

Pro-inflammatory gene expression in adipose tissue of patients with atherosclerosis

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Short title: Adipose tissue gene expression

Summary

Inflammatory changes, both in the arterial wall and adipose tissue, play a crucial role in the development of atherosclerosis. We measured the gene expression of tumor necrosis factor-alpha (TNF α), monocyte chemoattractant protein-1 (MCP-1), and interleukin 6 (IL-6) in adipose tissue (AT) of living kidney donors (LKD) and patients with peripheral arterial disease (PAD). Quantitative polymerase chain reaction (qPCR) and flow cytometry analyses were performed in subcutaneous (SAT), visceral (VAT), and perivascular adipose tissue (PVAT).

Data of PAD patients showed significantly higher expression in VAT in all three genes (TNF α 5-fold, $p < 0.05$; MCP-1 3.6-fold, $p < 0.05$; IL-6 18.8-fold, $p < 0.001$). The differences in PVAT and SAT were less significant. Total body pro-inflammatory status was documented by higher TNF α concentration in patients (4.86 ± 1.4 pg/ml) compared to LKDs (2.14 ± 0.9 pg/ml; $p < 0.001$), as was hsCRP (11.8 ± 7.0 in PAD; 1.5 ± 0.48 in LKDs; $p = 0.017$). We found no age-dependent relationship between gene expression vs. TNF α and hsCRP concentrations in both compared groups. No effect of the atherosclerosis score on gene expression and circulating inflammatory markers within the PAD group was observed.

Our results suggest that the AT of PAD patients infiltrated with macrophages produces more cytokines involved in the development of inflammation and atherosclerosis.

Key words: atherosclerosis, adipose tissue, gene expression

Introduction

Adipose tissue (AT) inflammation is typical for insulin resistance and dyslipidaemia triggering atherogenic subclinical inflammation (Alexopoulos et al., 2014). Traditionally, AT is distributed into two main different deposits: subcutaneous AT (SAT) and visceral AT (VAT). Although abdominal obesity was implicated as a risk factor of diabetes and atherosclerosis development as early as the 1950s (Vague, 1956), its role in these processes is still not fully understood. The extent of endocrine, paracrine, and autocrine activities involved in atherosclerosis development was first described in the 1990s (Funahashi et al., 1999). These activities do not only occur in adipocytes but, also, take the form of macrophages infiltrating AT (Suganami and Ogawa, 2010; Chawla et al., 2011), which may be a link between obesity and development of cardiovascular disease. Adipose tissue produces a variety of pro- and anti-inflammatory bioactive substances, such as monocyte chemoattractant protein 1 (MCP-1), leptin, adiponectin, interleukins (IL-1, IL-6, IL-8, etc.), and tumor necrosis factor-alpha (TNF α) (Sopasakis et al., 2005).

Not only the number of infiltrated macrophages but, also, the imbalance between pro- and anti-inflammatory phenotypes of macrophages might be involved in the risk of developing pro-inflammatory status and, subsequently, risk of developing cardiovascular disease (Ridker et al., 1997). Cytokines released from macrophages stimulate monocyte/macrophage infiltration and contribute to further cytokine production. All these changes induce inflammatory status of the body.

In general, VAT is considered to have a higher impact on pro-atherogenic changes, as it correlates with higher oxidative stress, chronic low-grade inflammation, and insulin resistance (Fox et al., 2007; Pou et al., 2007). Likewise, VAT is more infiltrated by macrophages (Jonas et al., 2015), which contributes to the more prominent inflammatory changes compared to SAT. Perivascular AT (PVAT) is a special subtype of VAT that surrounds blood vessels. While this type of VAT was long considered only a connective tissue supporting blood vessels, it has been recently suggested it might play a direct role in atherosclerotic plaque formation (Chang et al., 2013; Britton et al., 2012; Brown et al., 2014). Similar to VAT, PVAT produces many cytokines, including MCP-1, TNF α , IL-6, and IL-8. In addition, paracrine activity can have an effect on vessels; however, studies are conducted mostly in animal models (Brown et al., 2014).

