Charles University in Prague

Third Faculty of Medicine

Molecular adaptations of human adipose tissue in response to dietary treatment of obesity

by

Mgr. Michaela Tencerová

Department of Sports Medicine

Prague 2010

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PhD thesis

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Supervisor**: Doc. MUDr. Vladimír Štich, PhD.**

Specialization: **Molecular and cell biology, genetics and virology**

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“Running in circles...”

DECLARATION

Hereby, I declare that this thesis has been written by me and it was based on work that I have done during my PhD studies in collaboration with the colleagues from the Franco-Czech laboratory.

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Prague, 2010 .....

Mgr. Michaela Tencerová

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CONTENTS

LIST OF ABBREVIATIONS

AdipoR: adiponectin receptor

AdPLA: phospholipase A2

AMPK: AMP-activated proteinkinase

ANP: atrial natriuretic protein

apM1: adiponectin

APPL1: adiponectin receptor interacting protein

AT: adipose tissue

ATP: adenosine triphosphate

ATGL: adipocyte triglyceride lipase

ATM: adipose tissue macrophages

ATP: adenosine triphosphate protein

BMI: Body mass index

cAMP: cyclic adenosine monophosphate

CideN: cide-domain-containing proteins

CLS: crown like structure

CRP: C-reactive protein

CXCL5: C-X-C motif chemokine 5

DAG: diacylglycerol

DI: dietary intervention

ER: endoplasmic reticulum

ERK: extracellular signal-regulated kinase

FA: fatty acids

FABP: fatty acid binding protein

FFA: free fatty acid

FOXO: forkhead box protein O

GI: glycemic index

Grb2: growth receptor binding protein 2

GSK3: glycogen synthase kinase-3

GLUT4: glucose transporter 4

HMW: high molecular weight

HOMA: homeostasis model assessment

HSL: hormone sensitive lipase

IDF: International Diabetes Federation

IGF: insulin-like growth factor

IKK-β: inhibitor of NFκB kinase subunit beta

iNOS: inducibile nitric oxide synthase

IR: insulin receptor

IRS: insulin receptor substrate

JAK: Janus protein kinase

JNK: c-Jun-N-terminal kinase

LCD: low calorie diet

LMW: low molecular weight

LPL: lipoprotein lipase

LPS: lipopolysacharid

LEP: leptin

LEPR: leptin receptor

LXR: liver X receptor

MAG: monoacylglycerol

MAPK: mitogen activated protein kinase

MGL: monoglyceride lipase

MCP-1: monocyte chemoattractant protein 1

MCR4: melanocortin 4 receptor

MIP-1α: macrophage inflammatory protein-1α

MMP-9: metalloproteinase-9

MMW: medium molecular weight

mTOR: mammalian target of rapamycin

NFκB: nuclear factor kappa B

PBEF: pre-B cell colony enhancing factor

PC1: prohormone convertase 1

PDK: phosphoinositide-dependent protein kinase 1

PEPCK: phosphoenolpyruvate carboxykinase

PTP: phosphatase

PI3K: phosphatidylinositol 3-kinase

PIP_3: phosphatidylinositol 3-phosphate

PH: pleckstrin-homology

PKA: protein kinase A

PKC: protein kinase C

POMC: proopiomelanocortin

PPAR: peroxisome proliferator-activated receptor

PTB: phosphotyrosine-binding domain

RAR: retinoic acid receptor

RBP4: retinol binding protein 4

ROS: reactive oxygen species

RXR: retinoic acid-X receptor

SAA: serum amyloid A

SAT: subcutaneous adipose tissue

SHC: Src-homology 2 containing protein

SIRT: sirtuin

SNP: single nucleotide polymorphism

SOCS: suppressor of cytokine signalling

STAT: signal transducer and activator of transcription

SVF: stroma-vascular fraction

TAG: triacylglycerol

TLR: toll like receptor

TNF-α: tumor necrosis factor alpha

TNFR: tumour necresis factor receptor

TTR: transthyretin

TZD: tiazolidindiones

UPR: unfolded protein response

VAT: visceral adipose tissue

VLCD: very low caloric diet

VLDL: very-low-density lipoprotein

WHO: World Health Organisation

WM: weight maintenance

WS: weight stabilisation

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CHAPTER 1

OVERVIEW OF THE CURRENT KNOWLEDGE

1.1 OBESITY

Obesity is a multisystem disorder that belongs to a major health problem of the 21st century with increased prevalence reaching an alarming rate all over the world. According to the World Health Organisation (WHO) it is characterized as a global epidemic, also called "globesity", affecting people in both developed and developing countries. The last WHO estimation in 2005 has revealed that approximately 1.6 billion adults (aged 15 and older) in the world were overweight and at least 400 million of them were considered clinically obese. It is expected that by 2015 2.3 billion adults will be overweight and more than 700 million adults will be obese. In the European region, the prevalence of obesity has tripled since the 1980s. In 2005 it reached 5-20% in men and up to 30% in women; specifically in the Czech Republic 18.5% males and 20.7% females suffered from obesity [1, 2] (www.who.int). The alarming fact is that obesity is rapidly spreading among children and adolescents with a high impact on their health in later age. Obesity represents a risk factor for other metabolic and cardiovascular complications including insulin resistance, diabetes mellitus, atherosclerosis, cardiovascular diseases, hypertension, traditionally embodied in one term known as "metabolic syndrome", and several types of cancers. [3, 4] (Table 1). Moreover, it has been proven that obesity affects longevity and the quality of life. Also, it is associated with an increased rate of mortality [5, 6].

Obesity is a complex disease caused by the interaction between different factors: genetic, environmental, behavioural, cultural, and socio-economical acting through the physiological mediators of energy intake and expenditure. Individual's exposure to "obesogenic" environment high in sugars and fats together with sedentary life style (lower physical activity) could unmask latent/silent genetic factors leading to excessive weight gain. Also, it has been published that nutrition during prenatal development can contribute to earlier prevalence of obesity in adolescent [7]. Thus, obesity denotes a multifaceted problem in health care with no easy finding a solution.

Based on these facts, it is clear that obesity needs to have appropriate management that could decrease the health burden associated with treatment of obesity-related complications. It has been shown that optimizing a balanced diet and increasing physical activity are the best tools in the treatment of obesity. Many clinical studies observed a positive effect on health already after 5-10% weight loss [8]. However, there are still some individuals with no response to these kinds of interventional programmes. A better understanding of physiological and molecular mechanisms controlling body weight could contribute to shed a light on one of the biggest health challenges of the 21st century and may bring substantial health benefits.

Organ system	Type of diseases associated with obesity
Cardiovascular	Hypertension, atherosclerosis, cardiomyopathy
Gastrointestinal	Gall bladder disease, non-alcoholic steatohepatitis
Metabolic	Insulin resistance, diabetes mellitus, dyslipidemia
Respiratory	Sleep apnoea, hypoventilation syndrome
Musculoskelatal	Osteoarthrosis
Reproductive	Polycystic ovary syndrome, early puberty
All	Cancer of breast, prostate, gall bladder, colon

Table 1 List of obesity-linked comorbidities.

1.1.1 Characterization and classification of obesity

Overweight and obesity are defined as an abnormal or excessive accumulation of body fat mass caused by an imbalance in energy intake and expenditure that may have an adverse effect on health. According to WHO, a generally used index for measurement of overweight and obesity is the body mass index (BMI), which is calculated as the ratio of weight in kilograms over height squared in meters (kg/m^2). It defines overweight as BMI ≥ 25 kg/m^2 and obesity as BMI ≥ 30 kg/m^2. This classification is valid for both genders, male and female, and for all adult age groups (Table 2). However, there are some limitations to use BMI as a measure of obesity. For children, BMI cannot be used because it changes rapidly over time. Hence, obesity in

childhood is defined according to growth curves dependent on sex and age [9]. Furthermore, there are differences in BMI cut-off points among ethnic groups, e.g. the Asian population has a high risk of cardiovascular diseases and metabolic disorders at BMI lower than 25 kg/m^2 [10]. Therefore, the question is raised whether waist circumference would not be a better indicator for obesity-related complications because BMI does not reflect the differences of body fat mass distribution amongst individuals. It is known that preferential accumulation of fat in the upper part of the body, also called "central (abdominal) obesity", is associated with an increased risk of morbidity [4, 11]. According to the International Diabetes Federation (IDF), central obesity is defined in the European population as a waist circumference ≥ 94 cm in men and ≥ 80 cm in non-pregnant women. Taking all these aspects into account, further epidemiological surveys are required for estimation of the interaction between BMI, waist circumference and prevalence of health complications in different populations.

Category	**BMI (kg/m^2)**
Underweight	< 18.5
Normal range	18.5 – 24.9
Overweight	25.0 – 29.9
Obese class I	30.0 – 34.9
Obese class II	35.0 – 39.9
Obese class III	≥ 40.0

Table 2 The International classification of underweight, overweight, and obesity according to body mass index (Adapted from WHO www.who.int)

1.1.2 Genetics of obesity

Human obesity is not a single disorder, but it is a complex of heterogeneous conditions ("obesities") caused by interactions among genetic, environmental and behavioural influences (Figure 1). Evidence for potent genetic contributions to body weight has been provided by family studies and the studies on twins and adopted children [12, 13]. These studies have revealed a heritability for the magnitude of fat mass between 40-70% [14]. The influence of genetic predisposition on the aetiology of

obesity may be attenuated or amplified by non-genetic factors. There is a "susceptible-gene hypothesis" that a certain set of genes is more sensitive to the development of obesity complications than another. Therefore, people with this genetic outfit are more prone to obesity in any given environmental inductors [3]. This "disadvantage", however, could be beneficial early in the human evolution, since these genes could protect their holders in periods of famine (so called "thrifty gene" hypothesis). However, in the 21st century of modern diet full of fats they could contribute to the onset of obesity [15]. Heritability of obesity in most cases is not Mendelian, but it involves interaction of multiple genes localized on different loci (polygenic obesity). Monogenic causes of obesity (mutation in single gene) are very rare with early onset of weight gain and hyperinsulinemia in childhood. Screening approach of candidate genes based on results from animal models revealed a mutation in 6 genes associated with obesity: gene for leptin (LEP), leptin receptor (LEPR), proopiomelanocortin (POMC), melanocortin 4 receptor (MC4R), prohormone convertase 1 (PC1), and neutrophin receptor (TrkB) [16]. They are involved in signalling pathways regulating metabolism and food intake according to signals coming from the hypothalamus. Other types of obesity represent less common recessive syndromes (more than 25 have already been identified), e.g. Prader-Willi syndrome or Bradet-Biedl syndrome with an incidence of 1 in 15000-25000 and 1 in 150000 live births, respectively.

Recently, all findings including the interindividual genetic polymorphisms associated with obesity have been summarized and published under the name "The Human Obesity Gene Map" available for researchers on the website www.obesitygene.pbrc.edu with an annual update [17]. Nowadays, it consists of more than 600 genes, different chromosomal regions and DNA sequences. Another approach represents the "Hap-map" project concerning on single nucleotide polymorphisms (SNP) in relation to obesity development. The recent discovery in this field has revealed SNP in FTO gene associated with an increased prevalence of abnormal fat accumulation and type 2 diabetes. Although the rapid pace in the development of modern technologies used for the wide-genome research helps to cover the "gaps" in human genome, there is still not enough information about linkage between SNPs and expression of obesity phenotype. Since, the obesity-related SNPs explain only less than 1% of the prevalence of obesity; it is obvious that a lot of undisclosed factors contribute to the development of this modern epidemic and they are waiting for their discovery.

