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Epicardial adipocyte hypertrophy: Association with M1-polarization and toll-like receptor pathways in coronary artery disease patients

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KEYWORDS
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Abstract *Background and aims:* In coronary artery disease (CAD) epicardial adipose tissue (EAT) shows an elevated inflammatory infiltrate. Toll-like receptors (TLRs) are important mediators of adipose tissue inflammation and they are able to recognize endogenous products released by damaged cells. Because adipocyte death may be driven by hypertrophy, our aim was to investigate in CAD and non-CAD patients the association between EAT adipocyte size, macrophage infiltration/polarization and TLR-2 and TLR-4 expression.

Methods and results: EAT biopsies were collected from CAD and non-CAD patients. The adipocyte size was determined by morphometric analysis. Microarray technology was used for gene expression analysis; macrophage phenotype and TLRs expression were analyzed by immunofluorescence and immunohistochemical techniques. Inflammatory mediator levels were determined by immunoassays.

EAT adipocytes were larger in CAD than non-CAD patients and do not express perilipin A, a marker of lipid droplet integrity. In CAD, EAT is more infiltrated by CD68-positive cells which are polarized toward an M1 state (CD11c positive) and presents an increased pro-inflammatory profile. Both TLR-2 and TLR-4 expression is higher in EAT from CAD and observed on all the CD68-positive cells.

Conclusions: Our findings suggested that EAT hypertrophy in CAD promotes adipocyte degeneration and drives local inflammation through increased infiltration of macrophages which are mainly polarized towards an M1 state and express both TLR-2 and TLR-4.

Introduction

Coronary artery disease (CAD) is a clinical condition characterized by stenosis of coronary vessels due to atherosclerotic plaque formation. Recently, CAD onset and progression has been related to epicardial adipose tissue (EAT), a visceral fat depot surrounding the heart and sharing with myocardium the same microcirculation [1].

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With increasing thickness, EAT is able to locally produce reactive oxygen species, cytokines and chemokines which may create a local toxic and pro-inflammatory environment [2–5]. In particular, visceral obese patients display an increased EAT thickness and the tissue is mostly infiltrated by macrophages which may amplify the local pro-inflammatory response by producing itself additional mediators [6,7].

Immunohistochemical identification of macrophages is usually performed with antibody against CD68 antigen. These cells may be further characterized according to their polarization state as pro-inflammatory CD11c-positive macrophages, known as M1 [8,9], and anti-inflammatory CD206-positive macrophages, known as M2 [10]. The polarization process depends on different local stimuli [11,12].

Recently, the presence of stressed or dying adipocytes, releasing different cellular components, like residual lipid droplets, has been suggested as an additional stimulus inducing macrophage infiltration and M1 polarization, probably through the activation of toll-like receptors (TLRs) which play a primary role in the innate immune response [13,14]. Although TLRs are mainly known for their ability to recognize different pathogen-associated molecular patterns, recently it has been shown that they can also recognize endogenous molecules, such as heat shock proteins or cell debris [15], called damage-associated molecular patterns (DAMPs) [16].

In the field of CAD, recent studies suggested that macrophage EAT infiltration is also one of the main causes of the chronic inflammatory state associated with the disease [17,18]. Moreover, it has been reported that TLR-2 and TLR-4 are the main TLR isoforms involved in atherosclerosis and their expression is up-regulated in circulating monocytes [19–21].

Currently, less is known about the mechanisms promoting macrophage polarization in EAT in CAD patients. In the present study our aim was to investigate in CAD and non-CAD patients the association between EAT adipocyte size, macrophage infiltration/polarization and TLR-2 and TLR-4 expression.

