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# The number and phenotype of myocardial and adipose tissue CD68+ cells is associated with cardiovascular and metabolic disease in heart surgery patients

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Type 2 diabetes mellitus;
Coronary artery disease

**Abstract** *Background and aims:* CD68+ cells are a potent source of inflammatory cytokines in adipose tissue and myocardium. The development of low-grade inflammation in adipose tissue is implicated in the pathogenesis of obesity-associated disorders including type 2 diabetes mellitus (T2DM) and cardiovascular disease. The main aim of the study was to characterize and quantify myocardial and adipose tissue CD68+ cells and adipose tissue crown-like structures (CLS) in patients with obesity, coronary artery disease (CAD) and T2DM.
*Methods and results:* Samples were obtained from the right atrium, epicardial (EAT) and subcutaneous adipose tissue (SAT) during elective heart surgery (non-obese, n = 34 patients; obese, n = 24 patients). Immunohistochemistry was used to visualize CD68+ cells. M1-polarized macrophages were visualized by immunohistochemical detection of CD11c. The proportion of CD68+ cells was higher in EAT than in SAT (43.4 ± 25.0 versus 32.5 ± 23.1 cells per 1 $mm^2$; p = 0.015). Myocardial CD68+ cells were more abundant in obese patients (45.6 ± 24.5 versus 27.7 ± 14.8 cells per 1 $mm^2$; p = 0.045). In SAT, CD68+ cells were more frequent in CAD patients (37.3 ± 23.0 versus 23.1 ± 20.9 cells per 1 $mm^2$; p = 0.012). Patients having CLS in their SAT had higher average BMI (34.1 ± 6.4 versus 29.0 ± 4.5; p = 0.024).
*Conclusions:* Regional-based increases in the frequency of CD68+ cells and changes of their phenotype in CLS were detected in obese patients and CAD patients. Therapeutic modulation of adipose tissue inflammation may represent a target for treatment of obesity.


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## Introduction

Macrophages are specific immunocompetent cells involved in local inflammation in virtually all tissues. While the local clinical inflammation is relatively easy to diagnose, the local subclinical inflammation can still be only predicted and can be confirmed by performing a biopsy [1]. Macrophages are involved in many different diseases, which are associated with such low-grade chronic inflammation [2]. In tissues, macrophages are traditionally subdivided into M1 and M2 populations that have different properties and can be differentiated based on certain markers [1]. M1 or classically activated macrophages are considered pro-inflammatory, while so called alternatively activated M2 macrophages are anti-inflammatory and are involved in maintaining tissue homeostasis in processes such as wound healing, angiogenesis and resolution of inflammation [2].

A low-grade state of chronic inflammation is also involved in the pathogenesis of obesity-associated disorders including type 2 diabetes mellitus (T2DM) and coronary artery disease (CAD) [5]. Obesity is manifested by accumulation of adipose tissue consisting of several discrete anatomical depots [3]. Most of them belong to subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT), which also includes epicardial adipose tissue (EAT). Based on sex-specific differences, VAT accounts for 6–20% and SAT for 80–90% of whole body adipose tissue [4]. Even though the most characteristic component of adipose tissue are unilocular adipocytes, these cells are outnumbered by stromal vascular cells including preadipocytes, endothelial cells, pericytes and various immune cells [3]. Under physiological conditions, adipose tissue performs intricate metabolic functions such as safe accumulation of surplus lipids and secretion of beneficial adipokines [5]. These adipokines, including adiponectin, are also secreted by healthy EAT, which has an intimate connection with myocardium as there is no fascia separating theses layers of the heart wall [6]. During obesity, white adipose tissue may become severely dysfunctional, which is manifested by elevated free fatty acids and altered adipokine profile [3]. Collectively, these events lead to insulin resistance and development of diabetes mellitus [5]. Epicardial fat, as a special adipose tissue depot, has a significantly increased volume in subjects with metabolic syndrome than in those without and this increase is largely associated with the presence and severity of CAD [7]. Dysfunctional EAT produces adipokines and inflammatory cytokines which can potentiate atherogenic processes in coronary arteries [8,9]. Since first observations of immune cell infiltration into adipose tissue during obesity [10,11], there is a growing interest in characterization of inflamed fat phenotype in obese patients. Previous studies have compared the incidence of immunocompetent cells including macrophages in epicardial adipose tissue and subcutaneous adipose tissue [12,13] in various pathological conditions, particularly in obese patients and T2DM patients. More recently, arrangement of macrophages in a "crown-like structure" (CLS) around single adipocytes exhibiting features of necrosis has been reported in obese subjects [14,15]. The presence of CLS has been proposed as a hallmark of chronic fat tissue inflammation [16].

To further expand the knowledge about the role of adipose tissue low-grade chronic inflammation in metabolic disease we focused on CD68+ cell and CLS numbers as well as macrophage phenotype in a population of patients characterized by a combination of several pathological conditions (obesity, CAD and T2DM), who underwent elective heart surgery. To allow for multiple comparisons, several anatomical regions were evaluated, namely SAT in the sternal region, EAT and the myocardium of right atrium (RA). Qualitative and quantitative histological findings from these locations were correlated with multiple clinical and biochemical parameters.

