The density of Crown-Like Structures in epicardial adipose tissue could play a role in cardiovascular diseases

Alexis Elias Malavazos
Istituto Policlinico San Donato: IRCCS Policlinico San Donato
Angelica Di Vincenzo
Università Politecnica delle Marche: Universita Politecnica delle Marche
Gianluca Iacobellis
University of Miami Department of Medicine
Sara Basilico
IRCCS San Donato Hospital: IRCCS Policlinico San Donato
Carola Dubini
IRCCS San Donato Hospital: IRCCS Policlinico San Donato
Lelio Morricone
IRCCS San Donato Hospital: IRCCS Policlinico San Donato
Lorenzo Menicanti
IRCCS San Donato Hospital: IRCCS Policlinico San Donato
Tonia Luca
University of Catania: Universita degli Studi di Catania
Antonio Giordano
Università Politecnica delle Marche: Universita Politecnica delle Marche
Sergio Castorina
University of Catania: Universita degli Studi di Catania
Michele Carruba
Università degli Studi di Milano: Universita degli Studi di Milano
Enzo Nisoli
Università degli Studi di Milano: Universita degli Studi di Milano
Stefano Del Prato
Università di Pisa: Universita degli Studi di Pisa
Saverio Cinti (saverio.cinti@icloud.com)
Polytechnic University of Marche: Universita Politecnica delle Marche https://orcid.org/0000-0003-0362-5017

Research Article

Keywords: epicardial adipose tissue, crown-like structures, inflammation, cardiovascular diseases, open-heart surgery.

Posted Date: April 7th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1520579/v1

Abstract

Purpose The visceral fat of patients affected by abdominal obesity is inflamed, and the main histopathologic feature is the high density of crown-like structures (CLS). Epicardial adipose tissue (EAT) is a visceral fat of paramount importance for its relationships with coronary vessels and myocardium. Its inflammation in patients with abdominal obesity could be of clinical relevance, but histopathological studies on CLS density in EAT are lacking. This study aimed to assess the histopathology of EAT biopsies obtained from patients undergoing open-heart surgery. Methods We collected EAT biopsies from 10 patients undergoing open-heart surgery for elective coronary artery bypass grafting (CABG) (n=5) or valvular replacement (VR) (n=5). Biopsies were treated for light microscopy and immunohistochemistry. We quantify the CLS density in each EAT sample. Results Despite all patients having abdominal obesity, in EAT samples, no CLS were detected in the VR group; in contrast, CLS were detected in the CABG group (about 17 CLS/104 adipocytes vs. 0,0 CLS/104 adipocytes, CABG vs. VR group, respectively). An impressive density of CLS (100 times that of other patients) was found in one patient (LS) in the CABG group that had a relevant anamnestic aspect: relatively rapid increase of weight gain, especially in abdominal adipose tissue, coincident with myocardial infarction. Conclusion CLS density could be an important predictive tool for cardiovascular diseases. Furthermore, the LS case implies a role for timing in weight gain.

Level of evidence: No level of evidence; this is a basic science study.

What Is Already Known On This Subject?

The visceral fat of patients affected by abdominal obesity is inflamed, and the main histopathologic feature is the crown-like structures (CLS) density. Epicardial adipose tissue (EAT) is a visceral fat of paramount importance for its relationships with coronary vessels and myocardium. Its inflammation in patients with abdominal obesity could be of clinical relevance, but histopathology studies focusing on CLS density in EAT are lacking.

What does this study add?

This report is the first study that describes and quantifies the CLS density in human EAT samples. CLS density, found only in patients with abdominal obesity and CAD, could be an important predictive tool for cardiovascular diseases.

Introduction

Excess of visceral fat is characterized by chronic low-grade inflammation [1]. This inflammation can cause metabolic consequences, such as insulin resistance and type 2 diabetes mellitus, and local consequences, including cancers [1]. The cause of inflammation seems to be linked to adipocytes death, probably due to their pathologic hypertrophy engaging mechanisms ending with death for pyroptosis at a critical size [2]. Dead adipocytes are surrounded by scavenger macrophages reabsorbing their debris and

forming the classic crown-like structures (CLS) [3]. Visceral fat in mice shows a critical death size lower than that of subcutaneous adipocytes, suggesting that the excess of visceral fat has worse metabolic consequences than subcutaneous fat [4]. Epicardial adipose tissue (EAT) is a visceral fat with a peculiar anatomical contiguity with the myocardium. A positive correlation between EAT thickness and cardiac diseases has been shown [5,6].