Methods

Study population

Fifty male patients (30 CAD, undergoing coronary artery bypass grafting surgery, and 20 non-CAD, referring to hospital for valvular replacement surgery), aged 18–65 years, were enrolled at I.R.C.C.S. Policlinico San Donato. Exclusion criteria were: impaired left ventricular ejection fraction, congestive heart failure, acute myocardial infarction (<6 month), presence of pace-maker, type-1 diabetes mellitus, neoplasm, prior major abdominal surgery, renal/liver diseases and unstable (>5% change) body weight in the least 6 months. CAD and non-CAD patients had similar body mass index (BMI, kg/m^2) (26.52 ± 2.59 *vs.* 27.11 ± 4.17) and AST (U/L) (31.36 ± 31.12 *vs.* 30.44 ± 42.50). ALT (U/L) (38.30 ± 31.42 *vs.* 17.33 ± 7.39), LDL-cholesterol (mg/dL) (85.00 ± 33.54 *vs.* 110.82 ± 37.05) and triglycerides (mg/dL) (130.10 ± 64.80 *vs.* 139.80 ± 71.27) were increased in CAD patients ($p < 0.05$). HDL cholesterol was reduced (36.51 ± 10.41 *vs.* 48.00 ± 11.13; $p < 0.05$). The study protocol was approved by the local Ethics Committee (ASL Milano Due, protocol number 2516) and patients gave their written informed consent, conducted in accordance with the Declaration of Helsinki, as revised in 2013.

EAT and blood collection

EAT biopsy samples were harvested adjacent to the proximal right coronary artery prior to initiation of cardiopulmonary bypass pumping. For gene expression analysis, samples were stored in Allprotect Tissue Reagent (Qiagen, Hilden, Germany). For histological assays, tissues were fixed in 4% paraformaldehyde. EDTA plasma samples were collected and stored at −20 °C until analysis.

Adipocyte size quantification

Three mm^3 EAT biopsies were fixed in 4% paraformaldehyde for 16 h at 4 °C, dehydrated in graded scale of ethanols and paraffin embedded. Sections of 4 μm were obtained using rotary microtome (RM2245, Leica Microsystems GmbH, Wetzlar, Germany) and stained with hematoxylin-eosin (Sigma–Aldrich, Milan, Italy). The adipocyte size was evaluated on 10× images acquired using Nikon Eclipse 80i microscope equipped with digital camera Nikon DS-5Mc (Nikon, Tokyo, Japan) and image acquisition software (ACT-2U). Diameter and area of adipocytes were measured using image processing software (Image Pro Plus version 4.5.019; USA Media Cybernetics Inc; Maryland, USA).

Immunohistochemical staining

Deparaffinized EAT sections were rehydrated and antigen retrieval was performed by autoclaving in sodium citrate buffer (0.01 M, pH 6) for 5 min at 120 °C. After quenching of endogenous peroxidases (0.3% H_2O_2 for 20 min), and blocking with swine serum (Dako Cytomation), sections were incubated with anti-human primary antibodies:mouse monoclonal CD68 (1:100, 1 h) (Biocare Medical, Concord, CA), mouse monoclonal CD11c (1:200, over night, (on)) (Proteintech, Manchester, UK), mouse monoclonal CD206 (1:20, on) (R&D Systems, Minneapolis, MN, USA), rabbit polyclonal PLIN1 (1:200, on) (LifeSpan Bioscience, Albuquerque, NM, USA), polyclonal rabbit TLR-2 (1:400, Bio-Rad, Milan, Italy) and polyclonal rabbit TLR-4 (1:20,0 LifeSpan BioSciences). Amplification of immune signal was performed using anti-mouse and anti-rabbit HRP-polymer complex (MACH 1 Universal HRP-Polymer detection, Biocare Medical). Betazoid DAB (Biocare

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Medical) was used for color development. Sections were counterstained with Mayer's hematoxylin and mounted with Mowiol 4–88 (Calbiochem, La Jolla, CA, USA). Negative controls were performed by replacing primary antibodies with PBS. Immunohistochemical reactions were observed and acquired with Nikon Eclipse 80i microscope.

