bioRxiv preprint doi: https://doi.org/10.1101/485318; this version posted December 3, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Isolation of adipose tissue derived regenerative cells from human subcutaneous tissue with or without the use of an enzymatic reagent

Nick Valenzuela[1], Christopher Alt[1], Glenn E. Winnier[1], Eckhard U. Alt[1-4]

[1] *InGeneron, Inc., Houston, TX, USA*
[2] *Heart and Vascular Institute, Department of Medicine, Tulane University Health Science Center, New Orleans, LA, USA*
[3] *Sanford Health, University of South Dakota, Sioux Falls, SD, USA*
[4] *Isar Klinikum Munich, Munich, Germany*

Background: Freshly isolated, unmodified autologous adipose derived regenerative cells (ADRCs) (also named Stromal Vascular Fraction; SVF) have emerged as a promising tool for regenerative cell therapy. Due to regulatory concerns recently the use of cells recovered by non-enzymatic processing of adipose tissue has been suggested. The Transpose RT system (InGeneron, Inc., Houston, TX, USA) is a commercially available system for isolating ADRCs from adipose tissue. This system makes use of the proprietary, enzymatic Matrase Reagent for isolating cells. It is the aim of this study to address the question how and to which extent the use of Matrase Reagent would influence cell yield, cell recovery, cell viability, biological characteristics, physiological functions or structural properties of the ADRCs in the final cell suspension when compared to isolation of ADRCs from adipose tissue under otherwise identical conditions, but without the use of the Matrase Reagent.

Methods: Subcutaneous adipose tissue from 12 subjects undergoing elective lipoplasty was processed using the Transpose RT system. Identical samples of 25 g tissue were processed either with or without the use of Matrase Reagent. Then, characteristics of the ADRCs in the respective final cell suspensions were evaluated with SVF yield statistics, microscopic inspection, CFU assays, embryoid body formation, differentiation assays and quantitative PCR.

Results: Cell yield in numbers of viable cells/gram of processed lipoaspirate was approximately twelve times higher in the final cell suspension when ADRCs were isolated from lipoaspirate with the use of Matrase Reagent ($p<0,001$), compared to the final cell suspension that was obtained when ADRCs were isolated from lipoaspirate just mechanically without the use of enzyme. Furthermore, cells isolated from lipoaspirate with the use of Matrase Reagent formed on average 16 times more CFUs per g lipoaspirate compared to cells processed without enzyme. On the other hand, cells isolated with the use of Matrase Reagent exhibited no statistically significant differences in the expression of regenerative cell-associated genes such as Oct4, Hes1 and Klf4 compared to cells isolated without enzyme. Also, cells isolated from lipoaspirate both with and without the use of Matrase Reagent were independently able to differentiate into cells of all three germ layers (i.e., into the adipogenic, osteogenic, hepatogenic and neurogenic lineages).

Conclusion: The process of isolating ADRCs from lipoaspirate with the use of Matrase Reagent significantly increased the yield in cell numbers and cell viability compared to just mechanical processing. Biological characteristics, physiological functions or structural properties relevant for the intended use (i.e., regeneration, repair or replacement of weakened or injured tissue) were not altered or induced using Matrase Reagent. The use of enzyme did not manipulate the cells as expression of embryonic genes and pluripotent differentiation capacity was equally present in cells recovered with or without enzyme.

Correspondence
Eckhard U. Alt MD, PhD
Heart and Vascular Institute
Department of Medicine
Tulane University
Health Science Center
1340 Tulane Ave.
New Orleans
LA 70112, USA
Phone: +1 (504) 988 3040
Mobil: +1 (832) 853 3898
E-mail: ealt@tulane.edu
ealtmd@aol.com

bioRxiv preprint doi: https://doi.org/10.1101/485318; this version posted December 3, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

INTRODUCTION

Regenerative cell therapy, which refers to the therapeutic application of stem cells to repair diseased or injured tissue, has received increasing attention from basic scientists, clinicians and the public. Stem cells hold significant promise for tissue regeneration due to their innate ability to provide a renewable supply of progenitor cells that can form multiple cell types, whole tissue structures, and even organs.

Intensive basic research over the last decade has shown that the following factors mainly influence the effects of cell-based therapies: cell type, cell dose, cell delivery route, cell delivery timing and potential augmentation of cellular survival and/or function by physical stimulation, chemical/pharmacological treatment and genetic modification (e.g., Madonna et al., 2016; Wu et al., 2018). However, it is critical to note that particularly the selection of cell types and the augmentation of cellular survival and/or function are restricted by human safety issues. For example, embryonic stem cells (ESCs), which can be derived from the blastocyst stage of mammalian embryos, exhibit remarkable developmental plasticity that enables them to differentiate into all types of cells present in the body (Vazin and Freed, 2010). However, several major issues, including ethical concerns and the risk of teratoma formation, have limited research and clinical application of therapies based on ESCs (c.f., e.g., Fujikawa et al., 2005; Swijnenburg et al., 2005; King and Perrin, 2014). Substantial safety concerns such as tumorigenesis also severely limit the clinical translation of induced pluripotent stem (iPS) cells (Ahmed et al., 2011; Zhang et al., 2011). Moreover, clinical studies applying allogenic cells in cell-based therapies reported the production of donor-specific antibodies (Panés et al., 2016; Alvaro-Gracia et al., 2017), which is not the case when using autologous cells.

Primarily due to these issues a major interest now largely shifted to stem cells derived from postnatal and adult tissues. Most prominently, researchers have identified in the vascular location of all organs and tissues a universal type of adult stem cell, commonly referred to as mesenchymal stem cells (MSCs) that exhibit significant potential for differentiation into a number of cell types (c.f., e.g., da Silva Meirelles et al., 2006; Bai et al., 2007; Ilmer et al., 2014). Although these adult stem cells are essentially ubiquitous, isolation from critical organs such as bone marrow, skin, skeletal muscle, liver, heart or brain has limited practicality. This is due to the fact that such isolation carries a potential risk of damage to the donor and often does not yield enough cells. As such, it is typically required to expand cells in culture in order to obtain the desired number of cells for the respective therapeutic application.

A highly attractive alternative with great clinical significance is to isolate these adult stem cells from adipose tissue. Cells derived from adipose tissue, either freshly isolated (named Stromal Vascular Fraction (SVF) or more aptly Adipose Derived Regenerative Cells (ADRCs)), or culture-expanded (named Adipose Derived Stem Cells (ASCs)) have emerged as a promising tool for regenerative cell therapy. Both, ADRCs and ASCs provide certain advantages over other cell types for cell-based therapies. Specifically, adipose tissue can have a significantly higher stem cell density than bone marrow (5 to 10% vs. 0.1%) and harvesting adipose tissue can be less invasive than harvesting bone marrow (Aust et al., 2004; Yu et al., 2017). Compared to ASCs, the use of fresh, uncultured ADRCs allows immediate usage at point of care, combined with low safety concerns as no culturing or modification are applied.

A number of enzymatic and non-enzymatic systems for isolating ADRCs were developed (for a current overview see Oberbauer et al., 2015). The reported cell yield after some of these different procedures varied considerably (Van Dongen et al., 2018). Furthermore, it was shown that in general, enzymatic isolation of ADRCs yielded more cells than non-enzymatic (mechanical) isolation (Condé-Green et al., 2016). However, to our knowledge no study has addressed the question how and to which extent the use of an enzymatic reagent in isolating ADRCs from adipose tissue would influence the cell yield, cell recovery, cell viability, biological characteristics, physiological functions or structural properties of the ADRCs in the final cell suspension when compared to isolation of ADRCs from adipose tissue with identical laboratory hardware but without the use of an enzymatic reagent.

The aim of the present study was to test the hypothesis that isolation of ADRCs from adipose tissue with the use of an enzymatic reagent results in significantly higher cell yield, cell recovery and cell viability than isolation of ADRCs from adipose tissue with identical laboratory hardware but without the use of an enzymatic reagent, but would not influence biological characteristics, physiological functions or structural properties of the ADRCs in the final cell suspension. The study was carried out with the commercially available, proprietary Matrase Reagent and the Transpose RT system (both from InGeneron, Inc., Houston, TX, USA).

MATERIALS AND METHODS

Isolation of cells from subcutaneous adipose tissue

Subcutaneous adipose tissue was obtained by a medical practitioner from subjects via lipoaspiration undergoing elective lipoplasty according to standard procedures with informed consent. N=12 subjects (and three additional subjects for testing of residual collagenase activity in the final cell suspension) were consented according to the IntegReview IRB approved protocol #200601001 (IntegReview IRB, Austin, TX, USA). Subjects' ages

bioRxiv preprint doi: https://doi.org/10.1101/485318; this version posted December 3, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

ranged between 32 and 59 years (Table 1).

Table 1 | Subjects' demographics

Subject #	Age	Sex	Race
1	36	Female	Caucasian
2	59	Female	Caucasian
3	46	Female	Caucasian
4	49	Female	Caucasian
5	37	Female	Caucasian
6	36	Female	Caucasian
7	39	Female	Black
8	36	Female	Caucasian
9	32	Female	Hispanic
10	32	Female	Hispanic
11	38	Female	Caucasian
12	42	Female	Caucasian

1

2

3

A sample of recovered lipoaspirate from each subject was divided into two equal parts of 25 ml each, and was processed for 30 minutes either with the use of Matrase Reagent (InGeneron) or only mechanically without the use of Matrase Reagent under otherwise identical conditions with the Transpose RT System of InGeneron, Inc. Processing was performed as described in the tissue processing procedure section found in the 11011E Transpose RT Instructions for Use (11011-01 IFU; InGeneron, Inc.).