This fat depot's morphological and molecular characteristics are different from those of other visceral depots, and adipocytes have been described as intermediate between white and brown [7]. Although inflammation of EAT can be suspected by molecular approaches [8] and different imaging techniques [6], histopathologic studies focusing on CLS density are scarce [9]. Thus we studied the histopathology of ten EAT biopsies from patients undergoing open-heart surgery.

Methods

We analysed EAT from 10 with obesity or overweight abdominal obesity undergoing open-heart surgery: five patients for elective coronary artery bypass grafting (CABG) and five for valve replacement (VR). VR patients did not show any sign of coronary artery disease (CAD) in the pre-operative coronary angiographic examination. EAT biopsy samples were harvested adjacent to the proximal right coronary artery prior of cardiopulmonary bypass pumping and immediately fixed in paraformaldehyde 4% in phosphate saline buffer (PBS) at pH 7.4. After overnight fixation, tissue was dehydrated in ethanol, cleared in xylene, and embedded in paraffin.

All samples were stained with H&E and immunostained with antibody anti-CD68: to reveal the presence of macrophages widespread in adipose parenchyma and/or organized to form CLS (3). In brief, 3 μm paraffin tissue sections were obtained for each sample, and immunohistochemical staining was performed. Sections were rehydrated, reacted with 3% H_2O_2 (in dH_2O for 5 min), rinsed with PBS, and incubated with 2% blocking solution (in PBS for 20 min). Then they were incubated overnight at 4° C with the primary CD68 antibody (Dako #M0814; 1:200; antigen retrieval method by citrate buffer pH6). After some rinsing in PBS, they were incubated with the biotinylated secondary antibody (in PBS for 30 min), rinsed in PBS, and incubated in Vectastain ABC kit (Vector Laboratories) for 60 min, washed several times in PBS and lastly incubated with 3,3'-diaminobenzidine tetrahydrochloride (0.05% in 0.05 M Tris with 0.03% H_2O_2; 5 min) as the substrate. Sections were finally counterstained with haematoxylin, dehydrated, and mounted in Eukitt (Merck)., Negative control was included in each reaction by omitting the primary antibody, to assess the specificity of the antibody. All observations were performed using Nikon Eclipse E800 light microscope.

The size of adipocytes (area) was measured in all subjects. Five fields from each stained slide were captured at 10x magnification with a Nikon DXM 1220 camera. One hundred adipose cells for each paraffin tissue slide were counted using the morphometric program ImageJ (RRID:SCR_003070).

The density of CLS identified for their specific aspect (defined as a large lipid droplet mimicking an adipocyte (3) surrounded by CD68+ cells for at least 50% of its circumference) was calculated as previously described [10]. In brief, for each patient CLS density per 10^4 adipocytes was determined using ImageJ.

Data are presented as mean value ± standard error (SEM); the Student's t-test was used to compare CLS density of patients for VR and those for CABG. Additionally, one-way ANOVA compared the CLS density between the CABG patient (SL) alone and the other two groups. We used the GraphPad Prism 6.0 software and considered significant a $p < 0.05$.

We used Fisher's exact test for categorical variables and the t-test or Wilcoxon rank-sum test to compare unpaired means in normally or non-normally continuous distributed variables for clinical data comparisons.

Results And Discussion

The main demographic, anthropometric, clinical, and biochemical characteristics of the 10 patients (5 CABG and 5 VR) are reported in Table S1 (Supplementary Information). The mean age was 65.7±12.7 years. Even if CABG patients were older than VR patients (71.6±6.8 years vs. 59.8±14.3 years, respectively), no statistical difference was seen. The majority of patients were males (n=9).

