Thymus fat as an attractive source of angiogenic factors in elderly subjects with myocardial ischemia

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Abstract Aging negatively affects angiogenesis which is found to be linked to declined vascular endothelial growth factor (VEGF) production. Adult human thymus degenerates into fat tissue (thymus adipose tissue (TAT)). Recently, we described that TAT from cardiomyopathy ischemic subjects has angiogenic properties. The goal of our study was to analyze whether aging could also impair angiogenic properties in TAT as in other adipose tissue such as subcutaneous (subcutaneous adipose tissue (SAT)). SAT and TAT specimens were obtained from 35 patients undergoing cardiac surgery, making these tissues readily available as a prime source of adipose tissue. Patients were separated into two age-dependent

groups; middle-aged ($n=18$) and elderly ($n=17$). Angiogenic, endothelial, and adipogenic expression markers were analyzed in both tissues from each group and correlations were examined between these parameters and also with age. There were no significant differences in subjects from either group in clinical or biological variables. Angiogenic markers VEGF-A, B, C, and D and adipogenic parameters, peroxisome proliferator-activated receptors (PPAR γ 2), FABP4, and ADRP showed elevated expression levels in TAT from elderly patients compared to the middle-aged group, while in SAT, expression levels of these isoforms were significantly decreased in elderly patients. VEGF-R1, VEGF-R2, VEGF-R3, Thy1,

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CD31, CD29, and VLA1 showed increased levels in TAT from the elderly compared to the middle-aged, while in SAT these levels displayed a decline with aging. Also, in TAT, angiogenic and endothelial parameters exhibited strong positive correlations with age. TAT appears to be the most appropriate source of angiogenic and endothelial factors in elderly cardiomyopathy subjects compared to SAT.

Keywords Human aging · Ischemic cardiomyopathy · Adult thymus adipose tissue · Subcutaneous adipose tissue · Angiogenic factors · Endothelial markers

Introduction

Angiogenesis, the formation of new vessels from existing vasculature, becomes impaired with age. The formation of capillaries was first noted to be decreased in middle-aged rats relative to young rats (Arthur et al. 1998a), a phenomenon that has been confirmed subsequently in other animal models and in humans where it has been shown that functional impairment of endothelial progenitor cells from elderly subjects has been shown to correlate with endothelial dysfunction (Heiss et al. 2005). Human and murine adipose-derived stromal cells have been shown to release many potent angiogenic factors and differentiate into endothelial cells (Miranville et al. 2004; Moon et al. 2006), which makes adipose tissue an attractive option and has tremendous potential in the field of therapeutic neovascularization.

It has been shown that the thymus grows rapidly during embryonic life and during childhood, reaching its maximum absolute size in puberty; thereafter, growth ceases and involutes gradually until old age, when the gland is often smaller than at birth (Hale 2004). This age involution is shown by a decrease in the overall weight of the organ, associated lymphoid tissue atrophy and replacement by mature adipose tissue (Hale 2004). For this reason, all the studies carried out on adult thymus involution have been focused primarily on the immunological aspect; however, it has never been considered interesting enough to be studied as a potential source of humoral and/or angiogenic factors, even its differential characteristics, with respect to other adipose tissues, have

never been studied before. Part of this tissue is discarded in aortic cannulation procedures of the ascending aorta in cardiovascular surgery in patients in need of a cardiopulmonary bypass (CPB), making this tissue a readily available prime source of adipose tissue.

Vascular endothelial growth factor (VEGF) is the first member of angiogenic factors to be cloned and remains today the best-characterized angiogenic growth factor (Ferrara et al. 2003). VEGF was first described as a vascular permeability factor (Dvorak et al. 1999) and is recognized as being a potent stimulator of endothelial proliferation and migration (Wang et al. 2008). Gene targeting studies have demonstrated the critical importance of VEGF for both vasculogenesis and angiogenesis (Ferrara et al. 1996). It has been proven that white adipose tissue produces and secretes many different types of proangiogenic factors, such as VEGF-A and VEGF-B, the two key angiogenic factors produced by adipocytes (Saiki et al. 2006). Other adipose-tissue derived factors with pro-angiogenic properties are VEGF-C and VEGF-D, which have been found to be important in the proper formation and maintenance of the lymphatic network (Asano et al. 2001). As a result, VEGF has been used in numerous preclinical and early clinical gene or protein therapy trials with varying degrees of success (Sosman and Mier 2003). Substantial changes in the adhesive interactions between cells and the extracellular matrix (ECM) take place to permit endothelial cell migration during angiogenesis. These interactions are mediated by integrins such as CD29, CD31, CD49, CD73, CD90, etc. Interactions between these specific integrins and growth factor receptors such as VEGF-Rs mediate pro-angiogenic effects (Serini et al. 2008).

