Differences in the neovascular potential of thymus versus subcutaneous adipose-derived stem cells from patients with myocardial ischemia

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ABSTRACT

Adipose tissue-derived multipotent mesenchymal cells (ASCs) participate in the formation of blood vessels under hypoxic conditions. It is probable that the susceptibility of ASCs to the influence of age and aging-associated pathologies compromises their therapeutic effectiveness depending on the adipose tissue (AT) depot. Our aim was to examine the neovascular potential under hypoxic conditions of ASCs-derived from thymic (thymASCs) and subcutaneous (subASCs) AT from 39 subjects with and without type 2 diabetes mellitus (T2DM) and of different ages who were undergoing coronary bypass surgery (CBS).

We confirmed a significant decrease in the percentage of $CD34^{+}CD31^{-}CD45^{-}$ subASCs in the cell yield of subASCs and in the survival of cultured endothelial cells in the medium conditioned by the hypox-subASCs with increasing patient age, which was not observed in thymASCs. While the length of the tubules generated by hypox-subASCs tended to correlate negatively with patient age, tubule formation capacity of the hypoxic thymASCs increased significantly. Compared with subASCs, thymASCs from subjects over age 65 and without T2DM showed higher cell yield, tubule formation capacity, VEGF secretion levels and the ability to promote endothelial cell survival in their conditioned medium. Deterioration in subASCs neovascular potential relative to thymASCs derived from these subjects was accompanied by higher expression levels of NOX4 mRNA and fibrotic proteins.

Our results indicate that thymASCs from patients over age 65 and without T2DM have a higher angiogenic potential than those from the other patient groups, suggesting they may be a good candidate for angiogenic therapy in subjects undergoing CBS.

INTRODUCTION

Adipose tissue-derived multipotent mesenchymal cells (ASCs) differentiate into endothelial cells, improve postnatal neovascularization in ischemic tissue (Cao et al., 2005; Miranville et al., 2004; Moon et al., 2006; Planat-Benard et al.,2004), prevent vessel regression (Traktuev et al., 2009) and contribute to endothelial repair (Takahashi et al., 2010). Given their accessibility, abundance and differentiation into tissues of mesodermal and non-mesodermal origin, ASCs represent a useful tool in biotechnology and an alternative source of autologous adult stem cells (Fraser et al., 2006; Mizuno et al., 2012)

Because of the high prevalence of cardiovascular diseases in older populations (Go at al., 2013), the therapeutic effectiveness of ASCs could be limited since it has been observed that aging can negatively impact proliferative, adipogenic (Schipper et al., 2008) and osteogenic potential (Zhu et al., 2009). In addition, the reduction in the proliferation rate and multilineage differentiation ability with age is accompanied by decreased superoxide dismutase activity (Choudhery et al., 2014), higher reactive oxygen species production and decreased telomere length (Efimenko et al., 2011) with negative implications to proangiogenic factor secretion (Efimenko et al., 2014).

Studies on age-related changes in regional fat distribution have shown that aging has distinct effects on preadipocytes from different fat depots with subcutaneous depots being particularly affected and contributing to fat redistribution from subcutaneous to visceral depots (Tchkonia et al., 2004). This ectopic fat accumulation not only is related to the inability of subcutaneous adipose tissue to recruit new adipose cells through adipogenesis, but it also contributes to the risk of developing type 2 diabetes mellitus (T2DM) and cardiovascular disease (Gustafson and Smith, 2015). According to an analysis from the Framingham Heart Study, the proportion of cardiovascular disease attributable to diabetes increased over the second half of the twentieth century (Fox et al, 2007). Indeed, numerous studies have documented a diabetes-associated increase in mortality due to coronary artery disease, and this disease is the most common cause of death in adults with diabetes (Bonow and Gheorghiade, 2004).

Furthermore, age-dependent thymic atrophy is also associated with the replacement of thymic stroma by adipose tissue (Hale, 2004) and microarray analyses have revealed that the majority of the specific genes found to change with thymic aging were quite distinct from those of other organ systems (Taub and Longo 2005). In fact, experiments from our laboratory have demonstrated that thymus fat from elderly subjects is angiogenically active tissue, since adipogenic gene expression has been positively associated with VEGF isoform mRNA levels (Tinahones et al., 2009) and thymus fat extracts can induce proliferation and migration of endothelial cells in vitro (Salas et al., 2009). Importantly, thymus ASCs (thymASCs) from elderly subjects showed higher adipogenic and angiogenic capacity compared to those from middle-aged subjects, whereas in the subcutaneous adipose tissue the opposite was observed (Coín-Aragüez et al., 2013; Oliva-Olivera et al., 2015).

Considering that the growth of adipose depots depends on the availability of blood vessels (Cao, 2007), we assume that thymic adipose tissue expansion during aging could be favored by the neovascular properties of the thymASCs. Thus, in the present study we aimed to evaluate the neovascular potential of the thymASCs and subcutaneous ASCs (subASCs) from patients undergoing coronary artery bypass surgery. In addition, we examined the status of the ASCs according to yield and migratory capacity as well as the mRNA expression levels of genes involved in both cellular redox balance and tissue fibrosis.

MATERIALS AND METHODS

2.1 Subjects

The study included 39 participants recruited at Carlos Haya Hospital (Malaga, Spain), according to the declaration of Helsinki, who received a coronary-artery bypass graft with a cardiopulmonary bypass intervention during the period 2012-2014. The hospital ethics committee approved the study and the informed consent of all participants was obtained. The study patients were individuals with stable situations and without severe ischemic injury, since they had no previous myocardial infarction or had not had a myocardial infarction in the six months before surgery. Paired subcutaneous (specifically from the chest incision) and thymus adipose tissue biopsies were obtained at the beginning of the procedure before cardioplegic arrest. Since the population ≥ 65 years of age is largely considered older adults, the study patients were grouped as subjects without T2DM < 65 years of age (Adult) or ≥ 65 years age (Elderly). A third group of patients included subjects with T2DM, independent of their age. Due to the limited availability of subcutaneous adipose tissue, we were unable to conduct the experiments on all biopsies provided by all the study subjects. The number of experiments with cells from different donors (n) is specified in the respective assays. **Table S1** shows the age and the gender of the patients used to generate this specific set of data.

2.2. Isolation, yield and expansion of stromal vascular fraction derived from thymus and subcutaneous adipose tissue.

Cells from adipose tissue samples were isolated by enzymatic digestion. Briefly, adipose biopsies were finely dissected and treated with a solution containing 0.150% collagenase type I and 1.0% bovine serum albumin (BSA) at 37°C for 70 min. The resulting tissue suspension was centrifuged for 10 minutes at 500×g and floating adipocytes were discarded by decanting. After the stromal vascular fraction was filtered, resuspended in erythrocyte lysis buffer and centrifuged, the resulting cell pellet was plated in 25 cm^2or 75 cm^2 culture flasks with growth medium consisting of Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12), supplemented with fetal bovine serum (FBS) (0.1mL/mL), streptomycin (100µg/mL), penicillin (100U/mL) and L-glutamine (2mM). Approximately 16 hours after seeding, the first medium change was carried out and the cells were

allowed to proliferate under standard culture conditions at 37°C in a humid atmosphere with 5% CO_2 during eight days. After this period, cells were harvested with trypsin/EDTA (pH 7.0-7.6) and counted using the trypan blue exclusion assay with neubauer chamber to know the yield or number of cells per gram of tissue. The number of cells needed to perform the different assays was achieved by cell subculturing under standard culture conditions, with two or three growth medium changes per week and up to 90% confluence.