After 30 minutes initial processing time, the processed lipoaspirate solution was filtered through a 200 µm filter and centrifugated for 5 minutes in order to separate cells from the rest of the tissue. The SVF/ ADRCs were extracted using a 3 ml syringe and washed twice with lactated Ringer solution by repetitive centrifugation for 5 minutes. The final cell suspension was analyzed for cell yield, cell viability and cell size. The respective differentiation capacity into the three germ layers was assessed for both ADRCs that were isolated from lipoaspirate with the use of Matrase Reagent and without the use of Matrase Reagent.

SVF yield statistics, expansion, and cryopreservation

Fresh SVF was centrifuged at 600g at room temperature for 5 minutes and resuspended in 2 ml of media for counting. Cell counts and viability were determined using the NucleoCounter NC-200 device (ChemoMetec Inc., Bohemia, NY, USA) as described by the manufacturer's protocol.

For expansion, SVF was plated at a cell density of $7x10^6$ in T75 tissue culture flasks in 15 ml of MesenCult MSC basal media (Stem Cell Technologies, Cambridge, MA, USA) supplemented with MesenCult™ MSC Stimulatory Supplement, PenStrep (Gibco, Waltham, MA, USA), Fungizone (Life Technologies, Carlsbad, CA, USA), L-Glutamine (Corning Life Sciences, Tewksbury, MA, USA), at 37° C under 5% CO_2. Cells were passed once reaching 75% confluency using 0.25% Trypsin (Sigma-Aldrich, St. Louis, MO, USA) for 5 min at 37° C. All non SVF passages were plated at a cell density of $7.5x10^5$ in T75 tissue culture flasks in 15 ml of complete MSC media. Cells were cryopreserved by resuspending ASC cell pellets in Prime-XV MSC FreezIS DMSO-free media (Irving Scientific, Santa Ana, CA, USA) at cell densities between $2x10^6$ and $5x10^6$ cells/ml. The samples were frozen overnight at -80 °C in freezing containers designed to cool at a rate of -1° C/min (Mr. Frosty, Thermo Scientific, Waltham, MA, USA).

Microscopy

Brightfield images were obtained using a Zeiss Axio inverted microscope (Carl Zeiss Microscopy, Jena, Germany) equipped with 10x and 20x objectives, Leica DMC4500 camera (2560 × 1920 pixels; Leica Microsystems, Wetzlar, Germany) and Leica Application Suite software (version X 3.3.3.16958; Leica). The final figures were constructed using Corel Photo-Paint X7 and Corel Draw X7 (both versions 17.5.0.907; Corel, Ottawa, Canada). Only minor adjustments of contrast and brightness were made using Corel Photo-Paint, without altering the appearance of the original materials.

CFU assays

Freshly isolated SVF/ADRCs from each subject were plated into a 6-well plate (Corning Life Sciences) at two different cell densities. The cells were plated at densities of 50,000 and 100,000 total cells in 2 ml of complete MSC media in each well; these experiments were repeated in triplicate. Cells were incubated at 37° C under 5% CO_2 for 14 days to allow CFUs to form. Media was changed every 2-4 days. After day 14 the media were aspirated, cells were washed twice in PBS and fixed in 2 ml of 10% formalin at room temperature for 30 min with gentle rocking. Cells were then washed three times with DI water and stained with 2 ml hematoxylin (Bio-Rad) for 15 min at room temperature. The cells were then washed with warm tap water until the wash ran clear. CFUs were quantified by manually counting the entire well; each sample was counted at both cell concentrations and in triplicate. Colonies containing > 50 fibroblast colony-forming units (CFU-F) were counted. CFU-F frequency was calculated by dividing the number of colonies by the number of seeded cells.

Embryoid Body formation

Embryoid bodies are defined as spherical clusters of both pluripotent and committed stem cells that can organize in a developmental-specific manner and give rise to mature cells from any differentiation lineage (reviewed in, e.g., Brickman and Serup, 2017).

bioRxiv preprint doi: https://doi.org/10.1101/485318; this version posted December 3, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

To form embryoid bodies, freshly isolated ADRCs were plated into ultra-low adherent flasks (Corning Life Sciences) at a density of 60,000 cells/cm^2 in GMEM (Gibco) supplemented with 2 mM L-Glutamine (Corning Life Sciences), non-essential amino acids (Sigma-Aldrich), B27 (Life Technologies), 0.6% glucose (Sigma-Aldrich), 10 ng/ml human bFGF (Invitrogen Life Technologies), 20 ng/ml human EGF (Life Technologies), 1 U/ml human thrombin (EMD Millipore, Burlington, MA, USA) and 2 µg/ml ciprofloxacin (Sigma-Aldrich). Cells were incubated at 37° C under 5% CO_2 and half of the media was changed every 3 days.

Differentiation Assays

ASCs on their 3rd passage were assayed for differentiative potential into adipogenic, osteogenic, hepatogenic and neurogenic cell lines.

Adipogenic Differentiation – ASCs on their 3rd passage were plated onto a 12 well plate at 40,000 cells per well in 1.5 ml of complete MSC media and allowed to grow for 2 days. On day 3 all of the media was aspirated and replaced with either complete MSC or StemPro Adipogenic differentiation medium (Life Technologies) and incubated for 2 weeks, changing media every 3-4 days. Then, the presence of intracytoplasmic lipids (triglycerides) was assessed with Oil red-O staining as previously described (Yoshimura et al., 2006). Percentage of adipocytes was calculated by microscopic inspection. The percentage of adipocytes was determined by calculating the ratio of Oil red-O positive cells versus total cells.

Osteogenic Differentiation – ASCs on their 3rd passage were plated onto a 12 well plate at 20,000 cells per well in 1.5 ml of complete MSC media and allowed to grow for 2 days. On day 3 all of the media was aspirated and replaced with either complete MSC or StemPro Osteogenic Differentiation media (Life Technologies) and incubated for 2 weeks, changing media every 3-4 days. Then, the presence of calcific deposits was investigated with Alizarin red staining (Alfa Aesar, Haverhill, MA, USA) as described in the protocol.

Hepatogenic Differentiation – ASCs_on their 3rd passage were plated onto a 12 well plate at 20,000 cells per well in 1.5 ml of MesenCult media (Stem Cell Technologies) and allowed to grow for 2 days. Hepatogenic differentiation was achieved using the Human Mesenchymal Stem Cell Hepatogenic Differentiation Medium kit (Cyagen, Santa Clara, CA, USA). Then, the presence of structures containing a high proportion of carbohydrate macromolecules (glycogen, glycoprotein, proteoglycans) was investigated with Periodic Acid Schiff staining (Sigma-Aldrich) as described in the protocol.

Neurogenic Differentiation – ASCs_on their 3rd passage were plated onto a 12 well plate at 40,000 cells per well in 1.5 ml of MesenCult media (Stem Cell Technologies) and allowed to grow for 2 days. The media was then replaced with DMEM (Sigma-Aldrich) supplemented with 10 µM Forskolin (Stem Cell Technologies), 10 ng/ml bFGF (Life Technologies), 10ng/ml NGF (Invitrogen), and 1% FBS. Media was replaced every 3 days for 1 week. Cells were visualized by hemotoxylin (Sigma-Aldrich) staining as described in the protocol.

RNA isolation and Quantitative PCR

RNA isolation was performed using Trizol (Life Technologies) in accordance with the manufacturer's protocol. Total RNA was purified using the Direct-Zol RNA miniprep kit (Zymo Research, Irvine, CA, USA) as described in the protocol. cDNA was generated using iScript Reverse Transcriptase SuperMix (Bio-Rad Laboratories, Hercules, CA, USA) and relative mRNA levels were measured using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories) according to the manufacturer's protocols. Qualification of RNA and cDNA was performed using a NanoDrop spectrometer (Thermo Fisher Scientific). Primer probe sets were custom oligos (all from Sigma-Adrich) (Table 2).

Table 2 | Primer probe sets used in the present study

Gene	Oligos
Oct4	Oct4-F: 5′-GCAAGCCCTCATTTCACCA-3'
	Oct4-R: 5′-GCCCATCACCTCCACCAC-3′
Klf4	Klf4-F: 5'-AAGAGTTCCCATCTCAAGGCACA-3'
	Klf4-R: 5'-GGGCGAATTTCCATCCACAG-3'
Hes1	Hes1-F: 5'-CCTGTCATCCCCGTCTACAC-3'
	Hes1-R: 5'-CACATGGAGTCCGCCGTAA-3'
GAPDH	GAPDH-F: 5'-CGCTCTCTGCTCCTCCTGTT-3'
	GAPDH-R: 5'-CCATGGTGTCTGAGCGATGT-3'

GAPDH was used for normalization.

Testing for residual collagenase activity in cell preparations prepared with the use of Matrase Reagent

In this experiment ADRCs were isolated from lipoaspirate from n=3 additional subjects using the ARC system (InGeneron) and Matrase Reagent following the manufacturer's instructions for use. The resulting cell preparations were tested for collagenase activity using a commercially available assay (EnzChek™ Gelatinase/Collagenase Assay Kit; Invitrogen, Carlsbad, CA, USA).

