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RESEARCH ARTICLE

Metalloproteinase 2 and 9 Activity Increase in Epicardial Adipose Tissue of Patients with Coronary Artery Disease

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Abstract: ***Background:*** Epicardial adipose tissue (EAT) is a visceral adipose tissue (AT) surrounding and infiltrating myocardium and coronary arteries. Increased EAT may represent a chronic inflammatory injury and a link with coronary artery disease (CAD). Metalloproteinases (MMPs) are involved in expansion of AT.

Objective: To evaluate MMP-2 and -9 behaviour in EAT from CAD patients.

Methods: In EAT and subcutaneous AT (SAT) from patients undergoing coronary artery bypass graft (CABG, n=26) or valve replacement (No CABG, n=18), MMP-2 and -9 activity and localization, inflammatory cells and vascular endothelial growth factor (VEGF) levels were determined.

Results: In EAT from CABG, MMP-2 and -9 activity was increased compared with No CABG (p=0.041 and p=0.027, respectively) and compared with SAT (p=0.005 and p=0.048, respectively). In CABG patients EAT showed higher infiltration of macrophages and T lymphocytes than SAT (p=0.01 and p=0.002, respectively). In No CABG patients no sign of cellular retention was observed in EAT or SAT. Vascular density was higher in EAT from CABG than No CABG (p=0.015) and it was directly correlated with MMP-2 (p=0.006) and MMP-9 (p=0.02). VEGF levels in EAT were directly associated with MMP-2 (p=0.016).

Conclusion: In EAT from CABG patients the increase of MMP-2 and -9 activity and the presence of inflammatory cells would be partially responsible for ECM remodeling and major vascular density necessary for EAT expansion. Improved knowledge of EAT behaviour may allow identify new therapeutic targets for the treatment of CAD.

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INTRODUCTION

Obesity and inflammation have been related to cardiovascular disease (CVD) and growing evidence also focused attention on the association between visceral adipose tissue and increased risk of CVD [1]. More recently, attention has been directed to epicardial adipose tissue (EAT), a metabolically active visceral adipose tissue surrounding and infiltrating the myocardium and coronary arteries. Due to the close anatomical proximity to the heart and the absence of fascial boundaries, EAT may interact locally with the myocardium and coronary arteries through paracrine secretion of pro-inflammatory and pro-atherogenic adipokines [2,3]. Hence an excessive amount of EAT may represent a chronic inflammatory injury for the cardiovascular tissue and it could also play an active role in the relationship between adiposity, inflammation and CVD, mainly coronary artery disease (CAD) [4].

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EAT can be measured by imaging techniques. Several studies have shown that EAT thickness is significantly and independently related to the presence and severity of CAD, and presence of coronary calcification, independently of obesity [5-7]. Expansion of adipose tissue is associated with extensive modifications involving adipogenesis, angiogenesis and extracellular matrix (ECM) remodeling. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases able to degrade ECM components [8]. MMPs play an important role during physiological tissue remodeling in embryonic development [9] and angiogenesis [10], as well as in pathophysiological conditions such as atherosclerotic plaque development and vulnerability [11] and adipose tissue expansion [12-14]. MMPs are involved in two important events during fat mass development, the control of proteolysis and adipogenesis [13]. Therefore, different studies have shown that fibrosis of adipose tissue, defined as an excessive accumulation of ECM components, are also tightly regulated by MMPs [15].

In reference to the direct effect of EAT on atherosclerotic plaque, it has been hypothesized that adipocytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-6, IL-8 and monocyte chemotactic protein (MCP)-1 from EAT promote atheromatous plaque formation by passing into the myocardium via the vasa vasorum [16-17]. The expansion of EAT in CAD, as a result of the ECM degradation, could be a consequence of increased MMPs activity. Adipocytes also secrete vascular endothelial growth factor (VEGF). The main function of VEGF is angiogenesis, being recognized as the most important factor increasing blood capillaries in the adipose tissue by stimulating endothelial cell growth [18]. In the literature, there are no reports about the association between VEGF and MMPs in human EAT from CAD patients.

To our knowledge, only one study reported that EAT from CAD patients secretes more MMPs than SAT, leading to myocardial fibrosis [19]; however MMP activity and localization have not been evaluated in EAT and there is no evidence about MMPs behaviour in CAD compared with No CAD patients.

Our hypothesis is that in EAT from CAD patients inflammatory infiltrate and MMPs activity increase promoting ECM remodeling and major vascular density necessary for EAT expansion. Therefore, our aim was to evaluate for the first time MMP-2 and MMP-9 activity and localization and VEGF levels in EAT obtained from patients with CAD in comparison with No CAD patients and the relationship of these parameters with histological EAT characteristics.