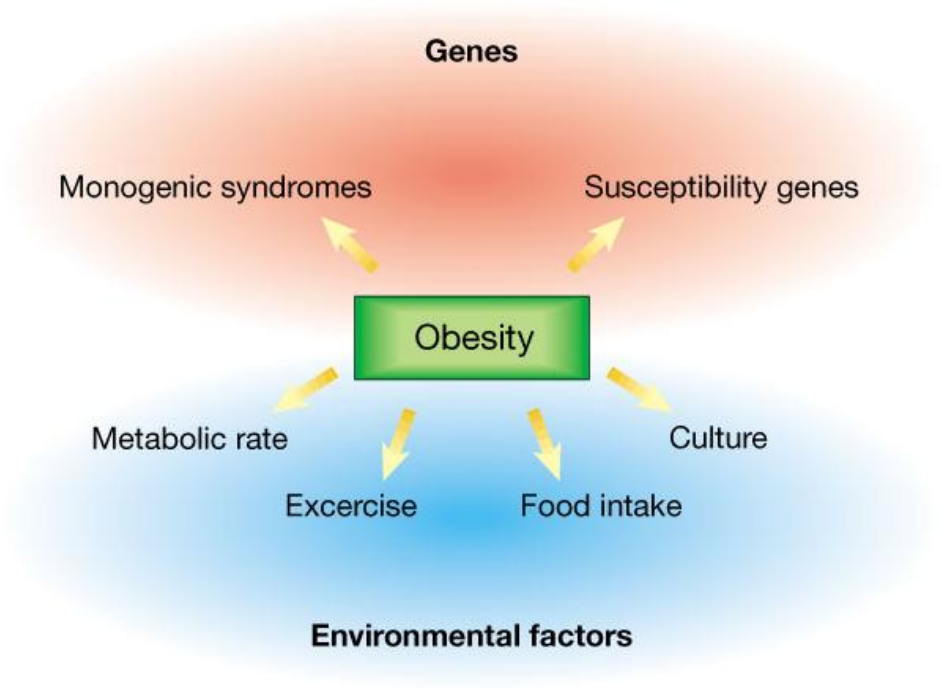

Figure 1 Genetic and environmental factors of obesity (adapted from Kopelman et al., Nature 2000) [3]

1.2 PHYSIOLOGY OF ADIPOSE TISSUE

1.2.1 Adipose tissue as a heterogeneous organ

Adipose tissue (AT) is a connective tissue primarily served as a storage site for fat in the form of triglycerides. The main type of AT in human body is white AT; another type is brown AT which can be found only in human neonates. However, recent data have revealed that active brown AT may exist also in adults under certain rare conditions (in the supraclavicular region and neck)[18]. White AT represents a heterogeneous organ composed mainly of mature adipocytes (60-70%), which are held in compact form by collagen fibers. Mature adipocytes are unilocular cells that contain a single large lipid droplet (consisting of triglycerides from 95%) surrounded by a phospholipid monolayer, pushing the cell nucleus into the peripheral edge and cytoplasm with organelles. The remainig cell populations in AT, so called “stroma-vascular fraction” (SVF) include different cell types, mainly fibroblasts, endothelial cells, preadipocytes, and immune cells [19] (Figure 2). It has been proven that this non-adipocyte cell fraction plays a crucial role in the pathophysiology of obesity (more detailed in chapter 1.2.4) [20, 21]in relation to its secretory activity. Flow cytometry and immunohistochemistry analysis of human AT showed higher content of immune cells

(i.e. resident macrophages, lymphocytes and neutrophils) in positive correlation with adiposity[22-26]. Endothelial cells participate in building a functional capillary network within AT and a microcirculation around each adipocyte that is necessary for adequate oxygen and nutrient supply during AT development. Moreover, they serve as a surface for regulating leukocyte infiltration into AT [27]. Mature adipocytes are derived from preadipocytes, i.e. progenitor cells which differ from mesenchymal stem cells (characterized by expression of CD34+/CD31-) [28, 29]. Recently, it has been demonstrated that preadipocytes are able to proliferate and contribute to the expansion of fat mass during obesity [30]. There are two main changes in the structure of AT occurring in obesity: size enlargement (hypertrophy) and increasing the number of progenitor cells (hyperplasia) [31]. Mature adipocytes are not able to proliferate [32], but they can expand their volume. The size of adipocytes (in diameter) varies from 20 to 200μm dependent on gender or location. During obesity, adipocytes are exposed to an environment high in lipids which leads to their hypertrophy accompanied by neovascularization. After reaching a critical cell size, new adipocytes are differentiated from stimulated progenitor cells. The mechanisms occuring in expanded AT involve not only structural changes, but also an interplay between adipocytes and the SVF immune cells that influence signalling pathways in AT through their secretory products. Therefore, it is necessary to know the cellular composition of AT in relation to the influence on AT secretory function with respect to different metabolic complications.

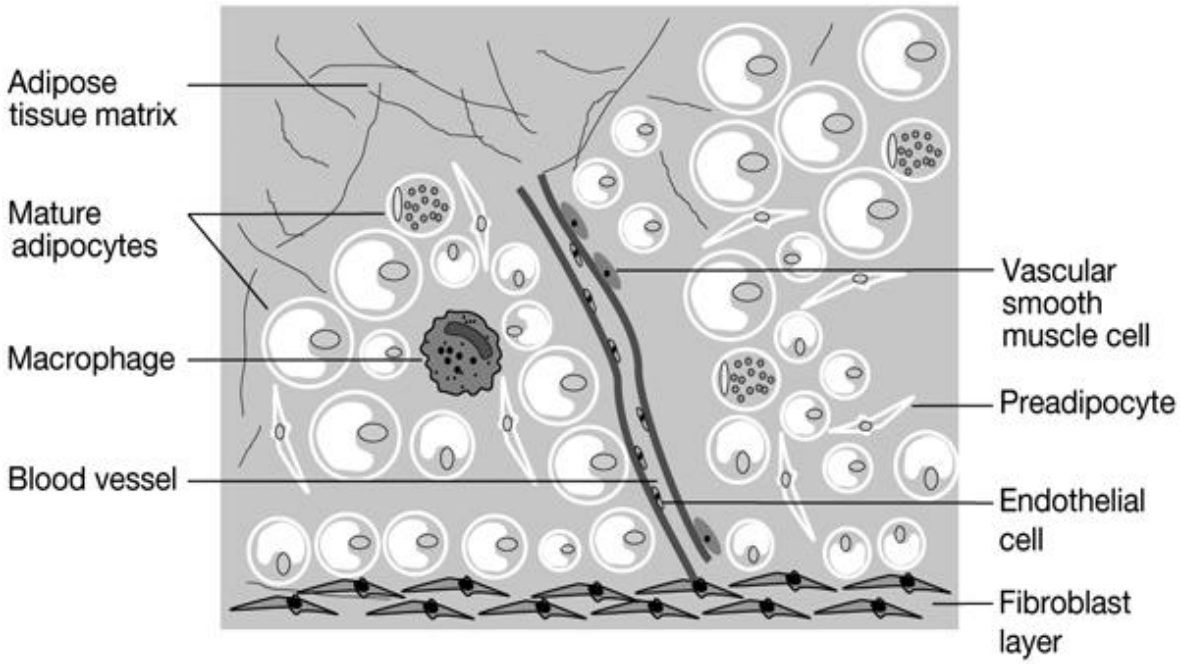

Figure 2 Cellular composition of adipose tissue (adapted from Schaffler et al. 2005, *Nat Clin Pract Gastroenterol Hepatol* 2: 103–111) [33]

1.2.2 Physiological function of adipose tissue (metabolism of fatty acids)

AT represents the largest energy organ that stores energy in the form of triacylglycerols (TAG) during energy excess/load (postprandially) and releases in the form of free fatty acids (FFA) during times of energy need (e.g. during fasting or physical activity) [34-36]. TAG serves as a long-term metabolic fuel with a high energy content, e.g. 1kg of AT contains 800g of TAG what is about 7000 kcal of energy [19]. This fat energy mobility is coordinated by two processes in AT: synthesis of TAG (lipogenesis) and breakdown of TAG (lipolysis), which is tightly regulated by hormonal and endocrine factors.

Lipogenesis, besides AT, occurs also in the liver (production of very-low-density lipoproteins (VLDL)) and is positively regulated by insulin. TAG synthesis in adipocytes proceeds either in *de novo* lipogenesis from non-lipid precursors (glucose) or uptake of fatty acids (FA) circulating in the blood. However, *de novo* lipogenesis is rare in humans because of the low activity of ATP citrate lyase [37]. More often TAG arise from long chain FA in the form of chylomicrons coming from intestinal absorption of dietary fat or VLDL particles secreted by the liver, which are hydrolyzed to FFA by lipoprotein lipase (LPL) expressed in the adipocytes and anchored in the luminal

surface of the endothelial cells via glycosaminoglycans. Released FFA are translocated into the adipocytes by specific fatty acid translocase CD36 [38] or by fatty acid binding proteins (FABP) [39] where are together with glycerol 3-phosphate esterified to TAGs (Figure 3). Translocation of FFA across the membrane is accompanied by their esterification with co-enzyme A to form fatty acyl-CoA [40]. Glycerol 3-phosphate is produced from glucose by glycolysis in the adipocyte during a saturated state.

Lipolysis (hydrolysis of TAG) is an opposite process occurring in adipocytes, which predominates over lipogenesis in times of starvation/energy supply. It is regulated on different levels, but the limiting factor for its rate is the hydrolysis of TAG by lipases (Figure 3). For long time, it has been considered that the initial enzyme for TAG degradation is hormone sensitive lipase (HSL). However, recent studies [41-43] have revealed another TAG hydrolase, known as adipocyte triglyceride lipase (ATGL) or desnutrin with a higher substrate specificity for TAG. According to these findings, the lipolytic process is initiated by ATGL/desnutrin, which hydrolyses TAG into diacylglycerol (DAG) and releases one molecule of FA. DAG is further hydrolyzed by HSL to monoacylglycerol (MAG) and generates a second FA, and MAG is finally hydrolyzed by monoglyceride lipase (MGL) to gain the third FA and glycerol [36]. Generated FFAs can be used for β-oxidation in the mitochondria and to form adenosine triphosphate (ATP) or they can be released into the circulation, and thus be transported to other organs. Glycerol is transported to the liver for gluconeogenesis.

Lipolysis is stimulated by catecholamines (adrenaline and noradrenaline) that bind to β-adrenoreceptors which in turn activate adenylyl cyclase to produce cyclic adenosine monophosphate (cAMP) from ATP. cAMP subsequently activates protein kinase A (PKA), which in turn phosphorylates and activates HSL. Other stimulators of lipolysis are natriuretic peptides (e.g. ANP) [44], growth hormone [45], glucocorticoids [42] or tumour necrosis factor-α (TNF-α) [46]. Insulin has inhibitory effect on lipolysis via phosphodiesterase 3B, which reduces the concentration of cAMP. Additionally, α2-adrenoreceptors [47], prostaglandins [48], adenosine receptors [49], neuropeptide Y/peptide YY and nitric oxide [50] belong to the anti-lipolytic group of mediators. Recently, new molecules with an important role in the regulation of lipolysis have been determined: lipid droplet proteins (perilipin) [51, 52], caveolin-1 [53], aquaporin-7 [54], cide-domain-containing proteins (CideN) [55], and sirtuins (SIRT) [56].