## Methods

### *Patients*

All patients (n = 58) were scheduled for elective cardiovascular surgery. All participants signed a written informed consent prior to the enrollment into the study. The study was approved by Human Ethics Review Board, First Faculty of Medicine and General University Hospital, Prague, Czech Republic (protocol number 41/14 Grant VES 2015 AZV VFN) and was performed in accordance with the guidelines proposed in Declaration of Helsinki (2000) of the World Medical Association. In this study group, there were obese (n = 24) and non-obese (n = 34), CAD patients (n = 26) and non-CAD patients (n = 22) and T2DM (n = 19) and non-T2DM patients (n = 39). Adult age and coronary artery disease or valvular disorder scheduled for elective coronary artery bypass graft implantation, valvular replacement, or valvuloplasty were selected as study inclusion criteria, while all acute cardiosurgical procedures along with the inability or refusal to provide written informed consent were defined as exclusion criteria. We used 31 samples of the RA, 44 samples of EAT, and 47 samples of SAT obtained from heart surgery. Characteristics of the study population and comparison between non-obese (BMI< 30) and obese (BMI> 30) group are shown in Table 1. The comparisons of study subjects divided according to the presence or absence of CAD and T2DM are shown in the Suppl. Tables 1 and 2, respectively. For cytometric and mRNA expression analyses, 14 non-obese and 11 obese subjects, 8 non-CAD subjects and 17 CAD subjects and 17 non-T2DM and 8 T2DM subjects were used. All subjects were from General University Hospital, Prague, Czech Republic.

### *Blood and tissue sampling*

Blood samples were taken at the beginning of surgery after overnight fasting. Histopathological and morphological analyses were performed blindly with respect to biochemical and biometric parameters as well as to clinical diagnosis.

**Table 1** Characteristics of patients: baseline clinical and laboratory parameters.

| Characteristics | All patients (n = 58) | Non-obese (n = 34) | Obese (n = 24) | p-Value |
|---|---|---|---|---|
| Age (range) | 67.2 (39.6 −84.3) | 69.9 (39.6 −84.3) | 63.5 (40.7 −75.1) | 0.008* |
| Males, n (%) | 43 (74) | 25 (74) | 18 (75) | 0.242 |
| Weight (kg) | 89 ± 17 | 79 ± 12 | 102 ± 16 | <0.001* |
| BMI (kg/m$^2$) | 29.6 ± 5 | 26.3 ± 2.5 | 34.3 ± 3.9 | <0.001* |
| Waist circumference (cm) | 105 ± 13 | 98 ± 10 | 114 ± 11 | <0.001* |
| Hip circumference (cm) | 108 ± 9 | 103 ± 6 | 114 ± 8 | <0.001* |
| WHR | 0.97 ± 0.08 | 0.95 ± 0.08 | 1.0 ± 0.07 | 0.113 |
| Atrial fibrillation (%) | 5 (9) | 4 (12) | 1 (4) | 0.310 |
| Arterial hypertension (%) | 39 (67) | 21 (62) | 18 (75) | 0.290 |
| CAD (%) | 36 (62) | 22 (65) | 14 (58) | 0.622 |
| T2DM (%) | 19 (33) | 10 (29) | 9 (38) | 0.518 |
| HDL (mmol/l) | 1.1 ± 0.4 | 1.1 ± 0.5 | 1.0 ± 0.3 | 0.257 |
| LDL (mmol/l) | 2.2 ± 0.8 | 2.0 ± 0.6 | 2.5 ± 0.9 | 0.039* |
| Triglycerides (mmol/l) | 1.5 ± 0.8 | 1.4 ± 0.5 | 1.7 ± 0.02 | 0.241 |
| Total cholesterol (mmol/l) | 3.95 ± 0.9 | 3.76 ± 0.8 | 4.25 ± 1.0 | 0.098 |
| Fasting glucose (mmol/l) | 6.8 ± 2.1 | 6.5 ± 1.8 | 7.3 ± 2.4 | 0.248 |
| $HbA_{1c}$ (mmol/mol) | 43 ± 10 | 41 ± 7 | 46 ± 13 | 0.127 |
| CRP (ug/ml) | 7.1 ± 10.2 | 6.5 ± 9.9 | 7.8 ± 10.9 | 0.732 |
| Atherogenity index | 2.6 ± 0.9 | 2.5 ± 1.0 | 2.9 ± 0.7 | 0.154 |

Values are mean ± standard deviation (SD) unless otherwise indicated. AF = atrial fibrillation; BMI = body mass index; WHR = waist-to-hip ratio, HDL = high-density lipoprotein; LDL = low-density lipoprotein; CRP = C-reactive protein; ApoB = apolipoprotein B; $HbA_{1c}$ = glycated hemoglobin. The significant results are marked by *. Student's t-test and Chi-square test.

Analogously, samples of RA, SAT and EAT were obtained at the beginning of surgery from approximately the same location in all patients and from tissue not previously traumatized mechanically or by cauterization to avoid the influence of local damage. EAT was taken from the anterior interventricular sulcus or the right margin of the heart and SAT was obtained from the sternotomy site. For flow cytometry and RNA expression analysis, approximately 1−2 g of SAT and EAT were obtained at the beginning of surgery immediately after sternotomy. Freshly collected specimens in PBS buffer (0.01 M PBS, pH 7.4) were used for flow cytometry, and aliquots in RNAlater® solution (Ambion® - Invitrogen, Carlsbad, California, USA) were stored at −80 °C and subsequently used for determination of mRNA expression.