We have recently found that thymus fat produces a variety of angiogenic factors such as angiotensin, Tie2, VEGF, VEGF-R1, and VEGF-R2. Furthermore, human umbilical cord endothelial cells cultured in the presence of thymus fat extract displayed enhanced proliferative and migratory responses, which are two relevant steps in angiogenesis (Salas et al. 2009). We have also shown that thymus adipose tissue (TAT) displays similar inflammatory and anti-inflammatory mechanisms to those observed in other adipose tissues, including subcutaneous adipose tissue (Tinahones et al. 2009). These findings lead us to believe that TAT

is an interesting source of angiogenic factors which may, in the future, have a relevant role in promoting angiogenesis and tissue repair. The goal of our study was to analyze and compare the variation of expression levels of angiogenic endothelial and adipogenic parameters in human thymus and subcutaneous adipose tissues in relation to the increase of age, and then to investigate whether aging impairs the angiogenic properties in TAT as in subcutaneous.

Material and methods

Patients and adipose tissue collection

The study recruited patients who received a coronary-artery bypass graft with a CPB in the Carlos Haya Hospital. The mean number of grafts used was 3.1 per patient. The subjects operated on and recruited in this study were individuals with stable situations and without severe ischemic injury. Therefore, these subjects were without infarct or with a previous infarct at least 6 months before surgery. Moreover, none the subjects of this study suffered from ischemic peripheral arteriopathy of the legs which could also alter gene expression of angiogenic factors. The number of patients recruited for RNA and protein expression analysis was $n=35$. The study was carried out on two groups of patients: the middle-aged group, aged from 45 to 65 ($n=18$), and the elderly group of patients with an age of 70 or over. For the flow cytometry study, the number of patients with similar clinicopathological characteristics was 12 ($n=6$ middle aged, and $n=6$ elderly). This study was approved by the local ethical committee of the Carlos Haya Hospital, and signed informed consent was obtained from all participating patients by the Spanish Society of Thoracic and Cardiovascular Surgery (SECTCV).

The relevant clinical and metabolic characteristics of these subjects are shown in Table 1. Both subcutaneous and thymus adipose tissues were obtained at the beginning of the procedure and before heart arrest. The site of subcutaneous fat harvest was from the chest incision. Tissues were immediately stored at -80°C for RNA and protein expression analysis. Fresh tissues were processed for flow cytometry.

RNA extraction

Total RNA was isolated from adipose tissue using the Trizol RNA isolation method (Invitrogen, Carlsbad, CA) and purified with the RNeasy Lipid kit (Qiagen, Valencia, CA)

Real-time PCR

The amplifications were performed using a MicroAmp optical 96-well reaction plate (PE Applied Biosystems) on an ABI 7500 real-time PCR system (Applied Biosystems). RT qPCR reactions were carried out for all genes using specific TaqMan gene expression assays. During PCR, the Ct values for each amplified product were determined using a threshold value of 0.1. The specific signals were normalized by constitutively expressed cyclophilin signals using the formula $2^{-\Delta\text{Ct}}$.

