# Charles University in Prague

## Third Faculty of Medicine

### Department of Sport Medicine


# Role of immune and adipose cells in the development of adipose tissue inflammation induced by stress associated with obesity

Doctoral thesis


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# DECLARATION

I, hereby, declare that this thesis is based on experiments performed at the Department of Sport Medicine, 3rd Faculty of Medicine, Charles University in Prague during my Ph. D. studies and it was written by me while all sources of information are recorded in the list of references. This thesis was not used to obtain any other or similar degree.
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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ACACA | acetyl-CoA carboxylase |
| ACLY | ATP-citrate lyase |
| ACP5 | acid phosphatase 5 |
| AdipoQ | adiponectin |
| AdipoR | adiponectin receptor |
| AGPAT | acylglycerol-3-phosphate acyltransferase |
| AMPK | 5'-AMP-activated protein kinase |
| AT | adipose tissue |
| ATF | activating transcription factor |
| ATGL | adipose triglyceride Lipase |
| ATP | adenosin triphosphate |
| BAT | brown adipose tissue |
| C/EBPs | CCAAT/enhancer-binding proteins |
| CCL | chemokine (C-C Motif) ligand |
| CCL2/MCP1 | chemokine (C-C Motif) ligand 2/monocyte chemoattractant protein 1 |
| CCL5/RANTES | chemokine (C-C Motif) ligand 5 |
| CCR2 | chemokine (C-C motif) receptor 2 |
| CD | cluster of differentiation |
| CRP | C-reactive protein |
| CTRP | C1q/Tumor Necrosis Factor Related Protein |
| CXCL | chemokine (C-X-C motif) ligand |
| DAG | diacylglycerol |
| DGAT | diacylglycerol acyltransferase |
| DI | dietary intervention |
| DNAJC3 | DnaJ (Hsp40) homolog, subfamily C, member 3 |
| DNL | *de novo* lipogenesis |
| DPP4 | dipeptidyl-peptidase 4 |
| EDEM1 | ER degradation enhancer, mannosidase alpha-like 1 |
| ELOVL | fatty acid elongase |
| ER | endoplasmic reticulum |
| ERS | endoplasmic reticulum stress |
| FA | fatty acid |
| FASN | fatty acid synthase |
| FCGBP | Fc fragment of IgG binding protein |
| MSR1 | macrophage scavenger receptor 1 |
| FoxP3 | forkhead box P3 |
| GATA3 | GATA binding protein 3 |
| GDR | glucose disposal rate |
| GLUT | glucose transporter |
| GPAT | glycerol-3-phosphate acyltransferase |
| HFM | high fat meal |
| HG | hyperglycemia |

| | |
|---|---|
| HMW | high molecular weight adiponectin |
| HOMA-IR | homeostasis model assessment of insulin resistance |
| HSL | hormone sensitive lipase |
| HSPA5 | heat shock 70kDa protein 5 |
| ICAM | intercellular adhesion molecule |
| IL | interleukin |
| IL10Rα | interleukin receptor alpha subunit |
| IL1Ra | intracellular interleukin1 receptor antagonist |
| INFγ | interferon gamma |
| IR | insulin resistance |
| IRE1 | inositol-requiring protein 1 |
| IRS | insulin receptor substrate |
| LCD | low calorie diet |
| LMW | low molecular weight adiponectin |
| LPL | lipoprotein lipase |
| MAG | monoacylglycerol |
| MGL | monoglyceride lipase |
| MMP9 | matrix metalloproteinase 9 |
| MMW | medium molecular weight adiponectin |
| mRNA | messenger RNA |
| NFκB | nuclear factor kappa-light-chain-enhancer of activated B cells |
| NK | natural killer |
| PAI1 | plasminogen activator inhibitor 1 |
| PBMC | peripheral blood mononuclear cells |
| PERK | protein kinase RNA-like ER kinase |
| PI3K | phosphatidylinositol-4,5-bisphosphate 3-kinase |
| PLA2G7 | phospholipase A2, group VII |
| PPARγ | peroxisome proliferator-activated receptor gamma |
| qRT-PCR | quantitative real time polymerase chain reaction |
| RBP4 | retinol binding protein 4 |
| RORC | RAR-related orphan receptor C |
| SAAT | subcutaneous abdominal adipose tissue |
| SCD | stearoyl-CoA desaturase |
| SFRP5 | secreted frizzled-related protein 5 |
| SGAT | subcutaneous gluteal adipose tissue |
| SPP1 | secreted phosphoprotein 1 |
| SVF | stromal vascular fraction |
| T2DM | diabetes mellitus type II |
| TACE | TNFα-converting enzyme |
| TAG | triacylglycerol |
| TBX21 | T-Box 21 |
| $T_C$ | cytotoxic T lymphocytes |
| TGFβ | transforming growth factor beta |
| $T_H$ | helper T lymphocytes |
| TIMP3 | tissue inhibitor of metalloproteinases 3 |

| | |
|---|---|
| TLR | toll-like receptor |
| TNFα | tumor necrosis factor α |
| $T_{REG}$ | regulatory T lymphocytes |
| UCP1 | uncoupling protein 1 |
| UPR | unfolded protein response |
| VAT | visceral adipose tissue |
| VCAM | vascular cell adhesion molecule |
| VEGF-A | vascular endothelial growth factor A |
| VLCD | very low calorie diet |
| WAT | white adipose tissue |
| WHO | World health organization |
| WM | weight maintenance phase of dietary intervention |
| XBP1 | X-box binding protein 1 |

# 1. INTRODUCTION

Obesity is characterized as an excessive accumulation of adipose tissue (AT) due to the imbalance between calorie intake and energy expenditure. It is defined as body mass index (BMI, i.e. ratio of weight in kg divided by the square of height in meters) above 30 kg/m$^2$. In western countries, the upsurge of obesity is presumably driven by high availability of low-cost energy-dense food abundant in fat and sugar in connection with a dramatic shift from physical to sedentary work/leisure activities. Nowadays similar changes in lifestyle are observed also in developing countries [1]. According to World health organization (WHO) prevalence of obesity increased more than two times since the 1980s and in the European region more than 50% of people are overweight or obese (prevalence of obesity in Czech republic is higher than 60%) (http://www.who.int/topics/obesity/en/). This means that in 2008 (when the data for the most recent WHO report were gathered) more than 1.4 billion adults were overweight and more than half a billion were obese. Importantly, overweight and obesity are associated with the development of metabolic comorbities and additional disorders, for example 44% of cases of type II diabetes mellitus (T2DM), 23% of ischaemic heart disease and 7–41% of certain cancers are globally attributable to overweight and obesity (http://www.who.int/topics/obesity/en/). Classification of the degrees of obesity according to BMI and their connection with severity of comorbidities that have to be treated by medications (high blood pressure, diabetes, stroke, etc.) is summarized in Table 1.

**Table 1:** Classification of human weight according to BMI (adapted from www.who.int).

| Classification | BMI [kg/m$^2$] | Risk of comorbidities | |
|---|---|---|---|
| Underweight | >18.5 | Malnutrition, osteoporosis | |
| Normal weight | 18.6-24.9 | - | |
| Overweight | 25-29.9 | Cardiovascular diseases, hypertension, atherosclerosis, insulin resistance, T2DM and others | Moderate |
| Obesity I class | 30-34.9 | | Increased |
| Obesity II class | 35-39.9 | | Severe |
| Obesity III class | ≥40 | | Very severe |

Accordingly, in a recent systematic review of the economic burden of obesity worldwide, Withrow and colleagues concluded that obese individuals have medical costs 30% higher than those with normal weight and that obesity accounts for 0.7–

2.8% of a country’s total health-care costs [2]. Moreover, at least 2.8 million people die each year as a result of being overweight or obese and thus overweight and obesity are linked to more deaths worldwide than underweight.
Therefore, obesity became one of the major health and socio-economic problems of 21st century [3]. Alarmingly increasing numbers of people suffering from weight excess, and amounts of money that are spent on medical care connected to obesity, are very strong reasons to deepen our understandings of AT function and its obesity-associated changes leading to deterioration of health. Then, new findings can be used to improve public education about prevention and treatment of obesity. The introduction of this thesis thus provides a concise insight into current knowledge of AT function with emphasis on its immune properties in lean and obese subjects.

## 1.1 Adipose tissue

A major and primary function of AT is to accumulate lipids upon energy excess and to release energy-rich substrates in response to energy needs. Nevertheless, last three decades of intensive research have contributed to the appraisal of AT as a truly multifunctional organ with important immune, endocrine and paracrine, regenerative, mechanical, and thermal function [4]. This multifunctionality is based on AT specific cellular composition and also various anatomical localizations. Accordingly to its complex properties and also size, AT is now recognized as an organ greatly contributing to the whole body metabolic homeostasis.

### 1.1.1 Distribution of adipose tissue

AT is one of the largest organ of the body (it represents 10-20% of body weight in lean up to 70% of body weight in obese [5]). The major anatomical AT depots (shown in Figure 1) are:

- upper-body subcutaneous depot
- intra-abdominal (omental and mesenteric depots, also termed visceral fat)
- lower-body (gluteal, subcutaneous leg or gluteo-femoral depot)
- ectopic fat deposited in atypical locations

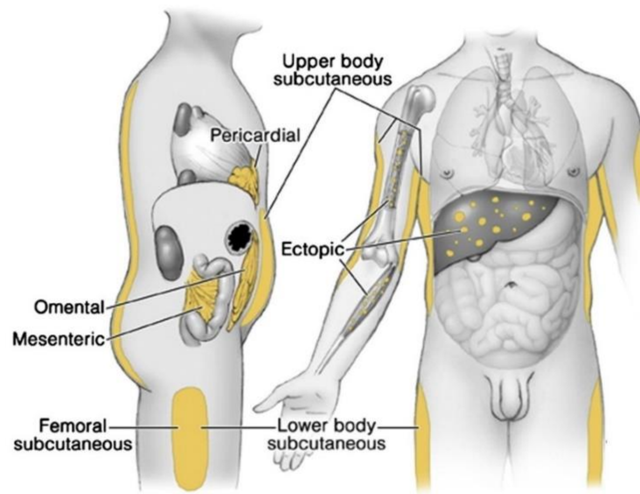


**Figure 1:** Adipose tissue depots (adapted from Tchokina 2013 Mechanisms and Metabolic Implications of Regional Differences [4])

The distribution of AT is affected by race, gender, aging and disease states, or physiological condition (e. g. starvation) and in response to drugs and hormones. Importantly, different anatomical depots of AT exhibit diverse ability to respond to external signal (e. g. insulin, lipolytic agents), secretory profile, as well as different composition of stromal vascular fraction (mainly AT resident immune cells) [4]. These variances result in distinct metabolic properties of the depots. Therefore, the distribution and size of AT depots in each individual can have an impact on his/her overall metabolic health.

An accumulation of AT in abdominal (upper) region is associated with an increased risk of displacement of AT into visceral region and ectopic depots and thus with an increased risk of cardiovascular and metabolic disorders development, as well as liver steatosis [6-8]. On the other hand, gluteal depot seems to be metabolically inert with low blood flow and low rate of fatty acid (FA) release [9]. A higher accumulation of AT in gluteo-femoral depot was shown to be linked with the reduction of metabolic [10], cardiovascular risk [11-14] and with lower morbidity and mortality [15-17]. Exact reason of predominantly protective role of gluteo-femoral depot is mostly unknown and only few studies paid attention to this topic. Recently, it was hypothesized that the differences in metabolic impact of gluteal when compared to abdominal AT depot are related to the differences in the inflammation-related characteristics, which were suggested as one of the key player in obesity-related health disturbances [18-20]. Hence, the characterization of the immune status of these two

Different subcutaneous AT depots (abdominal and gluteal) is one of the issues of this thesis.

### 1.1.2 Adipocytes

AT is composed mainly of its functional units, i.e. adipose cells or adipocytes, and mixture of other cells together called stromal vascular fraction (SVF). SVF contains preadipocytes, mesenchymal stem cells, endothelial cell and various immune cells [21] (details on immune cells of SVF are provided in Chapter 1.1.5.5, page 17).

Mature adipocytes capable of lipid storage arise from fibroblast-like AT precursors - preadipocytes - through multistep process called adipogenesis (Figure 2). Crucial determinants of this process are transcriptional factors peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs) [22]. During adipogenesis PPARγ orchestrates a cascade of several changes in morphology and gene expression resulting in activation of many enzymatic pathways necessary for adipocyte metabolic function. Ability of preadipocytes to undergo adipogenesis is important not only for the development of AT but also for its regeneration or hyperplastic expansion. The regulation of this process in adult humans seems to be highly important for the functionality of AT but it remains mostly unknown.

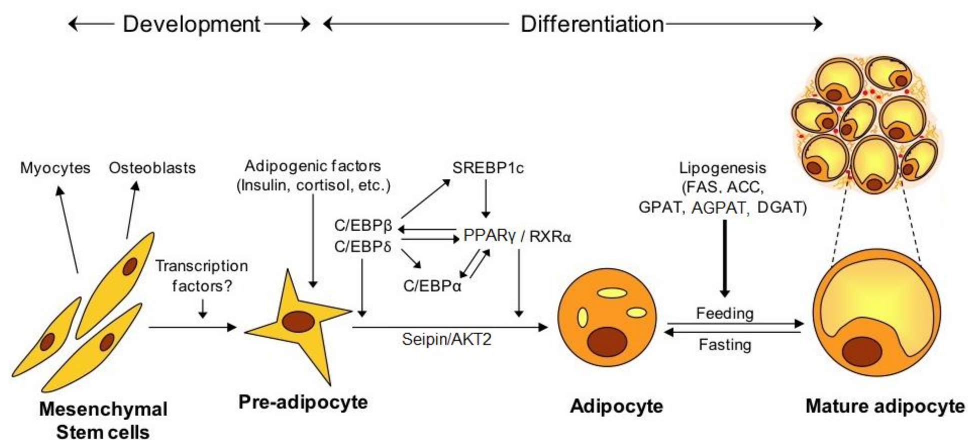


**Figure 2:** Schematic presentation of adipocyte differentiation (adapted from Garg 2011 Lipodystrophies Genetic and Acquired Body Fat)

In mammals different types of adipocytes are described: “classical” white adipocytes (characteristic for so called white adipose tissue, WAT) and brown adipocytes (located

in brown adipose tissue, BAT). These adipocytes are structurally and functionally divergent and are of different cellular origins [23, 24].

White adipocytes are nucleated cells comprising a lipid droplet which occupies most of the cell and a thin rim of cytoplasm displaced to the periphery [25]. WAT is responsible for energy-handling function of AT and represent major type of AT found in humans.

Brown adipocytes are multilocular, i.e. they contain several smaller lipid droplets, and they have higher mitochondria content. These mitochondria are specific by high expression of uncoupling protein 1 (UCP1) that allows dissipation of the proton electrochemical gradient generated by respiration in the form of heat [26]. Thus, BAT plays crucial role in nonshivering thermogenesis. BAT is mainly presented in small and hibernating mammals and human newborns, but the studies performed in last decade documented BAT activity also in adults [27].

Recently a “third” type of adipose cells was described; beige or brite adipocytes arise in WAT depots but represent rather BAT phenotype with plenty of lipid droplets and high mitochondrial activity [28, 29].

### 1.1.3 Lipid metabolism in adipose tissue

Lipids are extensive group of bio-functional, structurally heterogeneous molecules that play essential roles in multiple spheres of vital processes. Importantly, lipids serve as a source of energy that can be efficiently stored. Lipids from food are transported to AT through the blood stream in the form of chylomicrons and lipoproteins. FA are released from them by lipoprotein lipase (LPL), enzyme secreted by adipocytes and present on endothelial cells [30-32] and then transported inside the cells. FA are then used for synthesis of triglycerides (TAG). FA and subsequently TAG can be in adipocytes synthetized also from the carbohydrates in the process called *de novo* lipogenesis (DNL, Figure 3).

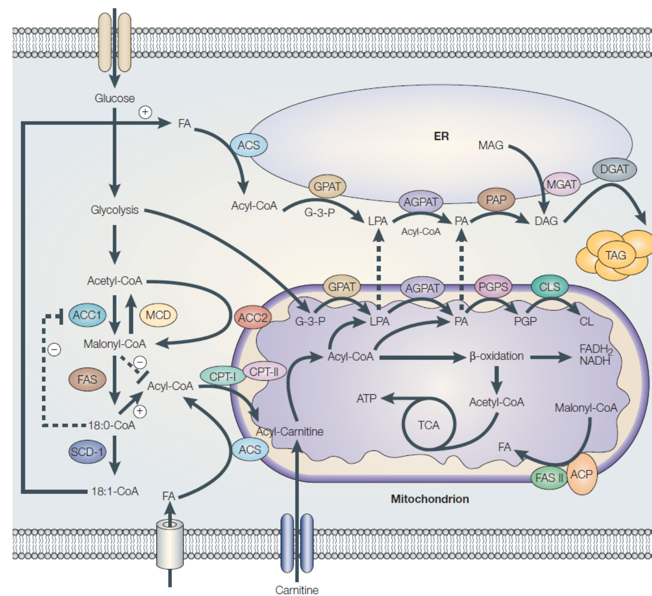


**Figure 3:** De novo lipogenesis (adapted from Shi 2004 Lipid metabolic enzymes: emerging drug targets for the treatment of obesity [33]). DNL pathway includes Krebs cycle (tricarboxylic acid cycle, citric acid cycle) and its product – acetyl coenzyme A – that is incorporated into the newly synthetized FA in the cytoplasm. Enzymes involved in transformation of citrate to FA are ATP-citrate lyase (ACLY), acetyl-CoA carboxylase (ACACA) and fatty acid synthase (FASN). FASN is the key rate-limiting enzyme of DNL. Elongation of FA chain is catalyzed by FA elongases (ELOVL) and desaturation is under the control of stearoyl-CoA desaturase (SCD). FA from food or *de novo* synthesized are subsequently esterified to glycerol to form TAG. The initial esterification step is under control of enzyme glycerol-3-phosphate acyltransferase (GPAT). The esterification of second FA is processed by acylglycerol-3-phosphate acyltransferase (AGPAT) and finally diacylglycerol acyltransferase (DGAT) connect third FA. Both saturated (palmitate, stearate, myristate,) and unsaturated FA (oleate, palmitooleate) can be incorporated into lipids. [34]

In the opposite process called lipolysis, TAG are hydrolyzed to FA and glycerol (Figure 4). These metabolites are then released into the blood stream and delivered to peripheral tissues (especially skeletal muscle and heart), where they are used for ATP production in the process of β-oxidation. Three key lipases participate in lipolysis within AT. Adipose triglyceride lipase (ATGL) selectively performs the first and rate-limiting step hydrolyzing TAG to generate diacylglycerol (DAG) and FA [35]. Hormone sensitive lipase (HSL) is a multifunctional enzyme capable of hydrolyzing preferentially DAG but also TAG and monoacylglycerol (MAG) [36, 37]. Finally, monoglyceride lipase (MGL) efficiently cleaves MAG into glycerol and non-esterified FA [38].

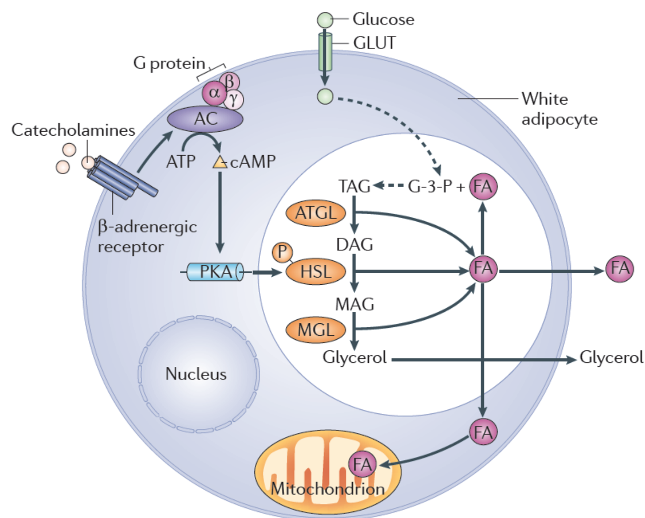


**Figure 4:** Schematic view of lipolysis (adapted from Altarejos 2011 CREB and the CRTC co-activators: sensors for hormonal and metabolic signals [39])

Both, lipogenesis and lipolysis, are under tight physiological control including hormonal and other means of regulation and usually their activation are mutually exclusive. For instance, insulin released from β cells after food intake inhibits lipolysis and via insulin receptor stimulates glucose transport into the cell and FA formation. Other signals regulating TAG formation/degradation (metabolism) are glucagon, catecholamines, glucocorticoids, cytokines, glucose and FA levels.

### 1.1.4 Adipose tissue as endocrine organ

Since the discovery of AT endocrine potential, this aspect of AT has been under huge scientific interest that resulted in an assembly of an extensive list of AT produced molecules important for metabolic and immune homeostasis. These cytokines and/or adipokines affect several organs responsible for lipid handling on central (brain) and peripheral (liver, muscle) level as well as immunologically active cells and AT itself [40]. Key adipokines are described further and summary of AT secretory products is presented in Table 2.

**Table 2:** Major factors secreted by adipose tissue and its predominant effect on immune system

| Category | Factors |
|---|---|
| Anti-inflammatory | AdipoQ [41], apelin [42, 43], CD163 [44, 45], IL1Ra [46], IL10 [47], IL13 [48], Omentin [49], Vaspin [50], CTRP [51, 52] |
| Pro-inflammatory | Leptin [53], adipsin [54], Visfatin [55, 56], Resistin [57, 58], TNFα [40], IL1β [59], IL6 [60], IL8 [59], PAI1 [61], CCL5 (or RANTES) [62], CCL2 (or MCP1) [63], TGFβ [64], RBP4 [65], DPP4 [66], VEGF-A [67] |
| Ambivalent effect | SFRP5 [68] |

Adiponectin (AdipoQ); chemokine (C-C Motif) ligand (CCL); cluster of differentiation (CD); C1q/Tumor Necrosis Factor Related Protein (CTRP); dipeptidyl-peptidase 4 (DPP4); interleukin (IL); intracellular interleukin1 receptor antagonist (IL1Ra); plasminogen activator inhibitor 1 (PAI1); retinol binding protein 4 (RBP4); secreted frizzled-related protein 5 (SFRP5); transforming growing factor β (TGFβ); tumor necrosis factor α (TNFα); vascular endothelial growth factor A (VEGF-A)

#### 1.1.4.1 Leptin

Leptin was discovered in 1994 as a factor enabling communication between AT and hypothalamus and thus crucial for the regulation of the appetite and consequently the size of body fat depot [53]. It is a 16 kDa protein product of *ob* gene. Leptin is produced almost exclusively by adipocytes [53] (weak production from stomach [69], lungs [70], placenta [71] and brain [72]). In hypothalamus, leptin activates receptors in anorexigenic neurons [73] and thus reduces appetite and increases energy expenditure [74]. The concentration of leptin in circulation is tightly and positively related to amounts of AT, and thus its levels are elevated in obese subjects. Prolonged exposure to increased leptin levels however leads to leptin resistance, which results in inability of higher leptin concentration to suppress efficiently appetite in obese. Leptin receptors are present not only in brain but also in peripheral organs and tissues, such as the liver, skeletal muscle, AT, heart and pancreas [75, 76]. Importantly, leptin receptors are expressed also by T cells [77], B cells [78], monocytes and macrophages [74] and, in these cells, effects of leptin are mainly pro-inflammatory (see Chapter 1.1.5, page 14 for more details).

#### 1.1.4.2 Adiponectin

One year after the discovery of leptin, in 1995, another important adipokine adiponectin (AdipoQ, apM1 - adipose most abundant gene transcript 1, GBP28 -

gelatin-binding protein, or Acrp30 - adipocyte complement-related protein 30) was identified concurrently by four independent research groups [79-82]. AdipoQ is a 30 kDa protein product of *adipoQ* gene secreted from mature adipocytes into the circulation where it reaches a concentration much higher (3–30 μg/ml) than typical for other cytokines [80, 83]. AdipoQ is secreted in distinct homo-multimeric isoforms including trimeric low molecular weight (LMW), hexameric medium molecular weight (MMW), and oligomeric high molecular weight (HMW) complexes [84, 85]. The major biological effects of AdipoQ are attributed to HMW isoform [86, 87]. AdipoQ acts as an insulin sensitizing and anti-inflammatory agents and plays a role in the regulation of glucose and lipid metabolism in muscle and liver. Although AdipoQ is produced mainly by adipocytes, its levels are dramatically diminished in obese subjects [88]. This suppression is associated with worsening of whole body insulin sensitivity and consequently with higher fasting glucose levels [89]. Signaling of AdipoQ is mediated via two main forms of its receptor with different affinity to isoforms of AdipoQ. AdipoR1 is expressed in many tissues and its activation stimulates 5'-AMP-activated protein kinase (AMPK) signaling pathway. AdipoR2 is predominantly expressed in liver and mediates AdipoQ effect on PPARγ pathway implemented in inhibition of inflammation and oxidative stress [90-92].

#### 1.1.4.3 Cytokines/chemokines

Produced by both adipocytes and SVF, cytokines represent important proportion of AT secretory output. The whole body and AT pro-inflammatory state is suggested as one of important contributors to metabolic disorders (insulin resistance (IR), T2DM) associated with obesity [93]. In general, levels of AT cytokines with pro-inflammatory properties are elevated in obesity. This group of cytokines/chemokines includes for example TNFα [40], CCL2 also known as monocyte chemoattractant protein 1 (MCP1) [63, 94], IL6 [60] and IL8 [59].

TNFα plays a role not only in acute and chronic inflammation but also in necrosis, apoptosis and other processes [95]. It is a first AT-derived cytokine found to directly cause IR. Levels of TNFα are elevated in obese subject and indeed correlate with the degree of IR [96]. Importantly, TNFα potentiates secretion of other inflammatory cytokines [97]. CCL2 attracts macrophages into the site of inflammation and similarly to TNFα, its association with IR and T2DM was shown [98, 99].

On the other hand, AT is also a source of anti-inflammatory factors. Their levels decrease with obesity (e. g. omentin [49]) or they go hand in hand with the levels of pro-inflammatory cytokines (e. g. apelin [100], IL1Ra [46], IL10 [101]). The later suggests a negative feedback regulation, which is typical for a resolution phase of acute inflammation [102, 103].

### 1.1.5 Adipose tissue as immune organ

#### 1.1.5.1 General aspects of immune system and reactions

Immune system consists of numerous cellular as well as non-cellular compounds and is essential for homeostasis maintenance. In healthy conditions, its key role is to defend organism against exogenous pathogens as well as endogenous potentially dangerous elements (e. g. cancer cells, necrotic cells). This is achieved by a co-operation of multiple components of immune system in highly regulated machinery. It seems that similar type of cooperation occurs also in AT in response to metabolic stress. Nevertheless, its exact steps and regulation have still not been fully elucidated and are solved in this Ph. D. thesis.

Different types of classification of immune components/processes are possible:

- humoral vs. cell-mediated
- innate vs. adaptive
- pro- vs. anti-inflammatory

The last mentioned type of classification appears to be the most appropriate for this thesis, which is focused on pro-inflammatory processes in response to obesity. In general but also specifically in AT, inflammatory response is characterized by the accumulation of phagocytic cells (macrophages, neutrophils) and presence of higher levels of pro-inflammatory cytokines including TNFα, IL6 etc. released from the inflamed tissue in order to attract effector immune cells. Anti-inflammatory response is mediated especially by $T_H2$ and B lymphocytes. The details about immune cells and their contribution to AT immune status are described in the following chapters.

#### 1.1.5.2 Monocytes and macrophages

Monocytes, hematopoietic cells from myeloid lineage, circulate in the blood stream. After adequate stimulation, they are able to move through the vascular wall, infiltrate the infected or damaged tissue and transform into effector cells – macrophages – capable of phagocytosis and secretion of a large spectrum of cytokines. From broad range of functionally and metabolically different groups of macrophages the major are described: classical "M1" macrophages are considered as one of the key elements in inflammation, while patrolling or alternative "M2" macrophages are involved in healing, tissue repair and regeneration [104-106]. The polarization of macrophages into these two phenotypes is regulated mainly by cytokines. Human M1 polarization is induced *in vitro* by IFNγ or IFNγ in combination with lipopolysaccharide or TNFα. After polarization, M1 macrophages produce cytokines and other molecules (reactive oxygen and nitrogen intermediates) to activate and recruit additional immune cells or to kill pathogens [107, 108]. M1 palette of cytokines consists of interleukins (IL): IL1β, IL6, IL12, chemokines: CCL2, chemokine (C-X-C motif) ligand (CXCL) 9, CXCL10 and classic inflammatory cytokines: TNFα. M2 alternative activation of macrophages is triggered by IL4, IL10 and IL13. M2 macrophages highly express for example interleukins: IL1Ra, IL10, cytokines: TGFβ and chemokines: CCL24 and CCL17 [109, 110]. Nevertheless, it should be mentioned that while the *in vitro* polarization is induced via effect of one or two cytokines, *in vivo* cells are exposed to highly complex and constantly changing mixture of cytokines, which stimulates their differentiation into several intermediate phenotypes [106]. The phenotypes of macrophages can be distinguished by the expression of specific surface markers. In rodents, M1 and M2 macrophages can be easily distinguished by the presence or absence of CD11c membrane marker [111]. In humans, phenotype-specific markers of macrophages are however not so clear. Mannose receptor (CD206) was suggested as one of the possible M2 markers [109], but it also represents a marker of tissue resident macrophages [112-115]. Other possible M2 markers in humans are dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-Sign, CD209) [46], hemoglobin-haptoglobin scavenger receptor (CD163) and Fc Gamma Receptor III (CD16) [116-118]. All three markers exhibit anti-inflammatory properties associated with M2 phenotype of macrophages, but for instance CD163 is expressed also on blood monocytes. On the other hand, CD86, one of the possible M1 marker is also expressed in comparable levels on M2 macrophages [44]. Similarly,

another putative M1 marker CD11c, which was clearly defined in rodents, is expressed on more than 80% of human monocytes (not shown, Kračmerová 2014).

#### 1.1.5.3 Granulocytes

Granulocyte group consists of three different cell types: basophils, eosinophils and neutrophils. All three types are rich in cytoplasmic granules that, after signal, are spilled outside the cells to defense against pathogens. Basophils contain histamine and serotonin in their granules and often are connected to allergic reactions. Neutrophils, as most abundant immune cells, play primary role in pro-inflammatory response, thus it is not a surprise that numbers of infiltrating neutrophils are elevated in obese subjects [119]. However, study of granulocytes is out of focus of this thesis.

#### 1.1.5.4 Lymphocytes

Lymphoid lineage of immune cells includes T and B lymphocytes, natural killer (NK) cells and NK T cells.

T lymphocytes are divided into two main groups according to their function and to the expression of typical surface markers. Cytotoxic T ($T_C$) lymphocytes express a unique marker T-cell surface glycoprotein CD8 (further only CD8). $T_C$ are part of innate immune system and are able to induce apoptosis in cells infected with intracellular pathogens and damaged cells. In contrast, T helper ($T_H$) lymphocytes express specific membrane marker glycoprotein CD4 and their function is “to help” other cells to acquire fully active phenotype. Pro-inflammatory $T_H1$ lymphocytes assist M1 macrophages to destroy phagocyted/engulfed pathogens, while $T_H2$ lymphocytes are anti-inflammatory and, by coordination with B lymphocytes, participate on antibodies-mediated immune response. It was shown that balance between $T_H1$ and $T_H2$ lymphocytes is crucial for the proper functionality and homeostasis of immune system. There are several minor groups of $T_H$ lymphocytes with both pro- and anti-inflammatory effects. In last few years, attention was paid mainly to two groups of $T_H$ lymphocytes - $T_H17$ and T regulatory ($T_{REG}$) cells. $T_H17$ differentiation is induced by TGFβ and IL6 and these cells are characterized with high production of IL17. Proposed role of $T_H17$ cells is to potentiate inflammation via stimulation of pro-inflammatory cytokines release from several types of cells, i. e. macrophages, epithelial and endothelial cells. Moreover, IL17 stimulates production of chemokines

(e. g. CXCL1, CXCL5, IL8) that are responsible for recruitment of neutrophils to the site of inflammation [120, 121]. Indeed, $T_H17$/IL17 levels are increased in autoimmune inflammatory diseases, such as Crohn's disease, rheumatoid arthritis, type I diabetes mellitus and others. In opposite, $T_{REG}$ cells, characterized by high expression of α subunit of receptor for IL2, were suggested to play an essential role in self-tolerance mechanisms and inhibition of auto-reactive species of lymphocytes [122]. Lowered levels of $T_{REG}$ are associated with autoimmune disease development, while higher levels are related to increased risk of cancer, due to immunological suppression. Similarly as described for $T_H1$/$T_H2$, the ratio between $T_H17$/$T_{REG}$ seems to play a key role in the balance between autoimmune diseases and immune suppression [123, 124].

Major function of B lymphocytes is, in collaboration with $T_H2$ lymphocytes, a production of antibodies as a part of anti-inflammatory immune reaction [125]. Other members of lymphoid lineage - NK cells – are considered as cells contributing to innate immunity. Its role is especially in the protection against viral infections and tumor patrolling [126]. Although role of B lymphocytes and NK cells in immune defense is essential, this work is in particular based on study of pro-inflammatory alterations of macrophages and T lymphocytes, thus B lymphocytes and NK cells are not described in more details.

#### 1.1.5.5 Immune cells and adipose tissue

AT is infiltrated with a panoply of immune cells including both innate and adaptive components [97]. Phenotype and activity of them is affected by cytokines that are direct products of adipocytes. For instance, leptin stimulates macrophage activation and induces proliferation of $T_H1$ lymphocytes [127, 128]. It also triggers a release of pro-inflammatory cytokines (TNFα, IL1β, IL6) from various immune cells [77, 129]. On the other hand, AdipoQ exhibits anti-inflammatory properties, such as ability to suppress NFκB target genes (C-reactive protein (CRP), TNFα, and IL6) and to induce secretion of anti-inflammatory cytokines by macrophages. Moreover, AdipoQ stimulates macrophage switch toward M2 phenotype [130-134]. Effects of metabolites frequently deregulated in obesity, such as FA and glucose, on immune cells polarization are one of the problems investigated in this thesis and are in more detail described further (Chapter 1.1.6.3, page 22)

In accordance with the above described modulatory effects of AT cytokines, AT immune cells from lean subjects exhibit anti-inflammatory phenotype, as mainly alternative M2 macrophages [44], eosinophils [135], $T_H2$ and $T_{REG}$ lymphocytes are presented [136]. On the other hand in obese/metabolically unhealthy subjects a switch to pro-inflammatory classical M1 macrophages and $T_H1$ phenotype occurs and numbers of cytotoxic $T_C$ lymphocytes are amplified (see Figure 7). For a long time it was assumed that the main and first players in AT ”colonization” by immune cells in obesity are macrophages that are attracted by dysfunctional or dying adipocytes [111, 137]. Contrary to expectations, Duffaut et al. [138] showed that the accumulation of macrophages in AT is preceded by T lymphocytes infiltration in response to high fat diet. Lymphocytes react to metabolic disturbances earlier than macrophages and thus they may regulate subsequent macrophage infiltration and activity [86, 138-141]. Analysis of dynamics of immune cells infiltration into AT under obesity-related circumstances is one of the aims of this thesis.

