

## **Xeno-free enzymatic products for the isolation of human adipose-derived stromal/stem cells**

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**Running title:** Xeno-free collagenase for hASCs isolation

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***Abbreviation list:***

ASCs - Adipose-derived stromal/stem cells

BM – Bone marrow

BMSCs – Bone marrow stromal/stem cells

BSA - Bovine serum albumin

CD - Cluster of differentiation

FBS - Fetal bovine serum

GMP – Good Manufacturing Practices

hASCs – Human adipose-derived stromal/stem cells

MSCs – Mesenchymal stem cells

PBS - Phosphate buffered saline

SD – Standard deviation

SVF - Stromal vascular fraction

***Abstract:***

Human adipose stromal/stem cells (ASCs) are an abundant, readily available population of adult stem cells that reside in adipose tissue and that have a great potential utility for tissue engineering and regenerative medicine therapeutic applications. Several preclinical studies have shown that ASCs have therapeutic applicability, but a standardized isolation and expansion methodology for clinical cell therapy has yet to be established. ASC are typically isolated and expanded using reagents with xenogenic components and this may pose certain risks and safety issues, such as exposure to infectious agents and immune reactions, creating further obstacles to the translation of ASC based therapies to clinical scenarios. The objective of this study was to determine the suitability and efficacy of various alternative enzymatic products, CLS1 (Worthington), CLSAFA (Worthington), NB4 (SERVA), and Liberase (Roche), for the digestion of adipose tissue and subsequent isolation of ASCs, assessing cells functionality concerning their proliferation and differentiation ability. Results show that there are no statistically significant differences on yield and proliferation of cells isolated after enzymatic digestion with any of the studied products. The differentiation potential of the cells was not affected and cell surface

markers expression was similar amongst all products. We concluded that clinical grade products can replace current research grade products effectively in our cell isolation protocols without any negative effect in the yield or function of human ASCs.

### ***Introduction:***

Adipose-derived stromal/stem cells (ASCs) have been applied pre-clinically in diverse fields due to their abundance and therapeutic suitability, but standard isolation and expansion methods have not yet been established for obtaining clinical-grade cells. When compared with bone marrow-derived mesenchymal stromal/stem cells (BMSCs) that are present at low frequency in the bone marrow (BM), ASCs can be retrieved in high numbers from either subcutaneous lipoaspirates or resected adipose tissue, and can be easily expanded *in vitro*[1]. Standard methodologies for isolation and *in vitro* cultivation of ASCs utilize collagenase, fetal bovine serum (FBS), trypsin, and other reagents such as serum albumin and growth factors of animal origin. The use of animal-derived reagents in clinical cell therapies is sub-optimal because of safety issues. There have been reports of severe anaphylaxis and immune reactions in patients transplanted with human cells exposed to animal-derived reagents[2, 3]. Other possible risks include viral or bacterial infections, prions and as yet unidentified zoonoses[3, 4].

Different stem cell therapy studies using ASCs are under way[5, 6], which calls for a strong focus on the safety, reproducibility and quality of transplanted *in vitro* - expanded stem cells. By replacing collagenase and other animal-derived products with animal protein-free reagents the safety and quality of the transplanted stem cells may be enhanced significantly[7-9]. Tissue

dissociation enzymes are critical reagents that affect the yield and quality of isolated cells required for cell based research and clinical transplantation. Most published ASC isolation protocols employ collagenase yet contradictory results regarding its use have been reported[10-12]. For some collagenase products, the enzymatic composition shows lot-to-lot variability that may affect reproducibility[13-15]. The current study set out to compare the effect of different xeno-free enzymatic alternatives on human ASC with respect to cell proliferation frequency, morphology, multilineage differentiation capacity[16, 17], and surface marker expression profiles. To our knowledge, this is among the first comprehensive comparisons on ASCs processed using different collagenase products, and can be a helpful tool for the optimization and standardization of ASC based research amongst different research centers.

### ***Material and Methods:***

#### Donor Demographics

Adipose tissue specimens were obtained with informed consent from women undergoing elective liposuction ( $n=5$ ), under a previously established protocol with Hospital da Prelada, Porto, Portugal. The age range was 33–57 years (mean  $42.6 \pm 9.4$ ) years and body mass index (BMI; weight in kg/height in  $m^2$ ) 21.9 – 36.6 (mean  $29.2 \pm 6.7$ ).

