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Article

The composition of adipose-derived regenerative cells isolated from lipoaspirate using a novel point of care system does not depend on the subject's individual age, gender, body mass index and ethnicity

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Abstract: Uncultured, unmodified, autologous, adipose-derived regenerative cells (UA-ADRCs) are a safe and effective treatment option for various musculoskeletal pathologies. However, it is unknown whether the composition of the final cell suspension systematically varies with the subject's individual age, gender, body mass index (BMI) and ethnicity. UA-ADRCs were isolated from lipoaspirate from n=232 subjects undergoing elective lipoplasty using the Transpose RT system (InGeneron, Inc.; Houston, TX, USA). The UA-ADRCs were assessed for the number of nucleated cells, cell viability and the number of viable nucleated cells per gram of adipose tissue harvested. Flow cytometry was used to further characterize the UA-ADRCs. UA-ADRCs isolated with the Transpose RT system (InGeneron) can be recommended for all ages, genders, BMIs and ethnicities. Furthermore, it was found that for all experimental methods and commerically available systems described in the literature to isolate UA-ADRCs or systems that provide non-enzymatic, mechanical dissociation of lipoaspirate, the Transpose RT system yielded the highest mean relative numbers of CD45- / CD31- / CD34+ cells (adipose tissue derived progenitors), CD45- / CD31+ / CD34+ cells (endothelial progenitors) and CD45+ / CD206+ cells (M2 macrophages). Application of these cell types may significantly contribute to tissue regeneration.

Keywords: adipose-derived regenerative cells; flow cytometry; microfragmented fat; NucleoCounter; regenerative medicine; stem cells; UA-ADRCs.

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1. Introduction

Uncultured, unmodified, autologous, adipose-derived regenerative cells (UA-ADRCs) are a safe and effective treatment option for various musculoskeletal pathologies, including tendon injuries [1-3], bone defects [4], facet joint syndrome [5] and knee osteoarthritis [6]. Treatment with UA-ADRCs is a point of care procedure. In the same surgical setting and within a very short time span (usually less than three hours), adipose tissue can be obtained by mini-liposuction, and the UA-ADRCs can be isolated and injected to the point in the body where they are needed [1,7,8].

UA-ADRCs belong to the larger group of orthobiologics. One can divide orthobiologics into (i) cell-free orthobiologics (including platelet rich plasma (PRP) [9], exosomes [10] and amniotic fluid [11]), (ii) orthobiologics that are based on allogeneic cells (including allogeneic mesenchymal stem cells (MSCs) derived from respectively placenta, umbilical cord or umbilical cord blood [12-14], allogeneic bone marrow-derived MSCs (BM-MSCs) [15] and allogeneic adipose-derived stem cells (ADSCs) [16]), (iii) orthobiologics that are based on autologous cells (including autologous UA-ADRCs [1], autologous ADSCs [17], autologous, micro-fragmented fat (from liposuction) [18], bone marrow aspirate concentrate [19], autologous BM-MSCs [20]), chondrocyte transplants [21] and autologous, activated peripheral blood stem cells [22]), and (iv) other orthobiologics (including tissue-engineered patches [23], cadaver grafts [24] and modulation of the immune system [25]).

For the purpose of tissue regeneration, enzymatically isolated UA-ADRCs have important advantages over all other injectable orthobiologics (Table 1) [3,5,7,8].

Table 1. Features and resulting potential disadvantages of orthobiologics that do not apply to UA-ADRCs.

Feature	Disadvantage	Orthobiologics that are affected by this disadvantage	References (examples)
Lack of cells in an orthobiologic	Impossibility of the contribution of integration of new cells derived from injected stem and progenitor cells into the host tissue in tissue regeneration	All cell-free orthobiologics (PRP, exosomes, amniotic fluid)	[8-11]
Limited amount of MSCs in an orthobiologic	Limited ability for regeneration of musculoskeletal tissue	Bone marrow aspirate concentrate	[7,8,26,27]
Need for culturing cells	Reduction of the life span of the cells by shortening the telomeres following repetitive cell divisions, and possible negative effects on the safety of the cells as a medicinal product	ADSCs, BM-MSCs, MSCs derived from respectively placenta, umbilical cord, umbilical cord blood or any musculoskeletal tissue	[7,8,28]
Selection of cells	Limited functionality of the cells*	All cultured cells	[7,8,29]
Allogenicity (cells taken from different individuals of the same species)	Inability of new cells derived from stem and progenitor cells to integrate into the host tissue because of immunological incompatibility	All allogeneic cells	[7,8,30]
Limited number of living cells in the final cell suspension	Potential risk of inflammatory reactions	Non-enzymatic, mechanical dissociation (microfragmentation) of lipoaspirate	[31-33]
Incomplete detachment of cells from the extracellular matrix	Potential limitations of the functionality of the transplanted cells	Non-enzymatic, mechanical dissociation (microfragmentation) of lipoaspirate	[3,7,31,34]

Abbreviations: PRP, platelet rich plasma; ADSCs, adipose-derived stem cells; BM-MSCs, bone marrow-derived mesenchymal stem cells; MSCs, mesenchymal stem cells. *,For example, unlike cultured ADSCs, fresh UA-ADRCs express those growth factors that are needed to stimulate ADSCs towards tenogenic differentiation in culture [29].

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Some authors have proposed to use stem cells derived from embryonic stem cells or induced pluripotent stem cells (iPS cells) for regeneration of musculoskeletal tissues [35-40]. However, these cell types have distinct disadvantages, hence the clinical application of these cell types for regeneration of musculoskeletal tissues will most likely not be introduced in routine clinical practice in the foreseeable future (Table 2) [3,5,7,8].

Table 2. Features and resulting potential disadvantages of embryonic stem cells and iPS cells that do not apply to UA-ADRCs.

Feature	Disadvantage	Cell types that are affected by this disadvantage	References
Need for destruction of human embryos	Ethical concerns	Embryonic stem cells	[41]
Continued expression of survivin (an anti-apoptotic oncofetal gene) upon differentiation	Potential risk of development of teratomas	Embryonic stem cells	[42-44]
Similarities between induced pluripotency and oncogenic transformation	Potential risk of malignant transformation of the cells (i.e. the development of cancer)	iPS cells	[45-47]

Abbreviation: iPS cells, induced pluripotent stem cells.

A recent randomized controlled trial on treatment of symptomatic, partial-thickness rotator cuff tears (sPTRCT) with allogeneic, cultured adipose-derived stem cells (ADSCs) [48] has demonstrated that the features and possible disadvantages of orthobiologics listed in Table 1, which do not apply to UA-ADRCs, are indeed of clinical relevance. Specifically, no benefit of treating sPTRCT with cultured ADSCs was found for 24 months post-treatment [48], and the results obtained after injection of cultured ADSCs did not differ from the results obtained after injection of saline in [48]. In contrast, treatment of sPTRCT with UA-ADRCs led to improved shoulder function without adverse effects, and subjects treated with injection of UA-ADRCs showed statistically significantly higher mean ASES total scores at 6 and 12 months post-treatment than subjects treated with injection of corticosteroid ($p < 0.05$) [1] (Figure 1).

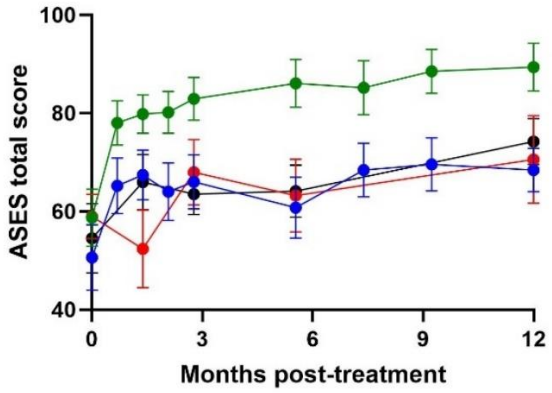

Figure 1. Mean and standard error of the mean (SEM) of the American Shoulder and Elbow Surgeons Standardized Shoulder Assessment Form (ASES) total score at baseline and during 12 months post-treatment after treating symptomatic, partial-thickness rotator cuff tears with injection of respectively (i) UA-ADRCs [1] (green dots and lines), (ii) allogeneic, cultured ADSCs [48] (red dots and lines), (iii) corticosteroid (control treatment in [1]) (blue dots and lines) or (iv) saline (control treatment in [48] (black dots and lines).

The discrepancy between the outcome of these studies [1,48] may be explained by the need for culturing cells and selection of cells [48], and/or by the fact that allogeneic cells were used in [48].

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The fact that treatment with UA-ADRCs is a real point of care procedure [1,7] raises the question whether the composition of the final cell suspension of UA-ADRCs depends on the subject's individual age, gender, body mass index (BMI) and ethnicity, which may cause interindividual differences in clinical results. Although more than 25 different experimental methods and commercially available systems used to isolate UA-ADRCs or systems that provide non-enzymatic, mechanical dissociation of lipoaspirate have been described in the literature [31,34,49-55], this important question has not been addressed so far.

In the present study, the Transpose RT system (InGeneron, Inc., Houston, TX, USA) was used to address this issue [1,2,4]. Of all experimental methods and commercially available systems to isolate UA-ADRCs or systems that provide non-enzymatic, mechanical dissociation of lipoaspirate described in the literature, the Transpose RT system (InGeneron) resulted in the highest number of living cells per ml lipoaspirate [31].

2. Materials and Methods

The present study was approved by ADVARRA IRB (Columbia, MD, USA) (Protocol #200601001; approval issued on October 16, 2006; last continuing review approval issued on August 24, 2022). ADVARRA is a fully AAHRPP (Association for the Accreditation of Human Research Protection Programs, Inc.) accredited IRB that provides ethical review for all phases of industry-sponsored and federally funded research in the U.S.

Subcutaneous adipose tissue was recovered by a medical practitioner via lipoaspiration from subjects undergoing elective lipoplasty according to standard procedures with informed consent. Lipoaspirate was harvested from the abdomen, bilateral flanks and/or medial thigh.

Adipose tissue from n=232 subjects was processed for isolation of UA-ADRCs using the Transpose RT system (InGeneron) (Figure 2) as described in the Tissue Processing Procedure section found in the 11011E Transpose RT Instructions for Use (11011-01 IFU; InGeneron) (Figure 3).