Statistical analysis

Mean and standard error of the mean (SEM) were calculated for all variables. The D'Agostino and Pearson omnibus normality test was used to determine whether the distribution of the investigated variables of the cells

bioRxiv preprint doi: https://doi.org/10.1101/485318; this version posted December 3, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

isolated with Matrase Reagent and the cells isolated without Matrase Reagent were consistent with a Gaussian distribution. Differences between the groups of cells were tested with nonparametric Wilcoxon matched-pairs signed rank test. In all analyses, an effect was considered statistically significant if its associated p value was smaller than 0.05. Calculations were performed with GraphPad Prism (Version 5; Graph Pad Software, San Diego, CA, USA).

RESULTS

Cell yield, cell viability and cell size

Compared to the isolation of cells from lipoaspirate without the use of Matrase Reagent, isolation of cells from lipoaspirate with the use of Matrase Reagent resulted in the following, statistically significant differences in the final cell suspension (all values given as mean ± SEM): 1) approximately nine times higher mean number of cells per gram of lipoaspirate (cell recovery: $7.2\times10^5 \pm 0.90\times10^5$ cells/g with the use of Matrase Reagent, and $0.84\times10^5 \pm 0.10\times10^5$ cells/g without the use of Matrase Reagent; $p < 0.001$; n=12 matched pairs of samples) (Fig. 1A); 2) approximately twelve times higher mean live cell yield per gram of lipoaspirate ($6.25\times10^5 \pm 0.79\times10^5$ cells/g with the use of Matrase Reagent, and $0.53 \pm 0.08 \times 10^5$ cells/g without the use of Matrase Reagent; $p < 0.001$; n=12 matched pairs of samples each) (Fig. 1B); and 3) approximately 41% higher mean cell viability (85.9% ± 1.1% with the use of Matrase Reagent, and 61.7% ± 2.6% without the use of Matrase Reagent; $p < 0.001$; n=12 matched pairs of samples) (Fig. 1C).

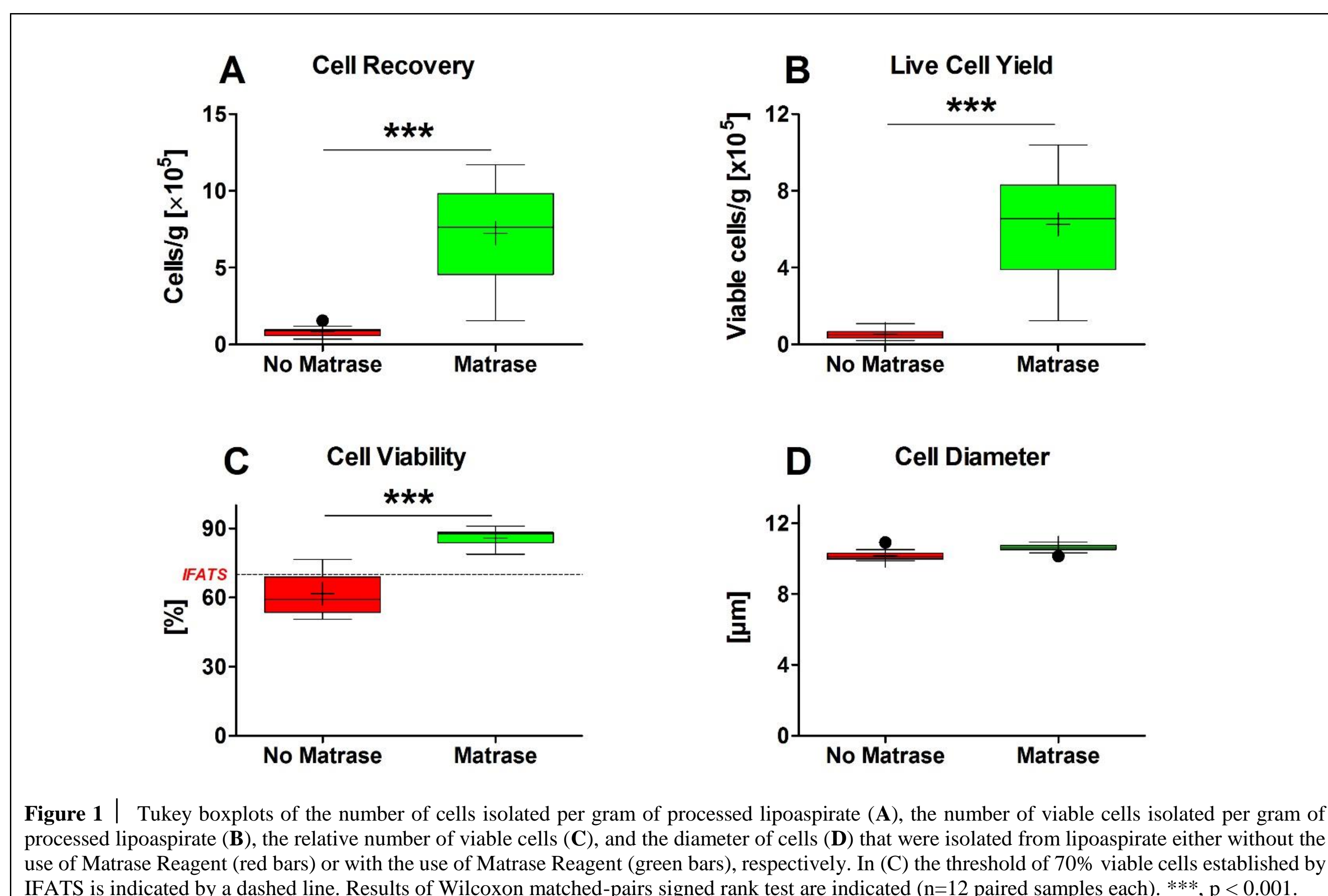

Figure 1 | Tukey boxplots of the number of cells isolated per gram of processed lipoaspirate (**A**), the number of viable cells isolated per gram of processed lipoaspirate (**B**), the relative number of viable cells (**C**), and the diameter of cells (**D**) that were isolated from lipoaspirate either without the use of Matrase Reagent (red bars) or with the use of Matrase Reagent (green bars), respectively. In (C) the threshold of 70% viable cells established by IFATS is indicated by a dashed line. Results of Wilcoxon matched-pairs signed rank test are indicated (n=12 paired samples each). ***, $p < 0.001$.

Of importance, the mean relative number of viable cells obtained by isolating ADRCs from lipoaspirate with the use of Matrase Reagent (85.9%) exceeded the proposed minimum threshold for the viability of cells in the SVF of 70 % established by the International Federation for Adipose Therapeutics and Science (IFATS) (Bourin et al., 2013), whereas the mean relative number of viable cells obtained by isolating ADRCs from lipoaspirate without the use of Matrase Reagent (61.7%) did not (Fig. 1C).

The difference in mean cell diameter between the cells obtained by isolating ADRCs from lipoaspirate with the use of Matrase Reagent and the cells obtained by isolating ADRCs from lipoaspirate without the use of Matrase Reagent was only approximately 4% (10.6µm ± 0.1µm

bioRxiv preprint doi: https://doi.org/10.1101/485318; this version posted December 3, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

with the use of Matrase Reagent, and 10.2μm ± 0.1μm without the use of Matrase Reagent; p = 0.05; n=12 matched pairs of samples) (Fig. 1D).

Accordingly, both the number and viability of cells in the final cell suspension were statistically significantly higher after isolating ADRCs from lipoaspirate with the use of matrase reagent than after isolating cells from lipoaspirate without the use of matrase reagent.

Colony-Forming Unit assay

ASCs derived from ADRCs that were isolated from lipoaspirate with the use of Matrase Reagent formed on average 16 times more CFUs per g lipoaspirate than ASCs derived from ADRCs that were isolated from lipoaspirate without the use of Matrase Reagent (4973±836 CFUs in case of the former ASCs and 307±68 CFUs in case of the latter ASCs; p = 0.002; n=10 matched pairs of samples) (Fig. 2).

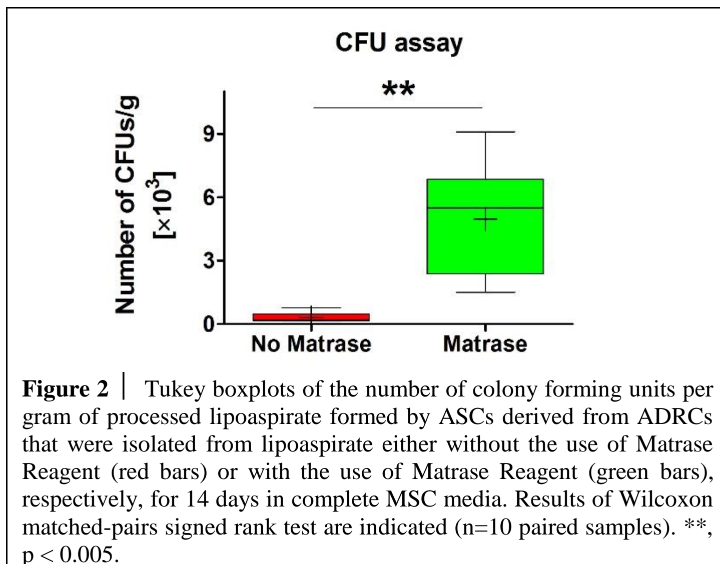

Figure 2 | Tukey boxplots of the number of colony forming units per gram of processed lipoaspirate formed by ASCs derived from ADRCs that were isolated from lipoaspirate either without the use of Matrase Reagent (red bars) or with the use of Matrase Reagent (green bars), respectively, for 14 days in complete MSC media. Results of Wilcoxon matched-pairs signed rank test are indicated (n=10 paired samples). **, $p < 0.005$.