For the semi-quantitative evaluation of IHC staining of CD68, CD11c, CD206, TLR-2 and TLR-4, slides were reviewed and scored by a double-blind analysis performed by two independent scientists. We adopted the German semi-quantitative scoring system, slightly modified for our purpose, in considering the staining intensity and area extent, which has been largely accepted and used in previous studies [22]. The intensity of the staining was quantified using the following scores: 0 = negative, 1 = weakly positive, 2 = moderately positive, 3 = strongly positive. The extent of the staining was quantified by evaluating the percentage of the positive staining areas in relation to the whole areas of the section, where a score of 0 was given for 0–1% reactivity, 1 point was assigned for 1–10% reactivity, 2 points were assigned for 11–25% reactivity, 3 points were given for 26–50% reactivity, 4 points were given for 51–80% reactivity, and samples with >80% reactivity were assigned a total of 5 points. Since macrophages are localized in the spaces between adipocytes, we could just evaluate a maximum of 3 points for the extent of the staining. The final immunoreactive score was determined by multiplying the intensity score by the extent score with the minimum score attainable being 0 and a maximum score of 9. The 9-tier scoring was also simplified by combining scores 8–9: strong expression (+++), 6–7: intermediate expression (++), 2–5: weak expression (+), and 0–1: negative NRIP expression (−).

Double-immunofluorescence staining

Deparaffinized and rehydrated sections were blocked with swine serum (Dako Cytomation, Milan, Italy) for 30 min at room temperature (RT) and incubated on with polyclonal rabbit TLR-4 (1:200) or with polyclonal rabbit TLR-2 antibodies (1:400) and then with secondary TRITC-conjugated donkey anti-rabbit IgG (R&D Systems) for 1 h at RT. Sections were secondly incubated with monoclonal mouse CD68 (1:100, 2 h), and then with FITC-conjugated donkey anti-mouse IgG (1:200, R&D Systems, 1 h). After 5 min of DAPI incubation (AbDSerotec, Puchheim, Germany), sections were mounted with Mowiol 4-88. Negative controls were performed by replacing primary antibodies with PBS. Images were acquired with fluorescence microscope (Nikon Eclipse 80i).

RNA extraction and gene expression analysis of EAT

Total RNA was extracted from tissue with the RNeasy Lipid Tissue Kit (Qiagen). RNA concentration was quantified by NanoDrop 2000 (ThermoScientific, Wilmington, Germany) and RNA integrity was assessed using the Agilent RNA 6000 Nano kit and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Gene expression analysis was performed by one color microarray platform (Agilent). Hybridization was performed using Agilent Gene Expression hybridization Kit and scanning with Agilent G2565CA Microarray Scanner System. Data were processed using Agilent Feature Extraction Software (10.7) with the single color gene expression protocol and raw data were analyzed with ChipInspector Software (Genomatix, Munich, Germany).

Statistical analysis

Data were expressed as mean ± standard deviation (SD) and analyzed by GraphPad Prism 5.0 program (GraphPad Software, Inc., San Diego, CA). The normality of data distribution was assessed by the Kolmogrov–Smirnoff test. Comparison between groups was performed using Student's two-tailed unpaired *T*-test or Mann–Whitney *U*-test, as appropriate. A p value < 0.05 was considered statistically significant.

Results

EAT adipocytes are hypertrophic in CAD

Morphometric analysis of EAT adipocytes suggested that CAD patients (Fig. 1a) have bigger adipocytes than non-CAD (Fig. 1c). In CAD, EAT adipocytes displayed both longer diameter (Fig. 1b) and larger area (Fig. 1d) than non-CAD (diameter: 81.92 ± 17.19 μm *vs.* 61.81 ± 5.83 μm; area: 257.10 ± 56.45 μm^2 *vs.* 191.40 ± 18.82 μm^2; $p < 0.05$ for both).

Perilipin A immunoreactivity is absent in EAT from CAD

Perilipin A, a lipid droplet protein involved in lipid trafficking, has been used as a marker of degenerative lipid droplets and cell death. In CAD, no perilipin A immunoreactivity was observed in EAT (Fig. 2a). Contrarily, a positive immunoreactivity was observed in non-CAD (Fig. 2b). Perilipin A mRNA was also reduced in CAD (fold change: −1.53, $p < 0.01$) (Fig. 2c).