The enzymatic products that were used in this study were the following:

- *Collagenase, Type 1 (CLS1)*. This is a crude collagenase product that contains average amounts of assayed activities (collagenase, caseinase, clostripain, and tryptic activities). Being a

research grade product, it may contain animal based components. [Worthington Biochemical Corporation, USA; Cat.No. LS004194]

- *Collagenase (Animal Origin Free)-A (AFA)*. This product is prepared in cultures grown in medium completely devoid of animal based components and designed for bioprocessing applications where introduction of animal derived pathogens must be prevented. [Worthington Biochemical Corporation, USA; Cat.No. LS004152].

- *Collagenase NB 4 Standard Grade (NB4)*. Purified blend of a balanced ratio of crude collagenase and other proteases. NB 4 Standard Grade is used in routine research protocols and is usually used to estimate efficacy of clinical grade GMP products that have similar enzymatic composition and properties, but are usually more expensive, such as Collagenase NB 6 GMP Grade. [SERVA Electrophoresis GmbH, Germany; NB 4 Cat.No. 17454; NB 6 Cat. No. 17458].

- *Liberase TM (Liberase)*. Xeno-free white lyophilizates consisting of aseptically filled, highly purified collagenase and neutral protease enzymes (isoforms I and II), and a small quantity of buffer salts. [Roche Applied Science, Portugal; Cat.No. 05401119001].

All samples of the enzymatic products were kindly provided by Worthington (USA), SERVA (Germany), and Roche (Portugal).

#### ASCs isolation and culture

ASC were isolated from human subcutaneous adipose lipoaspirate according to published methods with some minor modifications[18]. Lipoaspirate tissues were harvested by tumescent liposuction without ultrasound assistance from the abdominal region of subjects undergoing elective plastic surgery at Hospital of Prelada (Porto, Portugal). All protocols were conducted

with informed patient consent under a protocol approved and supervised by the Hospital Ethical Committee.

The tissue samples were transferred to the research laboratory within a closed plastic container maintained at room temperature in phosphate buffered saline (PBS) with 10% antibiotic/antimycotic and processed within 4 hrs after the surgical procedure which has been demonstrated to be within the optimal window of time[19]. . The lipoaspirate tissue was extensively washed with PBS at 37 °C to remove erythrocytes and then digested in PBS supplemented with the enzymatic product. The same volume of each collagenase was prepared to a final concentration of 0.2 Wünsch units (or 200 CDU) per milliliter of solution, according to manufacturer indications. Solutions of the enzymatic products selected for this study (described below), were used to digest equal volumes of lipoaspirate for 1 h at 37 °C with gentle agitation. Following room temperature centrifugation at 300 g for 5 min and re-suspension in stromal medium [DMEM/Hams F-12 medium (Hyclone, Logan, Utah, USA) supplemented with 10% FBS (Gibco, Invitrogen, USA) and 1% antibiotic/antimycotic], the stromal vascular fraction (SVF) cell pellet was plated at a density equivalent to 35 ml of lipoaspirate digest per T175 flasks (0.2 ml/cm<sup>2</sup>). In parallel, 10 ml of SVF were filtered (40 µm) and total nucleated cell counts were determined using a hemacytometer; all cell counts were performed uniformly by a single individual to avoid any variability in this parameter (P.P.C.).

After 24 h of incubation at 37 °C, 5% CO<sub>2</sub>, the adherent cells were washed with PBS at 37 °C and maintained in stromal medium until 80–90% confluence. This procedure was repeated for a total of 5 donors. Since the objective of this study is to evaluate cell recovery and behavior as a function of enzymatic digestion with each product, each tissue served as its own control, reducing the influence of donor to donor variability.

