

Appendix 1

Materials and methods*Patient characteristics*

A total of 32 patients affected by dry age-related macular degeneration (dryAMD), 15 males (46.8%) and 17 females (53.2%), with mean age 69.4 years (range, 50–83 years), undergoing elective surgery were recruited for the study. They were identified and enrolled according to several characteristics. The inclusion criteria were as follows:

- (I) Participants of whites classified as well-nourished and Caucasian race;
- (II) dryAMD diagnosis with spectral domain optical coherence tomography (SD-OCT), fundus autofluorescence imaging, and fluorescein angiography in the presence of drusen, atrophy, and irregularities of retinal pigment epithelium in at least 1 eye;
- (III) Good storage extrafoveal areas;
- (IV) Measurable visual acuity;
- (V) Normal intraocular pressure;
- (VI) Acceptance of the clinical protocol by signing the informed consent.

The exclusion criteria from the study for possible cross-related interference with the test were as follows:

- (I) Patients with signs of exudative AMD;
- (II) Myopia with spherical equivalent >6 diopters;
- (III) Other ocular disorders such as cataract, macular pucker or neovascular membrane-associated, glaucoma, optic neuritis, ocular trauma, and high refractive errors;
- (IV) Insufficient compliance in individuals affected by medical problems, such as hypovitaminosis, multiple sclerosis, epilepsy, Parkinson's disease, diabetes, hypertension, vasculitis, renal and hepatic diseases, gastrointestinal malabsorption, hypothyroidism, malignant tumors, and other systemic chronic diseases.

For each patient, we evaluated the best corrected visual acuity (BCVA) for far and near distances. BCVA was measured with early treatment diabetic retinopathy study charts at 4m in the logarithm of the minimum angle of resolution (logMAR) and visual acuity for near vision (close-up) in points (Pts). Therefore, we recorded the electrical cell activity through electroretinography (electromedical system of ocular electrophysiology, Retimax; C.S.O. Srl, Scandicci, Italy). The diagnosis was confirmed by confocal scanning laser ophthalmoscope Nidek F10 (Nidek Inc, Fremont, CA, USA), SD-OCT Cirrus (Carl Zeiss Meditec AG, Jena, Germany), and microperimetry Maia 100809 (CenterVue S.p.A., Padova, Italy) or standard automated perimetry Octopus 900 (Haag-Streit AG, Koeniz, Switzerland).

Cell harvesting and processing

The study was performed at Vista Vision between September 2018 and December 2019. Human adipose-derived stem cells (ASCs) included in stromal vascular fraction (SVF) were obtained from patients under the full approval of Low Vision Academy's Institutional Review Board and under the tenets of the 1964 Declaration of Helsinki (No. 2017/MC057, 4 May 2017). Informed consent was obtained from all subjects involved in the study. The harvest of the adipose tissue of the abdomen was surgically removed from patients during elective ocular surgery by PL, MD, in the operating room of Vista Vision (8). The harvest and the post-harvest fat processing method were conducted according to the Coleman technique by two trained doctors, CL and MT (9,10). Briefly, 10 mL of fat is harvested from the abdominal subcutaneous layer of each patient using a 3-mm blunt cannula connected to a locking syringe. The lipoaspirate is then centrifugated at 1,500 g of speed for 5 min at 20 °C to eliminate nonviable components, including cellular debris and oil (9,10). Finally, the bottom fluid portion of lipoaspirate from the centrifugated fat was drained, discarding blood, water, and aqueous components (10). This method has been demonstrated to obtain a higher concentration of adipose-derived mesenchymal stem cells within aspirate, reporting no damage to the structural integrity of adipose tissue and increased content of growth factors (9,10).

Cell isolation

SVF was obtained from patients affected with eye diseases who underwent elective surgery and were isolated under fresh conditions. With the patient under local anesthesia, adipose tissue was suctioned using a cannula with a 3-mm inner diameter connected to a locking syringe, according to the Lawrence and Coleman technique (9,10). The collected liposuction aspirate, to which was added 30 mL balanced salt solution, was used for isolation of the SVF.

The isolation of SVF was manually performed from each patient's lipoaspirate in a clean room near the operating room, according to a previously described method. Briefly, the adipose portion of the lipoaspirate was washed with phosphate-buffered saline (PBS; Biological Industries, Milan, Italy) and mixed with 2.5 mg/mL collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ, USA) for the enzymatic digestion. The collagenase/adipose mixture was placed in a 37 °C water bath for 30 min. to create a single-cell suspension and then filtered through a 100 µm cell strainer and finally to a 45 µm mesh. It was centrifugated for 5 min at 1,200 ×g to collect the cellular SVF as a pellet.