This study focuses on comparisons of the pro-inflammatory gene expression in SAT, VAT and PVAT of patients with atherosclerosis (documented by angiography of the peripheral arteries) to healthy living kidney donors (LKDs). Out of the many potential genes that contribute to the inflammatory environment in AT, we focused on three cytokines: TNF α , MCP-1, and IL-6. TNF α is one of the most

important pro-inflammatory cytokines (Kleinbongard et al., 2010), MCP-1 contributes mainly to monocyte adhesion and tissue infiltration (Kanda et al., 2006; Suganami and Ogawa, 2010) whereas IL-6 is involved predominantly in lipid metabolism (Litvinova et al., 2014; Sadashiv et al., 2015) and is considered the most important player in inflammatory processes. Cytokine production of adipocytes and preadipocytes (Zdychova et al., 2012) is probably influenced by infiltrated macrophages. Given the above facts, we analysed gene expression together with macrophage infiltration in both studied groups.

Methods

Adipose tissue was obtained intraoperatively either from LKDs during kidney transplantation, or during peripheral arterial tree reconstruction in patients with angiographically documented peripheral arterial disease (PAD). Visceral AT and PVAT of LKDs were obtained during cleansing of isolated kidney (VAT from the area outside Gerota's fascia, PVAT surrounding the renal artery). Similarly, these samples were obtained intraoperatively in the patient group (with the exception of 30% of PVAT obtained from periaortic AT). Subcutaneous AT was obtained from similar sites in both groups.

All healthy LKDs and patients scheduled for arterial tree reconstruction were informed about the study in detail and signed informed consent forms. The study was approved by the Institute's Ethics Committee.

Stromal vascular fractions of SAT, VAT, and PVAT were isolated for macrophage analysis by flow cytometry. The stromal vascular fraction was separated using a procedure which was in accordance with Zuk (Zuk et al., 2001) with a minor modification. Briefly, all visible blood vessels and connective tissues were removed and cut into small pieces (2 mm). About 2 grams of the AT obtained was exposed to collagenase ($c=0.002$ g/ml, Sigma-Aldrich, St. Louis, MO, USA) and then repeatedly filtered and purified. The obtained stromal vascular fractions were analysed on the same day using a CyAn ADP 9C flow cytometer using the Kaluza software (Beckman Coulter, Brea, CA, USA) and monoclonal antibody (CD14 – Phycoerythrin-(PE)-cyanine 7, Beckman Coulter, Brea, CA, USA) was used to identify monocytes/macrophages. The number of CD14+ monocytes/macrophages per gram of the AT used was finally counted.

Luminex performance assays (R&D Systems Inc, Minneapolis, MN, USA) were used to measure cytokine concentrations in blood plasma samples of the LKDs and PAD patients. The Luminex assay is a multiplex bead-based immunoassay, which allows for simultaneous measurement of multiple analytes using a library of antibody-coupled colour-coded beads. The analysis was done according to the manufacturer's instructions. Plates were read using a Luminex 100 analyzer (PerkinElmer Life Inc., Boston, MA, USA) and xPONENT software (Luminex, Austin, TX, USA). The Mann-Whitney test was used for statistical analysis. High-sensitivity C-reactive protein (hsCRP) was measured using an immunoturbidimetric assay with an autoanalyzer (Cobas Mira Plus, Roche, Basel, Switzerland).

Using common criteria and a scoring system, PAD patients were stratified into three groups (A, B, C) based on the severity of stenosis or clog/blockage of the aorta, common iliac artery, and external iliac artery (Hardman et al., 2014). Our PAD group was then divided into two subgroups only, A and

B, as only 2 individuals were assigned to group C; hence our analysis subsequently involved two groups with arteries showing mild or severe involvement.