No significant difference in the measurements of weight, height, body mass index (BMI), and waist circumferences was observed between CABG and VR patients.

According to BMI, an indicator of general fatness, eight patients were affected by obesity and two by overweight; while adopting waist circumference as an indicator of abdominal fat distribution, all patients were affected by abdominal obesity.

No difference was observed in smoking status, and only one patient in the CABG group was affected by type 2 diabetes mellitus. The percentages of antiplatelet, statins, angiotensinogen-converting enzyme inhibitors/angiotensin receptor blockers and oral glucose-lowering drugs were similar in the two groups; no difference was observed in echocardiographic EAT thickness between the two groups. Adipocytes have a dynamic endocrine role by expressing and secreting many factors, including hormones and cytokines, that depend on cell size [6]. Several studies have recently paid attention to EAT because its specific anatomical location. In this way, regional differences in adipocyte hypertrophy and inflammatory function might suggest a different metabolic response in patients with cardiovascular disease. The size of adipocytes in EAT is significantly smaller than in other adipose tissues [6].

In line with data from other studies (6), in our study, the size of adipocytes of both groups was around 3000 μm^2 (2940 ± 188 vs. 3260 ± 299 VR vs. CABG group respectively, p= 0.39 ns). Thus, the size was about one-half found in visceral fat (omentum) of patients with obesity of a different case series but measured with the same methods [10].

Inflamed visceral adipose tissue is marked by the presence of CLSs, where scavengers' macrophages surround dead adipocytes [3, 4]. Although all patients had abdominal (visceral) obesity, in EAT samples, no CLS were detected in the VR group who did not show any sign of CAD in the pre-operative coronary angiographic examination; in contrast, CLS were detected in 3 patients of the CABG group (about 17 CLS/10^4 adipocytes vs. 0.0 CLS/10^4 adipocytes, CABG vs. VR group respectively), although no significant differences in adipocyte size were seen in the two groups (Figure 1). Interestingly, the density of CLS was about three times higher than that found in visceral fat (omentum) of patients with obesity in the above-cited study [10]. One of the CABG patients (SL) had an extraordinary density of CLS (Figure 2); therefore, to avoid a false picture of the degree of inflammation in the CABG group, his data were not included. CLS density in SL was about 100 times the average value observed in other CABG patients. We observed such a density of CLS only in visceral fat of mice with severe obesity [11 and unpublished data], but never in humans [10]. Of note, the clinical data of LS differed from all other cases mainly for one important clinical aspect: he had a recent episode of myocardial infarction and a quite rapid (few years) weight gain of about 13 kg, almost entirely in the abdominal region, with an increase in abdominal circumference of 9 cm.

The specific unique nature of EAT with intermediate characteristics between white and brown adipocytes, also known as beige or brite adipose tissue, [6,7] could explain the higher inflammation found in EAT when compared with omental fat of another case series studied with the same methods [10]. In addition, the age of LS (78 years) must also be taken into account as, with ageing, epicardial adipocytes become more susceptible to environmental, metabolic, and haemodynamic factors, which gradually change the function of EAT from thermogenesis to energy storage [6].

Indeed, EAT brown fat-like activity decreases substantially with age. The changes are not only functional but also structural. The proportion of brown adipocytes decreases in favour of more unilocular white adipocytes in older individuals. This finding suggests that the transition from brown fat to beige fat is a feature of EAT in adults [6]. In line with these data, we recently showed that whitening of brown fat induces a high degree of inflammation with a high density of CLS in this depot (12)

The absence of CLS in the VR group could be explained by the small adipocytes size difference (about 10%), but mainly by the different ages of the two study groups, as the CABG group was more than ten years older than the VR group, who did not show any sign of CAD in the pre-operative coronary angiographic examination. Aging has been recently reported as an important factor worsening EAT chronic inflammation (13).