1.3. Immunophenotypic characterization by flow cytometry

ThymASCs and subASCs grown in passage zero were immunophenotypically characterized by flow cytometry according to cell surface markers CD31-Allophycocyanin (APC) (Miltenyi Biotec, Bergisch Gladbach, Germany), CD34-Fluorescein Isothiocyanate (FITC) (eBioscience, Santa Clara, California, USA) and CD45-Phycoerythrin Cyanine7 tandem fluorochrome (PE-Cy7) (eBioscience). ASCs in passage two incubated for 72 hours under normoxic (21% $O_{2(g)}$)(normo-thymASCs, normo-subASCs) or hypoxic (1.0% $O_{2(g)}$)(hypox-thymASCs, hypox-subASCs) conditions were also characterized immunophenotypically by flow cytometry according to the cell surface markers CD44-FITC (Miltenyi Biotec), CD140B- Phycoerythrin (PE) (R&D Systems, Minneapolis) and CD184-PE-Cy7 (eBioscience). During immunophenotypic characterization, ASCs were detached with trypsin/EDTA, washed with PBS, resuspended in blocking buffer (PBS supplemented with 3.0% BSA), and incubated for ten minutes on ice. Aliquots of 1×10^5 cells each were dispensed into polypropylene tubes and monoclonal mouse antibody solution against the respective cell surface markers, conjugated with their corresponding fluorochrome, was added according to the manufacturer's instructions. One tube was for labeling the corresponding IgG1-PE, IgG1-APC, IgG1-FITC (Miltenyi Biotec) and IgG1-PE-Cy7 (eBioscience) isotope controls. All tubes were incubated for 30 minutes on ice and protected from light. The cells were washed twice with blocking buffer then resuspended in 1000 µL of PBS to acquire 1×10^4 events per tube using a CyAn™ ADP High-speed Analyzer (Beckman Coulter, Brea, California, USA).

1.4. Chemotaxis assay of ASCs induced by stromal cell derived factor 1α

ASCs in passage two previously incubated for 72 hours under normoxic (21% $O_{2\ (g)}$) or hypoxic (1% $O_{2(g)}$) conditions were resuspended in endothelial basal medium (EBM) (PromoCell, Heidelberg, Germany) supplemented with fetal calf serum (FCS) (0.05 mL/mL) and distributed in duplicate 3×10^4 cells on the upper chamber of the 6.5 mm transwell plates with an 8 µm pore size (Corning, NY, USA). The lower chambers were occupied by either EBM supplemented with FCS (0.05 mL/mL) and100 ng/mL of stromal cell derived factor 1α (SDF1α) (R&D Systems, Minneapolis) or EBM supplemented with FCS (0.05 mL/mL) only. They were then allowed to migrate, incubating them for 24 hours under standard cell culture conditions. After this time, the non-migratory cells retained on the upper surface of the migration chamber were carefully removed using a cotton tip applicator and the cells that migrated to the undersurface of the transwell chamber were washed with PBS and fixed by incubating them with neutral buffered formalin for 15 minutes. Finally, the nuclei were labeled by incubating the cells with 4',6-diamidino-2-phenylindole (DAPI) (2.0 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA) and phase contrast and fluorescence microscopy (Nikon, Japan) with a 10× objective was used to examine the migrated cells, randomly selecting and photographing five fields per replicate. The number of migrated cells per experimental condition was determined using a Nikon NIS Elements image processor and the number of cells that migrated under the chemotactic effects of SDF1α was calculated by dividing the number of cells detected in the presence of SDF1α by the number observed in the absence of SDF1α for cells previously cultured under hypoxia or normoxia.

1.5. Tubule formation by hypox-ASCs

A total of 50 µL/cm^2 growth factor-reduced matrigel (BD Biosciences, San Jose, CA, USA) was added to 48-well cell culture plates and incubated for 30 minutes at 37°C allowing the matrigel to solidify. ASCs in passage one were resuspended in EBM (PromoCell) supplemented with FCS (0.05 mL/mL), seeded in duplicate at a density of 1×10^5 cells/cm^2, and incubated for six hours under conditions of 1% $O_{2(g)}$, 94% $N_{2(g)}$ and 5% $CO_{2(g)}$ provided by hypoxia incubator (Thermo Scientific, Waltham, MA, USA). After six hours, phase contrast and fluorescence microscopy (Nikon, Japan) with a 4× objective was used to examine tubules, randomly selecting and photographing four fields per replicate. Subsequently, the length of the tubules and number of branching points were

quantified using a Nikon NIS Elements image processor, considering as tubules those whose length exceeded four times the width.

1.6. Gene expression by Real-time quantitative PCR

RNA from cultured cells was isolated and purified using STAT-60 reagent (Amsbio, Abingdon, Oxon, UK) and reverse transcribed to cDNA using reverse-transcriptase enzyme (Transcriptor Reverse Transcriptase 20U/µL; 03531287001; Roche) in a 2720 Thermal Cycler (Applied Biosystems, Foster City, USA). Real-time quantitative PCR (RT-qPCR) was performed with 10 ng cDNA for NOX4 (Hs00418356_m1, NM_001143836.1), superoxide dismutase 2, mitochondrial (SOD2)(Hs00167309_m1, NM_000636.2), superoxide dismutase 3, extracellular (SOD3) (Hs00162090_m1, NM_003102.2), catalase (Hs00156308_m1; NM_001752.3), collagentype I alpha 1 (Col1a1) (Hs 00164004_m1, RefSeq. NM_000088.3), collagen type III alpha 1 (Col3a1) (Hs00943809_m1, RefSeq.NM_000090.3).The amplifications were performed using a MicroAmp optical 96-well reaction plate (PE Applied Biosystems) on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems). RT-qPCRs were carried out for all genes using specific TaqMan gene expression assays and the procedure was performed as recommended by the manufacturer. Specific signals were normalized with respect to endogenous control ribosomal protein L13A (RPL13A) (Hs 04194366-g1, NM_001270491.1) according to the $2^{-\Delta Ct}$ formula.

1.7. Collection of ASC-conditioned medium and Enzyme-Linked Solid Phase Immunosorbent Assay

ASCs in passage one were resuspended in growth medium, seeded in six-well plates at a density of 1.5×10^4 cells/cm^2 and maintained in standard culture conditions for ten days. Subsequently, this medium was replaced with EBM supplemented with FCS (0.05 mL/mL) and incubated under normoxic (21% $O_{2(g)}$). or hypoxic conditions (1% $O_{2(g)}$). After 72 hours of culture, the conditioned medium from normo-ASCs or hypox-ASCs was collected, centrifuged at 300×g for five minutes and reserved in aliquots at -80ºC until use. Quantikine enzyme-linked solid phase immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, USA) was used to detect vascular endothelial growth factor

(VEGF) and thrombospondin-1 (TSP1) according to the manufacturer's instructions. Data are expressed as mean ± standard deviation picograms or nanograms, respectively, of the cytokine per 10^6 cells at the time of harvest.