Samples for gene expression analysis were frozen immediately in liquid nitrogen. Total RNA was isolated from the whole visceral, perivascular and subcutaneous adipose tissue of LKDs and patients with angiographically documented atherosclerosis. Approximately 200 mg of each tissue was used for total RNA extraction with TRIzol Reagent (Molecular Research Centre, Cincinnati, OH, USA). 100 ng of total isolated RNA was used for reverse transcription. After DNase I (Sigma Aldrich, St. Louis, MO, USA) treatment to eliminate DNA from the RNA preparations, cDNA was generated according to the manufacturer's instructions using the High Capacity RNA-to-cDNA Master Mix kit (Life Technologies, Carlsbad, CA, USA). Levels of gene expression of the cytokines and adipokines of interest were subsequently determined on the Corbett Life Science Rotor Gene 3000 (Qiagen, Venlo, the Netherlands) using the QuantiTect SYBR Green PCR Kit (Qiagen, Venlo, the Netherlands). The nucleotide sequences of the primer pairs of TNF α (tumor necrosis factor α , NM_000594.3), MCP-1 (CCL2, C-C motif chemokine ligand 2, NM_002982.3) and IL-6 (Interleukin 6, NM_000600.4) used in the study are given in Table 1. Beta-2-microglobulin (B2M, NM_004048.2) levels were used as endogenous controls for normalization.

Relative gene expression was calculated using the $\Delta\Delta C_t$ method (Pfaffl, 2001) compared to the appropriate AT. The Kruskal-Wallis test with Dunn's post multiple comparison test was used for statistical analysis of differences between the groups compared, the Mann-Whitney test for two-group comparison, and the Friedman test for paired comparisons. The results were considered statistically significant with a $p < 0.05$.

Results

When comparing the characteristics of the group of PAD patients and that of healthy LKDs (Table 2), it was evident that the patients were significantly older compared to LKDs. To analyse this possible limitation, we compared individuals below and over 50 years of age in all parameters analysed within the LKDs group and found no significant differences when younger and older subgroups were compared in all three gene expression categories as well as in intra-vassal inflammatory marker concentrations. Living kidney donors were slightly leaner but this difference was not statistically significant. It is understandable that the prevalence of smoking as well as that of hypertension and type 2 diabetes was substantially higher in patients with proven atherosclerosis compared to healthy donors. The prevalence of smoking in the group of patients with PAD was almost three times, and the prevalence of hypertension more than twice that compared to LKDs. Only one living kidney donor had a higher fasting glucose concentration (>7 mmol/l), but the presence of diabetes was not clinically confirmed. It should be noted that the main difference was observed in the prevalence of smoking so the fact that almost all patients were smokers may have played an important role in this finding.

The concentration of TNF α in blood plasma was significantly higher (4.86 ± 1.4 pg/ml) in the PAD group compared to LKDs (2.14 ± 0.9 pg/ml) with a high level of significance ($p < 0.001$). A similar pattern was found in hsCRP levels between the groups (11.79 ± 7.0 in PAD and 1.5 ± 0.48 in LKD), but the significance was rather small ($p = 0.017$) because of high variation in both groups. The concentration of IL-6 was also higher in the PAD group, but this parameter was under the sensitivity limit of the method in the majority of LKDs (0.54 pg/ml). In agreement with accelerated pro-inflammatory status of patients compared to LKDs, measured by hsCRP concentration, also concentration of both pro-inflammatory cytokines (TNF α , IL-6) was higher in PAD patients.

As the production of cytokines whose expression was analysed is supposed to be predominantly in macrophages settled in the AT, their number per gram of each type of AT was analysed and compared between LKDs and the PAD patient group. There was no significant difference in the number of macrophages (CD14+) of the groups compared in VAT (11001 ± 10351 in LKDs, and 11457 ± 12462 in PAD patients) as well as in PVAT (11494 ± 12347 in LKDs and 15500 ± 13222 in PAD patients). The only difference of borderline significance was found in SAT (7411 ± 8527 in LKDs and 20721 ± 23312 in PAD patients). It is necessary to stress that the number of macrophages in any AT did not play a substantial role in the analysed gene expression. A quite high variation in the number of macrophages per gram in each of the compared groups was found.