EAT infiltrating macrophages are M1-polarized in CAD

Immunohistochemical analysis revealed that CD68-positive cells were prevalent in CAD and appear as aggregates. Only scattered CD68-stained cells were present in non-CAD (Fig. 3a and b).

Regarding macrophage polarization, CD11c-positive cells were mostly observed in CAD (Fig. 3c and d), whereas few CD206-positive macrophages appeared mainly in non-CAD (Fig. 3e and f). Semi-quantitative analysis performed with German scoring system confirmed that, in CAD, macrophages are shifted toward

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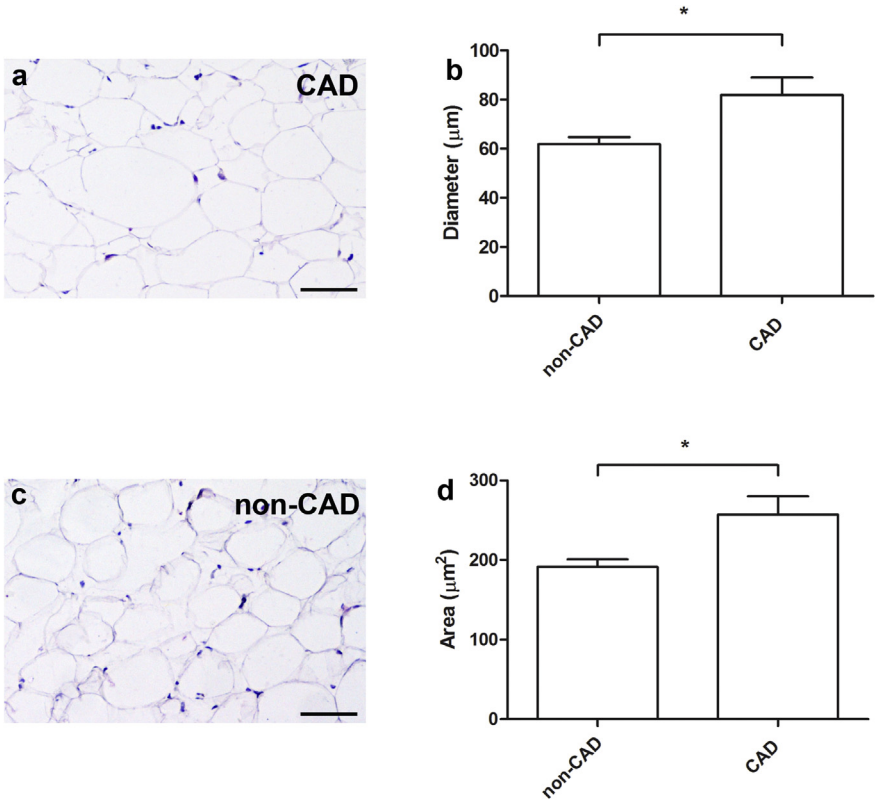

Figure 1 Morphometric analysis of adipocyte sizes in CAD and non-CAD EAT samples. Hematoxylin-eosin staining of CAD (a) and non-CAD (c) EAT sections. Bars: 60 μm. Quantification of adipocyte diameter (b) and area (d) in CAD and non-CAD patients. Shown are mean values ± SD. $^{*}p < 0.05$.

