Increased Number of Mast Cells in Epicardial Adipose Tissue of Cardiac Surgery Patients With Coronary Artery Disease

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Summary

Chronic inflammation of adipose tissue is associated with the pathogenesis of cardiovascular diseases. Mast cells represent an important component of the innate defense system of the organism. In our work, we quantified mast cell number in epicardial adipose tissue (EAT), subcutaneous adipose tissue (SAT), and right atrial myocardium (RA) in patients undergoing open heart surgery (n=57). Bioptic samples of EAT (n=44), SAT (n=42) and RA (n=17) were fixed by 4 % paraformaldehyde and embedded into paraffin. An anti-mast cell tryptase antibody was used for immunohistochemical detection and quantification of mast cells. We also demonstrated immunohistochemically the expression of CD117 and chymase markers. In EAT of patients with coronary artery disease (CAD), higher incidence of mast cells has been found compared to patients without CAD (3.7±2.6 vs. 2.1±1.2 cells/mm^2). In SAT and RA, there was no difference in the number of mast cells in CAD and non-CAD patients. Mast cells in SAT, EAT and RA expressed CD117 and chymase. An increased incidence of mast cells in EAT of CAD patients may indicate the specific role of these inflammatory cells in relation to EAT and coronary arteries affected by atherosclerosis.

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Introduction

Under pathological conditions such as obesity or coronary artery disease (CAD), adipose tissue is in the state of low-grade chronic inflammation (Dam *et al.* 2016). Such inflammation is manifested by an altered secretion of adipokines and infiltration by several different populations of inflammatory cells (Mraz and Haluzik 2014). White adipose tissue is distributed in the body in the form of several distinct depots, which are traditionally divided into subcutaneous (SAT) and visceral adipose tissue (VAT), which also includes epicardial adipose tissue (EAT) (Lee *et al.* 2013). The

cell type characteristic for this tissue is the unilocular adipocyte, but these cells are accompanied by a huge number of stromal cells including preadipocytes, endothelial cells, perivascular cells and there is a discrete population of immune cells even in normal conditions. EAT is a special adipose tissue depot, due to its intimate contact with coronary arteries and its vicinity to myocardium directly underneath epicardial fat tissue layer without any additional connective tissue barrier (Matloch *et al.* 2018a). Like adipose tissue in other locations, EAT is during healthy conditions a source of beneficial adipokines such as adiponectin (Fitzgibbons and Czech 2014). In CAD, EAT increases its size and becomes dysfunctional as it starts to produce a different set of adipokines and pro-inflammatory cytokines that can further potentiate atherogenic process in the coronary arteries (Matloch *et al.* 2018b, Mazurek *et al.* 2003, Iacobellis 2016). These cytokines are mainly produced by infiltrating inflammatory cells such as macrophages and lymphocytes, which are elevated in EAT of CAD patients (Hirata *et al.* 2011, Mraz *et al.* 2019). Mast cells are cells of hemopoetic origin and constitute an important part of the innate immune response (da Silva *et al.* 2014). These cells are found in most organs and are mainly associated with the connective tissue, often near blood vessels. Mast cells are traditionally described as cells involved in allergic response due to their Fc receptors for IgE immunoglobulins, but their physiological and pathological functions extend beyond this activity. Their role is mainly mediated by secreted factors, some of which are together with histamine and heparin stored as preformed substances in granules and many others including various cytokines and growth factors (eg. IL-1beta, CCL2, TNF-alpha or bFGF) are synthesized *de novo* upon activation (Krystel-Whittemore *et al.* 2015). Based on the content of chymase, tryptase or both, they can be divided into MC_T population typical for the mucosal lining of the respiratory tract and MC_{TC} population found in the connective tissue of the skin (da Silva *et al.* 2014). Importantly, mast cells have been also implicated in chronic low-grade inflammation of adipose tissue and in atherosclerotic cardiovascular disease (Kovanen and Bot 2017, Zelechowska *et al.* 2018). An increase of mast cell numbers in SAT was reported in obese patients (Liu *et al.* 2009, Spencer *et al.* 2014). Previous study suggested that mast cells accumulate in EAT (Mazurek *et al.* 2003) of CAD patients. The quantification of mast cell population in EAT of CAD patients have not been performed yet. Thus, the objective of this study was to determine the amount and phenotype of mast cells in EAT, SAT and myocardium of CAD patients and non-CAD patients, who underwent elective heart surgery. In addition, we evaluated the effect of other concomitant pathological conditions, namely obesity and type 2 diabetes mellitus (T2DM). We hypothesized that similarly to other inflammatory cells, mast cell numbers are increased in EAT of CAD patients and this way they can contribute to the chronic low-grade inflammation in EAT.