All of the data related to gene expression are presented in Figs 1, 2, and 3. Generally, the expression of all the analysed genes tended to be higher in the PAD group compared to LKDs. Only the VAT of the PAD group showed significantly higher expression in all three genes analysed (TNF α 5-fold, $p < 0.05$; MCP-1 3.6-fold, $p < 0.05$; IL-6 18.8-fold, $p < 0.001$). Only TNF α gene expression in the PVAT of PAD patients was significantly higher compared to LKDs (4.6-fold, $p < 0.01$) whereas no other differences between the groups compared were statistically significant. We found no difference of gene expression in SAT.

When the LKD group was divided into subgroups of below and over 50 years of age, no difference was seen in gene expression. Despite a significant difference in age between LKDs and PAD patients, it is most unlikely that this fact might have played an important role in gene expression of the two groups.

When analysing the gene expression of the PAD patient group from the point of view of severity of the disease, measured by the degree of arterial stenosis and blockade, no significant difference in the gene expression of TNF α , MCP-1 as well as IL-6 was found, with the implication being that, within the rather small group of PAD patients, there were no differences in gene expression between mild or severe atherosclerotic lesions of peripheral arteries.

A comparison of LKD smokers and non-smokers did not reveal any differences.

Discussion

The main result of our study is documented higher gene expression of pro-inflammatory cytokines in AT in consistent with the blood plasma levels of pro-inflammatory cytokines in the PAD group compared to healthy individuals (living kidney donors). This finding could support the importance of AT in the development of atherosclerosis, in particular the role of VAT in this process. It has been repeatedly proved that the main changes affecting the pro-inflammatory status in AT are associated with monocyte/macrophage infiltration and pro-inflammatory changes of long-lasting residents within macrophage populations (Suganami and Ogawa, 2010; Chawla et al., 2011). These pathological changes produce not only a shift towards decreased sensitivity of AT to insulin but, also, induce further changes within the whole body (Suganami and Ogawa, 2010). It is still not clear if pro-inflammatory status is the result of insulin resistance or the other way round (Blüher, 2016). Whichever way it is, a role of AT macrophages in atherosclerotic changes in the arterial wall (Suganami and Ogawa, 2010; Alexander et al., 2003) has been definitely confirmed. The bulk of data describing these pro-atherosclerotic changes comes from animal models but adequate information about this pro-atherogenic process in humans is still lacking. Generally, there is little information about the inflammatory activity of human AT of non-obese individuals.

To assess the role of the indirect effect of AT on the general pro-inflammatory status in humans, we analysed the expression of three pro-inflammatory genes produced both by whole AT of healthy individuals (LKDs) and patients with PAD. The difference in the pro-inflammatory status of both groups was documented by higher concentrations of TNF α and hsCRP in the blood of patients.

Our data of increased expression of pro-inflammatory cytokines in PAD patients are essentially consistent with the data of Cheng et al. (2008). They found higher expression of IL-6 and TNF α in the PVAT and VAT of patients with coronary artery disease compared to those without coronary artery disease. The differences in the gene expression of visceral fat of patients with coronary artery disease reported by Cheng et al. are very similar to our data whereas, in PVAT, we documented a significant difference only in TNF α . It should be stressed that this similarity is present despite the very different design of both studies.

While, in our group of PAD patients, the expression of all three genes and all three types of AT was in mean data higher compared to that of LKDs, statistically significant differences were found only in all three genes in VAT, and TNF α in PVAT. The differences were present although the number of macrophages infiltrating VAT and PVAT was no higher (Figs 1-3). The absence of a change in the gene expression in SAT in our study is in line with data presented by Gerhard (Gerhard et al., 2014). Also

Bilgic Gazioglu (Bilgic Gazioglu et al., 2015) reported higher gene expression of the TNF α gene in patients with coronary artery disease compared to controls but their data may have been biased by obesity of patients. When summarising the differences in gene expression between LKDs and PAD patients it should be stressed that we did not find any effect of smoking or age on all three genes in either group.