TaqMan® gene expression assay probes

VEGF-A	Hs00173626_m1
VEGF-B	Hs00957984_m1
VEGF-C	Hs00153458_m1
VEGF-D	Hs01128659_m1
VEGF-R1/FLT1	Hs01052935_m1
VEGF-R2/(KDR/Flk-1)	Hs00176676_m1
VEGF-R3/FLT4	Hs01047674_m1
CD90/Thy-1	Hs00264235_s1
CD31/PCAM-1	Hs01065279_m1
CD49a/ VLA1	Hs00168433_m1
CD29	Hs00559595_m1
Cyclophilin	4326316E

Western blot analysis

Total proteins from TAT and SAT were extracted by NE-PER Nuclear and Cytoplasmatic Extraction Reagents protocol (Pierce). Protein extracts (30 μg) were separated by SDS-PAGE, blotted onto a PVDF membrane and then incubated with specific antibodies (anti-VEGF-A SC-152, anti-VEGF-B SC8042, anti-VEGF-C SC25783, anti-VEGF-D SC 80447, anti-VEGF-R3/FLT4 (C-20) SC321, anti-VEGF-R2/FLK1 (A-3) SC6251, anti-VEGF-R1/FLT1 (C-17) SC316, anti-FABP4 SC271529 and anti-adipose-differentiation-related protein (ADRP) SC32888 from Santa Cruz

Table 1 Clinical and biological variables of patients

	Middle-aged (<i>n</i> =18)	Elderly (<i>n</i> =17)	<i>P</i>
Age (years)	45-65	≥70	
Glucose (mg/dL)	131.40±16.94	123.71±19.80	NS
Triglycerides (mg/dL)	132.50±16.30	121.29±17.54	NS
Values are presented as means ± SEM	Cholesterol (mg/dL)	146.33±9.01	NS
Relationships between both groups were analyzed using the Mann–Whitney <i>U</i> test	HDL-c (mg/dL)	32.67±2.43	NS
<i>BMI</i> body mass index, <i>HDL-c</i> high-density lipoprotein cholesterol, <i>LDL-c</i> low-density lipoprotein cholesterol	LDL-c (mg/dL)	95.00±5.54	NS
	Uric acid (mg/dL)	5.46±0.39	NS
	Creatinine (mg/dL)	1.00±0.10	NS
	Hb1Ac (%)	6.27±0.35	NS
	BMI (kg/m ²)	31.52±1.44	NS

Biotechnology). Anti-PPAR γ 2 MBS603559 was obtained from My Biosource. Protein signals were detected by electrochemiluminescence detection Quantity One[®] software (Bio-Rad Laboratories).

Isolation and expansion of cell derived stromal vascular fraction from human thymic fat and subcutaneous adipose tissue

Isolation and expansion of human cell derived stromal vascular fraction from adipose tissue was carried out using a procedure modified from Zuck et al. (2001). Briefly, adipose tissue samples were harvested from patients who underwent a coronary-artery bypass graft and transported in Hank's balanced salt solution supplemented with 3 % of penicillin and streptomycin. Adipose tissue biopsies were washed with PBS to remove cellular debris and dissected into small pieces with enzymatic digestion in a solution of 0.150 % collagenase type I and 1.0 % bovine serum albumin (BSA) for 1 h at 37°C on a shaking water bath.

Tissue suspension obtained from enzymatic digestion was centrifuged at 500×*g* for 10 min and floating adipocytes were separated from precipitated stromal vascular fraction by decanting, which was filtered through a 100-μm mesh and centrifuged at 300×*g* for 5 min. The cell pellet was re-suspended in erythrocytes lysis buffer for 10 min at room temperature and, after further centrifuging at 300×*g* for 5 min, re-suspended in DMEM/F12 supplemented with 10 % fetal bovine serum, 100 μg/mL streptomycin, 100 U/mL penicillin, 2 mM L-glutamine, and 1 μg/mL of amphotericin B. Cells were plated in tissue culture flasks and incubated at 37°C in humid atmosphere with

5 % of CO₂ for approximately 7 days until 90 % confluence was reached.

Immunophenotype characterization by flow cytometry

Cultured stromal vascular fraction cells (passage zero and three) were characterized by flow cytometry according to a panel of cell surface markers, including Thy1/CD90, CD29, and CD49a (eBioscience). Briefly, 1×10⁶ cells/mL were re-suspended in a blocking buffer solution, containing PBS supplemented with BSA to 3.0 %, and incubated for 10 min on ice. Cells (1×10⁵) were aliquoted per polypropylene tube and according to the instructions of the manufacturer, 10 or 20 μL of mouse antibody solution was added, against the respective cell surface markers, together with its corresponding fluorochrome (phycoerythrin [PE], fluorescein isothiocyanate, allophycocyanin, and phycoerythrin coupled to the cyanine dye Cy7 [PE-Cy7]). One of the tubes was destined for the marking of corresponding isotype controls. All tubes were incubated on ice and protected from light for 30 min. The cells were washed twice in a blocking buffer solution and finally re-suspended in 500 μL of PBS for acquisition of 1×10⁴ events by tube through a CyAnTM cytometer ADP high-speed analyzer (Beckman Coulter).