Methods

Patients

All patients (n=57) were scheduled for elective cardiovascular surgery. All participants signed 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 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 patients (n=33), CAD (n=35) and non-CAD patients (n=22) and T2DM (n=18) and non-T2DM patients (n=39). Adult age and coronary artery disease or valvular disorder scheduled for elective coronary artery bypass graft implantation (CABG), 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 17 samples of the RA, 44 samples of EAT, and 42 samples of SAT obtained from heart surgery. Characteristics of the study population and comparison between non-CAD and CAD (scheduled for CABG) group are shown in Table 1. The comparisons of study subjects divided according to the presence or absence of T2DM (based on the diagnosis in the patient's documentation) are shown in the Table 2. Characteristics of the study population and comparison between non-obese (BMI< 30) and obese (BMI> 30) group are shown in Table 3.

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=57)	Non-CAD (n=22)	CAD (n=35)	p-value
Age (range)	67.2 (39.6 - 84.3)	66.7 (39.6 - 77.4)	67.5 (54.7 - 84.3)	0.789
Males, n (%)	42 (74)	15 (68)	27 (77)	0.454
Weight (kg)	88±17	89±20	88±16	0.805
BMI (kg/m^2)	29.6±5	29.5±4.6	29.7±5.2	0.915
Waist circumference (cm)	104±13	105±14	104±13	0.759
Hip circumference (cm)	108±9	107±9	108±9	0.753
WHR	0.97±0.07	0.98±0.07	1.0±0.08	0.458
Atrial fibrillation (%)	4 (7)	1 (5)	3 (9)	0.562
Arterial hypertension (%)	38 (67)	12 (55)	26 (74)	0.124
T2DM (%)	18 (32)	7 (32)	11 (31)	0.975
HDL (mmol/l)	1.1±0.4	1.0±0.3	1.1±0.4	0.247
LDL (mmol/l)	2.2±0.7	2.5±0.9	2.1±0.6	0.115
Triglycerides (mmol/l)	1.5±0.8	1.5±0.6	1.4±0.9	0.689
Total cholesterol (mmol/l)	3.97±0.9	4.2±1	3.9±0.8	0.301
Fasting glucose (mmol/l)	6.9±2	7.2±2.4	6.8±1.7	0.541
HbA1c (mmol/mol)	43±10.1	43.2±10.6	42.9±9.9	0.935
CRP (µg/ml)	7.1±10.1	3.3±2	8.8±11.7	0.162
Atherogenity index	2.6±0.9	3±1.2	2.5±0.7	0.175

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; HbA1c = glycated hemoglobin. * significant results. Student's t-test and Chi-square test.

Table 2. Characteristics of patients - baseline clinical and laboratory parameters.

Characteristics	All patients (n=57)	Non-T2DM (n=39)	T2DM (n=18)	p-value
Age (range)	67.2 (39.6 - 84.3)	67.1 (39.6 - 84.3)	67.4 (54.7 - 78.3)	0.876
Males, n (%)	42 (74)	29 (74)	13 (72)	1.000
Weight (kg)	88±17	87±18	91.5±16.4	0.370
BMI (kg/m^2)	29.6±5	28.8±4.9	31.2±5.1	0.109
Waist circumference (cm)	104±13	100.9±12.5	109.2±12.1	0.041*
Hip circumference (cm)	108±9	106±8.3	109.9±9.3	0.159
WHR	0.97±0.07	0.95±0.08	1.0±0.06	0.073
Atrial fibrillation (%)	4 (7)	4 (10)	0	0.297
Arterial hypertension (%)	38 (67)	22 (56)	16 (89)	0.018*
CAD (%)	35 (61)	24 (62)	11 (61)	1.000
HDL (mmol/l)	1.1±0.4	1.1±0.3	1.2±0.5	0.322
LDL (mmol/l)	2.2±0.7	2.2±0.9	2.2±0.5	0.989
Triglycerides (mmol/l)	1.5±0.8	1.4±0.5	1.6±1.0	0.359
Total cholesterol (mmol/l)	4±0.9	3.9±1	4.0±0.8	0.576
Fasting glucose (mmol/l)	6.9±2	5.8±0.8	8.6±2.1	<0.001*
HbA1c (mmol/mol)	43±10.1	36.8±3.9	52.8±9.1	<0.001*
CRP (µg/ml)	7.1±10.1	6.7±10.4	7.8±10.3	0.776
Atherogenity index	2.6±0.9	2.6±0.9	2.7±1.0	0.761