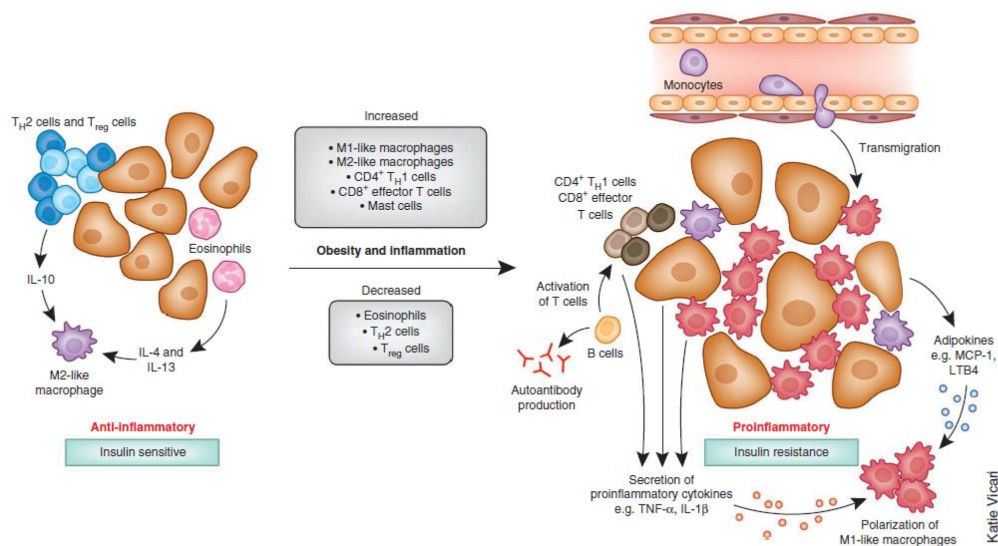


**Figure 7:** Adipose tissue immune cells in lean and obese (adapted from Osborn 2012 The cellular and signaling networks linking the immune system and metabolism in disease [142])

### 1.1.6 Adipose tissue dysfunction in obesity

During energy surplus, there are two strategies used by AT to handle excess nutrients. Hyperplastic expansion is characterized by enlargement of AT depot via recruitment of new preadipocytes with high potential to store lipids. This type of expansion is not associated with disrupted AT functionality and metabolic complications but is rather limited in adulthood [143-145]. The most common type of AT expansion in adults is therefore based on hypertrophic growth of adipocytes. Hypertrophied adipocytes are

however exposed to stressful conditions; e. g. increased mechanical tension, local hypoxia or increased needs for synthesis of lipids. Consequently, hypertrophied adipocytes exhibit signs of stress, such as expression of markers of endoplasmic reticulum stress (ERS) and higher activation of pathways that may contribute to inflammation [146-148]. These stress-activated pathways probably trigger worsening of adipocyte, and consequently AT function and thus prime adverse changes of the whole body metabolism. They are described in following chapters.

#### 1.1.6.1 Insulin resistance

Insulin is a hormone produced solely by pancreatic β cells and its major function is the regulation of carbohydrates metabolism. Postprandially increased levels of glucose induce a production/secretion of insulin. The action of insulin in the cells is mediated via its receptor with intrinsic tyrosine kinase activity [149]. After autophosphorylation of insulin receptor, insulin receptor substrates (IRS1/2) are recruited. These initial tyrosine phosphorylations convey insulin signals to a complex network of intracellular lipid and serine-threonine kinases that mediate the specific insulin biological effects. One of them is the triggering of membrane expression of glucose transporter 4 (GLUT4), a high-affinity glucose transporter that is expressed in the insulin sensitive tissues, such as AT and skeletal muscle [150] (see Figure 8). Except the glucose transport, insulin stimulates glycolysis, glycogen synthesis and inhibits the rate of glycogenolysis and gluconeogenesis in the liver. These effects are essential for the maintenance of normoglycaemia. Insulin also affects lipid metabolism, e. g. decreases the rate of lipolysis in AT and FA oxidation in liver and muscle, stimulates FA and TAG synthesis and TAG uptake into the AT and muscle [151].

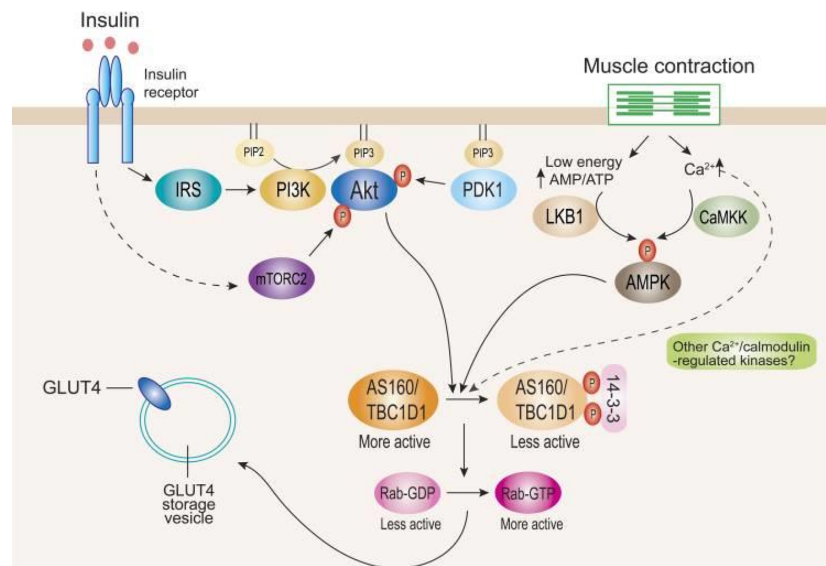


**Figure 8:** Insulin signaling pathway (adapted from Sakamoto 2008 Emerging role for AS160/TBC1D4 and TBC1D1 in the regulation of GLUT4 traffic [152])

Insulin resistance (IR) is defined as a lower capacity of cells to respond to insulin than expected for a given insulin concentration [153]. IR is thus frequently associated with increased plasma insulin levels and longer postprandial hyperglycemias (HG). Because of less efficient import of glucose, cells utilize other energy-rich molecules as a fuel and glucose remains longer in the circulation. Due to higher glucose values, β cells produce more insulin and prolongation of this state can lead to β cells damage and subsequently to T2DM development [153, 154]. Although impaired insulin sensitivity was observed also in lean subjects, obese are in much higher risk of IR development [155].

Nevertheless, it is only partially known why obesity frequently leads to whole body IR. Several mechanisms were suggested: dietary fluctuations, elevated FA levels and inflammatory changes [156]. Alterations in circulating FA and inflammatory cytokines are indeed consequences of impaired AT function and secretory production [157]. AT products can affect insulin action in the cells directly via disruption of insulin signaling pathway. This was documented on several occasions. TNFα levels influence transcription of insulin signaling molecules (insulin receptor, IRS1, and GLUT4) in adipocytes [158, 159]. Similarly, levels of other inflammatory cytokines (e. g. IL1β [160], IL6 [161], CCL2 [162]) are associated with worsening of IR. Another AT product, RBP4 reduces phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signaling in muscle [163]. On the other hand, levels of AdipoQ, which was shown to have insulin sensitizing and anti-inflammatory effects, decline during

obesity. Elevated FA are associated with a reduction IRS1 phosphorylation and IRS1-associated PI3K activity and may directly damage β cells [156, 157]

#### 1.1.6.2 Endoplasmic reticulum stress

In healthy adipocytes, endoplasmic reticulum (ER) is responsible for protein folding, maturation, quality control, trafficking and importantly also for lipid synthesis. In hypertrophic adipocytes, there is an augmented demand on ER synthesis of proteins and lipids. Overwhelming of ER capacity leads to ERS that is characteristic by the activation of unfolded protein response (UPR), [164] (see Figure 9). UPR has three arms that are dependent on ER-located transmembrane proteins: inositol-requiring protein 1 (IRE1), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6). Upon their activation, signal is then transduced into the nucleus via spliced form of messenger RNA (mRNA) coding X-box binding protein (XBP1; IRE1 arm), maturated ATF6 (ATF6 arm) and ATF4 (PERK arm). These transcription factors increase expression of proteins that are involved in the ER folding machinery to augment protein folding and to reduce the load in the ER. Prolonged exposition to ERS can however cause cell death [165].

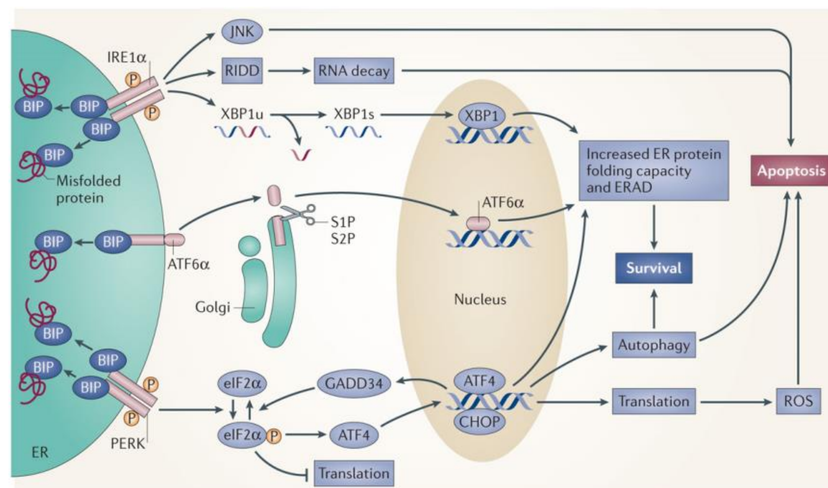


**Figure 9:** Unfolded protein response in ER stress (adapted from Wang 2014 The impact of the endoplasmic reticulum protein-folding environment on cancer development [166])

Importantly, UPR also triggers a variety of inflammatory and stress signaling systems including NFκB and Jun N-terminal kinase pathway, as well as networks activated by oxidative stress, all of which can influence metabolism [147, 167-169].

As suggested previously, ERS in adipocytes and immune cells could be caused by exposure of cells to saturated lipids and a high concentration of glucose [147, 170]. Nevertheless, putative effects of high postprandial levels of nutrients on UPR activation have not been elucidated *in vivo* in humans yet. Therefore one of the subjects of this thesis is to follow up the relationship between postprandial inflammation in both myeloid and lymphoid lineages of human peripheral blood mononuclear cells (PBMC) and ERS.

#### 1.1.6.3 Role of elevated glucose and lipid metabolites in obesity associated inflammation

As noted earlier, western diet is abundant of energy-dense food with high levels of glucose and lipids. Food intake *per se* is associated with temporarily altered levels of nutrients (glucose, TAG, FA) and insulin but also with temporary inflammatory response, also known as postprandial inflammation. It is manifested as an increase of plasma concentration of pro-inflammatory cytokines (IL6, PAI1) [171, 172]. In obese (human and animal) subjects this postprandial inflammation is however prolonged. The protraction of postprandial inflammation in obese with already higher basal levels of pro-inflammatory cytokines may represent a "Molotov cocktail" igniting the development of T2DM.

It was shown previously that both high levels glucose and FA affects various types of cells including immune cells, adipocytes and pancreatic β cells [170, 173-176]. Detrimental effects of hyperglycemia, in humans defined as fasting blood levels of glucose above 5.5 mmol/l or 100 mg/dl [177], might be mediated through induction of oxidative stress and through the activation of inflammatory pathways resulting in increased secretion of pro-inflammatory cytokines[175, 178]. Still, only a few reports addressed responses of cells of adaptive and innate immunity to this metabolic stimulus *in vivo* in obese individuals [179, 180].

Role of FA in the development of pro-inflammatory state and macrophage accumulation have been investigated predominantly in experimental animal models or in cell cultures *in vitro*. *In vitro*, saturated FA induce increased mRNA expression and secretion of pro-inflammatory cytokines and chemokines (CCL2, IL6, IL8) in adipocytes, macrophages and other cell types [181, 182]. In rodents, FA regulate macrophage accumulation in AT [183]. Saturated FA were found to activate classical inflammatory responses in immune cells and to regulate secretion of pro-inflammatory

cytokines in both, immune cells and adipocytes. Furthermore, long saturated FA induce ERS stress in β cells and thus mediate their apoptotic death *in vivo* and *in vitro* [181, 184, 185].

Studies in human subjects are rare and monitor mainly the impact of glucose and FA on adipokines levels in plasma. Therefore *PART ONE* of this thesis is based on *in vivo* human experiments focused on influence of acutely altered levels of nutrients on pro-inflammatory status and immune system response in blood and AT.

## 1.2 Treatment of obesity

As discussed above, health problems related to obesity are closely linked with the impaired function of hypertrophied AT. Therefore, weight loss (based on the reduction of AT) is an obvious strategy to treat obesity-related metabolic disturbances.

Modification of life style represents a physiological approach with the lowest health risks compared to medical or surgical intervention and therefore it is usually a first-choice method to reduce weight. Indeed, it was observed that even moderate diet-induced weight loss (5-10%) has beneficial effects on metabolic parameters [186-188]. This can be achieved by various types of diets (for overview see Table 3). *PART TWO* of this thesis is focused on the immune response of AT to the modest weight loss induced by multiphase dietary interventions (DI) in obese women.

**Table 3:** Types of dietary interventions (according to Tsigos 2008 [189])

| Type of dietary intervention | Daily energy intake |
|---|---|
| Hypocaloric balanced diet | ≥1200 kcal/day |
| Low calorie diet (LCD) | 800 – 1200 kcal/day |
| Very low calorie diet (VLCD) | ≤800 kcal/day |
| Multiphase diet | Combination of the above listed diets |

# 2. AIMS

General aim of this thesis was to elucidate the connection among impaired levels of nutrients/metabolites and pro-inflammatory state, immune system activation and metabolic status in healthy (obese and lean) subjects. In the Part one, acute effects of experimentally increased levels of nutrients on content and phenotype of immune cells in both circulation and AT were elucidated. Part two of thesis is focused on the effects of weight reduction on secretory state of adipocytes and immune cells in AT in relation to the improvement of insulin sensitivity.

**Specific aims:**

*PART ONE*

- To analyze the inflammation induced by a single high fat meal (HFM) in peripheral blood mononuclear cells including cells of innate and adaptive immunity and to test whether this HFM-induced inflammation is linked with ERS
- To elucidate the effect of short term interventions simulating levels of nutrients and lipid metabolism products (i. e. hyperglycemia, hypertriglyceridemia) seen in metabolically unhealthy obese on inflammation and immune system activation in blood and AT in healthy obese subjects

*PART TWO*

- To compare expression of pro-inflammatory markers in subcutaneous abdominal and gluteal adipose tissue in steady state and during weight reducing dietary intervention
- To clarify the relationship between serum concentrations of soluble form and adipose tissue mRNA levels of macrophage marker CD163 and to evaluate its possible utilization as a marker of insulin resistance in cross-sectional design and during weight reducing dietary intervention
- To compare the secretory profile of adipocyte precursors before and after weight reducing dietary intervention

# 3. RESULTS AND DISCUSSION

## 3.1 List of original publications

*PART ONE*

1. **Postprandial inflammation is not associated with endoplasmic reticulum stress in PBMC from healthy lean men**

Jana Kračmerová, Eva Czudková, Michal Koc, Lucia Mališová, Michaela Šiklová, Vladimír Štich and Lenka Rossmeislová

British Journal of Nutrition, 2013, August, 112(4):573-582. IF 3.3

2. **Experimental hyperglycemia induces an increase of monocyte and T-lymphocyte content in adipose tissue of healthy obese women**

*Michaela Tencerová, *Jana Kračmerová, Eva Krauzová, Lucia Mališová, Zuzana Kováčová, Zuzana Wedellová, Michaela Šiklová, Vladimir Štich and Lenka Rossmeislová

PLoS ONE, 2015, March, 10 (4): e0122872. IF 3.5

*These authors contributed equally to this work.

3. **Acute hyperlipidemia initiates pro-inflammatory and pro-atherogenic reaction in obese women**

Eva Krauzová, Jana Kračmerová, Michaela Tencerová, Lucia Mališová, Zuzana Kováčová, Lenka Rossmeislová, Vladimir Štich and Michaela Šiklová

Submitted to Arteriosclerosis, Thrombosis, and Vascular Biology. IF 5.6

4. **Expression of inflammation-related genes in gluteal and abdominal subcutaneous adipose tissue during weight-reducing dietary intervention in obese women**

Lucia Movisová, Lenka Rossmeislová, Zuzana Kováčová, Jana Kračmerová, Michaela Tencerová, Dominique Langin, Michaela Šiklová-Vítková and Vladimír Štich

Physiological Research, 2014, March, 63(1): 73-82. IF 1.5

5. **Soluble CD163 is associated with CD163 mRNA expression in adipose tissue and with insulin sensitivity in steady-state condition but not in response to calorie restriction**

Jana Kračmerová, Lenka Rossmeislová, Lucia Mališová, Zuzana Kováčová, Michaela Tencerová, Eva Klimčáková, Jan Polák, Vladimír Štich, Dominique Langin and Michaela Šiklová

Journal of Clinical Endocrinology and Metabolism, 2014, March, 99(3): 528-535. IF 6.3

6. **Weight loss improves the adipogenic capacity of human preadipocytes and modulates their secretory profile**

Lenka Rossmeislová, Lucia Mališová, Jana Kračmerová, Michaela Tencerová, Zuzana Kováčová, Michal Koc, Michaela Šiklová-Vítková, Nathalie Viquerie, Dominique Langin and Vladimír Štich

Diabetes, 2013, June, 62(6): 1990-1995. IF 7.9

## 3.2 Comments to the results and discussion

Since obesity and its associated metabolic comorbidities are one of the major health problems of 21st century, research of links leading from impaired function of AT in obese patients to IR development is in the center of interest of many research groups. It was shown that hypertrophied adipocytes release higher amounts of FA as well as pro-inflammatory cytokines and other molecules that disrupt sensitivity of cells to insulin. In contrast, amount of released insulin sensitizing molecules, such as AdipoQ, is reduced. In studies on cell cultures and rodent models, negative effect of high levels of glucose and FA [183] on immune status of AT cells was indicated, but exact influence of their action in humans was not elucidated yet. Therefore, the aim of the first part of my thesis was to investigate effects of experimentally increased levels of glucose and lipid metabolites (FA, TAG) on immune cells in blood and AT and systemic markers of inflammation in human volunteers.

In contrast to pro-inflammatory effects of overfeeding and high circulating levels of glucose and lipids, even moderate weight loss has beneficial impact on AT function and secretory profile, as well as on whole body immune status and insulin sensitivity, although the mechanisms of these benefits are not clear. Thus, the goal of the second part of this thesis was to improve our understanding of relationship between changes of immunity-related characteristics of AT and improvement of metabolic parameters triggered by weight loss.

*PART ONE*

Obese subjects have impaired function of AT manifested by its insufficient ability to store energy that leads to increased levels of nutrients, such as glucose and lipid compounds (free FA and glycerol) in blood stream. The raised levels of these metabolites are one of possible triggers of increased inflammatory state of obese subjects that can finally lead to impairment of insulin signaling and T2DM development. Nevertheless, even in lean subjects, plasma levels of glucose and FA, TAG etc. increase after meal consumption. This rise is associated with inflammatory state (so called postprandial inflammation), that is manifested by increased plasma levels of inflammatory cytokines and leukocyte activation [190, 191]. This effect of meal, especially of meal with high levels of nutrients, is however protracted in obese subjects and may contribute to aberrant immune system activation. Indeed, prolonged

exposure to nutrients can cause ERS that may activate classic inflammatory regulatory molecules such as NFκB and Jun N-terminal kinase [168]. Nevertheless, it remains unknown whether ERS is prerequisite for the development of postprandial inflammation. Therefore, **in the first study**, effect of HFM on PBMC and inflammatory state was examined, with emphasis on association between ERS and postprandial inflammation.

10 lean men consumed a high energy, high-fat meal (McDonalds, Prague, Czech Republic, 6151 kJ (1469 kcal), 32.8% carbohydrates, 47.4% lipids, 11.3% proteins) within 15 minutes. Blood samples were drawn each hour up to the $4^{th}$ hour. Activation of immune system was monitored by flow cytometry of peripheral blood and by quantitative real time polymerase chain reaction (qRT-PCR) of mRNA from CD14+ cells (monocytes). These cells were separated from PBMC isolated by Histopaque/Accuspin density system.

In this study, we confirmed the postprandial elevation of IL6 levels in plasma as was shown previously by several studies [192-194]. Also in accordance with previous studies [191, 195], HFM intake induced postprandial increase of all main leukocyte groups - granulocytes, monocytes and lymphocytes – in blood. Moreover we confirmed the finding by Gower et al.[196] showing increased CD11c expression on the surface of monocytes after ingestion of the HFM by healthy volunteers. CD11c is considered as an activation marker of monocytes because it enhances their adhesion to endothelial cells and the potential to migrate into target tissues. Importantly, high-fat diet feeding results in the infiltration of CD11c+ monocytes into AT in mice [111, 197], and these monocytes/macrophages exhibit a pro-inflammatory M1 phenotype. CD11c expression has also been found to increase in blood monocytes of obese subjects and to positively correlate with homeostasis model assessment of insulin resistance (HOMA-IR) [198]. We then focused on gene expression in CD14+ monocytes from peripheral blood i.e. cells that are intimately exposed to metabolite fluctuations and upon activation may contribute to the development of AT inflammation. Remarkably, the mRNA expression of all tested pro-inflammatory cytokines was in CD14+ monocytes enhanced after the HFM challenge. As noted already for CD11c expression, postprandial changes in the expression pro-inflammatory cytokines were similar to the changes in their expression associated with obesity [199, 200]. Therefore, a single HFM may activate monocytes in a similar direction to a long-term overfeeding or obesity.

To determine whether postprandial inflammation could be triggered by enhanced ERS, we analyzed ERS markers representing all three arms of UPR: heat shock 70kDa protein 5 (HSPA5), ATF4, ER degradation enhancer, mannosidase alpha-like 1 (EDEM1), XBP1 (spliced vs. total) and DnaJ (Hsp40) homolog, subfamily C, member 3 (DNAJC3). Following the HFM challenge, mRNA expression of a majority of ERS markers was not altered in PBMC. Thus, the classic activation of UPR does not seem to be the driver of the postprandial increase in the expression levels of inflammatory cytokines in CD14+ monocytes. The only ERS marker whose expression was postprandially elevated was ATF3. It mostly acts as a transcriptional repressor and may thus be part of a counterbalance system in healthy individuals, protecting them from over-activation of pathways induced by stress [201-203]. Therefore, it could be envisioned that this counterbalance system is impaired in obese and/or diabetic subjects who suffer from intensified and prolonged postprandial inflammation [146, 190, 204]. ATF3 is, however, activated not only by ERS but also by other various stresses [205], and the absence of the upregulation of ATF4 (classic UPR pathway, which in turns induces expression of ATF3 [206]) in the analyzed CD14+ cells suggests that the up-regulation of ATF3 is not associated with the activation of UPR. In conclusion, we demonstrated that inflammation induced by the HFM challenge in CD14+ monocytes was not accompanied by an activation of classic UPR. These results are presented in Paper 1 (Page 40).

Consumption of high fat/high energy meal is the most physiological but difficult-to-control experimental way to increase plasma levels of glucose and lipid metabolites. Thus, to test the effect of a single nutrient, a preferred experimental approach is an intravenous infusion of the selected compound. It was shown that acute hyperglycemia can activate inflammatory pathways in various cells resulting in increased secretion of pro-inflammatory cytokines [173-175]. Therefore, the objective of **the second study** was to investigate whether acute experimental HG, imitating increased glycaemia found in obese with metabolic syndrome , has an impact on phenotype and relative content of monocytes/macrophages and lymphocytes in circulation and the subcutaneous abdominal AT (SAAT).

30 healthy obese premenopausal women without signs of metabolic syndrome were recruited and divided into 3 groups (n=10 per group): one was exposed to hyperglycemic- euinsulinemic clamp (where the endogenous insulin release was

blocked by octreotide infusion) and two control groups were exposed to the infusion of octreotide or saline. SAAT was obtained using needle biopsy. Blood and SAAT samples were collected before and after the 3-hours lasting intervention and used for flow cytometry analysis. Moreover, SAAT was used to examination of mRNA levels of chemokines (CCL2, CCL5, CXCL12, IL1β, IL8, TNFα), markers of macrophages (CD14, CD206, matrix metalloproteinase 9 - MMP9, toll-like receptor (TLR) 2, TLR4) and T lymphocytes subtypes (CD3g, CD4, T-Box 21 - TBX21/$T_{H}1$, GATA binding protein 3 - GATA3/$T_{H}2$, RAR-related orphan receptor C - RORC/$T_{H}17$, forkhead box P3 - FoxP3/$T_{REG}$) by qRT-PCR.

We documented that HG induced an increase in CD45+/14+ monocyte/macrophage population in SAAT. It was shown previously that HG treatment of monocytes in vitro increases expression of Toll-like receptors [207] and also monocytes from patients with T2DM show a higher expression of TLR2 and TLR4 compared to healthy subjects [180] , thus the expression of these two receptors was investigated. In SAAT, only TLR4+ monocyte population was increased. This selective effect of HG on TLR4+ monocyte population could point to a specific physiological function of this subtype of monocytes in HG-affected SAAT. Indeed, recent findings suggest that TLR4 and TLR2 activation in macrophages results in the differential expression and secretion of pro-inflammatory cytokines [208, 209]. In accordance, HG clamp induced increase of mRNA expression of TLR4 along with TNFα, which has been shown to be up-regulated after TLR4 but not TLR2 stimulation in macrophages [208].

Contrary to monocyte population, a population of resident AT macrophages did not show any changes in response to HG in terms of relative content and TLRs expression (i.e. content of CD45+/14+/ +/TLR2+ and TLR4+). Therefore, it seems that SAAT microenvironment, changed by HG, activated only monocytic cells that are not fully differentiated into macrophages. Such a population of CD206- monocytic cells was described by Wentworth et al. [113] and was shown to be elevated in human obesity. It is plausible that these monocytes represent “the newest arrivals” into AT but then later can mature into CD206+ macrophages. Nevertheless, CD206 marker used to identify resident AT macrophages was previously suggested to be preferentially expressed by M2 macrophages [112], and thus it is also possible that observed increase in CD45+/14+/206- population could be attributed to M1 macrophages.

Lymphocytes play a key role in infiltration of immune cells into AT [139, 210, 211]. We found an increased content of total T lymphocytes and both major subpopulations

of T lymphocytes, i.e. $T_H$ CD4+ and $T_C$ CD8+ in SAAT of obese women in response to short-term HG. In animal studies, CD8+ T cells direct macrophage infiltration into AT [210] and CD4+ T cells have both anti- and pro-inflammatory roles based on their further specialization [212]. In line with previous data showing that HG modulates expression of genes related to immune response in SAAT of lean subjects [179, 213], we observed that mRNA levels of CD3g, CD4 and CD8a increased in the experimental condition of HG in obese women, which nicely supports the FACS results. Furthermore, we found the up-regulation of TBX21, GATA3 and FoxP3 mRNA levels in SAAT (corresponding to $T_H1$, $T_H2$ and $T_{REG}$ subtypes) after HG condition in obese women. It has been shown that $T_H1$, $T_{REG}$ are increased and $T_H2$ subpopulation is decreased with obesity [214, 215]. Based on our results, one can hypothesize that HG enhanced infiltration of both pro- and anti-inflammatory T cells in order to maintain immune homeostasis in AT.
In summary, our results show that the short-term HG induces an increase in the content of monocytes and T lymphocytes in SAAT of healthy obese women and thus may contribute to the worsening of an immune status of AT in obese individuals. These results are presented in Paper 2 (Page 51).

Beside glucose, another possible contributor to inflammation development appears to be elevated levels of FA. In mice, FA blood concentration and increased mobilization of FA during fasting was associated with increased macrophages content in AT [183]. *In vitro*, saturated FA induce increased mRNA expression and secretion of pro-inflammatory cytokines and chemokines (CCL2, IL6, IL8) in adipocytes, macrophages and other cell types. Objective of **the third study** was to describe the impact of artificially increased circulating concentration of FA on immune system activation in blood and SAAT.
17 obese premenopausal women were recruited into the intervention: 10 subjects were included in the treatment group with 7 hours lasting infusion of 20 % Intralipid solution (lipid emulsion of soya-bean oil (20%) stabilized with egg yolk phospholipids (1.2%) and glycerol (2.5%). The FA composition of Intralipid is as follows: palmitic acid 11.3%, stearic acid 4.9%, oleic acid 29.7%, linoleic acid 46.0% and linolenic acid 8.1%). 7 subjects participated in the control trial with infusion of glycerol (2.5%). To determine the effect of FA on relative content and phenotype of immune cells in blood and SAAT, blood and biopsied SAAT samples were collected before and after the

interventions and analyzed by flow cytometry. Moreover, SAAT was used to examination of mRNA levels of chemokines (CCL2, IL8), angiogenesis marker (VEGF-A), markers of macrophages activation (CD14, CD206, TLR4) and T lymphocytes subtypes (TBX21/$T_H$1, GATA3/$T_H$2, RORC/$T_H$17, FoxP3/$T_{REG}$) by qRT-PCR.

In our study, we found a trend to increased relative content of total T lymphocytes and an increase of $T_H$ subpopulation in blood in response to lipid infusion. This observation is in agreement with previous studies showing that FA modulate T cells proliferation [216] and that lymphocyte counts increase postprandially in healthy as well as in hyperlipidemic subjects with coronary artery disease [217, 218]. Intralipid-induced increase of $T_H$ lymphocyte content in blood is of particular interest as $T_H$ cells appear to be essential players in the development of atherosclerosis [219]. Despite no detectable changes in the content of lymphocytes in SAAT (evaluated by flow cytometry), we have observed an upregulation of SAAT mRNA expression of RORC ($T_H$17 marker). As mentioned earlier, $T_H$17 cells are pro-inflammatory and its increased numbers in AT of metabolically unhealthy obese subjects or in diet-induced obesity in mice were observed [220]. Thus, $T_H$17 lymphocytes could be the first cells in AT responding to elevated FA concentration. Together, our data suggest that acute hyperlipidemia induced by Intralipid infusion induce pro-inflammatory changes in lymphocyte populations.

In contrast to lymphocytes, the relative blood content of CD45+/CD14+ monocytes and the subpopulations of "non-classical" CD16+ activated monocytes was decreased. This trend could be possibly explained by the enhanced adherence of monocytes to the endothelial surface of vascular wall after the lipid infusion. Likewise, the enhanced adhesion of monocytes was observed in response to postprandial hypertriglyceridemia in rats [221]. Indeed, levels of soluble adhesion molecules (intercellular adhesion molecule - ICAM, vascular cell adhesion molecule - VCAM) and angiogenic factor VEGF-A, expressed by the endothelial cells [222], were increased in response to lipid infusion. Increased levels of adhesion molecules moreover suggest that lipid infusion in obese induced the endothelial activation, which is the first step in the development of atherosclerosis [196].

In SAAT, the relative content of monocytes was not changed, however the subset of CD45+/14+/206+/16+ resident macrophages was decreased. In some studies, CD206 is, similarly as CD16, considered as marker of non-classical –"M2" activated

macrophages [44]. Therefore we can hypothesize that Intralipid infusion stimulated the switch of macrophages to "classical" activated pro-inflammatory phenotype. This switch could be supported by increased mRNA expression of CCL2 and IL6 cytokines.

In conclusion, the acute hyperlipidemia induced by Intralipid infusion was associated with pro-inflammatory and pro-atherogenic changes in monocyte and lymphocyte populations and soluble mediators in blood in obese women. Moreover, pro-inflammatory changes – represented by a decrease of M2 macrophages content and increased expression of inflammatory cytokines and marker of $T_H17$ cells - were observed in SAAT.

These results thus point at the processes that could contribute to the initiation of atherosclerosis and worsening of AT immune status in obese patients exposed to high circulating lipid levels. These results are presented in Paper 3 (Page 65).

*PART TWO*

The obesity-related metabolic disturbances are linked with pro-inflammatory state of AT characterized by enhanced recruitment of macrophages into AT and modified AT secretion of cytokines [18-20]. Weight reduction induced by hypocaloric diet is the key approach for non-pharmacological treatment of obesity. Already a moderate loss of initial body weight induces an adaptation of human AT associated with improvement of whole-body metabolic status and suppression of low-grade inflammation [223, 224]. Therefore, purpose of **the fourth study** was to elucidate impact of weight reduction on macrophage content and cytokines production in two different subcutaneous fat depots: subcutaneous gluteal AT (SGAT) and SAAT. Additional goal was to evaluate whether previously described protective role of SGAT is attributable to different inflammation-related characteristics of this depot.

14 pre-menopausal women underwent 6 months DI consisting of 3 periods: 1 month of VLCD, 2 months of LCD, followed by 3 months of weight maintenance (WM) phase. The paired samples of subcutaneous AT were obtained from the abdominal and gluteal region using needle biopsy in three phases of DI and used for RNA isolation. A gene expression of 17 genes related to immune status of AT was analyzed by qRT-PCR. Genes were selected according to their origin or function: cytokines (IL6, TNF, CCL2, CXCL1, IL10, TGFβ1, IL receptor α subunit - IL10Rα) and cytokine receptor (chemokine (C-C motif) receptor 2 - CCR2) and macrophage markers (secreted

phosphoprotein 1 - SPP1, CD68, macrophage scavenger receptor 1 - MSR1, phospholipase A2, group VII - PLA2G7, acid phosphatase 5 - ACP5, Fc fragment of IgG binding protein - FCGBP, CD14, TLR4, TLR2).

Although protective role of AT accumulation in the lower body was suggested [10, 12, 15], our data, similar to findings of other groups [6, 225], did not show major difference in cytokines and macrophage markers between these depots in basal state, with exception of two macrophage markers ACP5 and MRS1 and two cytokines IL10Rα and CCL2. Taken together, our and previously published [226] results do not support the hypothesis of the lower pro-inflammatory profile of sGAT. This is in line with the finding reported by Tchoukalova et al. [225] that subcutaneous abdominal and femoral fat depot did not differ in number of macrophages in lean men and women.

