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Macrophage Phenotypes in the Adipose Tissue of Postmenopausal Women

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Summary

Atherosclerosis pathology is the interplay between high intravascular LDL particle concentration and monocyte/ macrophage presence within the sub-endothelial space of the artery. In this project, phenotypes of macrophages connected with subclinical inflammation in adipose tissue of living kidney donors were studied. Samples of subcutaneous adipose tissue of living kidney donors (n=36) were exposed to collagenase. Stromal vascular fraction (SVF) was eluted from the samples, then labeled with monoclonal antibodies (anti-CD14 and anti-calprotectin), conjugated with fluorochromes and analyzed by flow cytometry. The positive correlation between the number of total macrophages and calprotectin-positive macrophages with BMI in the subcutaneous adipose tissue of postmenopausal women was demonstrated ($p<0.05$; $R=0.43$ and $p<0.01$; $R=0.60$), whereas no positive correlation in premenopausal women and men was shown. In conclusion, we documented a significant effect of BMI increase on the presence of total macrophages in adipose tissue of postmenopausal women, in contrast to premenopausal women. This difference was much more pronounced when proinflammatory macrophages with membrane-bound calprotectin were analyzed.

Key words

Menopause • Adipose tissue • Inflammation • Macrophages • Calprotectin

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Introduction

Atherosclerosis pathology is the interplay between high intravascular low-density lipoprotein (LDL) particle concentration and monocyte/macrophage presence within the sub-endothelial space of the artery (Libby *et al.* 2011). Relationship and causality of increased cholesterol has been described in detail (Goldstein and Brown 2015). Although atherosclerosis is rare in premenopausal women, incidence increases after menopause (Rosano *et al.* 2007). These two phases might be related to inflammation as we found that concentration of high-sensitivity C-reactive protein (CRP) starts to increase only after menopause, whereas it increases continuously with age in men (Lorenzová *et al.* 2006). However, the mechanism of inflammation changes and the participation of macrophages in this process after menopause are not fully understood yet.

Recent evidence highlights the role of adipose tissue enlargement in the development of the systemic pro-inflammatory state, which might influence several pathologies like dyslipidemia, type 2 diabetes mellitus, hypertension and metabolic syndrome (Berg and Scherer

2005). It has been found that individuals with adipose tissue enlargement more often have metabolic disorders as well as elevated plasma lipoprotein concentrations, free fatty acids and cholesterol in the blood, hypertension and hyperinsulinemia. Adipose tissue enlargement, which is associated with low-grade inflammation (sub-clinical inflammation) and characterized by a slight increase of pro-inflammatory cytokines (Medzhitov 2008), plays a central role in the development of insulin resistance (Heilbronn and Campbell 2008). It has been documented that individuals with adipose tissue enlargement also exhibit higher levels of proinflammation markers and cytokines in the blood, such as CRP and IL-6, compared to non-obese individuals (Cottam *et al*. 2004).

Adipose tissue consists of adipocytes (mature fat cells) and stromal vascular fraction (SVF) cells formed by the extracellular matrix, pre-adipocytes, fibroblasts and endothelial and immune cells (Curat *et al.* 2004). Adipose tissue immune cells comprise almost the full spectrum of immune cell types (Schipper *et al.* 2012) and adipose tissue macrophages represent the largest subpopulation (Weisberg *et al.* 2003). Immune cells play an important role in tissue housekeeping, removal of apoptotic cells and tissue-homeostasis maintenance (Schipper *et al.* 2012). Initial evidence for the importance of infiltration of adipose tissue macrophages in experimental models with adipose tissue enlargement appeared in 2003 in the works of Weisberg *et al.* (2003). The same effect was then confirmed in human visceral adipose tissue. The proportion of adipose tissue macrophages in lean visceral fat is around 1-5 % of all cells, whereas in obese adipose tissue the proportion is several times higher (Harman-Boehm *et al.* 2007).