Randle et al. [57] has demostrated that glucose (except of FA) can also enroll in these energy processes. It is known as the “glucose-fatty acid cycle” representing the

substrate competition between FA and glucose as a fuel for oxidation in the mitochondrion. During nutritional overload the energy homeostasis in the body is impaired and leads to "metabolic inflexibility" accompanied by decrease of responsiveness to insulin that consequently affect its controlling feature in lipid and glucose metabolism. The strict regulation of the balance between release and utilization of FA is necessary to avoid the development of metabolic complications. On the one hand, a higher lipolytic activity of adipocytes could reduce the total fat mass, however on the other hand, an increased levels of FA in the circulation, which are not effectively used by tissues, could impair carbohydrate and fat homeostasis in the body and lead to "lipotoxicity", i.e. ectopic accumulation of fat within non-fat tissues such as muscle or liver and cause insulin resistance. However, recent findings in knock-out mice in adipose specific phospholipase A2 (AdPLA) revealed that increased lipolysis in AT does not necessarily lead to elevated serum FA levels [58]. There could be a preference for FA utilization and oxidation for energy expenditure which avoids the onset of obesity. Therefore, an efficient manipulation of lipolytic pathways in AT could serve as a therapeutic tool "in a never-ending fight with obesity", but before this point there are still questions that need to be resolved.

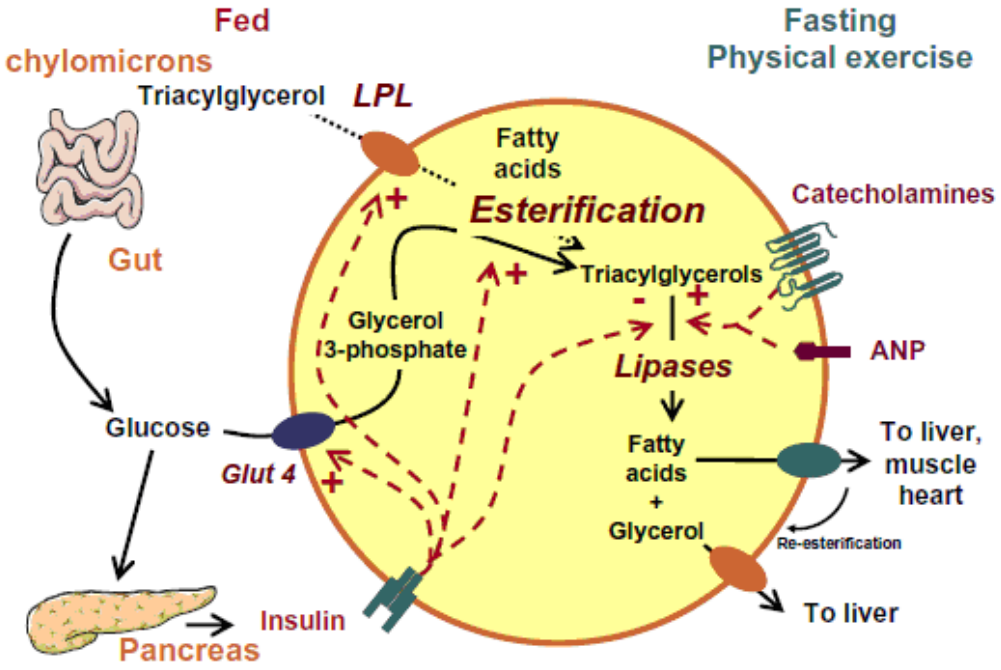

Figure 3 Metabolism of fatty acids in the adipose tissue (adapted from Lafontan and Langin, 2009, Progress in Lipid Research 48: 275-297). In saturated state (after fed), FA are stored in adipocytes in form of TAG (lipogenesis stimulated by insulin) induced by insulin, whereas in a fasted state or during physical exercise, TAG from adipocytes are broken down into FA and glycerol in a lipolytical process, which proceeds spontaneously or is activated by various factors, such as catecholamines, ANP or indirectly by pro-inflammatory cytokines (e.g. TNF-α).
LPL (liporotein lipase), ANP (atrial natriuretic peptide), GLUT4 (glucose transporter 4)

1.2.3 Insulin signalling pathway and adipose tissue (metabolism of glucose)

Insulin signalling pathway is important for the regulation of metabolic homeostasis in the body (besides cell differentiation and growth). Insulin is a hormone secreted by the pancreatic β-cells. Its secretion is regulated by the glucose concentration in the blood and is involved in glucose uptake (to lower blood glucose levels), lipolysis and hepatic gluconeogenesis. Insulin acts through its single receptor that is localized on the cell membrane of energy organs, such as skeletal muscle, AT, liver, or other organs, e.g. brain[59], pancreas[60], placenta [61] etc. The density of insulin receptor (IR) coverage on a target cell is around 200 000 to 300 000 molecules/cell. In the liver,

insulin inhibits the process of glycogenolysis (glycogen breakdown to glucose) and gluconeogenesis. Even though, the skeletal muscle is a main insulin-sensitive organ that uses glucose from the circulation as a primary source of energy, AT (through insulin activity) has an important role in controlling the whole-body glucose homeostasis.

On post-receptor level insulin signalling network is complex and divergent. IR (also able to bind insulin-like growth factor 1 (IGF-1)) belongs to the family of tyrosine kinase receptors that is composed of two α- and two β-subunits linked together by disulfide bonds. Binding of a ligand to the extracellular α-subunits promotes autophosphorylation and dimerization of a receptor that leads to binding and activation (i.e. phosphorylation) of other signalling molecules, including insulin receptor substrate proteins (IRS), but also Src-homology-2-containing protein (SHC) and proto-oncogene c-Cbl (Cbl). The family of IRS proteins consists of 6 tissue specific isoforms [62-66]. However, only IRS1 and IRS2 have a key role in insulin cascade in humans. IRS1 regulates anabolic function of insulin mainly in the muscle and AT, whereas IRS2 works in the liver [67-69]. IRS molecules contain pleckstrin-homology (PH) and phosphotyrosine-binding domain (PTB), which are necessary for their affinity to IR. IRS take in up to 20 different tyrosine phosphorylation sites that serve as docking sites for proteins with Src-homology-2 (SH2) domain, e.g. p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K), growth receptor binding protein 2 (Grb2), or cytoplasmic tyrosine kinase (Fyn). IR/IRS binding partners activate two different signalling pathways: PI3K – serine/threonine protein kinase B (PKB or AKT) pathway, which is responsible for most of the metabolic actions of insulin and Ras - mitogen activated protein kinase (MAPK) cascade, which control cell differentiation and growth [70]. PI3K activates second messenger, phosphatidylinositol 3-phosphate (PIP_3), on plasmatic membrane, which downstream activates additional signalling molecules with PH domain, such as 3-phosphoinositide-dependent protein kinase 1 (PDK1) and further AKT/PKB or protein kinase C (PKC). Activation of PKB leads to phosphorylation of other target molecules, such as glycogen synthase kinase-3 (GSK3) necessary for glucose synthesis, forkhead box protein O (FOXO) involved in hepatic gluconeogenesis, mammalian target of rapamycin (mTOR) essential for the differentiation of preadipocytes, as well as Rab GTPase-activating protein (AS160) responsible for the translocation of glucose transporter 4 (GLUT4) to plasmatic membrane and glucose uptake into the target cell [71].

In this complicated network there are 3 critical nodes necessary for the regulation of the insulin action (Figure 4): IR/IRS, PI3K and AKT/PKB [71]. The initial event, i.e. phosphorylation of IRS proteins, can be negatively regulated by tyrosine phosphatases (e.g. PTP1B) or serine phosphorylation via protein kinases, such as extracellular signal-regulated kinase (ERK), c-Jun-N-terminal kinase (JNK). In IRS1 molecule there are over 70 serine phosphorylation sites (e.g. Ser307, Ser270, Ser612) with an inhibitory effect. Serine phosphorylation has been observed in the obese states and has been associated with insulin insensitivity [72, 73]. Another manner to regulate insulin cascade is ligand-induced down-regulation of IR/IRS complex mediated by the suppressor of cytokine signalling-1 (SOCS1) and SOCS3 via ubiquitin-degradation of IR[74]. PI3K can be negatively regulated at the level of PIP3 by phosphatases such as PTEN or SHIP2 or by the concentration of its regulatory subunit. Additionally, the activity of AKT/PKB is also under control of phosphatase (PP2A). Importantly, it has been revealed that the down-regulation of the insulin signalling pathway may be induced by different extracellular stimuli, e.g. FFA, pro-inflammatory cytokines and adipokines (e.g. TNF-α, IL-6, leptin, RBP4) or nutrient components [75-77] that in an inappropriate rate leads to the development of insulin resistance. An insufficient activity of the insulin pathway during nutrients overload accompanied by a prolonged increased glucose concentration in the circulation has a detrimental effect on the secretory function of β-cells and could further spread in type 2 diabetes.

Insulin resistance is a characteristic feature of many obesity-related complications. However, the exact causes of insulin resistance are not clear. There is a body of evidence about various culprits that could contribute to its development. The summary scheme of possible mediators of insulin resistance is shown in Figure 5. In obesity, the elevated energy intake of the adipocytes leads to hypertrophy and expansion of fat mass which in turn activates the pro-inflammatory signalling pathways e.g. through nuclear transcription factor NF-κB and serine/threonine kinases JNK, which increases the production of pro-inflammatory cytokines, adipokines and chemokines, such as leptin, TNF-α, IL-6, MCP-1 (“humoral theory” of insulin resistance) that finally inhibit insulin action in AT. The release of chemoatractants into the circulation subsequently causes the elevated recruitment of immune cells, i.e. macrophages into the inflamed tissue that amplify the inflammatory response (“immune cells-induced insulin resistance”, discussed more detailed in 1.3). Regarding the hypertrophied adipocytes, it has been shown that these cells are faced to hypoxic condition because of insufficient

oxygen delivery to the tissue leading to the stimulation of inflammatory pathways [78]. Recently, a new hypothesis has been proposed about the endoplasmic reticulum (ER) stress, which plays a key role in the activation of many inflammatory pathways [79, 80]. The ER represents an important sensor for the nutrient balance in the cell. When its function is impaired, the unfolded protein response (UPR) arises. The activation of UPR pathway leads to decrease of protein synthesis and initiation of protein degradation. The ER stress is also a source of reactive oxygen species (ROS) contributing to insulin resistance. Another explanation for the origin of insulin resistance might be the increased oxidative stress induced by mitochondrial dysfunction that also activates inflammatory and stress molecules [81, 82]. At the beginning, the overload of FFA and glucose in the circulation induces a metabolic stress accompanied by insulin resistance and inflammation locally, in each insulin-sensitive organ (skeletal muscle, liver and AT) that could further spread via endocrine effects of cytokines, adipokines to systemic, whole body insulin resistance and inflammation. As shown in Figure 5, the vicious circle among putative culprits of insulin action impairment is very tangled and it is really hard to determine the first one, which triggers the cellular machinery in the obese state. Therefore, further functional studies are necessary to find a solution for a treatment of insulin resistance that could be potentially used in pharmacological therapy.