The main novelty of this study lies in the comparison of gene regulation in SAAT vs. SGAT during dynamic condition represented by two phases of a 6 months' DI. The bi-phasic pattern of the expression of macrophage markers and cytokines derived predominantly from the SVF cells observed in this study is in line with previous results obtained in SAAT in different cohorts of subjects [187, 188]. In both groups of genes (macrophage markers and cytokines), pattern of gene expression did not differ markedly in SAAT vs. SGAT, except for three cytokines – IL6, IL10 and CCL2. The reason for differential depot-related response of the three cytokines remains unknown but we speculate that it might be linked to differential response of endocannabinoid system [227]. In fact, endocannabinoids were shown to inhibit production of several pro-inflammatory cytokines in primary human Muller cells and it was reported that the expression of cannabinoid receptor type 1 during the weight reducing diet was different between SGAT and SAAT [228]. In light of our and others results demonstrating the absence of major differences between SGAT and SAAT it has been suggested that the deleterious effect of upper body obesity could be mediated by the excess of visceral adipose tissue (VAT) and not excess of SAAT. Furthermore, it should be noted that the present study compared SGAT and SAAT on transcriptional level and that the results of this study are limited to women. Female AT shows different metabolic and endocrine characteristics [229, 230] when compared with men. Moreover, the initial fat distribution in our set of women (mean waist/hip ratio 0.861±0.0) might play a role in the diet-induced response of the two fat depots

although the reports on the effect of initial fat distribution on the body fat reduction are not unequivocal [231, 232].

In conclusion, we did not find major differences in mRNA levels of macrophage markers and cytokines between SAAT and SGAT at baseline condition or in the pattern of their regulation in response to two phases of hypocaloric weight-reducing DI. Therefore, our results do not bring evidence of an altered pro-inflammatory status or an altered “responsiveness” of immune cells in SGAT when compared with SAAT. These results are presented in Paper 4 (Page 87).

**The fifth study** was focused on macrophage marker CD163 and its soluble form sCD163. CD163 is predominantly expressed by tissue macrophages and it is cleaved and released to circulation by similar enzyme as TNFα – TNFα converting enzyme (TACE, also known as ADAM Metallopeptidase Domain 17) [233]. Thus, circulating sCD163 could be produced by AT macrophages similarly as soluble TNFα. Levels of sCD163 were shown to be elevated in obese subjects and were found to represent a marker of IR due to its association with impaired insulin sensitivity [234, 235]. Moreover, sCD163 concentration was predicted as a marker of macrophages infiltration to AT, but this hypothesis was not validated. Hence, the aim/goal of this study was to extend our knowledge about coherence of CD163 in circulation and AT with IR not only in steady state, but also during dynamic weight reducing conditions that are associated with the improvement of metabolic health.

Two cohorts of subjects were examined in the study. Cohort 1 included 42 women with a wide range of BMI (17–48 kg/m2) divided into three groups according to their BMI and presence or absence of metabolic syndrome (lean, obese, obese with metabolic syndrome). Samples of VAT and SAAT were obtained during abdominal surgery. The values of glucose disposal rate (GDR; determinant/index of insulin sensitivity) were acquired from the euglycemic-hyperinsulinemic clamp method performed according to De Fronzo et al. [236].

Cohort 2 included 27 obese women who followed a DI consisting of 1 month of a VLCD and 5 months of a weight-stabilization period (consisted of 2 months of LCD and 3 months of a WM period). The biopsied samples of SAAT were obtained in three phases of DI. A gene expression of two macrophage markers (CD163, CD68), classical inflammatory marker TNFα and two genes responsible for sCD163 shedding

(TACE and tissue inhibitor of metalloproteinases 3 - TIMP3) in SAAT was analyzed by qRT-PCR.

In a Cohort 1, our finding supported previous suggestion that plasma sCD163 levels are associated with mRNA expression in SAAT [234, 237] and furthermore a similar association of sCD163 and VAT was found. Moreover, the correlation of sCD163 levels with mRNA expression of macrophage marker CD68 suggests that serum sCD163 might be perceived as a possible indicator of macrophage activation in AT. Next, we documented a strong relationship between insulin sensitivity, expressed as GDR, and circulating sCD163. These results extend the previously reported findings of a close relationship between sCD163 and HOMA-IR [234, 238]. In our study, insulin sensitivity correlated also with CD163 mRNA expression in both SAAT and VAT depots. Thus, we confirmed a validity of sCD163 and CD163 expressed in AT as a biomarker of insulin sensitivity at steady-state condition.

However, in a dynamic condition represented by the weight-reducing hypocaloric diet in Cohort 2, the above-mentioned associations were not present: the diet-induced change of sCD163 showed different pattern and did not correlate with the change of CD163 mRNA levels in SAAT either during the initial dynamic phase of the DI (VLCD) or during the WM phase. The mRNA CD163 expression pattern was in line with magnitude of mRNA CD68 in AT and other macrophage markers analyzed in our previous publications [187, 188, 239]. The discrepancy between the dynamics of soluble and AT mRNA levels of CD163 could be based on the translational or posttranslational regulation of expression. Among factors influencing sCD163 production could be the efficiency of shedding of the CD163 from the macrophage surface that is mediated/regulated by the enzyme TACE and its inhibitor TIMP3 [240]. However, no relevant change of TACE or TIMP3 mRNA expression in AT throughout the DI was found. Therefore, the changes in the shedding of CD163 in AT during DI probably do not contribute to the changes of sCD163 in circulation. Other possible explanation of this discrepancy is that CD163 is expressed in several other tissues, such as liver, muscle, kidney [235, 241-243], and also in blood monocytes [244]. Unfortunately, to our knowledge, there are no studies that evaluate direct contribution of other tissues to circulating levels of sCD163 or investigating CD163 expression in other tissues or cells during DI.

In this study the evolution of sCD163 during weight-reducing DI paralleled that of the GDR measured by a hyperinsulinemic clamp. However, the direct correlations

between the diet- induced changes of sCD163 and those of GDR were not found. Similarly, no correlation was found between the diet-induced changes of CD163 mRNA expression and insulin sensitivity. These findings suggest that circulating levels of sCD163 and AT mRNA expression of CD163 are probably not in a cause-effect relationship with insulin sensitivity.

In conclusion, in this study we demonstrated a quantitative association between the circulating levels of sCD163 and mRNA expression of macrophage markers CD163 and CD68 in SAAT and VAT in the steady-state condition. Furthermore, in the steady-state condition, we found a negative correlation between sCD163 levels and insulin sensitivity. However, in a dynamic condition represented by a weight-reducing DI, there is no such relationship between the diet-induced changes of the above-mentioned variables. Thus, there is no evidence that sCD163 might be used as a quantitative biomarker of the diet-induced changes of AT CD163 expression or changes of insulin sensitivity. These results are presented in Paper 5 (Page 98).

Worsening of metabolic state in obesity is associated with impaired endocrine function of adipocytes. The current knowledge on intrinsic endocrine potential of these cells is based on and limited to cross-sectional studies. We hypothesized that cell cultures of adipose precursors established from SAAT acquired before and after the diet-induced weight loss would reflect two distinct metabolic and nutritional stages of the donor and could provide information about the intrinsic endocrine potentials of obese and post-obese AT. Thus, **in the sixth study**, effect of moderate weight loss on the secretory profile of adipocyte precursors was examined.

23 premenopausal women underwent 5-6 lasting weight reducing intervention consisted of 3 months of LCD and subsequent 3 months of WM phase. Paired cell cultures of human preadipocytes were established from SAAT samples obtained by needle biopsy before and after the entire DI. To determine whether weight loss affects the intrinsic secretory potential of adipocytes, the secretion and mRNA expression of several cytokines (CCL2, IL6, IL8) and adipokines (AdipoQ, leptin) was measured in *in vitro* differentiated preadipocytes.

We showed that secretory capacity of *in vitro* cultured preadipocytes derived from adipocyte precursors is affected by moderate weight loss. This was documented by comparing secretion and expression of IL8, CCL2, leptin, and AdipoQ by cells isolated from paired subcutaneous AT biopsies from obese women undergoing long-

term DI. In obesity, hypertrophied adipocytes produce prevalently pro-inflammatory cytokines and chemokines such as TNFα, IL6 and CCL2 [245]. These cytokines may affect the phenotype of the macrophages already residing in the AT and stimulate infiltration and activation of macrophages from circulation [246]. On the other hand, secretion of insulin sensitizing AdipoQ is diminished [88] in obese subjects.

We observed an increase of expression and secretion of AdipoQ and its HMW form in adipocytes after DI. Leptin mRNA levels were also elevated in adipocytes after weight loss. In contrast, CCL2 and IL8 mRNA levels in adipocytes obtained after DI were reduced compared to baseline.

Importantly, the secretion of adipokines with the exception of leptin by *in vitro* cultivated adipocytes reflected in general changes seen at the level of AT explants [188]. Lower secretion of CCL2 from adipocytes reprogrammed by weight loss could contribute to a lower infiltration of macrophages into AT described earlier [187, 247]. Selective increase of HMW AdipoQ secretion might underlie beneficial effects of weight loss on insulin sensitivity.

Studies performed on cell culture models may be influenced by culture conditions. Although we cannot completely exclude possible effects of sub-cultivation on secretory potential of cells, it has been shown that in vitro conditions preserve the original phenotype of a donor as shown previously for preadipocytes and adipocytes [248, 249]. Moreover, sub-cultivation of stromal vascular cells eliminates contaminating cells like macrophages and results in more homogenous population than primary cells [250, 251]. It is also unlikely that the observed differences were based on dissimilar starting numbers of cells as there was no difference in the length of cultivation or yield of cells before and at the end of DI.

In conclusion, our study shows that weight loss alters secretory potential of preadipocytes. This effect may be associated with the improvement of the metabolic status of obese. We believe that the analysis of a distinct cellular population, such as preadipocytes subjected to uniform in vitro conditions, can offer a focused and unique image of an intrinsic adaptation of AT to weight loss. These results are presented in Paper 6 (Page 107).

# ORIGINAL ARTICLES

*PART ONE*

## Paper 1

Jana Kračmerová, Eva Czudková, Michal Koc, Lucia Mališová, Michaela Šiklová, Vladimír Štich and Lenka Rossmeislová

**Postprandial inflammation is not associated with endoplasmic reticulum stress in PBMC from healthy lean men**

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# Postprandial inflammation is not associated with endoplasmic reticulum stress in peripheral blood mononuclear cells from healthy lean men

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## Abstract

The consumption of lipids and simple sugars induces an inflammatory response whose exact molecular trigger remains elusive. The aims of the present study were to investigate (1) whether inflammation induced by a single high-energy, high-fat meal (HFM) is associated with endoplasmic reticulum stress (ERS) in peripheral blood mononuclear cells (PBMC) and (2) whether these inflammatory and ERS responses could be prevented by the chemical chaperone ursodeoxycholic acid (UDCA). A total of ten healthy lean men were recruited to a randomised, blind, cross-over trial. Subjects were given two doses of placebo (lactose) or UDCA before the consumption of a HFM (6151 kJ; 47·4 % lipids). Blood was collected at baseline and 4 h after the HFM challenge. Cell populations and their activation were analysed using flow cytometry, and plasma levels of inflammatory cytokines were assessed by ELISA and Luminex technology. Gene expression levels of inflammatory and ERS markers were analysed in $CD14^{+}$ and $CD14^{-}$ PBMC using quantitative RT-PCR. The HFM induced an increase in the mRNA expression levels of pro-inflammatory cytokines (*IL-1β*, 2·1-fold; *IL-8*, 2·4-fold; *TNF-α*, 1·4-fold; monocyte chemoattractant protein 1, 2·1-fold) and a decrease in the expression levels of *miR181* (0·8-fold) in $CD14^{+}$ monocytes. The HFM challenge did not up-regulate the expression of ERS markers (*XBP1*, *HSPA5*, *EDEM1*, *DNAJC3* and *ATF4*) in either $CD14^{+}$ or $CD14^{-}$ cell populations, except for *ATF3* (2·3-fold). The administration of UDCA before the consumption of the HFM did not alter the HFM-induced change in the expression levels of ERS or inflammatory markers. In conclusion, HFM-induced inflammation detectable on the level of gene expression in PBMC was not associated with the concomitant increase in the expression levels of ERS markers and could not be prevented by UDCA.

Key words: **Peripheral blood mononuclear cells: Ursodeoxycholic acid: Postprandial inflammation: Endoplasmic reticulum stress**

The pandemic of obesity in the Western world has been attributed to the lack of physical activity and availability of highly palatable, easily digestible and energy-dense food. Palatability is based on a high content of lipids and simple sugars. However, the overconsumption of lipids and simple sugars is associated with the exaggeration of postprandial blood glucose and lipid levels[1]. The protracted elevations of blood metabolites are the signs of postprandial dysmetabolism associated with so-called postprandial inflammation[1–3]. Postprandial inflammation is manifested by increased plasma levels of inflammatory cytokines and leucocyte activation[4,5], although the precise contribution of blood monocytes and lymphocytes to these pro-inflammatory changes remains unknown. While in healthy people, postprandial inflammation is transient, it is prolonged in obese people and in subjects with type 2 diabetes[2,3,6]. Thus, prolonged postprandial inflammation has been suggested to promote insulin resistance and atherosclerosis. The exact molecular trigger of postprandial inflammation is not fully elucidated yet. Nevertheless, it has been shown previously that exposure of cells to saturated lipids and a high concentration of glucose may cause endoplasmic reticulum stress (ERS), as documented by the increased mRNA levels of several ERS markers or by the increased activity of an ERS-responsive LacZ reporter

**Abbreviations:** ATF, activating transcription factor; DNAJC3, DnaJ (Hsp40) homolog, subfamily C, member 3; EDEM1, ER degradation enhancer, mannosidase alpha-like 1; ERS, endoplasmic reticulum stress; HFM, high-fat meal; HSPA5, heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa); MCP1, monocyte chemoattractant protein 1; miRNA, microRNA; PBMC, peripheral blood mononuclear cell; RANTES, regulated on activation, normal T-cell expressed and secreted; TLR, Toll-like receptor; UDCA, ursodeoxycholic acid; UPR, unfolded protein response; XBP1, X-box binding protein 1; XBP1s, X-box binding protein 1 spliced.

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system[7–9]. ERS leads to the activation of pathways that primarily decrease the burden of endoplasmic reticulum or eliminate the affected cell. Meanwhile, however, it leads to the stimulation of classic inflammatory regulatory molecules such as NF-κB and Jun N-terminal kinase[10]. Thus, postprandial inflammation could be triggered by ERS. Notably, ERS-induced inflammation may be alleviated by chemical chaperones such as bile acids[11]. One such chemical chaperone, ursodeoxycholic acid (UDCA), currently used therapeutically for the treatment of cholestasis, has been shown to prevent chemically induced ERS *in vitro*[12,13]. Given these facts, we analysed inflammation induced by a single high-fat meal (HFM) in two subpopulations of peripheral blood mononuclear cells (PBMC) representing cells of innate and adaptive immunity, and tested whether this HFM-induced inflammation is associated with ERS. Furthermore, we investigated whether the inflammatory or ERS response may be modified or prevented by the non-toxic chemical chaperone UDCA.

## Experimental methods

### *Subjects and study design*

A total of ten healthy lean male subjects were recruited to a randomised, blind, cross-over trial consisting of two 1 d studies, separated by at least 1 week (when the subjects followed their habitual diet and level of exercise). Exclusion criteria were as follows: weight changes of >3 kg within the 3 months before the start of the study; participation in other trials; hyperbilirubinaemia; smoking; alcohol or drug abuse. The characteristics of the subjects are provided in Table 1. Subjects were given 10 mg/kg of placebo (lactose) or UDCA (Ursosan; PRO.MED.CS) in gelatin capsules with the last evening meal (20.00 hours) before the experimental day. Upon admission (08.00 hours), a catheter was placed in the antecubital vein. After baseline blood sampling, subjects were given 15 mg/kg of placebo or Ursosan. Within 15 min, they consumed a high-energy, HFM consisting of a breakfast sandwich with pork meat and egg omelette, French fries, ketchup, Nutella spread, croissant, ice tea (McDonalds; 6151 kJ; 32·8 % carbohydrates, 47·4 % lipids and 11·3 % proteins). After the meal was consumed, blood was drawn each hour up to the 4th hour. During the intervention, subjects had free access to drinking-water. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the ethical committee of the Third Faculty of Medicine of Charles University in Prague, Czech Republic. Written informed consent was obtained from all subjects before the study.

**Table 1.** Characteristics of the subjects

(Mean values with their standard errors, *n* 10)

| | Mean | SEM |
|---|---|---|
| Age (years) | 26·3 | 1·04 |
| BMI (kg/m²) | 23·11 | 0·59 |
| Weight (kg) | 77·51 | 2·48 |
| Waist circumference (cm) | 81·5 | 1·96 |
| Fat mass (%) | 13·46 | 1·06 |
| Glucose (mmol/l) | 4·77 | 0·11 |
| Insulin (mU/l) | 5·4 | 0·66 |
| HOMA-IR | 1·16 | 0·15 |
| TAG (mmol/l) | 0·82 | 0·13 |
| HDL-cholesterol (mmol/l) | 1·59 | 0·15 |
| Total cholesterol (mmol/l) | 4·81 | 0·28 |

HOMA-IR, homeostasis model assessment of the insulin resistance index.

### *Determination of plasma levels of biochemical parameters*

Plasma glucose levels were determined using the glucose oxidase technique (Beckman Instruments, Inc.). Plasma insulin level was measured using an Immunotech Insulin Irma kit (Immunotech). Homeostasis model assessment of the insulin resistance (HOMA-IR) index was calculated as follows:

$$\text{HOMA-IR} = (\text{fasting insulin (mU/l)} \times \text{fasting glucose (mmol/l)})/22{\cdot}5.$$

Plasma levels of glycerol, NEFA and TAG were measured by colorimetric enzymatic assays using kits from Randox.

### *Flow cytometry analysis*

To determine the absolute numbers of cells in the blood, TruCOUNT tubes containing defined numbers of beads detectable by flow cytometry were used according to the manufacturer's protocol (BD Biosciences). Subpopulations of blood cells representing lymphocytes, granulocytes and monocytes were analysed according to their size and granularity. To detect specific surface antigens, whole-blood samples were stained with fluorescence-labelled monoclonal antibodies (fluorescein isocyanate-conjugated antibodies: CD4, CD14, CD16 and CD36; phycoerythrin-conjugated antibodies: CD3, CD11c, CD14, Toll-like receptor (TLR)2 and TLR4; allophycocyanin-conjugated antibodies: CD8 and CD56) or the appropriate isotype controls (BD Biosciences) for 30 min at room temperature. After cell staining, erythrocytes were lysed by erythrocyte lysis buffer for 15 min at room temperature. The cells were then washed with PBS and analysed on a FACSCalibur flow cytometer and CellQuest Pro Software (BD Biosciences). The number of immune cells in the analysed populations was expressed as a percentage of gated events or the absolute numbers calculated from data obtained by TruCOUNT analysis. Background was set up to 5 % of positive cells of the isotype control.

### *Isolation of peripheral blood mononuclear cells and CD14⁺ cells*

PBMC were isolated by gradient centrifugation. Briefly, 9 ml of uncoagulated blood were diluted in PBS to 16 ml and applied onto Leucosep tubes (Greiner Bio-One) filled with 3 ml of Histopaque-1077 separation medium (Sigma-Aldrich). After centrifugation for 15 min at 800 ***g***, plasma was discarded and PBMC located above the frit were transferred to a tube containing endothelial cell basal medium (PromoCell). The cells

were washed three times, diluted in isolation buffer (PBS supplemented with 0·1 % bovine serum albumin and 2 mM-EDTA, pH 7·4) and counted. Up to 10 million cells were mixed with 25 µl CD14 Dynabeads (Invitrogen) and incubated on a rotator for 20 min at 4°C, and then $CD14^+$ PBMC were separated with a magnet and lysed in RLT (Qiagen). $CD14^-$ PBMC were collected by centrifugation and lysed in RLT. Both fractions of PBMC were then used for RNA isolation. Separation efficiency was confirmed by both fluorescence-activated cell sorting and quantitative RT-PCR analysis (data not shown).

### Gene expression analysis

Total RNA was isolated using a miRNeasy Mini Kit (Qiagen). Genomic DNA was removed by DNase I treatment (Invitrogen). Complementary DNA was obtained by reverse transcription (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems) of 300 or 600 ng of total RNA. Complementary DNA equivalent to 5 ng of RNA was used for real-time PCR analysis using the Gene Expression Master Mix and Gene Expression Assay for heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa) (*HSPA5*) (Hs99999174_m1), activating transcription factor 4 (*ATF4*) (Hs00909569_g1), *ATF3* (Hs00231069_m1), ER degradation enhancer, mannosidase alpha-like 1 (*EDEM1*) (Hs00976004_m1), DnaJ (Hsp40) homolog, subfamily C, member 3 (*DNAJC3*) (Hs00534483_m1), regulated on activation, normal T-cell expressed and secreted (*RANTES*; Hs00174575_m1), *IL-1β* (Hs01555410_m1), *IL-8* (Hs00174103_m1), monocyte chemoattractant protein 1 (*MCP1*; Hs00234140_m1), *PPARα* (Hs00947539_m1), *PPARγ* (Hs01115513_m1), *TLR2* (Hs00152932_m1) and *TLR4* (Hs01060206_m1) (Applied Biosystems). *TNF-α*, X-box binding protein 1 (*XBP1*) total and *XBP1* spliced (*XBP1s*) were detected by specific primers (TNF-α: forward 5′-TCTCGAACCCCGAGTGACA-3′ and reverse 5′-GGCCCGGCGGTTCA-3′; *XBP1* total: forward 5′-CGCTGAGGAGGAAACTGAA-3′ and reverse 5′-CACTTGCTGTTCCAGCTCACTCAT-3′; *XBP1s*: forward 5′-GAGTCCGCAGCAGGTGCA-3′ and reverse 5′-ACTGGGTCCAAGTTGTCCAG-3′) using a SYBR Green technology (Power SYBR® Green Master Mix; Applied Biosystems). The microRNA (miRNA) were transcribed by a miScript II RT kit (Qiagen) without prior DNase I treatment. Complementary DNA equivalent to 1 ng of RNA was used for real-time PCR analysis using the miScript SYBR Green PCR Kit and miScript Primer Assay for ***miR146a*** and ***miR181a*** (Hs_miR-146a*_1 and Hs_miR-181a*_1; Qiagen). All samples were run in duplicate on a 7500 Fast ABI PRISM instrument (Applied Biosystems). Gene expression of target genes was normalised to the expression of ribosomal protein S13 (*RPS13*) (mRNA, Hs01011487_g1) or RNA, U6 small nuclear 2 (*RNU6-2*) (miRNA, Hs_RNU6-2_1) (Qiagen), and expressed as fold changes calculated using the $\Delta\Delta C_t$ method.

### Plasma cytokine analysis

Plasma levels of leptin and adiponectin were measured by ELISA (DuoSet; R&D Systems), with a limit of detection of 62·5 pg/ml. Plasma TNF-α, IL-6, IL-1β and IL-8 levels were measured by the MILLIPLEX MAP Human High Sensitivity Cytokine Panel (Merck), with a limit of detection of 0·13 pg/ml.

### Statistical analyses

Statistical analyses were performed using GraphPad Prism 6 and SPSS 12.0 for Windows (SPSS, Inc.). Data of plasma metabolites, gene expression ($\Delta C_t$) and flow cytometry-derived variables were log transformed, and normality of the data was assessed by the Shapiro–Wilk normality test. The effects of the HFM in the placebo and UDCA treatments were tested using the one-way and two-way ANOVA with Bonferroni *post hoc* analysis. Correlations among the relative mRNA levels were analysed using Spearman's correlation. Data are presented as means with their standard errors. Differences at the level of $P<0·05$ were considered to be statistically significant.

## Results

### Postprandial changes in plasma metabolites

Evolution of postprandial plasma levels of glycerol, NEFA, TAG, glucose and insulin in response to the HFM challenge is shown in Fig. 1. NEFA levels declined after the consumption of the HFM and then gradually increased during the time course of the experiment but not above the fasting levels (Fig. 1(a)). Glycerol and TAG concentrations reached peak values 3 h after ingestion of the HFM (Fig. 1(b) and (c)). Glucose levels did not alter significantly during the whole intervention (Fig. 1(d)), whereas insulin levels increased 1 h after ingestion of the HFM and remained elevated above the fasting levels (Fig. 1(e)). Baseline plasma levels of NEFA and glycerol were lower in the UDCA treatment, though this difference did not reach a significant level. Thus, no differences in baseline or postprandial plasma levels of the tested metabolites between the placebo and UDCA treatments were detected.

### Postprandial changes in blood cell populations

At the fasting state, numbers of leucocytes per µl of blood were not different between the placebo and UDCA treatments (placebo: 9821 (SE 704) cells/µl; UDCA: 9380 (SE 763) cells/µl). The HFM challenge significantly increased the absolute numbers of monocytes, lymphocytes and granulocytes and the total numbers of leucocytes (Fig. 2(a) and (b)). This increase was similar in the presence of UDCA. In addition, the relative distribution of two main leucocyte populations, namely lymphocytes and granulocytes, in the blood changed postprandially, i.e. the relative proportion of lymphocytes decreased, while that of granulocytes decreased reciprocally in response to the test meal in the placebo treatment (data not shown). The relative proportion of monocytes within the whole leucocyte population remained unaltered in response to the HFM challenge. Given that both the relative distribution of the leucocyte population and the absolute counts of cells were affected by the consumption of the test meal, the numbers of events representing gated cells were normalised by

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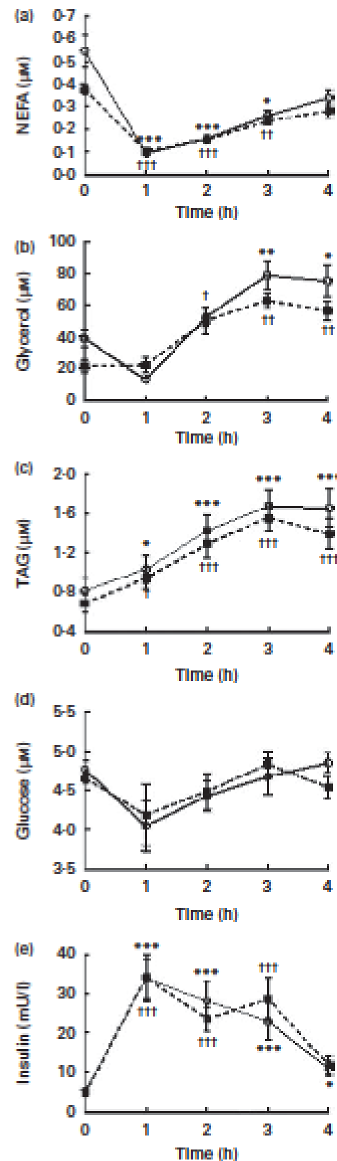


**Fig. 1.** Evolution of plasma levels of (a) NEFA, (b) glycerol, (c) TAG, (d) glucose and (e) insulin following a high-fat meal challenge. Values are means, with their standard errors represented by vertical bars. Mean value was significantly different from that of baseline levels in the placebo (-○-) treatment: * $P<0·05$, ** $P<0·01$, *** $P<0·001$. Mean value was significantly different from that of baseline levels in the ursodeoxycholic acid (-■-) treatment: † $P<0·05$, †† $P<0·01$, ††† $P<0·001$.

TruCOUNT data (the percentage of positive cells multiplied with the absolute number of events in either the monocyte, lymphocyte or granulocyte gate).

The HFM increased the counts of CD14$^+$/CD11c$^+$ and CD14$^+$/TLR2$^+$ monocytes in both placebo and UDCA treatments. The counts of CD14$^+$/TLR4$^+$ monocytes were increased after ingestion of the test meal in the placebo treatment only. However, only in the UDCA treatment, the HFM challenge increased the counts of CD4$^+$ and CD8$^+$ lymphocytes (Fig. 2(c) and (d)).

The evaluation of the expression levels of individual surface markers (expressed as geometric mean fluorescence intensity) revealed that the HFM enhanced the expression levels of the activation marker CD11c in monocytes. This increase was significant in both placebo and UDCA treatments (Fig. 2(e)).

### *Postprandial changes in plasma adipokines and inflammatory cytokines*

Plasma levels of leptin, adiponectin, IL-8 and TNF-α did not alter during the HFM intervention in either the placebo or UDCA treatment (data not shown). Plasma IL-6 levels increased gradually over the 4 h period in both placebo and UDCA treatments (Fig. 3). However, in most samples, plasma levels of IL-1β were under the detection limit.

### *Postprandial changes in the gene expression levels of cytokines in peripheral blood mononuclear cells*

At baseline levels, CD14$^+$ cells expressed substantially higher mRNA levels of *IL-1β*, *IL-8*, *MCP1* and *TNF-α* and lower mRNA levels of *RANTES* compared with the CD14$^-$ cell population (Fig. 4(a)). Therefore, the effect of the HFM on the expression levels of *IL-1β*, *IL-8*, *MCP1* and *TNF-α* was analysed in CD14$^+$ cells, and of *RANTES* in CD14$^-$ cells.

In CD14$^+$ cells, gene expression levels of all the measured cytokines were increased in response to the HFM challenge (Fig. 4(b)–(e)). This increase was similar in both treatments except for TNF-α that was not altered in response to the HFM challenge in the UDCA treatment. Subsequently, the expression levels of two miRNA (***miR181a*** and ***miR146a***) implicated in the negative regulation of the expression of TLR2/4 pathway members were analysed (Fig. 4(f) and (g)). The expression level of ***miR181a***, but not ***miR146a***, was decreased by the consumption of the test meal in both placebo and UDCA treatments. The mRNA expression level of *RANTES*, a cytokine produced by CD8$^+$ lymphocytes, was decreased in CD14$^-$ cells after ingestion of the HFM in the UDCA treatment only (Fig. 4(h)). This result was also confirmed when the expression of *RANTES* was normalised to the pan T-lymphocyte marker *CD3g* (data not shown). However, the changes in the mRNA expression levels of all the measured cytokines in response to the HFM challenge were not different between the placebo and UDCA treatments as revealed by the two-way ANOVA.

The expression levels of other genes potentially activated by dietary fatty acids (i.e. *TLR4*, *TLR2*, *PPARα* and *PPARγ*) were

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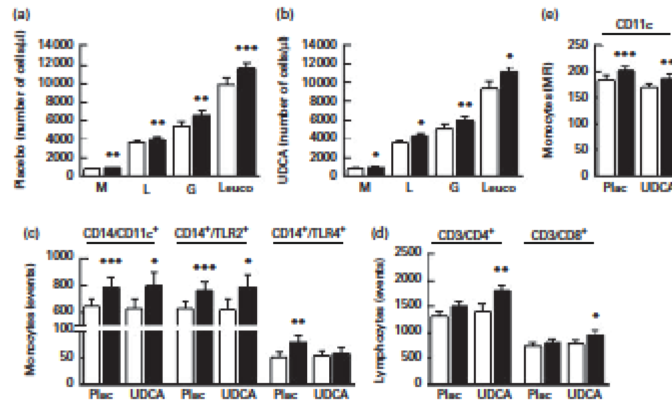


Fig. 2. Effect of the test meal on the numbers and activation of leucocytes. The absolute numbers of leucocytes at the fasting (baseline, □) state were compared with the numbers of leucocytes 4 h after a high-fat meal (■) challenge in the (a) placebo (Plac) and (b) ursodeoxycholic acid (UDCA) treatments. The number of cells in the subpopulations of (c) monocytes and (d) lymphocytes out of 10 000 events in both Plac and UDCA treatments. (e) Mean fluorescence intensity (MFI) for CD11c in monocytes. Values are means, with their standard errors represented by vertical bars. Mean value was significantly different from that of baseline levels: * $P<0·05$, ** $P<0·01$, *** $P<0·001$. M, monocytes; L, lymphocytes; G, granulocytes; leuco, total leucocytes; TLR, Toll-like receptor.

not altered significantly in response to the HFM challenge (Fig. 4(i)–(l)).

### *Postprandial changes in the gene expression of endoplasmic reticulum markers in CD14+ and CD14− peripheral blood mononuclear cells*

First, we compared the expression levels of ERS markers between the two subpopulations of PBMC. Compared with the CD14− cell population, CD14+ cells expressed higher mRNA levels of *ATF4*, *HSPA5* and *DNAJC3*, while both cell populations expressed the levels of *EDEM1* and *XBP1* to the same degree (Fig. 5(a)). The expression of *ATF3* was restricted to CD14+ cells. In response to the HFM challenge, PBMC did not alter the expression levels of *HSPA5*, *ATF4*, *EDEM1*, *XBP1* (spliced *v.* total) and *DNAJC3* in either the placebo or UDCA treatment (Fig. 5(b)–(f)). Nevertheless, the HFM challenge led to a significant increase in the mRNA levels of *ATF3* in CD14+ cells in both placebo and UDCA treatments (Fig. 5(g)). The relative change in *ATF3* expression induced by the test meal correlated with that in *IL-8* expression ($R$ 0·745, $P=0·017$), but did not correlate with the change in the expression of the other cytokines. In addition, baseline mRNA levels of *DNAJC3*, *EDEM1*, *ATF4*, *XBP1s* and *HSPA5* correlated with those of *RANTES* (all correlations reached $R>0·7$, $P<0·03$; Fig. 5(h)).

## Discussion

The aims of the present study were to (1) examine a potential association between inflammatory and ERS responses to a HFM in two subpopulations of PBMC representing cells of innate and adaptive immunity and (2) assess the potential of UDCA, a chemical chaperone, to modify or prevent these responses. Postprandial responses to the test meal were studied in healthy lean male subjects to model the situation that precedes and could contribute to the development of obesity and the metabolic syndrome.

First, we documented the effects of the test meal, which was selected as a typical example of a Western 'fast food' type of diet, on postprandial plasma changes in major metabolites. The evolution of NEFA plasma concentration followed a known pattern in response to a single mixed meal, i.e. an immediate sharp decrease in NEFA levels due to the antilipolytic action of insulin, followed by increased NEFA levels dependent on the spillover fatty acids from chylomicron TAG[14]. In contrast, glucose levels remained unaltered in response to the HFM challenge, as described previously[15–18], even though some published studies[19–21] have shown peak glucose levels after a 30 to 60 min period following a mixed meal challenge. The observed blunted hyperglycaemic response could be caused by significant absolute and relative

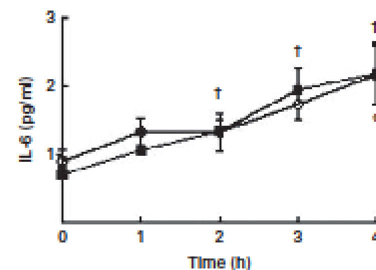


Fig. 3. Evolution of plasma levels of IL-6 following a high-fat meal challenge. Values are means, with their standard errors represented by vertical bars. * Mean value was significantly different from that of baseline levels in the placebo (–○–) treatment ($P<0·05$). † Mean value was significantly different from that of baseline levels in the ursodeoxycholic acid (–■–) treatment ($P<0·05$).