Expression of regenerative cell-associated genes in adipose-derived stem cells derived from uncultured, autologous adipose-derived regenerative cells

Embryoid body formation was observed after culturing ASCs for seven days in serum free media (Fig. 3). The embryoid bodies had a spherical appearance and defined borders. The majority of the embryoid bodies were small in diameter (<100 μm), some were medium (100-200 μm), and a few were large (>200 μm) in diameter. No statistically significant differences were observed in the formation or the size of the embryoid bodies derived from ADRCs that were isolated from lipoaspirate with or without the use of Matrase (data not shown).

Furthermore, mRNA was collected from ASCs that were cultured in conventional monolayer cultures or as embryoid bodies and was analyzed for expression of Oct4 (a transcription factor associated with self-renewal; e.g., Alt et al., 2011), Klf4 (a marker of stemness; e.g., Alt et al., 2011) and Hes1 (another known stem cell marker; e.g., Kageyama et al., 2007) using qPCR (all samples were analyzed in triplicate). It was found that the use of Matrase Reagent in the process of isolating cells from lipoaspirate had no statistically significant impact on the relative levels of mRNA for the regenerative cell-associated genes Oct-4, Klf4 and Hes1 for both conventional monolayer cultures (Fig. 4A,C,E) and embryoid body cultures (Fig. 4B,D,F) (mean and SEM of relative gene expression values as well as corresponding p-values are summarized in Table 3). Accordingly, the use of Matrase Reagent in processing lipoaspirate with the InGeneron Transpose RT System did not alter expression of regenerative cell-associated genes in the final cell suspension.

Differentiation potential of adipose-derived stem cells

To test the hypothesis that ADRCs isolated from lipoaspirate with or without the use of Matrase Reagent have the differentiation capacity to differentiate into tissue from all three germ layers, ASCs (obtained by culturing ADRCs) on their 3rd passage were exposed to specific adipogenic, osteogenic, hepatogenic and neurogenic induction media (or non-inductive media as a control).

Adipogenic differentiation potential – ASCs on their 3rd passage (derived from ADRCs isolated from lipoaspirate with or without the use of Matrase Reagent) were cultured for two weeks in adipogenic differentiation medium or control medium. Then, the presence of intracytoplasmic lipids (triglycerides) was assessed with Oil red-O staining, and relative numbers of Oil red-O positive cells were evaluated under a brightfield microscope. It was found that the use of Matrase Reagent in the process of isolating ADRCs from lipoaspirate had no impact on the visual appearance of the cells after induction of adipogenic differentiation (Fig. 5), and no statistically significant impact on the relative number of Oil red-O positive cells (Fig. 6).

Osteogenic differentiation potential – ASCs on their 3rd passage (derived from ADRCs isolated from lipoaspirate with or without the use of Matrase Reagent) were cultured for two weeks in osteogenic differentiation medium or control medium. Then, the presence of calcific deposits was investigated with Alizarin red staining. It was found that the use of Matrase Reagent in the process of isolating ADRCs from lipoaspirate had no impact on the visual appearance of the cells after induction of osteogenic differentiation (Fig. 7).

bioRxiv preprint doi: https://doi.org/10.1101/485318; this version posted December 3, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Hepatogenic differentiation potential – ASCs on their 3rd passage (derived from ADRCs isolated from lipoaspirate with or without the use of Matrase Reagent) were cultured for ten days in hepatogenic differentiation medium or control medium. Then, the presence of structures containing a high proportion of carbohydrate macromolecules (glycogen, glycoprotein, proteoglycans) was investigated with Periodic Acid Schiff staining. It was found that the use of Matrase Reagent in the process of isolating ADRCs from lipoaspirate had no impact on the visual appearance of the cells after induction of hepatogenic differentiation (Fig. 8).

Neurogenic differentiation potential – ASCs on their 3rd passage (derived from ADRCs isolated from lipoaspirate with or without the use of Matrase Reagent) were cultured for one week in neurogenic differentiation medium or control medium. Then, the morphology of the cells was investigated after staining cells with hematoxylin. It was found that the use of Matrase Reagent in the process of isolating ADRCs from lipoaspirate had no impact on the visual appearance of the cells after induction of neurogenic differentiation (Fig. 9).

Residual collagenase activity in cell preparations prepared with the use of Matrase Reagent

It was found that the collagenase activity was below the detection limit of the used assay (Fig. 10).

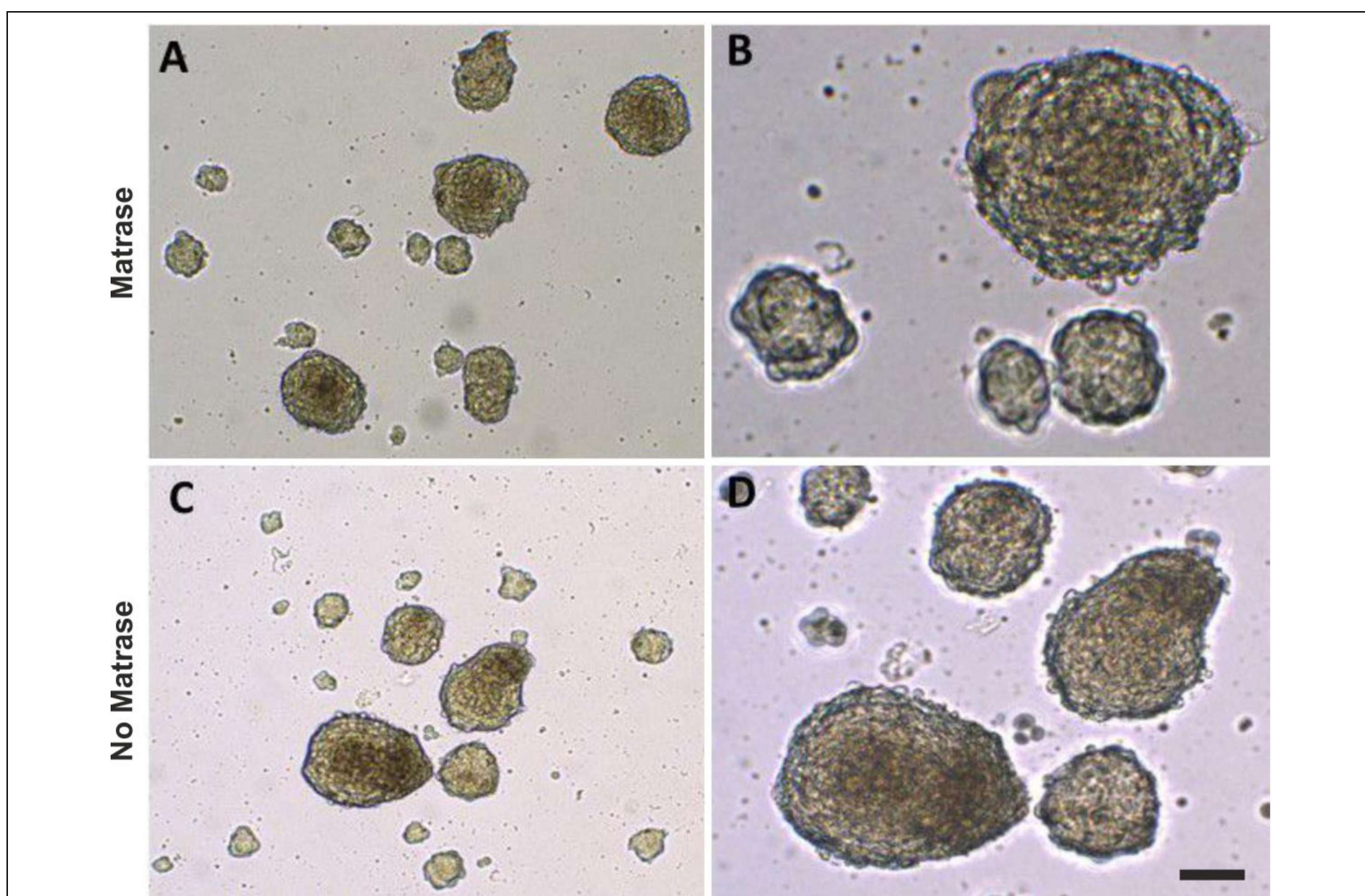

Figure 3 | Formation of embryoid bodies after culturing ASCs derived from ADRCs that were isolated from lipoaspirate either with the use of Matrase Reagent (**A**, **B**) or without the use of Matrase Reagent (**C**, **D**), respectively, for seven days in serum-free media. The scale bar in (D) represents 100 µm in (A, C) and 50 µm in (B, D).

bioRxiv preprint doi: https://doi.org/10.1101/485318; this version posted December 3, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