Flow cytometry

Once isolated, characterization of the cell composition of freshly-isolated SVF has been accomplished through multi-color flow cytometry (CytoFLEX Flow Cytometer, Beckman Coulter, Brea, CA, USA) that allows the identification of the surface marker expression of the cells *in vitro*. The panel of cell surface antigens was chosen in agreement with International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT) recommendations (3). The immunophenotypic analysis is performed to confirm the mesenchymal nature of isolated cells. The following fluorochrome-labeled monoclonal antibodies were used for SVF analysis: CD31-PE, CD34-PC, and CD45-APC (Beckman Coulter) (*Table S1*).

- ❖ CD31, or platelet endothelial cell adhesion molecule (PECAM-1), is normally found on endothelial cells, platelets, macrophages and Kupffer cells, granulocytes, lymphocytes, megakaryocytes, and osteoclasts.
- ❖ CD45 or protein tyrosine phosphatase, receptor type, C (PTPRC), leukocyte common antigen (LCA), panhematopoietic marker expressed on all human white blood cells.
- ❖ CD34, cell-cell adhesion molecule, a marker for pluripotent stem cells, expressed on all hematopoietic progenitor cells.

The markers were used in combination with ViaKrome (Beckman Coulter), which determines cell viability, excluding debris and dead cells induced by the isolation protocol. Cells were incubated with specific mAbs for 15 min. At least 10⁵ cells were acquired from each sample. The software CytExpert Version 2.2.0.97, CytoFLEX (Beckman Coulter) was used to create dot plots and to calculate the cell composition percentages according to the profile of the surface marker expression.

Statistical analysis

All statistical analyses were performed using the software SPSS Statistics (version 20.0, SPSS Inc., Chicago, IL, USA). Data were summarized with the mean ± standard deviation and minimum and maximum (min-max) values were also reported. The student's paired *t*-test was used to compare values between stained cells and unstained controls. A P value <0.001 was considered statistically significant.

Results

ASCs immunophenotypic characterization

The liposuction procedure was successfully performed in all cases. For immunophenotypic characterization of ASCs within the SVF, freshly isolated cells were examined for surface antigen expression using flow cytometry. Flow cytometry data were gated (*Figure S1*) to identify possible stromal cell subpopulations. The SVF yielded a mean of 73.32%±10.89% cell viability evaluated with CALCEINA-FITC, i.e., cell-permeant dye, to reduce the analysis distortion caused by the possible presence of dead or apoptotic cells. The ASCs were positive for CD34 and negative for CD31 and CD45. The frequency of ASCs estimated according to the panel of cell surface markers used was 51.06%±5.26% versus the unstained ASCs subpopulation which was 0.74%±0.84% (P<0.0001). The ASCs events/µL were 1602.13/µL±731.87/µL. The results of this analysis are shown in *Figures S1-S3* and *Table S2*.

Table S1 Antibodies used in the study

Fluorochrome-labeled monoclonal antibodies	Target protein	Supplier	Catalog No.
CD31-PE	PECAM-1	Beckman Coulter, CA, USA	1F11, 100 Tests, RUO IM2409
CD34-PC7	gp105-120		581, k 100 Tests, CE, A21691
CD45-APC	LCA		J33, 100 Tests, CE IM2473

The following fluorochrome-labeled monoclonal antibodies were used for stromal vascular fraction (SVF) analysis: CD31-PE, CD34-PC, and CD45-APC. CD31, or platelet endothelial cell adhesion molecule (PECAM-1), is normally found on endothelial cells, platelets, macrophages and Kupffer cells, granulocytes, lymphocytes, megakaryocytes, and osteoclasts. CD45 or protein tyrosine phosphatase, receptor type, C (PTPRC), Leukocyte common antigen (LCA), panhematopoietic marker expressed on all human white blood cells. CD34, cell-cell adhesion molecule, a marker for pluripotent stem cells, expressed on all hematopoietic progenitor cells. CD, cluster of differentiation.

Table S2 Phenotype characterization by flow cytometry of cell fractions in the stromal vascular fraction

Population	Staining	Events, mean \pm SD	Percentage (%)	Events/ μ L	P value
CD34	No	31.86 \pm 20.27	0.69 \pm 0.36	2.33 \pm 1.73	*<0.0001
	Yes	5,702.33 \pm 1,158.78	80.16 \pm 1.73	1,644.98 \pm 724.57	
CD31	No	7,034.00 \pm 927.88	98.95 \pm 0.76	4,771.37 \pm 1,086.62	0.01877
	Yes	5,516.50 \pm 1,347.28	84.69 \pm 14.57	3,043.13 \pm 429.95	
CD45	No	6,888.46 \pm 899.32	99.30 \pm 0.63	1,938.61 \pm 935.47	0.9860
	Yes	6,155.43 \pm 1,264.57	99.27 \pm 0.59	2,084.74 \pm 793.71	
ASCs	No	83.10 \pm 91.82	0.74 \pm 0.84	26.675 \pm 29.25	*<0.0001
	Yes	3,095.00 \pm 815.46	51.06 \pm 5.26	1,602.13 \pm 731.87	

Values were compared by Student's paired *t*-test. *, statistical significance with P=0.0001. All events: 10,000. Live cells in %: 73.32 \pm 10.89.