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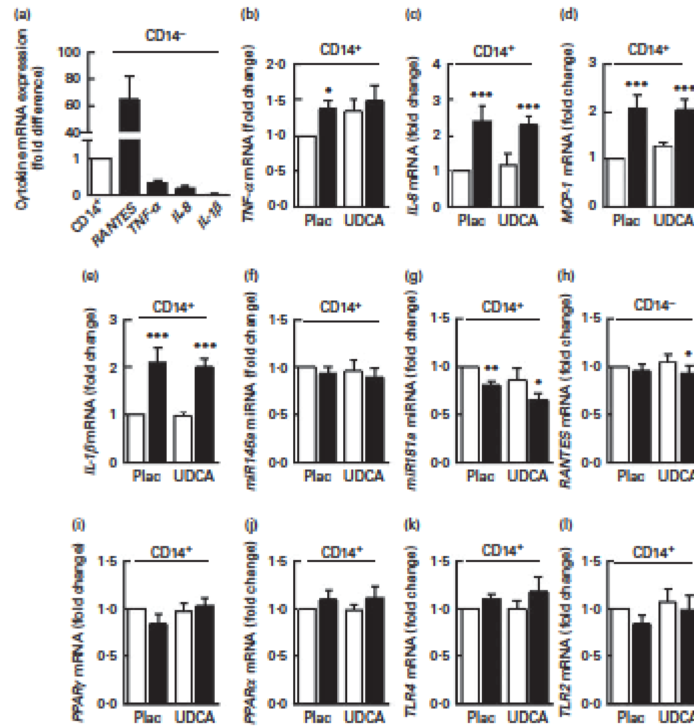


**Fig. 4.** Effect of the test meal on gene expression in CD14$^+$ and CD14$^-$ peripheral blood mononuclear cells (PBMC). (a) Comparison of mRNA expression levels of selected inflammatory cytokines between the CD14$^+$ and CD14$^-$ cells. Quantitative RT-PCR (qRT-PCR) analysis of cytokines (b–e, h) and miRNA (f, g) implicated in the regulation of inflammatory pathways in PBMC collected before and 4 h after a high-fat meal (HFM, ■) challenge. (i–l) qRT-PCR analysis of genes potentially activated by NEFA in CD14$^+$ cells collected before and 4 h after the HFM challenge. Values are means, with their standard errors represented by vertical bars. Mean value was significantly different from that of baseline (□) levels: * $P<0·05$, ** $P<0·01$, *** $P<0·001$. *RANTES*, regulated on activation, normal T-cell expressed and secreted; Plac, placebo; UDCA, ursodeoxycholic acid; *MCP1*, monocyte chemoattractant protein 1; miRNA, microRNA; TLR, Toll-like receptor.

amounts of fat and proteins in the test meal that have been shown to reduce postprandial glucose metabolism probably due to delayed gastric emptying[17,22]. Thus, the complexity of the meal, despite its high absolute (not relative) carbohydrate content, may lead to the paradoxical suppression of postprandial glucose plasma concentration.

In accordance with previous studies[4,23], postprandial leucocytosis was observed in the present study. In line with the results by Hansen *et al.*[4], the test meal used in the present study increased the absolute numbers of granulocytes in the blood. These fast changes observed in granulocyte numbers are probably caused by the release of cells from the marginal pool (cells residing in the slow-flowing lining fluid of the vasculature)[24]. We have also observed an increase in the absolute counts of lymphocytes and monocytes in the blood. It should be noted that the increase in lymphocyte counts may be associated with the circadian rhythm[25,26]. Nevertheless, the meal used in the present study had higher total energy, carbohydrate and protein contents than meals used in the previously cited studies by van Oostrom *et al.*[25,26]. Thus, these metabolic variables may have a more important role in the observed activation of lymphocytes and monocytes than in the circadian rhythm.

Postprandial inflammation was previously characterised by the increased circulating levels of several inflammatory cytokines[5]. We confirmed the postprandial elevation of IL-6 levels. Postprandial increases in plasma IL-6 levels were reported by others[27,28]. As mRNA levels of *IL-6* were barely detectable in CD14$^+$ or CD14$^-$ cells (data not shown), the elevation of IL-6 levels in the circulation was driven by other IL-6-producing cells or tissues.

Concerning HFM-induced changes in blood cells, we confirmed the finding by Gower *et al.*[29] showing increased CD11c expression on the surface of monocytes after ingestion of the HFM by healthy volunteers. CD11c is considered as an activation marker of monocytes because it enhances their adhesion to endothelial cells and the potential to migrate into target tissues. Importantly, high-fat diet feeding results

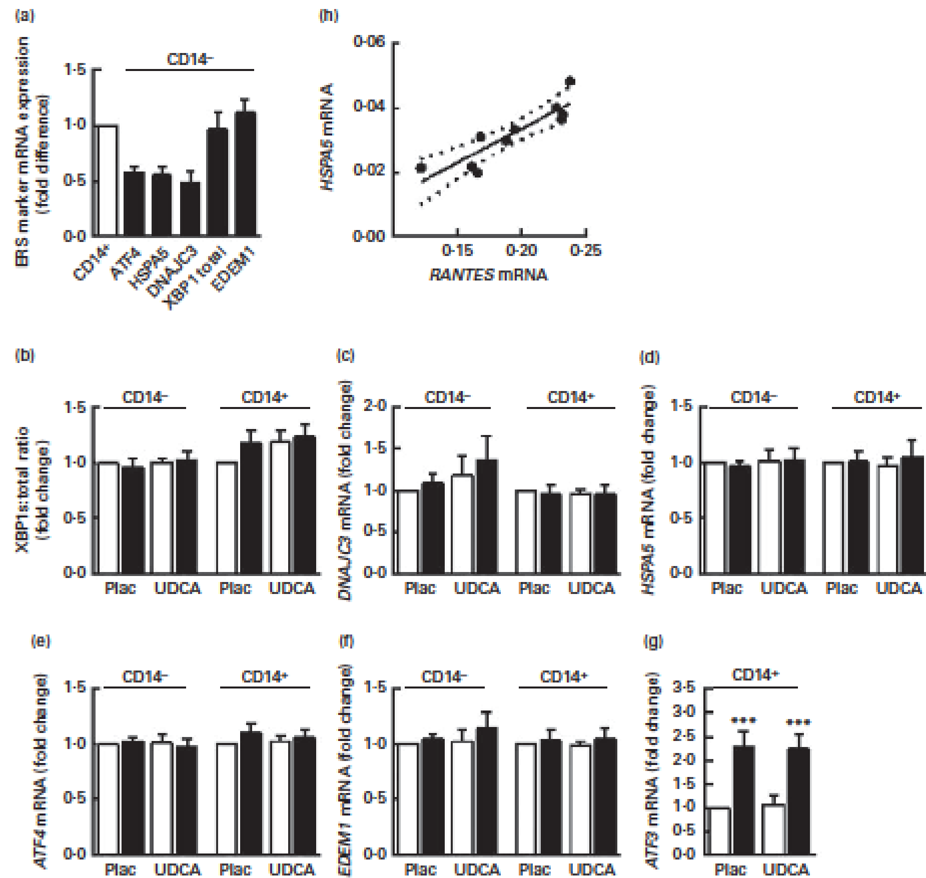


**Fig. 5.** Effect of the test meal on gene expression in CD14$^+$ and CD14$^-$ peripheral blood mononuclear cells (PBMC). (a) Comparison of mRNA expression levels of selected endoplasmic reticulum stress (ERS) markers between CD14$^+$ and CD14$^-$ cells. Quantitative RT-PCR analysis of ERS markers (b–g) in PBMC collected before and 4 h after a high-fat meal (■) challenge. Values are means, with their standard errors represented by vertical bars. *** Mean value was significantly different from that of baseline levels (□) ($P<0·001$). (h) Linear regression between mRNA levels of regulated on activation, normal T-cell expressed and secreted (*RANTES*) and *HSPA5* in CD14$^-$ cells at the fasting state ($R^2$ 0·792, $P=0·0006$). ATF, activating transcription factor; HSPA5, heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa); DNAJC3, DnaJ (Hsp40) homolog, subfamily C, member 3; XBP1, X-box binding protein 1; EDEM1, ER degradation enhancer, mannosidase alpha-like 1; XBP1s, X-box binding protein 1 spliced; Plac, placebo; UDCA, ursodeoxycholic acid.

in the infiltration of CD11c$^+$ monocytes into adipose tissue in mice[30,31], and these monocytes/macrophages exhibit a pro-inflammatory M1 phenotype. CD11c expression has also been found to increase in blood monocytes of obese subjects and to positively correlate with homeostasis model assessment of the insulin resistance index[32]. Therefore, a single HFM may activate monocytes in a similar direction to long-term overfeeding or obesity. This observation is important with respect to the fact that a majority of European and North American people are in a postprandial state most of the day, and therefore they might be exposed to a potentially harmful condition long before they become obese.

We then focused on gene expression in CD14$^+$ (monocytes) and CD14$^-$ (lymphocytes) PBMC, i.e. cells that are intimately exposed to metabolite fluctuations, but upon activation also contribute to the development of inflammation in adipose tissue in response to overfeeding. Until now, changes in gene expression induced by a meal were analysed only in the whole-PBMC population[15,16,18]. Analysis of such a mixture of cell types could mask the possible differences between the postprandial responses of mononuclear cells of innate and adaptive immunity. Therefore, we opted to separate these two categories of PBMC before gene expression analysis. Remarkably, the expression levels of all the tested pro-inflammatory

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cytokines were enhanced after the HFM challenge in CD14$^{+}$ monocytes. Moreover, we also detected decreased expression levels of *miR181a*, a negative regulator of the TLR4/NF-κB pathway[33]. This decrease in *miR181a* expression following the HFM challenge could reinforce the synthesis of pro-inflammatory cytokines. The observed down-regulation of *miR181a* expression may be specific for inflammation induced postprandially, given that the expression level of another miRNA, *miR146a*[34], involved in the negative regulation of several pro-inflammatory cytokines remained unaltered. As noted already for CD11c expression, postprandial changes in the expression of *miR181a* and pro-inflammatory cytokines were similar to the changes in their expression associated with obesity[33,35].

Interestingly, we did not detect any changes in the expression of genes potentially activated by dietary fatty acids (*PPARγ* and *PPARα*) in CD14$^{+}$ cells, although these cells were postprandially exposed to high levels of lipids. Indeed, it was reported previously that a fatty meal induced an increase in the content of TAG in leucocytes[23], suggesting the uptake of NEFA by leucocytes. However, the present data suggest that several hours of exposure to dietary lipids are not sufficient to induce substantial expression changes in the regulators of lipid metabolism in CD14$^{+}$ cells. The mRNA levels of *TLR2* and *TLR4* were not altered in CD14$^{+}$ monocytes by the HFM challenge, even though we detected higher counts of CD14/TLR2- and CD14/TLR4-positive monocytes in the blood. Nevertheless, the level of fluorescence (mean fluorescence intensity) of TLR2 and TLR4 on the monocyte surface was not altered (data not shown), which confirms the results of mRNA analysis.

To determine whether postprandial inflammation could be triggered by enhanced ERS, we analysed ERS markers representing all three arms of unfolded protein response (UPR). The activation of inositol-requiring enzyme 1 (IRE) leads to XBP1 splicing, which in turn stimulates the expression of *DNAJC3* and *EDEM1* and partially *HSPA5*[36]. HSPA5 is primarily a target of the ATF6 UPR arm[37]. The activation of PRKR-like endoplasmic reticulum kinase (PERK) is associated with the up-regulation of *ATF4*, which in turn induces the expression of *ATF3*[38]. Following the HFM challenge, mRNA expression of a majority of ERS markers was not altered in PBMC. Thus, the classic activation of UPR does not seem to be the driver of the postprandial increase in the expression levels of inflammatory cytokines in CD14$^{+}$ monocytes. The absence of XBP1 splicing was rather surprising as it can be stimulated by insulin[39], and insulin levels were raised in response to the HFM challenge. It was also reported that higher activation of XBP1 is detectable in monocytes from obese subjects and subjects with the metabolic syndrome[9]. The finding that the HFM challenge does not initiate ERS in PBMC also explains the minor effects of UDCA on the expression levels of inflammatory cytokines. These minor effects could not be based on the low bioavailability of UDCA in the blood as pharmacokinetic data show that UDCA reaches a peak concentration at 60 min after oral administration and its half-life is more than 3 d. The ability of UDCA to modulate the expression levels of inflammatory cytokines observed in the case of *TNF-α* in CD14$^{+}$ cells and *RANTES* in CD14$^{-}$ cells is therefore probably unrelated to its chaperone-like property. Importantly, UDCA has been shown to have an immunosuppressive potential different from its effect on ERS due to its ability to activate glucocorticoid receptors and to inhibit the TLR signalling pathway[12]. UDCA may also influence blood cells through binding to the G-protein-coupled bile acid receptor TGR5[40]. However, these effects were tested mostly *in vitro* or in patients with primary biliary cirrhosis, and therefore they cannot be easily extrapolated to an *in vivo* condition in healthy men.

The only ERS marker whose expression was postprandially elevated was *ATF3*. It mostly acts as a transcriptional repressor and may thus be part of a counterbalance system in healthy individuals, protecting them from overactivation of pathways induced by stress[41–43]. Therefore, it could be envisioned that this counterbalance system is impaired in obese and/or diabetic subjects who suffer from intensified and prolonged postprandial inflammation[2,3,6]. Indeed, careful evaluation of differences in the expression levels of any putative regulator of postprandial inflammation between lean and obese subjects will be crucial for identification of mechanisms leading to pathological deregulation of this process in metabolically impaired individuals.

Interestingly, the change in *ATF3* expression induced by the HFM challenge correlated specifically with a change in *IL-8* expression. IL-8 has recently been described as a cytokine whose expression is altered specifically by the HFM challenge[15]. ATF3 is, however, activated not only by ERS but also by other various stresses[44], and the absence of the up-regulation of ATF4 in the analysed CD14$^{+}$ cells of ATF3 in the classic UPR pathway suggests that the up-regulation of *ATF3* is not associated with the activation of UPR. Moreover, the lack of an increase in blood glucose concentration after the HFM challenge suggests that hyperglycaemia-induced oxidative stress is not the trigger of *ATF3* expression.

Although we did not find a relationship between HFM-induced changes in the expression levels of inflammatory cytokines and most ERS markers, the striking co-regulation of mRNA expression levels of *RANTES* and all ERS markers opens the question as to whether the higher ERS levels in CD14$^{-}$ cells (probably CD8$^{+}$ T cells that are the main producers of RANTES[45]) could be a marker of their activation as was previously suggested for the conditions of acute pathogen infection[46].

In conclusion, we demonstrate the evidence that inflammation induced by the HFM challenge in CD14$^{+}$ monocytes was not accompanied by an activation of a majority of the investigated ERS markers (*HSPA5*, *XBP1*, *DNAJC3*, *EDEM1* and *ATF4*). Administration of UDCA before the consumption of the HFM did not alter the expression levels of these ERS markers. The putative molecular trigger of postprandial inflammation remains to be established.

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The authors' contributions were as follows: L. R. was the guarantor of the study and, as such, had full access to all the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis, and also designed the study, performed the data analysis and wrote the manuscript; J. K., E. C., M. K. and L. M. performed the experiments and data analysis and contributed to the discussion; E. C. organised the clinical part of the study; M. S. and V. S. contributed to the discussion and the writing of the manuscript. All authors read and approved the final manuscript.

The authors declare that there are no conflicts of interest.

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**Paper 2**

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**Experimental hyperglycemia induces an increase of monocyte and T-lymphocyte content in adipose tissue of healthy obese women**

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*These authors contributed equally to this work.

PLOS ONE

RESEARCH ARTICLE

# Experimental Hyperglycemia Induces an Increase of Monocyte and T-Lymphocyte Content in Adipose Tissue of Healthy Obese Women

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## Abstract

### Background/Objectives

Hyperglycemia represents one of possible mediators for activation of immune system and may contribute to worsening of inflammatory state associated with obesity. The aim of our study was to investigate the effect of a short-term hyperglycemia (HG) on the phenotype and relative content of immune cells in circulation and subcutaneous abdominal adipose tissue (SAAT) in obese women without metabolic complications.

### Subjects/Methods

Three hour HG clamp with infusion of octreotide and control investigations with infusion of octreotide or saline were performed in three groups of obese women (Group1: HG, Group 2: Octreotide, Group 3: Saline, n=10 per group). Before and at the end of the interventions, samples of SAAT and blood were obtained. The relative content of immune cells in blood and SAAT was determined by flow cytometry. Gene expression analysis of immunity-related markers in SAAT was performed by quantitative real-time PCR.

### Results

In blood, no changes in analysed immune cell population were observed in response to HG. In SAAT, HG induced an increase in the content of CD206 negative monocytes/macrophages ($p<0.05$) and T lymphocytes (both T helper and T cytotoxic lymphocytes, $p<0.01$). Further, HG promoted an increase of mRNA levels of immune response markers (CCL2, TLR4, TNFα) and lymphocyte markers (CD3g, CD4, CD8a, TBX21, GATA3,

**Competing Interests:** The authors have declared that no competing interests exist.

FoxP3) in SAAT ($p<0.05$ and 0.01). Under both control infusions, none of these changes were observed.

## Conclusions

Acute HG significantly increased the content of monocytes and lymphocytes in SAAT of healthy obese women. This result suggests that the short-term HG can modulate an immune status of AT in obese subjects.

## Introduction

Obesity represents a high risk factor for the development of various metabolic and cardiovascular diseases such as insulin resistance, type 2 diabetes, liver steatosis or atherosclerosis. The common feature of these complications is a low-grade inflammation characterized by increased circulating levels of pro-inflammatory cytokines and chemokines (e.g. IL-6, TNF-α, CCL2, CCL5) and enhanced accumulation of immune cells (macrophages, lymphocytes) in adipose tissue (AT) [1–3].

In a previous study focused on subcutaneous abdominal AT (SAAT), we found a progressive increase in the mRNA expression of macrophage markers from obese towards obese with metabolic syndrome (MS) individuals [4]. Similar findings based on a comparison of insulin-resistant with insulin-sensitive subjects were presented by other laboratories [5–7]. However, the cause of higher AT inflammation in obese subjects with metabolic syndrome compared to metabolically healthy obese remains only partly elucidated. The altered control of glycaemia on the obese background might be one factor that plays a role in the further deterioration of AT functions. Indeed, it was suggested that the deterioration of postprandial glucose control precedes long-term elevation of fasting glucose concentration [8,9]. Moreover, it was shown that fluctuations in glucose levels are more harmful than chronic hyperglycemia (HG) *per se* [10]. Detrimental effects of acute HG might be mediated through induction of oxidative stress (via production of glycosylation end product and activation of protein kinase C) and through the activation of inflammatory pathways in various cells resulting in increased secretion of pro-inflammatory cytokines [10–13]. Still, only a few reports addressed responses of cells of adaptive and innate immunity to this metabolic stimulus *in vivo* in obese individuals [14,15].

Therefore, the objective of this study was to investigate whether acute experimental HG has an impact on phenotype and relative content of monocytes/macrophages and lymphocytes in circulation and the SAAT of healthy obese women.

## Subjects and Methods

### Subjects

The co-author and the head of the Department of Sport Medicine, Vladimir Stich, MD, PhD, recruited subjects for this study among the subjects consulting at the Obesity unit of the University Hospital Kralovske Vinohrady. 30 healthy obese premenopausal women were recruited and divided into 3 groups (n = 10) matched for BMI and age (group 1- HG clamp with octreotide infusion, group 2—octreotide infusion, group 3- saline infusion study). The subjects were matched for BMI and age (range 27–32 kg/m$^2$ and 40–44 years, respectively) and then they were assigned to one of the three experimental procedures without systematic randomization. All women were drug-free and without signs of metabolic syndrome [16], except for obesity.

To exclude subjects with metabolic syndrome we followed NCEP-ATP III guidelines (https://www.nhlbi.nih.gov/files/docs/guidelines/atglance.pdf), i.e. only women exerting less than 3 out 5 risk factors (waist circumference > 88cm, TAG > 1.7mmol/l, HDL-cholesterol < 1.3mmol/l, blood pressure > 130/85mm Hg, fasting glucose ≥ 5.6mmol/l) were admitted to the study. Their body weight had been stable for 3 months prior to the examination. Participants signed a written informed consent before the study. The study was performed according to the Declaration of Helsinki and approved by the Ethical Committee of the Third Faculty of Medicine (Charles University in Prague, Czech Republic).

## Design of clinical investigation

Clinical investigation was performed before intervention in the fasting state and at the end of the 3-hour HG clamp, or octreotide, or saline infusion. Anthropometric measurements and blood processing were performed as previously reported [17,18]. Body composition was assessed using multi-frequency bioimpedance (Bodystat, Quad scan 4000, Isle of Man, British Isles). 1–2 ml of un-coagulated blood samples was used for flow cytometry analysis. SAAT was obtained by needle biopsy carried out in the abdominal region (10 cm laterally from umbilicus) under local anesthesia (1% Xylocain) as previously described [17]. Biopsies were performed 30 min before the start of the experimental infusions and within the last 15 min of infusions on the contralateral side of abdomen. 1–2 g of SAAT was used for isolation of stromal vascular fraction (SVF) cells to perform flow cytometry analyses. In a subgroup of 6 women in HG and in 9 women from the two remaining experimental groups, 0.1 g of SAAT was immediately frozen in liquid nitrogen and stored at -80°C until RNA isolation.

## Hyperglycemic clamp

A bolus injection of 0.33 g/kg glucose followed by a varying 20% glucose infusion was used to achieve steady-state plasma glucose concentrations of 15 mmol/l for 180 minutes. Continuous infusion dose was adjusted every 5 to 10 minutes according to the measured plasma glucose. 5 minutes before the priming glucose, octreotide (Sandostatin, Novartis) infusion was started in order to block the release of endogenous insulin. The initial 25 μg IV bolus administered over 1 min was followed by an infusion at the rate 30 ng/min/kg body weight. To prevent hypokalemia, 0.26 mmol/l KCl was added to the glucose infusion.

To exclude any direct effect of infusion itself or infusion of octreotide on circulating cells and on SAAT characteristics, 2 groups of subjects as control groups (n = 10 per each group) different from those participating in the HG clamp received infusion of saline or octreotide alone (i.e. in the absence of the glucose infusion) at the duration, resp. dose identical to the hyperglycemic condition.

## Isolation of SVF cells

SAAT was washed with saline, further minced and digested with type I collagenase 300 U/ml in PBS/ 2%BSA (SERVA, Heidelberg, Germany) for 1h in 37°C shaking water bath. Digested tissue was subsequently centrifuged at 200 g for 10 minutes and filtered through 100- and 40-μm sieves to isolate SVF cells.

## Flow cytometry analysis

The whole blood and freshly isolated SVF cells were used for immediate flow cytometry analyses. SVF cells were resuspended in 100 μl PBS solution containing 0.5% BSA and 2 mM EDTA and incubated with fluorescence-labeled monoclonal antibodies (FITC-conjugated antibody

CD14, CD4; PE-conjugated antibody CD14, TLR2, TLR4, CD3; PerCP-conjugated antibodies CD45 and APC-conjugated antibodies CD206 and CD8) or the appropriate isotype controls (BD Bioscience, Bedford, MA) for 30 min at 4°C according to protocol of Curat et al. [19]. The whole blood samples were stained with the same set of fluorescence-labelled monoclonal antibodies as used for SVF cells (except for CD206) for 30 min at room temperature. After staining, erythrocytes were lysed by erythrocyte lysis buffer for 15 min at room temperature. Cells were washed with PBS and analysed on FACS Calibur flow cytometer with CellQuest Pro Software (BD Biosciences, NJ, USA). The number of immune cells belonging to specified populations was expressed as percentage of gated events.

### Quantitative real time PCR (RT-qPCR)

Total RNA extraction and reverse transcription (RT-PCR) were performed as previously described [17]. Before reverse transcription, genomic DNA was eliminated by DNase I (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR (RT-qPCR) was performed using an ABI PRISM 7000 and 7500 instrument (Applied Biosystems, Foster City, CA, USA). Primers and TaqMan probes were obtained from Applied Biosystems. Results are presented as fold change values calculated by ΔΔ Ct method normalized to geometric mean of two endogenous controls (18S rRNA and GUSB).

### Determination of plasma levels of biochemical parameters

Plasma glucose and insulin were determined using the glucose-oxidase technique (Beckman Instruments, Fullerton, CA) and an Immunotech Insulin Irma kit, resp. (Immunotech, Prague, Czech Republic). Homeostasis model assessment of the insulin resistance index (HOMA-IR) was calculated as follows: ((fasting insulin in mU/l) x (fasting glucose in mmol/l) / 22.5). Circulating levels of selected bioactive molecules were measured by commercial ELISA kits: RANTES/CCL5 (Duoset, R&D Systems, Minneapolis, MN, USA) and MCP-1 (Ready-SET-Go, eBioscience, San Diego, CA, USA). Plasma levels of other parameters were determined using standard biochemical methods.

### Statistical analyses

Statistical analysis was performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, Inc., San Diego, California, USA). The data were log-transformed for the analyses. The effect of HG clamp or octreotide/saline infusion was tested using parametric t-test. Differences of baseline clinical data between the three groups of patients were analysed by one-way ANOVA with Tukey multiple comparison tests. To compare the effect of HG vs. control infusions, the data were analysed by two-way ANOVA with repeated measures. Data are presented as mean ± SEM. Differences at the level of $p < 0.05$ were considered statistically significant.

## Results

### Clinical characteristics of obese subjects

The clinical data of subjects participating in three short-term interventions are shown in Table 1. There were no significant differences in anthropometric and laboratory parameters (including fasting blood glucose, plasma insulin levels, and HOMA-IR) between HG and octreotide group of subjects. Fasting glucose levels were lower in the saline group (vs. HG group) but no other differences were found between the two groups.

**Table 1. Characteristics of obese subjects in experimental groups.**

| Characteristics | Hyperglycemia (HG) | Octreotide | Saline |
|---|---|---|---|
| N | 10 | 10 | 10 |
| Age (years) | 42 ± 1 | 42 ± 2 | 44 ± 2 |
| Weight (kg) | 86.9 ± 2.7 | 87.5 ± 4.4 | 89.0 ± 2.2 |
| BMI (kg/m$^2$) | 30.8 ± 0.8 | 31.9 ± 1.5 | 31.6 ± 1.1 |
| Fat (kg) | 33.2 ± 1.6 | 34.5 ± 3.0 | 35.8 ± 2.1 |
| Waist circumference (cm) | 95.2 ± 2.6 | 100.8 ± 3.4 | 98.5 ± 2.3 |
| Systolic blood pressure (mm Hg) | 117. ± 4.6 | 124.3 ± 3.3 | 124.4 ± 3.5 |
| Diastolic blood pressure (mm Hg) | 76 ± 3.2 | 77.2 ± 1.5 | 79.5 ± 2.3 |
| Glucose (mmol/L) | 5.4 ± 0.1 | 5.2 ± 0.1 | 5.0 ± 0.1 [a] |
| Insulin (mU/L) | 6.5 ± 0.9 | 7.3 ± 0.8 | 6.6 ± 0.8 |
| C-peptide (mU/L) | 0.7 ± 0.1 | 0.8 ± 0.1 | 0.7 ± 0.1 |
| HOMA-IR | 1.6 ± 0.2 | 1.7 ± 0.2 | 1.4 ± 0.2 |
| Cholesterol (mmol/L) | 4.7 ± 0.2 | 4.4 ± 0.2 | 4.8 ± 0.3 |
| Triglycerides (mmol/L) | 0.9 ± 0.1 | 1.2 ± 0.2 | 1.1 ± 0.1 |
| HDL-C (mmol/L) | 1.3 ± 0.1 | 1.4 ± 0.1 | 1.4 ± 0.1 |

Data are presented as mean ± SEM

[a] $p < 0.05$

hyperglycemia vs saline; BMI: body mass index; HOMA-IR: homeostasis model assessment of the insulin resistance index; HDL-C: HDL cholesterol

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## Plasma insulin, C peptide and glucose levels during hyperglycemic clamp, octreotide and saline infusions

Throughout the HG clamp plasma glucose was maintained at 15 mmol/l (coefficient of variation 7.2 ± 0.7%), being approximately three times higher compared with baseline values. The addition of octreotide prevented hyperglycemia-stimulated endogenous production of insulin except at the end of the 3-hours hyperglycemia when plasma insulin and C-peptide concentrations were modestly increased (insulin 6.45 ± 0.86 mU/l at baseline vs. 10.76 ± 2.1, $p < 0.05$, C-peptide 0.72 ± 0.06 mU/l at baseline vs. 1.08 ± 0.18, $p < 0.05$).

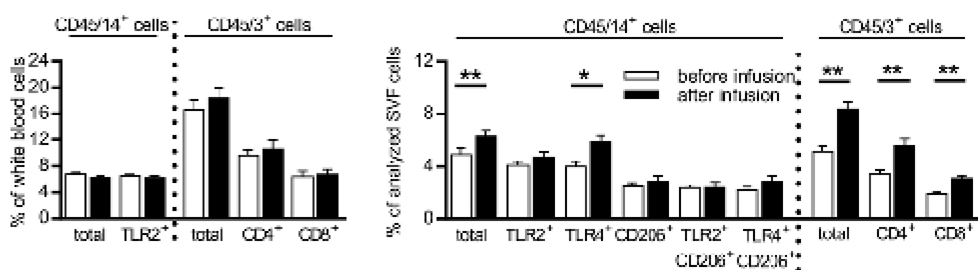
The infusion of octreotide alone decreased plasma insulin and C-peptide below basal levels (insulin, 7.34 ± 0.84 mU/l at baseline vs. 2.42 ± 0.39 mU/l at the end of infusion, $p < 0.001$, C-peptide 0.76 ± 0.84 mU/l at baseline vs. 0.27 ± 0.04 mU/l at the end of infusion, $p < 0.001$) and this was accompanied with a slight elevation of glucose levels (baseline 5.23 ± 0.11 mmol/l, end of infusion 5.95 ± 0.27 mmol/l, $p < 0.01$).

Glucose, insulin and C-peptide levels remained stable during the saline infusion (data not shown).

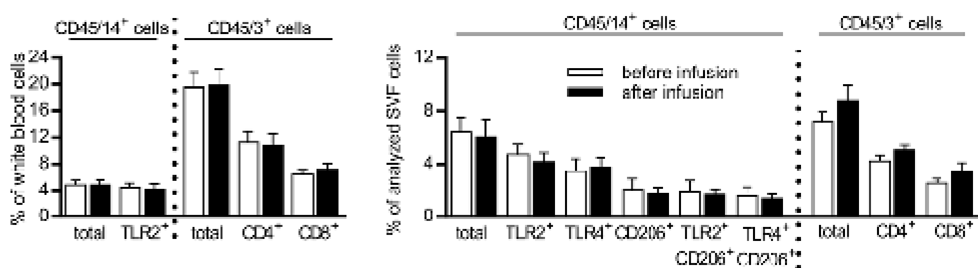
## Monocyte/macrophage and T lymphocyte content in peripheral blood and SAAT of obese women in response to hyperglycemic clamp, octreotide and saline infusion

The content of monocytes/macrophages characterized by expression of CD45+/14+ did not change in response to HG in blood but significantly increased in SAAT (Fig 1A). Similarly, no changes in relative content of monocytes/macrophages expressing Toll-like receptor (TLR) 2 and 4 were induced by HG in blood, while there was a significant HG-induced increase in relative content of CD45+/14+/TLR4+ population in SAAT (Fig 1A). These changes were independent of the content CD45+ cells with high granularity (granulocytes), in SAAT biopsy samples,

A) Hyperglycemic clamp




B) Octreotide infusion




C) Saline infusion

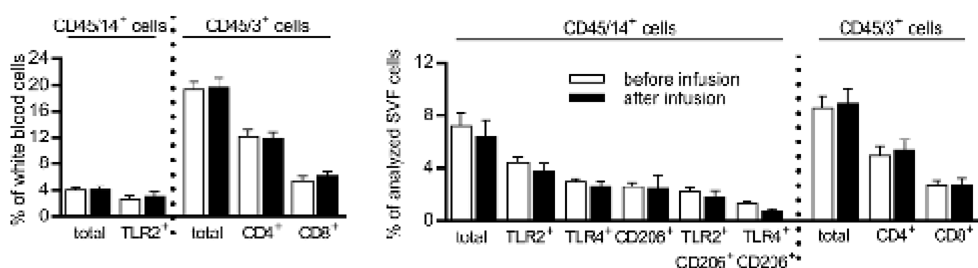



**Fig 1. Effect of hyperglycemic clamp (A), octreotide infusion (B), and saline infusion (C) on relative content of monocyte/macrophage and T-lymphocyte populations in peripheral blood and stromal vascular fraction (SVF) of subcutaneous abdominal adipose tissue of obese women.** A population of TLR4+ monocytes in blood is not shown due to a low frequency. White bars- before infusion, black bars- after infusion. Data are presented as mean ± SEM, each investigated group n = 10, *$p < 0.05$; ** $p < 0.01$: before vs after clamp.

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because their content was not different before or at the end of the HG clamp or other experimental infusions (17.8 ± 2.3% before and 17.4 ± 1.9% after infusion, n = 30). Resident AT macrophage populations were identified by the expression of mannose receptor CD206 on CD45+/CD14+ cells (i.e. CD45+/14+/206+, CD45+/14+/206+/TLR2+ and CD45+/14+/206+/TLR4+ cells) in SAAT (Fig 1A) and they were not affected by HG.

Populations of T lymphocytes (CD45+/3+ cells; T helper subpopulation-CD45+/3+/4+; T cytotoxic subpopulation- CD45+/3+/8+) remained unchanged in response to HG in blood but

significantly increased in SAAT (Fig 1A). The ratio between subpopulations of T helper and T cytotoxic lymphocytes (CD4+/CD8+) in SAAT did not change during HG clamp (data not shown). Importantly, neither octreotide nor saline infusion had a significant effect on relative content of monocyte/macrophage and T lymphocyte populations in blood and/or SAAT (Fig 1B and 1C). Of note, HG-specific increases in total T cell and T helper cell content in SAAT was confirmed by two-way ANOVA.

### SAAT mRNA levels of macrophage, lymphocyte and inflammatory markers in response to hyperglycemic clamp, octreotide and saline infusion

To extend the results of flow cytometry, mRNA levels of chemokines/cytokines (CCL2/MCP1, CCL5/RANTES, CXCL12/SDF-1α, IL8, IL1β, TNFα), markers of macrophages (CD14, CD206), Toll like receptors (TLR2, TLR4), lymphocyte markers (CD3g, CD4, CD8a) and remodeling marker (MMP9) were analysed in SAAT. Levels of CCL2 and CCL5 chemokines were also evaluated in plasma. The mRNA levels of CCL2, TLR4, TNFα and all measured T lymphocyte markers (CD3g, CD4, CD8a) including Th1 (TBX21), Th2 (GATA3) and T regs (FoxP3) markers significantly increased in response to HG (Fig 2A) but not after octreotide or saline infusion (Fig 2B and 2C, confirmed also by two-way ANOVA).