Our data documenting an important role of VAT and PVAT in atherosclerosis development are in agreement with results of tissue cultures reported by Zdychova (Zdychova et al., 2012) and the findings of one experimental model (Chatterjee et al., 2009).

Certainly, one of the limitations of our data is the highly significant difference between the groups in age. When comparing younger and older individuals within the group of LKDs (cut-off point 50 years) we did not find any difference either in the blood plasma cytokine concentration or in the gene expression of any of the genes in any AT. The absence of any age-dependent difference is not surprising as we observed (Kralova Lesna et al., 2015b) no correlation with pro-inflammatory macrophages in the AT of male LKDs, and a higher number of infiltrated macrophages population was found in women which increases after menopause (Králova et al., 2015a) (only 5 of the whole LKD group).

In summary, the increase in pro-inflammatory genes in VAT is likely due to pro-inflammatory polarisation of macrophages resident in this AT of patients with advanced atherosclerosis. The anticipated pro-inflammatory changes in VAT are in agreement with intra-vessel concentrations of the two pro-inflammatory markers, TNF α and hsCRP. Our data thus confirm the importance of AT in the process of atherogenesis. However, a more substantial question is whether or not the pro-inflammatory changes in AT, as documented in the affected group, were due to the pathology or if the pro-inflammatory status had existed long before in individuals prone to atherosclerosis development.

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Declaration of interest: The authors have no conflict of interest to declare.

Gene of interest	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
IL-6	AGCAGCAAAGAGGCACTGGCA	GCACAGCTCTGGCTTGTTCTCA
MCP-1	AGAAGCTGTGATCTTCAAGACC	AGCTGCAGATTCTGGGTTG
TNF α	CTCTGGCCCAGGCAGTCAGATCA	GGGCAGCCTTGGCCCTTGAA
Beta-2-microglobulin	TCTCTCTTTCTGGCCTGGAG	AATGTCGGATGGATGAAACC

Table 1: Nucleotide sequences of primer pairs used in this study

	Living kidney donors (n = 35)	Patients with PAD (n = 24)	Significance
Age (years)	46 \pm 10.5	61 \pm 12.9	p<0.0001
BMI	26.3 \pm 3.8	27.4 \pm 4.8	p=0.489
Smoker, n (%)	10 (28.6 %)	19 (79.2 %)	p<0.0001
Hypertension, n (%)	9 (25.7 %)	19 (79.2 %)	p<0.0001
Diabetes type 2, n (%)	0 (0 %)	7 (29.2 %)	p<0.001

Table 2: Characteristics of the analyzed populations

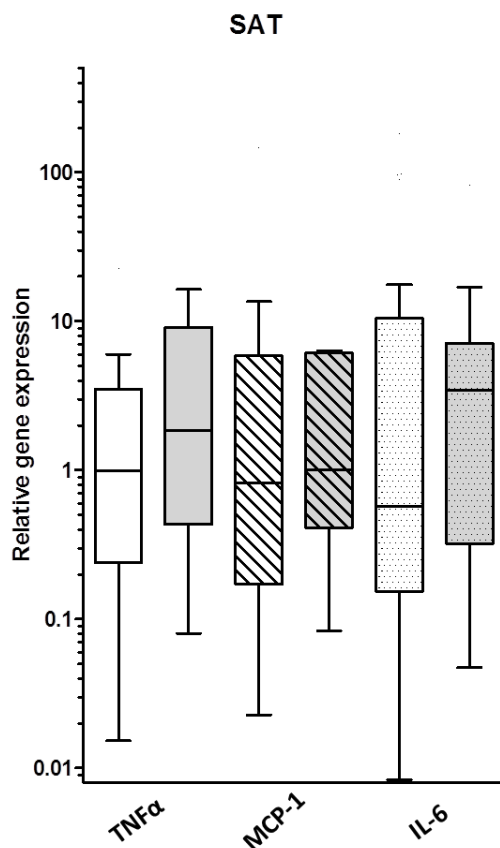


Fig. 1: Relative gene expression of TNF α , MCP-1, and IL-6 in SAT of living kidney donors (n=35) and patients with PAD (n=24). Comparison of gene expression between LKDs and patients is presented as Turkey boxplot.