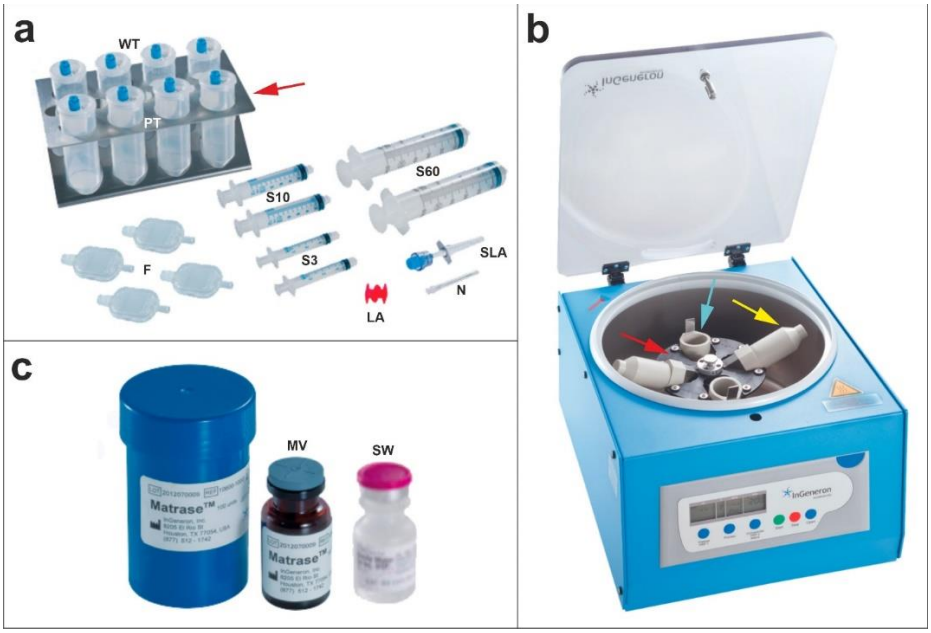

Figure 2. The Transpose RT system (InGeneron). The system consists of (**A**) the Transpose Ultra kit (all disposable components) and the Autoclavable Tube Rack (red arrow in A), (**B**) the Processing Unit and the Processing Unit Rotor (red arrow in B), and (**C**) the pharmaceutical-grade Matrase Reagent. Abbreviations: WT, wash tubes; PT, processing tubes; F, 200 µm filters; S10, 10 mL syringes; S60, 60 mL syringes; LA, luer adapter; SLA; spike luer adapter; N, needle; MV, Matrase vial; SW, sterile water. The blue and yellow arrows in (C) are explained in the legend of Figure 3.

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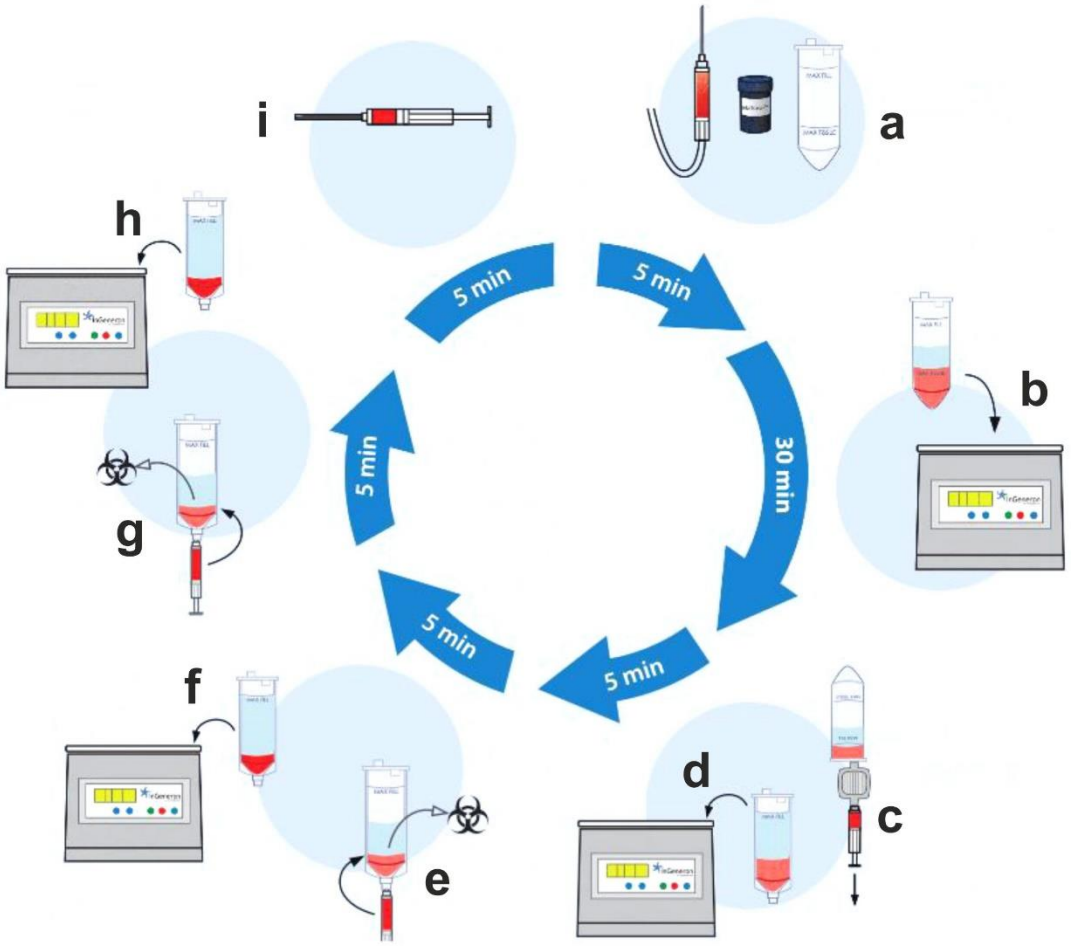

Figure 3. Isolation of UA-ADRCs from lipoaspirate using the Transpose RT system (InGeneron) shown in Figure 2 (taken from [1]). (**A**) Recovered lipoaspirate (25 ml) is loaded together with 2.5 ml reconstituted Matrase Reagent and lactated Ringer's solution (LRS) (preheated to 39° C) into a processing tube up to the MAX FILL line. (**B**) The filled processing tubes are subjected in an inverted position (indicated by the yellow arrow in Figure 2C) inside the Processing Unit to repetitive acceleration and deceleration for 30 minutes at 39° C. (**C**) The processed lipoaspirate solution is filtered through a 200 µm filter and transferred into a wash tube. (**D**) After filling the wash tube with saline (room temperature) up to the MAX FILL line, the cells are separated from the rest of the tissue by centrifugation at 600g for 5 minutes at room temperature (position of the wash tubes at the beginning of centrifugation indicated by the blue arrow in Figure 2C) . (**E**) The UA-ADRCs (approximately 2 ml) are extracted through a swappable luer vial adapter at the bottom of the wash tube, and the remaining substances (fat, debris and liquid) are discarded. (**F**) The cells are returned into the empty wash tube and (after adding fresh saline up to the MAX FILL line) centrifugated again for 5 minutes. (**G**,**H**) The previous washing step is repeated. (**I**) The concentrated UA-ADRCs (approximately 3 ml) are extracted and slowly pushed through a luer coupler into a new sterile syringe. At this point the UA-ADRCs are ready for application to the subject.

Age, gender, BMI and ethnicity of the subjects as well as the tissue volume/weight used for four tubes and the processing kit used (Transpose RT original or Transpose RT ultra; InGeneron) are summarized in Table 3.

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Table 3. Age, gender, BMI and ethnicity of the n=232 subjects whose lipoaspirate from elective lipoplasty was analyzed for characterization of UA-ADRCs isolated from lipoaspirate using the Transpose RT system (InGeneron), as well as the amount of tissue used for four tubes (Tissue) and the kit used.

Variable	Mean	SD	SEM	Minimum	Median	Maximum
Age	42.3	9.9	0.7	19	42	77
BMI	28.5	4.8	0.3	19.1	28.2	44.3
Tissue [g]	88.8	4.5	0.3	66.4	89.0	100.3
Gender	Female:	n=207				
	Male:	n=25				
Ethnicity	Caucasian:		n=153			
	Hispanic:		n=43			
	Black:		n=22			
	Asian:		n=7			
	African American:		n=3			
	Arabic:		n=2			
	Unknown:		n=2			
Kit used	Transpose RT original:			n=60		
	Transpose RT ultra:			n=172		

Abbreviations: SD, standard deviation; SEM, standard error of the mean; BMI, body mass index; g, grams.

The final cell suspension (c.f. Figure 3I) was analyzed for the following variables: V1, number of nucleated cells; V2, cell viability (%); V3, number of viable nucleated cells; V4, number of nucleated cells per gram tissue; and V5, number of viable nucleated cells per gram tissue. Cell counts and viability were determined using the FDA approved NucleoCounter NC-200 device (ChemoMetec Inc., Bohemia, NY, USA) as described by the manufacturer's protocol.

Statistical analysis of the obtained results was performed using univariate ANOVA, with gender, ethnicity and the kit used as fixed factors, and the subject's age, subject's BMI and the tissue volume/weight used for four wash/ processing tubes (c.f. Figure 2A) as covariates. *Post hoc* analysis was performed using linear regression analysis, Kolmogorov-Smirnov test and Fisher's exact test. Statistical analysis was performed using IBM SPSS Statistics for Windows (Version 28.0.0.0; IBM Corp., Armonk, NY, USA) and GraphPad Prism (Version 9.4.1 for Windows; GraphPad Software, San Diego, CA, USA).

UA-ADRCs from n=37 subjects were investigated using flow cytometry. The primary criterion for including a certain subject's UA-ADRCs into this analysis was the availability of a sufficient number of cells after having performed the NucleoCounter analysis. Based on the working hypothesis during the present study that the number of viable nucleated cells obtained would be related to the amount of tissue used for isolating UA-ADRCs, flow cytometry was preferentially performed on larger lipoaspirate specimens (note that flow cytometry was performed on fresh cells immediately after isolation; i.e., it was not possible to collect all lipoaspirate specimens first, followed by selection of lipoaspirate specimens for characterizing UA-ADRCs using flow cytometry).

To lyse red blood cells prior to labeling freshly isolated UA-ADRCs with primary antibodies for flow cytometry, cells were incubated with 1X BD PharmLyse lysing solution (BD Biosciences, San Jose, CA, USA) prepared and used according to the manufacturer's instructions. Afterwards, the cells were washed in FACS buffer (PBS containing 1% BSA and 0.05% sodium azide), followed by incubation for 30 minutes on ice with the conjugated, mouse-anti-human, primary antibodies listed in Table 4.

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Table 4. Primary antibodies used for flow cytometry.

CD	Clone	Isotype	Conjugate	Provider	Catalog #
CD3	OKT3	IgG2a, kappa	PE	eBioscience/ Thermo Fisher	12-0037-42
CD4	RPA-T4	IgG1, kappa	FITC	eBioscience/ Thermo Fisher	11-0049-42
CD14	61D3	IgG1, kappa	PE	eBioscience/ Thermo Fisher	12-0149-42
CD16	CB16	IgG1, kappa	FITC	eBioscience/ Thermo Fisher	11-0168-42
CD19	HIB19	IgG1, kappa	APC	eBioscience/ Thermo Fisher	17-0199-42
CD25	BC96	IgG1, kappa	PE	eBioscience/ Thermo Fisher	12-0259-42
CD31	WM59	IgG1, kappa	PE	eBioscience/ Thermo Fisher	12-0319-42
CD33	WM53	IgG1, kappa	FITC	eBioscience/ Thermo Fisher	11-0338-42
CD34	581	IgG1, kappa	PE-Cy	BD Pharmingen/ BD Biosciences	555823
CD45	HI30	IgG1	PerCP	Thermo Fisher	MHCD4531
CD45	HI30	IgG1, kappa	PerCP-eFluor71m	eBioscience/ fisher scientific	50-245-943
CD73	AD2	IgG1, kappa	APC	eBioscience/ Thermo Fisher	17-0739-42
CD90	5E10	IgG1, kappa	PE	eBioscience/ Thermo Fisher	12-0909-42
CD105	MEM-226	IgG2a	FITC	Thermo Fisher	MA1-19594
CD117	YB5.B8	IgG1, kappa	PE	eBioscience/ Thermo Fisher	12-1179-42
CD127	eBioRDR5	IgG1, kappa	APC	eBioscience/ Thermo Fisher	12-1179-42
CD144	16B1	IgG1	Alexa Fluor 488	eBioscience/ Thermo Fisher	53-1449-42
CD146	P1H12	IgG1, kappa	FITC	eBioscience/ Thermo Fisher	11-1469-42
CD206	19.2	IgG1, kappa	APC	eBioscience/ Thermo Fisher	17-2069-42

Manufacturers and providers: eBioscience / Thermo Fisher (Waltham, MA, USA); BD Pharmingen / BD Biosciences (San Jose, CA, USA). Abbreviations: CD, cluster of differentiation; PE, phycoerythrin; FITC, fluorescein isothiocyanate; APC, allophycocyanin; PE-Cy, R-phycoerythrin-cyanine complex; PerCP; peridinin-chlorophyll-protein complex.