Statistical analysis

The results are given as the mean ± standard error means (SEM). All clinical parameters are summarized by descriptive statistics. Relationships

between both groups (elderly and middle-aged) were analyzed using the Mann–Whitney *U* test. Correlation analysis was performed with Spearman's correlation coefficient test (*r*). Partial correlations for controlling for confounders (BMI, glucose, triglycerides, and cholesterol) were also performed. In all cases, the rejection level for a null hypothesis was $\alpha = 0.05$ for two tails. The statistical analysis was carried out with the SPSS software program (Version 15.0 for Windows; SPSS, Chicago, IL).

Results

Clinical and biological variables of both patient groups

Table 1 shows that there were no significant differences in clinical and biological variables between the two groups.

The comparisons of both TAT and SAT VEGF-A, VEGF-B, VEGF-C, and VEGF-D gene and protein expression levels between middle-aged adults and elderly cardiomyopathy ischemic patients.

qPCR analysis showed that TAT VEGF-A gene expression was significantly increased in elderly patients compared to the middle-aged group. In contrast, gene expression of VEGF-A in SAT was significantly decreased in elderly patients compared to middle-aged (Fig. 1a). Also, TAT VEGF-B gene expression was increased (not significantly) in elderly patients compared to middle-aged, while in SAT this expression was significantly decreased in elderly patients (Fig. 1b). VEGF-C and VEGF-D, which showed similar expression levels in both SAT and TAT in the middle age group, were significantly increased in elderly patients TAT, while in SAT the levels of these two isoforms were similar in both groups (Fig. 1c, d).

VEGF isoform protein expressions were also analyzed in both TAT and SAT from middle-aged and elderly patients. Figure 1e shows evidence of the presence of protein synthesis of the four VEGF isoforms in both TAT and SAT in all subjects. Protein analyses confirmed the results obtained by real-time PCR, clearly showing the increase of VEGF isoforms in TAT from elderly compared to middle-aged. While in SAT, protein levels of these isoforms were maintained similar or even decreased in elderly patients compared to middle-aged.

The comparisons of TAT and SAT endothelial marker gene and protein expression levels between middle-aged adults and elderly cardiomyopathy ischemic patients

All members of the VEGF family stimulate cellular responses by binding to tyrosine kinase receptors (the VEGFRs) on the cell surface. VEGF-R1 (Flt-1), VEGF-R2 (Flk-1/KDR), and VEGF-R3 (Flt-4) are known to mediate vasculogenesis (R1 and R2) and lymphangiogenesis (R3). Figure 2a, b, d show that VEGF-R1 and VEGF-R2 are highly expressed in TAT compared to SAT. Furthermore, expression levels of VEGF-R1 and VEGF-R2 in TAT were higher in elderly compared to middle-aged patients. In contrast, in SAT, expression levels of these two endothelial markers did not show any significant differences between elderly and middle-aged subjects. VEGF-R3 expression levels in TAT were similar in both elderly and middle-aged patients, while in SAT, the gene expression level of this receptor was significantly lower in elderly compared to middle-aged patients (Fig. 2c), even though no significant differences in protein expression were observed between the different groups and between TAT and SAT (Fig. 2d). It is well-known that the adhesive interactions between cells and the extracellular matrix take place to permit endothelial cell migration during angiogenesis. These interactions are mediated by integrins which bind to ligands in the ECM-promoting endothelial cell motility and proliferation. Here, we assessed whether the enhancement of angiogenic factor gene expression was accompanied by an increase in the expression of genes codifying for markers of endothelial progenitors cells controlling endothelial differentiation. Figure 3 shows that TAT CD90/Thy-1, CD31/PCAM-1, and CD29 gene expression levels were significantly increased in elderly patients compared to middle-aged patients, while these levels were decreased in the SAT of these individuals compared to middle-aged patients (Fig. 3a–c). VLA1/CD49a integrin alpha 1 chain, which is known to combine with beta1 subunit CD29 to form a receptor for collagen and laminin, was observed to be highly expressed at both age stages in TAT showing similar levels in the elderly and middle-aged, while the levels of this integrin were much lower in SAT compared to TAT, also, in SAT, these levels were diminished in elderly compared to middle-aged patients (Fig. 3d). To analyze