## Flow Cytometry

After the isolation procedure and subsequent 7 days in culture (confluence ~90%), cells were harvested using TrypLE Express without phenol red (Invitrogen, USA) [20], washed with PBS three times and aliquots of  $150 \times 10^3$  cells were incubated with monoclonal antibodies directed against: CD29 (eBioscience Cat. No. 14029982), CD 31 (Citomed Red Cat. No. FAB3567A), CD34 (BD Biosciences Cat. No. 555822), CD44 (BD Cat. No. 555479), CD45 (BD Cat. No. 555482), CD49f (eBioscience Cat. No. 17-0495-82), CD73 (BD Biosciences Cat. No. 550257), CD90 (BD Biosciences Cat. No. 559869), CD105 (eBioscience Cat. No. 16-1057-82) and CD146 (eBioscience Cat. No. 11-1469-42) for 20 min in the dark before being washed with PBS supplemented with 1% bovine serum albumin three times and fixed in 1% formaldehyde overnight at 4 °C [21-23]. For each sample,  $20 \times 10^3$  events were collected on a Becton Dickinson FACScalibur flow cytometer using CELLQuest acquisition software (Becton Dickinson) and analyzed using CELLQuest software (Becton Dickinson). This antibody panel was selected, in part, based on the International Society for Cell Therapy (ISCT) position paper on the criteria for defining mesenchymal stem cells/multipotent stromal cells (MSCs) [24].

## Differentiation Potential

### *Adipogenic differentiation*

Confluent cultures of ASCs (P1) were induced with adipogenic differentiation medium [DMEM/Hams F-12, 3% FBS, 1% antibiotic/antimycotic, 0.5 mM isobutylmethylxanthine, 33 mM biotin, 17 mM pantothenate, 5  $\mu$ M troglitazone (Sigma), 1 mM dexamethasone, 10 mM insulin] for 3 days before being converted to adipocyte maintenance medium (identical to adipogenic differentiation medium without isobutylmethylxanthine and troglitazone) [18, 25]. Cells were maintained for 9 days before fixation and oil red O staining.

#### *Osteogenic differentiation*

Other confluent cultures of ASCs were converted to osteogenic medium (DMEM/Hams F-12 or DMEM, 10% FBS, 1% antibiotic/antimycotic, 10 mM  $\beta$ -glycerophosphate, 50  $\mu$ g/ml sodium 2-phosphate ascorbate,  $10^{-8}$  M dexamethasone) and maintained in culture for 12 days with medium changes every third day. These cultures were rinsed 3 times with 150 mM NaCl, fixed in 70% ethanol, and stained with alizarin red [26].

#### *Chondrogenic differentiation*

For chondrogenic differentiation,  $5 \times 10^5$  ASC (P1) were resuspended in chondrogenic medium and centrifuged at 800 G for 5 min, in order to obtain cell pellets. Pellets were cultured in the same medium for 21 days at 37  $^{\circ}$ C and 5 % CO<sub>2</sub>, in 15 ml falcon tubes, with medium changes twice a week. Chondrogenic medium was composed of DMEM High Glucose, sodium bicarbonate, 1% antibiotic/antimycotic, 0.17 mM ascorbic acid, 10 ng/ml TGF- $\beta$ 3 (Human Transforming Growth Factor Beta 3), 10% Insulin–Transferrin–Selenium (ITS), 35 mM L-proline and 0.1 M sodium pyruvate. After fixation with formalin for 30 minutes, the pellets of

ASC were kept in PBS at 4 °C until being embedded in paraffin and sliced for histological staining with Hematoxylin-Eosin, Safranin O and Alcian Blue/Neutral Red.

### Statistical Methods

Values are presented as the mean  $\pm$  SD. Data was analyzed for normality using the Shapiro-Wilk test. Results were analyzed by ANOVA and Bonferroni posttest evaluated significance.

## ***Results:***

### ***Cell Yield and Viability***

Our first assessment compared the yield and viability of total nucleated cells recovered from the SVF and human ASC cultured for 7 days, until reaching 80-90% confluence, after enzymatic digestion of lipoaspirate samples with each collagenase product (total of 5 donors). The number of recovered cells and their viability were not significantly different amongst any of the products (Figure 1, A and B). Concurrently, cell morphology was observed by light microscopic examination (Figure 1, C). No morphologic differences were found in cells processed with different collagenase products throughout the entire culturing time.