Combinations of surface markers / primary antibodies and conjugates used for flow cytometry are summarized in Table 5.

Table 5. Combinations of surface markers / primary antibodies (M) and conjugates (C) used for flow cytometry.

Flow cytometry Tube 1		**Flow cytometry Tube 2**		**Flow cytometry Tube 3**		**Flow cytometry Tube 4**	
M	**C**	**M**	**C**	**M**	**C**	**M**	**C**
CD45	PerCP	CD45	PerCP	CD45	PerCP	CD45	PerCP
CD73	APC	CD4	FITC	CD34	PE-Cy	CD14	PE
CD90	PE	CD25	PE	CD105	FITC	CD16	FITC
CD105	FITC	CD127	APC	CD117	PE	CD206	APC
Flow cytometry Tube 5		**Flow cytometry Tube 6**		**Flow cytometry Tube 7**			
M	**C**	**M**	**C**	**M**	**C**		
CD45	PerCP	CD45	PerCP	CD45	PerCP		
CD3	PE	CD31	PE	CD31	PE		
CD19	APC	CD34	PE-Cy	CD34	PE-Cy		
CD33	FITC	CD146	FITC	CD144	Alexa Fluor 488		

After washing the cells twice with FACS buffer, flow cytometry was performed on a BD FACSCanto Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA) using BD FACSDiva Software (BD Bioscience). On average 13,671 ± 3,048 (mean ± standard deviation (SD)) (median, 13,308; range, 6,222 – 21,588) live cell events were acquired. Analysis was performed using FlowJo Software (FlowJo, LLC, Ashland, OR, USA).

Statistical analysis was performed using univariate ANOVA, with gender and ethnicity as fixed factors, and the tissue volume/weight used for four wash/ processing tubes

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(c.f. Figure 2A), the subject's age, subject's BMI, cell viability and the number of nucleated cells per gram tissue as covariates. *Post hoc* analysis was performed using linear regression analysis. Statistical analysis was performed using IBM SPSS Statistics for Windows (Version 28.0.0.0; IBM Corp.) and GraphPad Prism (Version 9.4.1 for Windows; GraphPad Software).

3. Results

3.1. Results of the investigations using the NucleoCounter (ChemoMetec)

The results of the investigations using the NucleoCounter (ChemoMetec) are summarized in Table 6.

Table 6. Mean, standard deviation (SD), standard error of the mean (SEM), minimum, median and maximum of the number of nucleated cells (V1), cell viability (V2), number of viable nucleated cells (V3), number of nucleated cells per gram tissue (V4) and number of viable nucleated cells per gram tissue (V5) of the UA-ADRCs isolated from lipoaspirate using the Transpose RT system (InGeneron) of n=232 subjects undergoing elective lipoplasty.

Variable	Mean	SD	SEM	Minimum	Median	Maximum
V1 [×10⁷]	5.38	2.33	0.15	1.1	5.0	14.3
V2 [%]	85.2	4.78	0.31	67.0	86.0	94.8
V3 [×10⁷]	4.59	1.98	0.13	0.9	4.4	12.4
V4 [×10⁵/g]	6.06	2.67	0.18	1.2	5.6	16.6
V5 [×10⁵/g]	5.18	2.28	0.15	1.0	4.9	14.4

Of note, the International Federation for Adipose Therapeutics and Science (IFATS) has determined a cell viability of at least 70% as a minimum criterion for UA-ADRCs [56]. This was achieved in 231 of the 232 (99.6%) investigated lipoaspirate specimens.

Furthermore, the results presented in Table 6 are in line with results reported in [1,31], which demonstrates the reproducibility of isolating UA-ADRCs from lipoaspirate using the Transpose RT system (InGeneron).

The results of the statistical analysis (univariate ANOVA with fixed factors and covariates) of the data shown in Table 6 are summarized in Table 7.

Table 7. Results (p values) of the statistical analysis (univariate ANOVA with fixed factors and covariates) of the data shown in Table 6.

	T	A	B	K	G	E	K×G	K×E	G×E	K×G×E
V1	0.155	0.077	0.075	0.707	0.141	0.139	0.582	0.857	0.979	0.154
V2	0.312	0.669	0.294	0.840	0.978	0.416	0.154	0.432	0.454	0.662
V3	0.216	0.085	**0.047**	0.719	0.125	0.085	0.655	0.807	0.963	0.146
V4	0.854	0.073	0.071	0.769	0.110	0.112	0.565	0.833	0.979	0.124
V5	0.738	0.081	**0.044**	0.773	0.097	0.066	0.638	0.773	0.961	0.117

P values < 0.05 are given boldface. Abbreviations: T, tissue volume/weight used for four wash/ processing tubes (c.f. Figure 2A); A, age; B, body mass index; K, kit used; G, gender; E, ethnicity; V1, number of nucleated cells; V2, cell viability; V3, number of viable nucleated cells; V4, number of nucleated cells per gram tissue; V5, number of viable nucleated cells per gram tissue.

ANOVA demonstrated a significant relationship between the subject's BMI (B in Table 7) and the number of viable nucleated cells (V3 in Table 7), as well as between the subject's BMI and the number of viable nucleated cells per gram tissue (V5 in Table 7). However, *post hoc* linear regression analysis did not support these findings of ANOVA (Figure 4).

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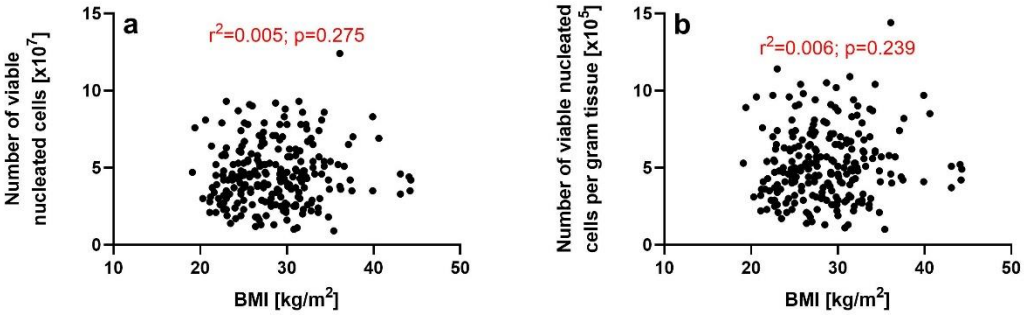

Figure 4. (**a**) Number of viable nucleated cells and (**b**) number of viable nucleated cells per gram tissue as a function of the subject's body mass index (BMI) of the UA-ADRCs isolated from lipoaspirate using the Transpose RT system (InGeneron) of n=232 subjects undergoing elective lipoplasty. The results of linear regression analysis are given in red. Abbreviation: r, Pearson's product moment correlation coefficient.

Collectively these data indicate that key characteristics of UA-ADRCs isolated from lipoaspirate using the Transpose RT system (InGeneron) (number of nucleated cells, cell viability, number of viable nucleated cells, number of nucleated cells per gram tissue and number of viable nucleated cells per gram tissue) do not depend on the subject's age, gender, BMI and ethnicity.

3.2. Validity of the lipoaspirate specimens investigated with flow cytometry as representative sample of the lipoaspirate specimens investigated with the NucleoCounter (ChemoMetec)

A working hypothesis during the present study was that the number of viable nucleated cells obtained would be related to the amount of tissue used to isolate UA-ADRCs. Accordingly, flow cytometry was preferentially performed on larger lipoaspirate specimens. However, *post hoc* analysis did not reveal a relationship between the number of viable nucleated cells obtained and the amount of tissue used for isolating UA-ADRCs (Figure 5).

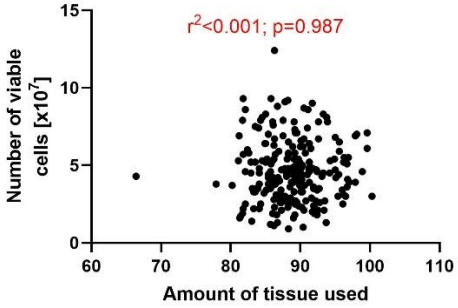

Figure 5. Number of viable nucleated cells as a function of the amount of tissue used for isolating UA-ADRCs from lipoaspirate using the Transpose RT system (InGeneron) of n=232 subjects undergoing elective lipoplasty. The results of linear regression analysis are given in red. Abbreviation: r, Pearson's product moment correlation coefficient.

However, this finding exerted no negative impact on the results of the flow cytometry analysis. This is due to the fact that *post hoc* analysis using the Kolmogorov-Smirnov test showed a statistically significant difference between the distributions of the amount of tissue used to isolate UA-ADRCs (Figure 6A), but no significant differences between the subjects whose UA-ADRCs were used for flow cytometry and those whose UA-ADRCs were not used for flow cytometry with respect to the distributions of the subject's age, subject's BMI, number of nucleated cells, cell viability, number of viable nucleated

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cells, number of nucleated cells per gram tissue and number of viable nucleated cells per gram tissue (Figure 6B-H).