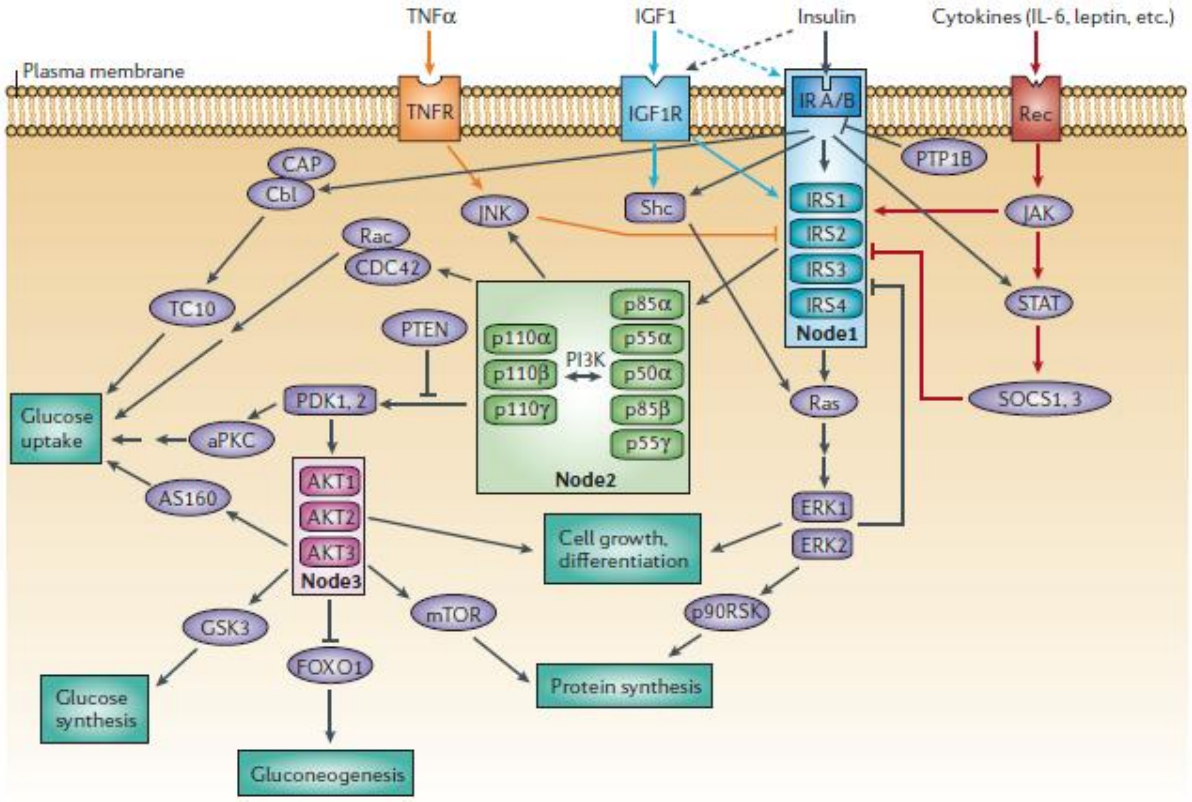

Figure 4 Insulin signalling pathway with 3 important highlighted nodes in its regulation: IRS, PI3K and AKT/PKB (adapted from Kido et al., 2001, J Clin Endocrinol Metab 86: 972-979)[83].

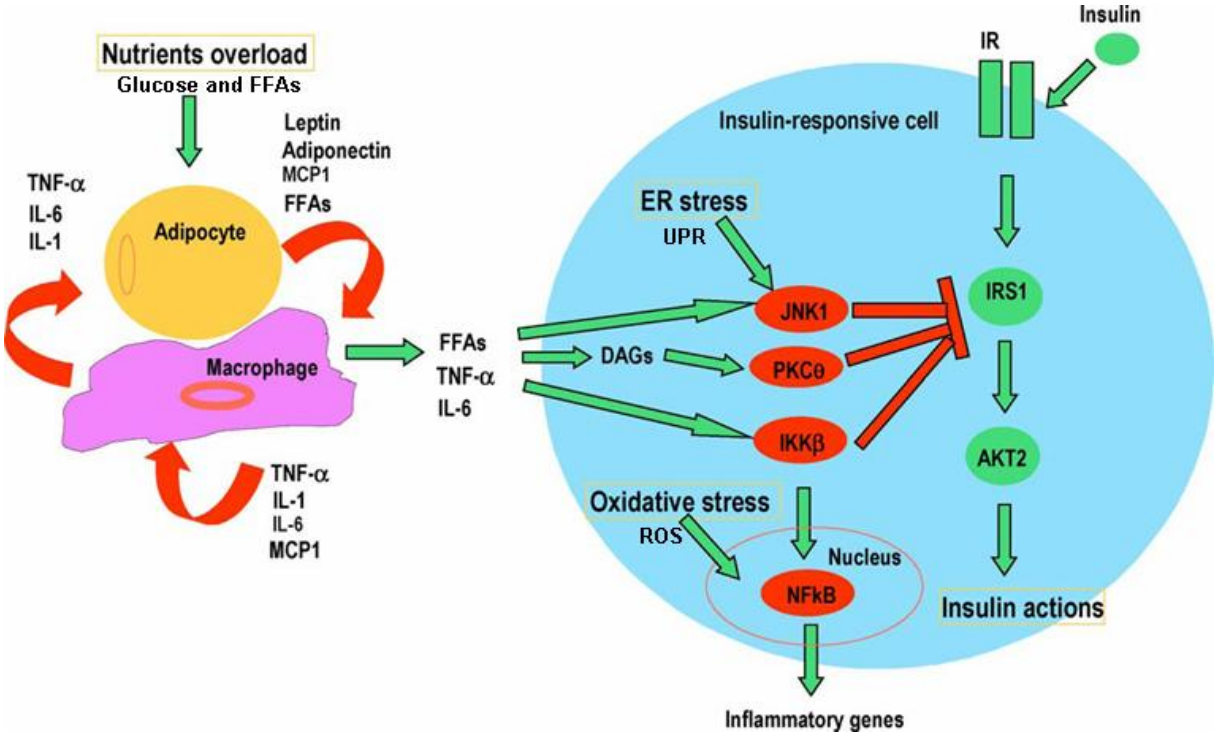

Figure 5 Possible culprits of the development of insulin resistance in an insulin-responsive cell (adapted from Chen et al., 2006, Pharmacological research 53: 469-477)[84]. Nutrient overload leads to hypertrophy of adipocytes, increased infiltration of macrophages, that activate pro-inflammatory signalling pathways through JNK/NFκB followed by an enhanced expression of pro-inflammatory molecules (e.g. TNF-α, IL-6, MCP-1) inhibiting insulin action. Nutrient overload could induce further molecular modulations in cellular organelles such as ER stress via UPR response or oxidative stress via ROS in insulin-sensitive cell, which finally also activate a pro-inflammatory signalling cascade and block insulin activity.

1.2.4 Endocrine function of adipose tissue

AT is no longer considered just as an inert energy storage reservoir, but as a real endocrine organ releasing, besides its classical metabolic products FFA and glycerol, also a large amount of bioactive molecules with paracrine and endocrine functions (Figure 6). In 1987, adipsin, a complement-related protein with serine protease activity, has been identified as one of the first proteins synthesized and secreted from the AT [85]. The theory about the endocrine function of the AT became apparent with the

discovery of leptin in 1994 [86], encoded in mice by the *ob* gene and in humans by the *LEP* gene, whose mutation was associated with the onset of obesity [87]. This discovery increased the interest of scientists to search for other substances released from AT in relation to obesity disorders. Today it is known that AT produces numerous bioactive peptides and cytokines, classified by the name adipocytokines [88] or adipokines [89], based on the site of their secretion, i.e. the adipocytes. However, recent studies determined that the majority of these molecules are also produced by the SVF cells [20, 21, 23], which changed the view upon the "adipocyte-centric" paradigm. Therefore, the classification of AT secreted proteins by the term adipokines with respect to the cellular origin might be confusing. There are only a few proteins (hormone-like proteins) that are almost exclusively secreted by the adipocytes, i.e. adiponectin and leptin. Other cytokines are produced by both adipocytes and the SVF cells. Nowadays, it has been characterized over 100 substances produced by AT with different functions [90, 91], including interleukins (e.g. IL-1, IL-1β, IL-6, IL-10) [92-95] interferons (e.g. IFN-β, IFN-γ) [96], TNF-α [97], chemokines (MCP-1, IL-8) [98-100], growth factors (IGF-1), peptides, such as resistin, apelin, visfatin, retinol-binding protein 4 (RBP4) [101-104], acute phase proteins (e.g. CRP, haptoglobin, SAA) [105-107] or very recently described vaspin, omentin, hepcidin [108-110]. It is expected that many of them are still waiting for their discovery or detailed functional analyses. Regarding the wide range of specific proteins, AT plays an important role in the integration of many physiological pathways. It is involved, besides energy homeostasis, in the thermogenesis, immune response, blood pressure control, haemostasis, neuro-endocrine regulation, bone marrow, thyroid and reproductive function [111]. Moreover, adipokines have been found to participate in the development of insulin resistance and obesity-related comorbidities ("humoral theory" about the origin of insulin resistance). It has been observed that their mRNA and protein levels are modulated in the obese state. Besides adiponectin, the release of the majority of pro-inflammatory cytokines in obesity is increased. However, as it has been summarized in the review of Klimcakova et al. [112], this fact is not confirmed in each clinical study. Because the AT is composed not only of adipocytes, but also of other cells, mainly macrophages contributing to the additional secretion of cytokines, it is important to determine the cellular origin of the production of these molecules. Adipokines participate also in the recruitment of immune cells, i.e. macrophages, lymphocytes into the AT that further contribute to inflammatory state in the AT and the induction of insulin resistance. In the following paragraphs I will only focus on the

description of the role of selected adipokines in relation to obesity, which were investigated in the clinical studies included in this work.

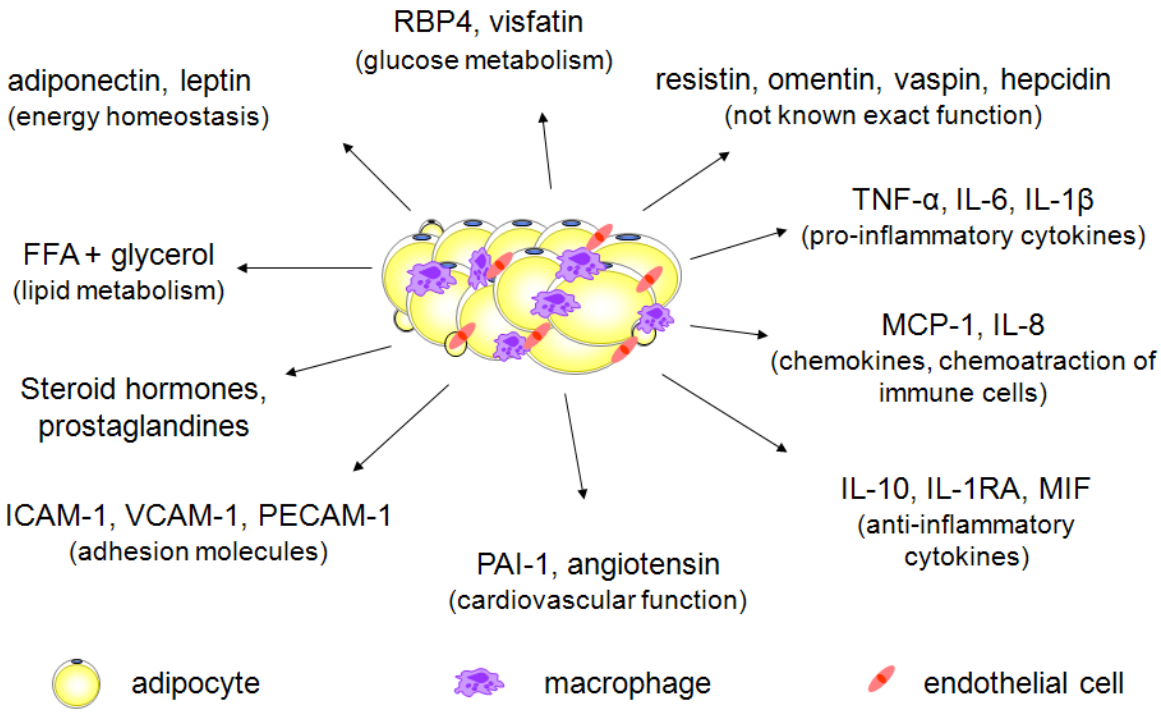

Figure 6 Adipose tissue as an endocrine organ. AT secretes a variety of bioactive molecules, such as hormones, cytokines, chemokines, FFA, which act in a paracrine or endocrine way and influence the physiology of AT as well as other tissues in the body. ICAM-1: intercellular adhesion molecule, VCAM-1: vascular cell adhesion molecule, PECAM-1: platelet/endothelial cell adhesion molecule, PAI-1: plasminogen activator inhibitor, MIF: macrophage inflammatory factor, MCP-1: monocyte chemoattractant protein, RBP4: retinol binding protein 4, TNF-α: tumour necrosis factor-α

1.2.4.1 Leptin

Leptin (originating from the greek word *leptos*, i.e. thin) is a 16kDa non-glycosylated peptide hormone, a member of the class I cytokine family, consisting of 167 amino acids. It is encoded by the *obese (ob)* gene in mice and by the *LEP* gene in humans [86]. It is mainly produced by adipocytes and in a limited proportion expressed by the stomach and placenta [113, 114]. Leptin binds to its receptor, encoded by the *db (*ObR*)* gene in mice and the *LEP-R* gene in humans. 6 different isoforms of leptin

receptor have been identified as a result of alternative splicing (ObRa, ObRb, ObRc, ObRd, ObRe, ObRf), which differ in the sites of their expression. The leptin receptors are localized in several organs and tissues, such as the liver, skeletal muscle, adipose tissue, heart, pancreas, hypothalamus [115, 116]. Leptin action is mediated mainly via the long isoform ObRb that subsequently transfers the signal via various pathway activating Janus kinase (JAK)/signal transducer and activator of transcription (STAT), MAPK, PI3K or AMPK [117, 118].