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; HbA1c = glycated hemoglobin. * significant results. Student's t-test and Chi-square test.

Table 3. Characteristics of patients - baseline clinical and laboratory parameters.

Characteristics	All patients (n=57)	Non-obese (n=33)	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)	0.006*
Males, n (%)	42 (74)	24 (73)	18 (75)	1.000
Weight (kg)	88±17	79±11	102±15.2	<0.001*
BMI (kg/m²)	29.6±5	26.2±2.4	34.3±3.8	<0.001*
Waist circumference (cm)	104±13	97.2±9.2	114±11	<0.001*
Hip circumference (cm)	108±9	103±6.1	114±7.6	<0.001*
WHR	0.97±0.07	0.95±0.07	1.0±0.07	0.034*
Atrial fibrillation (%)	4 (7)	3 (9)	1 (4)	0.631
Arterial hypertension (%)	38 (67)	20 (61)	18 (75)	0.394
CAD (%)	35 (61)	21 (64)	14 (58)	0.785
T2DM (%)	18 (32)	9 (27)	9 (38)	0.565
HDL (mmol/l)	1.1±0.4	1.2±0.5	1.0±0.2	0.262
LDL (mmol/l)	2.2±0.7	2.0±0.6	2.5±0.9	0.027*
Triglycerides (mmol/l)	1.5±0.8	1.3±0.5	1.7±1.0	0.165
Total cholesterol (mmol/l)	4±0.9	3.8±0.8	4.3±1.0	0.096
Fasting glucose (mmol/l)	6.9±2	6.6±1.7	7.3±2.3	0.287
HbA1c (mmol/mol)	43±10.1	41±6.9	46±13	0.105
CRP (µg/ml)	7.1±10.1	5±9.4	8.3±10.8	0.604
Atherogenity index	2.6±0.9	2.4±0.7	2.9±0.7	0.153

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; HbA1c = glycated hemoglobin. * significant results. 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.

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 mast cell tryptase and anti-CD117 antibodies. Sodium citrate (pH 6.0) was used for anti-chymase 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 mast cell tryptase (Dako, Denmark) diluted 1:20 000 in PBS + 1.5 % normal goat serum, goat polyclonal anti-mast cell chymase antibody (Abcam, UK) diluted 1:1000 in PBS + 1.5 % normal rabbit serum and rabbit polyclonal anti-human CD 117 antibody (Dako, Denmark diluted 1:2000 in PBS + 1.5 % normal goat serum were used as primary antibodies. Visualization was achieved using LSAB+ Dako REAL ™ Detection System, Peroxidase/DAB+, Rabbit/Mouse or using rabbit anti-goat biotinylated secondary antibody followed by Vectastain ABC kit peroxidase. The nuclei were counterstained by Harris's hematoxylin.

For dual immunofluorescence labeling of mast cell tryptase and chymase, we used anti-human mast cell tryptase antibody diluted 1:400 and goat polyclonal anti-mast cell chymase antibody diluted 1:400 in PBS + 1.5 %

normal rabbit serum. Secondary antibodies were rabbit anti-mouse Alexa Fluor 647 (Invitrogen) and rabbit anti-goat Cy3 (Jackson Immunoresearch, UK) diluted 1:500 in PBS + 5 % normal rabbit serum. The nuclei were stained with DAPI.