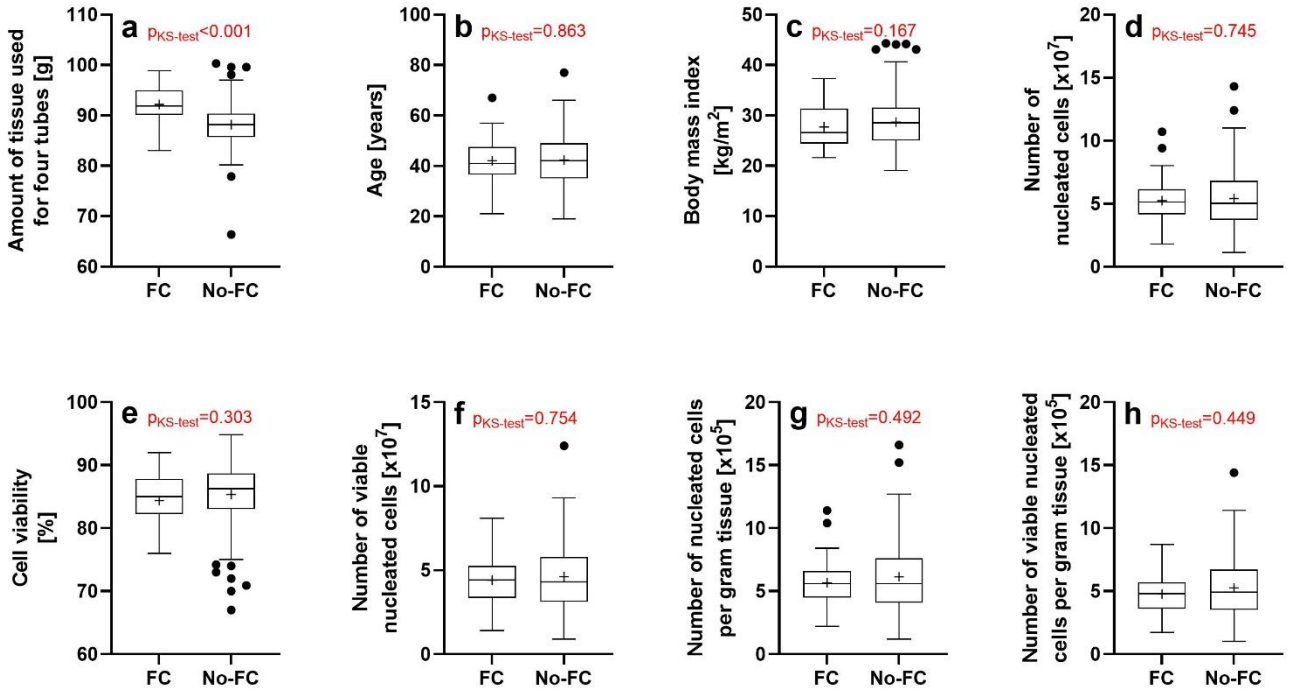

Figure 7. Tukey boxplots of (**a**) the amount of issue used, (**b**) subjects' age, (**c**) subjects' BMI, (**d**) number of viable nucleated cells, (**e**) cell viability, (**f**) number of viable nucleated cells, (**g**) number of nucleated cells per gram tissue and (**h**) number of viable nucleated cells per gram tissue of the subjects whose UA-ADRCs were used for flow cytometry (FC) and those whose UA-ADRCs were not used for flow cytometry (No-FC). The results of the Kolmogorov-Smirnov test (KS) are given in red.

Furthermore, Fisher's exact test showed no statistically significant difference in the distributions of gender (p = 0.143) and ethnicity (p = 0.324) between the subjects whose UA-ADRCs were used for flow cytometry and those whose UA-ADRCs were not used for flow cytometry (Table 8).

Table 8. Distributions of gender and ethnicity of the subjects whose UA-ADRCs were used for flow cytometry (FC) and those whose UA-ADRCs were not used for flow cytometry (No-FC).

		All subjects	FC subjects	No-FC subjects
Gender	Female	207	36	171
	Male	25	1	24
			$p_{Fisher's\ exact\ test}$ = 0.143	
Ethnicity	Caucasian	153	26	127
	Hispanic	43	3	40
	Black	22	5	17
	Asian	7	2	5
	African American	3	0	3
	Arabic	2	1	1
	Unknown	2	0	2
			$p_{Fisher's\ exact\ test}$ = 0.324	

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3.3. Results of the flow cytometry investigations

Table 9 summarizes mean, standard deviation, standard error of the mean, minimum, median and maximum of the relative numbers of cells expressing a given combination of surface markers.

Table 9. Combinations of surface markers / primary antibodies used for flow cytometry.

Surface markers	n	Mean	SD	SEM	Min	Med	Max
CD45-	37	58.0	7.7	1.3	42.1	57.6	72.1
CD45- CD73+ CD90+	37	32.5	8.7	1.4	20.4	30.0	61.4
CD45- CD73+ CD90+ CD105+	37	2.4	1.4	0.2	0.2	2.0	6.7
CD45- CD31+	37	19.3	6.5	1.1	7.5	17.4	37.0
CD45- CD31+ CD34+	37	15.3	4.5	0.7	7.3	14.4	28.1
CD45- CD31+ CD34+ CD146+	37	13.2	4.0	0.7	3.8	13.2	24.9
CD45- CD31-	37	36.5	5.5	0.9	28.3	35.6	51.8
CD45- CD31- CD34+	37	32.8	6.5	1.1	20.9	30.9	48.3
CD45- CD31- CD34+ CD146+	37	0.7	0.4	0.1	0.1	0.5	2.1
CD45- CD31- CD34-	37	9.2	4.0	0.7	2.2	8.7	18.3
CD45- CD31- CD34- CD146+	37	6.5	4.5	0.7	0.6	5.4	16.0
CD45+	37	42.0	7.7	1.3	27.9	42.4	57.9
CD45+ CD34+	27	1.7	1.4	0.3	0.6	1.4	7.8
CD45+ CD206+	37	16.4	4.3	0.7	8.1	16.0	26.1
CD45+ CD4+ CD25-	20	4.0	2.0	0.4	1.4	3.8	9.0
CD45+ CD4+ CD25+	20	4.3	1.9	0.4	0.7	4.8	8.6
CD45- CD73- CD90- CD105+	37	1.8	1.6	0.3	0.3	1.4	8.6
CD45- CD31+ CD34+ CD144+	37	11.9	4.2	0.7	4.5	10.9	22.0
CD45+ CD73+ CD90+ DC105+	37	6.9	3.8	0.6	0.5	7.3	13.7
CD45+ CD34+ CD105+ CD117+	9	0.2	0.1	0.0	0.1	0.2	0.5
CD45+ CD73- CD90- CD105+	36	4.2	2.8	0.5	0.6	3.7	13.3
CD45+ CD34- CD105+ CD117-	10	0.4	0.3	0.1	0.1	0.3	1.0
CD45+ CD14+ CD16+ CD206+	37	12.1	4.8	0.8	2.3	11.6	20.6
CD45+ CD31+ CD34- CD146-	37	19.4	5.9	1.0	6.1	18.9	37.0
CD45+ CD3+ CD19- CD33-	37	7.2	2.7	0.4	2.1	6.9	17.6
CD45+ CD3- CD19+ CD33-	37	0.7	0.4	0.1	0.1	0.6	1.9
CD45+ CD3- CD19- CD33-	37	4.1	1.5	0.2	1.9	3.7	8.7
CD14+	37	18.9	5.8	1.0	8.1	18.4	31.8
CD31+	37	49.6	5.8	0.9	39.1	50.8	58.8
CD34+	37	56.0	7.3	1.2	39.0	56.7	70.6
CD73+	37	46.5	7.1	1.2	28.8	46.8	59.4
CD90+	37	56.6	8.0	1.3	40.6	57.8	74.8
CD105+	37	24.1	6.1	1.0	2.3	24.4	38.1
CD45+ CD14+	37	17.8	5.3	0.9	7.5	16.9	29.6
CD45- CD90+	37	45.9	6.8	1.1	31.4	45.7	62.2
CD73+ CD90+	37	36.7	6.6	1.1	22.1	36.6	50.9
CD45+ CD73+ CD90+	37	8.1	4.2	0.7	1.3	7.4	16.2

Abbreviations: n, number of lipoaspirate specimens investigated; SD, standard deviation; SEM, standard error of the mean; Min, minimum value; Med, median value; Max, maximum value.

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Table 10 lists the combination of surface markers shown in Table 9, the respective cell type and the corresponding reference to the literature.

Table 10. Combination of surface markers shown in Table 9, respective cell type and corresponding reference to the literature.

Surface markers	Cell type	Reference
CD45-	CD45- cell group	
CD45- CD73+ CD90+	(ADSCs)	[57]
CD45- CD73+ CD90+ CD105+	ADSCs	[57]
CD45- CD31+	Endothelial cells	[54]
CD45- CD31+ CD34+	Endothelial progenitors	[54]
CD45- CD31+ CD34+ CD146+	Pericytes	[54]
CD45- CD31-	CD45- CD31- group	[54]
CD45- CD31- CD34+	Adipose tissue derived progenitors	[54,58]
CD45- CD31- CD34+ CD146+	Pericyte progenitors	[54]
CD45- CD31- CD34-	SVF nonprogenitors	[54]
CD45- CD31- CD34- CD146+	Pericytes	[54]
CD45+	CD45+ cell group	
CD45+ CD34+	Leukocyte progenitors	[54]
CD45+ CD206+	M2 macrophages	[54]
CD45+ CD4+ CD25-	Naïve T cells	[59]
CD45+ CD4+ CD25+	Regulatory T cells	[60]
CD45- CD73- CD90- CD105+	Endothelial cells	[61]*
CD45- CD1+ CD34+ CD144+	Endothelial cells	[61]*
CD45+ CD73+ CD90+ CD105+		[61]*
CD45+ CD34+ CD105+ CD117+	Hematopoietic stem cell precursors	[61]*
CD45+ CD73- CD90- CD105+	Macrophages/ Monocytes	[61]*
CD45+ CD34- CD105+ CD117-	Macrophages/ Monocytes	[61]*
CD45+ CD14+ CD16+ CD206+	Macrophages/ Monocytes	[61]*
CD45+ CD31+ CD34- CD146-	Macrophages/ Monocytes	[61]*
CD45+ CD3+ CD19- CD33-	T cells	[61]*
CD45+ CD3- CD19+ CD33-	B cells	[61]*
CD45+ CD3- CD19- CD33-	NK cells	[61]*
CD14+		[58]
CD31+		[62]
CD34+		[62]
CD73+		[62]
CD90+		[62]
CD105+		[62]
CD45+ CD14+		[63]
CD45- CD90+		[63]
CD73+ CD90+		[63]
CD45+ CD73+ CD90+		

*, according to the BD Biosciences Human and Mouse CD Marker Handbook [61] said cells would express the listed combination of surface markers. However, this does not imply that said combination of surface markers are specific for the corresponding cell type.

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The results of the statistical analysis (univariate ANOVA with fixed factors and covariates) of the data shown in Table 9 are summarized in Table 11.

Table 11. Results (p values) of the statistical analysis (univariate ANOVA with fixed factors and covariates) of the data shown in Table 9.