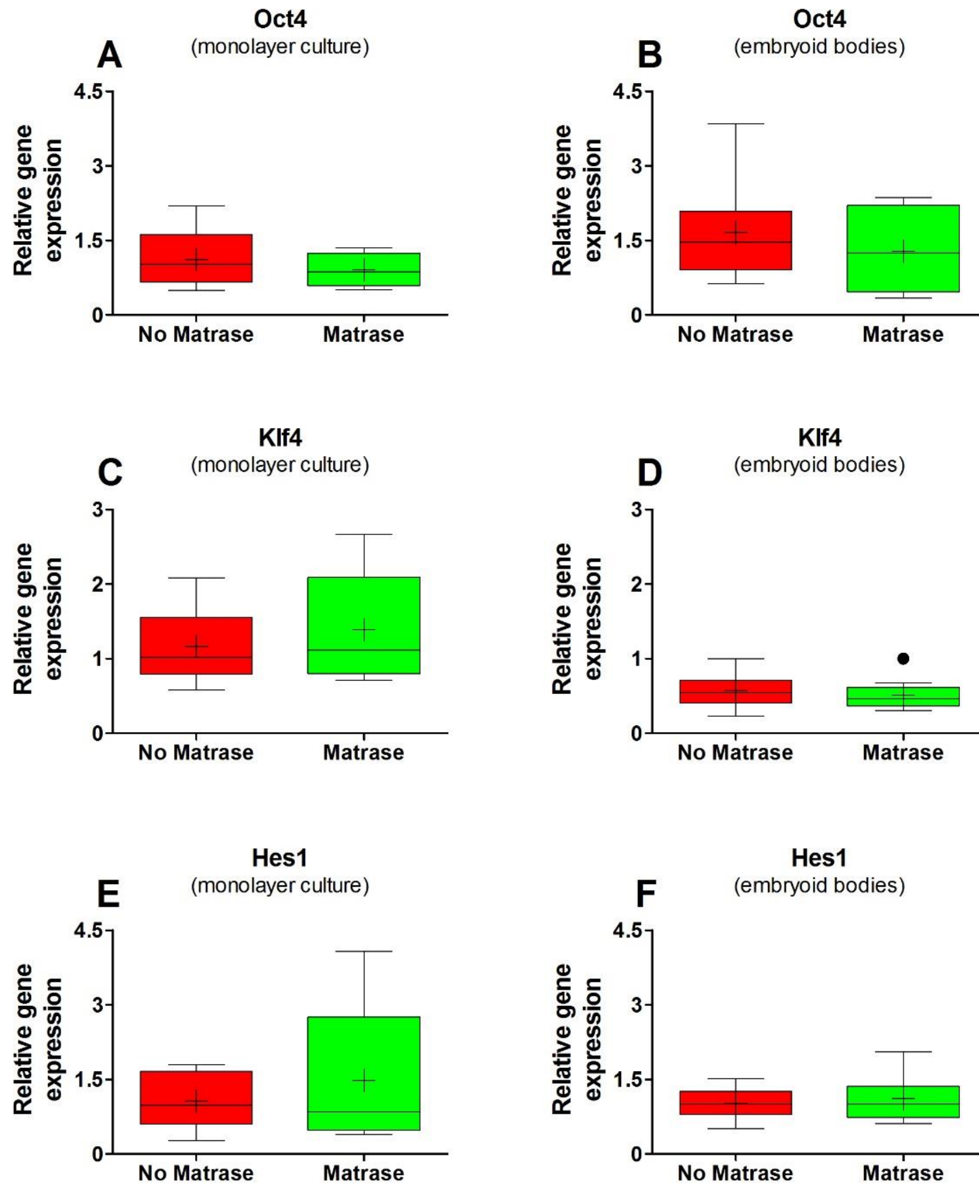

Figure 4 │ Tukey boxplots of relative gene expression (arbitrary units) of Oct4 (**A**, **B**), Klf4 (**C**, **D**) and Hes3 (**E**, **F**) of ASCs in conventional monolayer cultures (**A**, **C**, **E**) or obtained from embryoid bodies (**B**, **D**, **F**) after culturing ADRCs that were isolated from lipoaspirate either without the use of Matrase Reagent (red bars) or with the use of Matrase Reagent (green bars), respectively. The Wilcoxon matched-pairs signed rank test showed no statistically significant differences between the groups ($p > 0.05$; n=8 paired samples each).

bioRxiv preprint doi: https://doi.org/10.1101/485318; this version posted December 3, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Table 3 | Results of statistical analysis
The table shows the results of all statistical analyses that were performed in this study. All data are provided as {mean, standard error of the mean [SEM], number of paired samples}. P-values were obtained using nonparametric Wilcoxon matched-pairs signed rank test. Cell recovery and live cell yield data represent numbers of cells per gram processed lipoaspirate; relative gene expression data are provided in arbitrary units. Calculations were performed with GraphPad Prism (Version 5; Graph Pad Software, San Diego, CA, USA). Abbreviations: CR, cell recovery; LCY, live cell yield; CFUs, colony forming units; ASCs, adipose-derived stem cells; EB, embryoid bodies; OrO+, No. of Oil red-O positive cells

Variable	Isolation of cells without the use of Matrase Reagent	Isolation of cells with the use of Matrase Reagent	P value
Cell yield, cell viability and cell size			
CR [x10^5]	0.84, 0.10, 12	7.24, 0.89, 12	<0.001
LCY [x10^5]	0.53, 0.08, 12	6.25, 0.79, 12	<0.001
Cell viability [%]	61.7, 2.61, 12	85.9, 1.12, 12	<0.001
Cell diameter [µm]	10.2, 0.08, 12	10.6, 0.06, 12	0.05
Colony Forming Unit assay			
CFUs [number]	307, 68, 10	4973, 836, 10	0.002
Expression of regenerative cell-associated genes			
ASCs: Oct4	1.09, 0.20, 8	0.90, 0.11, 8	0.461
ASCS: Klf4	1.16, 0.18, 8	1.39, 0.26, 8	0.195
ASCs: Hes1	1.06, 0.20, 8	1.48, 0.49, 8	0.742
EB: Oct4	1.66, 0.31, 10	1.28, 0.25, 10	0.322
EB: Klf4	0.57, 0.07, 10	0.51, 0.07, 10	0.557
EB: Hes1	1.01, 0.11, 9	1.11, 0.15, 9	0.496
Adipogenic differentiation potential			
OrO+ [%]	33.1, 3.17, 7	38.7, 4.72, 7	0.109

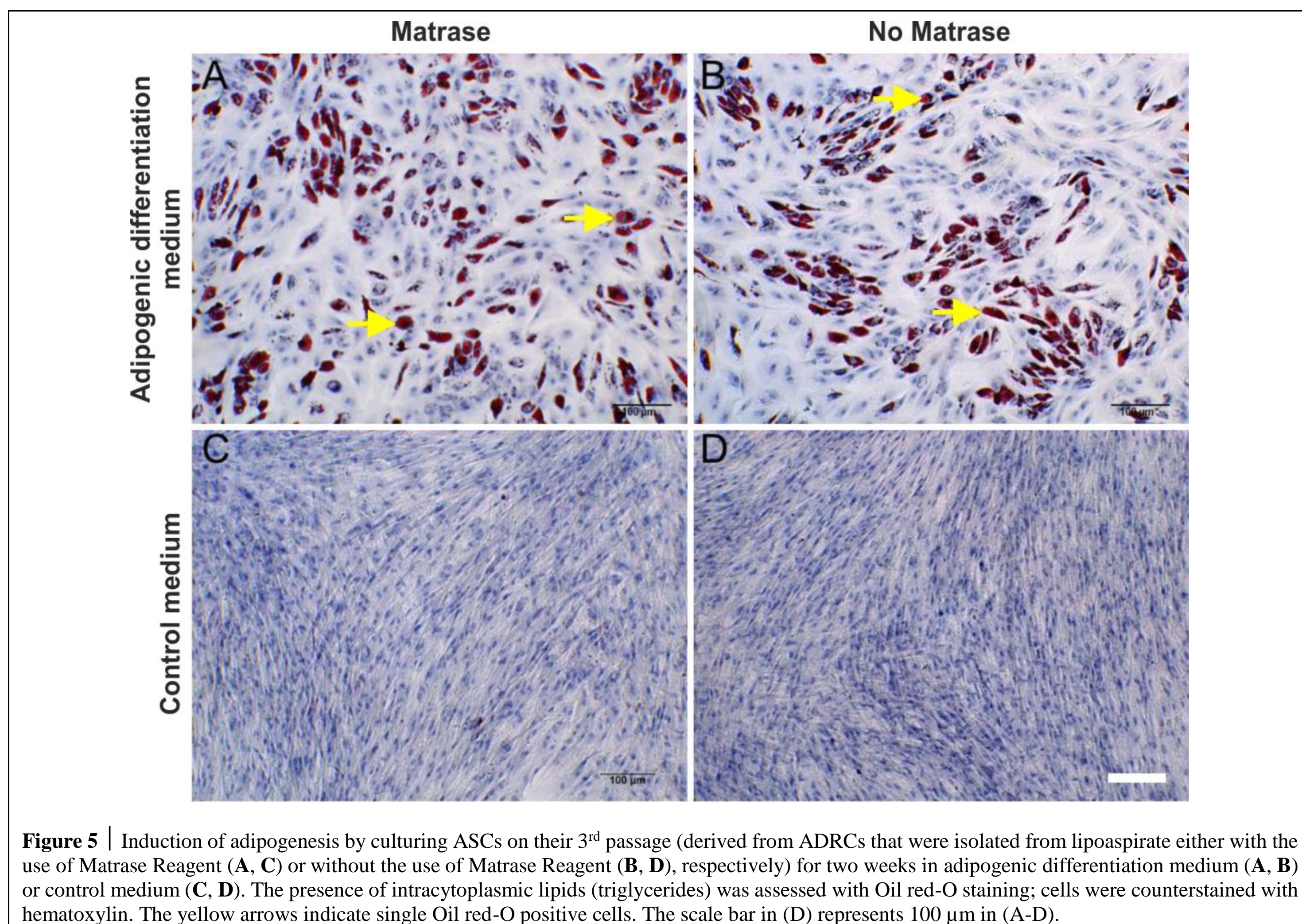

Figure 5 | Induction of adipogenesis by culturing ASCs on their 3rd passage (derived from ADRCs that were isolated from lipoaspirate either with the use of Matrase Reagent (**A**, **C**) or without the use of Matrase Reagent (**B**, **D**), respectively) for two weeks in adipogenic differentiation medium (**A**, **B**) or control medium (**C**, **D**). The presence of intracytoplasmic lipids (triglycerides) was assessed with Oil red-O staining; cells were counterstained with hematoxylin. The yellow arrows indicate single Oil red-O positive cells. The scale bar in (D) represents 100 µm in (A-D).