For dual immunofluorescence labeling of mast cell tryptase and CD117, we used anti-human mast cell tryptase antibody diluted 1:400 and rabbit polyclonal anti-CD117 antibody diluted 1:400 in PBS + 1.5 % normal goat serum. Secondary antibodies were goat anti-mouse Alexa Fluor 532 (Invitrogen) and goat anti-rabbit Cy5 (Invitrogen) diluted 1:500 in in PBS + 5 % normal goat serum. The nuclei were stained with DAPI.

Quantification of mast cells

For quantification, we used samples immunolabeled for mast cell tryptase. We obtained 40 images collected via a systematic random sampling from each tissue section (one tissue section/patient) using the 20x dry objective of the Leica DMLB microscope (Leica Microsystems GmbH, Wetzlar, Germany). The number of mast cells 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.

Measurement of adipocyte size

For the measurement of adipocyte size, we used the same slides as those for quantification of mast cells. The measurement included cells, which had their complete cell body in the image and the diameter was measured across the adipocyte cells cytoplasm intersecting with adipocyte nucleus. We measured diameter of approximately 30 cells in each section. One section represented one patient.

Statistical analysis

Statistical analysis was performed by unpaired t-test, Chi-square test, Mann-Whitney rank sum test. Statistical significance was assigned to $p < 0.05$.

Results

Clinical characteristics of patients

Clinical characteristics of the whole group and of non-CAD patients (n=22) and CAD patients (n=35) are summarized in Table 1. No statistically significant differences in the indicated parameters were detected between these 2 groups of patients.
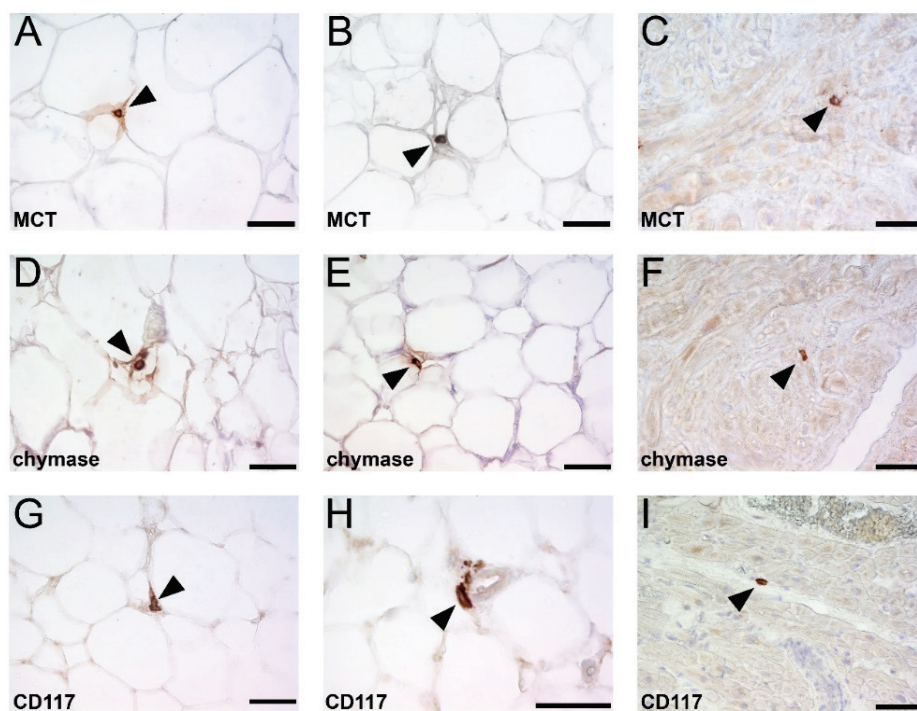