It is assumed that not just the number, but also the proportion of different macrophage phenotypes might play an important role. Because of macrophage diversity there has been an effort to categorize them by the M1/M2 system based mainly on their properties in *in vitro* systems (Mills 2012). M1 macrophages (classically activated) have pro-inflammatory potential, whereas M2 macrophages (alternatively activated) are mainly anti-inflammatory cells. In adipose tissue of lean individuals, macrophages that disperse among adipocytes mainly exhibit the M2 phenotype, whereas there is a shift from the anti-inflammatory M2 to the pro-inflammatory M1 phenotype in individuals with adipose tissue enlargement (Mills 2012). However, it seems that local micro-environmental changes can stimulate varying combinations of M1/M2 responses, resulting in overlaps and broad spectra between type 1 and type 2 macrophage phenotypes *in situ*. It is evident that the M1/M2 system (repeatedly mentioned in review articles) is too simplified to adequately describe the heterogeneity and function of macrophages in tissues (Zeyda *et al.* 2007). All macrophages in adipose tissue originate either from bone-marrow-derived monocytes, which infiltrate the tissue from circulation, or by *de novo* formation (Amano *et al.* 2014). Unfortunately, the difference between M1 and M2 phenotypes is the subject of on-going discussion and has not been clarified yet.

An alternative approach is to characterize resident and partially-differentiated macrophages by the presence of calprotectin (Stříž and Třebichavský 2004). Calprotectin, a complex of calcium- and zinc-binding proteins, is made up of S100A8/S100A9 (also known as migration inhibitory factor-related proteins 8 and 9 – MRP-8 and MRP-9) and is constitutively expressed and secreted by the myeloid cell lineage. Two forms of calprotectin exist – the soluble form, released and secreted mainly by neutrophils and activated monocytes, and the plasmatic membrane-integrated form. The main role of the membrane form of calprotectin seems to be cell adhesion (Mahnke *et al.* 1995), but several other roles have been described (Johne *et al.* 1997). It has been proven that calprotectin is expressed in monocytes and activated macrophages, but its amount decreases with increasing differentiation (Johne *et al.* 1997). As calprotectin is present on infiltrating tissue macrophages during acute inflammation but not on resident tissue macrophages (Stříž and Třebichavský 2004), it is assumed to be a marker of macrophage influx.

Recently, our transplantation program was substantially expanded by enlarging living kidney donor system. It makes possible to analyze the presence and phenotype of macrophages in human subcutaneous adipose tissue obtained per operatively. In the present study, we studied the relationship between macrophage phenotypes in subcutaneous adipose tissue and some risk factors of atherosclerosis. We focused on the association between monocyte number and phenotype on obesity in healthy living women kidney donors of different ages, reflecting premenopausal or postmenopausal statuses.

Methods

Study participants

Subcutaneous adipose tissue was obtained from living kidney donors during kidney removal

(n=36; 11 men, 15 premenopausal women and 10 postmenopausal women). Clinical data were collected from clinical documentation of enrolled subjects and from an interview targeted at life style factors. Prior to enrolment in the study, each subject was thoroughly informed about the study and an informed consent form was signed. The study was approved by the local Ethical Committee.

Immune cell isolation and analysis

Samples of approximately 2.5 g of subcutaneous adipose tissue were cleaned of connective tissue, blood vessels and blood residue. The samples were then minced with scissors and exposed to collagenase (c=0.002 g/ml, Sigma-Aldrich) and purified by subsequent filtration by two filters (150 μm and then 50 μm). The obtained stromal vascular fraction was then labeled with monoclonal antibodies conjugated with specific fluorochromes and subsequently analyzed by flow cytometry. The same labeling procedure was applied to the whole blood of studied subjects and analyzed after lysis of erythrocytes.

In order to analyze specific surface markers, the following antibodies were used: anti-CD14 – Phycoerythrin Cyanin 7 (PC7), Beckman Coulter and anti-calprotectin – Fluorescein isothiocyanate (FITC), the clone 27E10, Acris Antibodies. Corresponding isotype controls were used.

Incubation with antibodies lasted 20 min at room temperature in the dark. The Cyan ADP DAKO flow cytometer was used for flow cytometry analysis.

To delineate the gate for calprotectin-positive cells, analysis of peripheral blood monocytes was firstly completed and used for consequent SVF analysis. The initial gates were set on leukocytes based on side scatter (SSC) and forward scatter (FSC), from which the CD14 cell gate was set. Within the CD14 gate, calprotectin-positive cells were identified. When flow cytometry analysis was compared between peripheral blood and the subcutaneous adipose tissue of living healthy kidney donors, significant differences in the expression of the calprotectin marker were observed. High expression of calprotectin (around 40 %) in SVF macrophages of living kidney donors was found, whereas blood monocytes showed low expression of calprotectin (0-5 %). For further analysis, an absolute number of monocyte/ macrophage cell lines per gram of adipose tissue was used and the dilution and amount of adipose tissue applied was calculated. In this article, macrophages are defined as CD14-positive cells.