1.8. Effect of ASC-conditioned medium on HSaVECs survival and tubule formation

Human saphenous vein endothelial cells (HSaVECs) (Promega, Madison, WI, USA) at passage three were seeded at a density of 1 x 10^4 cells/cm^2 in dark 96-well plates (Sigma) in EBM (Promocell) supplemented with FCS (0.05 mL/mL) and incubated for 24 hours under standard cell culture conditions. Subsequently, culture medium was replaced with 200 µL of hypox-ASC-conditioned medium and incubated for 72 hours under standard cell culture conditions. Finally, the dead cells were discarded by washing with PBS, and the surviving cells that remained adhered to the culture surface were reserved at -80 for at least one week for later quantification by the Cyquant Cell Proliferation kit (Invitrogen, Carlsbad, CA, USA). Cell survival was estimated by fluorescence intensity, by dividing the emitted fluorescence value in the wells where HSaVECs were cultured in hypox-ASC-conditioned medium by the values emitted in the wells where HSaVECs were cultured in unconditioned medium.

To determine the effects of the conditioned medium on HSaVECs tubule formation, we coated 96-well plates (Corning) with 25 µL of growth factor-reduced matrigel and incubated them for 30 minutes at 37ºC. 25 × 10^3 HSaVECs were dispersed into ASC-conditioned culture medium according to the respective experimental conditions of hypoxia or normoxia. Cells were grown under standard conditions for 24 hours then capillary-like structures were examined as described above for ASCs. The rate of increase in tubule formation was calculated by dividing the mean length of the tubules produced by HSaVECs cultured in hypox-ASC-conditioned medium by the mean length of those generated by the HSaVECs cultured in normo-ASC-conditioned medium.

1.9. Statistical Analysis

The results were expressed as mean values ± standard deviation. The Shapiro-Wilk test was used to test for normality. Comparisons between more than two groups were performed using the nonparametric Kruskal-Wallis test and between two unpaired groups using the nonparametric Mann-

Whitney U test. In order to test if the differences between paired samples were significant, the Wilcoxon signed-rank test was performed. The correlation between variables was calculated with Spearman's Rho. All statistical analyses were done using SPSS (version 17.0; SPSS Inc, Chicago, IL). P values < 0.05 were considered statistically significant.

2. RESULTS

Anthropometric and clinical characterization of the patients

Table 1 shows the clinical characteristics of the patients. Statistical analysis confirmed significant differences in age between the Elderly and T2DM subjects compared to the Adult patients. Both patient groups also showed a statistically significant decrease in total cholesterol and LDL-cholesterol levels compared to the Adult subjects. In addition, the HDL-cholesterol concentration was significantly lower in the T2DM patients than in the Elderly and Adult subjects.

Decrease in percentage of $CD34^+CD31^-CD45^-$cells and subASC yield with increasing patient age

Immunophenotypic characterization of thymASCs **(Figure S1A)** and subASCs **(Figure S1B)** at passage zero confirmed the low percentage of endothelial cell $CD34^+CD31^+$ and macrophages $CD34^-CD45^+$, and revealed no significant differences in the percentage of $CD34^+CD31^-CD45^-$ cells between the different groups of patients **(Figure S2).** Although we observed a statistically significant positive correlation between the percentage of $CD34^+CD31^-CD45^-$ASCs derived from both tissues (**Figure 1A**), only the subASCs revealed a statistically significant decrease in the percentage of $CD34^+CD31^-CD45^-$cells with increasing patient age (**Figure 1B**). In addition, the number of thymASCs in passage zero obtained per gram of adipose tissue at eight days of culture (yield of ASCs) was significantly higher than that of the subASCs in the Elderly and T2DM subjects (**Figure 1C**). We also observed that the subASC yield decreased significantly with increasing patient age **(Figure 1D)**.

The thymASCs and subASCs of subjects undergoing coronary bypass do not differ in their ability to migrate under the chemotactic effects of SDF1α

Previous studies have confirmed the involvement of the CD140b receptor in the generation of mitochondrial ROS (Hye et al., 2015), the CD184 receptor in the chemotactic effects of SDF1α on human ASCs subjected to hypoxia (Thangarajah et al., 2009) and the CD271 receptor in the immunomodulatory properties of mesenchymal stem cells (Kuçi et al., 2010). Thus, we subsequently quantified the percentage of ASCs expressing these receptors.

We did not observe significant differences between the percentage of thymASCs **(Figure S3A)** and subASCs **(Figure S3B)** in passage two that expressed the immunophenotypic markers CD140b, CD184 and CD271 after having been cultured for 72 hours under normoxic or hypoxic conditions **(Table S2)**. However, only the subASCs cultured under normoxic or hypoxic conditions showed a significant negative correlation between the percentage of $CD271^+$ cells and patient age (**Figure 2A**).

Figure 2B shows the migratory effects of the chemokine SDF1α, CD184 receptor ligand and one of the chemokine expressed by ischemic tissue (Silvestre et al., 2013), on previously cultured ASCs under normoxic or hypoxic conditions. In agreement with the percentages of $CD184^+$ thymASCs and subASCs detected by flow cytometry, we found no significant differences between the migratory rate of the thymASCs and subASCs in any of the patient groups

The thymASCs from the Elderly patients show greater tubule length and number of branching points than those of the Adult patients

Considering that hypoxic conditions favor the neovascular activity of ASCs (Thangarajah et al., 2009) and that ischemic tissues are characterized by the decrease in partial oxygen pressures (Silvestre et al., 2013), we then focus our experiments towards the effects of hypoxia on ASCs.

Figure 3A shows the formation of capillary-like structures by the thymASCs and subASCs on growth factor-reduced matrigel for six hours under conditions of 1 % $O_{2(g)}$ hypoxia. We found no significant differences between the length of tubules generated by the thymASCs and subASCs in any of the patient groups, but the thymASCs from the Elderly subjects showed a statistically significant increase in the length of tubules with respect to the thymASCs from the Adult patients (**Figure 3B**). We also observed that while the length of the tubules generated by the subASCs tended to correlate negatively

with patient age, the tubule formation capacity of the thymASCs increased significantly with increasing patient age **(Figure 3C)**. Relevantly, the number of branching points generated by thymASCs from the Elderly patients was greater than that produced by thymASCs from the Adult and T2DM patients **(Figure 3D).**

The thymASCs from the Elderly patients show reduced levels of NADPH oxidase isoform NOX4 mRNA compared to the subASCs

It is widely recognized that oxidative stress is one of the phenomena involved in biological aging and multiple studies have shown that precursor cells treated with oxidants show decreased ability to form tubules (Ingram et al., 2007). Therefore, we next examined the expression levels of some of the genes involved in cellular redox balance.

The application of the qPCR technique allowed us to confirm a statistically significant decrease in the expression levels of NOX4 mRNA in the thymASCs with respect to the subASCs from the Elderly patients **(Figure 4A)**. In addition, evaluation of expression levels of antioxidant enzymes involved in catalyzing the dismutation of superoxide derived from NADPH oxidase activity revealed a statistically significant positive correlation between the levels of SOD3 expression with SOD2 and Catalase in the thymASCs **(Figure 4B)**, which was not observed in the subASCs **(Figure 4C)**.

The thymASCs derived from the Elderly patients show reduced levels of Col1a1 and Col3a1 expression compared to the subASCs

Because NADPH oxidase-dependent redox signaling has been shown to be involved in fibrotic response associated with tissue repair (Jiang et al., 2014) and considering the fibrogenic potential revealed by multipotent mesenchymal cells in *in vivo* experiments (Baertschiger et al., 2009; Kim et al., 2011), we then evaluated the mRNA expression levels of some collagen isotypes.

Although we found a statistically significant positive correlation between levels of expression of Col1a1 and Col3a1 with NOX4 mRNA in both the thymASCs **(Figure 5A)** and subASCs **(Figure 5B)**, the transcriptional levels of Col1a1 and Col3a1 in the subASCs from the Elderly patients were significantly higher than those recorded for the thymASCs **(Figure 5C)**.