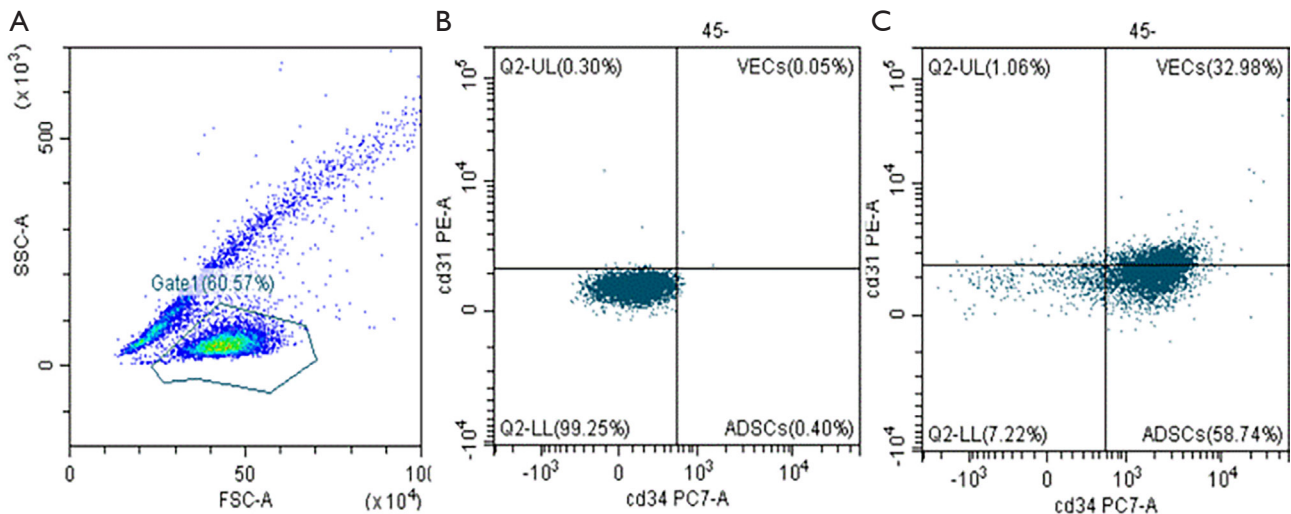


Figure S1 Representative flow cytometric histograms of adipose-derived stem cells within the stromal vascular fraction. Fluorescent dot plot showing cells within the freshly-isolated lipoaspirate (A). Dot plot showing cells unstained (B) and stained by antibodies directed against CD45, CD31, and CD34 (C). The results are presented in brackets as a percent of positively stained cells.