The main function of leptin is the regulation of food intake through its action, i.e. providing a satiety signal within the hypothalamus [119]. Moreover, it controls insulin sensitivity, glucose and lipid homeostasis through the activation of AMP-activated protein kinase (AMPK) in insulin-responsive organs [117] (Figure 7). Leptin is also involved in the energy homeostasis, neuro-endocrine control, reproduction, onset of puberty, or angiogenesis. It has been observed that in obese subjects circulating levels of leptin are higher and gender different (2-3 times higher in women than in men, even when adjusted for BMI)[120, 121]. The plasma level of leptin in humans is expressed in terms of ng/ml. It correlates with fat mass and BMI and decreases with weight reduction [92, 122-126]. These beneficial results are almost reached in each intervention study. Therefore, it is now used as a "reference adipokine" measured in weight loss programs. Mutations in leptin (*ob/ob* mice) or in leptin receptor in mice (*db/db* mice) induce obese phenotypes with increased appetite, weight gain and insulin resistance. In humans these rare genetic ablations cause early-onset morbid obesity [127] accompanied by hyperphagia, hyperinsulinemia and hyperleptinemia, which is contrary to the minimal plasma leptin levels in mice. Patients with this diagnosis can be treated with exogenous leptin, which decreases the detrimental effect of obesity[128]. However, in some of them this treatment fails. It might be explained by the development of "a leptin-resistant" state, which diminishes the effect of leptin on energy intake and expenditure [129]. Leptin resistance can lead to the activation of SOCS proteins that decrease the stimulation of leptin receptor in negative feedback of leptin signalling [130].

Besides its metabolic and endocrine function, leptin plays an important role in the modulation of the immune response. It has been observed that it has a protective effect on T lymphocytes from apoptosis, regulates T cells and monocytes activation, and induces the switching of immune phenotype toward Th1 response. Additionally, it can participate in an induction of oxidative stress and an up-regulation of adhesion

molecules in endothelial cells [131, 132]. Moreover, plasma leptin levels are elevated by inflammatory stimuli, such as IL-6, IL-1, LPS [133]. It has been revealed in *in vitro* studies that leptin contributes to increase the accumulation of monocytes into the AT[23, 134].

To summarize, it seems that leptin, besides its important role in the regulation of metabolic homeostasis, exerts also pro-inflammatory properties. Because of the "two-faced" (dual) character of this molecule, leptin represents a promising molecule that could contribute to the treatment of obesity.

1.2.4.2 Adiponectin

Adiponectin was discovered at the end of 20th century almost simultaneously by four groups and it was given different names, such as GBP28, apM1, Acrp30, or AdipoQ[135-138]. Adiponectin is encoded by the *apM1* gene and primarily produced by adipocytes, but also by skeletal muscle, cardiac myocytes and endothelial cells [139-141]. It is a 30 kDa protein structurally similar to the complement protein 1q, which is characterized by a N-terminal collagen-like domain and a C-terminal globular domain. After synthesis it undergoes various post-translational modifications necessary for its bioactivity [142]. Adiponectin is secreted from adipocytes into the circulation in different isoforms, i.e. the complexes of several sizes: trimer (also known as low-molecular-weight adiponectin (LMW), hexamer composed of two trimers connected by a disulphide bond (known as middle-molecular-weight adiponectin), and a high molecular weight (HMW) isoform consisting of up to 12-18 molecules [143, 144]. Adiponectin also exists in its cleavage form; known as globular adiponectin generated by leukocyte elastase [145] capable of trimerization, but not further polymerization. Each adiponectin complex exerts distinct biological functions. Recently it has been revealed that the HMW form has a more insulin-sensitizing effect and associates better with glucose tolerance than total adiponectin [143, 146, 147]. Moreover, the ratio HMW/LMW or HMW/total adiponectin has been suggested to be a good predictor of insulin resistance and metabolic complications [147, 148]. In respect to HMW, moderate weight loss or treatment with tiazolidindiones (TZD) selectively improves HMW levels in circulation [148, 149]. Adiponectin represents only 0.01% of all plasma proteins, but its normal plasma levels are higher compared to other adipokines (5-10 μg/ml). Contrary to almost all cytokines, adiponectin plasma levels are lower in obese

or insulin resistant subjects and inversely correlated with BMI and body fat [150-152]. Its production by adipocytes can be negatively regulated by various mediators, such as TNF-α, IL-6, insulin, glucocorticoids [153-155].

The distribution of adiponectin complexes into the circulation is controlled at the level of its secretion from the adipocytes. The assembling of adiponectin complexes is realized in the ER through its chaperones. This is a critical point in the formation of the biologically active protein. Nevertheless, it has been observed that the proportion of its particular isoforms is different between plasma levels and secretion media obtained from AT explants of obese patients [156]. There is also evidence that mutation in the adiponectin gene leads to the impairment of protein multimerization and decrease secretion into the circulation [157, 158]. All these aspects could participate in hypoadiponectinemia in diabetes, hypertension, atherosclerosis, or endothelial dysfunction [159, 160].

Adiponectin transfers its signal through two transmembrane receptors AdipoR1 and AdipoR2 [161]. AdipoR1 is preferentially expressed in the muscle, whereas AdipoR2 is predominantly expressed in the liver [162]. T-cadherin has recently been identified as a new molecule participating in the adiponectin signalling pathway, especially as a receptor for the MMW and HMW form of adiponectin [163]. Another member of this cascade is an adaptor molecule, called as an adiponectin receptor interacting protein (APPL1), which enhances the transduction of the signal downstream of the receptor and activates the AMPK and MAPK pathway [164], or peroxisome proliferator-activated receptor α (PPAR-α) [162]. Through the activation of AMPK (Figure 7), similar as leptin, adiponectin increases fatty acid oxidation in the skeletal muscle, GLUT4 translocation that leads to an enhanced insulin sensitivity [117]. Besides its beneficial metabolic functions, adiponectin also has an antiatherogenic and antiatherosclerotic effect [148, 163]. It inhibits the expression of adhesion molecules and scavenger receptors on monocytes [165]. Furthemore, it influences macrophage function by diminution of the expression of pro-inflammatory cytokines, such as IL-6, TNF-α and by increasing the production of the anti-inflammatory molecules IL-10, IL-1Ra [166, 167]. Adiponectin has also been observed to participate in the inhibition of macrophage transformation to foam cells, the formation o f atherosclerotic plaques, as well as macrophage infiltration to AT [168].

Taken together, adiponectin is one of the few molecules secreted by the AT with anti-inflammatory properties, which plays a key role in the modulation of the

inflammatory response and energy homeostasis in body. Thus it might be a target of new pharmacological therapy. However, it is still poorly understood how its secretion from adipocytes is regulated and what the reason for its diminished levels in obesity is.

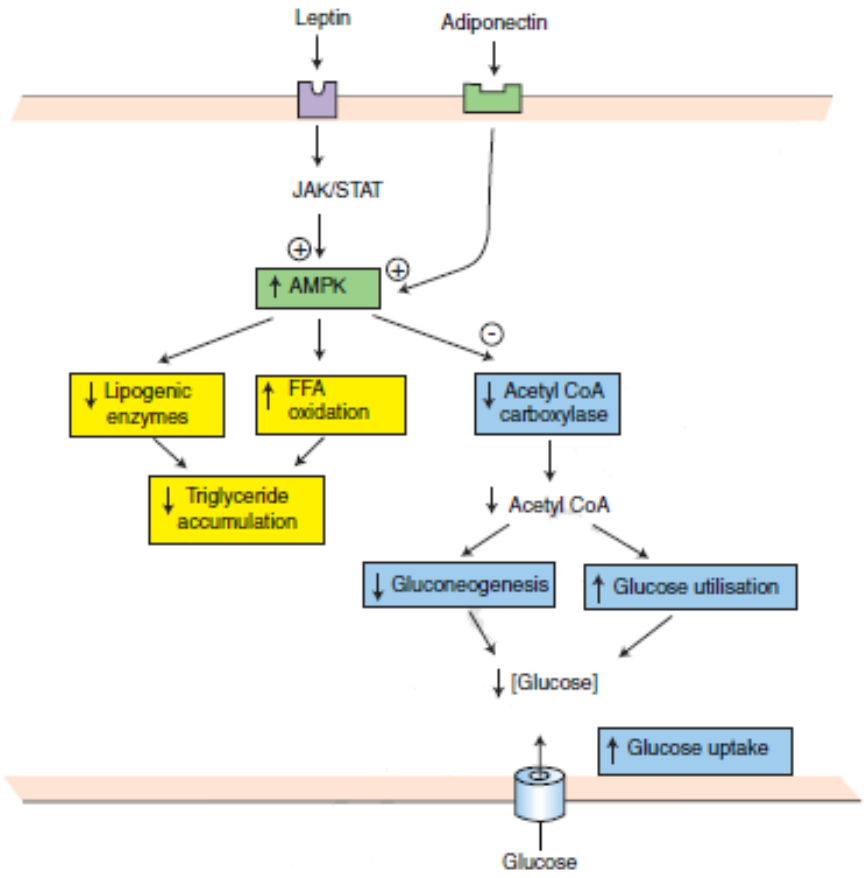

Figure 7 Role of leptin and adiponectin in the regulation of glucose and lipid metabolism through activation of AMPK, which downstream stimulates FA oxidation in the AT/muscle and glucose utilization in the liver (adapted from Langin et al., 2008, Obesity: Science to Practice)[19].

1.2.4.3 Tumour necrosis factor-α (TNF-α)

TNF-α was originally described as a cytokine inducing the necrosis of tumours after acute bacterial infection. Further, it has been identified as a factor inducing cachexia, also called cachectin in the catabolic disease and as a regulator of adipocyte metabolism, i.e. suppression of the expression of AT specific genes and lipogenesis[169, 170]. However, the first link between this pro-inflammatory cytokine and obesity came from a study of Hotamisligil in 1993, which revealed an increased

TNF-α expression in the AT of genetic obese (ob/ob) mice [97] leading to obesity-related insulin resistance. These findings led to the conception about obesity as a low-grade inflammatory condition.