An alternative, plausible explanation of the higher presence of CLS in EAT from CABG than VR patients is the downregulation of the EAT housekeeping genes transcriptome that we observed and reported previously (14). The hypothesis might be that end/stage or advanced coronary artery disease (CAD) causes downregulation of EAT genes due to fibrotic and apoptotic changes or, better, to the mounting pyroptotic changes following the continuous and chronic inflammatory insult (2). These processes would lead to adipocytes' death and consequent CLS formation. Based on this observation, we suggest that the

EAT adipocytes are “burned-out” (pyroptosis) in advanced CAD. CAD more than obesity plays a role in the higher EAT inflammation and related morphological changes. This study clearly confirms this.

In line with previous data showing that EAT plays a role in the progression and development of CAD, inflammation is the main feature of EAT in patients with obesity and CAD, showing dense macrophage infiltrates [5,6,8], as observed among adipocytes in all our studied patients with abdominal obesity and CAD (not shown).

To the best of our knowledge, only one recent paper described CLS in EAT of patients with obesity, CAD and type 2 diabetes mellitus [9]. They did not quantify the CLS density in each patient but described only if CLS were present or not. Surprisingly, in their case series of EAT biopsies studied by immunohistochemistry (n=16 patients affected by obesity and n=28 patients without obesity), the CLS were found only in 14% of patients without obesity, but with CAD; interestingly the prevalent phenotype of macrophages was the pro-inflammatory M1 [9]. It would be interesting to know if the patients without obesity but with CLS in EAT, in that case series, had an excess of visceral fat. Data presented show only an average (including patients without CLS) waist circumference within normal values.

Conclusions

Although our data cannot be considered significant from a statistical point of view for obvious numerical reasons, we thought worthwhile their description in order to stimulate the study of EAT as an important cardiovascular risk factor and therapeutic target for drugs with cardiovascular benefits such as glucagon-like peptide 1 receptor (GLP1R) agonists and sodium-glucose co-transporter 2 (SGLT2) inhibitors. Of note, it is well known that these drugs induce EAT reduction. If confirmed in a larger number of patients, CLS density could be an important predictive tool for cardiovascular diseases. Furthermore, the LS case outlines the importance of timing in weight gain as recently described also by another study [15].

Strength and limits

This study is the first analysis of CLS density in the human EAT sample, as far as we know. Nonetheless, some limitation is evident. Firstly, our patients’ number was small and underpowered. Secondly, unfortunately we do not have subcutaneous fat from the substernal site as control tissue. However, our and other studies clearly showed the difference between EAT and subcutaneous adipose tissue (10, 14).

Declarations

Funding: Funds to SC and AG by Progetti di Rilevante Interesse Nazionale (PRIN 2017, #2017L8Z2).

This study was partially supported by Ricerca Corrente funding from Italian Ministry of Health to IRCCS Policlinico San Donato

Acknowledgments: We apologize to those authors whose work we did not cite in the text due to space considerations.

Conflict of interest. All authors declare that they have no conflicts of interest.

Ethics approval. The study protocol was approved by the local Ethics Committee (ASL Milano Due, n° 2516).

Informed consent. Patients gave their written informed consent to the examination protocol, conducted in accordance with the Declaration of Helsinki, as revised in 2000.