MATERIALS AND METHODS

Subjects

We included 44 patients of both sexes, undergoing coronary artery bypass graft (CABG, n = 26) or valve replacement (No CABG, n = 18). The patients attended the Cardiac Surgery Division of Hospital de Clinicas José de San Martín, University of Buenos Aires. Clinical data was obtained upon admission to hospital before surgery. Diagnosis of CAD was based on previous coronary angiograms. Reductions in luminal coronary artery diameters > 70% were considered significant. No CABG patients were randomly selected among patients who did not undergo CABG intervention. These patients had no clinical signs of CAD and showed normal coronary arteries on angiography. Most of the patients were on treatment with statins, aspirin, β-blockers, ACE inhibitors or angiotensin receptor blockers (ARBs).

The weight and height of each participant were measured and body mass index (BMI) was calculated to evaluate obesity; blood pressure was recorded in all cases.

The following exclusion criteria were considered for both groups: previous heart surgery, concomitant infective diseases, alcohol intake >20 g/day, recent history of acute illness, diabetes, hypothyroidism, renal failure, liver disease, and any other condition that may interfere with inflammatory markers, such as Chagas disease and Human Immunodeficiency Virus patients.

Written informed consent was required from all the participants before inclusion in the study. The study was performed in accordance to the ethical guidelines of the Declaration of Helsinki of the World Medical Association for medical studies in humans. The study was approved by the Ethical Review Committee of the Faculty of Pharmacy and Biochemistry, University of Buenos Aires and by the Ethical Review Committee of the Hospital de Clínicas José de San Martín.

Blood Collection

After 10-12 h overnight fast, before cardiovascular surgery, peripheral venous blood samples were drawn. Serum was kept at 4°C within 48 h for the evaluation of glucose, lipids and lipoproteins, or stored at −70°C for further determination of insulin.

Adipose Tissue Biopsies

EAT (~ 0.1-1.0 g wet weight) and subcutaneous adipose tissue (SAT, ~ 2.0 g wet weight) samples were obtained before starting extracorporeal circulation. EAT biopsies were collected from the area near the proximal tract of the right coronary artery and SAT samples were harvested from the thorax. Tissue samples were aliquoted and immediately frozen at -70ºC until analysis; one sample was fixed in 4% formalin buffer, pH 7.0 and conserved at 4º C for histological evaluation.

Measurements

Total cholesterol, triglycerides (TG), and fasting glucose were measured using commercial enzymatic kits (Roche Diagnostics, Mannheim, Germany) and a Cobas C-501 autoanalyzer; intra-assay coefficient of variation (CV) <1.9%, inter-assay CV <2.4%. High density and low-density lipoprotein cholesterol (HDL-C and LDL-C) were determined by homogeneous colorimetric method; intra-assay CV <2.0 % and inter-assay CV <2.5%, respectively and Non HDL-cholesterol (Non HDL-C) was calculated as total cholesterol minus HDL-C. Serum apolipoproteins A-I (apoA-I) and B-100 (apoB-100) were determined by immunoturbidimetry (Roche Diagnostics, Mannheim, Germany); intra-assay CV <1.9%, and inter-assay CV <2.5% for both parameters. Insulin was measured with Immulite/Immulite 1000 Insulin (Siemens, USA); intra-assay CV <2.6%, and

inter-assay CV <3.9%. To estimate insulin-resistance (IR), the homeostasis model assessment for insulin resistance (HOMA-IR) index and the TG/HDL-C index were calculated.

Gelatinolytic Zymography

MMP-2 and MMP-9 activity from EAT and SAT was measured by gelatinolytic zymography as previously described [20-21]. Briefly, adipose tissue was homogenized in 50 ~~Mm~~ Tris buffer, pH 7.4,containing 5 mM $CaCl_2$, 1 µM $ZnCl_2$ and 1% Triton X-100. Fifteen µg of protein was applied to non-reduced sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis copolymerized with gelatin 0.1% (G-8150, Sigma), substrate of MMP-2 and MMP-9. Gels were run in a Mini Protean-3 (~~Bio-Rad Laboratories,~~ Bio-Rad Laboratories, Hercules, California, USA) and incubated for 18 h in 0.15M NaCl, 10 mM $CaCl_2$, TrisHCl pH: 7.4 at 37°C. After staining with Coomassie blue R-250 (B-0149, Sigma, Saint Louis, USA) and destained with acetic acid-methanol-water (1:3:6), enzyme activity was demonstrated by the absence of staining in areas where the gelatin has been degraded. MMP-2 (67 kDa, active form) and MMP-9 (84 kDa, active form) were identified by molecular weight prestained standards (Bio-Rad Laboratories, Hercules, California, USA). Conditioned media from the promyelocyte U-937 cell lines was used as activity standard. The intra-assay CV was < 4.8% and inter-assay CV < 8.6%. Because of the complexity of this assay, the CV is considered to be quite satisfactory. Band intensities were quantified using Sion-Image J software (Scion Corporation), and relative activity was expressed as a ratio to the internal standard.