Fig. 1 VEGF isoforms mRNA gene and protein expression in thymus adipose tissue (TAT) and subcutaneous adipose tissue (SAT) from middle-aged and elderly cardiomyopathy subjects. **a–d** VEGF-A, VEGF-B, VEGF-C and VEGF-D mRNA expression analysis was performed in TAT and SAT from two groups of middle-aged (45–65 years old, $n=18$) and elderly (70+ years old, $n=17$) cardiomyopathy subjects. mRNAs were normalized to cyclophilin levels. Results were obtained from triplicate and expressed as the

mean $\pm$ SEM. Relationships between both groups, elderly and middle-aged patients, were analyzed using the Mann–Whitney U test. * $p<0.05$. **e** Immunoblotting analysis of VEGF isoforms. The resulting blot of each isoform was compared to β -Actin constitutive protein. Density analysis was carried out by normalizing the samples to β -actin using NIH's Image-J software program. The blot is representative of three independent experiments with different samples, * $p<0.05$

whether gene expression differences were maintained in protein expression, endothelial marker

surface expression was analyzed by flow cytometry in stromal fraction of both adipose tissues. Table 2 shows

Fig. 2 Endothelial markers mRNA gene and protein expression in thymus adipose tissue (TAT) and subcutaneous adipose tissue (SAT) from middle-aged and elderly cardiomyopathy subjects. **a–c** Vascular endothelium growth factor receptors mRNA expression analysis was performed in TAT and SAT from two groups of middle-aged (45–65 years old, $n=18$) and elderly (>70 years old, $n=17$) cardiomyopathy subjects. mRNAs were normalized to cyclophilin levels. Results were obtained from triplicate and expressed as the mean $\pm$ SEM. Relationships

between both groups, elderly (Eld) and middle-aged (M-A) patients, were analyzed using the Mann-Whitney U test. * $P < 0.05$. Immunoblotting analysis of *VEGF-R1*, *VEGF-R2*, and *VEGF-R3*. The resulting blot of each isoform was compared to β -Actin constitutive protein. Density analysis was carried out by normalizing the samples to β -Actin using NIH's Image-J software program. The blot is representative of three independent experiments with different samples, * $p < 0.05$

that Thy1/CD90, CD29, and CD49a/VLA1 surface marker expression was increased in TAT from elderly patients compared to middle-aged, while in SAT, surface expression of these markers decreased in the elderly

compared to middle-aged group. In TAT, the differences observed in Thy1/CD90 and CD49a/VLA1 surface expression was significant, while the differences observed in CD29 expression were not.

Fig. 3 Integrin markers mRNA gene expression in thymus adipose tissue (TAT) and subcutaneous adipose tissue (SAT) from middle-aged and elderly cardiomyopathy subjects. **a–d** integrin markers (Thy1/CD90, CD29, and VLA1/CD49a) mRNAs expression analysis was performed in TAT and SAT from two groups of middle-aged (M-A) (45–65 years old, $n=18$)

and elderly (Eld) (>70 years old, $n=17$) cardiomyopathy subjects. mRNAs were normalized to cyclophilin levels. Results were obtained from triplicate and expressed as the mean $\pm$ SEM. Relationships between both groups, elderly and middle-aged patients, were analyzed using the Mann–Whitney U test. * $p<0.05$

The comparisons of TAT and SAT adipogenic gene and protein expression levels (PPAR γ 2, ADRP, and FABP4) between middle-aged adults and elderly cardiomyopathy ischemic patients

qPCR analysis showed that PPAR γ 2, ADRP, and FABP4 gene expression levels were significantly increased in elderly patient TAT compared to the levels observed in middle-aged TAT, while in SAT these levels were maintained or slightly diminished