The level of VEGF secreted by the thymASCs from Elderly patients and the survival of HSaVECs cultured in their conditioned medium was higher than that detected in the medium conditioned by the subASCs

The Adult subjects showed the highest concentration of TSP1 in the medium conditioned by the thymASCs and subASCs cultured for 72 hours under hypoxic conditions, followed by the Elderly and T2DM patient **(Figure 6A)**. The levels of VEGF secreted into the medium conditioned by the thymASCs followed a pattern similar to that of the TSP1, but we did not detect significant differences between the concentration values of the VEGF present in the medium conditioned by the subASCs from the three patient groups **(Figure 6B)**. However, VEGF levels secreted by the thymASCs were significantly higher than those secreted by the subASCs in the Elderly subjects group **(Figure 6B)**. Interestingly, we found a statistically significant positive correlation between the levels of VEGF and TSP1 secreted by the thymASCs **(Figure 6C)**, which did not reach statistical significance in the case of the subASCs **(Figure 6D)**.

Additionally, we confirm that the medium conditioned by the thymASCs and subASCs can support the formation of tubules by HSaVECs **(Figure S4)**. We did not detect significant differences in the rate of increase in tubule formation by the HSaVECs cultured for 24 hours in the medium conditioned by the thymASCs or subASCs **(Figure 6E)**. **Figure 6F** also shows that the survival of HSaVECs cultured for 72 hours in medium conditioned by the hypoxic thymASCs was significantly higher than that of the hypoxic subASCs in both the Adult and Elderly subjects groups. In fact, the survival of the cultured HSaVECs in the medium conditioned by the hypoxic subASCs decreased significantly with the increase in the age of the patients **(Figure 6G)**.

3. DISCUSSION

Recent results have emphasized the relevance of cell source selection in the development of autologous therapy as ASCs from different depots show intrinsic differences in the ability to differentiate into multiple lineages (Russo et al., 2014; Wang et al., 2014) and mesenchymal stem cells isolated from bone marrow, muscle, and adipose tissue are differentially influenced by aging according to proliferation, senescence, and chondrogenic response (Beane et al., 2014). Evidence suggests that the age-related decline in the regenerative potential of stem cells seems to be heterogeneous and could be attributed both to environmental influences and to the combined effects of replicative and chronological ageing (Rando, 2006). In the present study we observed a significant decrease in the percentage of $CD34^+CD31^-CD45^-$ subASCs with the increase in age of the patients undergoing coronary bypass, which in the thymASCs did not reach statistical significance. The decrease in the percentage of $CD34^+CD31^-CD45^-$ cells could compromise the availability of subASCs with angiogenic potential in patients of advanced age since it has been demonstrated that the nonendothelial population of $CD34^+$ cells from adipose tissue is able to differentiate *in vitro* into endothelial cells and enhance neovascularization in ischemic tissue (Miranville et al., 2004; Planat-Benard et al., 2004). In fact, it has been observed that the decrease in the availability of $CD45^-CD34^+CD133^+$ ASCs from visceral adipose tissue with the increasing age of the subjects was accompanied by a decline in their angiogenic function (Madonna et al., 2011).

Because in vitro experiments have corroborated that the $CD34^+$ fraction has a higher proliferative rate than the $CD34^-$ fraction (Suga et al., 2009), it is likely that the decrease in the percentage of $CD34^+CD31^-CD45^-$ subASCs associated with aging contributed to the decrease in cell yield observed in the subASCs with increasing patient age, as the subASCs from the Elderly and T2DM subjects showed lower yields than the thymASCs. Intrinsic differences in susceptibility to apoptosis may also have influenced variations in the cell yield between the ASCs since it has been described that ASCs from different depots respond differentially to apoptotic stimuli (Schipper et al., 2008)

Together with their *in vitro* expansion capacity, the migratory and integration capacity of precursor cells is another variable that influences the availability of a sufficient number of cells in reparable ischemic tissues. CD184 is the main receptor responsible for the migratory effects of SDF1α, one of the chemokines controlled by the HIF pathway and implicated in the cell recruitment in the context of ischemia (Silvestre et al., 2013). Although CD184 is usually absent on the surface of culture-expanded mesenchymal stem cells (Karp and Leng 2009), we detected $CD184^+$ cells in both the thymASCs and subASCs in passage two and confirm that they retain the ability to respond to the chemotactic effects of SDF1α. These results suggest that the migratory properties of the thymASCs and subASCs derived from patients undergoing coronary bypass can still be enhanced by techniques such as short-term spheroid formation (Cheng et al., 2013). However, we detected a statistically significant decrease in the percentage of $CD271^+$ subASCs with increasing patient age that might decrease the anti-inflammatory efficacy of subASCs, since in vitro experiments have shown that $CD271^+$ mesenchymal stem cells significantly inhibit the proliferation of allogenic T-lymphocytes (Kuçi et al., 2010)

In line with the results obtained by Zhang et al (Zhang et al., 2011), we also confirmed that ASCs from patients with cardiovascular disease retain their ability to form capillary-like structures. While the length of the tubules generated by the subASCs tended to decrease with increasing patient age, the length of those produced by the thymASCs increased significantly and the tubule length generated by the thymASCs derived from the Elderly subjects was significantly higher than that of the Adult subjects. Consistent with the possibility that thymASCs are involved in the expansion of thymic adipose tissue during aging, our laboratory has reported that thymASCs from elderly subjects also showed higher adipogenic capacity compared to those from middle-aged subjects (Oliva-Olivera et al., 2015). Similar results were revealed in another study in which adipogenic and angiogenic protein expression levels of thymic adipose tissue from elderly subjects were higher than those from middle-aged subjects, whereas in the subcutaneous adipose tissue the opposite was observed (Coín-Aragüez et al., 2013).

Although experiments carried out in murine models have corroborated that ASCs from diabetic mice are compromised in their proliferative potential, migration, secretion of angiogenic cytokines and ability to establish a vascular network (Cianfarani et al., 2013; Rennert et al., 2014; Cronk et al., 2015), subASCs from diabetic patients have shown secretion of VEGF, growth rates and tubule formation similar to those of subASCs from healthy subjects (Gu et al., 2012; Policha et al., 2014; Lafosse et al., 2016). Our results agree with those of these authors, as we similarly observed no significant differences between the subASCs from T2DM patients and the subASCs from the other patients. Considering the direct and indirect effects of glucose-lowering agents used as drugs for T2DM (Tahrani et al., 2016), it is logical to allow that the subASCs may be under the beneficial effects of glucose-lowering therapies used to treat T2DM patients. However, unlike the subASCs, the thymASCs from the T2DM subjects showed lower numbers of branching points and levels of VEGF secretion than those from the Elderly patients. Due to different tissues being exposed to variable concentrations of glucose-lowering agents (Tahrani et al., 2016), it is likely that the thymASCs from T2DM patients are less affected by the favorable effects of glucose-lowering agents than the subASCs, but this remain to be determined.