Histological Evaluation

The histological examination by light microscopy was performed in a blinded manner. Fixed EAT and SAT samples were dehydrated in ethanol, embedded in paraffin wax, and cut with a microtome Reichert (Austria). The resulting 5µ sections were stained with haematoxylin and eosin reagent and periodic acid-Schiff stain for the determination of size and density of adipocytes and vascular density, and Picrosirius red, a specific technique for collagen fibres [22]. Vascular density was determined by quantifying total blood vessels in 20 fields at 400 x, and expressed in number of vessels/mm^2. Blood vessel density was normalized to the adipocyte number. The quantification was performed in at high power field using a computerized image analyser (ImageProPlus, Media Cybernetics Corp, Maryland, USA).

Immunohistochemistry

To localize MMP-2 and MMP-9 and evaluate inflammatory cell infiltration, immunohistochemistry was performed. EAT and SAT sections were processed simultaneously under identical conditions. Streptavidin-biotin-peroxidase technique was applied. Slides were incubated in a humidified chamber with primary monoclonal antibodies against MMP-2 (dilution 1/50, clone 17B11,Novocastra, Leica microsystems), MMP-9 (dilution 1/50, clone 2C3, Vector Laboratories), CD3 (dilution 1/250, clone MRQ-39, Cell Marque, USA) or CD 20 (dilution 1/250, clone L 26, Dako, USA) for lymphocytes and CD 68 (dilution 1/100, clone Kp-1, Cell Marque, USA) for macrophages. The reaction was revealed with diaminobenzidine and contrasted with Harris´s haematoxylin. Negative controls were performed by omitting the primary antibody. Positive cells (reddish brown) were considered positive and counted in 40X high-power fields in each section and expressed as number of CD68-positive or CD3/20 cells/mm^2. The number of inflammatory cells was normalized to the adipocyte number.

Western Blotting

EAT and SAT were homogenized in 20 mMTris buffer, pH 7.4, containing 150 mM NaCl, 1% Triton X-100 and 2% protease inhibitor cocktail (Sigma Aldrich, Saint Louis, USA). Tissue homogenates were centrifuged and protein concentrations were determined by Lowry's method in the supernatant. Thirty mg of protein were separated in 12% SDS-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membrane. Blots were blocked with 5% skim milk for 1 h and incubated overnight at 4°C with a polyclonal rabbit IgG antibody anti-VEGF (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) or anti-β-actin (Sigma Aldrich, Saint Louis, USA). After washing with Tris buffer saline and Tween 0.1%, the blots were incubated with HRP conjugated secondary antibody (Bio-Rad, USA) for 1h at room temperature. The specific signals were visualized using the ECL Western Blotting Analysis System (ThermoScientific, Pierce, USA) enhanced chemiluminescence system. VEGF band at the expected size of 42 kDa was identified by the use of pre-stained molecular weight standards (Thermo Scientific, Pierce, USA), which was absent in the negative control experiments performed in the absence of primary antibody. The relative intensity of protein signal was quantified by densitometric analysis using Fluorchem program (Alpha Innotech Corp, California, USA). Results are expressed as VEGF protein/actin protein ratio.

Statistical Analysis

Data are presented as mean ± SD or median (range) according to normal or skewed distribution, respectively. Data distribution was tested by the Kolmogorov and Smirnov test. Differences between CABG and No CABG group were tested using the unpaired Student t test, χ^2 test or the Mann-Whitney U test, according to the data distribution. Statistical comparisons between EAT and SAT from the same group were tested by paired Student t test. Previously, each variable was examined for normal distribution, and abnormally distributed variables were log transformed. To verify the difference of MMPs activity between groups, we performed an analysis of covariance (ANCOVA), controlling for necessary confounders such as age. Only complete sets (i.e. pairs) of EAT and SAT results are reported. Pearson or Spearman analysis, for parametric or non-parametric variables, was used to determine correlations between parameters. The SPSS 19.0 software package (Chicago, IL) was used for statistical analysis. A two-tailed $p<0.05$ was considered significant.

RESULTS

The general characteristics of CABG and No CABG patients are shown in Table **1**. No significant differences in any

Table 1. General characteristics of patients with and without coronary artery bypass graft (CABG).