bioRxiv preprint doi: https://doi.org/10.1101/485318; this version posted December 3, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

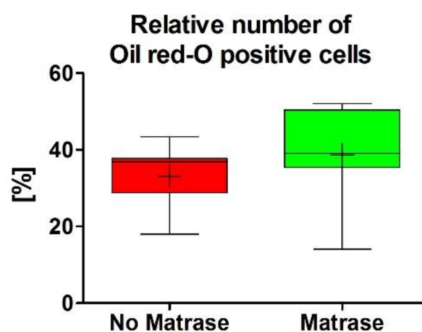

Figure 6 | Tukey boxplots of the relative number of Oil red-O positive cells obtained after culturing ASCs on their 3rd passage (derived from ADRCs that were isolated from lipoaspirate either without the use of Matrase Reagent (red bars) or with the use of Matrase Reagent (green bars), respectively) for two weeks in adipogenic differentiation medium. The Wilcoxon matched-pairs signed rank test showed no statistically significant differences between the groups (p = 0.109; n=7 paired samples).

Matrase

No Matrase

Osteogenic differentiation medium

Control medium

A B C D

100 µm

Figure 7 | Induction of osteogenesis by culturing ASCs on their 3rd passage (derived from ADRCs that were isolated from lipoaspirate either with the use of Matrase Reagent (**A**, **C**) or without the use of Matrase Reagent (**B**, **D**), respectively) for two weeks in osteogenic differentiation medium (**A**, **B**) or control medium (**C**, **D**). The presence of calcific deposits was investigated with Alizarin red staining; cells were counterstained with hematoxylin. Cells of an osteogenic lineage are stained bright to deep red and easily visible as dense red patches. The scale bar in (D) represents 100 µm in (A-D).

bioRxiv preprint doi: https://doi.org/10.1101/485318; this version posted December 3, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

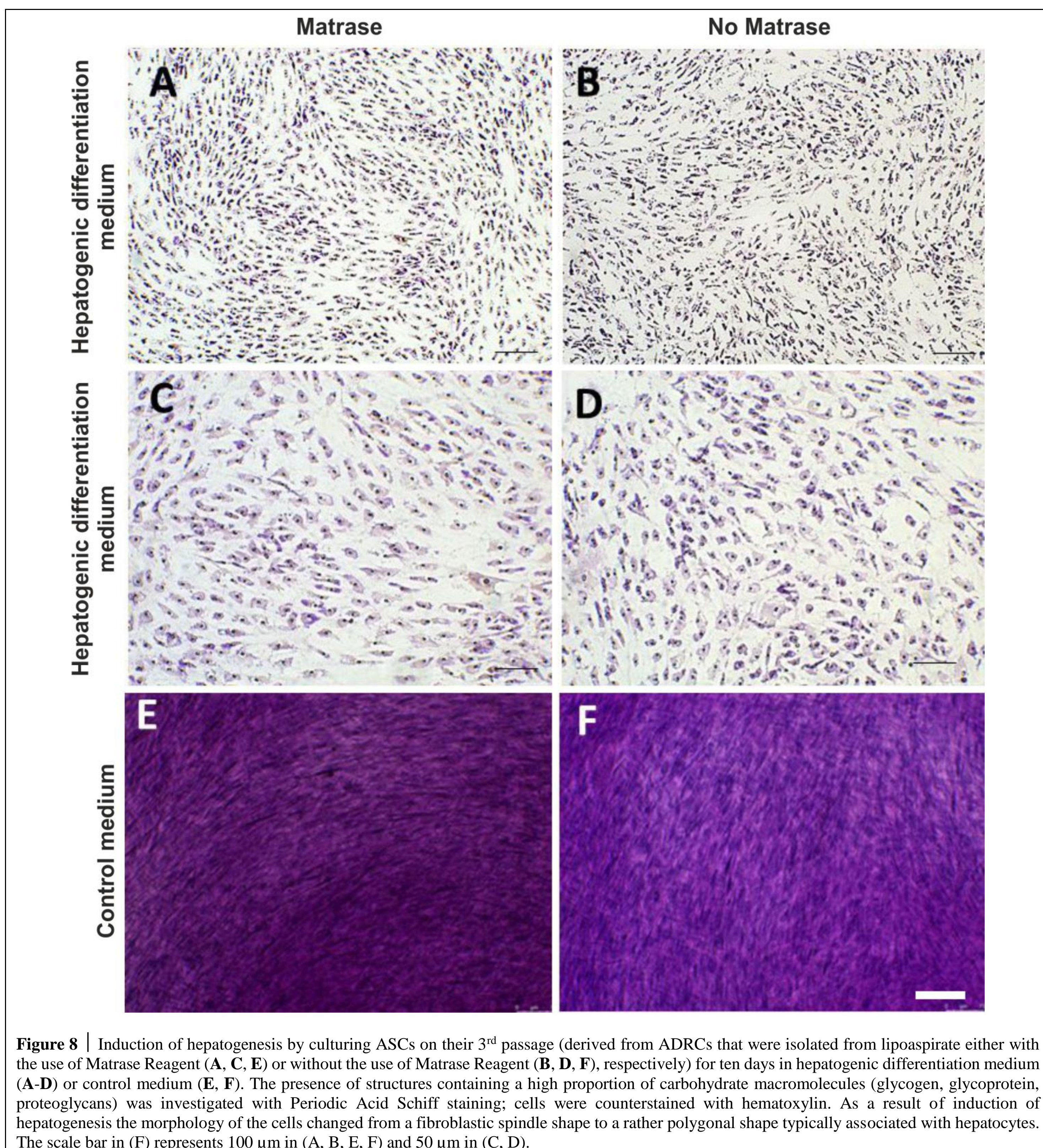

Figure 8 │ Induction of hepatogenesis by culturing ASCs on their 3rd passage (derived from ADRCs that were isolated from lipoaspirate either with the use of Matrase Reagent (**A**, **C**, **E**) or without the use of Matrase Reagent (**B**, **D**, **F**), respectively) for ten days in hepatogenic differentiation medium (**A**-**D**) or control medium (**E**, **F**). The presence of structures containing a high proportion of carbohydrate macromolecules (glycogen, glycoprotein, proteoglycans) was investigated with Periodic Acid Schiff staining; cells were counterstained with hematoxylin. As a result of induction of hepatogenesis the morphology of the cells changed from a fibroblastic spindle shape to a rather polygonal shape typically associated with hepatocytes. The scale bar in (F) represents 100 µm in (A, B, E, F) and 50 µm in (C, D).

bioRxiv preprint doi: https://doi.org/10.1101/485318; this version posted December 3, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