### Plasma levels of chemokines in response to hyperglycemic clamp, octreotide and saline infusion

Circulating levels of two chemokines involved in attraction of monocytes and lymphocytes, i.e. CCL2 and CCL5, were not changed in response to either conditions (data not shown).

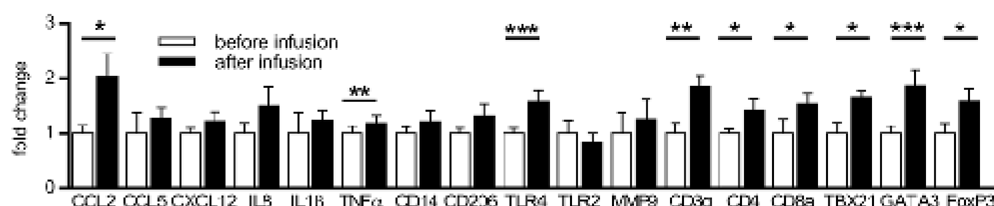
## Discussion

Obesity-related inflammation has been considered one of the major risk factors for the development of metabolic and cardiovascular diseases. Short-term HG represents one of the possible triggers to aberrant activation of the immune system [20]. This could contribute to the further worsening of the inflammatory state in obese subjects resulting in metabolic syndrome or type 2 diabetes. Thus, we investigated the effect of HG on immune cell phenotype and content in circulation and SAAT. The present study was carried out in healthy obese women representing an optimal model for studying the processes contributing to the deterioration of metabolic status of obese subjects.

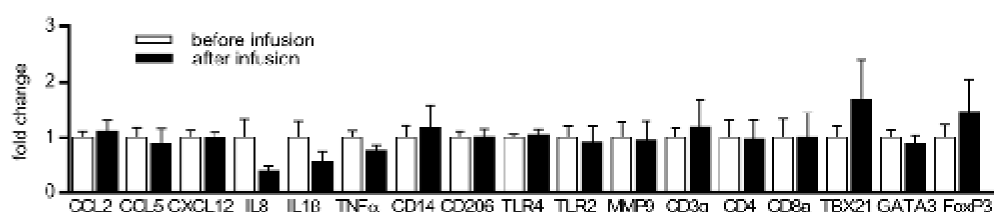
We documented that HG induced an increase in CD45+/14+ monocyte/macrophage population in SAAT. Upon the octreotide or saline infusion, no changes in monocyte/macrophage population in SAAT were detected; therefore the above-mentioned increased numbers of monocytes/macrophage in SAAT cannot be attributed to octreotide or infusion *per se*.

Since it was shown previously that HG treatment of monocytes *in vitro* increases expression of Toll-like receptors [21] and also monocytes from patients with type 2 diabetes show a higher expression of TLR2 and TLR4 compared to healthy subjects [15], we investigated the expression of these two receptors in circulating blood cells and SAAT in obese women. While the relative content of activated monocyte/macrophage population defined as a triple positive population CD45+/14+/TLR4+ was increased in response to HG, no significant changes in CD45+/14+/TLR2+ population were observed in SAAT. Thus, the selective effect of HG on TLR4+ monocyte/macrophage population could point to a specific physiological function of this subtype of monocytes/macrophages in HG-affected SAAT. Indeed, recent findings suggest that TLR4 and TLR2 activation in macrophages results in the differential expression and

A) **Hyperglycemic clamp**




B) **Octreotide infusion**




C) **Saline infusion**

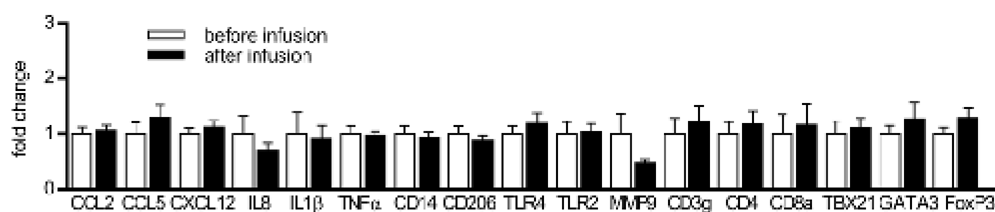



**Fig 2. Effect of hyperglycemic clamp (A), octreotide infusion (B), and saline infusion (C) on mRNA levels of selected immunity-related genes in subcutaneous abdominal adipose tissue of obese women.** White bars- before infusion, black bars- after infusion. Data are presented as mean fold change ± SEM. Relative mRNA levels are normalized to geometrical mean of 2 housekeeping genes 18S and GUSB, n = 6 (hyperglycemic clamp), n = 9 (octreotide and saline infusion), *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$: before vs after clamp.

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secretion of pro-inflammatory cytokines [22,23]. We found increased mRNA levels of TLR4 along with TNF in the AT of obese women after HG clamp, which has been shown to be up-regulated after TLR4 but not TLR2 stimulation in macrophages [23]. This mechanism of cytokine regulation is considered to be important for the control of migration and subsequent activation of inflammatory monocytic cells. Notably, we observed that the surface expression of both TLR2 and TLR4 was detectable in the majority of monocytes present in SAAT despite low expression of TLR4 in circulating monocytes (low expression of TLR4 on circulating

monocytes was also documented by Kashiwagi et al [24]). Thus, we speculate that the expression of TLR4 on monocytes could either be stimulated by the SAAT microenvironment or, alternatively, only those monocytes expressing TLR4 could reach the SAAT. However, further studies will be needed to clarify this hypothesis.

Contrary to monocyte population, a population of resident AT macrophages did not show any changes in response to HG in terms of relative content and TLRs expression (i.e. content of CD45+/14+/206+/TLR2+ and TLR4+). Therefore, it seems that SAAT microenvironment, changed by HG, activated only monocytic cells that are not fully differentiated into macrophages. Such a population of CD206- monocytic cells was described by Wentworth et al. [25] and was shown to be elevated in human obesity. It is plausible that these monocytes represent "the newest arrivals" into AT but then later can mature into CD206+ macrophages. Nevertheless, CD206 marker used to identify resident AT macrophages was previously suggested to be preferentially expressed by M2 macrophages [26], and thus it is also possible that observed increase in CD45+/14+/206- population could be attributed to M1 macrophages. This hypothesis however could not be tested as M1 macrophage marker CD40 [26] is expressed also on 60% of circulating monocytes (not shown).

The pro-inflammatory state associated with metabolic complications represents a bridging of innate and adaptive immune systems in AT physiology. Previous studies investigating the dynamics of immune cell infiltration of AT during the onset of obesity suggested that lymphocytes are the first players of immunity which infiltrate the AT [27–29]. In our study, we found an increased content of total T lymphocytes and both major subpopulations of T lymphocytes, i.e. T helper CD4+ and T cytotoxic CD8+ in SAAT of obese women in response to short-term HG. Importantly, the role of CD4+ and CD8+ T cells in modulating AT inflammation and overall metabolic status has been documented previously in both animal models and human. According to animal studies [28], CD8+ T cells direct macrophage infiltration into AT. CD4+ T cells have both anti- and pro-inflammatory roles based on their further specialization [30] and the balance between these individual CD4+ subpopulations is responsible for the control of metabolic inflammation [31]. Notably, at least two of the CD4+ subpopulations, i.e. Th1 and Th17 cells, are pro-inflammatory and their numbers are significantly elevated in AT of metabolically unhealthy obese subjects or in diet-induced obesity in mice [31,32]. Thus, we could speculate that the increase of CD4+ cells upon HG could be attributed to these two subpopulations (Th1 and Th17 cells) however this hypothesis has to be proven in further study.

In blood, short-term HG caused no alteration in relative content of immune cell populations or their phenotype, along with no change in circulating levels of chemokines, i.e. CCL2 and CCL5, involved in chemo-attraction of monocytes and lymphocytes. Thus, a short metabolic stimulus of 3-hour HG is probably insufficient to alter relative content of various leukocyte populations in circulation but it has a significant effect on immune response in SAAT of obese healthy women. In fact, relative content of immune cell populations in SAAT was analysed in the context of other cell types (i.e preadipocytes, endothelial cells) whose numbers in AT are presumably insensitive to short-term metabolic insults, which may facilitate a detection of even small changes in numbers of immune cells.

It was shown that HG modulates expression of genes related to immune response in SAAT of lean subjects [14,33]. We observed that mRNA levels of TLR4 (also expressed on adipocytes and endothelial cells [34]; [35]), CD3g, CD4 and CD8a increased in the experimental condition of HG in obese women, which nicely supports the flow cytometry results. Unlike circulating levels of CCL2, mRNA levels of CCL2 in SAAT were increased after a short-term HG. One could hypothesize that these local changes of immune response genes in the AT could affect monocyte/macrophage population. Indeed, recent paper of Amano et al. [36], suggested that CCL2 promotes proliferation of resident macrophages in AT in obesity. Likewise, other clinical

studies have reported an increased expression of activation markers on monocytes and neutrophils in type 2 diabetic patients [15,37,38].

Unlike other studies analysing T cell subpopulations in mice [39], we did not analyse among T cell subtypes by flow cytometry due to the limited numbers of SVF cells derived from needle biopsy samples. However, we found the up-regulation of TBX21, GATA3 and FoxP3 mRNA levels (representing major differentiation factors of Th1, Th2 and Tregs subtypes) in SAAT after HG condition in obese women. It has been shown that Th1, Tregs are increased and Th2 subpopulation is decreased with obesity [40,41]. Based on the combination of our results from flow cytometry and mRNA analysis, one can hypothesize that HG enhanced infiltration of both pro- and anti-inflammatory T cells in order to maintain immune homeostasis in AT. However, to determine a comprehensive picture of the sequence of the immune cells activation in circulation, accumulation in AT, and their role in the induction of the AT pro-inflammatory state further analyses need to be performed. In fact, the role of immune cell infiltration in AT is not unequivocal: it still remains unknown whether it reflects the dysfunction of AT metabolism or prevents this event. The study of Duffaul et al. [42] documented that early T cells infiltration into AT has protective role since it inhibits pro-inflammatory reaction of innate cells. Similar finding by Sultan et al. [43] showed that adaptive cells alone are not responsible for the impairment of insulin sensitivity in obesity.

For characterization of particular immune cell populations in blood and SAAT, we used flow cytometry. This method enables simultaneous detection of several surface markers and provides results superior over immunohistochemistry or gene expression analysis alone. However, the flow cytometry analysis of needle biopsy-derived samples may raise concerns of a possible contamination of SAAT sample by blood cells. Similar to our previous study [18], where this possible limitation was already discussed, the content of granulocytes, i.e. CD45+ cells with high granularity, in SAAT samples, was not different before or at the end of the HG clamp or other experimental infusions used in this study. This suggests that blood contamination does not affect the outcome of the flow cytometric data in SAAT. Another possible limitation of this study was a slight increase of plasma insulin levels at the end of the HG clamp. Noteworthy, this final concentration of insulin remained within the range of normal fasting levels and was negligible when compared with the usual postprandial concentrations. In addition, the reports showing an acute effect of insulin on the circulating levels of pro-inflammatory cytokines [44,45] were based on the exposure to 4 fold higher levels of insulin than those detected in the present study. Moreover, circulating resting T lymphocytes are devoid of insulin receptor [38]. Thus even though we cannot completely rule out the possibility that the slight increase of plasma insulin may contribute to the observed effect of HG on immune cells, it seems rather unlikely.

In summary, our results show that the short-term HG induces an increase in the content of monocytes and T lymphocytes in SAAT of healthy obese women and thus suggest that the oscillations in glycaemia levels may modulate an immune status of AT in obese individuals.

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## Author Contributions

Conceived and designed the experiments: MT VS LR. Performed the experiments: MT JK EK LM ZK ZW MS VS LR. Analyzed the data: MT JK LM ZK MS LR. Contributed reagents/materials/analysis tools: MT JK LM ZK LR. Wrote the paper: MT LR VS.

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## Paper 3

Eva Krauzová, Jana Kračmerová, Michaela Tencerová, Lucia Mališová, Zuzana Kováčová, Lenka Rossmeislová, Vladimir Štich and Michaela Šiklová

**Acute hyperlipidemia initiates pro-inflammatory and pro-atherogenic reaction in obese women**

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**ACUTE HYPERLIPIDEMIA INITIATES PRO-INFLAMMATORY CHANGES IN CIRCULATION AND ADIPOSE TISSUE IN OBESE WOMEN**

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## ABSTRACT

**Objective:** Increased levels of lipids in circulation represent one of the possible mediators for activation of immune system and may contribute to worsening of inflammatory state associated with obesity and endothelial dysfunction. The aim of our study was to investigate the effect of a short-term lipid infusion on the relative content and phenotype of immune cells and pro-inflammatory markers in circulation and subcutaneous abdominal adipose tissue (SAAT) in obese women.

**Approach and Results:** Seven-hour intravenous lipid infusion and control investigation were performed in two groups of obese women (n=10, n=7, respectively). Before and at the end of the infusion, samples of SAAT and blood were obtained and relative content and phenotype of immune cells were determined using flow cytometry. Analysis of immune cell markers, inflammation and angiogenesis markers was performed in SAAT by RT-PCR and in plasma by ELISAs. Relative content of monocytes (CD45+/14+ and CD45+/14+/16+ population) was reduced in circulation in response to high lipid levels, which suggested the increased adhesion of these cells to endothelium. In line with this, the levels of sICAM, sVCAM and VEGF-A in plasma were increased. Relative content of T-helper lymphocytes (CD45+/3+/4+) increased in blood. In SAAT the relative content of monocyte/macrophages subpopulation CD45+/14+/206+/16+ decreased. Intralipid infusion promoted an increase of RORC (pro-inflammatory Th17 lymphocytes), IL6, MCP1, VEGF-A and CD36 mRNA levels in SAAT.

**Conclusions**: Acute hyperlipidemia induces a pro-atherogenic response associated with altered relative content of immune cells in blood and with pro-inflammatory changes in SAAT in obese women.

**Keywords:** hyperlipidemia; free fatty acids; human adipose tissue; T-lymphocytes; monocytes; intercellular adhesion molecules;

## INTRODUCTION

Obesity represents a high risk factor for the development of cardiovascular diseases and atherosclerosis. The common feature of these complications is a low-grade inflammation and activation of immune cells. It was proposed that one of the triggers of these pro-inflammatory processes are circulating free fatty acids (FFA) and triglycerides (TG) [1-3]. Lipids, namely saturated FFA, were found to activate classical inflammatory responses in immune cells and to regulate secretion of pro-inflammatory cytokines in several types of cells [4-8]. The ability of lipids to activate immune cells was documented also in vivo upon the postprandial increase of lipid metabolites: the consumption of high-fat meal was accompanied with an increase of pro-inflammatory cytokine plasma levels [9-11] and increased circulating leukocyte counts [12, 13]. Importantly, the postprandial increase of circulating lipids (TG, FFA) as well as the signs of systemic postprandial inflammatory response were higher in obese [14-16].

The effects of lipids on immune processes within the whole adipose tissue are known only partially. Saturated FFA may induce an increased expression of pro-inflammatory cytokines in adipocytes similarly to what was shown in immune cells [17, 18]. Moreover, FFA appeared have been suggested as an important driver of macrophage accumulation in AT in mice [19]. Nevertheless, it remains unknown whether acute hyperlipidemia can alter the content and phenotype of immune cells in adipose tissue in humans and thus contributes to the progression of metabolic and cardiovascular disturbances associated with obesity.

Effects of FFA on cells can be mediated through binding to the receptors/sensors, such as toll like receptor 4 (TLR4) and fatty acid translocase (CD36), that control inflammatory signaling pathways [20]. Indeed, in humans, increased circulating levels of FFA were associated with increased expression of CD36 on monocytes, which led to lipid accumulation in these cells [21]. Such a lipid overload caused monocytes to form foam cells that are implicated in the development of atherosclerosis 22. In vivo and in vitro experiments provided evidence that the activation of monocytes by high FFA and TG levels led to an increased expression of receptors of adhesion molecules (ICAM, VCAM) and to foam cells formation [2, 13, 23, 24]. Furthermore, it was documented that postprandial triglyceridemia increases levels of soluble cell adhesion molecules (sICAM, sVCAM), which regulate the infiltration of monocytes to the endothelium [25, 26]. Therefore, the aims of the current study were to examine the effect of acute experimentally-induced hyperlipidemia on relative content and phenotype of

immune cells in circulation and in subcutaneous adipose tissue (SAAT) and on circulating levels of adhesion molecules in obese women. The results of this study suggest that the hyperlipidemia modulates systemic and adipose tissue inflammatory status and may contribute to the adipose tissue dysfunction and pro-atherogenic changes in obese.

## MATERIAL AND METHODS

### Subjects

Ten obese healthy premenopausal women were recruited into the intervention group with Intralipid infusion (age 40 ± 2 year, BMI 31.2 ± 0.8 kg/m$^2$) and seven obese women participated in the control group with infusion of Glycerol (age 43 ± 3 years, BMI 32.0 ± 1.0 kg/m$^2$). Exclusion criteria were weight changes of more than 3 kg within the 3 months before the study, hypertension, impaired fasting glucose, diabetes, hyperlipidemia, drug-treated obesity, drug or alcohol abuse, pregnancy or participation in other studies. Subjects did not take any medications and did not suffer from any disease except for obesity. All subjects were fully informed about the aim and the protocol of the study and signed an informed consent approved by the Ethical committee of the Third Faculty of Medicine (Charles University in Prague, Czech Republic).

### Experimental protocol

The subjects entered the laboratory at 7.00 a.m. after an overnight fast. A complete clinical investigation was performed, anthropometric parameters were measured and body composition was determined with multifrequency bioimpedance (Bodystat QuadScan 4000; Bodystat Ltd., Isle of Man, British Isles). Subsequently, the subjects were placed in a semi-recumbent position and a catheter was placed in the antecubital vein. To increase plasma FFA and TG concentration intravenous infusion of lipid emulsion was applied. Intralipid 20% (Fresenius Kabi, Bad Hamburg, Germany) consists of soya-bean oil (20%) stabilized with eggyolk phospholipids (1.2%) and glycerol (2.5%). The fatty acid composition was as follows: palmitic acid 11.3%, stearic acid 4.9%, oleic acid 29.7%, linoleic acid 46.0% and linolenic acid 8.1%. The infusion of Intralipid 20% was administered through cannula at a rate 60 ml/hod for one hour and then it was continued at constant rate 90 ml/h for following six hours. In

the control group, saline infusion with 2.5% glycerol was administered at the same rate for seven hours.

Before the start of infusions and every 60 minutes during infusions venous blood was collected into 50 µl of an anticoagulant and antioxidant cocktail (Immunotech SA, Marseille, France) and immediately centrifuged (1300 rpm, 4°C). The plasma samples were stored at -80°C until analyses.

The needle biopsies and 2 ml of un-coagulated blood samples were taken 30 min before the start of the experimental infusions and 15 min before the end of infusions. Needle biopsies of SAAT were obtained approximately 10-15 cm lateral to the umbilicus under local anesthesia (1% Mesocain, Zentiva, Prague, Czech Republic), as previously described[27]. Approximately 1g of SAAT and 2ml of blood were used for isolation of stroma-vascular fraction (SVF) to perform flow cytometry analyses. An aliquot of SAAT (approx. 0.1g) was immediately frozen in liquid nitrogen and stored at -80°C until gene expression analyses.

*Determination of plasma levels of biochemical parameters*

Plasma glucose was determined using the glucose-oxidase technique (Beckman Instruments, Fullerton, CA). Plasma insulin was measured using an Immunotech Insulin Irma kit (Immunotech, Prague, Czech Republic). Homeostasis model assessment of the insulin resistance index (HOMA-IR) was calculated as follows: ((fasting insulin in mU/l) x (fasting glucose in mmol/l) / 22.5). Plasma levels of FFA and TG were measured using enzymatic colorimetric kits (Randox, Crumlin, UK). The concentrations of sVCAM, sICAM, VEGF-A, IL8, IL6, TNFα and MCP1 in plasma were measured by ELISAs (eBioscience, San Diego, USA; R&D Systems, Minneapolis, USA) according to manufacturer's protocol. Plasma levels of other parameters were determined using standard clinical biochemical methods.

*Isolation of SVF cells*

SAAT was washed with saline, minced/cut into small pieces and digested with type I collagenase (SERVA, Heidelberg, Germany) for 1h in 37°C shaking water bath and subsequently centrifuged at 200 g for 10 minutes and filtered through 100- and 40-µm sieves to isolate SVF cells.

*Flow cytometry analysis*

The whole blood and isolated SVF cells were analyzed immediately after isolation for flow cytometry analyses as described before [12]. Briefly, $10^4$ SVF cells were resuspended in 100 µl PBS solution containing 0.5% BSA and 2 mmol/l EDTA and incubated with fluorescence- labelled monoclonal antibodies (FITC-conjugated antibody CD14, CD16, CD4; PE-conjugated antibody CD14, TLR4, CD3; PerCP-conjugated antibodies CD45 and APC-conjugated antibodies CD206 and CD8) or the appropriate isotype controls (BD Bioscience, Bedford, MA) for 30 min at 4°C.

The whole blood samples were stained with the same set of fluorescence-labelled monoclonal antibodies as used for SVF cells for 30 min at room temperature. After cell staining, erythrocytes were lysed by erythrocyte lysis buffer for 15 min at room temperature. Cells were washed with PBS and analyzed on FACS Calibur flow cytometer and CellQuest Pro Software (BD Biosciences, NJ, USA). The number of immune cell populations was expressed as percentage of gated events. Background was set up to 5% of positive cells of isotype control.

*Gene expression analysis*

Total RNA extraction and reverse transcription (RT-PCR) were performed as previously described [28]. Before reverse transcription, genomic DNA was eliminated by DNase I (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR (RT-qPCR) was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers and TaqMan probes were obtained from Applied Biosystems. Results are presented as fold change values based by the ΔΔ Ct method normalized to endogenous control GUSB.

*Statistical analyses*

Statistical analysis was performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, Inc., San Diego, California, USA). Differences of baseline clinical data between the two groups of patients were assessed using nonparametric Mann-Whitney test for unpaired observations. The effect of Intralipid infusion or control infusion on biochemical, gene expression and flow cytometry-derived variables was assessed using a nonparametric Wilcoxon test for paired observations. The changes of variables (fold change) during Intralipid

compared to control experiment were analyzed using Mann-Whitney non-parametric test. Correlations between anthropometric data and flow cytometry-derived variable was analyzed using Spearman`s correlation. Data are presented as mean ± SEM. Differences at the level of $p < 0.05$ were considered statistically significant.

## RESULTS

*Subject characteristics and levels of free fatty acids and triglycerides during the experimental infusions*

The anthropometric and biochemical data of subjects participating in the interventions are shown in Table 1. There were no significant differences in anthropometric and laboratory metabolic indices (including fasting blood glucose, plasma insulin levels, HOMA-IR, cholesterol, FFA and TG) between Intralipid and control group. Lipid infusion resulted in a continuous significant increase of plasma levels of FFA and TG ($p < 0.001$). In control intervention, no changes in FFA and TG levels compared to basal levels were observed (Fig. 1).

*Effect of lipid infusion on monocyte/macrophage content in blood and SAAT of obese women*

Monocyte/macrophage content was identified by CD45+ (common leukocyte antigen) and CD14+ (co-receptor of Toll like receptor 4, TLR-4) markers. Two populations of monocytes/macrophages were distinguished by CD16 expression. CD16- are defined as "classical" activated pro-inflammatory monocytes/macrophages (M1) and CD16+ as non-classical activated (M2)[29]. In blood, lipid infusion reduced the relative content of whole monocyte population and CD16+ monocytes, respectively (Fig. 2A). In SAAT, the relative content of total and CD16+ monocyte/macrophage population was not significantly changed (Fig. 2A). The relative content of resident macrophages in SAAT was evaluated using an expression of a mannose receptor CD206. The relative contents of resident populations CD206+ and 206+/CD16+ macrophages were reduced in response to lipid infusion (Fig. 2A), while the subset of non-resident CD206- cells had a tendency to increase (p=0.06) (Fig. 2A). Control infusion did not exert any effect on monocyte/macrophage content in SAAT (Fig. 2B).

*Effect of lipid infusion on T-lymphocyte content in peripheral blood and SAAT of obese women*

T-lymphocytes were identified by the combination of general leukocyte and T-cells marker CD45+/3+. Within the population of T lymphocytes, T-helper cells were detected by a presence of CD4+ marker and T-cytotoxic cells by CD8+ marker. The total T-cells population in blood had a tendency to increase in response to lipid infusion ($p=0.08$) (Fig. 2A). This increase was associated with a rise of T-helper cells sub-population ($p<0.05$) (Fig. 2A), while no change in T-cytotoxic cells population was observed. In SAAT, the increase of T-lymphocytes populations in response to lipid infusion was not consistent (Fig. 2A). The control infusion did not exert any effect on any T-cells content either in blood or in SAAT (Fig. 2B).

*Effect of lipid infusion on plasma levels of cytokines, VEGF-A and adhesion molecules*

The observed decrease of monocyte populations in blood led us to the hypothesis that, in response to hyperlipidemia, these cells adhere to endothelium of vascular wall. Therefore, we analyzed the plasma levels of molecules related to adhesion of immune cells to endothelial surface (sICAM, sVCAM) and to angiogenesis (VEGF-A). In response to lipid infusion the plasma levels of sICAM, sVCAM and VEGF-A markedly increased ($p< 0.001$), while no change was observed during control infusion (Fig. 3).

Moreover, we investigated plasma levels of selected cytokines and chemokines related to immune cells migration and activation (IL6, IL8, MCP1, and TNFα). Plasma levels of IL8, IL6 and MCP1 were increased in response to lipid infusion while no such change was observed during control infusion (Fig. 3). The levels of TNFα were not changed during either lipid or control infusion.

*Effect of lipid infusion on expression of genes related to immune response in SAAT*

To extend the results of flow cytometry analysis, mRNA expression of markers of monocytes/macrophages (CD14, CD206), and markers of $T_H$ lymphocytes subtypes ($T_H1$: TBX21, $T_H2$: GATA3, $T_{REG}$: FOXP3, $T_H17$: RORC) were determined in SAAT. Among these markers, the expression of RORC ($p<0.01$) – marker of $T_H17$ lymphocytes - was increased after lipid infusion. The other immune cells markers were not changed during either lipid or control infusion (Fig 4). Further, mRNA levels of chemokines/cytokines (MCP1, IL8, IL6, TNFα), angiogenic marker (VEGF-A), fatty

acid translocase (CD36) and toll like receptor 4 (TLR4) were analyzed. The mRNA levels of MCP1, IL6, CD36, ($p<0.05$), and VEGF-A ($p<0.01$) were increased in response to lipid infusion (Fig. 4), while the mRNA expressions of the other markers were not different from the baseline. No significant changes were observed in the control experiment.

## DISCUSSION

In this study, we have shown that the lipid infusion, associated with an increased levels of circulating FFA and TG, induced modifications of relative content of particular sub-populations of monocytes/macrophages and/or T-lymphocytes in blood and SAAT in obese women, modified expression of several pro-inflammatory cytokines in SAAT and changed circulating levels of cytokines and adhesion molecules.

Monocyte/macrophages and T-lymphocytes are among the most abundant immune cells invading both adipose tissue and atherosclerotic lesions and appear to be essential for the initiation and/or progression of the metabolic pro-atherogenic disturbances in obese [30, 31].

The present study provides evidence that acute systemic elevation of lipids induced by Intralipid infusion decreased the relative content of monocytes in circulation. This result led us to hypothesis that these cells adhere to the endothelium of vascular wall. This is in agreement, and further extends, the previous findings showing that monocytes in circulation increasingly adhered to the vascular wall in response to repeated postprandial hypertriglyceridemia in rats [24]. Similarly, it was shown in vitro that treatment of endothelial cells with TG-rich lipoproteins increased adherence of human monocytes to these cells 32 23. Moreover, the increased expression of surface adhesion molecules (CD11b, CD62L etc.) in immune cells was demonstrated postprandially in lean and obese men [2, 13, 33]. Among the two analyzed subpopulations of monocytes, we observed a decrease specifically in CD16+ subpopulation. These monocytes might be identified as “non-classical” activated cells (M2), i.e. exhibiting anti-inflammatory properties. Auffray et al. postulated that these “non-classical” activated monocytes patrol healthy tissue through crawling along the endothelium [34]. It was suggested that CD16+ monocytes/macrophages are present also as reparative mechanism in reaction to the damaged vessel in the early stage atherosclerotic lesions [30]. Thus, we may speculate that CD16+ monocytes represent the first cells that react

to an acute hyperlipidemia and that adhere to endothelium damaged by high concentrations of lipids in order to protect it. Indeed, the dysfunctional endothelium was suggested as one of the first steps in atherosclerosis development [30, 35, 36]. It has been shown previously, that the exposure to high concentration of FFAs and TG induces oxidative stress in endothelium leading to an impairment of its function [6, 37-39] and to elevated expression and secretion of chemokines and adhesion molecules (ICAM, VCAM) [24, 32]. Similarly, in this study we found an increase of plasma levels of soluble adhesion molecules (sICAM, sVCAM), chemokines (IL8 and MCP1) and also angiogenic factor VEGF-A in response to lipid infusion in obese subjects. The increased expression of angiogenic marker VEGF-A and chemokine MCP1 was detected also in SAAT. Moreover, the increased levels of adhesion molecules support the above mentioned hypothesis of monocytes adhesion to endothelia.

In SAAT, the relative content of CD16+ monocytes remained unchanged, but the subset of CD206+/16+ macrophages was decreased. CD206 is considered not only as a marker of AT resident macrophages but also a marker of "non-classical" activated, i.e. M2, macrophages [40], similarly as CD16. Therefore the decrease in these CD206+ and CD16+/CD206+ populations may suggest a switch from M2 to "classical" M1 activated pro-inflammatory phenotype of macrophages in response to lipid infusion. The switch to M1 pro-inflammatory phenotype in SAAT could be indeed supported by the observed upregulation of MCP1 expression in SAAT. In addition, the subpopulation of CD206- monocytes had a tendency to increase in response to lipid infusion. Such a population of CD206- monocytic cells was described by Wentworth et al. [41] and was shown to be elevated in human obesity. It is plausible that these monocytes represent "the newest arrivals" into AT and later can mature into pro-inflammatory macrophages.

In our study, we found an increase of T helper cell content and a trend to an increase of total T lymphocyte content ($p=0.08$) in circulation in response to lipid infusion. This finding is in line with previous studies, in which lymphocyte counts were shown to be increased postprandially in healthy as well as in hyperlipidemic subjects with coronary artery disease [2, 42]. Moreover, intra-venous administration of CD4+ cells enhanced atherosclerosis in immunodeficient ApoE knockout mice [43] and the development of atherosclerosis was significantly reduced in CD4/ApoE deficient mice [44]. Thus based on our data one can hypothesize that adaptive immune system in obese reacts to hyperlipidemia in the pro-atherogenic way.

In SVF of SAAT, no changes of T lymphocyte relative counts in response to Intralipid were detected. Similarly mRNA levels of the T lymphocyte markers that were used in FACS analysis, were not altered. However, mRNA analysis of SAAT revealed an increase in the expression of RORC, a TH17 lymphocyte marker. TH17 cells represent a subtype of CD4+ T-lymphocytes with a pro-inflammatory phenotype. Their numbers were found to be elevated in adipose tissue of metabolically unhealthy obese subjects or in adipose tissue of mice with diet-induced obesity [45, 46]. Therefore the increase of expression of TH17 marker (RORC) might suggest a selective activation/recruitment of TH17 lymphocytes, which may be interpreted as a pro-inflammatory change in SAAT in response to high lipid levels.
The activation and lipid overload in leukocytes were shown to be mediated by several scavenger receptors, i.e. SRA, CD36 [21, 47]. In this study, we observed increased mRNA expression of CD36 in SAAT in response to increased FFAs, similarly to previous studies on adipocytes 48 and leukocytes [21, 47]. This increased expression of CD36 could thus induce enhanced fatty acid flux and lipids accumulation in the immune cells and their switch to the pro-inflammatory phenotype [21, 22]. However, it is to be noted that the origin of the increased expression of CD36 in SAAT cannot be ascribed only to immune cells: changes in CD36 expression on preadipocytes and adipocytes have to be taken into account as well.
In conclusion, the acute hyperlipidemia induced by Intralipid infusion was associated with pro-inflammatory and pro-atherogenic changes in monocyte and lymphocyte populations as well in soluble mediators in obese women. The pro-inflammatory changes are represented by a decrease of M2 macrophages content and increased expression of several pro-inflammatory cytokines and of the marker of TH17 cells in SAAT.
Together, these results point at the processes that could contribute to the development of atherosclerosis and metabolic complications in obese exposed to higher chronically, as well as the postprandially, increased levels of FFA and TG.

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Contribution of authors: E.K. performed the experiments, researched data, and wrote the manuscript. J.K. performed the experiments, researched data, and edited manuscript. M.T. designed the study, performed the experiments, and researched data. L.M. performed the experiments. Z.K performed the experiments. L.R. researched data, and reviewed/edited manuscript. V.S. designed the study, and reviewed/edited the manuscript. M.S. researched data and wrote the manuscript.