(Fig. 4a–c). Western blotting analysis showed that even protein levels of PPAR γ 2 and ADRP were elevated in SAT from middle-aged subjects compared to TAT; these levels were significantly decreased in SAT from elderly compared to middle-aged, while in TAT the levels of these two adipogenic markers were increased in elderly subjects. FABP4 protein levels in middle-aged patients were higher in SAT than in TAT, but in elderly subjects these levels were significantly increased in TAT

Table 2 Flow cytometry analysis to quantify endothelial markers, Thy1/CD90, CD29, and CD49a/VLA1, cell-surface expression in stromal vascular fraction from both TAT and SAT from middle-aged and elderly cardiomyopathy subjects

	TAT		SAT	
	Middle-aged ($n=6$)	Elderly ($n=6$)	Middle-aged ($n=6$)	Elderly ($n=6$)
Thy1/CD90	90.95 $\pm$ 2.31	97.78 $\pm$ 3.35*	95.25 $\pm$ 9.41	83.95 $\pm$ 7.41*
CD29	96.31 $\pm$ 2.56	98.15 $\pm$ 6.91	97.17 $\pm$ 6.43	85.35 $\pm$ 13.65*
CD49a/VLA1	48.02 $\pm$ 5.21	53.95 $\pm$ 1.49*	26.02 $\pm$ 1.85	21.85 $\pm$ 2.95

Stromal vascular fraction cells were prepared for flow cytometry as described in “Material and methods”. Data represent the percentage of cell staining for cellular surface markers from stromal vascular fractions from both subcutaneous (SAT) and thymus (TAT) adipose tissues. Values presented in the table depict average $\pm$ SEM of three independent experiments. Relationships between both groups, elderly and middle-aged patients, were analyzed using the Mann–Whitney U test * $p<0.05$

Fig. 4 Adipogenic markers, PPAR γ , ADRP, and FABP-4 mRNA gene and protein expression in thymus adipose tissue (TAT) and subcutaneous adipose tissue (SAT) from middle-aged and elderly cardiomyopathy subjects. **a–c** PPAR γ , ADRP, and FABP-4 mRNA expression analysis was performed in TAT and SAT from two groups of middle-aged (M-A) (45–65 years old, $n=18$) and elderly (Eld) (>70 years old, $n=17$) cardiomyopathy subjects. mRNAs were normalized to cyclophilin levels. Results were obtained from triplicate and expressed as the mean $\pm$ SEM.

Relationships between both groups, elderly and middle-aged patients, were analyzed using the Mann–Whitney U test. * $p < 0.05$. **d** Immunoblotting analysis of PPAR γ , ADRP, and FABP-4. The resulting blot of each isoform was compared to β -Actin constitutive protein. Density analysis was carried out by normalizing the samples to β -Actin using NIH's Image-J software program. The blot is representative of three independent experiments with different samples, * $p < 0.05$.

reaching the levels detected in SAT, where protein levels of this adipogenic marker were maintained

similar in middle-aged and elderly patients (Fig. 4d).

Correlative analysis

Correlative analysis of cardiomyopathy ischemic patients age and angiogenic markers gene expression in TAT showed a strong positive and significant correlation between these parameters, especially CD31, VEGF-A, VEGF-C, VEGF-D, VEGF-R1, VEGF-R2, and VEGF-R3 which were highly correlated with age (Table 3). Next, partial correlation analysis was used to determine the strength of the association of these angiogenic markers gene expression levels in TAT with age, after controlling for other clinical covariates including glucose, BMI, triglyceride, and cholesterol (Table 4). Partial correlation analysis showed that expression levels of CD31, VEGF-R1, VEGF-R2, VEGF-A, and VEGF-C were independently correlated with age, indicating that this significant association was not mediated by glucose, BMI, triglycerides, or cholesterol. However, the correlations of VEGF-R3 and VEGF-D with age were eliminated when glucose, BMI, triglycerides and cholesterol were controlled.

Moreover, when we analyzed the correlation between adipogenic (PPAR γ 2, FABP-4, and ADRP) and angiogenic (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-R1, VEGF-R2, and VEGF-R3) markers in TAT, we observed strong positive correlations between these parameters (Table 5), while in subcutaneous adipose tissue these correlations were negative (data not shown).