Grey bars represent patients with PAD, white bars represent LKDs. TNF α – tumor necrosis factor-alpha; MCP-1 – monocyte chemoattractant protein 1 - stripes; IL-6 – interleukin 6 – dots.

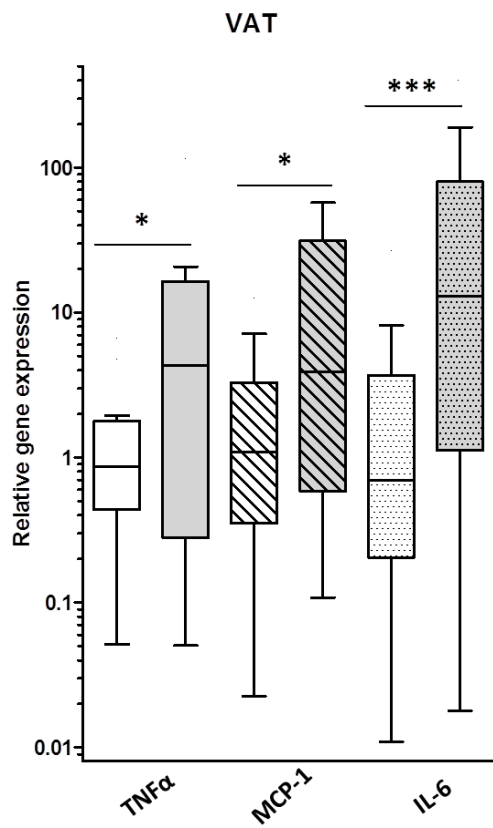


Fig. 2: Relative gene expression of TNF α , MCP-1, and IL-6 in VAT of living kidney donors (n=35) and patients with PAD (n=24). Comparison of gene expression between LKDs and patients is presented as Turkey boxplot. Grey bars represent patients with PAD, white bars represent LKDs. TNF α – tumor necrosis factor-alpha; MCP-1 – monocyte chemoattractant protein 1 - stripes; IL-6 – interleukin 6 – dots; * $p < 0.05$; *** $p < 0.001$.

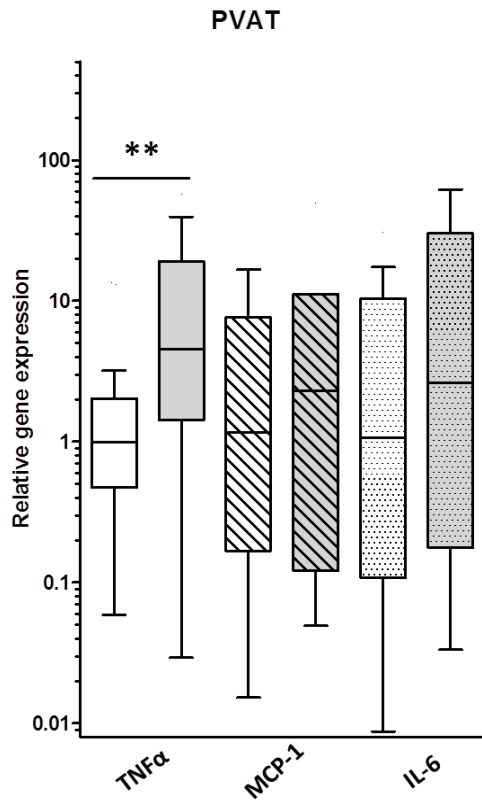


Fig. 3: Relative gene expression of TNF- α , MCP-1, and IL-6 in PVAT of living kidney donors (n=35) and patients with PAD (n=24). Comparison of gene expression between LKDs and patients is presented as Turkey boxplot. Grey bars represent patients with PAD, white bars represent LKDs. TNF α – tumor necrosis factor-alpha; MCP-1 – monocyte chemoattractant protein 1 - stripes; IL-6 – interleukin 6 – dots; ** p < 0.01.