Fig. 1. Immunohistochemical detection of mast cell tryptase, chymase and CD117 in SAT, EAT and RA. (**A-C**) Images showing a result of immunoperoxidase reaction for mast cell tryptase (MCT). (**A**) Solitary immunoreactive cell (arrowhead) in SAT is localized between adipocytes. (**B**) A single immunoreactive cell (arrowhead) close to a small blood vessel in EAT sample. (**C**) Solitary immunoreactive cell (arrowhead) in the myocardial interstitium of RA. Scale bar in A-C=50 µm. (**D-F**) Images showing a result of immunoperoxidase reaction for chymase. (**D**) A single immunoreactive cell (arrowhead) close to an arteriole in the sample of SAT. (**E**) Solitary immunoreactive cell (arrowhead) in EAT is localized between adipocytes. (**F**) Solitary immunoreactive cell (arrowhead) in the myocardial interstitium of RA. Scale bar in D-F=50 µm. (**G-I**) Images showing a result of immunoperoxidase reaction for CD117. (**G**) Solitary immunoreactive cell (arrowhead) in SAT is localized between adipocytes. (**H**) An immunoreactive cell with granules (arrowhead) close to a small arteriole in EAT sample. (**I**) Solitary immunoreactive cell (arrowhead) in the myocardial interstitium of RA. Scale bar in G-I=50 µm.
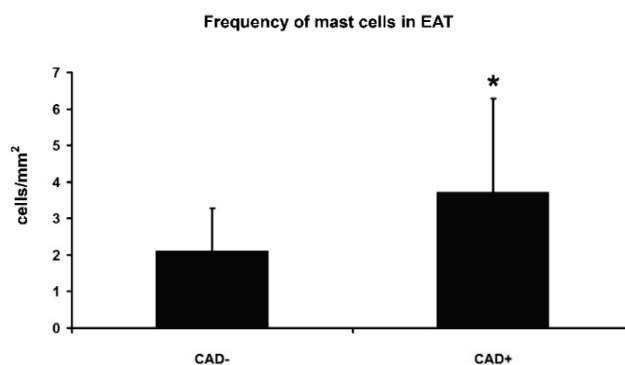
Fig. 2. Quantification of mast cell tryptase-positive cells in EAT. A graph showing frequency of mast cell tryptase-positive mast cells in EAT of CAD patients and non-CAD patients. An average number of mast cell-positive cells per square mm of cross-sectioned adipose tissue is given +/- SD. The significant result (p>0,05) is marked by *****. Mann-Whitney U test.

Mast cells in EAT, SAT and RA of patients with obesity, CAD and T2DM

Tryptase-positive mast cells were detected in all samples of EAT, SAT and RA regardless of patient group. These cells were usually scattered throughout adipose or myocardial tissue as single cells in the interstitium (Fig. 1A-C). Occasionally, small groups consisting of 2-3 cells, especially in regions of perivascular connective tissue, were detected. We quantified the number of tryptase-positive mast cells in EAT, SAT and RA samples and we compared the results between CAD and non-CAD patients, obese and non-obese patients and T2DM and non-T2DM patients (Table 4). We found a significantly higher number of tryptase-positive mast cells in EAT samples of CAD

Table 4. Quantification of mast cells in different patient groups and different localization.

	CAD	non-CAD	obese	non-obese	T2DM	non-T2DM
EAT	3.7±2.6*	2.1±1.2	3.6±2.6	2.9±2.0	2.7±1.6	3.3±2.5
SAT	3.3±2.3	3.2±2.0	3.5±2.3	3.2±2.2	3.7±2.2	3.1±2.3
RA	3.4±2.6	2.5±1.6	3.4±2.5	2.8±2.0	3.3±2.0	3.0±2.4