	CABG (*n*=26)	**No CABG (*n*=18)**	***P*=**
Age (years)	71 ± 9	70 ± 8	0.757
Gender (W/M)	11/15	8/10	0.459
BMI (Kg/m^2)	27.1 ± 2.8	25.8 ± 5.9	0.340
Systolic blood pressure (mmHg)	128 ±15	131 ± 18	0.665
Diastolic blood pressure (mmHg)	75.0 ± 11	69 ± 20	0.293
Risk factors (%)			
Hypertension	53.8	44.5	0.063
Dyslipidemia	11.5	16.7	0.714
Ex-smokers	30.8	38.9	0.890
Medications (%)			
Statins	38.5	44.4	0.867
ACE inhibitors/ARBs	44	46	0.369
β-blockers	19.2	11.5	0.652
Aspirin	70.0	7.1	0.001
TG (mmol/l)	1.8 ± 0.8	1.4 ± 0.9	0.543
Total-C (mmol/l)	3.9(2.6-9.3)	4.4(3.1-5.7)	0.370
LDL-C (mmol/l)	2.4(1.3-7.1)	2.8(1.9-3.9)	0.409
HDL-C (mmol/l)	1.0(0.3-1.4)	1.1(0.7-1.5)	0.481
No HDL-C (mmol/l)	3.0(1.7-8.2)	3.2(2.4-4.7)	0.509
Total-C /HDL-C	4.2(2.6-13.4)	4.1(2.6-7.3)	0.968
apoA-I (g/l)	1.1(0.8-1.6)	1.2(0.5-1.7)	0.655
apoB-100 (g/l)	0.7(0.5-1.1)	0.8(0.7-0.9)	0.604
Glucose (mmol/l)	5.3 (4.2-11.1)	5.7 (4.8-8.4)	0.113
Insulin (pmol/l)	39.5 ± 25.2	56.9 ± 10.4	0.229
HOMA-IR	1.8 ± 1.1	2.1 ± 0.5	0.664
TG/ HDL-C	4.0 ± 2.9	3.5 ± 3.3	0.796

Data are expressed as mean ± SD (Student-t test), median (range) (Mann-Whitney U test) or % (χ2 test) according to the data distribution. CABG indicates coronary artery bypass graft; W, women; M, men BMI, body mass index; ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; TG, triglycerides; Total-C, total cholesterol; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; No HDL-C, no high density lipoprotein cholesterol; apoA-I, apolipoprotein A-I; apoB-100, apolipoprotein B-100; HOMA-IR, homeostasis model assessment for insulin resistance index.

parameter were observed between CABG and No CABG patients, likely due to statin therapy in the CABG group. CABG patients had more aspirin use compared with the No CABG patients (p=0.001).

Gelatinases activity was significantly increased in EAT from CABG compared with No CABG patients (MMP-2: 1.65 ± 0.54 *vs* 1.31 ± 0.26 relative units (RU), p=0.049, MMP 9: 1.53±0.54 *vs* 1.18±0.22 p=0.024) (Fig. **1a** and **1b**). These differences remained significant after adjusting by age (F= 4.82, p=0.040 and 7.48, p=0.015). No differences in gelatinases activity were observed in SAT between groups. In the CABG group, there were no significant difference in MMP-2 and MMP-9 activities between patients on or without treatment with aspirin (p=0.525 and p=0.844, respectively).

Moreover, in EAT from CABG patients, we observed an increase in MMP-2 (1.65 ± 0.54 *vs* 1.25 ± 0.36 RU, p= 0.008) and MMP-9 (1.53 ± 0.54 *vs* 1.19± 0.27 RU, p= 0.049) activities compared with SAT (Fig. **1**). In No CABG group there were no differences in MMP-2 and MMP-9 activity between EAT and SAT.