Surface markers	A	G	BMI	E	T	V2	V4
CD45-	0.973	0.632	0.411	0.411	0.847	0.139	0.141
CD45- CD73+ CD90+	0.100	**0.002**	0.373	0.402	0.483	0.617	0.060
CD45- CD73+ CD90+ CD105+	0.858	0.547	0.769	0.762	0.464	0.156	0.295
CD45- CD31+	0.306	0.494	0.469	0.648	0.659	**0.025**	0.343
CD45- CD31+ CD34+	0.390	0.836	0.198	0.962	0.702	0.089	0.232
CD45- CD31+ CD34+ CD146+	0.625	0.930	0.455	0.998	0.755	0.458	0.686
CD45- CD31-	0.157	0.914	0.918	0.638	0.508	0.656	0.386
CD45- CD31- CD34+	**0.049**	0.908	0.462	0.397	0.742	0.186	0.819
CD45- CD31- CD34+ CD146+	0.976	0.724	0.456	0.688	0.706	0.731	0.441
CD45- CD31- CD34-	0.099	0.719	0.507	0.339	0.852	**0.035**	0.129
CD45- CD31- CD34- CD146+	0.145	0.680	0.114	0.179	0.578	**0.037**	0.545
CD45+	0.969	0.625	0.406	0.437	0.859	0.139	0.148
CD45+ CD34+	**0.045**	--*	0.186	0.600	0.073	0.521	0.976
CD45+ CD206+	0.747	0.483	0.409	0.781	0.657	0.313	0.488
CD45+ CD4+ CD25-	0.390	--*	0.188	0.487	0.697	0.182	0.956
CD45+ CD4+ CD25+	0.139	--*	0.572	0.278	0.645	0.108	0.501
CD45- CD73- CD90- CD105+	0.300	0.283	0.410	0.527	0.199	0.873	0.160
CD45- CD31+ CD34+ CD144+	0.254	0.886	0.314	0.882	0.987	0.084	0.220
CD45+ CD73+ CD90+ CD105+	0.140	0.576	0.995	0.139	0.452	0.106	0.445
CD45+ CD34+ CD105+ CD117+	0.218	--*	0.874	0.140	0.596	0.515	0.372
CD45+ CD73- CD90- CD105+	**0.043**	0.437	**0.028**	0.519	0.748	0.553	0.686
CD45+ CD34- CD105+ CD117-	0.678	--*	0.792	0.534	0.702	0.624	0.834
CD45+ CD14+ CD16+ CD206+	0.740	0.747	0.484	0.517	0.626	0.204	0.861
CD45+ CD31+ CD34- CD146-	**0.046**	0.706	0.124	0.392	0.425	0.747	0.375
CD45+ CD3+ CD19- CD33-	0.590	0.789	0.636	0.491	0.824	0.244	0.299
CD45+ CD3. CD19+ CD33-	0.318	0.335	0.883	0.371	0.389	0.245	0.841
CD45+ CD3- CD19- CD33-	0.088	0.890	0.982	0.167	0.979	0.307	**0.021**
CD14+	0.919	0.794	0.181	0.292	0.709	0.440	0.952
CD31+	**0.041**	0.931	0.757	0.372	0.454	0.362	0.327
CD34+	**0.008**	0.826	**0.035**	**0.030**	0.820	0.274	0.478
CD73+	**0.017**	0.671	0.553	0.073	0.934	0.293	0.617
CD90+	0.273	0.245	0.351	0.368	0.671	0.941	0.356
CD105+	0.590	0.903	0.601	0.610	0.854	0.664	0.267
CD45+ CD14+	0.821	0.739	0.224	0.352	0.784	0.328	0.892
CD45- CD90+	0.922	0.558	0.527	0.640	0.828	0.456	0.949
CD73+ CD90+	0.052	0.666	0.525	0.173	0.990	0.477	0.757
CD45+ CD73+ CD90+	**0.032**	0.573	0.478	**0.046**	0.185	**0.020**	0.166

P values < 0.05 are given boldface. Abbreviations: A, age; G, gender; BMI, body mass index; E, ethnicity; T, tissue volume/weight used for four tubes; V2, cell viability; V4, number of nucleated cells per gram tissue; *, no data available for male subjects.

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Statistically significant relationships between the relative number of a given cell type and any of the fixed factors and covariates used in the univariate ANOVA (p values that are given boldface in Table 11) were tested using *post hoc* linear regression analysis. The results of *post hoc* analysis are summarized in Figure 7 and Figure 8; statistically significant results are summarized in Table 12.

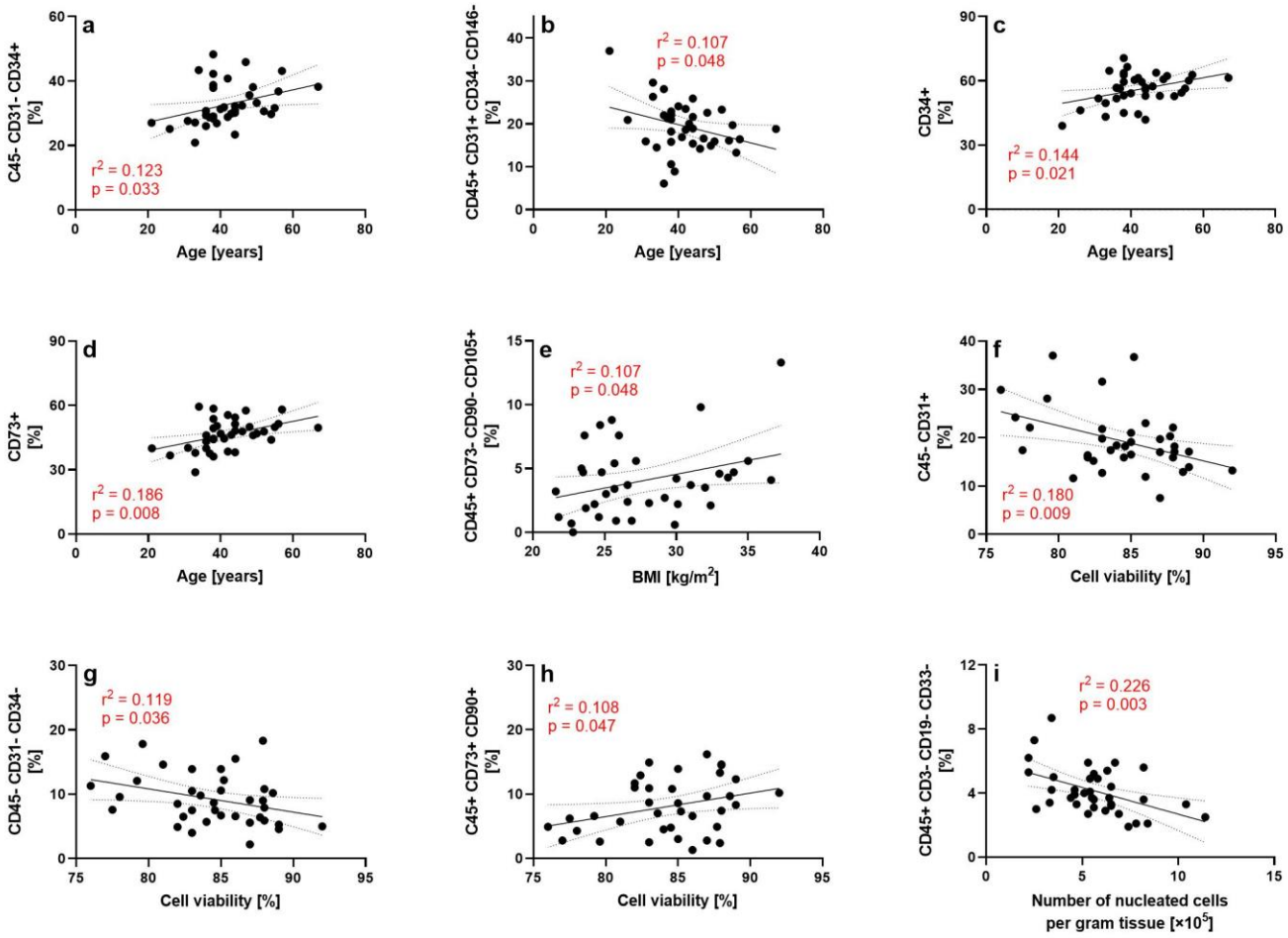

Figure 7. Individual relative numbers of cells of a certain cell type as a function of the (**a**-**d**) subject's age, (**e**) body mass index, (**f**-**h**) cell viability and (**i**) number of nucleated cells per gram tissue of the UA-ADRCs isolated from lipoaspirate using the Transpose RT system (InGeneron) of the subjects whose UA-ADRCs were used for flow cytometry. The relations shown here are those that were statistically significant in the univariate ANOVA (p values in Table 11 that are given boldface). The results of linear regression analysis are given in red. Abbreviation: r, Pearson's product moment correlation coefficient.

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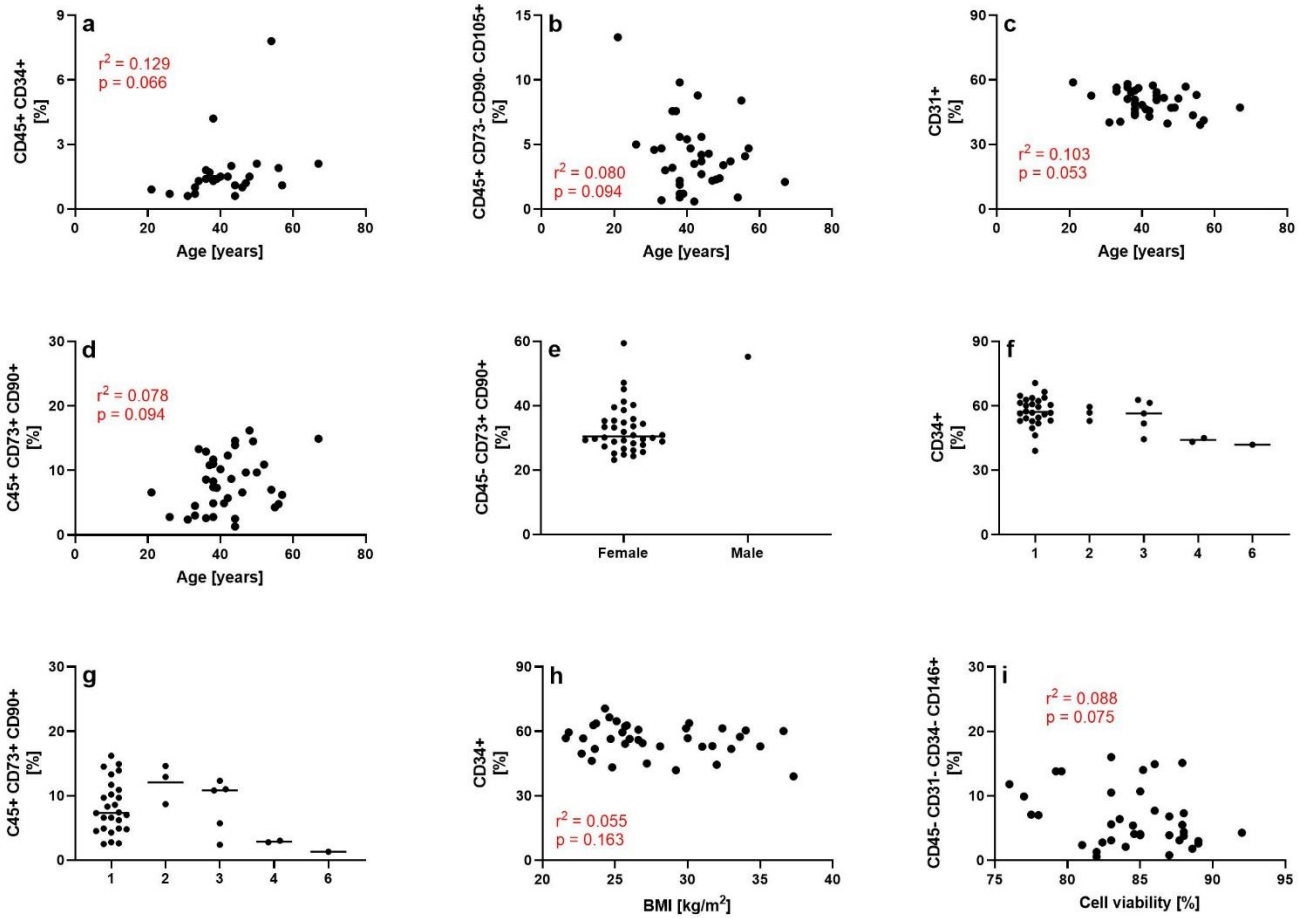

Figure 8. Individual relative numbers of cells of a certain cell type as a function of the (**a**-**d**) subject's age, (**e**) gender, (**f**,**g**) ethnicity, (**h**) BMI and (**i**) cell viability of the UA-ADRCs isolated from lipoaspirate using the Transpose RT system of the subjects whose UA-ADRCs were used for flow cytometry. The relations shown here are those that were statistically significant in the univariate ANOVA (p values in Table 11 that are given boldface). The results of the statistical analysis using linear regression analysis are given in red. Abbreviation: r, Pearson's product moment correlation coefficient. Ethnicity is coded in (F,G) as follows: 1, Caucasian; 2, Hispanic; 3, Black; 4, Asian; 6, Arabic.