### ***Immunophenotype***

Next, studies evaluated the immunophenotype of ASC cultured after being processed with each of the collagenase products. A subset of well characterized human ASC cell surface markers

(Figure 3) was examined by flow cytometry [21, 23, 27]. Consistent with previously published results [23], the human ASC displayed strong positivity for adhesion markers CD29 ( $\beta_1$  integrin), and CD105 (endoglin), CD73 (5'ecto-nucleotidase), and the extracellular matrix protein CD90 (Thy-1). More important, we could not find any significant difference in the expression of any of the analyzed markers amongst cultured ASCs independent of the collagenase products used for the initial tissue digestion.

### ***Differentiation potential***

We evaluated the differentiation potential of ASC isolated by tissue digestion with each collagenase product, by staining with Oil Red O (adipogenic), Alizarin Red (osteogenic), Safranin O and Alcian Blue/Neutral Red (chondrogenic). None of the enzymatic products used had any effect on the ability of ASC to differentiate along adipocyte, osteoblast or chondroblast lineage pathways (Figure 3). All observations, both microscopic and macroscopic were conducted by the same person (PPC) in order to minimize variability in the analysis.

### ***Discussion and Conclusion***

In order to use adult stromal/stem cells in human subjects for regenerative medicine therapies, we need to take into consideration several criteria, namely the safety, efficacy, reproducibility and quality control. Consequently, clinical cell based therapies that employ xenogenic reagents may prove to be an unsuitable option with respect to patient safety and represent a high risk of infection and severe immune reactions in the patient.

The results obtained from the present study indicate that human lipoaspirate tissue samples can be successfully processed using different enzymatic products, without substantial differences in

the yield of both SVF cells and ASCs. The total number of recovered cells and their viability were not significantly different amongst the different products used to digest adipose tissue samples. Although culture medium containing FBS was used to conduct this study, previous work[28] reported comparable results when using serum-free and xeno-free culturing conditions. Therefore, we believe that the FBS containing medium had no effect on ASCs behavior after the isolation process. Furthermore, the assessment of the total nucleated cells present in the SVF established the efficacy of the enzymatic products used to digest lipoaspirate samples.

Realizing that any of the products was equally effective retrieving ASCs from lipoaspirate samples, we needed to evaluate if cells would be altered by any of these products and the enzymatic digestion process itself. Flow cytometry assessed the immunophenotype of ASC obtained with the different enzymatic products, through a panel of cell surface markers. The human ASC displayed strong positivity for the stromal markers CD29 ( $\beta_1$  integrin), CD73 (5' ecto-nucleotidase), CD90 (Thy-1) and CD105 (endoglin) when processed with any of the enzymatic products. Moderate expression of CD 44, CD49f and the hematopoietic progenitor cell marker CD34 was found in all ASCs. Consistently with published reports [23] low expression of CD31, CD 45, and CD146 was found. The results were consistently similar amongst all products.

Further experiments were carried out for evaluation of the differentiation potential of ASC obtained upon tissue digestion with each product being assessed, by staining with Oil Red O (adipogenic), Alizarin Red (osteogenic), and Alcian Blue/Neutral Red and Safranin O (chondrogenic). The product used had no effect on the ability of ASC to differentiate along any of the different lineage pathways analyzed.

Extensive characterization of stem cells is essential prior to their clinical use, as different culture conditions may change the characteristics of the cells. The results obtained under the present study show that ASC isolated after enzymatic digestion using different collagenase products display similar phenotypes based on their differentiation ability and surface marker expression, in conformity with previously published reports [21, 29].

These results show that highly purified and non-animal sources of collagenase suitable for Current Good Manufacturing Process (cGMP) perform as effectively as our routine laboratory research grade products. Thus, these clinical grade reagents can be substituted into existing protocols without loss of yield or function of human ASC during isolation from adipose tissue samples. Furthermore, a more intensive production of these products due to their generalized use would provide more suitable cost-effective alternatives for stem cell research laboratories. To use adherent human stromal/stem cells clinically, it will be advantageous to eliminate all potential sources of xeno-protein contamination. These studies will have relevance for optimizing cGMP methods as the use of human ASC in tissue engineering and regenerative medicine grows.