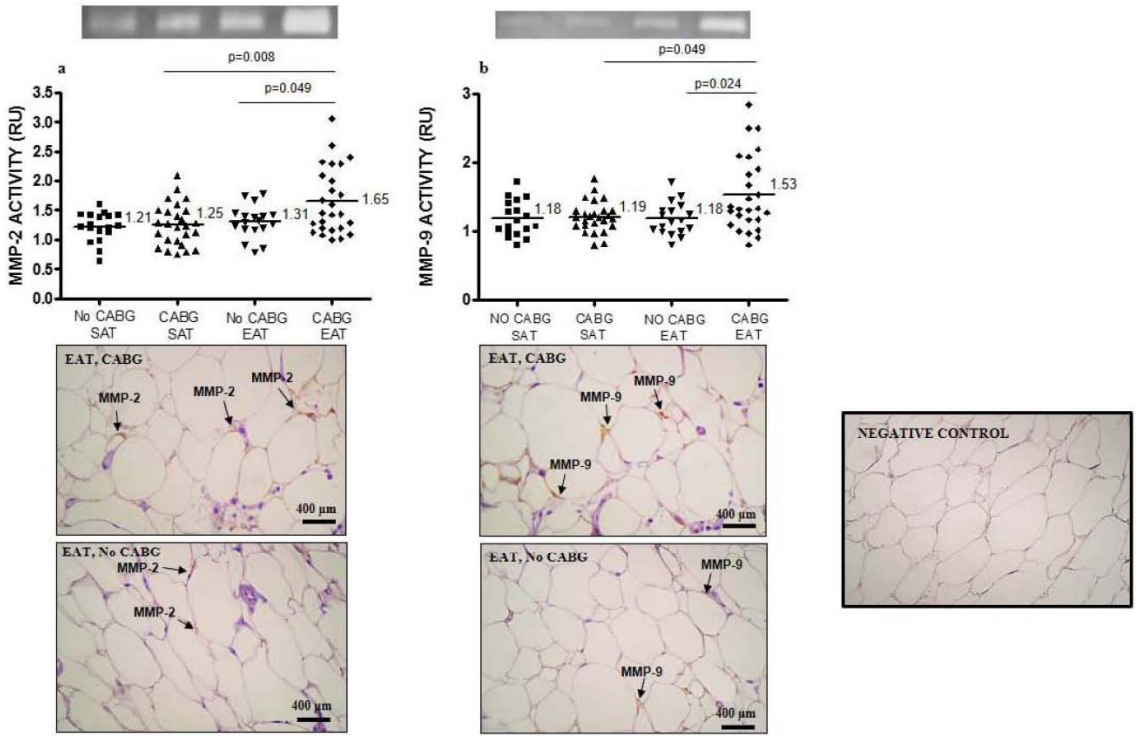

Fig. (1). MMP-2 (**a**) and MMP-9 (**b**) activity in epicardial (EAT) and subcutaneous (SAT) adipose tissue from patients with and without coronary artery bypass graft (CABG and No CABG). RU, relative units. Mean values are shown, Student t-test. Representative immunohistochemistry for MMP-2 and MMP-9 in EAT of CABG and No CABG by Immunoperoxidase technique.

In reference to MMPs localization, in EAT from CABG and No CABG, MMP-2 and -9 were mainly localized in perivascular connective stroma and in the basement membrane surrounding adipocytes (Fig. **1**). In SAT, similar results were observed. Adipocyte size, and adipocyte and vascular density of EAT and SAT from CABG and No CABG group ~~is~~ illustrated in Fig. **2**.

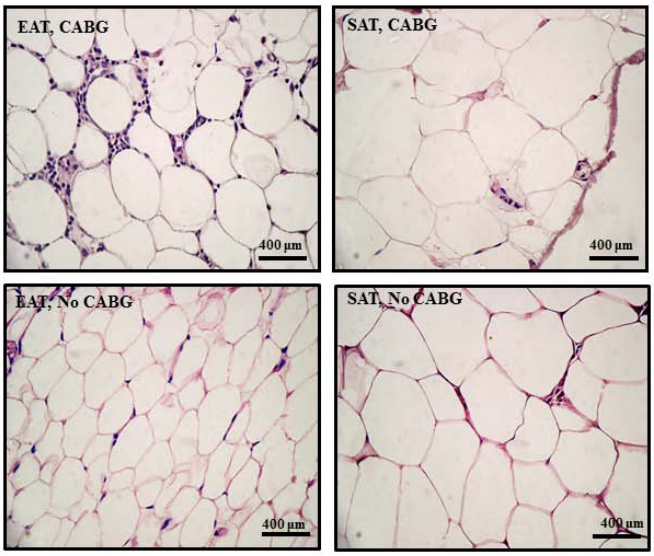

Fig. (2). Histological characteristics (adipocyte size and adipocyte and vascular density) of epicardial (EAT) and subcutaneous (SAT) adipose tissue patients with coronary artery bypass graft (CABG) and without coronary artery bypass graft (No CABG).

As seen in Table **2**, no differences in number and size of adipocytes were observed in EAT between groups. Regarding SAT, similar results were obtained. However, EAT presented higher number and smaller adipocytes than SAT in CABG (p=0.03 and p=0.043, respectively) and No CABG group (p=0.018 and p=0.008, respectively) (Table **2**).