Table 12. Statistically significant relations between the relative numbers of cells of a certain cell type and a certain covariate that were significant ($p < 0.05$) in both the univariate ANOVA (Table 11) and the *post hoc* linear regression analysis (Figure 7).

Surface markers	Cell type	Statistically significant relation
CD45- CD31- CD34+	Adipose tissue derived progenitors	Age
CD45+ CD31+ CD34- CD146-	Macrophages / Monocytes*	Age
CD34+		Age
CD73+		Age
CD45+ CD73- CD90- CD105+	Macrophages / Monocytes*	BMI
CD45- CD31+	Endothelial cells	Cell viability
CD45- CD31- CD34-	SVF non-progenitors	Cell viability
CD45+ CD73+ CD90+		Cell viability
CD45+ CD3- CD19- CD33-	NK cells*	Number of nucleated cells per gram tissue

*, according to the BD Biosciences Human and Mouse CD Marker Handbook [61] said cells would express the listed combination of surface markers. However, this does not imply that said combination of surface markers are specific for the corresponding cell type.

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Except for an age-related increase in the relative number of CD45- / CD31- / CD34+ cells (Adipose tissue derived progenitors in [54,56]) and a negative correlation between the cell viability and the relative numbers of CD45- / CD31+ cells (endothelial cells) and CD45- / CD31- / CD34- cells (SVF non-progenitors in [54]) the relative numbers of cells of the other cell types in UA-ADRCs isolated from lipoaspirate using the Transpose RT system (InGeneron) did neither depend on the subject's age, gender and ethnicity nor the tissue volume/weight used for four wash/ processing tubes (c.f. Figure 2A), the cell viability and the number of nucleated cells per gram tissue (note that the other combinations of surface markers listed in Table 12 are not considered here because they do not unequivocally indicate a certain cell type).

4. Discussion

This is the first study demonstrating, for a system designed to isolate UA-ADRCs from lipoaspirate (i.e., the Transpose RT system (InGeneron)), that key characteristics of the UA-ADRCs (number of nucleated cells, cell viability, number of viable nucleated cells, number of nucleated cells per gram tissue and number of viable nucleated cells per gram tissue) obtained using this system do not depend on the subject's age, gender, BMI and ethnicity. This result has important implications for the general applicability of UA-ADRCs isolated from lipoaspirate using this system in regeneration of musculoskeletal tissue. The same applies to the characterization of UA-ADRCs using flow cytometry, although the presented data are limited by the fact that 36 of the 37 investigated lipoaspirate specimens were from women.

Table 13 provides a comparison of key results of the present study with published data in the literature that have characterized the products from other commercially available systems to isolate UA-ADRCs or systems that provide non-enzymatic, mechanical dissociation of lipoaspirate. Of note, the relative number of lipoaspirate specimens from male subjects investigated in [54] (2 of 23 (87%)) was not statistically significantly different from the relative number of lipoaspirate specimens from male subjects investigated in the present study (1 of 37 (97%)) (Fisher's exact test; $p = 0.552$). In [52], all investigated lipoaspirate specimens were from female subjects (5 of 5). In [32], lipoaspirate specimens from 18 female and 15 male subjects were investigated, but no flow cytometry was performed.

Table 13. Comparison of the results of the present study with published data in the literature.

	Cell type	**Adipose progenitors [%]**	**Endothelial progenitors [%]**	**M2 macrophages [%]**	**Cell viability [%]**
	Surface markers	CD45- CD31- CD34+	CD45- CD31+ CD34+	CD45+ CD206+	
Reference	System/method				
This study†	A	32.8	15.3	16.4	85.2
[58]†	A*	20.0	--	--	--
[54]†	B	16.1	9.4	5.6	82.0
[52]†	C	10.7	--	--	84.0
[52]†	D	9.1	--	--	82.0
[52]†	B	8.9	--	--	69.3
[52]†	E	7.2	--	--	50.3
[32]‡	F	--	--	--	64.4

Shown are mean relative numbers of cells of specific cell types as well as the mean cell viability of (†) UA-ADRCs or (‡) cell suspensions generated by non-enzymatic, mechanical dissociation of lipoaspirate using different, commercially available systems. Abbreviations: A, Transpose RT system (InGeneron); B, GID SVF-2 system (GID Bio, Inc., Louisville, CO, USA); C, Cytori StemSource 900/MB system (Lorem Cytori USA, Inc., San Diego, CA, USA); D, PNC MultiStation (PNC Technologies Co., Ltd., Anyang, Republic of Korea); E, MediKhan Lipokit Platform (Medi Khan Inc., Seoul, Korea); F, Arthrex ACP double syringe system (Arthrex, Naples, FL, USA). *, analysis of frozen/thawed UA-ADRCs.

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Of all commercially available systems to isolate UA-ADRCs or systems that provide non-enzymatic, mechanical dissociation of lipoaspirate that have been described in the literature, use of the Transpose RT system (InGeneron) resulted in the highest mean relative numbers of CD45- / CD31- / CD34+ cells (adipose tissue derived progenitors), CD45- / CD31+ / CD34+ cells (endothelial progenitors) and CD45+ / CD206+ cells (M2 macrophages).

As in the present study, UA-ADRCs were isolated from lipoaspirate using the Transpose RT system (InGeneron) in [58]. The mean relative number of CD45- / CD31- / CD34+ cells (adipose tissue derived progenitors) of 20.0% in [58] (compared to 32.8% in the present study) may be explained by the fact that frozen/thawed UA-ADRCs were investigated in [58], whereas respectively fresh UA-ADRCs or non-enzymatically, mechanically dissociated lipoaspirate were investigated in the present study as well as in [32,52,54]. Furthermore, the evidence of a statistically significant negative association between the subject's BMI and the relative number of CD45- / CD31- / CD34+ cells (adipose tissue derived progenitors) in [58], that was not found in the present study (c.f. Table 11), may be explained by the facts that in [58] (i) a smaller sample was investigated than in the present study (n=24 lipoaspirate specimens in [58] vs. n=37 lipoaspirate specimens in the present study), and (ii) the statistical analysis in [58] was a simple linear regression analysis, whereas in the present study univariate ANOVA was applied, with gender and ethnicity as fixed factors, and the tissue volume/weight used for four tubes, subject's age, subject's BMI, cell viability and the number of nucleated cells per gram tissue as covariates. The mean relative number of CD14+ cells reported in [58] (22.5%) was similar to the mean relative number of CD14+ cells found in the present study (18.9%) (c.f. Table 9). Collectively, these data support the reproducibility of results of characterizing UA-ADRCs isolated from lipoaspirate using the Transpose RT system (InGeneron) with flow cytometry.

Adipose tissue derived progenitors and stem cells contained in UA-ADRCs may play a crucial role in regeneration of musculoskeletal tissue [1-5]. Regarding the regeneration of tendon tissue, for example, ten different aspects can be differentiated in this regard, as shown in the following text:

First, when positioning a decellularized human flexor tendon seeded with human, cultured ADSCs (tendon A) next to a decellularized human flexor tendon not seeded with ADSCs (tendon B) *in vitro*, the ADSCs migrated from tendon A into tendon B [64]. After injection of UA-ADRCs into a tendon defect, the cells can therefore migrate from the site of application to the site of tendon lesion, as suggested in [2].

Second, direct coculture of canine ADSCs and canine tenocytes *in vitro* induced enhancement of tenogenesis by intensive cell-cell interaction and exchange of vesicles [65]. These results are in line with the finding of cell-cell interactions and exchange of vesicles between human ADSCs and MDA-MB-231 cells (a commercially available human breast cancer cell line) *in vitro* [5], as well as with the finding of cell-to-cell connection of endothelial progenitor cells with cardiac myocytes by nanotubes [66]. Collectively, these results indicate an important mechanism for cell fate changes of stem cells and progenitor cells in tissue regeneration.

Third, direct co-culture of human ADSCs and human tenocytes *in vitro* induced synergistic proliferation and type I collagen production, whereas indirect co-culture of these cell types did not induce synergistic proliferation [67]. Furthermore, direct co-culture of human ADSCs and human tendon-derived cells (TDCs) *in vitro* resulted in increased gene expression and increased production of type I A1 collagen as well as decreased gene expression and decreased production of type III A1 collagen [68]. These results, which were not obtained by separate culture of human ADSCs and human TDCs [68], indicate that, after injection of UA-ADRCs into a tendon defect, direct contact between the UA-ADRCs and tenocytes may play a pivotal role in affecting the properties of the UA-ADRCs in terms of proliferation and differentiation based on cues from the local microenvironment.

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Fourth, after encapsulating human ADSCs in 3D collagen scaffolds and exposure to different uniaxial tensile strains and loading frequencies *in vitro*, the ADSCs showed increased gene expression of tenascin, scleraxis, tenomodulin, runX2, decorin, aggrecan, type I collagen and type III collagen, without increased expression of genes related to osteogenic, chondrogenic and myogenic differentiation [69]. Furthermore, after exposure of human ADSCs with mechanical tensile stimulation using a pneumatic microchamber device *in vitro*, the ADSCs showed increased proliferation and increased gene expression with interindividual variability (nanog, sox2 and oct4 in case of four of seven subjects and runX2 in case of three of seven subjects) [70]. These results indicate that, after injection of UA-ADRCs into a tendon defect, tensile stimuli on the cells may stimulate them towards active contribution to tendon regeneration.

Fifth, after injection of cultured, autologous ADSCs into a rabbit Achilles tendon defect/repair model in vivo, the cells differentiated into tenocytes and integrated into the host tissue [71]. Furthermore, after injection of cultured, autologous ADSCs into an experimentally induced tendon defect in horses in vivo, the ADSCs differentiated into cells that were integrated into new (tendon) tissue, with detection up to nine weeks post-treatment [72]. In addition, when seeding human, cultured ADSCs on a specific scaffold (Hyalonect meshes) *in vitro*, the ADSCs created a capillary network within the scaffold [73]. These results indicate that after injection of UA-ADRCs into a tendon defect, the cells may differentiate into other cell types that are necessary for tendon regeneration and integrate into the host tissue.

Sixth, indirect co-culture of human ADSCs and human tendon explants *in vitro* resulted in reduced production of type III collagen by the ADSCs and increased production of type I collagen by the cells in the tendon explants [74]. These results indicate that after injection of UA-ADRCs into a tendon defect, the cells may stimulate locally resident tenocytes towards the production of type I collagen (more precisely the production of type I procollagen, which is processed by enzymes outside the cell, followed by self-arrangement of the processed molecules into long, thin collagen fibrils that cross-link to one another in the extracellular space [2]). Furthermore, at the same time the locally resident tenocytes may prevent the UA-ADRCs from producing molecules that may not contribute to optimal tendon regeneration.