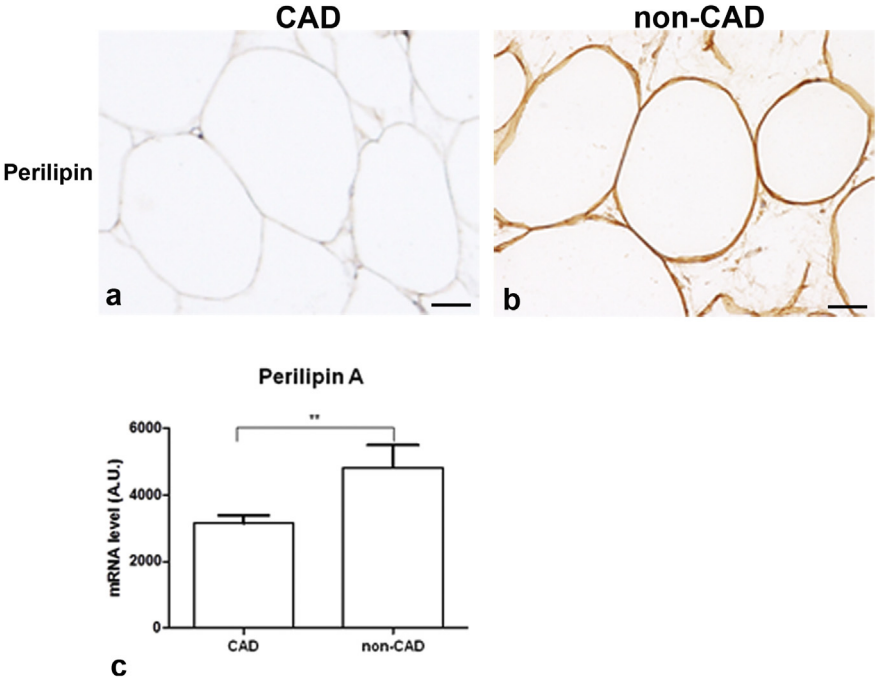

Figure 2 Perilipin A expression in EAT. Perilipin A immunoreactivity is absent in CAD (a), whereas it is present in non-CAD (b). Bars: 10 μm. Reduced mRNA level of perilipin A was observed in CAD (c). $^{**}p < 0.01$.

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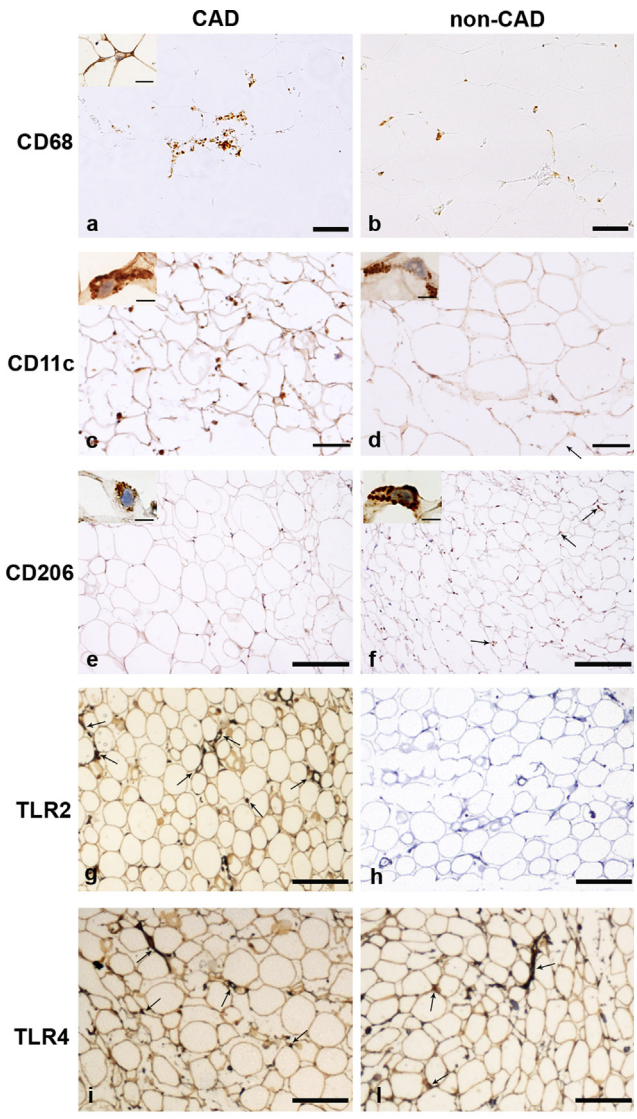

	CAD			non-CAD		
	intensity	extent	Final score	intensity	extent	Final score
CD68	3	3	**9** +++	3	2	**6** ++
CD11c	3	3	**9** +++	3	1	**3** +
CD206	3	1	**3** +	3	2	**6** ++
TLR2	3	3	**9** +++	0	0	**0** -
TLR4	3	3	**9** +++	2	1	**2** +