The viability of analyzed cells was measured for every sample using the 7-aminoaktinomycin D (7-AAD) method and only samples with a viability higher than 75 % were considered.

Statistics

Data are presented as means with standard deviations for continuous variables and percentages with standard deviations for categorical variables. Between-group comparisons of continuous and discontinuous variables were performed using the unpaired Student's t-test and Chi-Square test, respectively. Linear regression was used for modeling the relationship between the proportion of macrophages under study and BMI. In all tests, p values less than 0.05 were considered statistically significant.

Results

The total number of enrolled living kidney donors was 36 (11 men and 25 women). There was no difference in age between men (n=11) and premenopausal women (n=15) whereas ages of the postmenopausal women group (n=10) was obviously higher. The percentage of male smokers was higher compared to the whole group of women. There were less smokers in the postmenopausal group of women, which, importantly, seems to point to the possible higher proinflammatory status of smokers. Surprisingly, the prevalence of hypertension and hyperlipoproteinemia treatment was lower in men, whereas in whole Czech population this relationship is opposite (Cífková *et al.* 2010) (Table 1).

A weak positive correlation of the total number of macrophages to BMI in subcutaneous adipose tissue in the whole group of living kidney donors was found ($p<0.05$, $R=0.12$). When the marker of pro-inflammatory macrophages – calprotectin – was included, the correlation was higher ($p<0.02$, $R=0.15$). In subcutaneous adipose tissue of living kidney donors the average number of macrophages per gram was 10 440 and calprotectin-positive macrophages per gram 7 550 respectively.

When comparing the relationship of age and effect of smoking, we did not find substantial differences between smokers and non-smokers either in the whole group or in men and women separately.

Table 1. Characteristics of healthy kidney donors. Values are expressed as means and standard deviations (SD) and prevalence of factors involved.

	Men (n=11)	Premenopausal women (n=15)	Postmenopausal women (n=10)	Significance (pre x post)	Significance (pre x men)	Significance (post x men)
Age	43.7 (SD 14.0)	43.7 (SD 5.1)	55.3 (SD 5.0)	p<0.0001	n.s.	p<0.03
BMI [kg/m^2]	28.2 (SD 4.0)	24.9 (SD 3.7)	25.5 (SD 3.9)	n.s.	p<0.04	n.s.
% of smokers	45.5	33.3	20.0	n.s.	n.s.	n.s.
% of individuals with hypertension	9.1	20.0	30.0	n.s.	n.s.	n.s.
% of individuals on statin therapy	0	6.7	30.0	n.s.	n.s.	n.s.