Seventh, after application of cultured, autologous ADSCs in a canine tendon defect/repair model in vivo, increased expression of genes associated with anti-inflammatory M2 macrophages and decreased expression of genes associated with pro-inflammatory M1 macrophages were observed in the tendon tissue [75]. Furthermore, triple culture of mouse ADSCs, mouse tendon fibroblasts and mouse M1 macrophages *in vitro* resulted in conversion of M1 macrophages into M2 macrophages [76]. On the other hand, there was no impact of the ADSCs on interleukin 1 beta (IL-1β) induced up-regulation of pro-inflammatory factors and matrix degradation factors by cultured tendon fibroblasts [76]. These results indicate that after injection of UA-ADRCs into a tendon defect, the cells can exert additional, important effects in treatment of sPTRCT beyond tissue regeneration (the significance of M2 macrophages in the early phase of treatment is outlined in detail below).

Eighth, after treatment of an experimentally induced tendon defect in dogs with surgical repair and augmentation with cultured, autologous ADSCs and bone morphogenetic protein (BMP) 12 in a nanofiber/fibrin-based scaffold in vivo, induction of a negative inflammatory reaction was observed at the repair site, leading to poor healing (no distinction was made whether the ADSCs or BMP-12 were responsible for inducing the negative inflammatory reaction) [77]. Furthermore, no effect on tendon healing was observed after injection of cultured, autologous ADSCs into an equine tendon defect model in vivo [78]. These results indicate that treatment of tendon defects with injection of cultured, autologous ADSCs may not be sufficient, because important cell types are missing in cultured ADSCs compared with UA-ADRCs.

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Ninth, after treating an experimental rabbit supraspinatus defect/repair model with UA-ADRCs applied in fibrin glue in vivo, increased production of type I collagen, an increased ratio of type I / type III collagen and increased production of BMP-2 were observed in the supraspinatus tendon, as well as improved biomechanical properties of the treated tendon [79,80]. These results are in line with the results obtained after treating sPTRCT with UA-ADRCs presented in [1,2].

Tenth, after conditional overexpression of tenomodulin in cultured ADSCs obtained from a transgenic mouse line, the ADSCs showed increased gene expression towards tenogenic differentiation (i.e., increased gene expression of tenomodulin, scleraxis, tenascin-C, type I collagen, type III collagen and type IV collagen) and reduced gene expression towards osteogenic and chondrogenic differentiation (i.e., reduced gene expression of alkaline phosphatase, runx2, osteocalcin, sox9, type II collagen and aggrecan) [81]. Furthermore, after seeding human, cultured ADSCs on a 3D collagen scaffold supplemented with urea-extracted fraction of tendon extracellular matrix *in vitro*, uniaxial tension triggered increased proliferation and tenogenic differentiation as well as prevention of osteogenic differentiation of the ADSCs [82]. These results may explain why after injection of UA-ADRCs into a tendon defect no formation of ectopic bone and cartilage was observed in [1,2].

Considering all the data presented here, one can conclude that treatment of tendon defects with UA-ADRCs may indeed result in tendon regeneration without scar formation, as indicated in [2]. Furthermore, there is strong evidence indicating that the molecular and cellular mechanisms of action of UA-ADRCs in tendon regeneration are far beyond paracrine effects of the stem and progenitor cells contained in UA-ADRCs.

The potential role of endothelial progenitors in tendon and cartilage regeneration is still largely unknown. However, at least in the early phase of tissue regeneration provision of sufficient blood supply to the defect site appears mandatory. This is supported by the finding of immunolabeling of both CD34 (indicating endothelial cells) and Ki-67 (a marker of cell proliferation) in microvessels next to the region of active tendon regeneration in a biopsy from a human supraspinatus tendon that was taken ten weeks post-treatment of a traumatic sPTRCT with injection of UA-ADRCs [2]. Furthermore, endothelial progenitors were demonstrated to play a critical role at the site of tendon-bone integration in tendon regeneration [83].

M2 macrophages are mainly involved in anti-inflammatory responses [84]. Tears of the rotator cuff were shown to be associated with synovial inflammation and increased expression of the pro-inflammatory markers interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) [85,86]. Furthermore, exposure of cultured ADSCs with IL-1β and TNF-α resulted in decreased expression of the tenogenic transcription factor scleraxis [87]. The latter does not only play a pivotal role in promoting tenocyte proliferation and extracellular matrix (ECM) synthesis during embryonic tendon development but is also involved in promoting the initial expansion of newly committed tenocytes and the production of ECM proteins in adult tendons [88]. This is in line with the finding that exposure of postnatal tendon cells with IL-1β resulted in reduced anabolic activity (leading to abnormal ECM deposition and organization) and increased catabolic activity (leading to proinflammatory cues and ECM degradation) [89]. In summary, the presence of M2 macrophages in UA-ADRCs may significantly contribute to tendon regeneration. Furthermore, the presence of M2 macrophages in UA-ADRCs may explain the very early treatment success observed after treating sPTRCT with UA-ADRCs [1,2], which cannot be explained by the formation of new tendon tissue.

The importance of the highest possible cell viability of UA-ADRCs or cells in cell suspensions obtained using non-enzymatic, mechanical dissociation of lipoaspirate results from the fact that the injection of non-viable cells into tissue can lead to inflammatory reactions [33]. Furthermore, the data presented in Figure 7G demonstrate that, with decreasing cell viability, the mean number of CD45- / CD31- / CD34- cells (SVF nonprogenitors) increased. This finding implies that with decreasing cell viability those cell types

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that play an important role in tendon and cartilage regeneration are increasingly underrepresented in UA-ADRCs. This is in line with the finding in [52] that with decreasing average cell viability (Cytori > MultiStation > GID SVF-2 > Lipokit; details of these commercially available systems are provided in Table 13), there was a trend towards decreasing relative numbers of CD45- / CD31- / CD34+ cells (adipose tissue derived progenitors) (Cytori > MultiStation > GID SVF-2 > Lipokit). However, it would not be correct at this point to focus on just one cell type characterized by flow cytometry. This is due to the facts that (i) no such trend was found in the present study for the CD45- / CD31- / CD34+ cells (adipose tissue derived progenitors), and (ii) stem cells contained in UA-ADRCs are a heterogeneous population that cannot be fully characterized by flow cytometry [90]. Thus, full characterization of the stem cells (as well as of all other cell types) contained in UA-ADRCs will require large-scale single-cell transcriptomic sequencing, as recently performed for bone marrow aspirate concentrate [91]. Of note, corresponding data obtained for cultured ADSCs [92] are not suitable for drawing conclusions for UA-ADRCs, as stem cells contained in UA-ADRCs demonstrate rapid and marked changes in gene expression when subjected to standard tissue culture conditions [62,90].

The mean cell viability of UA-ADRCs isolated from lipoaspirate using the Transpose RT system (InGeneron) found in the present study (85.2%) is very similar to independent reports in the literature (88.5% in [1] and 85.9% in [31]). This indicates a high reproducibility of the composition of the cell suspension obtained using the Transpose RT system (InGeneron). In contrast, the mean cell viability of UA-ADRCs isolated from lipoaspirate using the GID SVF-2 system (GID Bio) was reported as 82.0% in [54] and [93], but only 69.3% in [32]. Furthermore, the mean relative number of CD45- / CD31- / CD34+ cells (adipose tissue derived progenitors) obtained using the GID SVF-2 system (GID Bio) was reported as 16.1% in [34] but only 8.9% in [52]. In this regard it is of note that in [54] the cell suspension produced using the GID SVF-2 system (GID Bio) was sequentially passed through a 100- and 60-µm pore filter before flow cytometry to reduce the presence of debris and large cell clumps; this additional step was not performed in [52]. Accordingly, different starting materials were investigated in [52] and [54] using flow cytometry, which may explain the discrepancy in the mean numbers of CD45- / CD31- / CD34+ cells (adipose tissue derived progenitors) reported in these studies. However, this does not explain the discrepancy in the mean cell viability reported in these studies.

In any case, the flow cytometry data reported in [54] may not characterize the UA-ADRCs isolated from lipoaspirate using the GID SVF-2 system (GID Bio) applied in clinical use, because the additional step of passing the cells through a 100- and 60-µm pore filter before flow cytometry is not part of the protocol for clinical use of this system [93]. However, this raises the general question of the microscopy appearance of the cell suspension applied to a patient. In this regard, Figure 9 shows a cell smear of UA-ADRCs isolated from lipoaspirate using the Transpose RT system (InGeneron).

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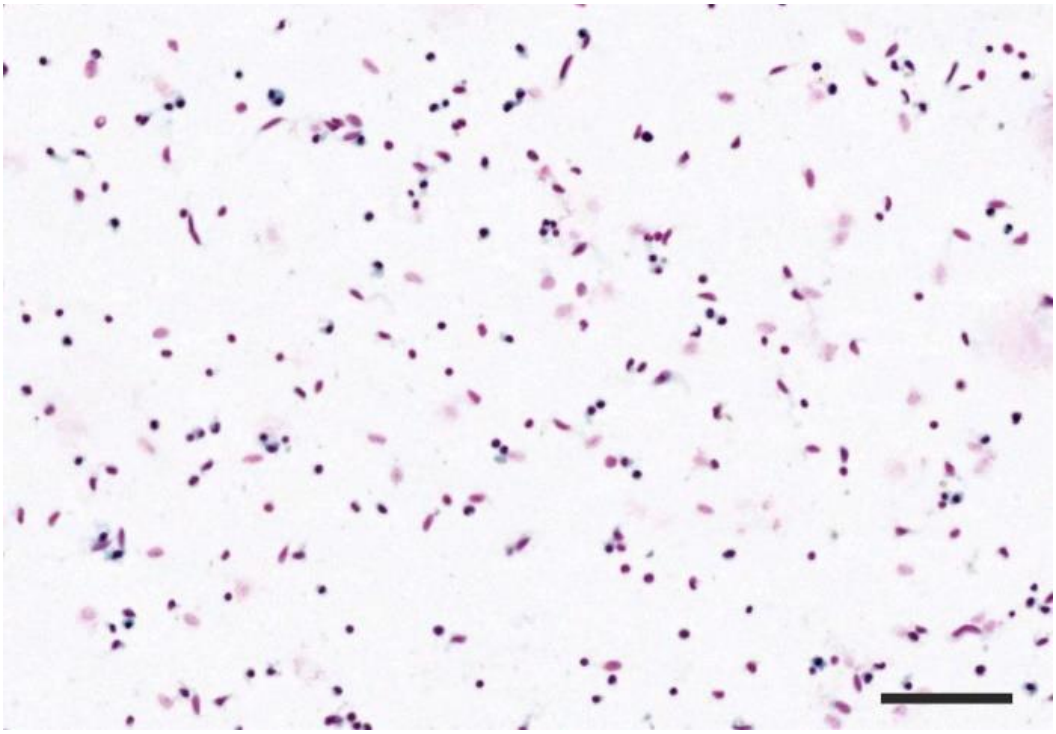

Figure 9. Smear of cells (Azan staining) isolated from lipoaspirate using the Transpose RT system (InGeneron). The scale bar represents 100 μm.

In contrast, Figure 3A in [34] shows the results of an experimental method for non-enzymatic, mechanical dissociation of lipoaspirate that shares key features with the commercially available system used in [32]. Unlike the Transpose RT system (InGeneron) the method used in [54] (and, thus, most probably also the system used in [32]) did not result in a cell suspension but in a suspension of cells and tissue fragments containing adipocytes. This may also explain why no data from flow cytometry have been published for the method and the system used in [32,54]. Furthermore, non-enzymatic, mechanical dissociation of lipoaspirate may also be the reason for the low mean viability of the cells reported in [32] (on average only 64.4%). When isolating UA-ADRCs from lipoaspirate using the Transpose RT system (InGeneron) with or without using the Matrase Reagent, the mean cell viability dropped from 85.9% with using the Matrase Reagent to 61.7% without using the Matrase Reagent [31].