Vascular density was significantly higher in EAT from CABG compared with No CABG (p=0.015) without differences in SAT between groups (Table **2**). This difference remained significant after normalizing for the adipocyte number (p=0.05). Moreover, in the CABG group, vascular density was significantly higher in EAT than in the SAT group (p=0.014) (Table **2**).

In EAT, vascular density directly correlated with MMP-2 and MMP-9 activity (r=0.690, p=0.008 and r=0.634, p=0.02, respectively) and it was inversely associated with adipocyte size (r=-0.631, p=0.016). Furthermore, vascular density, both in EAT and SAT, was inversely associated with insulin levels (r=-0.774, p=0.024 and r=-0.896, p=0.016, respectively).

In reference to fibrous tissue, there were no evidence of fibrosis in EAT of any group (Fig. **3**).

Only in CABG patients EAT showed higher dense inflammatory cell infiltrates than SAT. When specific inflammatory cell markers were used, the presence of T lymphocytes (CD 20/ CD3+) and macrophages (CD68+) was demonstrated (Fig. **4**). The magnitude of CD68+ and CD3/20+ cell infiltration into EAT was significantly higher than SAT from CABG patients (86±40 *vs* 69±6 CD68+ cells/mm^2, respectively, p=0.01; 30±18 *vs* 17±9 CD3/20+ cells/mm^2 respectively, p=0.002). However this difference did not remain significant after normalizing for the number of adipocytes.

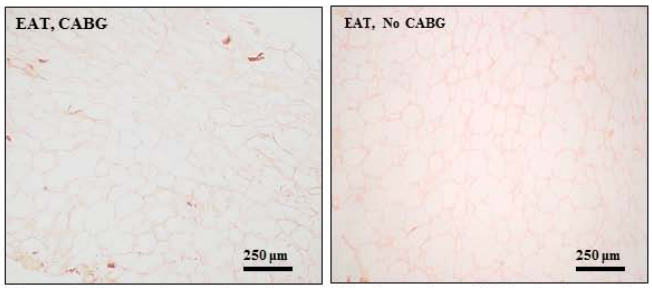

Fig. (3). Fibrosis characteristics in epicardial adipose tissue (EAT) of patients with coronary artery bypass graft (CABG) and without coronary artery bypass graft (No CABG) by Picrosirius red technique.

In No CABG patients no sign of inflammatory cell infiltration was observed in EAT and SAT.

VEGF expression did not show differences between CABG and No CABG group in EAT (1.17 (0.33-5.0) *vs* 2.48 (0.39-6.16) RU, p=0.714 and SAT (1.82 (0.34-5.23) *vs* 1.69 (0.73-7.33) RU, p=0.722, respectively), neither between EAT nor SAT from the same patient (Fig. **5**). In the whole population, VEGF levels in EAT were directly associated with MMP-2 activity (r=0.7119, p=0.014). No associations between VEGF levels and vascular density were found.

DISCUSSION

This study, to our knowledge, is the first to evaluate gelatinases activity in EAT from patients with and without CABG. Our main finding was that MMP-2 and MMP-9 activities were increased in EAT from patients with CABG, related with an augmented vascular density. MMP-2 and -9 were located mainly in perivascular connective stroma and in the basement membrane surrounding adipocytes.

Visceral adipose tissue expansion is directly related to cardiovascular risk more than subcutaneous adiposity [23]. A significant amount of evidence supports the association of EAT with the early stages of atherosclerosis and plaque formation in patients with CAD [24-28]. Moreover, it has been proposed that the expansion of EAT depots could be an independent causal factor for CAD [27] given the anatomic proximity and absence of a dividing facial plane with myo-

Table 2. Histological characteristics of EAT and SAT of patients with and without coronary artery bypass graft (CABG)

	CABG		No CABG	
	EAT	**SAT**	**EAT**	**SAT**
Adipocyte density (number/mm^2)	218 ± 47 $^{\alpha}$	161 ± 31	241 ± 53$^{\gamma}$	176 ± 47
Adipocyte size (x10^{3}µm^2)	4.37 ± 1.21$^{\beta}$	7.36 ± 2.74	4.32 ± 0.81$^{\delta}$	6.66 ± 1.84
Vascular density (blood vessels /mm^2)	51 ± 13*	37 ± 12	40 ± 8	38 ± 4
Normalized Vascular density	0.23 ± 0.05**	0.22 ± 0.05	0.17 ± 0.04	0.22 ± 0.04

Data are expressed as mean ± SD. Student t-test. CABG indicates coronary artery bypass graft; EAT, epicardial adipose tissue; SAT, subcutaneous adipose tissue. $^{\alpha}$p=0.03 and $^{\beta}$p=0.043 vs SAT CABG; $^{\gamma}$p=0.018 and $^{\delta}$p=0.008 vs SAT No CABG; *p=0.015 and **p=0.05vs EAT No CABG.