References

1. Rohm TV, Meier DT, Olefsky JM, Donath MY. Inflammation in obesity, diabetes, and related disorders. Immunity. 2022 Jan 11;55(1):31-55. doi: 10.1016/j.immuni.2021.12.013. PMID: 35021057; PMCID: PMC8773457.
2. Giordano A, Murano I, Mondini E, Perugini J, Smorlesi A, Severi I, Barazzoni R, Scherer PE, Cinti S. Obese adipocytes show ultrastructural features of stressed cells and die of pyroptosis. J Lipid Res. 2013 Sep;54(9):2423-36. doi: 10.1194/jlr.M038638. Epub 2013 Jul 8. PMID: 23836106; PMCID: PMC3735940.
3. Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS, Obin MS. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res. 2005 Nov;46(11):2347-55. doi: 10.1194/jlr.M500294-JLR200. Epub 2005 Sep 8. PMID: 16150820.
4. Murano I, Barbatelli G, Parisani V, Latini C, Muzzonigro G, Castellucci M, Cinti S. Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice. J Lipid Res. 2008 Jul;49(7):1562-8. doi: 10.1194/jlr.M800019-JLR200. Epub 2008 Apr 3. PMID: 18390487.
5. Malavazos AE, Ermetici F, Coman C, Corsi MM, Morricone L, Ambrosi B. Influence of epicardial adipose tissue and adipocytokine levels on cardiac abnormalities in visceral obesity. Int J Cardiol. 2007 Sep 14;121(1):132-4. doi: 10.1016/j.ijcard.2006.08.061. Epub 2006 Nov 13. PMID: 17107724.
6. Iacobellis, G. Epicardial adipose tissue in contemporary cardiology. Nat Rev Cardiol (2022). https://doi.org/10.1038/s41569-022-00679-9
7. Sacks HS, Fain JN, Bahouth SW, Ojha S, Frontini A, Budge H, Cinti S, Symonds ME. Adult epicardial fat exhibits beige features. J Clin Endocrinol Metab. 2013 Sep;98(9):E1448-55. doi: 10.1210/jc.2013-1265. Epub 2013 Jul 3. PMID: 23824424.
8. Iacobellis G, Malavazos AE, Corsi MM. Epicardial fat: from the biomolecular aspects to the clinical practice. Int J Biochem Cell Biol. 2011 Dec;43(12):1651-4. doi: 10.1016/j.biocel.2011.09.006. Epub 2011 Sep 28. PMID: 21967993.

9. Pierzynová A, Šrámek J, Cinkajzlová A, et al. The number and phenotype of myocardial and adipose tissue CD68+ cells is associated with cardiovascular and metabolic disease in heart surgery patients. Nutr Metab Cardiovasc Dis. 2019;29(9):946-955. doi:10.1016/j.numecd.2019.05.063
10. Camastra S, Vitali A, Anselmino M, Gastaldelli A, Bellini R, Berta R, Severi I, Baldi S, Astiarraga B, Barbatelli G, Cinti S, Ferrannini E. Muscle and adipose tissue morphology, insulin sensitivity and beta-cell function in diabetic and nondiabetic obese patients: effects of bariatric surgery. Sci Rep. 2017 Aug 21;7(1):9007. doi: 10.1038/s41598-017-08444-6. Erratum in: Sci Rep. 2018 May 22;8(1):8177. PMID: 28827671; PMCID: PMC5566429.
11. Razzoli M, Frontini A, Gurney A, Mondini E, Cubuk C, Katz LS, Cero C, Bolan PJ, Dopazo J, Vidal-Puig A, Cinti S, Bartolomucci A. Stress-induced activation of brown adipose tissue prevents obesity in conditions of low adaptive thermogenesis. Mol Metab. 2015 Nov 11;5(1):19-33. doi: 10.1016/j.molmet.2015.10.005. PMID: 26844204; PMCID: PMC4703853.
12. Kotzbeck P, Giordano A, Mondini E, Murano I, Severi I, Venema W, Cecchini MP, Kershaw EE, Barbatelli G, Haemmerle G, Zechner R, Cinti S. Brown adipose tissue whitening leads to brown adipocyte death and adipose tissue inflammation. J Lipid Res. 2018 May;59(5):784-794. doi: 10.1194/jlr.M079665. Epub 2018 Mar 29. PMID: 29599420; PMCID: PMC5928436.
13. Conte M, Petraglia L, Poggio P, Valerio V, Cabaro S, Campana P, Comentale G, Attena E, Russo V, Pilato E, Formisano P, Leosco D, Parisi V. Inflammation and Cardiovascular Diseases in the Elderly: The Role of Epicardial Adipose Tissue. Front Med (Lausanne). 2022 Feb 15;9:844266. doi: 10.3389/fmed.2022.844266. PMID: 35242789; PMCID: PMC8887867.
14. McAninch EA, Fonseca TL, Poggioli R, Panos AL, Salerno TA, Deng Y, Li Y, Bianco AC, Iacobellis G. Epicardial adipose tissue has a unique transcriptome modified in severe coronary artery disease. Obesity (Silver Spring). 2015 Jun;23(6):1267-78. doi: 10.1002/oby.21059. Epub 2015 May 9. PMID: 25959145; PMCID: PMC5003780.
15. Suh SH, Oh TR, Choi HS, Kim CS, Bae EH, Oh KH, Lee KB, Han SH, Sung S, Ma SK, Kim SW. Association of Body Weight Variability With Progression of Coronary Artery Calcification in Patients With Predialysis Chronic Kidney Disease. Front Cardiovasc Med. 2022 Jan 26;8:794957. doi: 10.3389/fcvm.2021.794957. PMID: 35155608; PMCID: PMC8826058.