Values are mean ± standard deviation (SD) unless otherwise indicated. EAT = epicardial adipose tissue, SAT = subcutaneous adipose tissue, RA = right atrial myocardium. * significant result (p<0.05), Mann-Whitney U test.
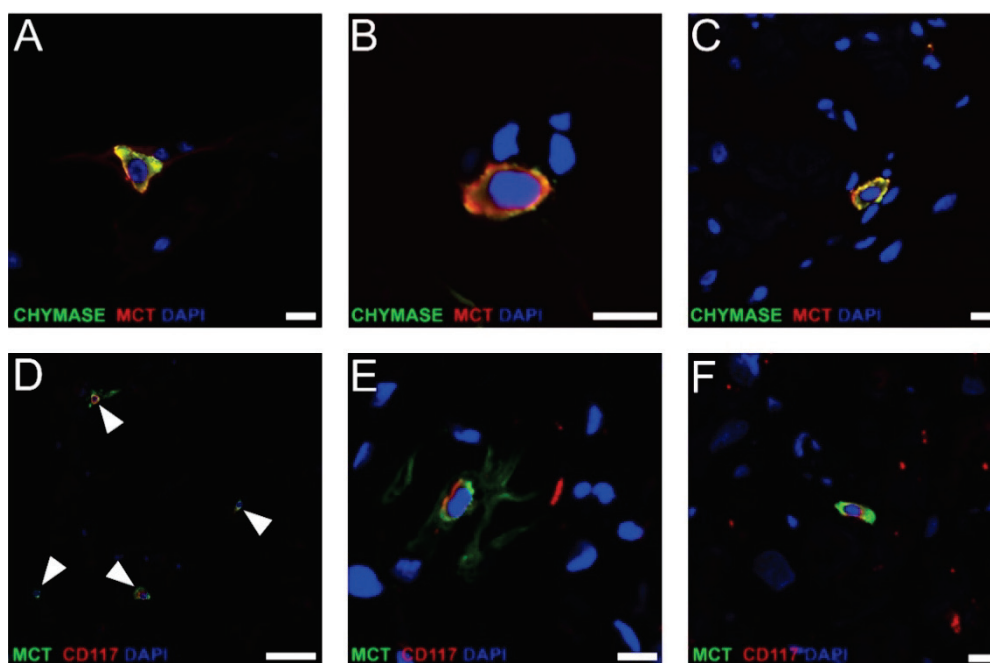

Fig. 3. Immuno-detection of chymase/mast cell tryptase and mast cell tryptase/CD117 double positive cells in SAT, EAT and RA. (**A-C**) Confocal images showing a result of dual immunofluorescence labeling for chymase and tryptase. (**A**) Chymase (green) is expressed in a mast cell tryptase-positive mast cell (red) localized in the interstitial spaces between adipocytes in SAT. (**B**) A confocal image of a mast cell in EAT co-expressing chymase (green) and mast cell tryptase (red). (**C**) Chymase (green) is expressed in a mast cell tryptase-positive mast cell (red) localized in the endomysium of RA myocardium. Nuclei are stained with DAPI (blue). Scale bar in A-C=10 μm. (**D-F**) Confocal images showing a result of dual immunofluorescence labeling for tryptase and CD117. (**D**) CD117 (red) is expressed in tryptase-positive (green) mast cells (arrowheads) localized in the interstitial spaces between adipocytes in SAT. Scale bar=50 μm. (**E**) A confocal image of a mast cell in EAT co-expressing CD117 (red) and mast cell tryptase (green). Scale bar=10 μm. (**F**) CD117 (red) is expressed in mast cell tryptase-positive mast cell (green) localized in the endomysium of RA myocardium. Nuclei are stained with DAPI (blue). Scale bar=10 μm.

patients compared to non-CAD patients (Table 4, Fig. 2). There was no statistically significant difference in the number of tryptase-positive mast cells in EAT when obese and non-obese and T2DM and non-T2DM patients were compared and neither in other anatomical locations we found significant differences between selected groups of patients (Table 4).

Further characterization of mast cell populations in EAT, SAT and RA

To characterize mast cell population in EAT, SAT and RA based on expression of some other mast cell markers we performed immunohistochemical detection of chymase and CD117. Chymase-positive cells had similar morphology and localization pattern like tryptase-positive mast cells. They were detected in all patient samples from all anatomical locations (Fig. 1 D-F). To confirm the identity of these cells, we performed a dual immunofluorescence labeling using antibodies against chymase and tryptase (Fig. 3A-C). Double-positive cells were detected in all tissue samples from all patients. No cells were positive for only one cell marker and all double-positive cells displayed mast cell morphology.

Using antibody against CD117 marker, we observed cells in the SAT, EAT as well as in RA that corresponded to mast cells based on their localization and morphology (Fig. 1G-I). To verify the localization of CD117 immunoreactivity, a dual immunofluorescence labeling of CD117 and mast cell tryptase were performed. In all samples of SAT, EAT and RA, only CD117/mast cell tryptase-double positive cells were found (Fig. 3D-F).