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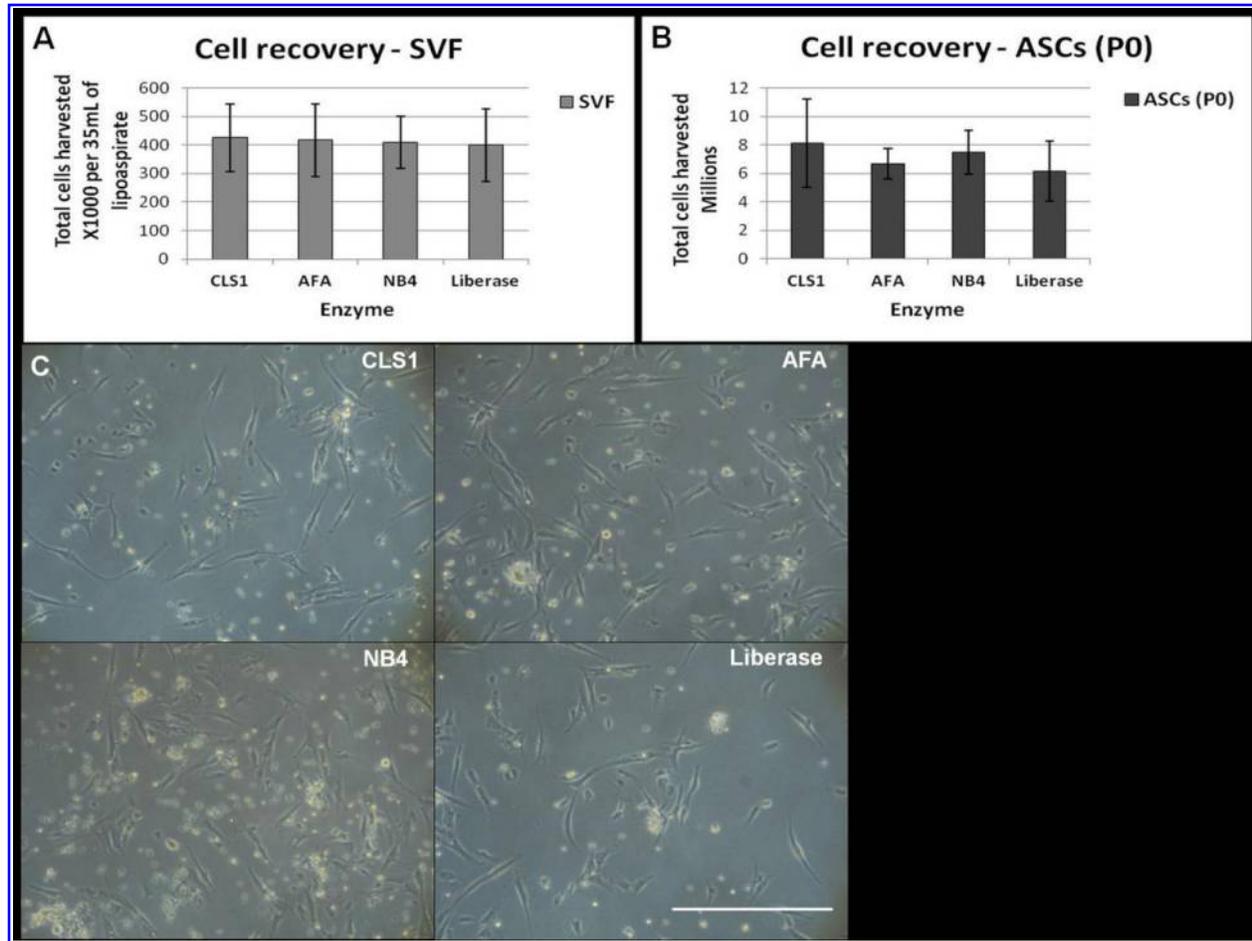
Germany; and Roche Applied Sciences, Portugal for kindly providing samples of their products for this study.