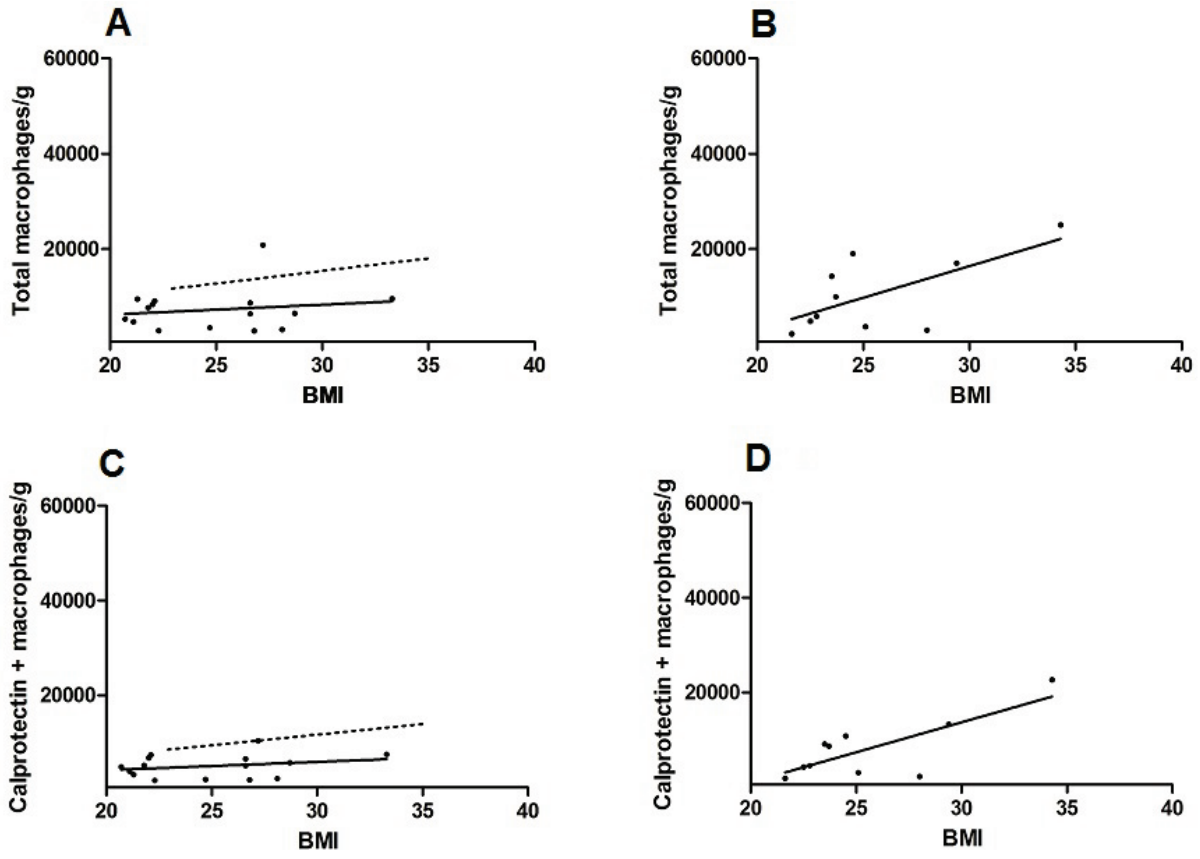

Fig. 1. Relation of total macrophages (per gram) to BMI in premenopausal women (**A**; n=15) and men (**A**; n=11) in subcutaneous adipose tissue. Relation of total macrophages (per gram) in postmenopausal women (**B**; n=10, p<0.05; R=0.43) in subcutaneous adipose tissue. Relation of calprotectin-positive macrophages (per gram) to BMI in premenopausal women (**C**; n=15) and men (**C**; n=11) in subcutaneous adipose tissue. Relation of calprotectin-positive macrophages (per gram) to BMI in postmenopausal women (**D**; n=10, p<0.01; R=0.60) in subcutaneous adipose tissue. In the case of men, the slope of line is illustrated by the dotted line.

Women were further divided into groups of premenopausal age (younger than 51 years, n=15) and postmenopausal age (51 years and older, n=10) (Lejsková *et al.* 2012). Menopausal status of women was later confirmed by analysis of follicle-stimulating hormone (FSH) concentration. All women in the postmenopausal group exceeded the cut of point for menopause with only one exception (in this instance the menopause of this individual was controlled by correspondence questionnaire). While the positive correlation (p<0.05; R=0.43) of total macrophages with BMI in subcutaneous adipose tissue was observed in postmenopausal women, no

such relationship was found in premenopausal individuals. The slope of line for this relationship in premenopausal women resembles the slope of line in the group of men (individual data are not presented) (Fig. 1A and 1B). These differences were more pronounced when the correlation of the number of calprotectin-positive macrophages to BMI was analyzed. There was no significant relationship between the number of calprotectin-positive macrophages and BMI in premenopausal women (resembling the same relationships in men) (Fig. 1C). The relationship between the number of calprotectin-positive macrophages and BMI in the subgroup of postmenopausal women was more pronounced ($p<0.01$; $R=0.60$) (Fig. 1D), compared to total macrophage analysis.

Discussion

In the present study, the group of living kidney donors and their stromal vascular fraction was analyzed to find a possible relationship between subclinical inflammation of adipose tissue and BMI. The group of living kidney donors represents more or less healthy individuals of the Czech population. There were no or negligible differences in BMI, but it must be noted that the entire group of healthy living kidney donors was leaner compared to the representative population sample of the Czech MONICA study (Cífková *et al.* 2010). Similar prevalence of smoking and hypertension was also documented and first stage hypertension was discovered as a result of a very detailed investigation into enrolled donors.