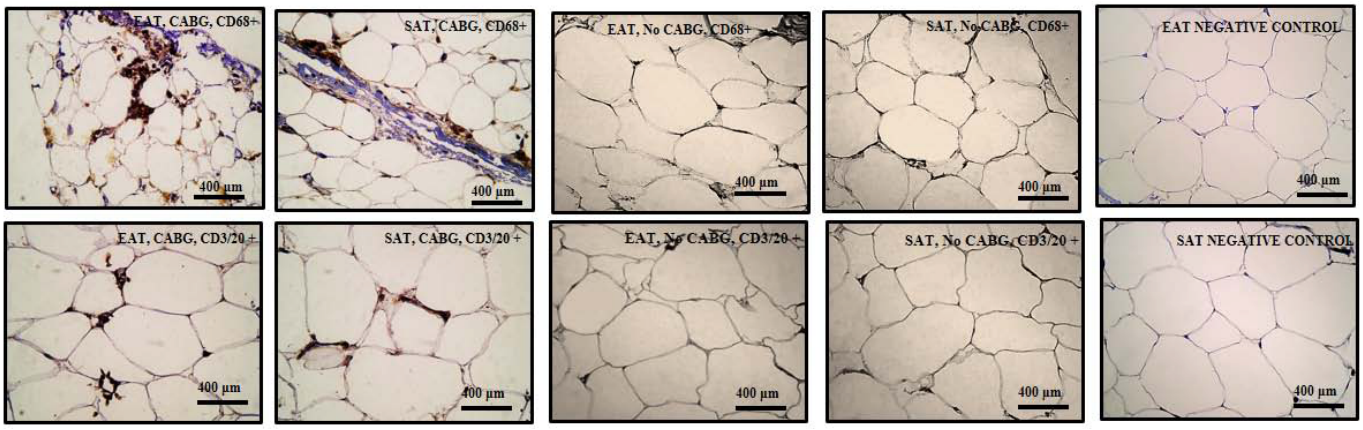

Fig. (4). Representative Immunohistochemistry for macrophages (CD68+) and T lymphocytes (CD 3/ CD20) in epicardial (EAT) and subcutaneous (SAT) adipose tissue from coronary artery bypass graft (CABG) and without coronary artery bypass graft (No CABG) patients. Brown staining corresponds to positive immunohistochemistry.

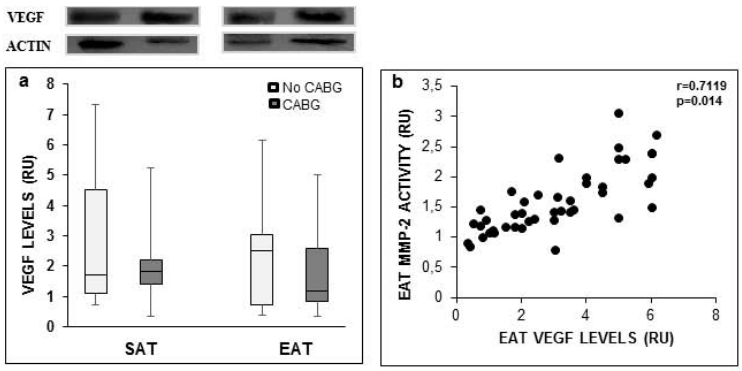

Fig. (5). a) Vascular endothelial growth factor (VEGF) in epicardial (EAT) and subcutaneous (SAT) adipose tissue from patients with and without coronary artery bypass graft (CABG and No CABG). RU, relative units. Median values are shown, Mann Whitney U-test. **b**) Association between MMP-2 activity and VEGF levels in whole population. Spearman test.

cardium [29]. Hyperplasia of fat tissue would require a flexible ECM; in this process, MMPs are involved in the control of proteolysis and adipogenesis [13]. The behaviour of MMPs in different visceral adipose tissue has been studied in cell cultures [29], animal models [21,14,30] and humans [31], and contradictory results have been reported. As MMPs are involved in the expansion of adipose tissue as well as in the atherosclerotic plaque development and vulnerability, we evaluated MMP-2 and -9 activity and localization in EAT from CABG patients and its relationship with the histological characteristics of this tissue. We observed that both gelatinase activities were increased in EAT from CABG and the increase in both activities was directly associated with the vascular density. These findings support the recent study that reported that EAT from CABG patients secretes MMPs, such as MMP-1, -8 and -9, more abundantly than SAT [19].

MMPs have been extensively studied in the pathogenesis of the atherosclerosis process and CVD because of their major significance in vascular remodeling. It is important to note that there are segments of the coronary arteries that are free from atherosclerosis and that this may be attributed to the lack of adipose tissue in such areas [32].