M.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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**Table 1**. Anthropometric and biochemical characteristics of two experimental groups of obese women

| | **Intralipid** | **Glycerol Controls** | **P** |
|---|---|---|---|
| Age (years) | 40.0 ± 2 | 43 ± 3 | NS |
| Weight (kg) | 87.7 ± 2.4 | 89.8 ± 2.3 | NS |
| BMI ($kg/m^2$) | 31.2 ± 0.8 | 32.0 ± 1.0 | NS |
| Waist circumference (cm) | 96.0 ± 2.1 | 96.3 ± 2.2 | NS |
| Waist-to-hip ratio | 0.8 ± 0.0 | 0.8 ± 0.0 | NS |
| Fat mass (%) | 38.9 ± 1.0 | 40.3 ± 1.6 | NS |
| Fat-free mass (%) | 61.1 ± 1.0 | 59.7 ± 1.6 | NS |
| Cholesterol (mmol/L) | 5.1 ± 0.2 | 4.9 ± 0.4 | NS |
| HDL-C (mmol/L) | 1.5 ± 0.1 | 1.4 ± 0.1 | NS |
| Triglycerides (mmol/L) | 1.2 ± 0.1 | 1.0 ± 0.1 | NS |
| Glucose (mmol/L) | 5.1 ± 0.1 | 5.0 ± 0.1 | NS |
| Insulin (mU/L) | 6.4 ± 0.7 | 6.1 ± 0.8 | NS |
| HOMA-IR | 1.3 ± 0.1 | 1.4 ± 0.2 | NS |

Data are presented as mean ± SEM;

BMI, body mass index; HOMA-IR, homeostasis model assessment of the insulin resistance index; HDL-C, HDL cholesterol

## FIGURES

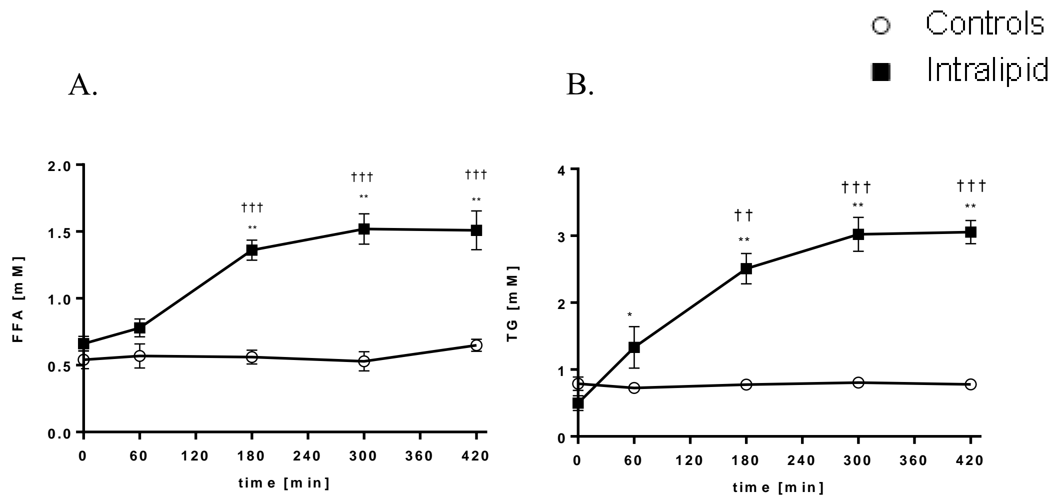
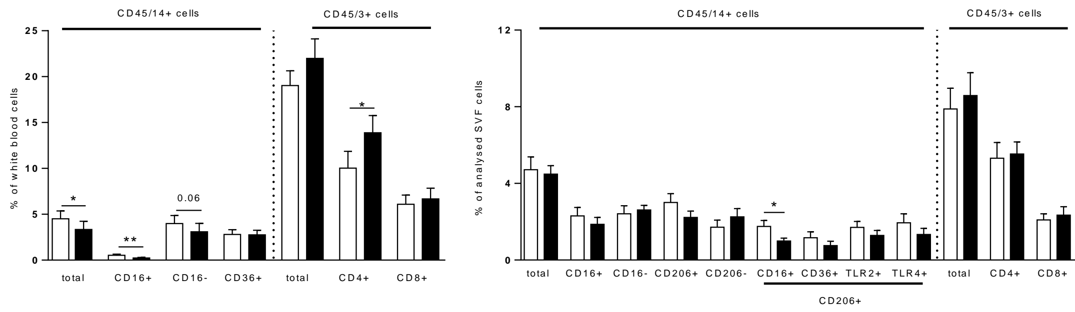



**Figure 1.** Evolution of plasma levels of (A) FFA, (B) TG during experimental condition. Values are means with their standard errors. Mean value was significantly different from that of basal state in Intralipid treatment: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Mean value was significantly different from that of controls:† $p<0.05$, †† $p<0.01$, ††† $p<0.001$.

A. Intralipid infusion




B. Control glycerol infusion

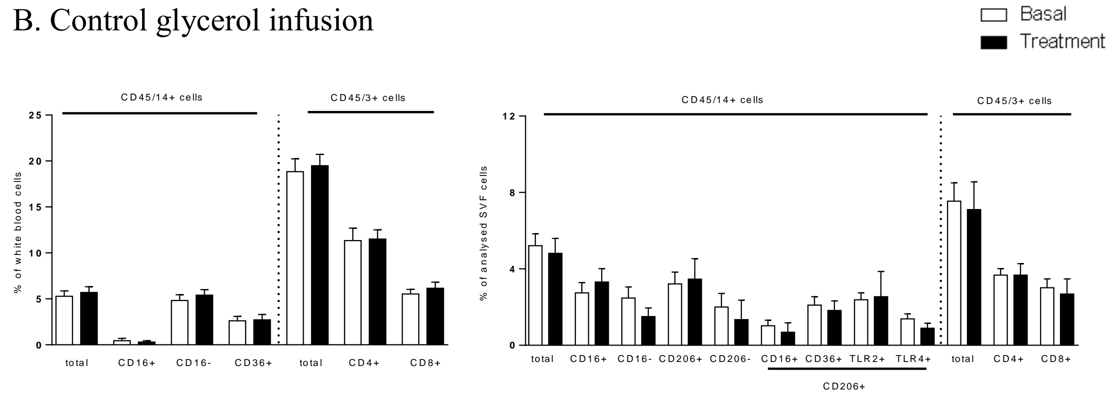



**Figure 2.** Relative content of monocyte/macrophage and T-lymphocyte populations in blood and subcutaneous adipose tissue (SAAT) after Intralipid (A) or glycerol (B) infusion in obese women. Data are presented as mean ± SEM, *$p < 0.05$; ** $p < 0.01$.

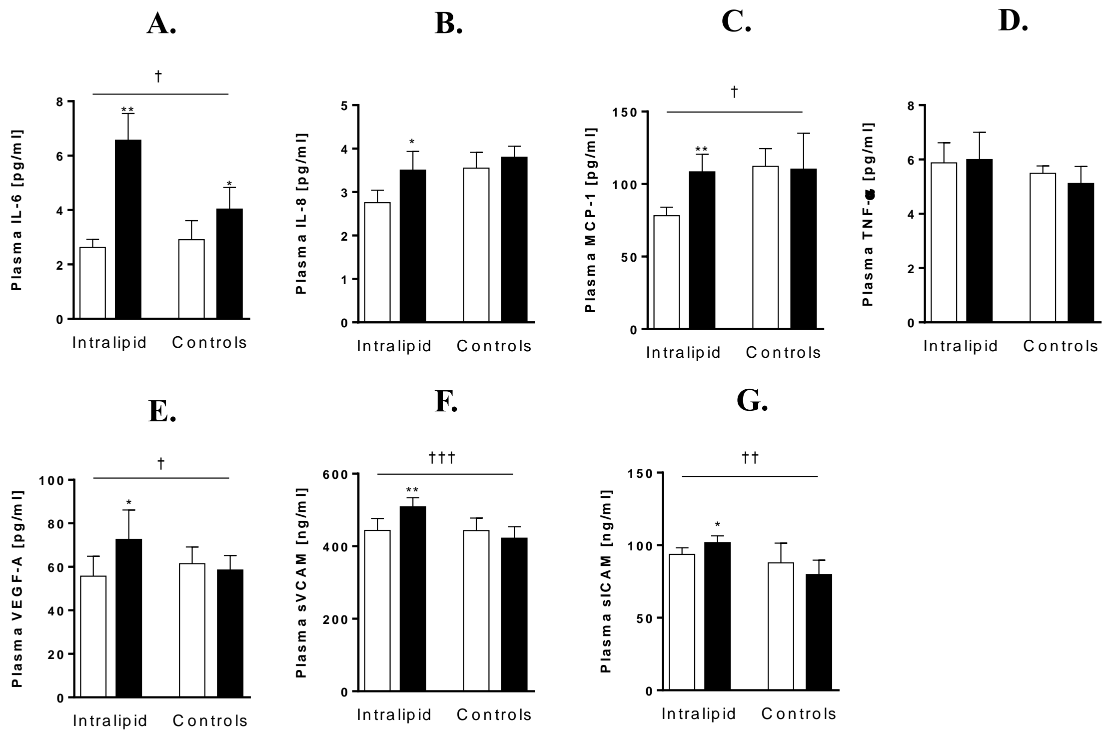



**Figure 3.** Evolution of plasma levels of cytokines (A) IL6, (B) IL8, (C) MCP1, (D) TNFα, (E) VEGF-A, (F) sVCAM, (G) sICAM during experimental conditions. Data are presented as mean fold change ± SEM. Mean value was significantly different from that of basal state: * $p<0.05$, *** $p<0.001$ or from control group: † $p<0.05$, †† $p< 0.01$, ††† $p<0.001$

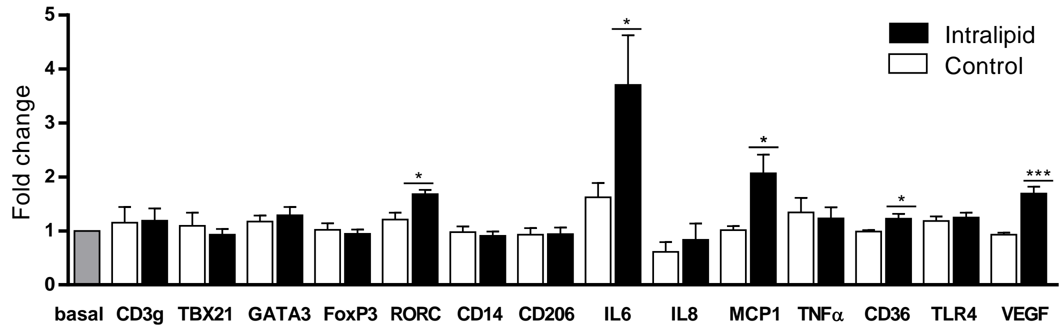



**Figure 4.** Effect of Intralipid on gene expression of selected macrophage and T lymphocytes markers in SAAT of obese women. Data are presented as mean fold change ± SEM. Relative mRNA levels are normalized to housekeeping gene GUSB. Mean value was significantly different from that of basal state: * $p<0.05$, *** $p<0.001$

*PART TWO*

## Paper 4

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**Expression of inflammation-related genes in gluteal and abdominal subcutaneous adipose tissue during weight-reducing dietary intervention in obese women**

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# Expression of Inflammation-Related Genes in Gluteal and Abdominal Subcutaneous Adipose Tissue During Weight-Reducing Dietary Intervention in Obese Women

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**Summary**

Accumulation of adipose tissue in lower body lowers risk of cardiovascular and metabolic disorders. The molecular basis of this protective effect of gluteofemoral depot is not clear. The aim of this study was to compare the profile of expression of inflammation-related genes in subcutaneous gluteal (sGAT) and abdominal (sAAT) adipose tissue at baseline and in response to multiphase weight-reducing dietary intervention (DI). 14 premenopausal healthy obese women underwent a 6 months' DI consisting of 1 month very-low-calorie-diet (VLCD), subsequent 2 months' low-calorie-diet and 3 months' weight maintenance diet (WM). Paired samples of sGAT and sAAT were obtained before and at the end of VLCD and WM periods. mRNA expression of 17 genes (macrophage markers, cytokines) was measured using RT-qPCR on chip-platform. At baseline, there were no differences in gene expression of macrophage markers and cytokines between sGAT and sAAT. The dynamic changes induced by DI were similar in both depots for all genes except for three cytokines (IL6, IL10, CCL2) that differed in their response during weight maintenance phase. The results show that, in obese women, there are no major differences between sGAT and sAAT in expression of inflammation-related genes at baseline conditions and in response to the weight-reducing DI.

**Key words**

Body fat distribution • Hypocaloric diet • Macrophages • Cytokines

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## Introduction

Obesity is associated with higher risk of metabolic and cardiovascular diseases. In addition to body mass index (BMI), body fat distribution plays a major role in the development of the above mentioned diseases. While upper body fat accumulation is associated with increased obesity-related health risk, the lower body fat accumulation was shown to be linked with the reduction of metabolic (Snijder *et al.* 2004), cardiovascular risk (Canoy *et al.* 2007, Faloia *et al.* 2009, Seidell *et al.* 2001) and with lower morbidity and mortality (Folsom *et al.* 1993, Pischon *et al.* 2008). The increased amount of lower body fat – expressed as hip circumference – was associated with lower triacylglycerol and higher HDL cholesterol levels (Ruige and Van Gaal 2009).

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Possible mechanisms that may contribute to the subcutaneous gluteal adipose tissue (sGAT) protective role have not been fully elucidated. Different uptake and release of fatty acids in the subcutaneous abdominal (sAAT) when compared to sGAT have been suggested as a candidate underlying cause (Berman *et al.* 1998, Berman *et al.* 2004). In several studies, the activity of lipoprotein lipase (LPL) was found to be higher (Arner *et al.* 1991, Ferrara *et al.* 2002) in sGAT in obese women. This might suggest a higher capacity of sGAT for lipid accumulation.

In addition to alteration of adipose tissue (AT) fatty acid handling, the obesity-related metabolic disturbances are linked with pro-inflammatory state of AT characterized by enhanced recruitment of macrophages in AT and modified AT secretion of cytokines (Klimcakova *et al.* 2011, O'Hara *et al.* 2009, Suganami and Ogawa 2010, Trayhurn and Wood 2004). Thus, it may be hypothesized that differences in the inflammation-related characteristics underlie the differences in metabolic role of sGAT when compared with sAAT. Few studies paid attention to this topic. Recently, Evans *et al.* (2011) showed that sGAT had, contrary to the expectation, greater mRNA expression of a set of pro-inflammatory genes than sAAT.

Thus the first aim of our study was to compare, in obese women, expression of wider range of cytokines and macrophage markers in sGAT vs. sAAT (selected according to our previous studies of Capel *et al.* 2009, Klimcakova *et al.* 2011, Siklova-Vitkova *et al.* 2012) and their association with indices of metabolic syndrome. Our second aim was to explore the regulation of expression of the above mentioned genes in a dynamic condition that was realized by a dietary intervention (DI) using hypocaloric diet. It was shown previously that hypocaloric diet-induced changes in adipocyte lipolysis (Mauriege *et al.* 1999) or in adipocyte size (Bjorntorp *et al.* 1975) were less pronounced in sGAT when compared with sAAT. We hypothesized that this impaired responsiveness, or "inflexibility", of sGAT might also appear in respect to the diet-induced modulation of gene expression of immunity-related genes. Thus, we measured mRNA expression of the respective genes in the paired samples of sGAT and sAAT obtained in obese women before and during multiple phases of a 6 months' hypocaloric DI.

## Materials and Methods

### *Subjects*

14 premenopausal obese women (BMI 34.2±2.6 kg/m$^2$, range 27-49 years) without medication and diseases except for obesity participated in this study. Their body weight had been stable for 3 months prior to the examination. The informed consent was obtained from each patient before the study. The study was performed according to the Declaration of Helsinki protocols and was approved by Ethical Committee of the Third Faculty of Medicine, Charles University in Prague.

### *Dietary intervention and clinical investigation*

The entire DI lasted 6 months. During the first dietary period, obese subjects received a very low calorie diet (VLCD) of 800 kcal/day (liquid formula diet, Redita, Promil, Czech Republic) for 1 month. The subsequent period consisted of a 2 months' low-calorie diet (LCD) followed by 3 months' weight maintenance (WM) diet. LCD was designed to provide 600 kcal/day less than the individually estimated energy requirement based on an initial resting metabolic rate multiplied by 1.3, the coefficient of correction for physical activity level. Patients consulted a dietician once a week during the first 3 months of the program and once a month during subsequent phase. They provided a written 3 days' dietary record at each dietary consultation.

Complete clinical investigation including anthropometric measurements, blood sampling and AT biopsies was performed in the morning in the fasting state before the beginning of the diet and at the end of VLCD and WM periods.

The whole body composition was evaluated by multi-frequency bioimpedance (Bodystat, Quad scan 4000, Isle of Man, UK). The blood was collected and centrifuged at 1300 RPM, 4 °C, separated plasma was stored at −80 °C until analysis. The paired samples of subcutaneous AT were obtained from the subcutaneous abdominal (10 cm lateral to the umbilicus) and gluteal (right upper quadrant) region using needle biopsy under local anesthesia (1 % Xylocaine). AT samples were obtained from superficial sAAT, as we verified on several occasions using ultrasonography. AT was washed in physiological saline, aliquoted, snap-frozen in liquid nitrogen and stored at −80 °C until processing.

### *Laboratory measurements*

Plasma glucose was determined using the glucose-oxidase technique (Beckman Instruments, Fullerton, CA). Plasma insulin was measured using an Immunotech Insulin Irma kit (Immunotech, Prague, Czech Republic). Homeostasis model assessment of the insulin resistance index (HOMA-IR) was calculated as follows: ((fasting insulin in mU/l) x (fasting glucose in

**Table 1.** Clinical characteristics of subjects and plasma levels of cytokines before dietary intervention (basal) and at the end of VLCD and weight maintenance (WM) phase of the dietary intervention.

| | Basal | VLCD | WM |
|---|---|---|---|
| *Age (year)* | 27-49 | | |
| *BMI (kg/m²)* | 34.2±0.2 | 31.5±0.2*** | 30.4±0.2††† |
| *Weight (kg)* | 93.5 ± 0.6 | 86.0±0.6*** | 83.1±0.7††† |
| *Waist circumference (cm)* | 102.3±0.4 | 95.4±0.6*** | 93.6±0.6††† |
| *Hip circumference (cm)* | 119.1±0.5 | 114.1±0.5*** | 112.1±0.5††† |
| *Waist to hip ratio (cm)* | 0.861±0.0 | 0.838±0.0* | 0.836±0.0†† |
| *Fat mass (%)* | 41.9±0.3 | 39.4±0.4*** | 37.2±0.4††† |
| *FFM (%)* | 58.1±0.3 | 60.1±0.4* | 63.0±0.5††† |
| *Glucose (mmol/l)* | 4.9±0.4 | 4.6±0.0* | 4.7±0.0 |
| *Insulin (mIU/l)* | 11.7±0.8 | 8.0±0.3* | 9.0±0.4† |
| *FFA (μmol/l)* | 842±60.1 | 1190±28.2* | 676±13.5† |
| *Triglycerides (mmol/l)* | 1.6±0.0 | 1.2±0.0 | 1.5±0.0 |
| *HDL cholesterol (mmol/l)* | 2.0±0.0 | 1.2±0.0 | 1.6±0.0 |
| *Total cholesterol (mmol/l)* | 5.2±0.0 | 4.3±0.0*** | 5.2±0.0 |
| *HOMA-IR* | 2.6±0.1 | 1.7±0.1* | 1.9±0.1†† |
| *hs-CRP (mg/l)* | 5.8±0.4 | 4.2±0.3 | 4.0±0.3† |
| *IL10 (pg/ml)* | 1.4±0.4 | 1.5±0.6 | 1.5±0.6 |
| *IL6 (pg/ml)* | 3.7±0.1 | 3.1±0.1 | 2.6±0.1 |
| *TNF (pg/ml)* | 2.0±0.1 | 2.3±0.1** | 2.0±0.1 |
| *CCL2 (pg/ml)* | 104.3±4.7 | 103.1±3.2 | 86.4±2.7† |

Values are means ± SEM, n=14. Significance was set as follows: VLCD vs. basal: * p<0.05, ** p<0.01, *** p<0.001, WM phase vs. basal: † p<0.05, †† p<0.01, ††† p<0.001. BMI, body mass index; CCL2, chemokine (C-C motif) ligand 2; FFM, fat-free mass; HOMA-IR, homeostasis model assessment of the insulin resistance index; hs-CRP, high-sensitivity C-reactive protein; IL6, interleukin 6; IL10, interleukin 10; FFA, free fatty acids; TNF, tumor necrosis factor

mmol/l) / 22.5). Plasma levels of other relevant substances were determined using standard clinical biochemistry methods. Plasma levels of cytokines were determined using multiplex human cytokines Milliplex panels (Millipore-Merck, Bedford, MA, USA).

*Gene expression analysis*

Total RNA was isolated from 100-300 mg aliquots of AT using RNeasy Lipid Tissue RNA Mini kit (Qiagen, Hilden, Germany). RNA concentration was measured by Nanodrop1000 (Thermo Fisher Scientific, Wilmington, Delaware, USA). Genomic DNA was removed by DNAse I treatment (Invitrogen, Carlsbad, CA, USA). cDNA was obtained by reverse transcription (High Capacity cDNA Reverse Transcription Kit, Applied Biosystem, Carlsbad, CA, USA) using 200 ng of total RNA. 1 ng of cDNA was then preamplified to improve detection of target genes during subsequent Real Time qPCR (16 cycles, TaqMan Pre Amp Master Mix Kit, Applied Biosystem). For the preamplification, 20 x TaqMan gene expression assays of all target genes were pooled together and diluted with 1x TE buffer to the final concentration 0.2x (each probe). The RT-qPCR was performed on Biomark Real Time qPCR system and 96x96 chip (Fluidigm, USA) in triplicates. This part of analysis was carried out as a paid service by Biotechnology Institute, AS CR.

Expression of 17 genes grouped according to their origin or function was measured: cytokines (IL6, TNF, CCL2, CXCL1, IL10, TGFβ1, IL10RA) and cytokine receptor (CCR2), macrophage markers (SPP1, CD68, MSR1, PLA2G7, ACP5, FCGBP, CD14, TLR4, TLR2). Macrophage markers were selected according to the work of Capel *et al.* (2009) and Klimcakova *et al.* (2011) and cytokines produced predominantly by cells of stromavascular fraction were chosen according to study of Siklova-Vitkova *et al.* (2012) so that comparisons of outcomes between this and our previous studies were enabled. Expression data were normalized to expression of reference gene, PPIA, and delta Ct was

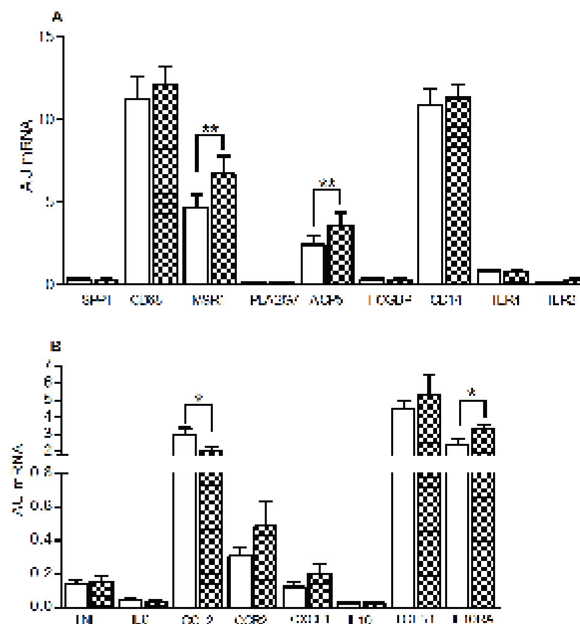


**Fig. 1.** Adipose tissue gene expression in basal state, before the diet. **A:** macrophage markers, **B:** cytokines. Subcutaneous abdominal adipose tissue (open columns), subcutaneous gluteal adipose tissue (cross-hatched columns). Each column represents mean of gene expression levels calculated as delta Ct (normalized to PPIA). * $p<0.05$, ** $p<0.01$

log-transformed for statistical analysis.

*Statistical analysis*

The data from RT-qPCR were analyzed with Genex software (MultiD Analysis AB, Sweden) and SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). For all the analyses the data were log-transformed. One way ANOVA was used for comparison of gene expression separately in each depot, the effect of sAAT vs. sGAT on the diet-induced changes was estimated by two way ANOVA with repetitive measures and Tukey's posthoc analysis. The level of significance was set at $p<0.05$. Clinical and anthropometrical data were analyzed with GraphPad Prism 5.0. (La Jolla, CA, USA). Correlations were performed using Pearson's parametric test.

## Results

*Effect of dietary intervention on anthropometrical and plasma variables*

The clinical data of the entire group of subjects at baseline and during the DI are presented in Table 1. When compared to baseline, the subjects' body weight decreased by 8.0 % after the VLCD and decreased further during subsequent period so that the weight loss at the end of WM represented 11.1 % of the original weight. BMI, fat mass, waist and hip circumference showed a similar pattern. The relative decrease of waist circumference was greater than that of hip circumference at the end of VLCD as well as at the end of the entire DI (VLCD: waist: −6.8±0.3 %, hip: −4.2±0.3 %, $P<0.05$, the end of DI: waist: −8.6±0.2 %, hip: −5.9±0.3 %, $P<0.01$). Plasma levels of insulin were lower at the end of VLCD when compared to baseline condition and remained lower at the end of WM. Glycemia and total cholesterol decreased after VLCD and returned to the baseline levels at WM. Free fatty acid levels were increased after VLCD and decreased below the baseline values at the end of the WM. Insulin resistance assessed by HOMA-IR decreased during VLCD and remained reduced at the end of WM phase. The changes of plasma levels of cytokines IL10, IL6, TNF, CCL2 were in line with our previous study (Siklova-Vitkova *et al.* 2012).

*Comparison of gene expression in gluteal and abdominal subcutaneous AT in obese women at pre-diet condition (Fig. 1)*

To compare gene expression profile in sAAT and sGAT, we measured mRNA expression of 17 genes divided into 2 functional groups: macrophage markers (9 genes), cytokines (8 genes), and reference gene PPIA. The expression of macrophage markers was similar in

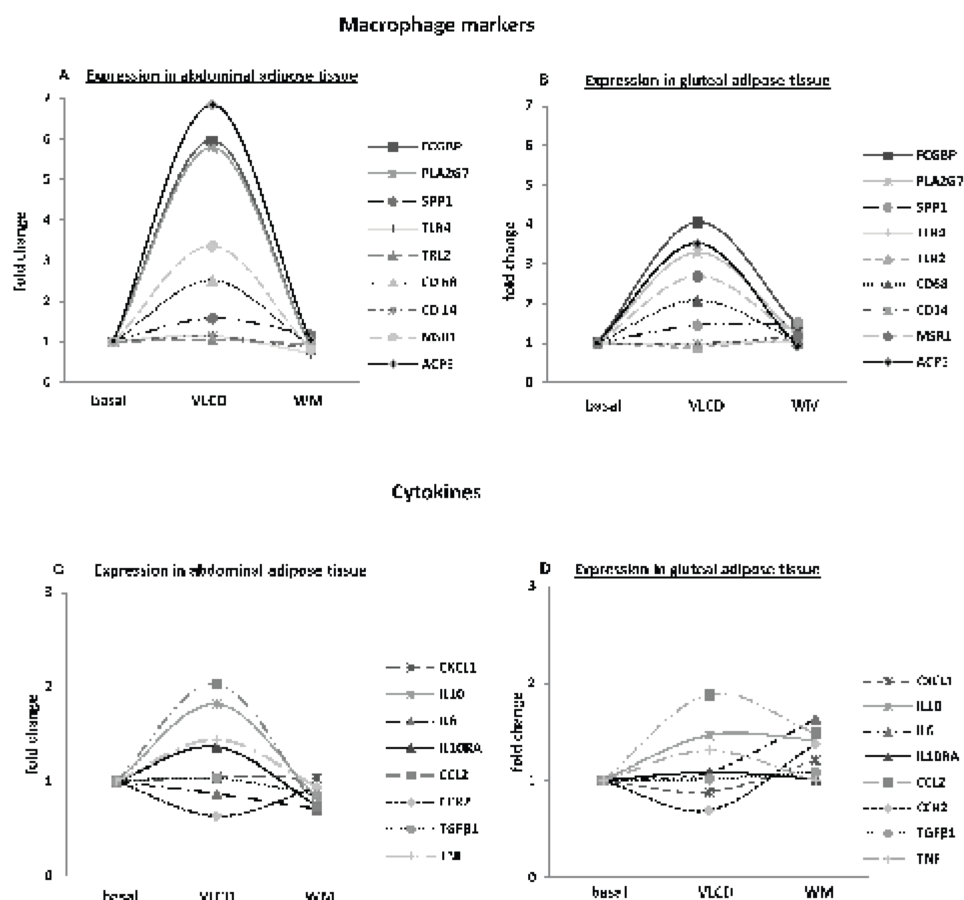


**Fig. 2.** Profile of gene expression in subcutaneous abdominal (sAAT) and gluteal (sGAT) adipose tissue during the two phases (VLCD and WM) of dietary intervention. **A** and **B**: Expression of macrophage markers in sAAT (**A**) and sGAT (**B**). **C** and **D**: Expression of cytokines in sAAT (**C**) and sGAT (**D**). The data are presented as fold change in respect to the pre-diet (basal) levels. Data are presented as means ± SEM.

both depots except for two genes, ACP5 and MSR1 that had higher expression in sGAT compared to sAAT (Fig. 1A). There were no depot-related differences in the expression of all measured cytokines (TNF, IL6, CCR2, CXCL1, IL10, TGFβ1) with exception of IL10RA (higher in sGAT) and CCL2 (lower in sGAT) (Fig. 1B).

*Effect of dietary intervention on gene expression in gluteal and abdominal subcutaneous AT (Fig. 2, Table 2)*

The evolution of the mRNA expression for individual genes during DI is shown in Figure 2. Schematic representation of the direction and significance of the diet-induced changes of gene expression during VLCD and during the entire DI in each depot is presented in Table 2.

*Macrophage markers (Fig. 2A-2B)*

The expression of 6 macrophage markers (CD68, ACP5, FCGBP, MSR1, PLA2G7, SPP1) increased during VLCD in both depots while expression of 3 remaining markers (CD14, TLR4, TLR2) was not changed in both depots. At the end of WM, the mRNA levels of all macrophage markers were not different from the baseline values in both depots (Fig. 2A-2B).

*Cytokines (Fig. 2C-2D)*

During VLCD, the mRNA levels of 5 cytokines (TNF, IL6, IL10RA, TGFβ1, CXCL1) did not change in either depot, CCL2 increased and CCR2 decreased in both depots and IL10 showed a significant increase

**Table 2.** Changes of gene expression in subcutaneous abdominal (sAAT) and subcutaneous gluteal (sGAT) adipose tissue during VLCD and weight maintenance (WM) phases of dietary intervention in relation to the pre-diet (basal) level.

| Name of gene | sAAT | | sGAT | |
|---|---|---|---|---|
| | VLCD | WM | VLCD | WM |
| ***Macrophage markers*** | | | | |
| *CD68* | **↑ | ↔ | *↑ | ↔ |
| *ACP5* | ***↑ | ↔ | **↑ | ↔ |
| *CD14* | ↔ | ↔ | ↔ | ↔ |
| *FCGBP* | ***↑ | ↔ | **↑ | ↔ |
| *MSR1* | ***↑ | ↔ | **↑ | ↔ |
| *PLA2G7* | **↑ | ↔ | *↑ | ↔ |
| *SPP1* | *↑ | ↔ | *↑ | ↔ |
| *TLR4* | ↔ | ↔ | ↔ | ↔ |
| *TLR2* | ↔ | ↔ | ↔ | ↔ |
| ***Cytokines*** | | | | |
| *TNF* | ↔ | *↓ | ↔ | ↔ |
| *IL10* | *↑ | *↓ | ↔ | *↑ (##) |
| *IL6* | ↔ | *↓ | ↔ | ↔ (##) |
| *IL10RA* | ↔ | **↓ | ↔ | ↔ |
| *CCL2* | *↑ | *↓ | *↑ | *↑ (##) |
| *CCR2* | **↓ | ↔ | **↓ | ↔ |
| *TGFβ* | ↔ | ↔ | ↔ | ↔ |
| *CXCL1* | ↔ | ↔ | ↔ | ↔ |

↑ denotes a higher level of gene expression at the end of respective dietary phase (VLCD or WM) in relation to the pre-diet (basal) level; ↓ denotes a lower level of gene expression in relation to the pre-diet (basal) level; ↔ denotes no change in gene expression in relation to the pre-diet (basal) level; *,**,*** denotes the level of significance ($p<0.05$, $p<0.01$, $p<0.001$, respectively) of the difference between the value at the end of respective dietary phase vs. pre-diet (basal) level; ## denotes the level of significance ($p<0.01$) of the difference between sGAT and sAAT in respect to the change of gene expression during respective dietary phase (as assessed by two-way ANOVA)

selectively in sAAT.

At the end of DI, IL10 and CCL2 mRNA levels were higher than baseline values in sGAT while, in sAAT, the mRNA levels of these two cytokines as well as those of IL6, IL10RA and TNF were lower when compared with the baseline.

The diet-induced changes of mRNA levels were similar in the two depots for all of the cytokine genes with 3 exceptions: during the entire DI, expression of IL10, IL6, and CCL2 decreased in sAAT while it increased or was unaltered in sGAT.

*Summary of the comparison of the diet-induced responses of the gene expression in sGAT and sAAT*

For majority of measured genes no differences in the diet-induced changes between sGAT and sAAT were found. Different responses were found only for the 3 cytokines (IL10, IL6, CCL2): their decrease during the entire DI was pronounced in sAAT but not in sGAT.

*Correlations*

No correlations between the diet-induced changes of mRNA expression of examined genes in sGAT vs. sAAT were found. No correlations were found between the diet-induced changes of plasma levels of IL10, IL6, TNF, CCL2 and the changes in the expression of these genes in either sGAT or sAAT. In addition, no correlations between the diet-induced changes of mRNA expression of examined genes and those of BMI or HOMA-IR were found.

## Discussion

It was hypothesized that the protective role of

AT accumulation in the lower body, in respect to cardiovascular risk and metabolic disturbances, might be based on the lower pro-inflammatory profile of sGAT. However, recent work of Evans *et al.* (2011) reported that the expression of several pro-inflammatory markers was higher in sGAT compared to sAAT in a mixed group of lean and obese black and white South African women. Our work extended the range of explored genes and showed that, in a wide group of cytokines and macrophage markers, there were, with exception of 4 genes, no differences between sAAT and sGAT at baseline. Taken together, our and Evans' results do not support the hypothesis of the lower pro-inflammatory profile of sGAT. This is in line with the finding reported by Tchoukalova *et al.* (2010) that subcutaneous abdominal and femoral fat depot did not differ in number of macrophages in lean men and women.

The main interest of this study lies in the comparison of gene regulation in sAAT vs. sGAT in dynamic condition represented by two phases of a 6 months' dietary intervention. The pattern of the expression of macrophage markers observed in this study, including the increase during initial VLCD phase is in agreement with our previous work carried out in sAAT in another cohort of subjects (Capel *et al.* 2009). The pattern is bi-phasic, characterized by an increased expression during VLCD and a decrease towards baseline values during subsequent weight maintenance phase of the diet. Increased expression of macrophage markers during VLCD might be associated with enhanced fatty acids release from adipocytes as a possible trigger of macrophage activation and infiltration mediated by TLR4 signaling as shown before (Kosteli *et al.* 2010, Suganami *et al.* 2005). Importantly, this bi-phasic response of macrophage markers expression was similar in sGAT when compared with sAAT (Fig. 2, Table 2) and the magnitudes of the diet-induced changes were not different in the two fat depots (Table 2). This finding suggests the same regulation of macrophage infiltration in sGAT and sAAT during weight-reducing dietary intervention.