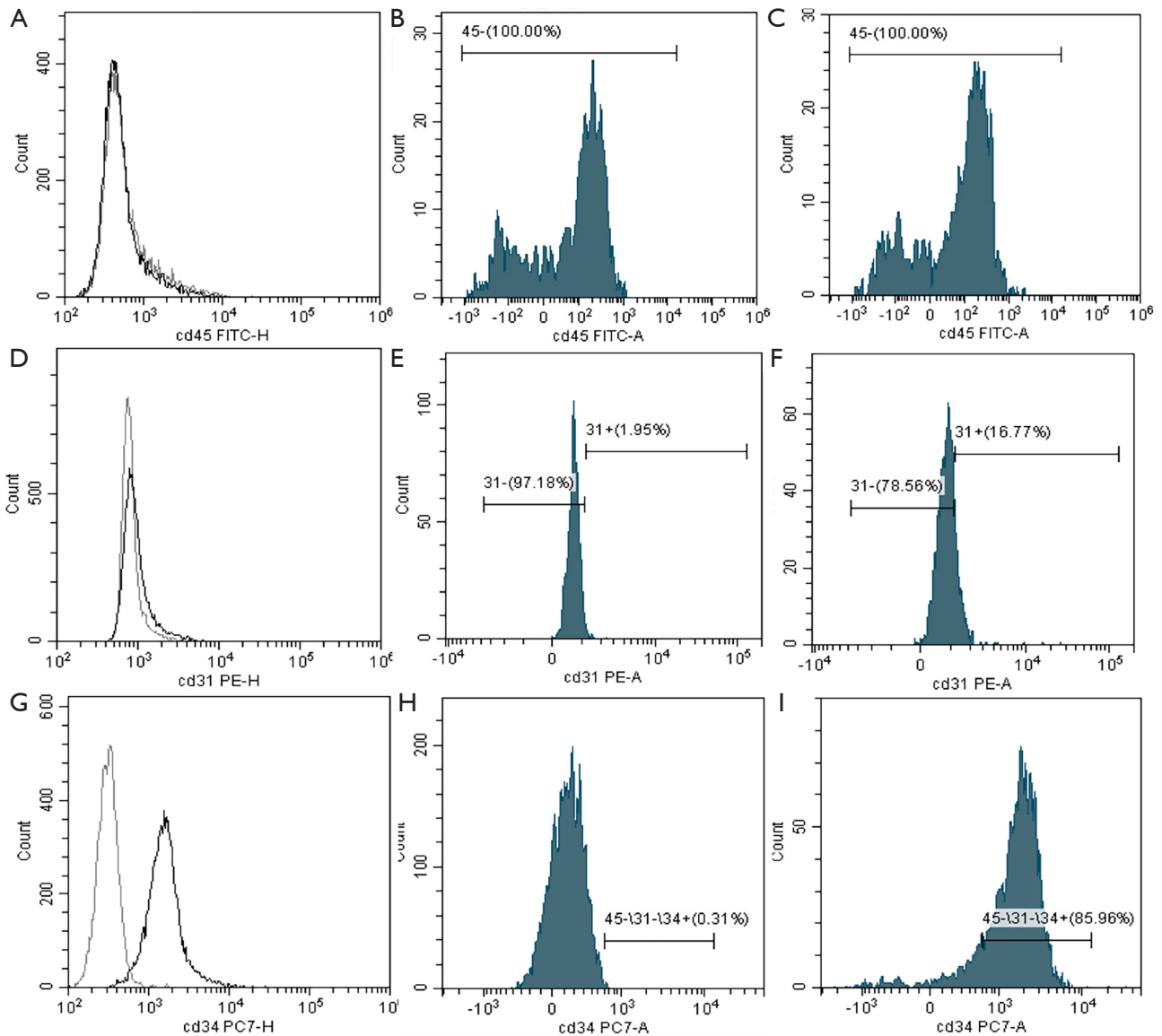


Figure S2 Immunophenotype characterization of ASCs represented by two-dimensional flow cytometry histograms. (A,D,G) Histograms represent an isotype-matched negative Ab control (gray line) and Abs against CD45, CD31, and CD34 respectively (black line); (B,E,H) histograms represent control antibody staining; Histograms displaying staining for CD45-FITC, the panhematopoietic marker (C), CD31-PE, found on endothelial cells, platelets and leukocytes (F) and CD34-PC, a marker for pluripotent stem cells (I). Insets indicate markers and percentages of positively stained cells. Specific-marker fluorescence intensity and cell counts are indicated in abscissa and ordinate respectively.

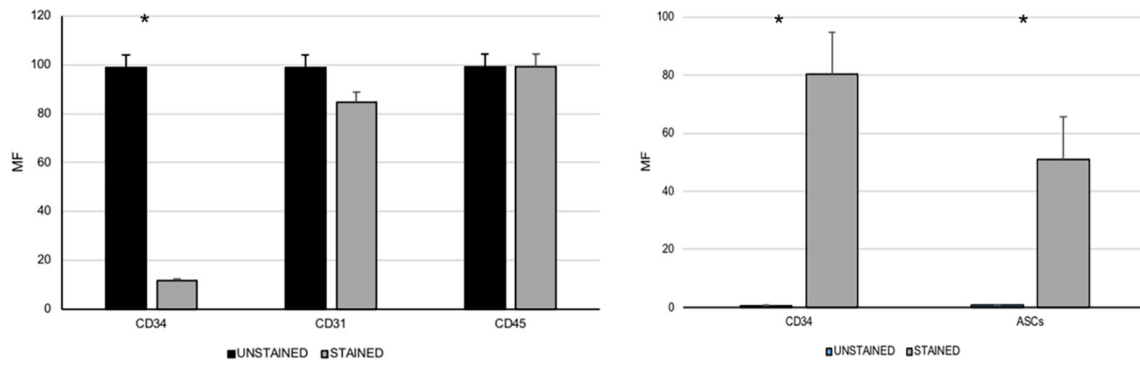


Figure S3 Phenotype characterization by flow cytometry of cell fraction in the stromal vascular fraction containing ASCs. Freshly isolated SVF cells were analyzed using three specific mAb antibodies labeled with three different fluorochromes: PE-labeled mAb anti-CD31, PC7-labeled mAb anti-CD34, APC-labeled mAb anti-CD45. The CD31⁻CD45⁻ cell fraction was gated to characterize the ASCs population that was CD34⁺. Data are expressed as mean and standard error. N=32. *, P value >0.0001 Unstained *vs.* Stained. MF, mean fluorescence.