The higher number of mast cells could possibly be a result of changes in adipocyte diameter in EAT during CAD that would lead to a relative expansion of interstitial connective tissue. We measured adipocyte diameter on fraction of samples and we did not find a significant difference between adipocyte diameter in EAT, when CAD and non-CAD patients were compared (data not shown).

Discussion

Immune cells of various types are part of white adipose tissue and their numbers change during chronic inflammation of adipose tissue, which accompanies obesity and some other related pathologies (Dam *et al.* 2016). In this study, mast cell numbers and phenotype were evaluated in SAT, EAT as well as in atrial myocardium of patients undergoing heart surgery. We found a higher infiltration of mast cells in EAT of patients with CAD compared with non-CAD patient group. In addition, mast cells in adipose tissue and myocardium displayed characteristic markers of MC_{TC} population and all mast cells were positive for CD117 marker. No other CD117+ cells were detected in these samples.

This is the first report of mast cells number in EAT of patients with CAD, obesity or T2DM. Mast cells were previously recognized as part of the inflammatory cell infiltrate in EAT of CAD patients but comparison of their number between CAD and non-CAD groups were not performed (Mazurek *et al.* 2003). The increased infiltration of mast cells in CAD patients corresponds to findings in other inflammatory cell populations. Macrophages are elevated in EAT of CAD patients as shown in previous reports (Hirata *et al.* 2011). The chronic inflammatory process in EAT during CAD is not only accompanied by an increase of the total number of macrophages, but there is also a shift towards a pro-inflammatory phenotype (Gurses *et al.* 2016, Vianello *et al.* 2016). Such pro-inflammatory macrophages form a typical morphological feature of adipose tissue called crown-like structures, which are characteristic for EAT during CAD (Pierzynova *et al.* 2019). In diet-induced obese mice, mast cells associate with CLS (Altintas *et al.* 2011), but in adipose tissue samples of CAD patients we did not observe such tendency. Also T-lymphocytes are elevated in EAT of patients with CAD (Mraz *et al.* 2019). Thus, based on these reports, the elevated mast cell numbers in EAT of CAD patients suggest their involvement in chronic adipose tissue inflammation together with other cells responsible for both innate as well as acquired immunity.

Mast cells were investigated in association with CAD due to their infiltration of the coronary artery wall (Kovanen and Bot 2017). They are implicated in several different steps along the progression of atherosclerotic process. Their numbers are increased in the intima and other layers of the coronary artery wall and they can act by secretion of both pre-formed as well as newly synthesized factors (Theoharides *et al.* 2011). The potential mechanisms that can be attributed to mast cell effects on atherosclerotic process are stimulation of leukocyte immigration, an increase of vascular permeability facilitating lipoprotein accumulation, foam cell formation and foam cell apoptosis, apoptosis of endothelial cells leading to vascular wall erosion and

thrombosis, effects promoting plaque destabilization and rupture such as apoptosis of smooth muscle cells, collagen proteolysis and stimulation of neoangiogenesis and intraplaque hemorrhage from fragile microvessels (Kovanen 2019). Based on our findings it is likely that the inflammatory process does not affect the vascular wall only, but it spreads throughout the periadventitial adipose tissue into the surrounding EAT. The main finding of our study is an increase of mast cell number in EAT of CAD patients and this can be the result of increased mast cell migration into EAT. Although the principle chemoattractant and driver of mast cell proliferation is stem cell factor (Okayama and Kawakami 2006), it was reported that also adipokines such as leptin can stimulate mast cell migratory response (Hristova 2001, Zelechowska et al. 2019). This is quite likely scenario, because leptin production is increased in EAT of CAD patients (Cheng et al. 2008, Gruzdeva et al. 2017). The question of mast cell stimulation during atherosclerosis is best answered in studies dealing with mast cell activity within the atherosclerotic coronary wall (Kovanen and Bot 2017). Coronary artery mast cells can be stimulated by immune complexes of IgG with oxidized LDL particles (Lappalainen et al. 2011), complement system (Laine et al. 2002), neuropeptides secreted from nerve endings in the coronary artery adventitia (Laine et al. 2000, Bot et al. 2010), CRH released during stress (Alevizos et al. 2014) and TLR-4 ligands such as oxidized LDL (Meng et al. 2013). Hypothetical stimuli of mast cells residing in the pericoronary EAT may include those mentioned above, but EAT mast cells can be also stimulated by an excess FFA, possibly acting thorough TLR binding of FFA (Agier et al. 2018, Rogero and Calder 2018, Iacobellis and Barbaro 2019). Furthemore adipokines secreted by EAT may also stimulate the secretory activity of mast cells (Zelechowska et al. 2019). The question whether the inflammation starts in the EAT or in the vascular wall cannot be resolved based on our observational study since we analyzed patients with a previous history of CAD. Most likely, there is a bidirectional signaling and inflammatory cell migration between the atherosclerotic artery and the surrounding adipose tissue. Increased infiltration of mast cells in EAT perpetuates a chronic inflammatory state of adipose tissue and while the activity of mast cells within the coronary artery wall have more direct influence on atherosclerotic process (Kovanen 2019), mast cells in EAT might act indirectly. Through secretion of several different factors, mast cells