Because NADPH oxidase is one of the main sources of reactive oxygen species in adipose tissue (Furukawa et al., 2004) and that alterations in cellular redox balance may affect the functions of precursor cells (Atashi et al., 2015; Case et al., 2008), it is likely that the significant differences detected in expression levels of NADPH oxidase isoform NOX4 mRNA between the thymASCs and subASCs derived from the Elderly subjects favor the differential effect of subject age on the properties of the ASCs. In fact, we observed that the expression levels of SOD3 correlated positively with the transcriptional levels of SOD2 and catalase only in the thymASCs, suggesting a more balanced redox state in the thymASCs than in the subASCs

Other effects associated with lower levels of NOX4 mRNA expression in the thymASCs from the Elderly subjects could be the significantly lower values of Col1a1 and Col3a1 mRNA that these cells showed with respect to the subASCs derived from the Elderly subjects since evidence suggesting that NOX4-derived reactive oxygen species facilitate TGF β signaling activation-mediated fibrosis by

inducing synthesis of extracellular matrix proteins (Jiang et al., 2014). According to this interpretation, we corroborate a statistically relevant positive association between NOX4 expression levels and levels of Col1a1 and Col3a1 mRNA in both the thymASCs and subASCs.

The success of cell-based therapies also depends on the secretion of paracrine signals that encourage survival of the cells in the ischemic environment (Szöke and Brinchmann, 2012). In our study, we detected that the highest concentrations of VEGF were in the medium conditioned by the hypox-thymASCs from the Adult patients. Particularly in the Elderly patients, the hypox-thymASCs secreted significantly higher VEGF levels than the hypox-subASCs. Unlike the hypox-subASCs, the hypox-thymASCs showed a statistically significant positive association between VEGF and TSP1 secretion levels, suggesting that the secretion of cytokines that stimulate and inhibit angiogenesis is more balanced in hypox-thymASCs than in hypox-subASCs.

Probably because the survival of the HSaVECs cultured in the medium conditioned by the hypox-subASCs was negatively associated with the age of the patients, the hypox-thymASCs derived from the Adult and Elderly patients showed greater ability to favor the survival of the HSaVECs than the respective hypox-subASCs. These results suggest that thymASCs from Elderly subjects are better able than subASCs to promote cell survival during use of the saphenous vein in patients undergoing coronary artery bypass surgery. Nonetheless, experiments *in vivo* models and a comprehensive characterization of the cytokine profile secreted by ASCs from patients undergoing coronary artery bypass surgery are still required. Because of the limited availability of subcutaneous adipose tissue, another of the limitations of our study is that we were unable neither to assess the antioxidant activity of the ASCs nor to confirm by immunoblotting the results on gene expression.

Overall, our study suggests that the aging and disease status associated with cardiovascular disorders in patients undergoing coronary artery bypass surgery exert a differential effect on the properties of the thymASCs and subASCs. In addition, the differences observed between these ASCs regarding their neovascular potential indicate that thymASCs from patients over 65 years and without type 2 diabetes could be one of the cell sources to be considered for angiogenic therapy in patients

undergoing coronary artery bypass surgery. The development of minimally invasive surgery and cell transplantation techniques could enable thymus adipose tissue to become a feasible cell source for angiogenic therapy in these patients.

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7. Authors' contributions

W.O.O. designed the experiment, researched data, and wrote the manuscript. L.C.A., S.L. researched data and contributed to the discussion. J.S. selected the patients and contributed to the discussion. F.J.T. and R.E.B. designed the experiment, reviewed, edited the manuscript and supervised the study. R.E.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis

Abbreviations

ASCs Adipose tissue-derived multipotent mesenchymal cells

BMI Body mass index

COL1A1 Collagen type I alpha 1

COL3A1 Collagen type III alpha 1

DAPI 4',6-diamidino-2-phenylindole

EBM endothelial basal medium

FCS Fetal calf serum

FITC Fluorescein Isothiocyanate

Hb1AcGlycated hemoglobin

HDL Cholesterol High-density lipoproteins cholesterol

HSaVECs Human saphenous vein endothelial cells

hypox-subASCs SubASCs cultured under hypoxic conditions

hypox-thymASCs ThymASCs cultured under hypoxic conditions

LDL Cholesterol Low-density lipoproteins cholesterol

NADPH Nicotinamide adenine dinucleotide phosphate

Normo-subASCs subASCs cultured under normoxia conditions

Normo-thymASCs ThymASCs cultured under normoxia conditions

PE Phycoerythrin

PE-Cy7 Phycoerythrin Cyanine7 tandem fluorochrome

ROS Reactive oxygen species

RPL13A Ribosomal protein L13A

SDF1α Stromal cell derived factor 1α

SOD2 Superoxide dismutase 2 mitochondrial

SOD3 Superoxide dismutase 3 extracellular

thymASCs Thymus ASCs

TSP1 Thrombospondin-1

VEGF Vascular endotelial growth factor

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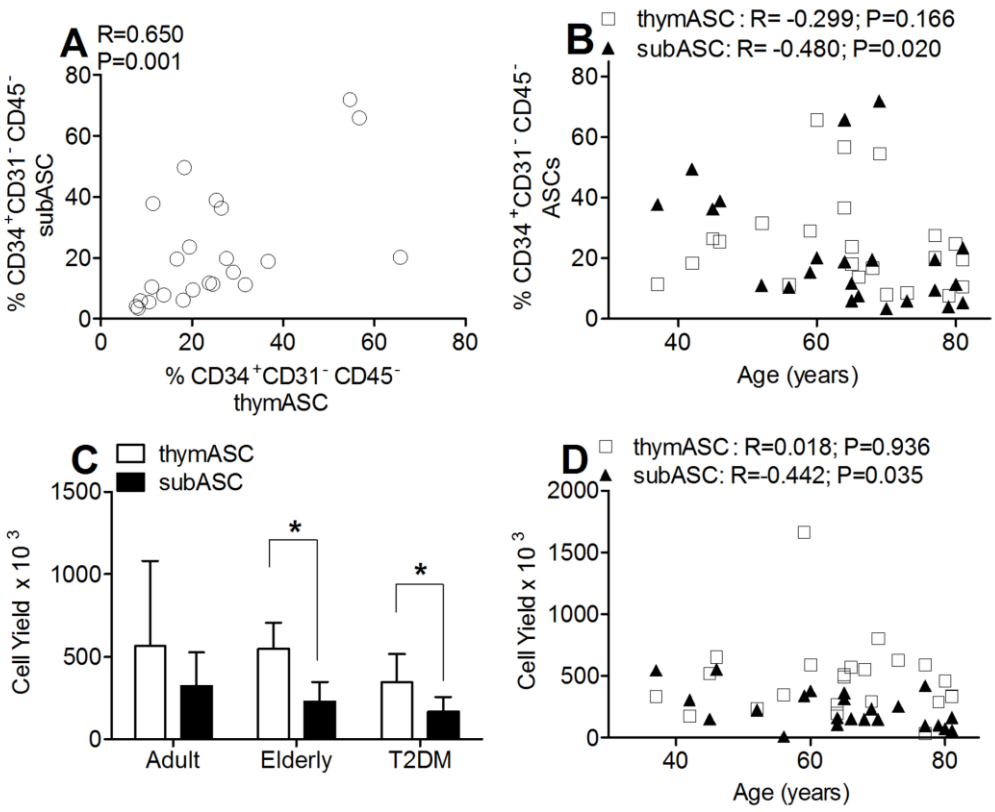

Figure 1. Percentage of CD34$^+$CD31$^-$CD45$^-$ cell fraction and cell yield of ASCs.