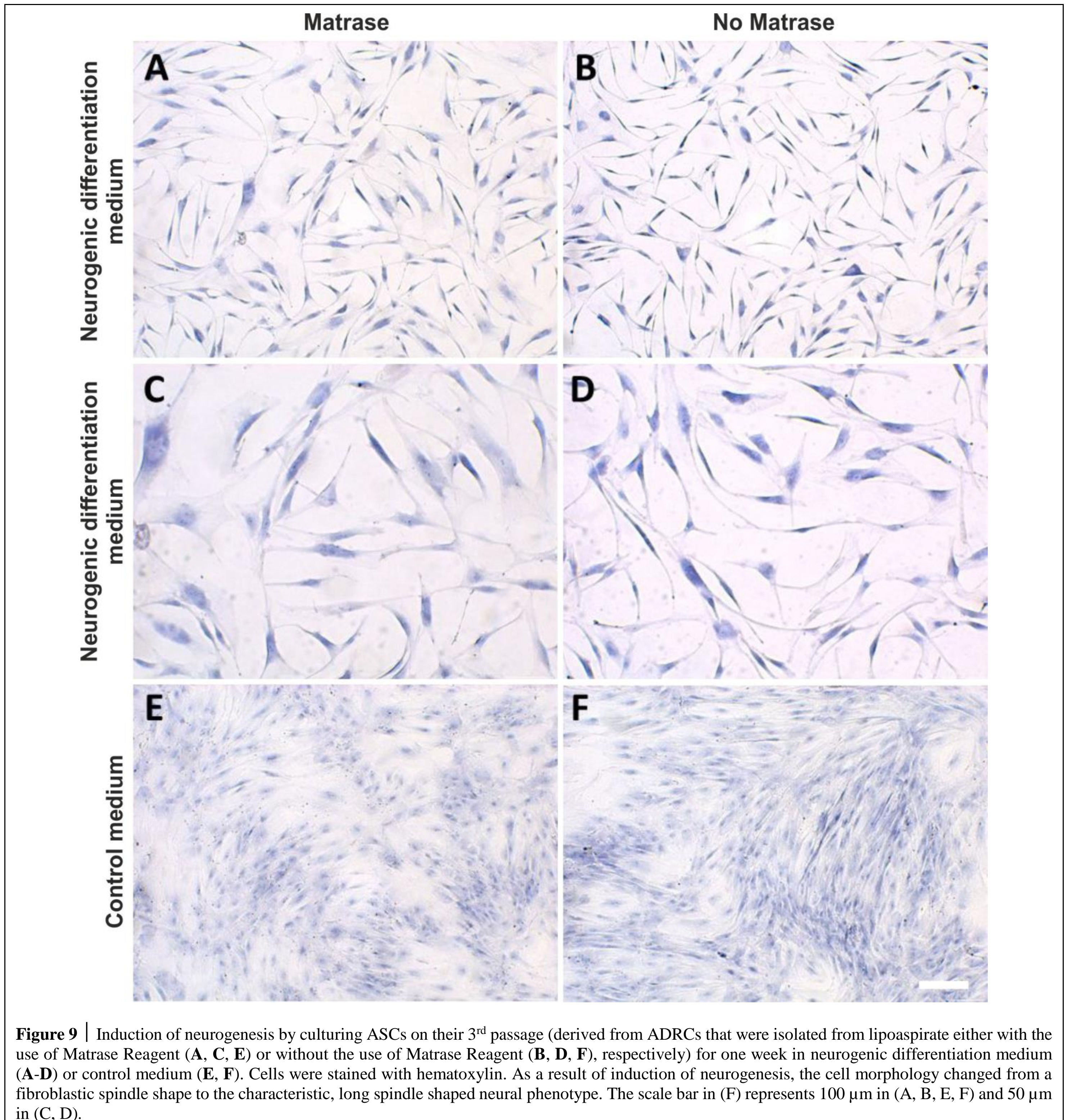

Figure 9 | Induction of neurogenesis by culturing ASCs on their 3rd passage (derived from ADRCs that were isolated from lipoaspirate either with the use of Matrase Reagent (**A**, **C**, **E**) or without the use of Matrase Reagent (**B**, **D**, **F**), respectively) for one week in neurogenic differentiation medium (**A**-**D**) or control medium (**E**, **F**). Cells were stained with hematoxylin. As a result of induction of neurogenesis, the cell morphology changed from a fibroblastic spindle shape to the characteristic, long spindle shaped neural phenotype. The scale bar in (F) represents 100 µm in (A, B, E, F) and 50 µm in (C, D).

bioRxiv preprint doi: https://doi.org/10.1101/485318; this version posted December 3, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

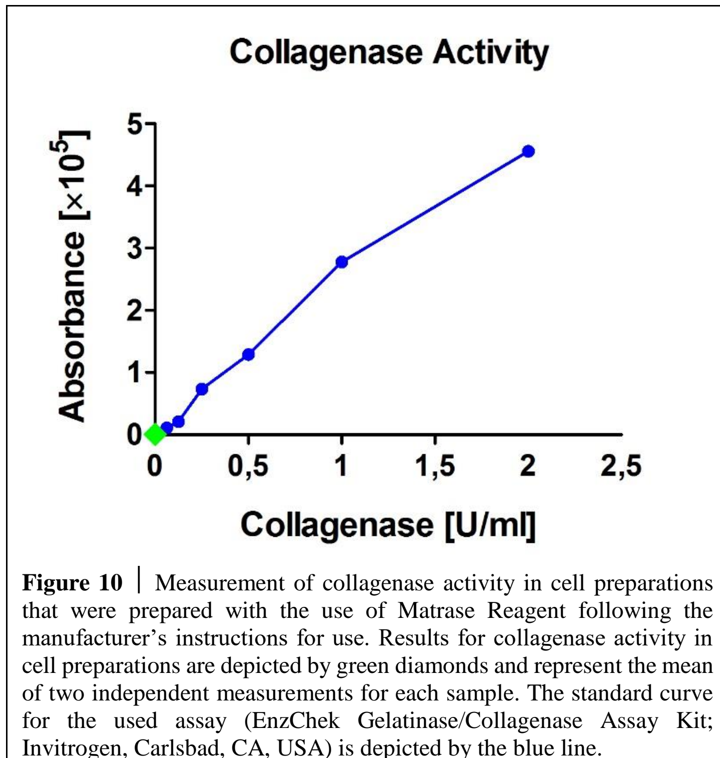

Figure 10 | Measurement of collagenase activity in cell preparations that were prepared with the use of Matrase Reagent following the manufacturer's instructions for use. Results for collagenase activity in cell preparations are depicted by green diamonds and represent the mean of two independent measurements for each sample. The standard curve for the used assay (EnzChek Gelatinase/Collagenase Assay Kit; Invitrogen, Carlsbad, CA, USA) is depicted by the blue line.

DISCUSSION

Due to regulatory concerns, there are currently several systems offered for clinical therapeutic usage that process adipose tissue without the use of an enzyme. It was the aim of this study to evaluate the effect on cells, efficiency and safety regarding cell viability in direct comparison between processing with or without the use of an enzyme under otherwise identical conditions. InGeneron's Transpose RT system utilizes a proprietary method for isolating ADRCs from adipose tissue. Specifically, this method uses the enzymatic activity of Matrase Reagent to release the cells from the extracellular matrix. We observed a substantial, statistically significant lower cell yield and cell viability in the final cell suspension isolated by just mechanical processing without the use of Matrase Reagent, due to less efficient release of cells from the extracellular matrix when no enzyme was used.

Specifically, the yield of viable cells/gram of processed lipoaspirate was approximately twelve times higher in the final cell suspension when ADRCs were isolated from lipoaspirate with the use of Matrase Reagent, compared to the final cell suspension that was obtained when cells were isolated from lipoaspirate without the use of Matrase Reagent, under otherwise identical processing conditions.

Of importance, the mean relative number of 86% viable cells obtained by isolating ADRCs from lipoaspirate *with* the use of Matrase Reagent exceeded the proposed minimum threshold for viability of cells in the SVF of 70 % established by IFATS (Bourin et al., 2013), whereas the mean relative number of 61% viable cells obtained by isolating ADRCs from lipoaspirate *without* the enzyme did not.

On the other hand, cells isolated from lipoaspirate were able to form CFUs independent of enzyme usage, indicating the presence of stem cells in both methods of cell recovery. However, cells isolated from lipoaspirate with the use of Matrase Reagent formed on average 16 times more CFUs per g lipoaspirate than cells isolated without the use of Matrase Reagent. These results are in line with previously reported studies comparing enzymatic to non-enzymatic extraction of ADRCs from lipoaspirate (Shaw et al., 2013) and with results of cells recovered from adipose tissue or just from the tumescence fluid portion (Yoshimura 2006).

For considerations of clinical usage, these findings have an important impact: the significantly lower viability of cells recovered just mechanically without enzyme means that nearly 40 % of the cells that would be transferred to a patient are not viable. In order to even match the number of spheroids formed from cells isolated with enzyme, 16 times more adipose tissue would be required as starting material for processing without enzyme. In other words, instead of 100 g patient derived adipose tissue for enzymatic processing, 1600 g for the just mechanical process would be required. This could mean a more complex recovery procedure and potentially higher morbidity, especially in patients at increased risk of bleeding.

Aside from these differences, cells isolated from lipoaspirate with the use of Matrase Reagent showed no statistically significant differences in the expression of regenerative cell-associated genes Oct4, Hes1 and Klf4 compared to ASCs derived from ADRCs that were isolated from lipoaspirate without the use of Matrase Reagent. Besides this, cells isolated from lipoaspirate both with and without the use of Matrase Reagent were able to differentiate into all three germ layers (i.e., into the adipogenic, osteogenic, hepatogenic and neurogenic lineages).

Of importance, after processing cells with Matrase Reagent following the manufacturer's instructions for use, the collagenase activity in the final cell suspension was below the detection limit of the used assay. This suggests that the enzyme has only a supportive function in releasing the cells but has no presence or effect in the final cell suspension.

In summary, this study demonstrates that isolating ADRCs with the use of Matrase Reagent did not subject the cells to substantial manipulation. Due to the high yield of viable, pluripotent cells in the final cell suspension recovered from lipoaspirate (or adipose tissue in general) with the Transpose RT system and the use of Matrase

bioRxiv preprint doi: https://doi.org/10.1101/485318; this version posted December 3, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Reagent, cells are neither required to be cultured nor expanded, nor is any genetic manipulation such as overexpression of embryonic genes as in the case of induced Pluripotent Cells , so called iPS cells, necessary.

The process of isolating ADRCs from lipoaspirate with the use of Matrase Reagent did not alter the biological characteristics, physiological functions or structural properties relevant for the intended use (i.e., regeneration, repair or replacement of weakened or injured tissue), as their ability to be able to differentiate into all three germ layers was *independent from the use of enzyme* and was definitely *not induced by the use of the enzyme*. This is further evidenced by the fact that cells recovered with or without the use of enzyme are equally able to express embryonic stem cells genes, both without any prior genetic manipulation.