### *Histology and immunohistochemistry*

Samples of RA, SAT and EAT were immediately fixed in 4% paraformaldehyde, processed, embedded in paraffin, sectioned and stained with hematoxylin-eosin. Type, amount and integrity of tissue was determined and in accordance with this evaluation, the specimens were used for immunohistochemistry.

For indirect immunohistochemical method, 7 μm thick sections were used. The sections were deparaffinized and immersed into preheated antigen-retrieval solution. Tris-chelaton III (pH 8.5) was used for the anti-human CD68-PG-M1, CD11c and DC-SIGN antibodies. Sodium citrate (pH 6.0) was used for the rabbit polyclonal anti-cleaved caspase 3 antibody. Then the samples were incubated at 98 °C for 10 min and allowed to cool to room temperature. The endogenous peroxidase activity and the non-specific antibody binding sites were blocked with 5% goat or bovine serum in Phosphate Buffered Saline (PBS). The mouse monoclonal anti-human CD68-PG-M1 (Dako, Denmark), CD11c (Novocastra™Leica, United Kingdom) and the rabbit polyclonal anti-human DC-SIGN (LifeSpan BioSciences, USA), Caspase 3 (Cell Signaling Technology, USA) and anti-cleaved lamin A (Cell Signaling Technology, USA) were used as primary antibodies (dilution shown in Suppl. Tab. 3). Visualization was achieved using LSAB+ Dako REAL ™ Detection System, Peroxidase/DAB+, Rabbit/Mouse according to previous publication [17]. The nuclei were counterstained by Harris's hematoxylin (Fig. 1).

For dual immunofluorescence labeling, we used anti-human CD68-PG-M1 antibody diluted 1:100 and anti-DC-SIGN antibody diluted 1:200 in the incubation buffer. Secondary antibodies were goat anti-mouse Alexa Fluor 555 (Invitrogen) and goat anti-rabbit Cy5 (Invitrogen) diluted 1:500 in the incubation buffer. The nuclei were stained with DAPI.

Control samples of human tonsil underwent the same staining (not shown). Omission of the primary antibodies gave the negative result.

### *Quantification of the CD68+ cells*

For quantification, we obtained 20 images collected via a systematic random sampling from each tissue section using the 40x dry objective of the Leica DMLB microscope (Leica Microsystems GmbH, Wetzlar, Germany). The number of macrophages was assessed by manual analysis of microscopic images using Image J 1.50i software (National Institutes of Health, USA). Dense connective tissue, empty fields and bigger blood vessels were excluded from the area of given image. We excluded also the samples, in which we found the ectopic tissue of thymus in involution.

### *Quantification of CLS*

Samples of SAT and EAT immunolabeled for CD68+ cell quantification were also used for quantification of CLS. CLS was defined as an adipocyte surrounded by CD68+ cells around at least 50% of its circumference. The whole cross-sectional area of all SAT and EAT sections was analyzed for the presence of CLS. A sample with at least one CLS was regarded as CLS positive.