A: Correlation between percentages of CD34$^+$CD31$^-$CD45$^-$cell fraction detected in thymASCs and subASCs of paired thymus and subcutaneous adipose tissue from subjects undergoing coronary artery bypass surgery. **B:** Correlation between CD34$^+$CD31$^-$CD45$^-$ thymASCs, subASCs and age of patients undergoing coronary artery bypass surgery. **C:** Cell yield of ASCs cultured as monolayer under standard conditions. After eight days, cells in passage zero were harvested with trypsin/EDTA and counted using the trypan blue exclusion assay with neubauer chamber. Data are expressed as mean number of cells per gram of adipose tissue. **D:** Correlation between cell yield or number of ASCs per gram of adipose tissue and age of patients. Values are reported as mean ± standard deviation (Adult, n = 7; Elderly, n = 7; T2DM, n = 9).*****, Significantly different results (Wilcoxon signed-rank test, $P< 0.05$)

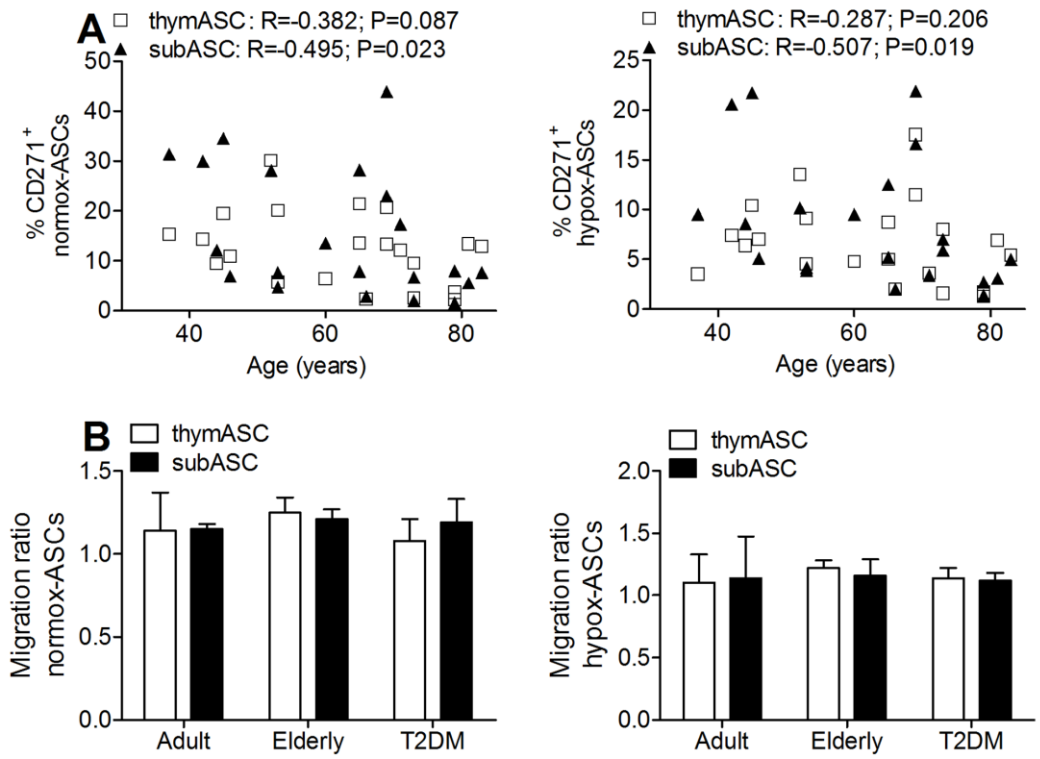

Figure 2. Percentage of CD271$^+$ ASCs and effects of SDF1α on migration of ASCs previously cultivated under 21% $O_{2(g)}$ or 1% $O_{2(g)}$. A: Correlation between CD271$^+$ thymASCs, subASCs and age of patients undergoing coronary artery bypass surgery. (Adult, n = 6; Elderly, n = 6; T2DM, n = 9).**B:** The migration rate was calculated by dividing the number of cells detected in the presence of SDF1α by those observed in the absence of SDF1α. The results are presented as mean migration rate values ± standard deviation (Adult, n = 3; Elderly, n = 3; T2DM, n = 3).

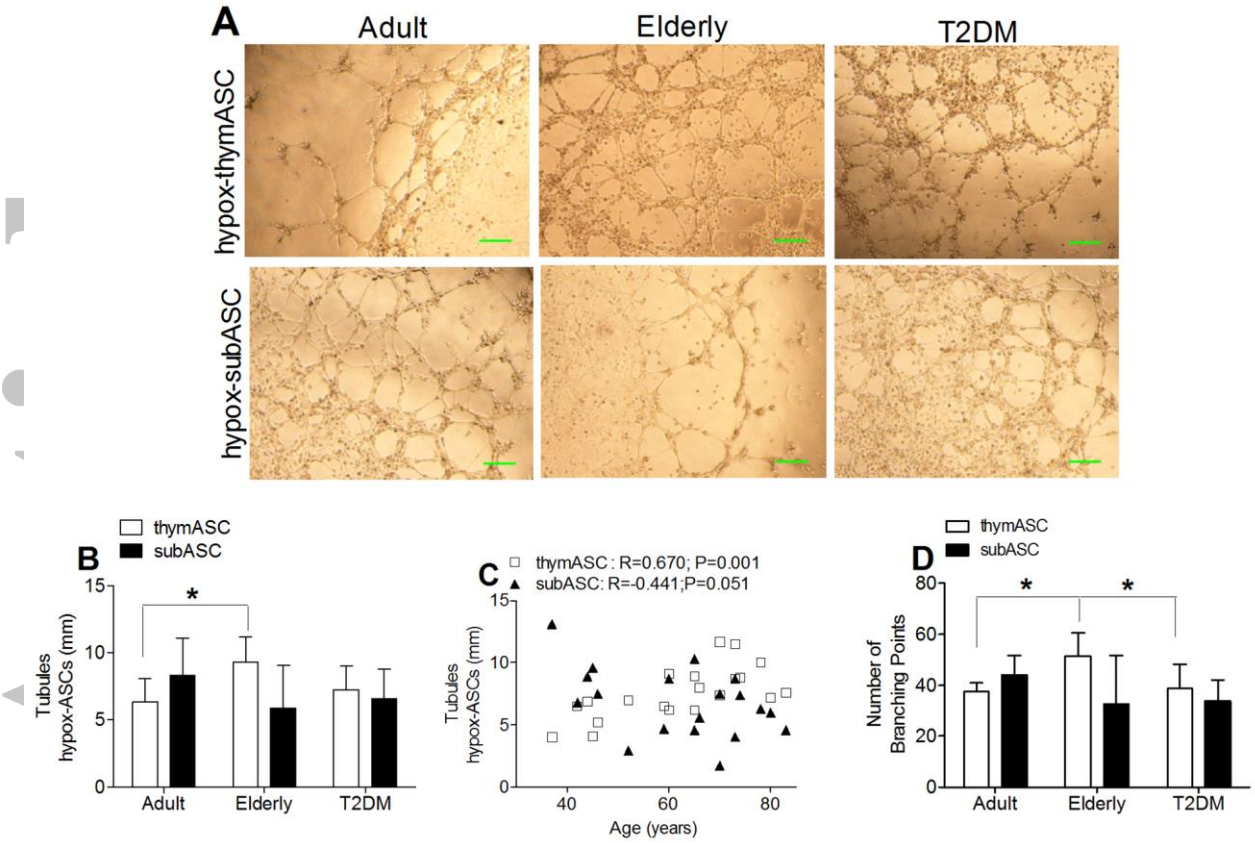

Figure 3.Capacity for tubule formation by hypox-ASCs. A: Morphology of capillary-like structures formed by hypox-thymASCs and hypox-subASCs on growth factor-reduced matrigel incubated under hypoxic conditions (1% $O_{2\,(g)}$) for six hours. ASCs in passage one were resuspended in EBM (PromoCell) supplemented with FCS (0.05 mL/mL), seeded in duplicate at a density of 1 × 10^5 cells/cm^2, and incubated for six hours under conditions of 1% $O_{2(g)}$, 94% $N_{2(g)}$ and 5% $CO_{2(g)}$ provided by hypoxia incubator. For all the study, cell density was the same. Bar = 250 µm. **B:** Values for the length of the tubules generated by hypox-thymASCs and hypox-subASCs from Adult, Elderly and T2DMsubjects. **C:** Correlation between length of the tubules generated by hypox-thymASCs, hypox-subASCs and age of patients undergoing coronary artery bypass surgery. **D:** Number of branching points generated by hypox-thymASCs and hypox-subASCs from Adult, Elderly and T2DM subjects.