TNF-α is a 26 kDa transmembrane monomer that is enzymatically cleaved to a 17 kDa soluble TNF-α molecule [171]. Both molecules are biologically active. The transmembrane form seems to exert autocrine and paracrine fuction, whereas the soluble molecule mediates endocrine effects [172]. TNF-α is produced by many types of cells, such as macrophages, lymphocytes, fibroblasts, and smooth muscle cells [173]. In AT it has been documented that the SVF cells (preadipocytes, immune cells) produce more TNF-α than adipocytes [20]. TNF-α mediates its signal in AT through two distinct transmembrane receptors: tumour necrosis factor alpha receptor 1 and 2 (TNFR1 and TNFR2) that after ligand binding trimerize. Both TNFRs, due to proteolytical cleavage, can be released to the circulation as soluble forms. They further activate other signalling molecules involved in the regulation of cell metabolism, such as cell differentiation (adipogenesis), proliferation, apoptosis of preadipocytes and adipocytes, as well as energy homeostasis [174-176]. Regarding obesity and IR, TNF-α inhibits insulin action through the activation of JNK or ERK phosphorylated Ser residues in IRS1 and NF-κB stimulating the expression of inhibitory molecules SOCS3, and generates increased lipolytic activity of AT leading to overload of FFA to the circulation that could further store in non-fat tissues, such as skeletal muscle, liver (ectopic storage of fat) and increase a risk of lipotoxicity [177-180]. TNF-α also down-regulates the expression of adiponectin, GLUT4, PPAR genes involved in glucose homeostasis [153, 181-183].

The role of TNF-α in the development of insulin resistance has been provided on mice lacking of TNF-α function, which were resistant to high fat diet-induced obesity and improved insulin sensitivity [184]. The similar association has been observed in humans by administration of anti-TNF-α antibodies[185]. Additionally, mRNA and plasma levels of TNF-α, as well as TNFRs, have been reported to be increased in obesity and type 2 diabetes compared to lean control and furthermore positively correlated with BMI and insulin levels [92, 185-187]. Weight loss was shown to decrease their productions [188, 189]. However, this expression profile is not always standard in each clinical study [112]. It could be explained by the fact that TNF-α is not secreted by AT into the circulation and therefore AT does not contribute directly to the increased plasma levels of TNF-α [190]. Maybe other cells e.g. macrophages circulating in plasma could contribute to elevated TNF-α production. Neutralization of TNF-α by

antibody increased glucose uptake in response to insulin in obese mice [97]. However, the treatment of obese, insulin resistant or diabetic patients with anti-TNF-α antibody did not lead to positive results [191, 192]. One of the possible explanations could be the fact that TNF-α does not play a dominant role in th einduction of insulin resistance and predominantly acts in paracrine way.

TNF-α is a typical pro-inflammatory cytokine that induces the production of other pro-inflammatory cytokines, such as IL-6, IL-1, IFNγ and also adhesion molecules, monocyte chemoattractant protein-1 (MCP-1) [21, 193]. It has been observed that it participates in a macrophage-induced inflammation in AT as a mediator of immune response to overload of FFA [76] and influences macrophage phenotype. Vice versa, the production of anti-inflammatory cytokines, such as IL-10, IL-1Ra, and adiponectin could diminish its effect. This interplay between molecules with different characters in AT leads to "the competition" of intracellular pathways that determine considering in which direction the cytokine production will be developed. Nevertheless, the triggers that induce the production of TNF-α in AT and its exact role in the onset of obesity require further investigation.

1.2.4.4 Monocyte chemoattractant protein-1 (MCP-1)

MCP-1, also known as chemokine (C-C motif) ligand 2 (CCL-2), belongs to the key factors mediating the chemoattraction and transendothelial migration of circulating monocytes and T lymphocytes to the site of inflammation via its receptor CCR2 on target cells. It exists in two predominant forms (9 and 13 kDa) due to different glycosylation with similar biological activity [194]. Its binding to the receptor activates the signalling pathway of PKC via formation of inositol triphosphate, release of intracellular Ca^{2+} and Rho family proteins leading to cell mobility [195]. MCP-1 is secreted by various cell types, such as fibroblasts, endothelial cells, vascular smooth muscle cells, monocytes and T cells [196]. Recently, it has been reported that MCP-1 is also produced by adipocytes and confirmed the findings about increased ATM infiltration during obesity [197]. However, it is important to note that the SVF cells contribute more to the production of MCP-1 than adipocytes [198]. Mice lacking MCP-1 or its receptor have a decreased macrophage recruitment in AT accompanied by the improvement of insulin sensitivity and are protected against weight gain induced by a high fat diet [199, 200]. Nevertheless, in another study this result has not been

confirmed [201]. Hence, it raises the suggestion that MCP-1 is not the only critical mediator for the macrophage recruitment. There are also other possible candidates, such as macrophage inflammatory protein-1α (MIP-1α) or osteopontin that might participate in this process [202, 203].

With respect to human obesity, it has been observed that obese patients have increased MCP-1 plasma levels compared to lean controls [98, 198] and after weight loss its circulating levels decreased [197]. Under normal conditions, however MCP-1 plasma levels are undetectable [91]. *In vitro* experiments on adipocytes documented that MCP-1 decreases insulin-stimulated glucose uptake and trigger leptin secretion [204, 205]. It has been determined that MCP-1 expression is regulated by TNF-α, IFNγ, platelet-derived growth factor (PDGF) or stress factors [206] via the activation of p38MAPK and NFκB pathways [207].

Furthermore, MCP-1 is involved in the recruitment of monocytes in the arterial wall and so contribute to atherogenesis [208]. Recently, it has been revealed that an MCP-1 polymorphism associated with a high risk for atherosclerosis [209]. Taken together, these findings suggest that MCP-1 could play an important role in the development of inflammatory diseases.

1.2.4.5 C-X-C motif chemokine 5 (CXCL5)

CXCL5, also known as epithelial neutrophil activating peptide (ENA-78), is a member of chemotactic cytokines sharing structural and biological features with IL-8 and Groα. It is involved in the chemoattraction of immune cells in response to inflammatory stimuli via its receptor CXCR2, which is expressed on various types of cells like endothelial, pulmonary, or intestinal epithelial cells [210, 211]. Recently, it has been reported that CXCL5 represents a novel adipocytokine mainly secreted by ATM, which participates in worsening of insulin action in muscle and is associates with obesity and type 2 diabetes [212]. Moreover, CXCL5 plasma levels are elevated in obese patients compared to lean subjects and decrease after VLCD. In addition, obese insulin-resistant subjects have higher serum levels of CXCL5 than non-resistant [212]. Increased levels of CXCL5 have been also observed in other infammatory states, such as rheumatoid artridis, Crohn`s disease, or pulmonary disease [211].

In vitro experiments revealed that CXCL5 inhibits insulin action in muscle via activating the JAK/STAT/SOCS signalling cascade [212]. Furthermore, in mice CXCL5

is up-regulated by TNF-α via NFκB stimulation and reduced by PPARγ agonist rosiglitazone [212]. Blocking of CXCL5 function through anti-CXCL5 antibody or antagonist to its receptor CXCR2 improved insulin sensitivity in obese mice. Moreover, CXCR2 receptor has been determined to be present in macrophages-rich intimae in atherosclerotic plaques and participate in the accumulation of these immune cells into lessions [213].

Altogether, it seems that CXCL5 contributes to the onset of insulin resistance and other obesity comorbidities. However, the exact mechanism how it acts and influences other insulin-sensitive organs it is not well known. What`s more, its association to the activation of macrophages has not been studied yet. Therefore, it could be an object of further investigations in this scientific field.

1.2.4.6 Visfatin/PBEF/NAMT

Visfatin, a 52 kDa protein, was originally isolated (1994) from B cell precursors as a cytokine enhancing its maturation, also called pre-B cell colony-enhancing factor (PBEF) [214]. A decade later it was discovered to be predominantly expressed in visceral AT, from which the name visfatin was devoted. It was reported to exert insulin-mimetic action via binding to IR at different site than insulin and enhance glucose uptake into cell [102] (Figure 8). This function has been observed in mice model. However, the subsequent studies in humans have not confirmed these findings and were partially retracted [215]. Nevertheless, this discovery stimulated a lot of researchers to study a role of visfatin in relation to metabolic complications. Recently, it has been shown that visfatin displays nicotinamide phosphoribosyltransferase (Nampt) activity essential for glucose homeostasis in pancreatic β-cell function [216]. Three different functions gave rise to three different names of unique protein.

With respect to visfatin production, besides AT it is also produced by skeletal muscle, liver, bone marrow, lymphocytes [217]. Especially in AT it is more expressed by SVF cells than adipocytes [218, 219]. Because of missing signal peptide in its structure visfatin is secreted from cell via non-classical secretory pathway.

In human studies, there have not been found any differences between two fat depots in visfatin expression as it was published in the initial study [219, 220]. Additionally, increased mRNA and plasma levels of visfatin have been observed in condition of obesity, insulin resistance and type 2 diabetes [220-222]. However, these

results are controversial in some clinical studies, which determined either increased mRNA or plasma levels in lean subjects or people with lower BMI [219, 223, 224]. Furthermore, it has been shown that weight loss in morbidly obese patients or exercise training in diabetic patients has positive impact on visfatin plasma levels [225-227]. According to the findings obtained in above mentioned studies, there are not unequivocally association between visfatin expression and BMI and insulin resistance. Based on results from *in vitro* studies, production of visfatin is regulated by several factors, such as IL-6, TNF-α, hormones [228-232]. However, there are also some discrepancies due to different types of experimental model used in the studies.

Regarding the visfatin action in glucose homeostasis, it has been shown that visfatin as a rate-limiting enzyme Nampt plays a key role in nicotinamide adenine dinucleotide (NAD) biosynthesis necessary for glucose-stimulated insulin secretion in pancreatic β-cells. This activation pathway involves SIRT-1 molecule, whose activity is reduced by age [216].

Recently, visfatin has been identified as an adipokine with immunomodulating properties because it induces chemotaxis, expression of IL-1β, TNF-α, IL-6 in human monocytes[233]. Moreover, it is strongly expressed in lipid-rich macrophages localized in atherosclerotic plaques, in which it enhances the expression of metalloproteinase-9 (MMP-9). Therefore, it is suggested that it may participate in plaque destabilization [234].

To sum up, visfatin works as intracellular enzyme, as well as extracellular cytokine. However, it is not clear if it acts as a pro- or anti-inflammatory molecule and what it is its role in the pathophysiology of obesity and insulin resistance.

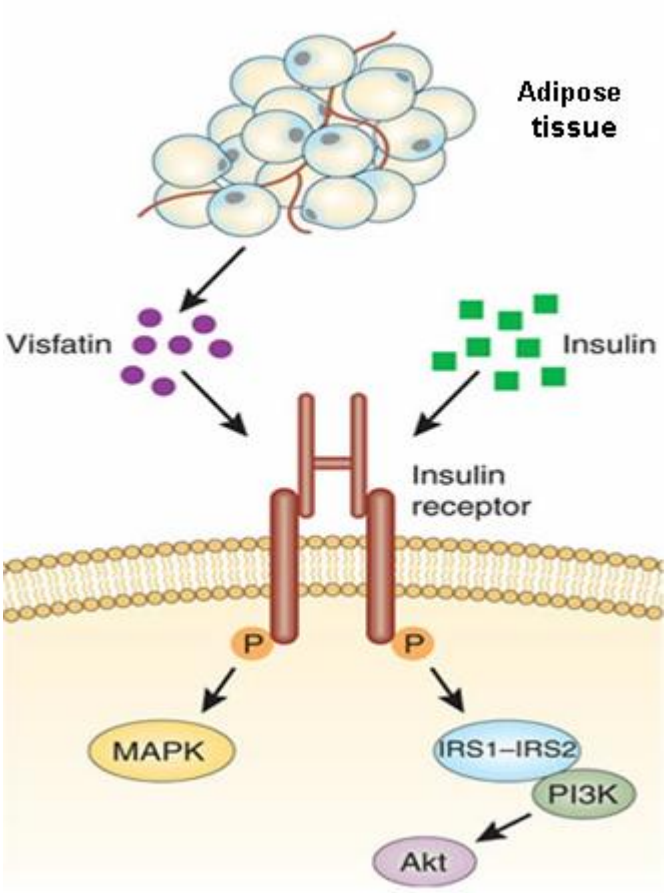

Figure 8 Role of visfatin in insulin signalling cascade (adapted from Murphy et al 2006, Nature Medicine 12: 32-33)[235]. Visfatin acts through IR receptor binding to the distinct site (different from insulin) and activates downstream signalling molecules.