The mean relative numbers of CD34+ cells and CD90+ cells found in the present study (56.0% and 56.6%; c.f. Table 9) are in line with mean relative numbers of CD34+ cells and CD90+ cells reported in [62] (60% and 54.8%). This indicates that, at least when single surface markers are considered, different systems used to isolate UA-ADRCs from lipoaspirate can lead to very similar outcome. On the other hand, the mean relative number of CD105+ cells reported in the present study (24.1%) is much higher than the relative number of CD105+ cells reported in [62] (4.9%). This difference may arise from the fact that in [62] cells were isolated from lipoaspirate using 0.1% collagenase I. Collagenase I cleaves type I, type II and type III collagen but not type IV collagen [94], the main collagen of the basement membrane [95]. Type IV collagen is cleaved by neutral protease [96] which is contained in the Matrase Reagent (InGeneron). CD105 (endoglin) is found (among other cell types) on endothelial cells, endothelial progenitors [97,98], ADSCs as defined by The International Society for Cellular Therapy (ISCT) (CD45- / CD73+ / CD90+ / CD105+) [57] and vascular-associated, pluripotent stem cells (vaPS cells) [5]. Adhesion of these cell types to the basement membrane is achieved by $\alpha 5\beta 1$ integrin [5,97,98]. Under physiological conditions SPARC (secreted protein acidic and rich in cysteine; also known as osteonectin) can mobilize the aforementioned cell types through its effect on integrin $\alpha 5\beta 1$, providing a functional basis for the regulation of the contribution of these cells to tissue

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and organ repair by SPARC [5,97,98]. The latter is synthesized by several cell types, including osteoblasts and odontoblasts, endothelial cells and fibroblasts, but also macrophages, infiltrating leukocytes and cancer cells [5]. Thus, SPARC may represent a key regulator in making endothelial progenitor cells, ADSCs as defined by ISCT and particularly vaPS cells a replacement source responsive to the signals of the surrounding tissue, and the neutral protease contained in the Matrase Reagent (InGeneron) may substitute the function of SPARC in isolation of UA-ADRCs from lipoaspirate.

CD105 (endoglin) is a TGF-β binding protein [99,100]. Several studies demonstrated that TGF-β1 and TGF-β3 stimulate tenogenic differentiation of cultured ADSCs *in vitro* [101-103] and, thus, may play a pivotal role in tendon healing.

Of note, SPARC does not release cells from the basement membrane by cleaving/destroying α5β1 integrin [98]. Rather, α5β1 integrin is released and can now take on other tasks. One of these tasks is the binding with CD105 (i.e., next to its function as a TGF-β binding protein, CD105 also acts as an adhesion molecule [104]). This binding is probably a cell-mediated signal for the stem cells to take up work and differentiate itself etc. [104]. It is tempting to speculate that if the cells now get an additional signal on CD105 (e.g. through TGF-β1 and TGF-β3), they may really be "on fire". Accordingly, a high relative number of CD105+ cells may represent an important quality criterion of UA-ADRCs. Furthermore, endothelial progenitors (which are contained in UA-ADRCs but not in cultured ADSCs) may play an important role in activating the stem cells contained in UA-ADRCs, beyond the formation of new capillaries.

Of note, isolation of UA-ADRCs from lipoaspirate using the Transpose RT system (InGeneron) resulted in the highest relative numbers of CD105+ cells and CD45- / CD31+ / CD34+ cells (endothelial progenitors) of all systems used to isolate UA-ADRCs from lipoaspirate that have been described in the literature.

Last but not least, a recent study [63] reported a novel, experimental method for non-enzymatic, mechanical dissociation of lipoaspirate that supposedly resulted in a higher cell viability (on average 85.8%) than enzymatic isolation of UA-ADRCs from lipoaspirate using collagenase NB6 (Serva Electrophoresis, Heidelberg, Germany) which contains collagenase I, collagenase II, neutral protease and clostripain (cell viability on average 82.9%). Since these data challenge the data summarized in Table 13, it is crucial to fully understand the mode of operation of the method used in [63]. This is the reason why the description of this method in [63] is fully cited here: "The lipoaspirate was transferred into syringes, connected to a closed unit, harnessing 3 different sets of blade grids on 3 luer-lock ports on a rotating canal. The lipoaspirate was placed in the first port, passed back-and-forth 10 times through the first blade grid containing multiple 1000-micron holes. The direction of the rotating canal was changed to the second port, and the lipoaspirate was passed through the second blade grid containing 750-micron holes and through the 500-micron holes blade grid for full dissociation." [63]. According to this description the method described in [63] is similar to the method described in [54] and the commercially available system described in [32].

Furthermore, compared with enzymatic isolation of UA-ADRCs, non-enzymatic, mechanical dissociation of lipoaspirate in [63] supposedly resulted in higher mean relative numbers of CD45- / CD31+ cells (named endothelial progenitors in [63]) (21.3% vs. 13.0%), CD45- / CD90+ cells (named ADSCs in [63]) (42.4% vs. 20.3%) and CD73+ / CD90+ cells (also named ADSCs in [63]) (52.2% vs. 31.0%), but a lower mean relative number of CD45+ / CD14+ cells (named monocytes/macrophages in [63]) (7.1% vs. 23.0%). However, the following should be considered for the interpretation of these results:

First, the average cell viability of 85.8% reported in [63] is among the highest values ever reported for both UA-ADRCs and cells in cell suspensions generated using non-enzymatic, mechanical dissociation of lipoaspirate (c.f. Table 9 and [31]), and is much higher than the average cell viability reported for other methods and systems for non-enzymatic, mechanical dissociation of lipoaspirate (e.g., 64.4% in [32]; more results are provided in [31]). However, it should be kept in mind that the cell viability was determined using a

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flow cytometer in [63]. Considering the tissue fragments shown in Figure 3B in [34], obtained after non-enzymatic mechanical dissociation of lipoaspirate in [34], it appears questionable that the results reported in [63] using a flow cytometer are representative of the entire suspension of cells and tissue fragments generated with the method described in [63]. Without the availability of a photomicrograph showing the tissue fragments obtained with this method it could also be that the results reported in [63] only represent a portion of the suspension of cells and tissue fragments generated with the method described in [63]. However, this would render the results reported in [63] clinically meaningless.

Second, Table 14 summarizes mean relative numbers of CD13+ cells, CD34+ cells, CD73+ cells, CD90+ cells and CD146+ cells that were reported in [63], the present study (Table 9) and [62].

Table 14. Comparison of mean relative numbers of CD13+ cells, CD34+ cells, CD73+ cells, CD90+ cells and CD146+ cells reported in [63], this study (Table 9) and [62].

Marker	Study	[63] – E	[63] – M	Present study – E	[62] – E
CD13+		14.2	24.7	--	37.0
CD34+		7.8	16.8	56.0	60.0
CD73+		5.2	14.1	46.5	25.0
CD90+		6.1	11.1	56.6	54.8
CD146+		7.5	17.3	--	21.4
CD45-/ CD90+		20.3	42.4	45.9	--
CD73+/ CD90+		31.0	52.2	36.7	--
CD45+/ CD73+ CD90+		--	--	8.1	--

Abbreviations: E, enzymatic isolation of UA-ADRCs from lipoaspirate; M, non-enzymatic, mechanical dissociation of lipoaspirate.

The relative numbers of these cell types reported in [63] are considerably lower than the corresponding data obtained in the present study (Table 9) and in [62]. Particularly the very low relative numbers of CD34+ cells, CD73+ cells and CD90+ cells obtained in [63] using enzymatic isolation of UA-ADRCs from lipoaspirate calls into question the validity of the data reported in [63]. Unfortunately, the data reported in [62] were not discussed in [63]. In any case, the data summarized in Table 9 demonstrate that the methods described in [63] offer no advantages over isolating UA-ADRCs from lipoaspirate using the Transpose RT system (InGeneron) or the method used in [62].

Third, according to [54], CD45- / CD31+ cells are endothelial cells rather than endothelial progenitors; the latter were defined as CD45- / CD31+ / CD34+ cells in [54]. Of course, opinions can differ when it comes to the exact characterization of certain cell types using surface markers. However, a critical discussion of one's own data against the background of the published literature should be a matter of course. Unfortunately, no discussion of the selected combination of surface markers was performed in [63], and [54] was not cited in [63].

Fourth, it appears invalid to characterize ADSCs as CD45- / CD90+ cells or CD73+ / CD90+ cells, as undertaken in [63]. As mentioned above, the ISCT defined ADSCs as CD45- / CD73+ / CD90+ / CD105+ cells [57], and there is a huge difference between the relative number of CD45- / CD73+ / CD90+ cells and the relative number of CD45- / CD73+ / CD90+ / CD105+ cells contained in UA-ADRCs (32.5% vs 2.4% in the present study; c.f. Table 9). Furthermore, the mean relative number of CD45+ / CD73+ / CD90+ cells was 8.1% in UA-ADRCs isolated from lipoaspirate using the Transpose RT system (Table 9). However, CD45+ cells are not ADSCs.

Fifth, the mean relative numbers of CD45- / CD90+ cells as well as of CD73+ / CD90+ cells reported in [63] exceeded the mean relative number of CD90+ cells in [63] (Table 14). However, the opposite must be true since CD45- / CD90+ cells and CD73+ / CD90+ cells are a subset of CD90+ cells (as is the case in the present study, as shown in Table 9 and

Table 14). Accordingly, the data reported in [63] are either invalid or related to different reference values, which was, however, not specified in [63].

The main limitation of the present study is the fact that 36 of the 37 lipoaspirate specimens investigated with flow cytometry were from women. This limitation is shared with other studies that reported results of investigating UA-ADRCs with flow cytometry [52,54]. Therefore, we will verify the results of this experimental study performed on lipoaspirate specimens from subjects undergoing elective lipoplasty on lipoaspirate specimens that are currently collected in the framework of a large, randomized controlled trial (n=246 subjects) on treatment of sPTRCT with injection of UA-ADRCs isolated from lipoaspirate using the Transpose RT system (InGeneron) vs. treatment with injection of corticosteroid [105].

5. Conclusions

The present study shows that, of all the experimental methods and commercially available systems available to isolate UA-ADRCs or systems that provide non-enzymatic, mechanical dissociation of lipoaspirate, the Transpose RT system (InGeneron) enables clinicians to isolate the most suitable UA-ADRCs for tendon and cartilage regeneration.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All raw data (including the files that were obtained from the BD FACSDiva Software (BD Bioscience)) are available from the corresponding author on reasonable request.

Conflicts of Interest: C.S. is Advisory Medical Director of InGeneron, Inc. (Houston, TX). C.A. is Director of Medical and Scientific Affairs of InGeneron. C.H. is Clinical Science Manager of InGeneron. E.U.A. is Executive Chair of InGeneron. InGeneron had no role in study design, data collection and analysis, interpretation of the data, and no role in the decision to publish and write this manuscript. No other potential conflicts of interest relevant to this article were reported.

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