Discussion

Currently, subcutaneous and visceral adipose tissues are the most suitable sources of angiogenic factors on offer (Rehman et al. 2004) and an appropriate candidate for tissue engineering and neovascularization (Kondo et al. 2009). However, recently,

most investigations indicate that aging seriously impairs the angiogenic properties of these adipose tissues (El-Ftesi et al. 2009; Efimenko et al. 2011). Even so, and because of ease of access, these adipose tissues remain the most suitable source of angiogenic factors and progenitor cells with a potential to improve neovascularization of ischemic tissues (Efimenko et al. 2011).

In this work and for the first time, we offer an attractive new source of adipose tissue and angiogenic factors, the adult thymus adipose tissue, which does not appear to impair its angiogenic properties with ageing; on the contrary these characteristics are significantly increased with aging.

To our knowledge, this is the first report that shows that in elderly cardiomyopathy ischemic subjects, whereas the expression levels of angiogenic and endothelial markers declined in subcutaneous adipose tissue, thymus fat showed an increase in gene expression of these parameters in comparison with middle-aged subjects. Likewise, adipogenic marker gene expression showed an increase in TAT from elderly subjects compared to middle-aged, while in SAT they showed a decrease. Moreover, in TAT, expression levels of CD31, VEGF-A, VEGF-C, VEGF-R1, and VEGF-R2 correlated significantly and positively with age independently of any confounder clinical covariates, including BMI, glucose, cholesterol and triglycerides. Interestingly, the associations of VEGF-D and VEGF-R3 with age were eliminated when controlling BMI, glucose, cholesterol, and triglycerides, suggesting that these clinical parameters have confounding effects on lymphangiogenic parameters, while angiogenic factors seem to be independent of these clinical covariates in TAT from these patients. On the other hand, in subcutaneous adipose tissue the correlations between all these angiogenic markers and age were negative. These latest data confirmed previous data showing that aging impairs angiogenesis and decreases expression levels of several angiogenic factors (El-Ftesi et al. 2009; Efimenko et al. 2011; Arthur et al. 1998b). In fact, it has been shown that adipose tissue angiogenic function is impaired in elderly subjects, suggesting that this decline may impair the use of adipose tissue as a source of autologous endothelial progenitor cells, potentially useful for cardiovascular repair in elderly patients (Madonna et al. 2011). Other studies carried out on rodents have shown that aged mice adipose tissue stem cells displayed decreased

Table 3 Analysis of correlation between the expression of angiogenic markers in TAT and age of cardiomyopathy ischemic patients

	Age (<i>r</i>)
CD31	0.446**
VEGF-A	0.469**
VEGF-C	0.339*
VEGF-D	0.397*
VEGF-R1	0.504**
VEGF-R2	0.463**
VEGF-R3	0.372*

Correlations were determined by Spearman's correlation coefficient test

* $p < 0.05$; ** $p < 0.01$

Table 4 Partial correlations between the expression of angiogenic markers in TAT, from cardiomyopathy ischemic patients, and age controlling for glucose, triglycerides, BMI, and cholesterol

Confounders covariates	Glucose	Triglycerides	BMI	Cholesterol
	Age (<i>r</i>)	Age (<i>r</i>)	Age (<i>r</i>)	Age (<i>r</i>)
CD31	0.647*	0.672**	0.448**	0.639*
VEGF-R1	0.727***	0.588**	0.529***	0.607*
VEGF-R2	0.678***	0.526**	0.482**	0.522**
VEGF-A	0.698**	0.529*	0.512**	0.525**
VEGF-C	0.574*	0.578*	0.395*	0.650*

* $p < 0.05$; ** $p < 0.01$; *** $p \leq 0.005$

angiogenic properties compared to adipose tissue stem cells from younger mice (Efimenko et al. 2011).