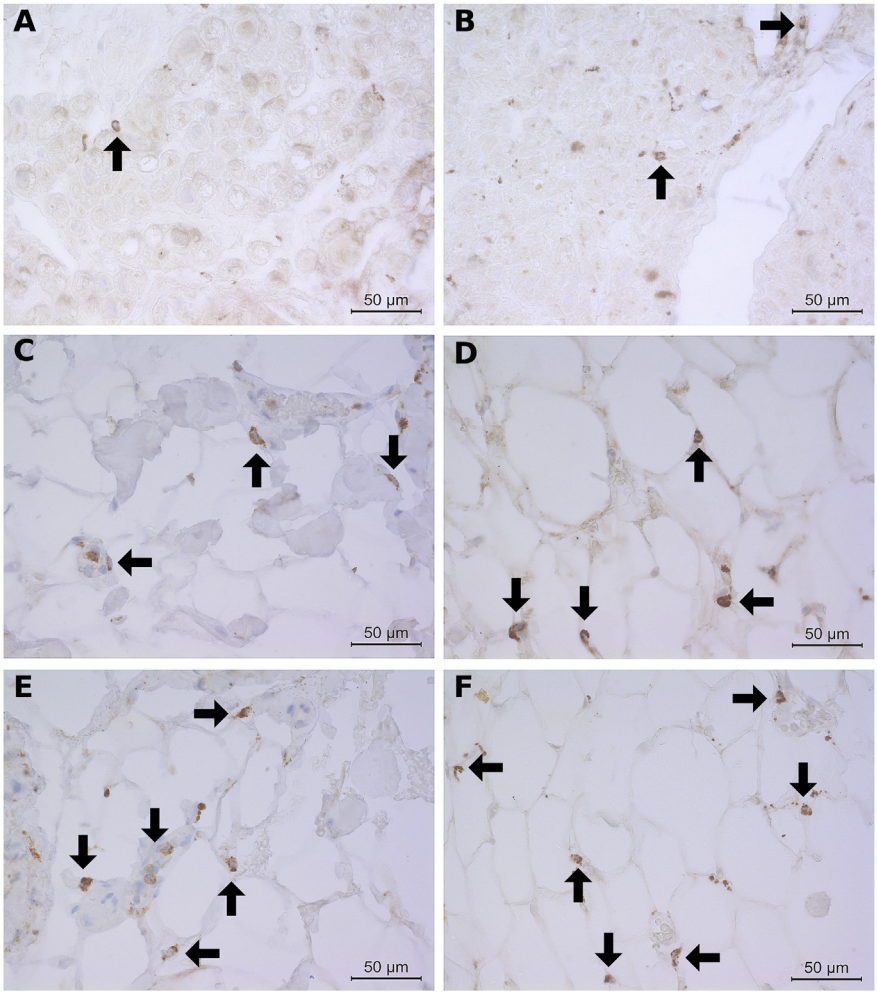


**Figure 1 Immunohistochemical detection of CD68+ cells in RA myocardium, EAT and SAT.** Samples of RA myocardial tissue of non-obese patient (A) and of an obese patient (B). Samples of SAT in non-CAD patient (C) and CAD patient (D). Samples of EAT in CAD patient (E) and in non-CAD patient (F). The arrows indicate CD68+ cells.

### Isolation of stromal vascular fraction from adipose tissue and flow cytometry

Adipose tissue was minced with sterile scissors and visible blood vessels were removed. Samples were washed in PBS, digested by 0.01% collagenase (Collagenase from Clostridium histolyticum, St. Louis, MO, USA) for 30 min at 37 °C and centrifuged for 12 min at 1200×*g*. Adipocytes were removed and the supernatant was repeatedly washed. Finally, samples were filtered through Falcon® 40 μm Cell Strainer (Becton, Dickinson and Company, Franklin Lakes, USA) to eliminate any remnant adipocytes. Flow cytometry was performed using freshly isolated and filtered stromal vascular fraction. A total amount of 100 μl of cell suspension with average $10^6$ cell content was labeled by monoclonal antibodies conjugated with FITC (fluorescein isothiocyanate), PE (phycoerythrin), PerCP (peridinin-chlorophyll protein complex), and APC (allophycocyanin). For labeling, single labeled antibodies in combination of CD206 FITC, CD163 PE and HLA-DR PerCP and CD14 APC, and combination of CD11c PE, HLA-DR PerCP and CD14 APC (Exbio Prague, a.s., Vestec, Czech Republic) were used. The samples were labeled in the dark for 30 min at 2–8 °C and then red cells were lysed using Excellyse I (Exbio Prague, a.s., Vestec, Czech Republic) according to manufacturer's instructions. Finally, labeled cells were analyzed on BD AccuriTM C6 (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Data analysis was performed using FlowJo X 10.0.7r2 software (FlowJo, LCC, Ashland, OR, USA). Gating strategy was as follows: doublets were excluded, macrophages (CD14+HLA-DR+ cells) were gated and then CD11c positive or CD163/CD206 positive cells were assessed (Suppl. Fig. 1). Percentage of gated macrophages were statistically compared and only data from one analysis are presented. Minimal count of acquired events was 50,000.

### *Quantitative real time PCR*

Samples of adipose tissue were homogenized on MagNA Lyser Instrument (Roche Diagnostics GmbH, Mannheim, Germany). Total RNA was extracted on MagNA Pure instrument (Roche Diagnostics GmbH, Mannheim, Germany). RNA concentration was determined from

absorbance at 260 nm on NanoPhotometer (Implen, Munich, Germany). Reverse transcription was performed using random primers according to the manufacturer's protocol of the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). The determination of gene expression was performed on the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). For reaction, a mix of TaqMan® Universal PCR Master Mix II, NO AmpErase® UNG (Applied Biosystems, Foster City, CA, USA), nuclease-free water (Fermentas Life Science, Vilnius, Lithuania) and specific TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) was used. Beta-2-microglobulin (B2M) was used as endogenous reference. The formula 2-ddCt was used to calculate relative gene expression.

### *Statistical analysis*

Statistical analysis was performed and graphs were drawn using program R version 3.3.1 (2016-06-21) and SigmaPlot 13.0 (SPSS Inc., Chicago, IL, USA). Results are expressed as mean ± standard deviation (SD). One way program R followed by unpaired t-test, Chi-square test, Mann–Whitney rank sum test, Spearman correlation test were used for the assessment of intergroup differences, as appropriate. Spearman correlation test was used to assess the association between CD68+ cells and other measured parameters. Baseline data of all study subjects were used for correlation analyses. Statistical significance was assigned to $p < 0.05$.

## Results

### *Clinical characteristics of patients*

Clinical characteristics of the non-obese patients (n = 34) and obese patients (n = 24) are summarized in Table 1. Body mass index (BMI) was significantly different between non-obese and obese group (26.3 ± 2.5 versus 34.3 ± 3.9 kg/m$^2$, respectively; $p < 0.001$). The non-obese patients were older than the obese patients. The frequency of CAD, arterial hypertension and T2DM was not significantly different between non-obese and obese group. LDL levels corresponded to already known differences between non-obese and obese patients.