Figure 3 Immunohistochemical and semi-quantitative analysis of macrophage phenotype and TLR-2 and TLR-4 expression in EAT. Panels a and b show CD68-positive cells, c and d CD11c-positive cells, e and f CD206 positive cells. C, d, e and f inserts are enlargements of macrophage staining. Panels g and h show TLR-2 positive cells; i and l show TLR-4 positive cells. Bars: a–e, 30 μm; f–l, 60 μm; inserts: a, 10 μm; c, d, e and f, 5 μm (a). The table shows the semi-quantitative analysis performed by German scoring of antigen presented in the figures. CD68, CD11c, TLR-2 and TLR-4 were most expressed in CAD (9-tier scoring for all antigens) than non-CAD patients. Contrarily, CD206 was lower in CAD than non-CAD (3-tier *vs.* 6-tier).

an M1 pro-inflammatory state (Fig. 3). At gene level we did not find any statistically significant difference in CD68, CD11c and CD206 levels between CAD and non-CAD (data not shown).

EAT infiltrating macrophages in CAD are immunoreactive for both TLR-2 and TLR-4

IHC analysis indicated an increased immunoreactivity for both TLR-2 and -4 in CAD (Fig. 3g–l) in stromal region. Due to their role in promoting inflammatory pathways in response to DAMPs, we then evaluated whether EAT infiltrating CD68-positive macrophages, usually localized in these stromal regions, were immunoreactive for TLR-2 and TRL-4. In CAD, we observed that all CD68-positive cells were also immunoreactive for both TLR-2 (Fig. 4a–c) and -4 (Fig. 4g–i). In non-CAD patients, TLR-2 was almost undetectable (Fig. 4d–f), whereas TLR-4 was faintly expressed (Fig. 4l–n).

CAD patients displayed increased level of pro-inflammatory mediators in EAT

The pro-inflammatory mediators MCP-1, TNF-α, PTX3, TLR-2 and -4, which are involved in the innate immunity response, as well as the anti-inflammatory cytokine adiponectin were evaluated in EAT. CAD patients displayed about 2-fold increase in MCP-1 and TNF-α levels ($p < 0.05$), a 1.6-fold increase in TLR-2 ($p < 0.01$) and 1.3 fold increase in TLR-4 ($p < 0.05$). A 4-fold increase in PTX3 level has also been observed ($p < 0.05$). Contrarily, adiponectin level was 1.7-fold decreased in CAD ($p < 0.001$) (Fig. 5).

Discussion

The novelty of our study is the observation that the increased inflammatory state observed in EAT in CAD seems to be induced by hypertrophic and damaged adipocytes and TLR-2 and TLR-4 up-regulation may represent one potential molecular link between adipocyte death and the activation of the immune response.

Previous studies suggested that the increased macrophage infiltration in obese visceral as well as subcutaneous adipose tissue may be promoted by stressed and/or dying adipocytes which release different cellular components, such as residual lipid droplets. Moreover, it has been found an association between macrophage infiltration and increased adipocyte dimension [13]. To our knowledge, our study is the first one exploring such relationship in EAT in CAD. We observed that in CAD, EAT adipocytes are hypertrophic compared to non-CAD and the loss of perilipin A immunoreactivity should suggest the lipid droplet degeneration. Perilipin A is a lipid-droplet associated protein involved in the regulation of adipocyte lipolysis [23]. Previous data indicated that the deletion of perilipin A results in leanness and reverses obesity in db/db mouse [24]. According to these data and our morphometric results, we expected to observe an increased perilipin A expression in CAD patients, as a