Figures

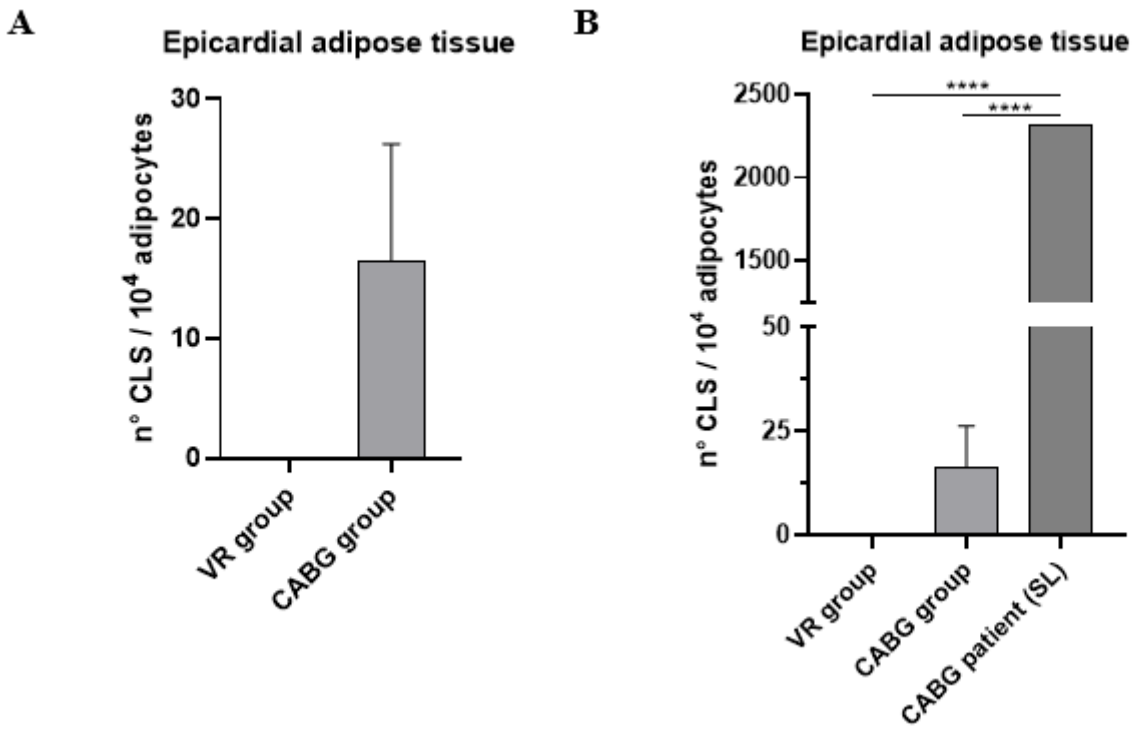

Figure 1

Crown-like structures (CLS) density in epicardial adipose tissue of patients undergoing open-heart surgery for valvular replacement (VR) or elective coronary artery bypass grafting (CABG) . A) Number of CLS per 10^4 adipocytes in VR group (n=5) vs. CABG group (n=4) without patient SL. B) CLS density/10^4 adipose cells in two groups of patients and in CABG patient (LS) with peculiar clinical data different from the other patients examined (recent heart attack after a period of relatively rapid increase of abdominal visceral fat).

****: P<0.0001

Figure 2

Light microscopy. Immunohistochemistry for CD68+. Representative picture of epicardial adipose tissue of CABG patient LS. An impressive amount of classic CLS (single and groups) are visible (arrows, some indicated). Bar: 30mm

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- STAB1.docx