### *CD68+ cells in EAT, SAT and RA of patients with obesity, CAD and T2DM*

CD68+ cells were mostly found as single scattered cells in the interstitial tissue surrounding adipocytes and in the endomysium of atrial myocardium (Fig. 1; Suppl. Fig. 2). We quantified the frequency of CD68+ cells in samples from EAT, SAT and RA and compared the results between patient groups divided according to the presence of obesity, CAD and T2DM (Table 2). When samples from all patients were compared, CD68+ cells were significantly more abundant in EAT compared to SAT (Table 2, Fig. 2a). Fig. 2c and Table 2 show that in SAT there were more CD68+ cells in CAD patients than in non-CAD patients. As shown in Fig. 2b and Table 2, in the atrial myocardium of obese patients there were more CD68+ cells than in atrial myocardium of non-obese patients.

### *Crown-like structures in EAT and SAT of patients with obesity, CAD and T2DM*

To further characterize CD68+ population in adipose tissue, we performed analysis of SAT and EAT sections and in fraction of samples, we identified CLS that consisted of CD68+ macrophages surrounding adipocytes (Fig. 3A–C). These CLS were either solitary (Fig. 3A, C) or we found multiple CLS in one section (Fig. 3E). Classical definition of CLS postulates that there is no apoptosis of adipocytes in these structures. To verify this in our material, we incubated the specimens with CLS with antibodies against cleaved caspase 3 and cleaved lamin A, which are markers of apoptotic cells. As shown in Suppl. Fig. 5A and C, no immunolabeling was detected in CLS, while we could observe apoptotic cells or apoptotic bodies in positive control sections of human tonsils (Suppl. Fig. 5B, D).

As a next step, we quantified CD68+ CLS in SAT as well as EAT samples and compared their frequency between different groups of patients (Suppl. Tab. 4). We found that all CLS EAT samples came from CAD patients. Furthermore, even though there was no significant difference in the frequency of CLS SAT samples between obese and non-obese patients, we found that patients with CLS SAT had in average higher BMI than patients with non-CLS SAT samples (34.1 ± 6.4 vs. 29.0 ± 4.5; $p = 0.024$). In contrast, there was no statistically significant difference in BMI between patients with CLS EAT and non-CLS EAT.

Next, we performed the immunohistochemical detection of M1 macrophage population marker CD11c in SAT and EAT specimens, which were CLS-positive or non-CLS. We found CD11c+ cells in all CLS samples and most of these cells localized to either solitary (Fig. 3B, D) or multiple CLS (Fig. 3F). In non-CLS samples, the CD11c+ cells were only rare. We also performed detection of another macrophage/dendritic cell marker DC-SIGN to find out whether it is specifically associated with CLS samples. We found DC-SIGN+ cells in all adipose tissue samples with localization similar to CD68+ cells (Suppl. Fig.3A) DC-SIGN immunoreactivity was also detected in solitary (Suppl. Fig. 3C) or multiple CLS (Suppl. Fig. 3D). In addition, dual immunolabeling of adipose tissue samples confirmed CD68+/DC-SIGN+ cells in isolated cells (Suppl. Fig. 4D–F) as well as in CLS (Suppl. Fig. 4G–I). Apart from adipose tissue, DC-SIGN positive cells were also detected in RA samples (Suppl. Fig. 3B), where they also co-expressed CD68 antigen (Suppl. Fig. 4A–C).

### Flow cytometric analysis of stromal vascular fraction and expression analysis of macrophage markers in EAT and SAT

A fraction of EAT and SAT samples was analyzed using flow cytometry as described in Methods section and as illustrated by Suppl. Fig. 1. The results of flow cytometric

**Table 2** CD68+ cells in different patient groups and different localization.

| Patients localization | Total CD68+ | non-obese | obese | p-Value | non-CAD | CAD | p-Value | non-T2DM | T2DM | p-Value |
|---|---|---|---|---|---|---|---|---|---|---|
| EAT (cells/mm$^2$) | 43.4 ± 25.0 † | 41.2 ± 23.5 | 47.0 ± 26.2 | 0.385 | 49.6 ± 27.8 | 39.2 ± 21.4 | 0.384 | 42.4 ± 23.8 | 47.0 ± 27.8 | 0.779 |
| SAT (cells/mm$^2$) | 32.5 ± 23.1 | 31.2 ± 25.3 | 34.3 ± 20.0 | 0.329 | 23.1 ± 20.9 | 37.3 ± 23.0 | 0.012* | 31.1 ± 22.4 | 35.3 ± 25.0 | 0.673 |
| RA (cells/mm$^2$) | 35.8 ± 21.4 | 27.7 ± 14.8 | 45.6 ± 24.5 | 0.045* | 36.6 ± 26.9 | 35.3 ± 18.6 | 0.695 | 31.0 ± 19.4 | 46.2 ± 22.7 | 0.712 |

Values are mean ± standard deviation (SD) unless otherwise indicated. EAT = epicardial adipose tissue, SAT = subcutaneous adipose tissue, M = myocardium. The significant results are marked by *. P-Value = 0.015 between the number of CD68+ cells in all EAT samples compared to all SAT is marked by †. Mann–Whitney U test.

analysis of stromal vascular fraction are shown in Suppl. Tab. 5. We found an increase of pro-inflammatory M1 macrophage population in EAT samples of patients with CAD in contrast with non-CAD group. Furthermore, in patients with CAD, there was a decrease of anti-inflammatory M2 macrophage population in SAT relative to non-CAD group.