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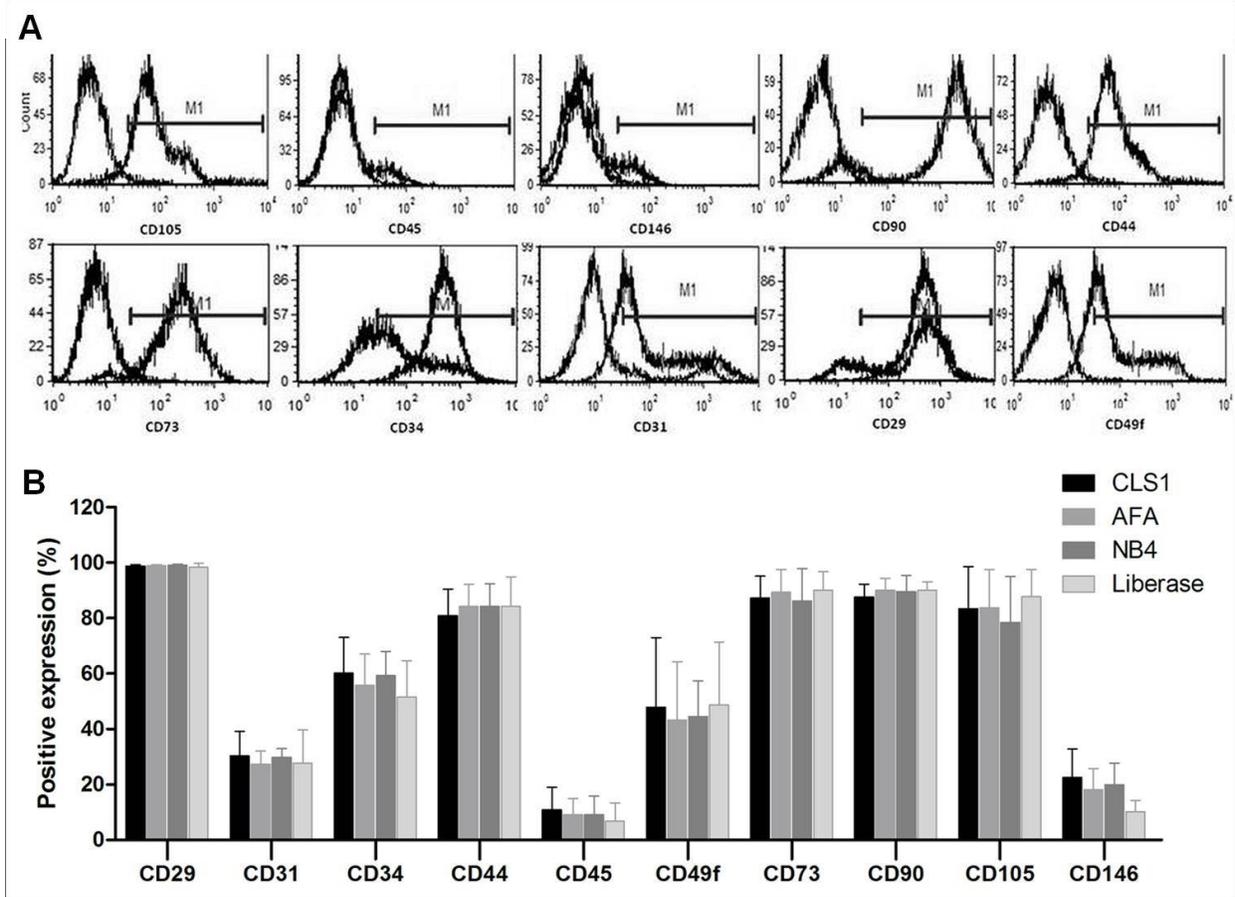
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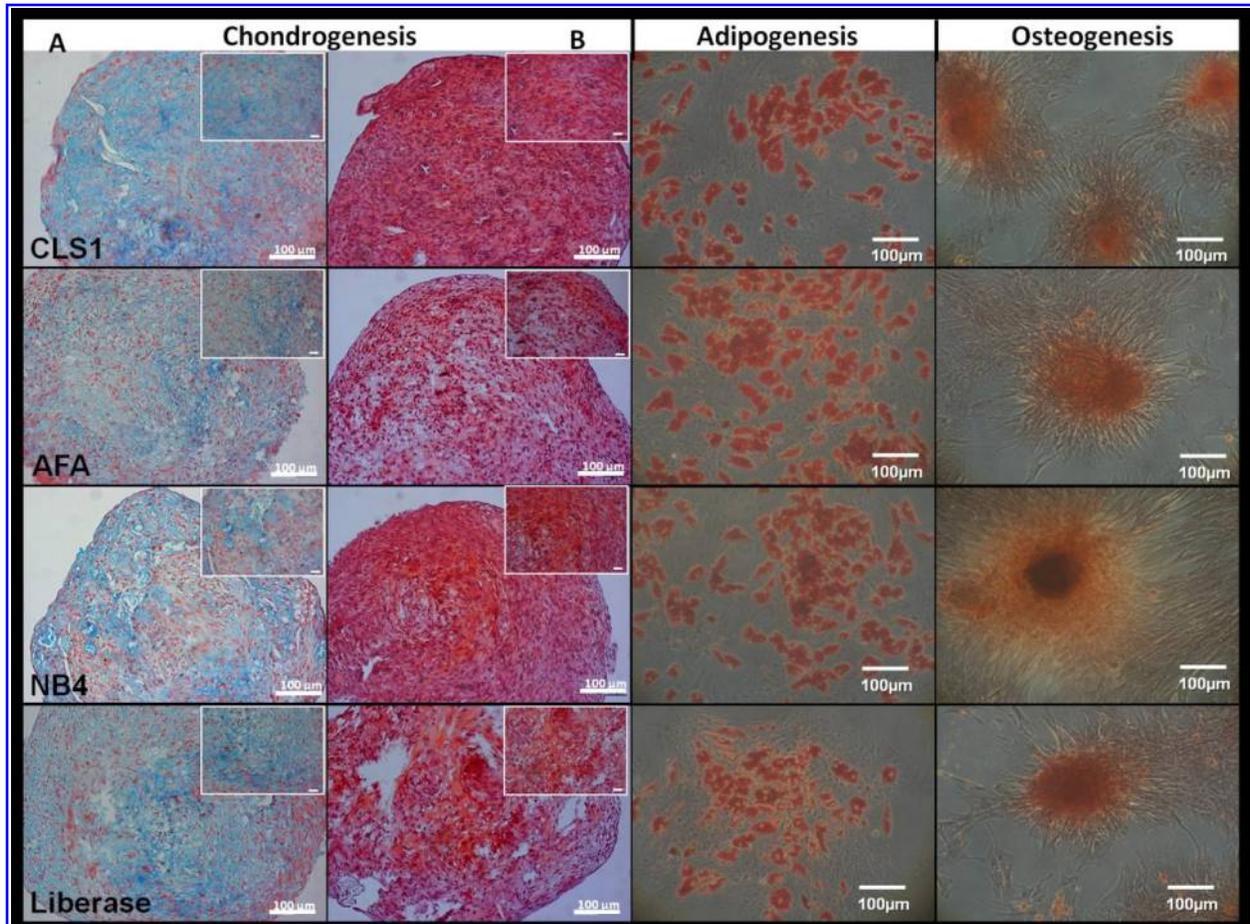