The bi-phasic pattern of the diet-induced regulation was found also in the expression of cytokines derived predominantly from the cells of stromavascular fraction. This pattern was in accordance with previous results obtained in sAAT in a different cohort of subjects (Siklova-Vitkova *et al.* 2012). The magnitudes of the diet-induced changes were not different between sGAT and sAAT – except for three cytokines – IL6, IL10 and CCL2. The observed variability in respect to the individual cytokine genes is, again, in agreement with the above mentioned study of Siklova-Vitkova *et al.* (2012): in that study the same three cytokine genes were the only ones that showed a significant decrease in sAAT at the end of dietary intervention. This partial differential depot-related response – limited to the expression of the three cytokines – might be linked to differential response of endocannabinoid system as observed in the study of Bennetzen *et al.* (2011). In fact, during the weight-reducing diet the authors found different change of expression of cannabinoid receptor type 1 in sGAT when compared with sAAT. And endocannabinoids were shown to inhibit production of several proinflammatory cytokines in primary human Muller cells (Krishnan and Chatterjee 2012). This mechanism could be taken into account, although no such regulation has been reported in adipose tissue. Moreover, in resting condition, Rantalainen *et al.* (2011) found differential expression of 12 % of measured microRNA (e.g. miR146-5b, miR-21, miR155) in sGAT when compared with sAAT which could imply a differential expression of targets of these microRNA such as IL10 (Quinn and O'neill 2011) in the two fat depots.

The lack of correlations in gene expression changes between sAAT and sGAT suggests that, in spite of the similar pattern of the diet-induced response of the gene expression in the two fat depots, there is no direct quantitative association of the magnitude of the change between these two depots. In light of our and others results demonstrating the absence of major differences between sGAT and sAAT it has been suggested that the deleterious effect of upper body obesity could be mediated by the excess of visceral adipose tissue (VAT) and not excess of sAAT. Nevertheless, several studies showed that both, VAT and sAAT, are associated with the increased risk of metabolic profile and pro-inflammatory status (although the association was stronger with VAT) (Fox *et al.* 2007, Oka *et al.* 2010, Pou *et al.* 2007).

Furthermore, it should be noted that the present study compared sGAT and sAAT on transcriptional level. Due to the lack of sufficient amount of adipose tissue we did not explore the protein levels of cytokines in adipose tissues or their levels of secretion. It is not excluded that the underlying causes of different physiological impact of sGAT vs. sAAT may be at the level of translation or post-translational regulations of cytokine production or release.

It is to be noted that the results of this study are limited to women. Female adipose tissue shows different metabolic and endocrine characteristics (Kern *et al.* 2003, Montague *et al.* 1997) when compared with men. Moreover, the initial fat distribution in our set of women (mean WHR=0.861±0.0) might play a role in the diet-induced response of the two fat depots although the reports on the effect of initial fat distribution on the body fat reduction are not unequivocal (Svendsen *et al.* 1995, Jones and Edwards 1999).

In conclusion, we did not find major differences in mRNA levels of macrophage markers and cytokines between sAAT and sGAT at baseline condition or in the pattern of their regulation in response to two phases of hypocaloric weight-reducing dietary intervention (with exception of 3 cytokines that were differentially regulated during weight maintenance phase). Therefore, our results do not bring evidence of an altered pro-inflammatory status or an altered "responsiveness" of immune cells in sGAT when compared with sAAT. Further research elucidating the molecular base of the protective role of sGAT fat depot is therefore warranted.

## Conflict of Interest

There is no conflict of interest.

## Acknowledgements

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**Paper 5**

Jana Kračmerová, Lenka Rossmeislová, Lucia Mališová, Zuzana Kováčová, Michaela Tencerová, Eva Klimčáková, Jan Polák, Vladimír Štich, Dominique Langin and Michaela Šiklová

**Soluble CD163 is associated with CD163 mRNA expression in adipose tissue and with insulin sensitivity in steady-state condition but not in response to calorie restriction**

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# Soluble CD163 Is Associated With CD163 mRNA Expression in Adipose Tissue and With Insulin Sensitivity in Steady-State Condition but Not in Response to Calorie Restriction

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**Context:** Soluble CD163 (sCD163) was suggested as a biomarker of insulin sensitivity and CD163 mRNA expression representing macrophage content in adipose tissue (AT).

**Objective:** The aim of this study was to investigate, in cross-sectional and prospective design, the relationship between sCD163 circulating levels and CD163 mRNA expression in adipose tissue and insulin sensitivity assessed by euglycemic-hyperinsulinemic clamp.

**Design, Setting, Participants, and Interventions:** Two cohorts of subjects were examined in the study. Cohort 1 included 42 women with a wide range of body mass index (17–48 kg/m²); cohort 2 included 27 obese women who followed a dietary intervention consisting of 1 month of a very low-calorie diet and 5 months of a weight-stabilization period.

**Main Outcome Measures:** Serum levels of CD163 and mRNA expression of CD163 and CD68 in sc and visceral (visc) AT were determined, and insulin sensitivity [expressed as glucose disposal rate (GDR)] was measured in cohort 1. In cohort 2, serum levels of CD163, mRNA expressions of CD163, CD68, and CD163-shedding factors [TNF-α-converting enzyme (TACE) and tissue inhibitor of metalloproteinase (TIMP3)] in sc AT were examined and GDR was measured before and during dietary intervention.

**Results:** In cohort 1, circulating sCD163 correlated with CD163 mRNA levels in both sc and visc AT. sCD163 and CD163 mRNA expression in both fat depots correlated with GDR. In cohort 2, the diet-induced changes of sCD163 levels did not correlate with those of CD163, CD68, TACE, and TIMP3 mRNA levels. Although the pattern of the diet-induced change of sCD163 paralleled that of GDR, there was no correlation between the changes of these two variables.

**Conclusion:** sCD163 correlates with CD163 mRNA expression in sc and visc AT and with whole-body insulin sensitivity in the steady-state condition. These associations are not observed with respect to the diet-induced changes during a weight-reducing hypocaloric diet. ***(J Clin Endocrinol Metab* 99: E528–E535, 2014)**

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Abbreviations: ADAM 17, a disintegrin and metalloproteinase domain 17; AT, adipose tissue; BMI, body mass index; Ct, cycle threshold; HOMA-IR, homeostasis model assessment of insulin resistance; MS, metabolic syndrome; sCD163, soluble CD163; TACE, TNF-α-converting enzyme; T2DM, type 2 diabetes mellitus; TIMP3, metalloproteinase inhibitor 3; VLCD, very low-calorie diet.



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Obesity is associated with systemic and adipose tissue low-grade inflammation that has been postulated to be involved in the development of insulin resistance, type 2 diabetes mellitus (T2DM) and other obesity-related comorbidities (1). This low-grade inflammation is characterized by an increased recruitment of monocytes/macrophages in adipose tissue (AT) (2). Macrophages in AT produce and secrete proinflammatory cytokines, eg, TNF$\alpha$ or IL-6. TNF$\alpha$ is expressed as a membrane-bound protein (3) and is cleaved into a soluble form by TNF-$\alpha$-converting enzyme (TACE) also called as a disintegrin and metalloproteinase domain 17 (ADAM 17) (3). Recently it was shown that TACE/ADAM 17 acts also as a shedding enzyme for haptoglobin-hemoglobin complex receptor CD163 (4, 5). This glycosylated protein expressed predominantly by tissue macrophages may be released from cellular surface as soluble CD163 (sCD163). Thus, circulating sCD163 could be produced by AT macrophages similarly as soluble TNF$\alpha$. sCD163 levels in circulation were found to be increased in obese and T2DM patients (6–8), and its serum levels correlated with body fat mass (6). Moreover, sCD163 was found to be a strong predictor of a risk of the development of T2DM (8, 9). Circulating levels of sCD163 were recently proposed as a biomarker of the whole-body insulin resistance and other indices of metabolic syndrome (MS) (8, 10). Moreover, based on the cellular origin of CD163, Parkner et al (10) hypothesized that circulating sCD163 levels are linked to CD163 expression and macrophage content in AT. However, up until today, no studies investigating this relationship are available.

Therefore, the first aim of this study was to investigate the relationship between serum sCD163 levels and CD163 mRNA levels in both cross-sectional and prospective design. Circulating sCD163 and CD163 mRNA expression in sc and visceral (visc) adipose tissue were determined in a cohort of women with a wide range of body mass index (BMI) and in a cohort of obese women submitted to long-term dietary intervention. In addition, to further examine the production of soluble form of CD163, mRNA expression of enzymes involved in the regulation of CD163 shedding [TACE and tissue inhibitor of metalloproteinase 3 (TIMP3)] was explored during dietary intervention. The second aim of the study was to investigate, in both cohorts, the association between circulating sCD163 and insulin sensitivity when measured by euglycemic-hyperinsulinemic clamp.

## Materials and Methods

### Subjects

Two cohorts of subjects participated in the study.

#### Cohort 1

Forty-two women with a wide range of BMI (aged 21–66 y, BMI 17–49 kg/m$^2$) scheduled to have abdominal surgery (laparoscopic cholecystectomy, hysterectomy, or gastric banding) were included in the study. Exclusion criteria were malignancy, inflammatory conditions, congestive heart failure, chronic liver or kidney disease, psychiatric disorders, and body weight fluctuations greater than 3 kg over the preceding 3 months. According to the BMI, the presence or absence of the MS [evaluated according to the International Diabetes Federation criteria (11)], 42 participants were stratified into three groups [lean (n = 10 subjects), obese (n = 18), and obese with MS (n = 14)].

#### Cohort 2

This cohort consisted of 27 obese premenopausal women (aged 38 ± 1 y; BMI 38 ± 2 kg/m$^2$). Exclusion criteria were malignancy, inflammatory conditions, hypertension, diabetes, hyperlipidemia treated by drugs, weight change of greater than 3 kg within the 3 months prior to the study, drug-treated obesity, pregnancy, participation in other trials, and alcohol or drug abuse. Written informed consent was obtained from all subjects. This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethical Committee of the Third Faculty of Medicine of Charles University in Prague, Czech Republic.

### Study design

#### Study 1 (cohort 1)

Clinical investigation was realized 7–14 days prior to the surgery. Anthropometric measurements, blood sampling, and euglycemic-hyperinsulinemic clamp were performed at rest after an overnight fast. Body composition was evaluated using bioelectrical impedance (QuadScan 4000; Bodystat). Visc and sc fat areas were assessed using computed tomography scans at the level L4–5 (12). The two-dimensional area calculation was used; three slides were taken into consideration. Blood samples were obtained before the clamp and plasma parameters were measured using standard procedures. Insulin sensitivity was assessed using euglycemic hyperinsulinemic clamp according to De Fronzo et al (13). During the surgical procedure, paired samples of sc abdominal and omental visc adipose tissue were obtained and processed immediately. AT was washed in physiological saline, homogenized in RLT lysis buffer (QIAGEN) and stored at −80°C until total RNA extraction.

#### Study 2 (cohort 2)

Participants underwent dietary intervention consisting of a 1-month very low-caloric-diet (VLCD) (800 kcal/d, liquid formula Redita; Promil) and a subsequent weight-stabilization phase. The latter consisted in a 2-month low-caloric diet (600 kcal/d less than the estimated energy requirement) followed by a 3-month weight-maintenance phase. Patients consulted a dietitian once a week during energy restriction (VLCD and low caloric diet) and once a month during the weight-maintenance phase. They provided a written 3-day dietary record at each dietary consultation during the weight stabilization.

Clinical investigation, anthropometric measurements, and blood sampling were performed in the morning at a fasting state at baseline (before the dietary intervention) and at the end of

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VLCD and weight-stabilization phases. Needle biopsy of sc abdominal AT was performed under local anesthesia (1% xylocaine; AstraZeneca PLC) from the abdominal region (14–20 cm lateral to the umbilicus) (14). In 23 women, the euglycemic-hyperinsulinemic clamp was performed according to the method of De Fronzo et al (13). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as fasting blood glucose (millimoles) × fasting insulin (milliunits per liter)/22.5.

### Blood analysis

Plasma levels of glucose, insulin, lipids, and C-reactive protein were determined using standard methods. Serum concentration of sCD163 was determined by a human CD163 quantikine ELISA kit (R&D Systems). The interassay precision of the current assay was 2.63% ± 0.9%, and the limit of detection was 1.55 ng/mL. The TNFα concentration in plasma was analyzed using a high-sensitivity human cytokine Lincoplex kit (Merck-Millipore).

### Quantitative RT-PCR

Total RNA isolation was performed as previously described (14). Genomic DNA was removed by deoxyribonuclease I treatment (Invitrogen). cDNA was obtained by reverse transcription (high capacity cDNA reverse transcription kit; Applied Biosystems). The mRNA expression of CD163, CD68, TNFα, TACE/ADAM17, and TIMP3 was assessed by real-time quantitative PCR on ABI PRISM 7900 or 7500 sequence detection system using custom TaqMan low-density arrays or a TaqMan gene expression assay (Applied Biosystems). The level of expression of the target genes was normalized to glucuronidase beta or peptidylprolyl isomerase A, and fold change of expression was calculated using the ΔΔ-cycle threshold (Ct) method.

### Statistical analysis

#### *Study 1*

To compare plasma and mRNA levels between the three groups of individuals, log-transformed data were analyzed by one-way ANOVA with Bonferroni post hoc analysis using GraphPad Prism version 6.00 for Windows (GraphPad Software). Correlations between the respective variables were assessed using the Pearson's parametric test.

#### *Study 2*

To evaluate the diet-induced evolution of clinical variables and plasma and mRNA levels, data were log transformed and analyzed using ANOVA with repeated measures. Correlations between the diet-induced relative changes of respective variables were assessed using the Pearson's parametric test (GraphPad Software). Data were expressed as means ± SEM. $P < .05$ was considered statistically significant.

## Results

### Study 1: cross-sectional

Clinical and metabolic characteristics of the three groups of subjects (lean, obese, and obese with MS) are shown in Table 1. The glucose disposal rate related to body weight or fat-free mass was lower in both obese and obese with MS, when compared with the lean.

Serum levels of sCD163 were higher in both obese and obese with MS than in the lean group (Figure 1A). The plasma levels of TNFα were not different between the

**Table 1.** Clinical and Metabolic Characteristics of Cohort 1: 42 Women Included in Cross-Sectional Study

| | Lean (n = 10) | Obese (n = 18) | Obese With MS (n = 14) |
|---|---|---|---|
| Age, y | 36 ± 4 | 42 ± 2 | 49 ± 3[a] |
| Weight, kg | 63 ± 2 | 102 ± 4[b] | 92 ± 2[b] |
| BMI, kg/m$^2$ | 22 ± 0.4 | 38 ± 1[b] | 34 ± 1[b] |
| Fat mass, % | 26.5 ± 5.8 | 45.4 ± 1.1[b] | 42.3 ± 1.2[b] |
| Fat-free mass, % | 73.5 ± 1.8 | 54.6 ± 1.1[b] | 57.8 ± 1.2[b] |
| CT visc/total fat | 0.19 ± 0.02 | 0.19 ± 1.2 | 0.27 ± 1.6[c] |
| Waist, cm | 75 ± 1 | 110 ± 3[b] | 107 ± 2[b] |
| Glucose, mM | 4.8 ± 0.1 | 5.4 ± 0.1[c] | 6.3 ± 0.5[c] |
| Insulin, pM | 33 ± 5 | 70 ± 10[c] | 98 ± 10[b] |
| Glycerol, μM | 81 ± 10 | 104 ± 9 | 130 ± 11[c] |
| Triacylglycerol, mM | 0.8 ± 0.04 | 1.2 ± 0.1[c] | 2.5 ± 0.4[c] |
| NEFAs, μM | 526 ± 66 | 635 ± 50 | 763 ± 67[a] |
| HDL-cholesterol, mM | 1.5 ± 0.1 | 1.4 ± 0.1 | 1.2 ± 0.1 |
| Total cholesterol, mM | 4.4 ± 0.2 | 4.6 ± 0.3 | 4.6 ± 0.4[c] |
| HOMA-IR | 1.0 ± 0.1 | 3.4 ± 0.9[b] | 4.0 ± 0.6[b] |
| $GDR_{kg}$, mg/kg·min | 7.0 ± 0.7 | 3.6 ± 0.4[b] | 2.8 ± 0.3[b] |
| $GDR_{FFM}$, mg/kg·min | 9.6 ± 1.0 | 6.7 ± 0.7[a] | 4.9 ± 0.5[b] |

Abbreviations: CT, computed tomography; $GDR_{FFM}$, glucose disposal rate related to fat-free mass; $GDR_{kg}$, glucose disposal rate related to body weight; HDL, high-density lipoprotein; MS, metabolic syndrome; NEFA, nonesterified free fatty acid.

[a] $P < .05$ compared with lean.

[b] $P < .001$ compared with lean.

[c] $P < .01$ compared with lean.

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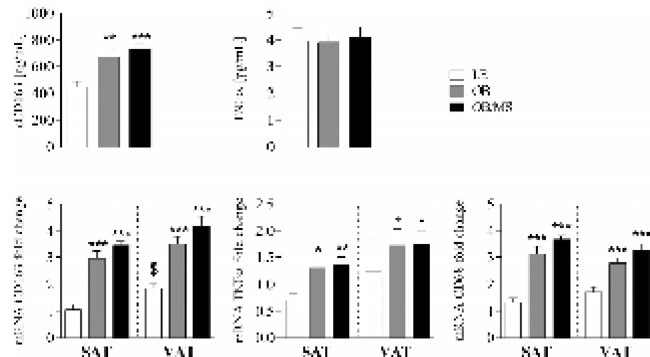


**Figure 1.** Study 1 (cross-sectional study): plasma/serum (A) and mRNA (B) levels of CD163, CD68, and TNF$\alpha$ of lean (LE; n = 10), obese (OB; n = 18), and obese patients with MS (OB/MS; n = 14). mRNA expression in sc (SAT) and visc (VAT) AT was normalized to GUSB, and fold change of expression was calculated using the $\Delta\Delta$Ct method. Values are means ± SEM. **, $P <$ .01 compared with lean; ***, $P <$ .001 compared with lean; $, $P <$ .05 compared with SAT.

groups. CD163, CD68, and TNF$\alpha$ mRNA levels were higher in obese and obese with MS subjects than in lean in both fat depots (Figure 1B).

### Correlations

Circulating sCD163 levels correlated positively with CD163 mRNA and also with CD68 mRNA expression in both visc and sc AT (Table 2 and Supplemental Figure 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). CD68 mRNA correlated with CD163 mRNA levels in both fat depots (Table 2). Interestingly, when adjusted to BMI, the correlation between sCD163 levels and mRNA expression in sc AT remained significant (r = 0.44; $P$ = .004), whereas the correlation between sCD163 and mRNA expression in visc AT disappeared (r = 0.23; $P$ = .8). Positive correlations were found between both sCD163 and CD163 mRNA expression in AT, and with most of the variables related to the amount of body fat mass, but not with the amount of visc AT (Table 2).

Importantly, inverse correlations were found between both sCD163 and CD163 mRNA levels in AT, and glucose disposal rate related either to the body weight or fat-free mass (Table 2 and Supplemental Figure 1).

### Study 2: dietary intervention

Clinical and metabolic characteristics of subjects in cohort 2 before and during dietary intervention are summa-

**Table 2.** Correlations Between sCD163 or CD163 mRNA Expression in Subcutaneous Abdominal and Visceral Adipose Tissues With Respect to Clinical Parameters in a Group of 42 Lean, Obese, and Obese With MS Women

| | sCD163 | CD163 mRNA Subcutaneous AT | CD163 mRNA Visceral AT |
|---|---|---|---|
| Weight, kg | 0.367 | **0.452**[a] | **0.598**[c] |
| BMI, kg/m$^2$ | **0.458**[a] | **0.578**[a] | **0.617**[a] |
| Fat mass, % | **0.460**[a] | **0.590**[a] | **0.593**[a] |
| Waist, cm | **0.467**[a] | **0.589**[b] | **0.618**[c] |
| CT % visc AT in total | 0.115 | 0.279 | 0.252 |
| Glucose, mM | 0.352 | 0.339 | **0.427**[a] |
| Insulin, pM | **0.454**[a] | **0.515**[a] | **0.434**[a] |
| NEFAs, $\mu$M | 0.213 | 0.279 | **0.453**[a] |
| HDL-cholesterol, mM | −0.283 | −0.339 | −0.324 |
| Triacylglycerol, mM | 0.305 | **0.409**[a] | **0.490**[a] |
| HOMA-IR | 0.314 | 0.370 | **0.405**[a] |
| $GDR_{kg}$, mg/kg·min | **−0.433**[a] | **−0.597**[b] | **−0.575**[b] |
| sCD163, ng/mL | | **0.586**[b] | **0.420**[a] |
| CD163 mRNA (SAT) | **0.586**[b] | | **0.575**[a] |
| CD163 mRNA (VAT) | **0.420**[a] | **0.575**[a] | |
| CD68 mRNA (SAT) | **0.543**[b] | **0.909**[b] | **0.574**[b] |
| CD68 mRNA (VAT) | 0.331 | **0.485**[b] | **0.917**[b] |
| TNF$\alpha$, pg/mL | 0.393 | −0.003 | 0.142 |
| TNF$\alpha$ mRNA (SAT) | 0.221 | **0.668**[b] | 0.365 |
| TNF$\alpha$ mRNA (VAT) | **0.463**[a] | **0.430**[a] | 0.255 |

Abbreviations: CT, computed tomography; $GDR_{kg}$, glucose disposal rate corrected for body weight; HDL, high-density lipoprotein; NEFA, nonesterified free fatty acid; SAT, sc AT; VAT, visc AT. Data are presented as Pearson's correlation coefficient. Bold represents statistically significant correlations.

[a] $P \leq .01$.

[b] $P \leq .001$.

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**Table 3.** Clinical Characteristics of Cohort 2: 27 Obese Women Before the Diet and at the End of VLCD and Weight Stabilization Phase of a 6 Months' Dietary Intervention

| | Basal | After VLCD | After WS |
|---|---|---|---|
| Age, y | 38 ± 1 | | |
| Weight, kg | 96 ± 2 | 88 ± 2[a] | 85 ± 2[a] |
| BMI, kg/m$^2$ | 35 ± 1 | 32 ± 1[a] | 31 ± 1[a] |
| Fat mass, kg | 40.6 ± 1.6 | 35.3 ± 1.5[a] | 32.3 ± 1.6[a] |
| Fat-free mass, kg | 55.5 ± 0.9 | 53.0 ± 0.8[a] | 52.6 ± 0.8[a] |
| Waist, cm | 103 ± 2 | 97 ± 2[a] | 95 ± 2[a] |
| Glucose, mM | 5.2 ± 0.1 | 4.9 ± 0.1[b] | 4.9 ± 0.1[b] |
| Insulin, pM | 87 ± 9 | 57 ± 5[a] | 56 ± 6[b] |
| Glycerol, μM | 170 ± 14 | 130 ± 10[b] | 135 ± 14 |
| Triacylglycerol, mM | 1.47 ± 0.1 | 1.19 ± 0.1[c] | 1.28 ± 0.1 |
| NEFAs, μM | 767 ± 45 | 968 ± 76[c] | 644 ± 41[c] |
| HDL-cholesterol, mM | 2.2 ± 0.3 | 1.1 ± 0.1[b] | 1.4 ± 0.1[c] |
| Total cholesterol, mM | 5.1 ± 0.2 | 4.2 ± 0.2[a] | 5.0 ± 0.2 |
| HOMA-IR | 2.9 ± 0.3 | 1.8 ± 0.2[a] | 1.8 ± 0.2[b] |
| $GDR_{kg}$, mg/kg·min | 3.0 ± 0.3 | 3.6 ± 0.4[c] | 4.1 ± 0.4[a] |
| $GDR_{FFM}$, mg/kg·min | 4.9 ± 0.5 | 5.7 ± 0.5[c] | 6.4 ± 0.5[b] |

Abbreviations: $GDR_{kg}$, glucose disposal rate corrected for body weight; HDL, high-density lipoprotein; NEFA, nonesterified free fatty acid; WS, weight stabilization. Data are presented as mean ± SEM. Glucose disposal rate was measured in a subgroup of 23 subjects.

[a] $P < .001$ when compared with baseline (prediet) values (ANOVA)

[b] $P < .01$ when compared with baseline (prediet) values (ANOVA).

[c] $P < .05$ when compared with baseline (prediet) values (ANOVA).

rized in Table 3. The body weight was decreased by 11.3% at the end of dietary intervention. The whole-body insulin sensitivity improved as measured by HOMA-IR or glucose disposal rate. The AT sensitivity to antilipolytic effect of insulin (measured as a decrease of plasma free fatty acids levels during hyperinsulinemic clamp) was not changed during the diet (data not shown).

Dietary intervention induced a decrease of sCD163 serum concentration at the end of the VLCD and weight stabilization when compared with the baseline values (Figure 2A). Plasma levels of TNFα were higher than baseline at the end of VLCD and decreased to the baseline levels at the end of weight stabilization (Figure 2A).

mRNA levels of CD163 and CD68 were higher at the end of VLCD when compared with the baseline and both of them decreased to the levels not different from baseline at the end of weight stabilization (Figure 2B). TNFα and TACE mRNA expression did not change significantly throughout the entire dietary intervention, whereas that of TIMP3 increased above the baseline at the end of weight stabilization (Figure 2B).

## Correlations

At baseline, serum sCD163 correlated positively with CD163 and CD68 mRNA in sc AT ($r = 0.579$, $P < .001$; $r = 0.383$, $P < .05$), similarly to Study 1. However, the diet-induced change of circulating sCD163 did not correlate with the CD163 or CD68 mRNA levels either during VLCD or during the entire dietary intervention (Table 4). The diet-induced changes of CD163 mRNA expression correlated with the mRNA changes of CD68, TNFα, TIMP3, or TACE (Table 4).

In addition, despite the fact that baseline values of sCD163 and CD163 mRNA correlated inversely with glucose disposal rate ($r = -0.625$, $P < .01$; $r = -0.845$, $P < .01$, respectively), the diet-induced changes of sCD163 and CD163 mRNA during the VLCD or during the entire dietary intervention did not correlate with the changes of insulin sensitivity (Table 4).

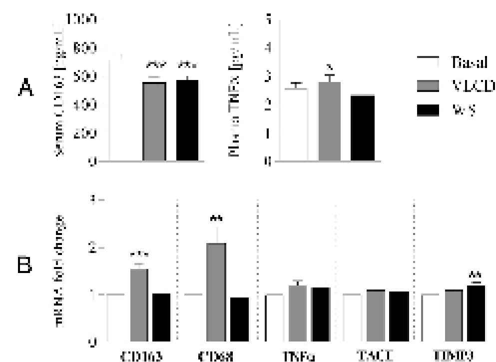


**Figure 2.** Study 2 (dietary intervention): plasma/serum levels (A) of CD163 and TNFα and mRNA levels (B) of CD163, CD68, TNFα, TACE, and TIMP3 in sc AT. mRNA expression was normalized to peptidylprolyl isomerase A, and fold change of expression was calculated using the ΔΔCt method. Data are means ± SEM (n = 27). **, $P < .01$ compared with baseline (before the diet); ***, $P < .001$ compared with baseline (before the diet).

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**Table 4.** Correlations (Pearson's) Between Relative Changes of sCD163 or CD163 mRNA Expression in Subcutaneous Abdominal AT With Relevant Changes of Glucose Disposal Rate, Circulating sCD163, and TNFα and mRNA Expression of sCD163, CD68, TNFα, TACE, and TIMP3

| | VLCD | | WS | |
|---|---|---|---|---|
| | **sCD163** | **CD163 mRNA** | **sCD163** | **CD163 mRNA** |
| sCD163 | | 0.16 | | 0.080 |
| CD163 mRNA | 0.160 | | 0.080 | |
| TNFα | −0.165 | | −0.005 | |
| TNFα mRNA | | **0.677**[a] | | 0.364 |
| CD68 mRNA | 0.038 | **0.827**[b] | −0.019 | **0.763**[b] |
| TACE mRNA | | **0.746**[a] | | 0.449 |
| TIMP mRNA | | **0.603**[a] | | **0.522**[a] |
| $GDR_{kg}$ | −0.393 | −0.37 | −0.22 | −0.22 |

Abbreviation: $GDR_{kg}$, glucose disposal rate corrected for body weight; WS, weight stabilization. Data are presented as Pearson's correlation coefficient. Bold indicates statistically significant correlations.

[a] $P \leq .01$.

[b] $P \leq .001$.

The diet-induced changes of CD163 mRNA levels also did not correlate with the changes of body weight, BMI, or fat mass (data not shown).

## Discussion

The aim of this study was to elucidate whether the concentration of sCD163 in blood reflects the CD163 mRNA expression in adipose tissue and whether sCD163 plasma concentrations and AT mRNA levels are associated with insulin sensitivity. In a cohort of women with a wide range of BMI and insulin sensitivity, we found a positive correlation between mRNA expressions of CD163 in both sc and visc AT, in respect to circulating sCD163 levels. Thus, we bring an evidence for a previous suggestion of a link between circulating sCD163 and CD163 expression in sc AT (10, 15), and, in addition, we find the same link with respect to CD163 expression in visc AT. Moreover, the correlation of sCD163 levels with mRNA expression of macrophage marker CD68 suggests that serum sCD163 might be perceived as a possible indicator of macrophage activation in AT. Interestingly, the correlation of sCD163 with CD163 mRNA expression in visc AT disappeared after adjustment to BMI, whereas the correlation with expression in sc AT remained significant. This finding may suggest that sc AT is a more important contributor to sCD163 when compared with visc fat.

Next, we documented a strong relationship between insulin sensitivity, measured by the gold standard method of euglycemic hyperinsulinemic clamp, and circulating sCD163. These results extend the previously reported findings of a close relationship between sCD163 and HOMA-IR (8, 10). In this study, insulin sensitivity correlated also with CD163 mRNA expression in both fat depots. Thus, we demonstrate the capacity of sCD163 as a biomarker of insulin sensitivity measured by the clamp method at steady-state condition.

However, in a dynamic condition represented by the weight-reducing hypocaloric diet, the above-mentioned associations were not present: the diet-induced change of sCD163 showed different pattern and did not correlate with the change of CD163 mRNA levels in sc AT either during the initial dynamic phase of the dietary intervention (VLCD) or during the phase of the weight stabilization. Indeed, although the sCD163 serum levels were lower than baseline at the end of both phases of the diet, the mRNA levels increased during VLCD and then decreased to the baseline at the end of the weight stabilization. The same pattern of evolution was found for the other macrophage marker CD68. This time course is in agreement with our previous results showing the diet-induced responses of macrophage markers in adipose tissue during multiphase dietary intervention (14, 16, 17). It is to be noted that CD163 is considered as M2 macrophage marker, similarly as lymphatic vessel endothelial receptor 1, which expression during dietary intervention exhibited the same pattern (data not shown). The increased expression of these markers after strong caloric restriction could be associated with active remodeling of AT during this intervention (18). However, in humans, the phenotypic diversity of AT macrophages is now known to include more than two types of macrophage states (19).

The discrepancy between the dynamics of CD163 in circulation and in AT could be caused by diet-induced adaptations at the translational or posttranslational level of the specific protein. Among factors influencing sCD163 production could be the efficiency of shedding of the CD163 molecule from the macrophage surface. Both CD163 and TNFα are shed from the cellular surface by TACE that is inhibited by the metalloproteinase inhibitor TIMP3 (20). However, no relevant change of TACE mRNA expression in AT throughout the dietary intervention was found, and the TIMP3 mRNA level slightly increased at the end of weight stabilization, ie, later than the decrease of sCD163 levels occurred. Therefore, the changes in the shedding of CD163 in AT during dietary intervention probably do not contribute to the changes of sCD163 in circulation.

The difference between the serum and expression of CD163 by AT macrophages during diet might be therefore related to contributions of other tissues to circulating sCD163. CD163 is expressed in macrophages resident in

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many nonadipose tissues, eg, liver, muscle, kidney (21–24), and also in blood monocytes (25). Unfortunately, to our knowledge, there are no studies that evaluate direct contribution of other tissues to circulating levels of sCD163 or investigating CD163 expression in other tissues or cells during dietary intervention.

In this study the evolution of sCD163 paralleled that of the glucose disposal rate when measured using a hyperinsulinemic clamp during weight-reducing dietary intervention. However, the direct correlations between the diet-induced changes of sCD163 and those of glucose disposal rate were not found. Similarly, no correlation was found between the diet-induced changes of CD163 mRNA expression and insulin sensitivity. Consequently, we conclude that, in a dynamic condition of a weight-reducing hypocaloric diet, the change of sCD163 in circulation and CD163 mRNA expression in sc AT is not a quantitative biomarker of the change of the whole-body insulin sensitivity. These findings suggest that circulating levels of sCD163 and AT mRNA expression of CD163 are probably not in a cause-effect relationship with insulin sensitivity. But we believe that it does not invalidate the assumption that sCD163 could be used as a biomarker of insulin resistance under steady-state conditions.

As mentioned above, the variations in CD163 mRNA expression in AT measured in this study reflect the macrophage content in adipose tissue as well as the very expression of CD163 in each of the macrophages resident in AT. Thus, no direct conclusions about the relationship between sCD163 and macrophage content in AT may be made based on this study. However, it is to be noted that the pattern of evolution of CD163 mRNA expression in AT in this study was similar to the pattern of macrophage marker CD68 and to other macrophage markers observed during the same multiphase dietary intervention in our recent study (14).

In conclusion, in this study we demonstrated a quantitative association between the circulating levels of sCD163 and mRNA expression of macrophage markers CD163 and CD68 in sc and visc adipose tissue in the steady-state condition. Furthermore, in the steady-state condition, we found a negative correlation between sCD163 levels and insulin sensitivity as assessed using a hyperinsulinemic euglycemic clamp. However, in a dynamic condition represented by a weight-reducing hypocaloric diet, there is no such relationship between the diet-induced changes of the above-mentioned variables. Thus, there is no evidence that sCD163 might be used as a quantitative biomarker of the diet-induced changes of AT CD163 expression or changes of insulin sensitivity. Therefore, further research of the translational or post-translational processes involved in sCD163 production during diet and other physiological stimuli is warranted.

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**Paper 6**

Lenka Rossmeislová, Lucia Mališová, Jana Kračmerová, Michaela Tencerová,
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Dominique Langin and Vladimír Štich

**Weight loss improves the adipogenic capacity of human preadipocytes and modulates their secretory profile**

Diabetes, 2013, June, 62(6):1990-1995.