(Adult, n = 6; Elderly, n = 6; T2DM, n = 8)**. ***, Significantly different results (Mann-Whitney test; $P < 0.05$).

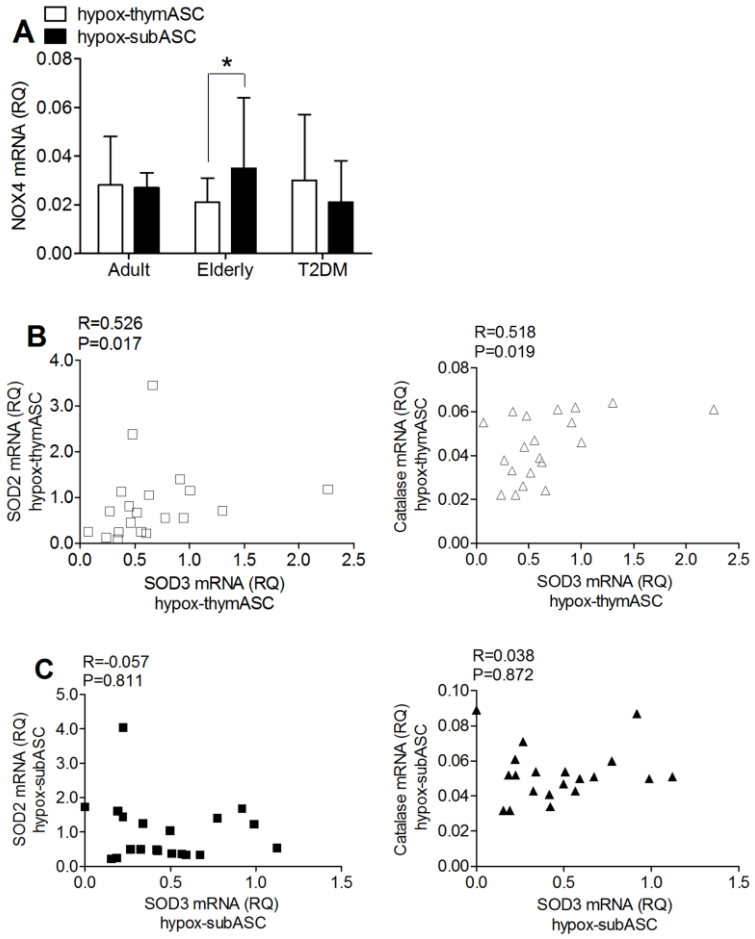

Figure 4. Expression of genes involved in cellular redox balance in hypox-ASCs exposed for 72 hours at 1% $O_{2\ (g)}$.A: Levels of NOX4 mRNA expression in hypox-thymASCs and hypox-subASCs from Adult, Elderly and T2DMsubjects. **B-C:** Correlation between transcriptional levels of SOD2, Catalase mRNA and SOD3 mRNA both in hypox-thymASCs (**B**) and hypox-subASCs (**C**) from patients undergoing coronary artery bypass surgery. Values are reported as mean ± standard deviation (Adult, n = 6; Elderly, n = 6; T2DM, n = 8).*****, Significantly different results (Wilcoxon signed-rank test, $P< 0.05$).

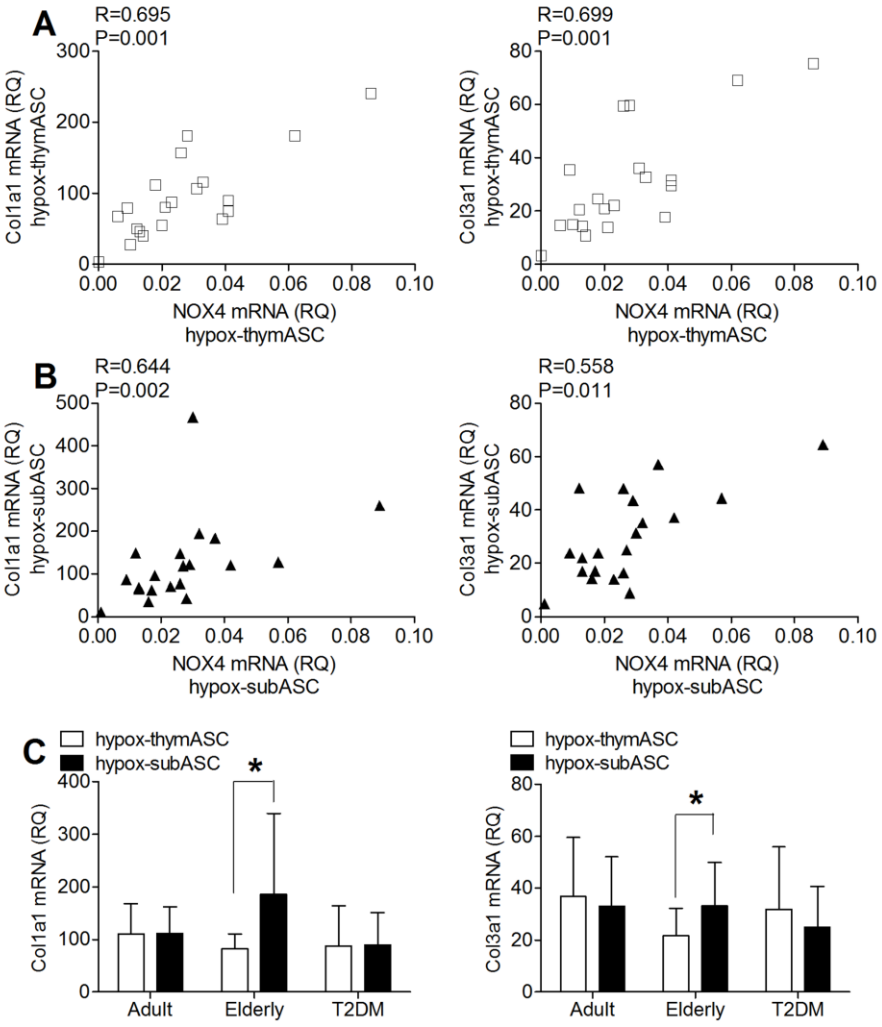

Figure 5.Expression of genes implicated in tissue fibrosis in ASCs exposed for 72 hours at 1% $O_{2\ (g)}$. A-B: Positive correlation between transcriptional levels of Col1a1, Col3a1 mRNA and NOX4 mRNA both in hypox-thymASCs (**A**) and hypox-subASCs (**B**) from patients undergoing coronary artery bypass surgery. **C:** Levels of Col1a1 and Col3a1 mRNA expression in hypox-thymASCs and hypox-subASCs from Adult, Elderly and T2DMsubjects. Values are reported as mean ± standard deviation (Adult, n = 6; Elderly, n = 6; T2DM, n = 8).*****, Significantly different results (Wilcoxon signed-rank test, $P< 0.05$).