1.2.4.7 Retinol binding protein 4 (RBP4)

RBP4 is another recently discovered adipokine (2005), which is connected with pathogenesis of obesity [236]. It has been originally identified as a 21 kDa transport protein for retinol (vitamin A) in circulation, encoded by the *RBP4* gene. The main source of RBP4 is liver, but it is also secreted by AT, especially by mature adipocytes [237, 238]. It has a binding partner, transthyretin (TTR) necessary for RBP4 renal clearance and regulation of its plasma concentration [239].

The first link between RBP4 and obesity-induced disorders has been determined in GLUT4 knockout mice (Glut4-/-), which had selectively increased expression of RBP4 in AT, but not in liver. In addition, transgenic overexpression or peritoneal injection of recombinant RBP4 in mice led to the development of insulin resistance.

Moreover, genetic ablation of RBP4 in mice (Rbp4-/-) improved insulin sensitivity[236]. Based on these animal studies it seems that GLUT4 impairment in adipocytes induces enhanced release of RBP4 into circulation (Figure 9). It was also confirmed in human study of Graham et al (2006), in which GLUT4 expression in AT was inversely correlated with RBP4 plasma levels [103]. Nevertheless, this finding has not been confirmed in each investigation [237]. Notably, increased RBP4 plasma levels activate hepatic expression of a retinoid-regulated gene, phosphoenolpyruvate carboxykinase (PEPCK) inducing gluconeogenesis in liver that further leads to increased glucose in plasma with negative consequence on insulin sensitivity in muscle [236]. The mechanism, how RBP4 influence insulin action may involve retinol-dependent binding of active retinol forms to retinoic acid receptors (RARs) and retinoic acid-X receptors (RXR) to regulate the expression of specific genes (e.g. PEPCK in direction of insulin resistance or PPARγ in favour of insulin sensitivity) or retinol-independent way via cell surface receptors, such as Megalin/gp320. *In vitro* experiments on human adipocytes have revealed that RBP4 inhibits the IRS-1 activation by phosphorylation at Ser307 [240].

In humans, increased circulating levels of RBP4 have been observed in subjects with insulin resistance, obesity, type 2 diabetes or non-alcoholic fatty liver disease [103, 236, 241-243]. In opposite to this, there are some studies, which did not find similar results [237, 244]. In some human studies it has been found positive correlation between plasma RBP4 and insulin resistance [103, 243, 245], whereas it has not been observed in other papers [237, 246-249]. Exercise training in insulin resistant subjects, weight loss induced by dietary intervention or gastric banding in morbidly obese subjects improves plasma levels of RBP4 [103, 250, 251]. Normalizing of circulating levels of RBP4 can be also reached by TZDs [236]. However, small decrease of weight loss did not reach positive effect on RBP4 production[237]. These discrepancies in the human studies could be caused by using different methods for the assessment of insulin sensitivity, different techniques for the measurement of RBP4 concentrations in plasma [103], or the composition of intervention group of subjects.

Besides its association with adiposity and insulin resistance, RBP4 has been also found to correlate with macrophage markers suggesting its possible implication in obesity-induced inflammation [249]. Recently, it has been demonstrated that people with *RBP4* haplotype are more susceptible to the development of type 2 diabetes [252, 253].

Based on these findings, it is clear that RBP4 participates in regulation of glucose homeostasis in body and can be a target for diabetic pharmaco-therapy. However, its physiological function in the development of insulin resistance in human is uncertain and needs further studies.

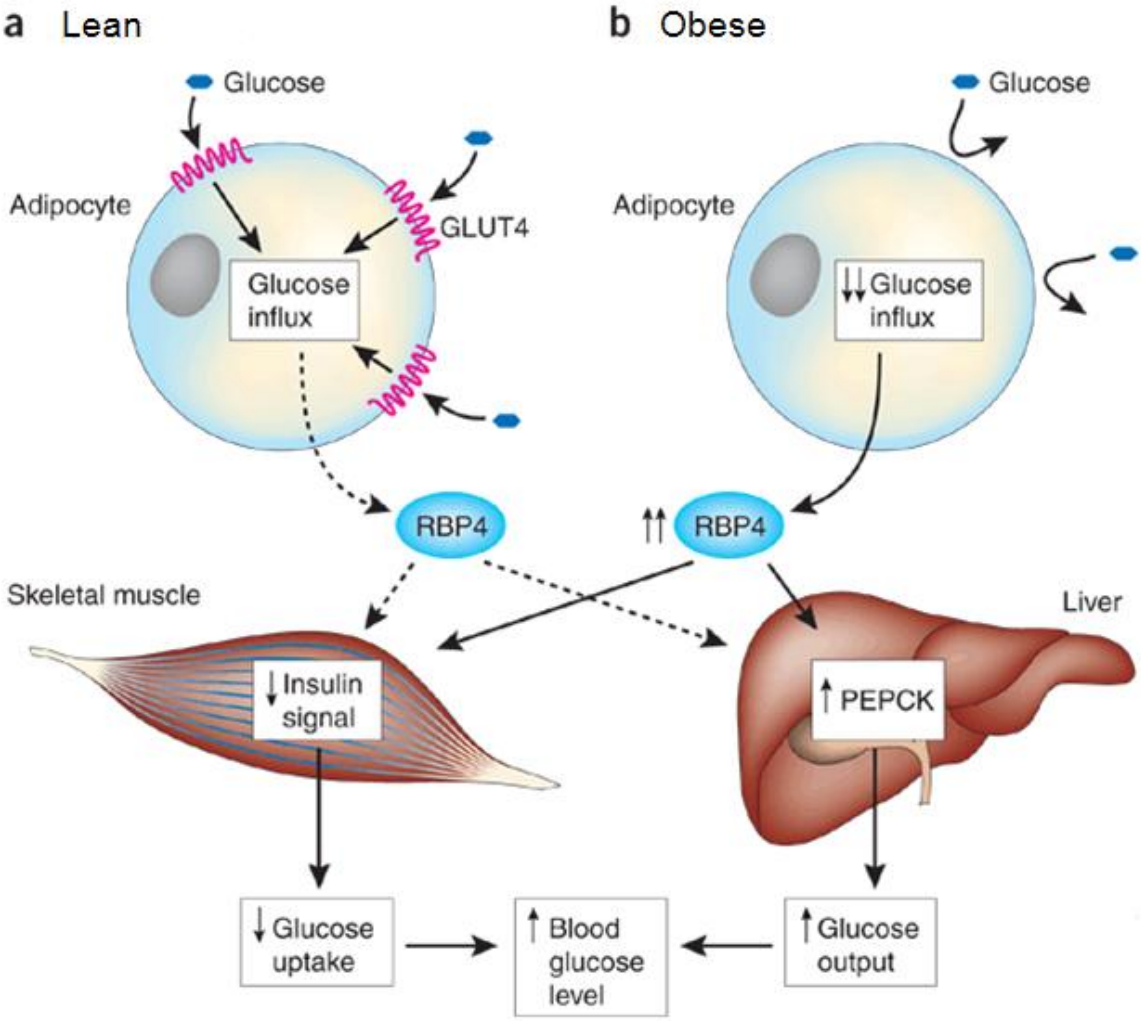

Figure 9 Role of RBP4 in the regulation of glucose homeostasis (adapted from Tamori et al. 2006, Nature Medicine 12: 30-31)[254]. A) In lean subjects, glucose is utilized via GLUT4 in the adipocytes and RBP4 secretion by AT is low. B) In obese subjects, glucose utilization into AT is decreased due to an inhibition of the insulin action leading to an increased secretion of RBP4. Consequently RBP4 diminishes the glucose uptake by the skeletal muscle and increases gluconeogenesis in the liver by stimulation of PEPCK expression. All these actions cause an elevated glucose level in the blood contributing to obesity-related complications.

1.3 OBESITY-INDUCED INFLAMMATION

Nowadays, obesity is characterized as a chronic low-grade inflammatory state of the body accompanied by an increased production of inflammatory bioactive molecules, such as cytokines (TNF-α, IL-6), chemokines (MCP-1, IL-8), and acute-phase proteins (C-reactive protein (CRP), serum amyloid A (SAA), haptoglobin). Since inflammation and metabolic disorders share the same signalling mediators, it gave rise to a new term for this condition i.e. "metaflammation" (metabolically-induced inflammation) [255]. The first note of the association between obesity, insulin resistance and inflammation in AT came from 1993, when Hotamisligl and his colleagues determined an increased expression of cytokine TNF-α in the AT of obese rodents and its negative effect on insulin sensitivity [97]. Ten years later, two research groups of Weisberg and Xu independently identified an elevated infiltration of macrophages in the AT of obese mice and humans and their contribution to worsen the AT metabolic activity [26, 203]. Recently, it has been revealed that other immune cells, such as lymphocytes and neutrophils also recruit into the AT and compete in the machinery of an inflammatory response inducing changes of morphology and physiological function in target tissues and their activation states in relation to obesity [24, 25]. Moreover, these immune cells appear to be a major source of increased levels of pro-inflammatory molecules in the circulation [20, 21]. It has been suggested that hypertrophied adipocytes or dying adipocytes releasing FFA into circulation could attract immune cells into AT by the expression of various chemoattractants (MCP-1 or IL-8). Cinti et al. [256], using immunohistochemical analysis, demonstrated the presence of AT macrophages (ATM) accumulating around dead or dying adipocytes and building so-called "crown-like structures". However, FFA themselves can serve as a common agent for an immune, as well as a metabolic response [255]. In obesity, due to abnormal lipolytic activity of AT, the FFA flux increases in the circulation and the FFA bind to toll-like receptors (TLRs, e.g. TLR4) on the adipocytes that downstream activate inhibitory Ser kinases JNK or ERK influencing IRS-1. In parallel with this mechanism, TLRs can also activate an inflammatory signalling pathway through IκB kinase (IKK-β), which further stimulates the transcription factor NF-κB subsequently inducing gene expression of other pro-inflammatory molecules, e.g. TNF-α [76, 257] enhancing lipolysis (Figure 10). Studies inhibiting JNK or IKK function indicate their central role in the insulin signalling pathway [258-260]. This complicated scheme of production of molecules with a different character triggers a vicious circle between immune and metabolic system, i.e.

adipocytes and macrophages. Additionally, MCP-1 and CRP have been also reported to induce Ser phosphorylation of IRS-1 [261, 262]. Regarding the crosstalk between these two types of cells, macrophages are very similar to adipocytes in term of lipid metabolism and secretion activity [263]. They share functions with adipocytes; e.g. macrophages are capable of lipid droplet accumulation and adipocytes can produce most of cytokines, such as TNF-α, IL-6. These two cell types express similar gene products e.g. FABP, perilipin, PPARγ, liver X receptor (LXR), matrix metalloproteinase (MMP) [264, 265] necessary for realization of their functions (Figure 11). The macrophage function, which consists of taking up lipids from the circulation and participating in cholesterol metabolism, is associated with atherosclerosis. It has been shown that activating pathways through TLRs inhibits LXR action and enhances the accumulation of cholesterol in macrophages leading to the formation of foam cells as a consequence of atherosclerotic complications. These evidences confirm the hypothesis about the integrative crosstalk between the immune and metabolic system.