# Weight Loss Improves the Adipogenic Capacity of Human Preadipocytes and Modulates Their Secretory Profile

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Calorie restriction–induced weight loss is accompanied by profound changes in adipose tissue characteristics. To determine the effect of weight loss on differentiation of preadipocytes and secretory capacity of in vitro differentiated adipocytes, we established cultures of these cells from paired subcutaneous adipose tissue biopsies obtained before and at the end of weight-reducing dietary intervention (DI) in 23 obese women. Based on lipid accumulation and the expression of differentiation markers, in vitro adipogenesis increased after weight loss and it was accompanied by enhanced expression of genes involved in de novo lipogenesis. This effect of weight loss was not driven by changes of peroxisome proliferator–activated receptor γ sensitivity to rosiglitazone. Weight loss also enhanced the expression of adiponectin and leptin while reducing that of monocyte chemoattractant protein 1 and interleukin-8 by cultured adipocytes. Thus, the weight-reducing (DI) increased adipogenic capacity of preadipocytes and shifted their secretion toward lower inflammatory profile. Reprogramming of preadipocytes could represent an adaptation to weight loss leading to partial restoration of preobese adipose tissue traits and thus contribute to the improvement of metabolic status. However, enhanced adipogenesis could also contribute to the unwanted weight regain after initial weight loss. *Diabetes* **62:1990–1995, 2013**

Worsening of metabolic health in obesity is associated with the hypertrophy of adipocytes (1). Indeed, the recruitment of new and small adipocytes improves insulin sensitivity (2). These cells have a high potential to store lipids and therefore alleviate peripheral lipotoxicity associated with whole-body insulin resistance. However, adipose stromavascular cells derived from obese donors exhibit impaired adipogenic capacity (3), and the factors influencing sensitivity of human preadipocytes to adipogenic stimuli in vivo remain unknown.

Weight reduction induced by hypocaloric diet is the key approach for treatment of obesity-related metabolic disturbances. A moderate loss of body weight induces an adaptation of human adipose tissue associated with improved whole-body metabolic status (4,5). We hypothesized that cell cultures of preadipocytes established from subcutaneous adipose tissue collected before and after a weight loss–inducing dietary intervention (DI) correspond to two distinct metabolic and nutritional stages of the donor. The current knowledge on intrinsic adipogenic and endocrine potential of these cells is based on and limited to cross-sectional studies. Here, we show that DI-induced weight loss increased the differentiation capacity of preadipocytes and shifted their secretion toward less inflammatory profile. This reprogramming of preadipocytes by weight loss could represent a cellular mechanism leading to the restoration of preobese traits of adipose tissue and correction of inflammatory status.

## RESEARCH DESIGN AND METHODS

**Subjects.** Obese premenopausal women ($n = 23$) were recruited at the Third Faculty of Medicine of Charles University and University Hospital Kralovske Vinohrady, Prague, Czech Republic. Exclusion criteria were set as previously described (6). The study was performed according to the Declaration of Helsinki and was approved by the ethics committee of the Third Faculty of Medicine of Charles University. Volunteers signed informed consent before participation in the study.

**DI and clinical investigation.** The DI lasted 5–6 months. Participants reduced their calorie intake by 600 kcal/day in relation to the individually estimated energy requirement (initial resting metabolic rate multiplied by 1.3, the coefficient of correction for physical activity). Weight loss was achieved within the first 3 months, and then women were advised to keep the diet leading to the weight maintenance. Subjects consulted a dietitian once a week during the first 3 months and once a month during the weight-maintenance phase.

Clinical investigation was performed after an overnight fast before and at the end of DI. Anthropometric measurements, blood sampling, and needle biopsy of adipose tissue were performed as previously described (6). Briefly, after administration of local anesthesia (1% xylocaine), a 1- to 2-mm incision was made 10 cm laterally from umbilicus and a 12G needle coupled with syringe was used to aspirate fragments of superficial subcutaneous adipose tissue. On average, 1.5 g tissue was obtained (0.6−2.5 g).

**Isolation and culture of preadipocytes.** Adipose tissue was digested in 1.5 volume of collagenase I (300 units/mL; Biochrom, Berlin, Germany) for 60 min in 37°C shaking water bath and processed as previously described (7). Digested tissue was diluted with PBS/gentamicin and spun at 1,300 rpm for 5 min. Cells were then shaken forcefully to complete the dissociation from mature adipocytes and centrifuged. Pellet containing cells from the stromavascular fraction was incubated in erythrocyte lysis buffer for 10 min at room temperature. Cells were centrifuged, and without any filtration step, they were resuspended in PM4 medium (8) with 132 nmol/L insulin. PM4 was replaced every other day. Cells were subcultivated at 70% confluence; experiments were performed at passage 3. Differentiation of 2-day postconfluent cells was induced by Dulbecco's modified Eagle's/F12 medium supplemented with 66 nmol/L insulin, 1 μmol/L dexamethasone, 1 nmol/L T3, 0.1 μg/mL transferrin, 0.25 mmol/L isobutylmethylxanthine, and 1 μmol/L rosiglitazone. After 6 days, rosiglitazone and isobutylmethylxanthine were omitted and dexamethasone was replaced with 0.1 μmol/L cortisol. The differentiation continued until day 12. Medium conditioned for 24 h was then collected, and cells were harvested for RNA and protein analysis. Protein concentration was determined by BCA assay (Pierce Biotechnology, Rockford, IL). For experiments focused on the

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effect of peroxisome proliferator–activated receptor (PPAR)γ activation, cells were induced to differentiate in the media containing either 1 μmol/L rosiglitazone or DMSO. As nondifferentiating controls, preadipocytes switched to serum-free medium supplemented with transferrin and insulin were used.

**Gene expression analysis.** Total RNA was isolated using an RNeasy Mini kit (Qiagen, Hilden, Germany). mRNA levels were measured using reverse transcription quantitative PCR (Applied Biosystems, Carlsbad, CA) (9). GUSB (glucuronidase beta) was used as an endogenous control. Results are expressed as $\Delta\Delta C_t$ (threshold cycle) values.

**Cytokine analysis.** Cytokines were measured by ELISA (interleukin [IL]-6 and monocyte chemoattractant protein [MCP]1, Ready-Go-sets, eBioscience, San Diego, CA; IL6 Quantikine HS, R&D Systems, Abingdon, U.K.; and adiponectin DuoSet, R&D Systems, Minneapolis, MN). Detection of adiponectin isoforms was performed by native polyacrylamide gel electrophoresis and Western blot (10). Chemiluminescent signal was detected on Kodak Image Station 4000R and analyzed by associated software.

**Oil Red O and BODIPY staining.** Cells were fixed, stained with Oil Red O (ORO), and analyzed as previously described (11) or stained with 1 μmol/L BODIPY 493/503 (Life Technologies) and DAPI. Standard curve from ORO stock was used to normalize data. The OD of eluates from 100% differentiated cells reached values of 40% of stock ORO. Images for BODIPY analysis were acquired on fluorescent microscope DMI6000 coupled with CCD camera (Leica Microsystems, Wetzlar, Germany).

**Statistical analysis.** Data were analyzed using GraphPad Prism 5.0 software with Wilcoxon matched-pair signed rank or Mann-Whitney *U* test, as appropriate. The level of significance was set at $P < 0.05$.

## RESULTS

**Clinical characteristics of obese subjects.** The clinical data of subjects before and after DI are listed in Table 1. Compared with baseline values, the subjects' body weight decreased by 9.7% and insulin resistance assessed by homeostasis model assessment of insulin resistance was reduced.

**Preadipocytes derived from adipose tissue after weight loss exhibit increased adipogenesis.** The stromavascular cells from adipose tissue samples were isolated, expanded, and differentiated into adipocytes. Cell cultures derived from the samples obtained after weight loss exhibited increased differentiation as evaluated by the ORO staining and mRNA expression of differentiation markers *aP2* and *PPARγ* (Fig. 1*A* and *B*). Moreover, the expression of stearoyl-CoA desaturase 1 (*SCD1*), diacylglycerol *O*-acyltransferase 2 (*DGAT2*), fatty acid synthase (*FASN*),

TABLE 1
Clinical characteristics of the subjects before and after dietary intervention

| | Baseline | DI | *P* |
|---|---|---|---|
| Age (years) | 40.7 ± 1.79 | | |
| Weight (kg) | 91.49 ± 2.12 | 82.5 ± 1.93 | **<0.001** |
| BMI (kg/m$^2$) | 32.97 ± 0.91 | 29.71 ± 0.82 | **<0.001** |
| Fat mass (%) | 39.71 ± 1.1 | 36.47 ± 1.11 | **<0.001** |
| Waist (cm) | 102.6 ± 2.24 | 93.04 ± 2.13 | **<0.001** |
| WHR | 0.86 ± 0.02 | 0.84 ± 0.02 | **0.011** |
| Glucose (mmol/L) | 5.42 ± 0.11 | 5.06 ± 0.13 | **0.012** |
| Insulin (mIU/L) | 9.62 ± 0.97 | 7.27 ± 0.92 | **0.002** |
| HOMA-IR | 2.36 ± 0.27 | 1.71 ± 0.27 | **0.002** |
| Total cholesterol (mmol/L) | 5.38 ± 0.27 | 4.63 ± 0.18 | **0.006** |
| HDL-C (mmol/L) | 1.66 ± 0.09 | 1.44 ± 0.07 | **0.007** |
| Triglycerides (mmol/L) | 1.19 ± 0.09 | 0.78 ± 0.05 | **<0.001** |
| IL-6 (pg/mL) | 0.86 ± 0.1 | 0.82 ± 0.09 | 0.381 |
| MCP1 (pg/mL) | 25.61 ± 2.72 | 23.73 ± 2.82 | 0.075 |
| Adiponectin (μg/mL) | 1.89 ± 0.12 | 1.92 ± 0.14 | 0.721 |

Data are means ± SEM. $n$ = 23. HDL-C, HDL cholesterol; HOMA-IR, homeostasis model assessment of the insulin resistance index; WHR, waist-to-hip ratio. *P* values in bold reached the level of significance.

ATP citrate lyase (*ACLY*), *ChREBPα*, and *GLUT4* was also upregulated, confirming higher capacity of cells for lipogenesis (Fig. 1*B*). Although mRNA levels of markers of mitochondrial biogenesis nuclear respiratory factor 1 and PPARγ coactivator 1α were not altered, *UCP1* expression was notably upregulated (Fig. 1*B*). Interestingly, cell differentiation into adipocytes was associated with an increase in total protein content that was more pronounced after weight loss (Fig. 1*C*). The ratio between protein content in adipocytes versus preadipocytes correlated with the degree of differentiation measured by ORO (Fig. 1*D*). Notably, there was a positive link between changes in protein content and mRNA levels of differentiation markers, lipogenic genes, and *UCP1* (Fig. 1*E* and Supplementary Table 1). The enhancement of adipogenesis was not caused by the alteration of proliferative capacity of preadipocytes, since there was no difference in the yield of the cells at passage 3 and length of cultivation period preceding the experiments (Supplementary Table 2).

To decipher the putative role of PPARγ in the reprogramming of preadipocytes induced by weight loss, preadipocytes were stimulated with differentiation medium supplemented either with DMSO (control) or 1 μmol/L rosiglitazone (PPARγ ligand). As expected, rosiglitazone enhanced markedly the expression of *FASN*, *SCD1*, and *aP2* compared with control cells (Fig. 2*A*); however, the upregulation of lipogenic markers *FASN* and *SCD1* in cells derived after the DI was more pronounced in the absence of rosiglitazone (Fig. 2*A*). Furthermore, the ratio of FASN and SCD1 expression under rosiglitazone versus control treatment was not different between the cells obtained before and after the DI (Fig. 2*B*). In addition, the percentage of cells accumulating neutral lipids after 6 days of differentiation in the presence or absence of PPARγ ligand was in both cases higher after weight loss (Fig. 2*C*). Again, the ratio between the numbers of cells differentiated in the presence of rosiglitazone versus DMSO was unchanged after the DI (not shown). These data suggest that weight loss did not alter the sensitivity of cells to rosiglitazone and, rather, affected pathways upstream of PPARγ. Indeed, the expression of *PPARγ* itself as well as the expression of *KLF9* (Kruppel-like factor 9), the transcription factors that regulate PPARγ transcription, was not different in preadipocytes derived before or after weight loss (Fig. 2*D*). However, cells derived after weight loss exhibited a marked downregulation of expression of runt-related transcription factor 2 (*RUNX2*), the transcription factor favoring osteogenic differentiation (12), both prior to and during differentiation (Fig. 2*D*).

**Expression and secretion of cytokines by in vitro differentiated preadipocytes are altered after weight loss.** For determination of whether weight loss affects the intrinsic secretory potential of adipocytes, the secretion and mRNA expression of several cytokines were measured in in vitro differentiated preadipocytes derived from adipose tissue before and after weight loss. Both expression and secretion of adiponectin and its high–molecular weight form were higher in adipocytes after DI compared with baseline (Fig. 3). However, when the secretion of total adiponectin was adjusted to the degree of differentiation assessed by ORO, the effect of DI was lost, suggesting a close relationship between adiponectin secretion and the differentiation state of adipocytes (not shown). Leptin mRNA levels were also elevated in adipocytes after weight loss (Fig. 3*A*), and this change was not related to the degree of differentiation. In contrast, *MCP1* and *IL-8* mRNA levels

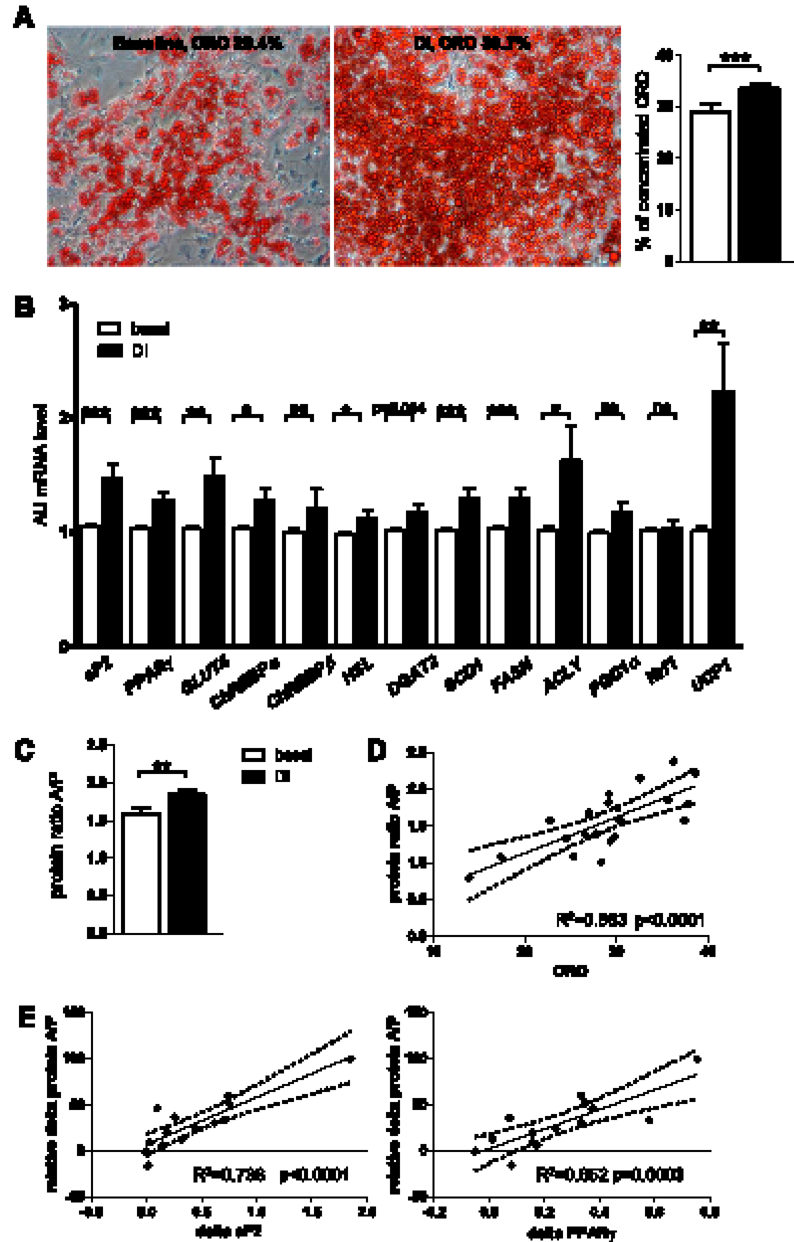


FIG. 1. Weight loss improves in vitro adipogenesis. Cells were differentiated for 12 days, and then accumulation of lipids, protein content, or gene expression was analyzed. *A*: Effect of weight loss on lipid accumulation. Representative images of adipocytes from one donor before and after DI stained with ORO and quantification of neutral lipid accumulation expressed as percent of stock ORO ($n = 22$). *B*: Effect of weight loss on gene expression. mRNA expression (arbitrary units [AU]) in adipocytes normalized to *GUSB* expression ($n = 15$). *C*: Effect of weight loss on protein content. Ratio between total protein content in adipocytes vs. preadipocytes ($n = 22$). Data are means ± SE; **P* < 0.05, ***P* < 0.01, ****P* < 0.001. *D*: Linear regression between ORO accumulation and protein content in adipocytes (A) vs. preadipocytes (P) at baseline. *E*: Linear regression between relative Δprotein (adipocytes vs. protein) and ΔmRNA expression of *aP2* and *PPARγ*.

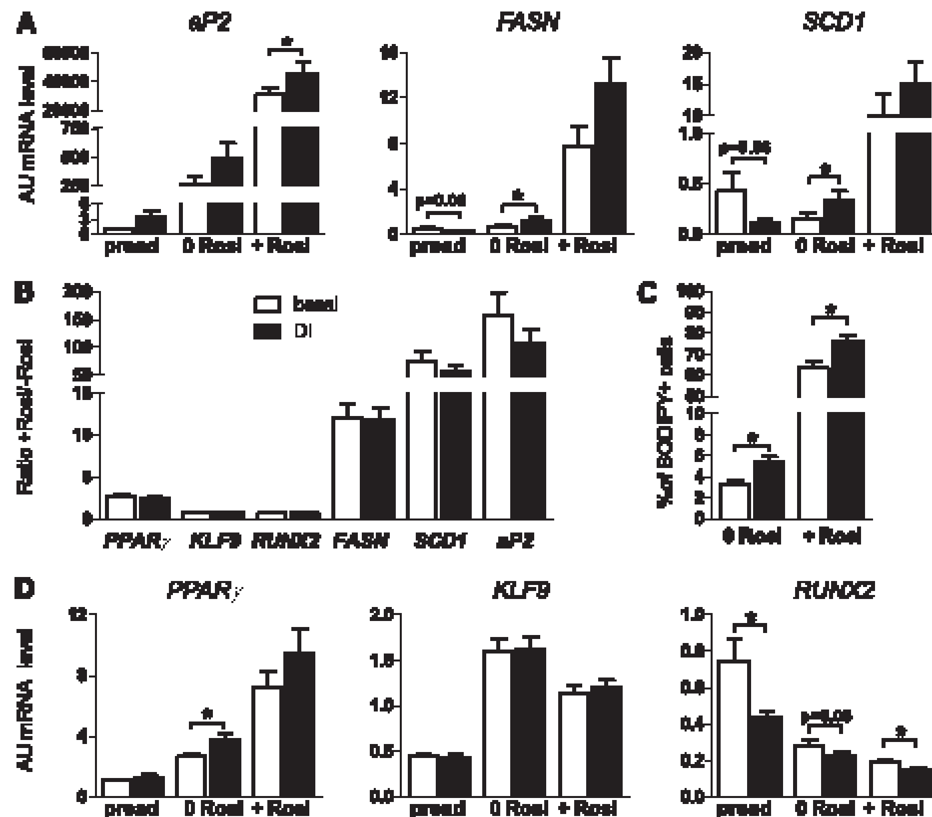


**FIG. 2. Weight loss does not enhance the sensitivity to PPARγ ligand rosiglitazone (Rosi) but is connected with the suppression of *RUNX2* expression. *A* and *B*: Cells were differentiated for 3 days in the presence or absence of 1 mmol/L rosiglitazone. Cells kept in serum-free medium supplemented with transferrin and insulin that did not undergo adipogenesis were used as controls. *A*: Relative mRNA levels of selected genes were detected by quantitative RT-PCR ($n = 6$). □, Baseline; ■, DI. Data are means ± SE; *$P < 0.05$. *B*: The ratio between expression of selected genes in cells differentiated in the presence and absence of rosiglitazone was calculated in cells derived before and after the DI. *C*: Cells were differentiated for 6 days in the presence or absence of 1 mmol/L rosiglitazone ($n = 4$). After staining with BODIPY, the 45 microscopy images encompassing an average of 2,700 cells were analyzed and numbers of BODIPY⁺ cells were counted. Chart represents the percentage of BODIPY⁺ cells within analyzed populations (each on average from 2,700 cells). Data are means ± SE; *$P < 0.05$. AU, arbitrary units; pread, preadipocytes.**

in adipocytes obtained after DI were reduced compared with baseline (Fig. 3*A*). Secretion of MCP1 was lower (Fig. 3*B*) compared with baseline even after adjustments to the degree of differentiation (not shown), but no significant changes in secretion or expression of IL-6 were observed (Fig. 3*A* and *B*).

## DISCUSSION

It has been hypothesized that worsening of metabolic health in obesity is related to dysfunction of hypertrophic adipocytes or diminished ability of adipose tissue to react to energetic surplus by the enhanced adipogenesis from available precursors. The latter is evidenced by *1*) insulin-resistant subjects exhibiting lower expression of adipogenic genes (13) and *2*) the insulin-sensitizing drugs thiazolidinediones alleviating insulin resistance by the recruitment of new adipocytes with a high potential to store lipids (14,15).

In this study using cells derived from paired subcutaneous adipose tissue biopsies from obese women undergoing long-term DI, we showed that adipogenic potential of preadipocytes was increased by moderate weight loss. Obesity was shown to be associated with lower differentiation capacity of preadipocytes (3,16). Our data obtained in the prospective study therefore not only are in agreement with the cross-sectional observations but also provide evidence that the lowering of adipose tissue mass is associated with higher preadipocyte differentiation capacity and sensitivity to adipogenic stimuli. This implies that the effect of weight loss on adipose tissue function should be ascribed not only to changes in size and metabolism of mature adipocytes and in proinflammatory potential/numbers of infiltrated immune cells (6,17) but also to reprogramming of preadipocytes. Lower *RUNX2* expression in cells derived after weight loss suggests that weight loss inhibits alternative lineage programs

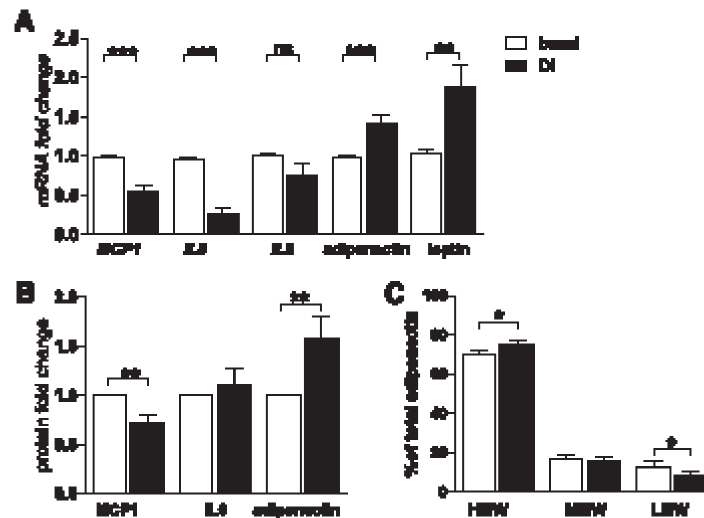


FIG. 3. Weight loss alters the expression and secretion of cytokines in adipocytes differentiated in vitro. Cells were differentiated for 12 days, and conditioned media and cells were collected after 24 h of incubation in freshly added media for analysis of cytokine expression and secretion. *A*: Effect of weight loss on cytokine gene expression. Quantitative RT-PCR analysis of mRNA for selected cytokines, normalized to *GUSB* expression ($n = 15$). *B*: Effect of weight loss on cytokine secretion. Fold change over the basal values for MCP1, IL-6, and adiponectin in conditioned media measured by ELISA, normalized to protein content ($n = 22$). *C*: Effect of weight loss on adiponectin isoform secretion. Quantification of adiponectin isoforms by native polyacrylamide gel electrophoresis and Western blot analysis ($n = 21$). Data are means ± SE; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. HMW, high molecular weight; LMW, low molecular weight, MMW, medium molecular weight.

(e.g., osteogenesis), which in turn favors the adipogenic differentiation.

Nevertheless, the impact of in vivo changes of preadipocytes, which are important for the maintenance or development of AT (17), remains unknown. It is tempting to speculate that upon fat mass reduction, a higher sensitivity of precursor cells to adipogenic stimuli could enhance fatty acid storage and therefore indirectly lower lipotoxicity at the whole-body level while improving insulin sensitivity. On the other hand, increased adipogenesis after previous weight loss could compromise long-term weight loss maintenance. Indeed, studies on obese and then calorie-restricted rats showed that short overfeeding after calorie restriction was accompanied with appearance of small adipocytes (18).

Development of mature adipocytes is dependent on active lipogenesis. In serum-free culture conditions, all accumulated lipids are synthesized de novo (19). De novo lipogenesis in adipose tissue, possibly orchestrated by ChREBPβ (20), was downregulated in subjects with hypertrophied adipocytes who are more insulin resistant compared with subjects with smaller adipocytes (21). Since we observed that weight loss was accompanied with a higher expression of lipogenic genes *FASN*, *DGAT2*, *SCD1*, *ACLY*, and *ChREBPα* (a regulator of ChREBPβ expression) in in vitro differentiated cells, it can be suggested that de novo lipogenesis capacity linked to higher insulin sensitivity represents intrinsic characteristics of adipocytes reprogrammable by weight loss.

In obesity, adipocytes produce more proinflammatory cytokines and chemoattractants while secretion of insulin-sensitizing adiponectin is diminished (22). In our study, we show that weight loss altered the capacity of in vitro differentiated adipocytes to express *IL-8*, *MCP1*, leptin, and adiponectin. Lower secretion of MCP1 from adipocytes reprogrammed by weight loss could contribute to a lower infiltration of macrophages into AT. Selective increase of high–molecular weight adiponectin secretion might underlie beneficial effects of weight loss on insulin sensitivity.

Studies performed on cell culture models may be influenced by culture conditions. Although we cannot completely exclude possible effects of subcultivation on adipogenic and secretory potential of cells, it has been shown previously that in vitro conditions preserve the original phenotype of a donor in preadipocytes and adipocytes (13,23). Moreover, subcultivation of stromavascular cells eliminates contaminating cells like macrophages and results in a more homogenous population than primary cells (3,24). It is also unlikely that the observed differences were based on dissimilar starting numbers of cells, as there was no difference in the length of cultivation or yield of cells before and at the end of DI.

In conclusion, our study shows that weight loss improves the adipogenic capacity of preadipocytes and alters their secretory potential. This effect may be associated with the improvement of the metabolic status of obese as well as with an increased tendency for weight regain. We believe that the analysis of a distinct cellular population, such as preadipocytes subjected to uniform in vitro conditions, can offer a focused and unique image of an intrinsic adaptation of AT to weight loss.

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L.R. designed the study, performed experiments and data analysis, and wrote the manuscript. L.M., J.K., M.T., Z.K., and M.K. performed experiments and contributed to discussion. M.S.-V., N.V., and D.L. contributed to discussion and to the writing of the manuscript. V.S. designed the study, organized the clinical part of the study, and contributed to discussion and the writing of the manuscript. L.R. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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# 4. CONCLUSIONS

This thesis analyzed immune status of AT and circulating leukocytes under various physiological and pathophysiological interventions in lean and obese humans. First part examined acute effects of elevated levels of nutrients on inflammation and representation of immune cells. Second part investigated beneficial effects of moderate weight reduction on immune attributes of AT, with respect to improvement of pro-inflammatory state and sensitivity to insulin action.

The major conclusions of this thesis are:

- Ingestion of HFM induced postprandial inflammation detectable on the level of gene expression in CD14+ PBMC. This inflammation was not associated with the concomitant increase in the expression levels of ERS markers.

- Acute short-term HG induced an increase in the content of monocytes and T lymphocytes in SAAT of healthy obese women.

- Acute hyperlipidemia induced a pro-inflammatory response associated with alteration of relative content of immune cells in blood and SAAT and enhanced release of pro-atherogenic mediators.

- No major differences were found in mRNA levels of selected immunity related genes between SAAT and SGAT in basal conditions. During weight reduction, majority of genes changed with similar pattern thus refuting the hypothesis that protective role of SGAT is given by lower expression of pro-inflammatory/immune system related genes.

- sCD163 correlated with CD163 mRNA expression in SAAT and VAT and with whole-body insulin sensitivity in the steady-state condition. These associations were not observed with respect to the diet-induced changes during a weight-reducing hypocaloric diet.

- Secretory potential of human *in vitro* cultured pre/adipocytes was altered to the less inflammatory after the weight reduction

# 5. SUMMARY

Obesity and overfeeding are associated not only with increased circulating levels of nutrients and metabolites, but also with increased risk of the development of additional disorders, such as cardiovascular diseases, cancer or insulin resistance. Plausible link between obesity and its comorbidities is inflammatory state, observed on the whole body level as well as in AT. As possible initiators of this inflammation, hypertrophied adipocytes were suggested. Adipocytes *per se* secrete a spectrum of heterogeneous molecules including cytokines. Under the stress conditions, adipocytes and subsequently AT resident immune cells switch to pro-inflammatory state and via secretory signaling attract additional immune cells. Furthermore, hypertrophic adipocytes release higher levels of metabolites that may also contribute to pro-inflammatory polarization of immune cells, mainly macrophages.

General aim of this thesis was to investigate connection between impaired levels of nutrients and pro-inflammatory statue and activation of immune cells in healthy (obese and lean) subjects.

In the Part one of this thesis, we analyzed acute reaction of immune cells in circulation and AT on artificially elevated levels of nutrients, imitating its increased values typical for metabolic syndrome. HFM ingestion led to inflammatory reaction detectable in circulating monocytes but not associated with ER stress. Similarly, short-term HG and hyperlipidemia induced a pro-inflammatory response associated with altered relative content of immune cells in blood and SAAT. Moreover, changes induced by acute hyperlipidemia were associated with enhanced release of pro-atherogenic mediators.

In the studies included in the Part two, we extended our knowledge about beneficial effects of weight reduction on pro-inflammatory and metabolic statue of obese patients. Moderate weight loss was accompanied by amelioration of levels of pro-inflammatory markers in circulation and in AT. The effect on mRNA levels of immunity-related markers was similar in abdominal and gluteal subcutaneous AT. Expression changes of one of these markers, CD163, which were induced by weight loss, were not associated with changes of insulin sensitivity. Furthermore, weight loss reprogrammed precursors of adipocytes and reduced their intrinsic inflammatory potential.

In conclusion, in short-term interventions we confirmed that impaired levels of glucose and lipid metabolites (FA, TAG) are associated with activation of immune

cells in humans. On the other hand, weight reduction led to improvement of secretory function of adipocytes *per se* and inflammatory status of AT on mRNA level. Results of this thesis thus contribute to understanding of obesity and overfeeding associated inflammation, even so further investigation of the functional changes in AT by nutrients and obesity is warranted.

# 6. SHRNUTÍ

Obezita, charakterizovaná zvýšenou akumulací tukové tkáně (TT), i přejídání jako takové jsou spojeny nejen se zvýšenými plasmatickými hladinami živin a metabolitů, ale i s narůstajícím rizikem vzniku dalších chorob, např. chorob kardiovaskulárního systému, rakoviny nebo insulinové rezistence. Pravděpodobným pojítkem mezi obezitou a chorob s ní spojených je zánětlivý stav organismu, pozorovaný jak na systémové úrovni jako zvýšené hladiny plasmatických cytokinů, tak na úrovni TT. Příčinou tohoto zánětlivého stavu může být narušený metabolismus TT. Adipocyty sekretují širokou paletu různorodých molekul včetně cytokinů. Za stresujících podmínek (hypoxie, stres endoplasmatického retikula) začnou adipocyty a následně i rezidentní imunitní buňky produkovat prozánětlivé cytokiny, které atrahují další imunitní buňky. Adipocyty mohou navíc uvolňovat zvýšené množství metabolitů (mastné kyseliny, glycerol), které rovněž přispívají k polarizaci imunitních buněk, zejména makrofágů.

Cílem této práce bylo nalézt spojení mezi zvýšenými hladinami nutrientů (glukosa, mastné kyseliny) a zánětlivým stavem, resp. aktivací imunitních buněk u zdravých (obézních i štíhlých) jedinců.

V první části této práce byla sledována akutní reakce imunitních buněk na experimentálně zvýšené hladiny nutrientů. Pokrm s vysokým obsahem tuku a energie způsobil postprandiální leukocytózu a vedl k prozánětlivé reakci detekované v krevních monocytech. Obdobně krátkodobá hyperglykemie a hyperlipidemie indukovaly prozánětlivou odpověď, spojenou se změnou zastoupení imunitních buněk na úrovni krve i TT. Změny indukované akutní hyperlipidemií byly navíc spojeny s uvolněním aterogenních mediátorů.

Cílem druhé části bylo prohloubit znalosti o pozitivních efektech redukce hmotnosti na prozánětlivý a metabolický stav obézních pacientů. Mírný váhový úbytek byl provázen snížením hladin cytokinů v plasmě a TT. Efekt redukce hmotnosti na imunitní markery byl obdobný jak v abdominálním tak gluteálním tukovém depu. V průběhu dietní intervence nebyla pozorována spojitost mezi změnami v hladinách jednoho ze stanovovaných markerů, CD163, a insulinovou sensitivitou. Mimoto se vlivem váhové redukce upravil i sekreční potenciál samotných adipocytů.

Lze shrnout, že na základě analýz efektů krátkodobých intervencí byla potvrzena hypotéza, že zvýšené hladiny glukosy a mastných kyselin v krvi jsou asociovány s

aktivací imunitního systému. Oproti tomu, redukce hmotnosti vedla ke zlepšení jak sekrečního profilu samotných adipocytů tak k snížení prozánětlivého stavu TT na úrovni mRNA, ale tyto změny nebyly přímo asociovány se zlepšením insulinové senzitivity. Výsledky této práce přispěly k pochopení prozánětlivých pochodů asociovaných s obezitou a přejídáním, i přesto je další výzkum funkčních změn na úrovni TT nezbytný.

# 7. ANNEX

**List of co-authored articles not included in the thesis:**

**Stress of endoplasmic reticulum modulates differentiation and lipogenesis of human adipocytes**

Michal Koc, Veronika Mayerová, Jana Kračmerová, Aline Mairal, Lucia Mališová, Vladimír Štich, Dominique Langin, Lenka Rossmeislová

Biochemical and Biophysical Research Communications. 2015 Mar. [Epub ahead of print]. IF 2.3

**Ursodeoxycholic acid but not tauroursodeoxycholic acid inhibits proliferation and differentiation of human subcutaneous adipocytes**.

Lucia Mališová, Zuzana Kováčová, Michal Koc, Jana Kračmerová, Vladimír Štich, Lenka Rossmeislová

PLoS ONE. 2013 Dec, 8(12): e82086. IF 3.5

**Adaptation of human adipose tissue to hypocaloric diet.**

Lenka Rossmeislová, Lucia Mališová, Jana Kračmerová, Vladimír Štich

International Journal of Obesity (London). 2013 May, 37(5): 640-50. IF 5.2

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