We observed that there were no differences in the number and size of adipocytes in EAT and SAT between CABG and No CABG patients; however in both groups EAT presented higher number and smaller adipocytes than SAT. It has been reported that blood vessels seen in EAT come from the vasa vasorum in the adventitia of coronary arteries [29]. When analysing vascular density, EAT from CABG patients presented a significant increased number of blood vessels/mm^{2}compared with SAT, as well as compared with EAT from the No CABG group. Given the direct association observed between vascular density and gelatinases activity in EAT, it could be suggested that the augmented vascularity would be, in part, consequence of increased in MMP-2 and -9 activity. It should be kept in mind that vascular density may be affected by the number and/or size of adipocytes as well as by the angiogenic activity [33]. In reference to fibrosis, in our study there were no signs of ECM accumulation in EAT and SAT from both groups, probably due to the effect of MMP activity on the ECM. In accordance with our results, histopathological studies from EAT in patients with CABG revealed dense inflammatory infiltrates [2,34]. In the CABG group, we observed that EAT presented dense inflammatory cell infiltrates, predominantly represented by macrophages. The presence of inflammatory cells in EAT could also reflect the response to plaque rupture and lead to amplification of vascular inflammation and plaque instability [35]. It has been reported that the increase in protein levels of inflammatory cytokines is associated with dense inflammatory infiltrates of macrophages, T cells and mast cells in EAT [3]. In EAT from CABG patients we observed the presence of T lymphocytes and macrophages but in No CABG patients no sign of cellular infiltration was observed. In obesity, adipose tissue inflammation and hypertrophy are two closely linked processes that aid in the recruitment of T-cells and macrophages contributing to IR [36]. In our study, although both groups presented a similar IR profile, ~~BMI and waist circumference,~~ EAT from CABG patients presented higher inflammatory profile.

It has been shown that both, adipocytes and macrophages, produce~~s~~ inflammatory cytokines and pro-angiogenic factors responsible of neovascularization [36]. Although previous studies reported lack of association between EAT volume and VEGF circulating levels [37], recently McKenney *et al*. demonstrated that EAT from pigs on a high fat diet presented increased gene expression of VEGF among other inflammatory and pro-angiogenic factors [39]. In our study we found a direct association between MMP-2 activity and VEGF levels. Previous studies have reported that MMPs can promote the expression of VEGF either *in vitro* or within tumours, enhancing angiogenesis in human EAT [39]. Regarding the lack of association between VEGF levels and vascular density reported in this study, other grow factors like platelet-derived growth factor, responsible of capillary maturation, should be studied [36]. This study demonstrates for the first time that EAT from CABG patients presents higher gelatinases activity than EAT from No CABG patients. This finding reveals a potential role of MMPs in EAT expansion, in an inflammatory environment, stressing the risk factor characteristics of EAT for CAD.

It has been reported that statins decrease MMP levels [40]. Atorvastatin therapy induced a reduction of computed tomography measured epicardial fat volume in hyperlipidaemic post-menopausal women [41]. Although data on epicardial fat are limited, lifestyle measures and cardiovascular drugs may improve EAT behaviour and decrease cardiovascular risk [42].

In our study, given that patients of both groups were on statin treatment, it was not possible to elucidate the impact of these drugs on MMPs behaviour in EAT. Further studies will be necessary to evaluate the effect of therapeutic treatments on MMPs and the reduction of EAT volume. We consider the present study as a basis for such further experiments to elucidate the influence of EAT on myocardium.

One limitation of this study is that specific tissue inhibitors of MMPs (TIMPs) were not evaluated. It must be taken into account that changes in TIMPs levels could be responsible for variations in MMPs activity. Another limitation is the sample size, however, it is important to highlight the difficulty to obtained human EAT. Regarding that the increase in EAT volume is a risk factor for CVD, the evaluation of EAT through image techniques would improve the study of these patients. Moreover, given that the SAT can be separated into a deep and superficial layer by a *fascia superficialis* with some different metabolic characteristics between them [43] and considering that the SAT samples evaluated in this study were from the thoracic area, it would be interesting to compare EAT with other SAT depots in future studies.

In summary, this study demonstrates for the first time that EAT from CABG patients presents higher gelatinases activity than EAT from No CABG patients. In EAT from CABG patients the increase of MMP-2 and -9 activity and the presence of inflammatory cells would be partially responsible of ECM remodeling and major vascular density necessary for EAT expansion. Improved knowledge of EAT behaviour may allow to identify new therapeutic targets for the treatment of CAD.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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