We recently showed that the VEGF isoform most expressed in TAT is VEGF-A and VEGF-B compared to other isoforms of these angiogenic factors (VEGF-C, VEGF-D, etc.) (Tinahones et al. 2009). These angiogenic factors are key regulators of angiogenesis and vasculogenesis (Oosthuysen et al. 2001; Carmeliet et al. 1996). In the present study, we observed that TAT, despite having lower expression levels in middle-aged subjects compared to SAT of VEGF-A and VEGF-B, these two angiogenic factors increased significantly in TAT from elderly subjects while in SAT this factor showed a clear and significant decrease. Moreover, whereas in SAT, VEGF-C, and VEGF-D expression levels did not show any difference with aging, TAT showed a clear increase in

elderly subjects compared to the middle-aged. Both isoforms are known to play a pivotal role in the rise of lymphangiogenesis (Karkkainen et al. 2004; Stacker et al. 2001). Besides, when analyzing endothelial marker gene expression, similar profiles, such as angiogenic factors, were observed showing a noticeable and significant increase of these parameters in TAT from elderly subjects while in SAT these levels were drastically or highly decreased. Additionally, most of the angiogenic and endothelial marker gene expression levels showed positive and significant correlation with age in TAT. All together, these data visibly indicate that in elderly subjects, TAT thymus fat is a promising and attractive source of angiogenic activity compared to subcutaneous adipose tissue.

In reality, age involution of the adult human thymus would consist of the replacement of its immunogenic

Table 5 Correlation analysis of angiogenic and adipogenic marker gene expression in cardiomyopathy ischemic TAT

(<i>r</i>)	VEGF-R1	VEGF-R2	VEGF-R3	PPAR γ 2	ADRP	FABP4	CD31
VEGF-A	0.955**	0.979**		0.456**	0.653**	0.744**	0.393*
VEGF-B				0.392*	0.306*	0.395*	
VEGF-C		0.320*		0.385*	0.389*	0.364*	0.371*
VEGF-D	0.461**		0.522**	0.338*	0.574***	0.565**	
VEGF-R1				0.386*			0.459**
VEGF-R2				0.417*		0.282*	
VEGF-R3							0.419*
PPAR γ 2	0.386*	0.417*					
FABP4							0.327*
ADRP							0.380*

Correlations were determined by Spearman's correlation coefficient test

ADRP adipose differentiation-related protein, FABP-4 fatty acid-binding protein 4, PPAR γ 2 peroxisome proliferator-activated receptor gamma2, VEGF vascular endothelial growth factor, VEGF-R vascular endothelial growth factor receptor

* $P < 0.05$; ** $P < 0.01$; *** $P \leq 0.005$

composition by adipose tissue. This fact is obviously appreciated by the increase of adipose thymus mass throughout life. This consideration could be corroborated here by the analysis of adipogenic factors which showed a significant increase of gene and protein expression of these parameters in TAT from elderly subjects, while in SAT, these markers showed a significant decrease remaining at lower levels than those detected in elderly TAT. Moreover, the fact that angiogenic factors showed significant strong positive correlations with adipogenic markers, leads us to believe that the increase of angiogenic properties could be due to the increase of adipose mass throughout life, as it is well-known that angiogenesis is linked to adipogenesis (Cao 2007).

Finally, one could wonder why thymus adipose tissue should be of interest as an angiogenic source, in view of easy access to other body adipose tissues such as subcutaneous. As we mentioned above, cardiovascular disease and age increase impairs angiogenic properties of adipose tissues, including subcutaneous and visceral, which make them insufficient candidates for angiogenic therapies and for neovascularization of ischemic tissues (El-Ftesi et al. 2009). Thymus fat could be an attractive and promising candidate for these therapies. To note that the largest sources of thymus adipose tissue are ischemic patients, which in the great majority are usually over the age of 60. Part of this tissue used to be discarded in aortic cannulation procedures of the ascending aorta in cardiovascular surgery in patients in need of a CPB (Rathore et al. 2009; Pacifico et al. 1986). Taking into account all data obtained in this study, we believe that thymus fat is slowly becoming of interest as the most appropriate source of angiogenic factors and probably endothelial progenitor cells, which leads us to consider motivating the study for its potential use in the regeneration if new vessels, especially in cardiomyopathy ischemic patients. These considerations may seem speculative, but we have faith that characterizing thymus adipose tissue-derived stromal cell population and endothelial progenitor cells in this adipose tissue, besides the ability of these endothelial cells to improve neovascularization, would clarify all these concerns and would establish whether this fat could have a relevant role in regenerative medicine.

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