According to the position the U.S. Food and Drug Administration (FDA) takes regarding the manipulation of cells by use of enzyme (U.S. Department of Health and Human Services, 2017), *examples of relevant biological characteristics of cells or nonstructural tissues include differentiation and activation state, proliferation potential, and metabolic activity*, and *processing that alters any relevant biological characteristics of cells or nonstructural tissues generally would be considered more than minimal manipulation.* Furthermore, according to the position the European Medicine Agency takes on advanced therapy medicinal products (ATMPs) per Regulation (EC) No 1394/2007 (The European Parliament and The Council, 2007), c*ells or tissues shall be considered 'engineered' if they fulfil at least one of the following conditions: 1) the cells or tissues have been subject to substantial manipulation, so that biological characteristics, physiological functions or properties relevant for the intended regeneration, repair or replacement are achieved. The manipulation listed in Annex I, in particular, shall not be considered as substantial manipulations; and 2) the cells or tissues are not intended to be used for the same essential function or functions in the recipient as in the donor.*

This definition of 'engineered' or 'manipulated' cells or tissues as an advanced therapy medicinal product (ATMP) has triggered the development of several methods to recover cells without use of enzyme for application in clinical practice. However, the results of the present study demonstrate that isolating cells from adipose tissue using the Transpose RT system and the Matrase Reagent does not render ADRCs to be classified as manipulated. This contradicts the position of some regulatory authorities that only tissue processed and cells recovered without the use of enzyme are considered "Non-Manipulated". For the practice of medicine our findings do not support the hypothesis that ADRCs should preferentially be isolated from adipose tissue without enzyme: in the present study just mechanically processed tissue showed significantly lower cell viability and cell yield. In order to obtain a comparable cell recovery just by mechanical processing without enzyme, significantly larger initial amounts of human tissue would be required; especially if under the aspect to avoid any further manipulation a "point of care" application without culturing would be considered. The significantly lower cell viability associated with just mechanical processing represent an additional point of concern.

REFERENCES

Ahmed RP, Ashraf M, Buccini S, Shujia J, Haider HKh. Cardiac tumorigenic potential of induced pluripotent stem cells in an immunocompetent host with myocardial infarction. Regen Med 2011;6(2):171-178.

Alt E, Yan Y, Gehmert S, Song YH, Altman A, Gehmert S, Vykoukal D, Bai X. Fibroblasts share mesenchymal phenotypes with stem cells, but lack their differentiation and colony-forming potential. Biol Cell 2011;103(4):197-208.

Álvaro-Gracia JM, Jover JA, García-Vicuña R, Carreño L, Alonso A, Marsal S, Blanco F, Martínez-Taboada VM, Taylor P, Martín-Martín C, DelaRosa O, Tagarro I, Díaz-González F. Intravenous administration of expanded allogeneic adipose-derived mesenchymal stem cells in refractory rheumatoid arthritis (Cx611): results of a multicentre, dose escalation, randomised, single-blind, placebo-controlled phase Ib/IIa clinical trial. Ann Rheum Dis 2017;76(1):196-202.

Aust L, Devlin B, Foster SJ, Halvorsen YD, Hicok K, du Laney T, Sen A, Willingmyre GD, Gimble JM. Yield of human adipose-derived adult stem cells from liposuction aspirates. Cytotherapy 2004;6(1):7-14.

Bai X, Sadat S, Gehmert S, Alt E, Song YH. VEGF receptor Flk-1 plays an important role in c-kit expression in adipose tissue derived stem cells. FEBS Lett 2007;581(24):4681-4684.

Bourin P, Bunnell BA, Casteilla L, Dominici M, Katz AJ, March KL, Redl H, Rubin JP, Yoshimura K, Gimble JM. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). Cytotherapy 2013;15(6):641-648.

Brickman JM, Serup P. Properties of embryoid bodies. Wiley Interdiscip Rev Dev Biol 2017;6(2). doi: 10.1002/wdev.259.

Condé-Green A, Kotamarti VS, Sherman LS, Keith JD, Lee ES, Granick MS, Rameshwar P. Shift toward mechanical isolation of adipose-derived stromal vascular fraction: review of upcoming techniques. Plast Reconstr Surg Glob Open 2016;4(9):e1017.

da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci 2006;119(Pt 11):2204-2213.

Fujikawa T, Oh SH, Pi L, Hatch HM, Shupe T, Petersen BE. Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing

bioRxiv preprint doi: https://doi.org/10.1101/485318; this version posted December 3, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

cells. Am J Pathol 2005;166(6):1781-1791.

Ilmer M, Vykoukal J, Recio Boiles A, Coleman M, Alt E. Two sides of the same coin: stem cells in cancer and regenerative medicine. FASEB J 2014;28(7):2748-2761.

Kageyama R, Ohtsuka T, Kobayashi T. The Hes gene family: repressors and oscillators that orchestrate embryogenesis. Development 2007;134(7):1243-1251.

King NM, Perrin J. Ethical issues in stem cell research and therapy. Stem Cell Res Ther 2014;5(4):85.

Madonna R, Van Laake LW, Davidson SM, Engel FB, Hausenloy DJ, Lecour S, Leor J, Perrino C, Schulz R, Ytrehus K, Landmesser U, Mummery CL, Janssens S, Willerson J, Eschenhagen T, Ferdinandy P, Sluijter JP. Position Paper of the European Society of Cardiology Working Group Cellular Biology of the Heart: cell-based therapies for myocardial repair and regeneration in ischemic heart disease and heart failure. Eur Heart J 2016;37(23):1789-1798.

Metcalf GL, McClure SR, Hostetter JM, Martinez RF, Wang C. Evaluation of adipose-derived stromal vascular fraction from the lateral tailhead, inguinal region, and mesentery of horses. Can J Vet Res 2016;80:294-301.

Oberbauer E, Steffenhagen C, Wurzer C, Gabriel C, Redl H, Wolbank S. Enzymatic and non-enzymatic isolation systems for adipose tissue-derived cells: current state of the art. Cell Regen 2015;4:7.

Panés J, García-Olmo D, Van Assche G, Colombel JF, Reinisch W, Baumgart DC, Dignass A, Nachury M, Ferrante M, Kazemi-Shirazi L, Grimaud JC, de la Portilla F, Goldin E, Richard MP, Leselbaum A, Danese S; ADMIRE CD Study Group Collaborators. Expanded allogeneic adipose-derived mesenchymal stem cells (Cx601) for complex perianal fistulas in Crohn's disease: a phase 3 randomised, double-blind controlled trial. Lancet 2016;388(10051):1281-1290.

Shah FS, Wu X, Dietrich M, Rood J, Gimble JM. A non-enzymatic method for isolating human adipose tissue-derived stromal stem cells. Cytotherapy 2013;15(8):979-985.

Swijnenburg RJ, Tanaka M, Vogel H, Baker J, Kofidis T, Gunawan F, Lebl DR, Caffarelli AD, de Bruin JL, Fedoseyeva EV, Robbins RC. Embryonic stem cell immunogenicity increases upon differentiation after transplantation into ischemic myocardium. Circulation 2005;112(9 Suppl):I166-I172.

The European Parliament and The Council. Regulation (EC) No 1394/2007 of the European Parliament and of The Council of 13 November 2007 on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004. Official Journal of the European Union 10.12.2017, L 324/121-137. Available at https://ec.europa.eu/health/sites/health/files/files/eudralex/vol-1/reg_2007_1394/reg_2007_1394_en.pdf (accessed on December 1st, 2018).

U.S. Department of Health and Human Services. Regulatory considerations for human cells, tissues, and cellular and tissue-based products: minimal manipulation and homologous use. Guidance for industry and Food and Drug Administration staff. Published December 2017. Available at https://www.fda.gov/ucm/groups/fdagov-public/@fdagov-bio-gen/documents/document/ucm585403.pdf (accessed on December 1st, 2018).

van Dongen JA, Tuin AJ, Spiekman M, Jansma J, van der Lei B, Harmsen MC. Comparison of intraoperative procedures for isolation of clinical grade stromal vascular fraction for regenerative purposes: a systematic review. J Tissue Eng Regen Med 2018;12(1):e261-e274.

Vazin T, Freed WJ. Human embryonic stem cells: derivation, culture, and differentiation: a review. Restor Neurol Neurosci 2010;28(4):589-603.

Wu R, Hu X, Wang J. Concise review: optimized strategies for stem cell-based therapy in myocardial repair: clinical translatability and potential limitation. Stem Cells 2018;36(4):482-500.

Yoshimura K, Shigeura T, Matsumoto D, Sato T, Takaki Y, Aiba-Kojima E, Sato K, Inoue K, Nagase T, Koshima I, Gonda K. Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates. J Cell Physiol 2006;208(1):64-76.

Yu H, Lu K, Zhu J, Wang J. Stem cell therapy for ischemic heart diseases. Br Med Bull 2017;121(1):135-154.

Zhang Y, Wang D, Chen M, Yang B, Zhang F, Cao K. Intramyocardial transplantation of undifferentiated rat induced pluripotent stem cells causes tumorigenesis in the heart. PLoS One 2011;6(4):e19012.