The same collection of samples was analyzed using quantitative real time PCR for expression of CD68, iNOS, CD163 and CD206 (Suppl. Tab. 6). No statistically significant differences were detected when we compared obese vs. non-obese, CAD vs. non-CAD and T2DM vs. non-T2DM groups.

### *Associations of adipose tissue histology with biometric and biochemical parameters*

We also evaluated the influence of biometric and biochemical parameters (Table 1, Suppl. Tab. 1, Suppl. Tab. 2) on the number of CD68+ cells in different tissues. There was a correlation between the number of CD68+ cells in myocardium of RA and BMI (Suppl. Fig. 6). The number of CD68+ cells in the myocardium of RA correlated positively with glycated hemoglobin ($HbA_{1c}$) in T2DM patients (Suppl. Fig. 7A). The number of CD68+ cells correlated negatively with high density lipoprotein (HDL) in the EAT of CAD patients (Suppl. Fig. 7B).

## Discussion

In this study, we quantified CD68+ macrophages in bioptic samples of atrial myocardium, EAT and SAT obtained from patients undergoing elective cardiovascular surgery. In addition, in samples of adipose tissue we characterized the CD68+ population with respect to CLS formation and CD11c expression as an indicative of pro-inflammatory macrophage polarization. Finally, correlation of these findings with biochemical and clinical parameters was performed. The novelty of the present study is that it examines simultaneously three different locations – SAT, EAT and myocardium of cardiovascular surgery patients, which allows for direct comparison between CD68+ cell frequency and phenotype across these anatomical regions. We found that CD68+ cell number and their polarization in specific anatomical sites is related to some concomitant pathological conditions of cardiovascular surgery patients, namely obesity and CAD (summarized in Suppl. Tab. 7). The major results were as follows: 1) Increased CD68+ cell number and their pro-inflammatory polarization in SAT was associated with CAD. 2) Increased CD68+ cell number in myocardium was associated with obesity. In addition, we found that EAT had in general higher CD68+ cell number when compared to SAT and that CLS-positive adipose tissue samples contained numerous pro-inflammatory CD11c+ macrophages in contrast to non-CLS adipose tissue samples.

Despite some recent controversies regarding markers of pro- and anti-inflammatory macrophages and slightly simplified phenotypic classification of macrophages into these two groups [18], the finding of CD11c+ macrophages participating in an obviously inflammatory process is in line with their pro-inflammatory activation. Furthermore, CD11c+ macrophages in CLS found in SAT were already demonstrated in one previous paper [19]. The results of

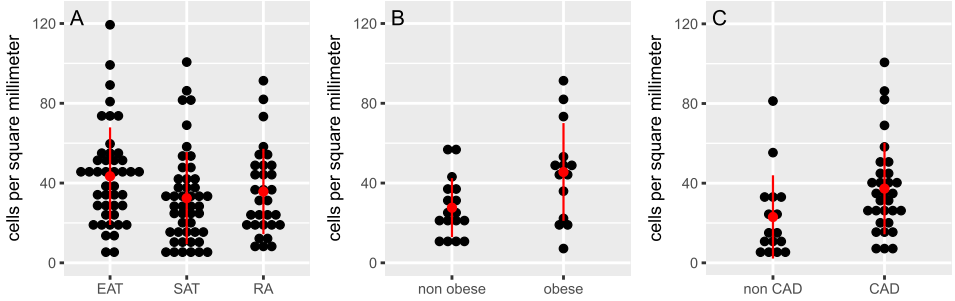


**Figure 2 Number of CD68+ cells in adipose tissue and myocardium of RA.** (A) Dot plot shows the number of CD68+ cells in all EAT samples compared to all SAT (43.4 ± 25.0 versus 32.5 ± 23.1 cells per 1 mm$^2$; p-value = 0.015). (B) Dot plot shows the number of CD68+ cells in the atrial myocardium in obese patients compared to non-obese patients (45.6 ± 24.5 versus 27.7 ± 14.8 cells per 1 mm$^2$; p-value = 0.045). (C) Dot plot shows CD68+ cells in SAT were more abundant in CAD patients than in non-CAD patients (37.3 ± 23.0 versus 23.1 ± 20.9 cells per 1 mm$^2$; p-value = 0.012).