**Fig. 1 – A and B - Chart representation of the total number of cells recovered from SVF and cultured ASCs.** Total of 5 donors ( $n = 5$ ); values presented as mean  $\pm$  standard deviation (error bars); no significant difference amongst any of the collagenase products ( $p < 0.05$ ). Y axis – total cells harvested per 35 mL of lipoaspirate. On the left, total number of nucleated cells recovered immediately after digestion with each enzymatic product; on the right, total number of ASCs recovered after 7 days in culture (80-90% confluence). **C– ASCs morphology.** Light microscopy photographs of ASCs at passage 0, after 3 days in culture. From left to right and up to down: cells obtained upon adipose tissue digestion with CLS1, AFA, NB4 and Liberase (representative of one donor). White bar represents 100  $\mu\text{m}$ .





**Fig. 2 – Immunophenotype by Flow Cytometry analysis.** Top panel (A): representative histograms of one donor for the CLS1 product. Low panel (B): chart with selected panel of cell surface markers; no significant difference across any of the collagenase products ( $p < 0.05$ ); values presented as mean  $\pm$  standard deviation (error bars); a total of 5 samples was analyzed ( $n = 5$  donors).



**Fig. 3 – Light microscopy photographs of ASCs induced to differentiate into different lineages.** **A-** ASCs pellets cultured for 21 days in chondrogenic medium, stained with Alcian Blue and counterstained with Neutral Red; **B** – same ASCs pellets stained with Safranin O (upper right corner showing higher magnification [white bar represents 30 μm]). Adipogenic differentiation is evidenced by Oil Red O staining. Osteogenic differentiation is evidenced by Alizarin Red staining.