can stimulate T-lymphocytes (Hershko and Rivera 2010) and they activate macrophages as well (Xu and Shi 2012). Both macrophages and T-lymphocytes were repeatedly implicated in chronic inflammation of EAT associated with CAD and other pathologies (Hirata et al. 2011, Gurses et al. 2016, Vianello et al. 2016, Mraz et al. 2019). In contrast to EAT, in the SAT it was found previously that mast cell numbers increase in certain pathological conditions and contribute to the chronic inflammation of adipose tissue (Zelechowska et al. 2018). The study of mast cells in adipose tissue during obesity was initiated both in humans as well as in experimental animals by observations of mast cell increase in obesity (Liu et al. 2009). In contrast to this and another report (Spencer et al. 2014), we did not find any difference in mast cell numbers between obese and non-obese patients undergoing heart surgery neither in SAT nor in EAT. This can be due to differences in the study population or the specific SAT depot used for analysis in our and other studies. Interestingly, in diet-induced obese mice there was no difference between mast cell numbers in SAT depots, but obese mice had more mast cells in mesenteric, perinephric and epididymal fat (Altintas et al. 2011). Furthemore, in a human study, no difference between mast cell numbers was found when both subcutaneous as well as omental adipose tissue was compared between obese and lean subjects (Divoux et al. 2012). Nevertheless, there was an increased mast cell infiltration of both adipose tissue depots in obese diabetic patients compared to lean and obese non-diabetic patients. In our study, we could not subdivide obese patient group according to presence of diabetes due to the low number of patients enrolled.

For the quantitative analysis we used tryptase as a well-accepted and ubiquitous marker of mast cells (da Silva et al. 2014). To further characterize mast cell population in the adipose tissue and myocardium, we also performed the detection of chymase and we document that mast cells in these locations belong to MC_{TC} population typically found for example in the skin. We also evaluated the expression of CD117 in the samples of EAT, SAT and in the myocardium. Although CD117 is also well-known marker of mast cells, there are reports of other CD117+ cells in the heart as well as in the epicardium, which are by some authors considered as a special type of cells called telocytes (Popescu et al. 2010, Varga et al. 2017). In addition, in the heart, CD117 marker was reported to be present in cardiac stem or progenitor cells (Bearzi et al. 2007). The use of CD117 as

a marker of telocytes or marker of cardiac stem cells was questioned in other reports (Zhou *et al.* 2010, Iancu *et al.* 2019) and based on the results of double-labeling immunohistochemistry performed in the present study, we can conclude that the great majority of CD117+ cells in SAT, EAT and atrial myocardium are mast cells.

This study focused on the quantitation of mast cells in EAT, SAT and myocardium. We characterized mast cell population only partially with respect to several mast cell markers. Nevertheless, these findings open up possibility of further studies focusing on detection of specific secretory products of mast cells in EAT during CAD.

In conclusion, our data support the concept that mast cells constitute an important part of inflammatory cell infiltration of EAT associated with CAD. The specific role of EAT-based mast cells during atherosclerotic process has to be further investigated, possibly through not only advanced experimental animal studies but also prospective human studies.

Conflict of Interest
There is no conflict of interest.

Acknowledgements

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