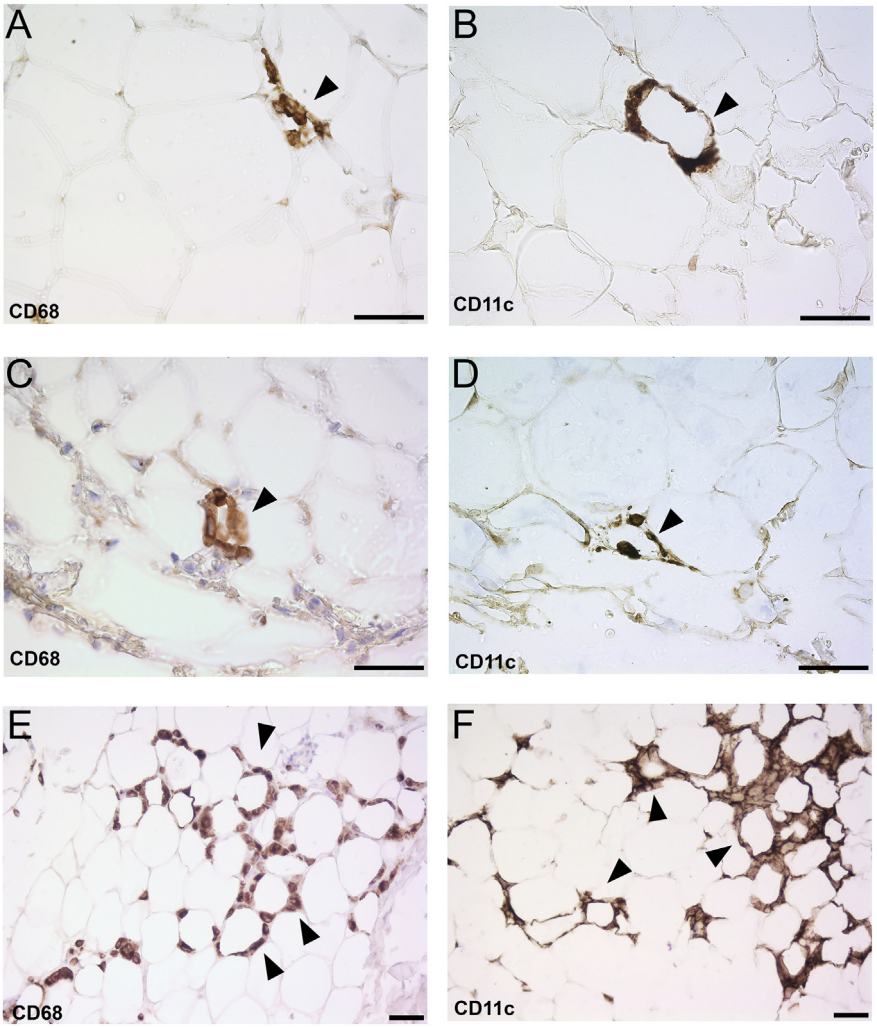


**Figure 3 Crown-like structures in and macrophage polarization in SAT and EAT.** (A, C, E) Images of showing a result of immunoperoxidase reaction for CD68 in SAT (A, E) and EAT (C). Immunoreactive cells (arrowheads) are localized to either solitary (A, C) or multiple CLS (D). (E–F) Images of SAT (B, F) and EAT (D)showing a result of immunoperoxidase reaction for M1 polarity marker CD11c. Arrowheads point to immunoreactive cells localized to either solitary (B, D) or multiple CLS (F). Scale bar in (A–F) = 50 μm.

the present study further expand these observations by showing that CD11c+ macrophages are tightly associated with CLS both in SAT as well as EAT. In contrast, another macrophage/dendritic cell marker DC-SIGN did not discriminate between CLS and non-CLS CD68+ cell populations as it was found in both CLS-associated as well as solitary macrophages. Based on negative detection of two apoptotic markers, cleaved-caspase-3 and cleaved lamin A, there was no apoptosis in CD68+ and CD11c+ cell aggregates around adipocytes, so we could conclude that these were CLS according to original characterization by Cinti and colleagues [14,15].

When macrophage numbers in SAT were compared in the study by Hirata et al., no difference was found when CAD and non-CAD patient populations were compared [20]. The data of the present study show that patients undergoing cardiovascular surgery due to CAD have more CD68+ macrophages in their SAT compared to their non-CAD counterparts. Interestingly, in the present study, CD68+ cell numbers in EAT were not different when EAT samples were compared between CAD and non-CAD patients. However, all CLS-positive EAT samples with pro-inflammatory CD11c+ macrophages came from CAD patients and also in SAT, there were more CLS+ samples from CAD patients compared to non-CAD patients. Correspondingly, in collection of samples analyzed by flow cytometry, we observed an increase of pro-inflammatory M1 macrophages in EAT of CAD patients compared to non-CAD patients. In addition, using the same method we found a lower number of M2 anti-inflammatory macrophages in SAT of CAD patients compared to non-CAD patients, thus illustrating a shift from anti-inflammatory towards pro-inflammatory state of adipose tissue macrophages in CAD. These findings further strengthen the importance of pro-inflammatory macrophage polarization in adipose tissue for the atherogenic process in coronary arteries as previously documented [20–22]. According to current concepts such effect might be mediated by the release of pro-inflammatory cytokines (TNF-alpha, IL-6) and adipokines by inflamed adipose tissue [6,8,9,23]. In

case of EAT this could be mediated by a more direct action of these factors through paracrine or vasocrine mechanism [24]. Dysregulation of SAT accompanied by chronic-low grade inflammation can influence the coronary artery atherogenesis by lipid metabolites, adipokines and cytokines transported over a longer distance via blood circulation [23,25].