Subclinical inflammation in adipose tissue and its relation to BMI as one risk factor of atherosclerosis has been repeatedly described (Gustafson 2010) with a focus on macrophages, the prevailing populations of adipose tissue immune cells. Nevertheless, definitions of pro-atherogenic cells (M1) and athero-protective cells (M2) are still not completely understood. Also, recent reviews describing two extreme macrophage phenotypes, M1 and M2, state that in spite of extensive efforts focused on different surface markers, gene expression and metabolic activities, results are not consistent enough to easily distinguish between these two types (Mills *et al.* 2015, Italiani and Boraschi 2014). Therefore, we analyzed the proinflammatory potential of macrophages in adipose tissue of healthy individuals based on the positivity of membrane-bound, calprotectin. Calprotectin has been repeatedly described to be a marker of macrophage influx (Johne *et al.* 1997) and it seems plausible that calprotectin-positive macrophages are related to acute proinflammation at a local level (Stříž and Třebichavský 2004, Šplíchal *et al.* 2002).

The number of total macrophages in subcutaneous adipose tissue of kidney donors is in agreement with the literature (Zeyda *et al.* 2007, Bourlier *et al.* 2008). When the total number of macrophages in this tissue was analyzed in a combined group of subjects, a slight but significant correlation to BMI was documented in agreement with earlier published data (Zeyda *et al.* 2007, Bourlier *et al.* 2008). Since we also found a positive correlation of total macrophage presence to age in women, we decided to include parameters of premenopausal and postmenopausal individuals.

The total number of macrophages per gram of adipose tissue in 15 premenopausal women did not correlate at all with BMI in the range of 21-33. Therefore, there was no effect of adipose tissue mass on macrophage infiltration. This relationship resembles situation we found in 11 men (Fig. 1A). On the other hand, the relationship of BMI and macrophage infiltration was more positive in the subgroup of postmenopausal women (Fig. 1B). Therefore, it is reasonable to assume that previously published data, in which age and sex have not been considered, have been unable to discover a possible effect of menopause.

At this time, no published data related to plasmatic membrane calprotectin expression in any adipose tissue type are available, even though this problem has been repeatedly studied in several inflamed tissues (Oshitani *et al.* 1997, Zheng *et al.* 1995). When calprotectin was included for analysis, the pattern of the number of calprotectin-positive macrophages to BMI resembled the data of total macrophage numbers in premenopausal women and men (Fig. 1C). The relationship between calprotectin-positive macrophage numbers and BMI in subcutaneous adipose tissue of postmenopausal women displayed higher significance ($p<0.01$; $R=0.60$) (Fig. 1D) compared to total macrophage numbers ($p<0.05$; $R=0.43$) (Fig. 1C).

As calprotectin-positive macrophages are supposed to be proinflammatory, our data suggest significant changes of proinflammatory status in adipose tissue after menopause. The relatively steeper increase of calprotectin-positive macrophages with the amount of increasing adipose tissue in postmenopausal women play a substantial role in the accerelation of proinflammatory status after menopause (Lorenzová *et al.* 2006). It is important to stress that the presented relationships were

documented in healthy individuals who were of normal weight or slightly overweight.

In conclusion, we documented a higher significant effect of BMI increase on total macrophage infiltration in adipose tissue of postmenopausal women, compared to premenopausal women. This difference was much more pronounced when the phenotypes of proinflammatory macrophages, tested for the presence of membrane-bound calprotectin, were analyzed. Therefore, calprotectin-positive macrophage infiltration might be an important part of the repeatedly documented higher risk of cardiovascular mortality in women after menopause.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Abbreviations

LDL, low-density lipoprotein; CRP, C-reactive protein; SVF, stromal vascular fraction; FITC, fluorescein isothiocyanate; PC-7, phycoerythrin cyanin-7; SSC, side scatter; FSC, forward scatter; 7-AAD, 7-aminoaktinomycin D; BMI, body mass index; SD, standard deviations; FSH, follicle-stimulating hormone; MRP, migration inhibitory factor-related protein.

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