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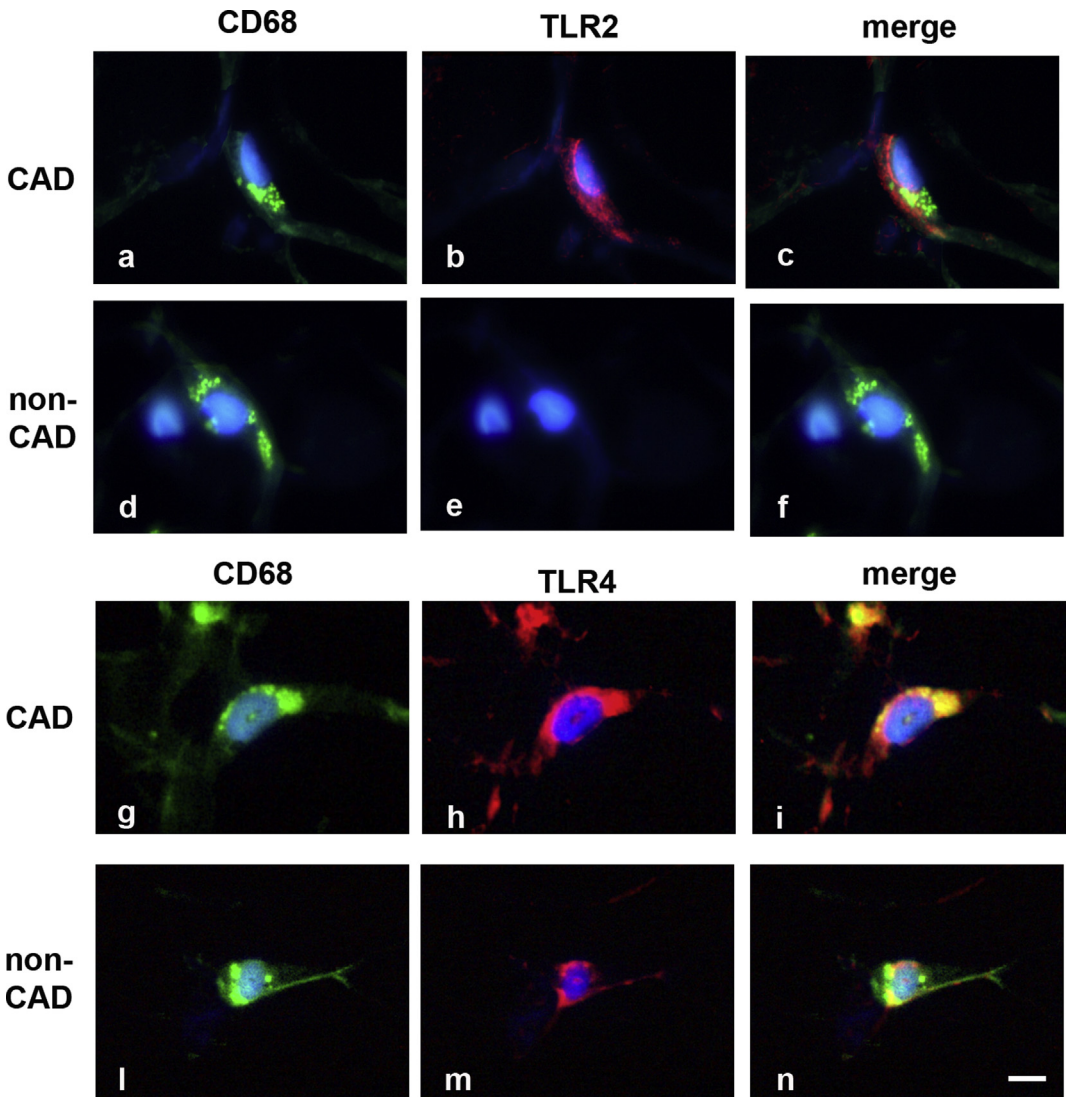

Figure 4 Double immunofluorescence staining of CD68-positive cells with anti-TLR-2 and -4 antibodies. Panels a, d, g and l show CD68 staining. Panels b and e represent TLR-2 and panels h and m TLR-4 staining. Panels c, f, i and n represent double staining showing the colocalization of CD68 with TLRs. Bar: 10 μm.