Some experimental studies on diet-induced obese mice describe increased macrophage infiltration of myocardium compared to controls [26]. In our study, we observed this phenomenon in a human population of obese cardiovascular patients, who had more CD68+ macrophages in the RA myocardium than non-obese patients. Importantly, in this study, RA CD68+ macrophage numbers correlated with BMI, when both obese and non-obese patients were taken into account. This further demonstrates the relationship between obesity and heart disease [25]. Mechanistically, obesity can possibly cause an increased macrophage infiltration via inflammatory cytokine and adipokine production by dysregulated SAT. This can lead to endothelial dysfunction of coronary microcirculation, endothelial cell activation and increased recruitment of monocytes differentiating into myocardial macrophages [25,27]. In addition, myocardial damage in obesity-associated cardiomyopathy might be another stimulus of macrophage infiltration [28].

There was no difference in the number of CD68+ macrophages in SAT when obese patients were compared to non-obese patients, which is in contrast to previous findings [14,29]. This difference is most likely reflecting a lower BMI of the obese patient group in the present study (Table 1). Nevertheless, we observed a greater tendency for CLS formation in SAT of obese patients. In addition, when both obese and non-obese individuals were included into statistics, patients with CLS-positive SAT had a higher BMI compared to patients with non-CLS SAT. This might indicate that the polarization of macrophages in SAT rather than their total number characterizes SAT of obese individuals in contrast to lean population, which corresponds to previous reports of an increased number of pro-inflammatory macrophages in SAT of obese patients [19,30]. The number of CLS-positive samples was in our study group rather low, which is in line with studies performed in moderately obese patients [12,31]. Much more CLS can be found in morbidly obese patients [32], but these individuals were not significantly represented in our study group. In contrast, in EAT there were no CLS-positive samples in obese patients and the only CLS-positive samples were obtained from non-obese patients. Since all of these non-obese patients with CLS-positive samples had CAD as well as dyslipidemia we can speculate that inflammatory process in EAT is more tightly associated with atherosclerosis of adjacent coronary arteries, which is affected by dyslipidemia independent of obesity. The effect of dyslipidemia can be illustrated by our finding of negative correlation between HDL plasma levels and CD68+ cell number in EAT of CAD patients. Even though, we did not measure EAT volume in the present study, it was published previously that epicardial fat thickness and CAD correlate independently of obesity [33]. We could also observe somewhat paradoxical finding in obese patients with unchanged CD68+ cell number in contrast to non-obese ones in EAT and an increased CD68+ cell number in RA myocardium of obese patients in contrast to non-obese. We can speculate that in this case the macrophage infiltration and pro-inflammatory activation of EAT vs. atrial myocardium has different causes, which can be partially independent as discussed above. For EAT, it would be mainly the vascular wall inflammation during coronary atherosclerosis that spreads through cytokine diffusion and inflammatory cell migration across adventitia into the surrounding EAT. For atrial myocardium, the cause could be dysregulation of myocardial metabolism during obesity and dysfunction of coronary microvasculature as mentioned above [27,28].

We did not find differences in CD68+ cell number in EAT, SAT and RA that would discriminate between T2DM and non-T2DM patients in our study group of subjects undergoing heart surgery most likely because of presence of other pathologies that affect the chronic low-grade inflammation in adipose tissue. Nevertheless, in patients with T2DM we observed a greater tendency for CLS formation in SAT in contrast to non-T2DM patients. Furthermore, in patients with T2DM there was a positive correlation between the plasma levels of HbA1c and CD68+ cell number in RA myocardium. This is in line with the inflammatory aspects of diabetic cardiomyopathy [34]. The association between insulin resistance or T2DM and chronic inflammation in EAT or SAT was reported previously. There was an increased inflammatory status in obese patients with T2DM compared to obese with normal glucose tolerance [35]. Another study showed that patients with CLS-positive SAT had worse metabolic parameters indicative of insulin resistance [16]. Regarding EAT, this adipose tissue depot can be considered relatively insulin resistant [36]. Recently, it was found that diabetic epicardial fat has a unique transcriptome accompanied by overexpression of inflammatory genes together with NF-kB pathway [37].

The finding of a higher CD68+ cell number in EAT compared to SAT is in line with a previous study by Hirata et al. performed in non-obese patients with and without CAD [20]. EAT is one form of visceral adipose tissue, which includes many different locations such as periaortic, mesenterial and omental visceral adipose tissue. The results of human studies comparing the level of inflammation and macrophage numbers in visceral vs. subcutaneous tissue are unequivocal. There are reports of a greater macrophage infiltration in omental vs. SAT [29,38] or greater macrophage numbers in omental and periaortic adipose tissue compared to SAT [13], but some studies also present data showing that there are more macrophages in SAT compared to visceral adipose tissue [31,35]. These differences can be partially explained by methodological variability in macrophage detection, but specific composition of assessed patient populations may also be the cause of such discrepancies.

In conclusion, data presented in this study further demonstrate an association between chronic low-grade inflammatory state of adipose tissue and cardiovascular and metabolic diseases of which macrophages are an important part. We also confirmed that adipose tissue inflammation is better characterized by abundant pro-inflammatory macrophage subpopulation rather than total CD68+ cell number. Further research, including experimental studies, should address the question of specific mediators of adipose tissue inflammation with the capability to influence the development of CAD, T2DM, insulin resistance and possibly other diseases, in which dysfunctional microvascular endothelium might play a role (e.g. heart failure, pulmonary hypertension) [27].

## Conflicts of interest

None declared.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.numecd.2019.05.063.

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