–4 million cells/1 g fat. Schafer [34] focused on the possibility of isolating fat-derived cells using ultrasonic energy/acoustic standing waves, ultimately producing small ($<50 \mu\text{m}$), but more vital pre-adipocytes and stimulating angiogenesis. Gimble et al. [35] suggested an interesting and simple procedure based on the shaking and washing of fat and related AD-MSCs and AD-SVFs. Their process yielded 2.5×10^6 cells per 100 mL of fat and maintained a high osteogenic and adipogenic differentiation capacity as compared to a classic enzymatic digestion procedure.

Currently, a large consensus about the best mechanical and/or enzymatic process to obtain the greatest number of AD-SVFs, lack. At the same time, longer-term studies related to the ratio between the different procedures of A-FG enrichment/purification and fat volume maintenance analysis (3 years and more) should be performed. This is the first article in the literature comparing fat volume maintenance with the different AD-SVFs amount obtained from four different procedures, but it is strictly focused on fat maintenance analysis obtained 1 year after the last treatment. Longer-term A-FG maintenance analysis (3 years) has been previously published by the author Gentile P. [7, 10]. In the first work [7], patients affected by facial defects treated with A-FG enriched with AD-SVF showed 61% of volume maintenance after 3 years compared with patients treated with FG not-enhanced, who showed 31% maintenance. In the second work [10], the patients affected by breast defects, treated with A-FG enriched with AD-SVF, showed 58% of volume maintenance after 3 years compared with the patients treated with A-FG not-enhanced, who showed only 29% maintenance. In both studies [7, 10] enrichment with AD-SVFs obtained only by enzymatic digestion was described. The results

obtained in terms of A-FG maintenance between the present study and past studies were similar. In the present study, the volume fat maintenance after 1 year obtained by enzymatic digestion (SG-1) was higher at 63% compared to 52% of centrifugation with filtration (SG-2) ($p < 0.0001$), to 60% of filtration (SG-3) ($p < 0.0305$), and to 39% of centrifugation (CG) ($p < 0.0001$). In the past studies, respectively, 61% [7] and 58% [10] of fat volume maintenance after 3 years were observed after the last A-FG enriched with AD-SVFs obtained by enzymatic digestion. At the end of this analysis, it does not seem to be a significant difference in terms of volume maintenance between 1 year (63%) and 3 years (61–58%) when the same procedure of enrichment was used.

As reported in the present study, and as analyzed from the above-mentioned investigations and related articles, a lack of a single standardized protocol both for the A-FG enrichment or purification and isolation methods of AD-SVFs, must be considered as a limitation, as prevents having an accurate prognosis/predictability of the results in terms of A-FG maintenance. Several current technologies and methods in modern plastic surgery for the assessment of prognosis (linked to all fields of plastic surgery, as well as regenerative surgery, flap congestion, and its salvage, wounds, and ortho-plastic surgery) have been proposed as recently reported [36–39]. In detail, Winter et al. [36] investigated the effectiveness of the LACE+ index (i.e., length of stay, acuity of admission, Charlson comorbidity index, and emergency department visits in the past 6 months) predictive tool in patients undergoing plastic surgery. Guarro et al. [37] modified the TIME-H, with the aim of assessing the impact of skin lesions and soft tissues on the surgical patient. Boissiere et al. [38] analyzed several articles comparing many methods for managing flap-venous congestion, without having found a scientifically valid conclusion about their effectiveness. Again, Guarro et al. [39] performed a pilot study focused on the statistical analysis of 32 cases of wound measurements conducted by both the traditional method (paper ruler) both the digital smartphone analysis. Three morphological parameters were evaluated: width (expressed in cm), length (expressed in cm), and area (expressed in cm^2). They concluded that digital measuring systems should be easily addressed as versatile tools that could be applied in daily clinical practice in the future [39]. In regenerative plastic surgery, and in the use of A-FG, the morphological aspects analysis (for example the ratio between recipient area volume and fat volume to inject, both expressed in cm^3), through digital and instrumental tools, as suggested by Guarro et al. [39], could improve the results predictability.

Conclusions

In vitro AD-SVFs cell analysis indicated that filtration was the most efficient system, between mechanical digestions procedures, thanks to the highest number of cells obtained 33.333 ± 5650 AD-SVFs/mL (SG-3) producing in vivo, the most A-FG volume maintenance ($60\% \pm 5.0\%$) after 1 year. Enzymatic digestion (SG-1) produced the best number of AD-SVFs ($50,000 \pm 6956$ AD-SVFs/mL) and the best fat volume maintenance after 1 year ($63\% \pm 6.2\%$). Confirmatory studies will need to be completed.

Author Contributions PG designed the studies, performed the procedures, analyzed the results, wrote the paper, wrote an editing review, dealt with methodology and validation, performed the data analysis, and conducted the study as the leader. MGS performed the in vitro procedures, analyzed in vitro results, wrote an editing review, dealt with methodology and validation, performed the data analysis. DDF and CC helped in supervision and validation; AO and VC dealt with methodology and validation, and final approval of the manuscript.

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Declarations

Conflict of interest The authors declared no potential conflicts of interest concerning the research, authorship, and publication of this article.

Ethical Approval The protocols adopted were performed following the European rules (1394/2007 EC) and EMA/CAT recommendations (June 20, 2014, EMA/CAT/600280/2010 Rev 1). The patients involved in the study compiled and signed a detailed informed consent including all risks, benefits, and alternative procedures, before any treatment. This paper has been the object of a research contract between the author P.G. and the “Tor Vergata” University, approved by Rectoral Decree R.D n. #1467/2017, continued in associate professor contract #13489/2021.

Informed Consent All participants provided informed consent.

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