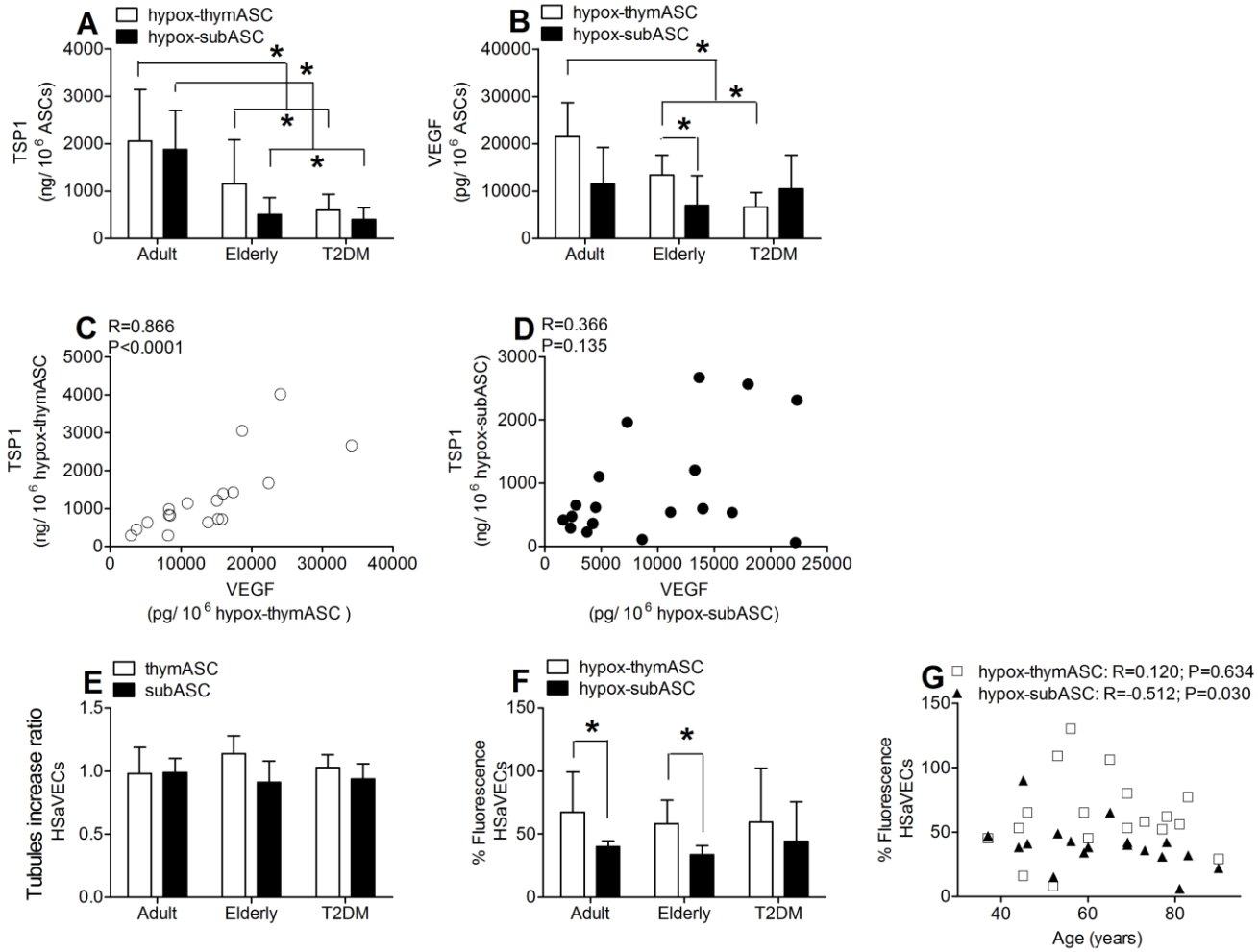

Figure 6. Secretion of TSP1, VEGF and bioactivity of the medium conditioned by hypox-ASCs cultured for 72 hours at 1% $O_{2(g)}$. A-D: Concentration of TSP1 (**A**) and VEGF (**B**) detected in medium conditioned by hypox-thymASCs and hypox-subASCs from Adult, Elderly and T2DMsubjects. Correlation between levels of TSP1 and VEGF secreted by hypox-thymASCs (**C**) and hypox-subASCs (**D**) from patients undergoing coronary artery bypass surgery. Concentrations of cytokines were determined by ELISA and their values normalized to 1×10^6 ASCs according to the number of cells present at the time of medium collection. Values are reported as mean ± standard deviation (Adult, n = 6; Elderly, n = 6; T2DM, n = 6).*****, Significantly different results (Mann-Whitney test; $P < 0.05$).

E: Rate of increase in the length of tubules generated by HSaVECs cultured in medium conditioned by thymASCs and subASCsfrom Adult, Elderly and T2DM subjects. The rate of increase in tubule formation was calculated by dividing the mean length of the tubules produced by HSaVECs cultured

in hypox-ASC-conditioned medium by the mean length of those generated by the HSaVECs cultured in normo-ASC-conditioned medium. **F:** Values of fluorescence emitted by the HSaVECs surviving after 72 hours of cultivate in medium conditioned by hypox-thymASCs and hypox-subASCsfrom Adult, Elderly and T2DM subjects. **G:** Correlation analysis between values of fluorescence emitted by the HSaVECs survivingin medium conditioned by hypox-ASCs and age of patients undergoing coronary artery bypass surgery. Cell survival was estimated by fluorescence intensity, by dividing the emitted fluorescence value in the wells where HSaVECs were cultured in hypox-ASC-conditioned medium by the values emitted in the wells where HSaVECs were cultured in unconditioned medium. Values are reported as mean ± standard deviation (Adult, n = 6; Elderly, n = 6; T2DM, n = 6).*****, (Wilcoxon signed-rank test, $P < 0.05$).

Table 1. Anthropometric and clinic characteristics of the study participants

	Adult (n=10)	Elderly (n=13)	T2DM (n=16)
Age, years	52.10 ± 9.21	74.92 ± 7.08*	68.44 ± 11.28*
Male/Female	8/2	8/5	12/4
BMI, kg/m^2	30.27 ± 4.47	27.32 ± 3.83	29.95 ± 3.90
Triglycerides, mmol/L	1.59 ± 0.39	1.46 ± 0.46	1.53 ± 0.50
Total cholesterol, mmol/L	5.61 ± 0.59	4.63 ± 1.13*	4.31 ± 0.64*
HDL cholesterol, mmol/L	1.17 ± 0.17	1.32 ± 0.31	1.02 ± 0.25*†
LDL cholesterol, mmol/L	3.71 ± 0.55	2.53 ± 0.61*	2.58 ± 0.58*
Creatinine, μmol/L	63.29 ± 13.85	79.30 ± 27.82	71.67 ± 22.91
Hb1Ac (%)	14.23 ± 1.34	13.99 ± 1.26	13.24 ± 1.95
Hematocrit (%)	43.20 ± 3.17	42.39 ± 3.42	40.33 ± 6.89

Values are means ± standard deviation. *Results significantly different (Mann-Whitney; $P < 0.05$) from NonElder individuals. †Results significantly different (Mann-Whitney; $P < 0.05$) from Elder patients. **Abbreviations:**; BMI, body mass index; HDL cholesterol, high-density lipoprotein cholesterol; LDL cholesterol, low-density lipoprotein cholesterol; Hb1Ac, glycated hemoglobin