potential mechanism promoting adipocyte hypertrophy. On the contrary, we observed an overall reduction in its level. In addition to its role in the regulation of lipolysis, the protein has also been acknowledged as a marker of lipid droplet integrity and its down expression/depletion is a sign of lipid droplet degeneration [13]. One potential explanation of EAT adipocyte death in CAD may be their increased size. In fact, it is known that adipocyte hypertrophy associated to deregulated cellular metabolism might promote adipocyte death [7,25]. Moreover, the hypertrophic state observed may be a peculiarity of CAD pathology regardless of the anthropometric characteristics of the patients. In fact, our study showed that EAT adipocytes resulted bigger in CAD than non-CAD despite the two groups were matched for BMI. Thus, the yet known inflammatory state previously described in EAT in CAD could be explained by our observation of a link between EAT hypertrophy and adipocyte death and the presence of macrophages polarizated toward a pro-inflammatory M1 state.

The novelty of our study is also the observation that macrophages infiltrating EAT express both TLR-2 and -4 which are the main players in the innate immune response. In fact, the activation of these receptors by endogenous products released by perilipin A-negative adipocytes may lead to NF-κB translocation [26] and up-regulation of pro-inflammatory mediators [26,27].

Since EAT and pericoronary fat amount have been related to the presence and extent of coronary artery plaques, coronary artery calcification and production of inflammatory mediators [28–30], future correlations between EAT adipocyte size, CAD severity and inflammation represent an interesting point to be addressed in future studies.

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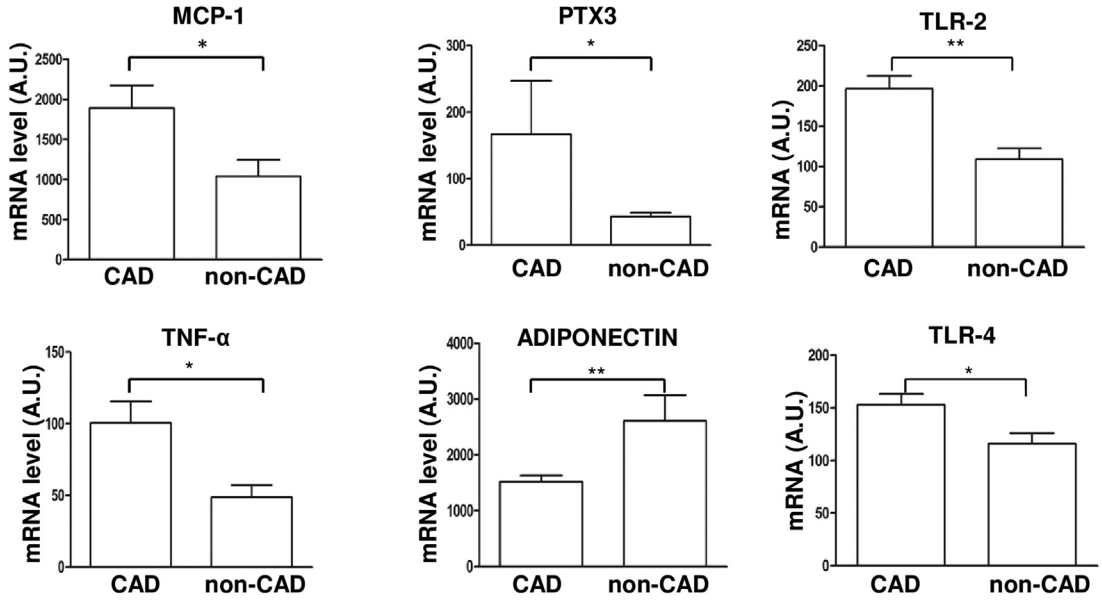

Figure 5 EAT expression of inflammation-related molecules in CAD and non-CAD. Gene expression analysis of mediators involved in inflammation in EAT was performed with Agilent system. Shown are mean values ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

In conclusion, our findings suggested that EAT hypertrophy in CAD promotes adipocyte degeneration and drives local inflammation through increased infiltration of macrophages which are mainly polarized towards an M1 state and express both TLR-2 and TLR-4.

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Appendix A. Supplementary material

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.numecd.2015.12.005.

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