In response to nutrient overload (glucose and lipids imbalance), other molecular processes could be stimulated within the insulin sensitive cell, such as ER, oxidative stress, or hypoxia, which contribute to the onset of obesity-induced inflammation via activation of pro-inflammatory NF-κB or Ser kinases [266]. Oxidative stress is caused by an inappropriate mitochondrial function to transform the excessive energy flux into the ATP molecule (during FA oxidation) leading to the accumulation of ROS and other metabolic intermediates e.g. DAG and ceramides having a detrimental effect on insulin action [267, 268]. ER stress is evoked by an accumulation of unfolded or misfolded proteins or facing to the environment with increased energy perturbation and toxic signals that also gives rise to ROS as a by-product [269, 270]. Regarding the ER activity, it might act as a sensor of metabolic stress, which translates into inflammatory response [79, 271]. Hypoxia is induced by an insufficient supply of oxygen to the tissue and by nutrients that consequently activated expression of hypoxic-inducible genes via the hypoxic inducible transcription factor (HIF) [272, 273]. It is important to highlight that all insulin-sensitive tissues like AT, muscle and liver are exposed to the condition of metabolic stress in obesity and so commonly contribute to systemic inflammatory state of the body.

The perspectives for therapy of obesity-related complications could be anti-inflammatory approaches. Chemicals targeting a single inflammatory molecule e.g. anti-TNF or anti-CCR2 (chemokine C-C motif receptor of MCP-1) have beneficial

results in animals, whereas their effect in humans is limited [191, 274]. Contrary to this application, the treatment with the recombinant anti-inflammatory molecule IL-1Ra in diabetic patients had a positive effect on glucose plasma levels and the improvement of insulin sensitivity [275]. Another category represents medicaments, such as salicylates, statins and TZDs, which have been shown to blunt the immune response in the body. However, their direct mechanism on the production of inflammatory cytokines in vivo is not clear yet [276, 277]. On the other hand, they could represent another possible tool in the treatment of the inflammatory state during metabolic disorders.

The big challenge in this field could be “an organelle therapy” directing to dysfunction in a particular organelle, i.e. the mitochondria or ER. It has been suggested that the use of chemical chaperones in obese mice leads to an increase of proper protein folding in the ER and an improvement of insulin sensitivity [278]. It is important to note that in applying prospective therapeutics with a beneficial outcome, one should not only look at a single targeting molecule, but also at the integrative implication [255].

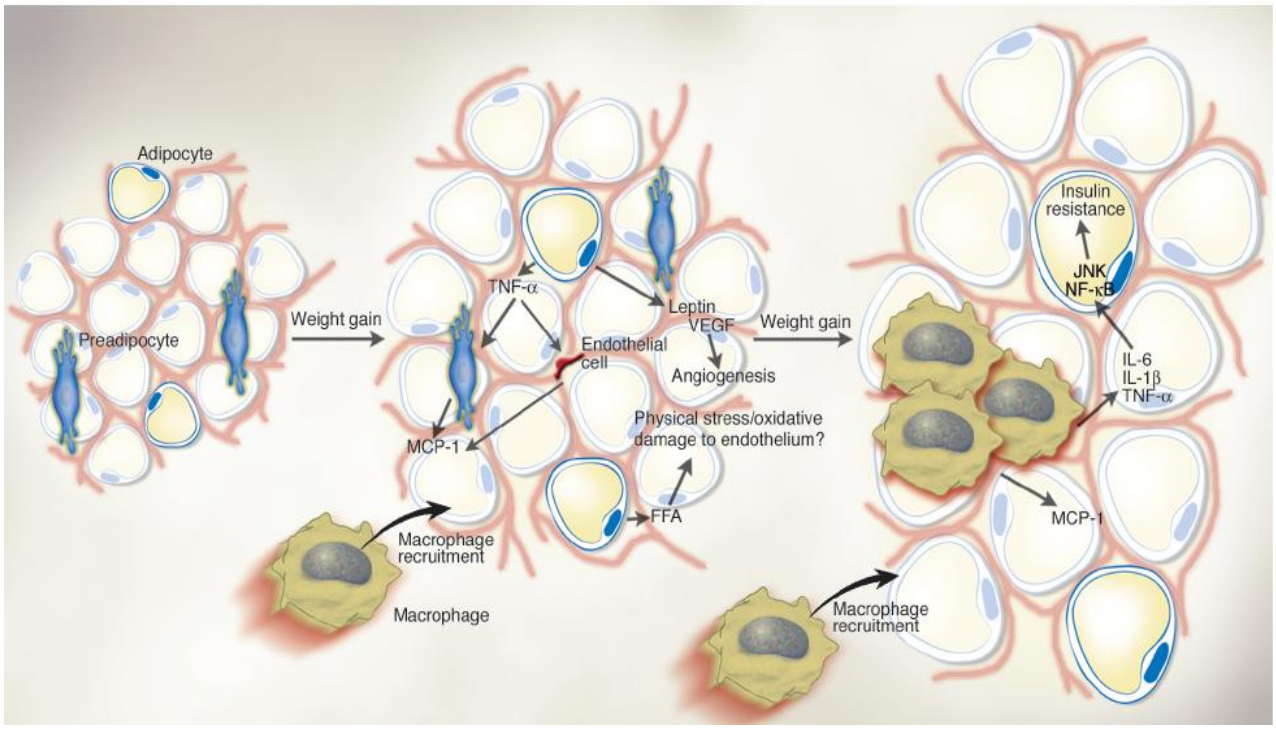

Figure 10 Macrophage-induced inflammation in obesity (adapted from Wellen et al. 2003, JCI 112: 1785-1788)[279]. During a long-term expose to nutrient overload, hypertrophied adipocytes release increased levels of pro-inflammatory molecules e.g. TNF-α, MCP-1 into the circulation that attract macrophages into the AT. ATM further stimulate inflammatory signalling pathways via NF-κB and JNK which further induce insulin resistance in the AT.

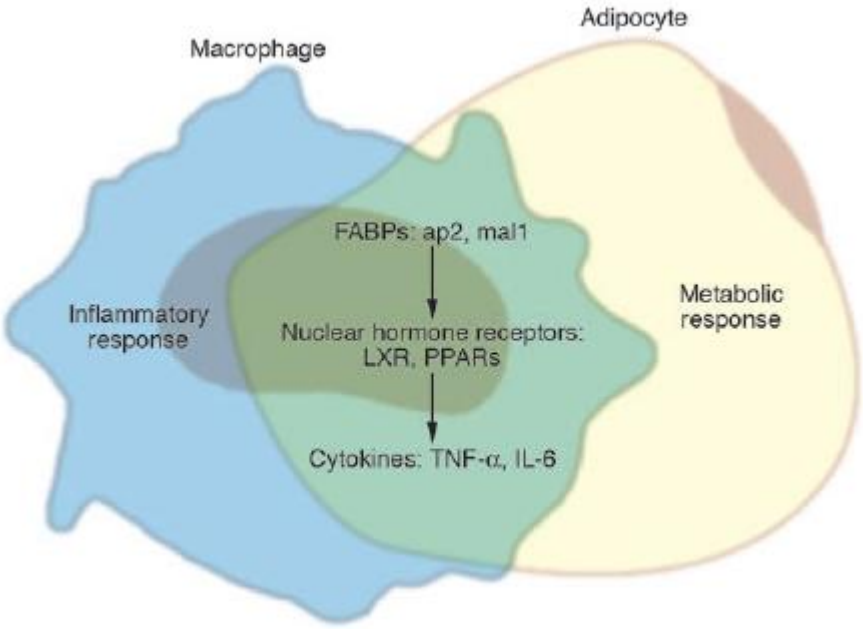

Figure 11 Interplay between adipocytes and macrophages through common metabolic and inflammatory mediators (adapted from Wellen et al. 2005, JCI 115: 1111-1119)[263]. They share similar functions, i.e. lipid metabolism and cytokine production that contribute to obesity-induced complication, such as insulin resistance. They are capable of lipid uptake due to FABP proteins (e.g. aP2, mal1), as well as secretion of inflammatory molecules (TNF-α, IL-6) via activation of transcription factors, such as LXR, PPAR.

1.3.1 Macrophages and their phenotype in relation to obesity

The attention of scientists to study macrophages in relation to different metabolic complications has been raised after the discovery in 2003 that obese AT is infiltrated by increased number of this kind of immune cells [26, 203]. These results have been subsequently confirmed in many animal and human studies [23, 280-284]. Moreover, the ATM content has been positively correlated with BMI and increased cytokine plasma levels [23, 281]. As it was mentioned above, ATM participate in the development of insulin resistance due to cytokine production and activation of inflammatory signalling pathways. The disruption of IKKβ or JNK1 gene in myeloid cells of obese mice protected against diet-induced obesity and insulin resistance [258, 285].

The general function of macrophages in the site of inflammation is to provide an immediate defence against a pathogen and to clear cellular debris by phagocytosis. Using flow cytometry analysis it has been demonstrated that the ATM content represents around 11% of the SVF cells isolated from AT of obese patients[23]. ATM differentiate from bone-marrow derived monocytes circulating in the blood capable of migration into the tissue in response to inflammation. However, recent evidences have revealed also other sources of ATM; they could be differentiated from preadipocytes [286] or are able themselves to proliferate *in situ* [280, 287]. Macrophage-induced inflammation could be realized through "a two hit process", in which tissue macrophages increase cytokines production leading to subsequent activation of inflammatory pathways in neighbouring cells (adipocytes in AT, hepatocytes in liver)[266].

The stimuli of ATM infiltration into insulin-sensitive tissues are not clear. Possible determinants of their recruitment might be: the size of adipocyte, altered adipokine secretion, FFA flux, local hypoxia, or nutritional status [134, 288]. Adipocyte size (altered AT morphology) seems to be the main integrative factor in the AT inflammatory cascade that builds a complex network amongst all these mediators. In obesity, larger (hypertrophied) adipocytes produce increased levels of cytokines and chemokines (MCP-1) attracting circulating monocytes into the tissue compared to smaller cells [289, 290]. *In vitro* experiments have revealed that leptin, SAA, MCP-1 enhance ATM infiltration into the AT, whereas adiponectin has the opposite effect [23, 218, 291, 292]. Indeed, the deletion of MCP-1 (or CCL-2) and its receptor CCR-2 in mice reduced the ATM content in AT and improved insulin sensitivity compared with wild type mice [199, 200]. Recently, a new chemokine, osteopontin has been shown to contribute to the infiltration of new macrophages into the AT [202, 293]. Furthermore, a long-term AT hypertrophy is accompanied by cellular physiological changes, such as hypoxia, ER stress or cell death, which all may participate in ATM recruitment. Immunohistochemical analysis has localized ATM around large dead or dying adipocytes suggesting the scavenge cell debris and lipid droplets [256, 283]. An increased FFA release from hypertrophied adipocytes into the circulation may also mediate the inflammatory response via TLR4 signalling pathway [257]. The ATM recruitment is very dynamic process that could be affected by the nutritional status as well. Weight loss induced by bariatric surgery in morbidly obese subjects decreased the

number of ATM accompanied by down-regulation of pro-inflammatory genes[281, 294].

Macrophages are a heterogeneous cell population with a high plasticity to differentiate into various phenotypes according to stimuli coming from the microenvironment [295, 296]. From a wide range of characterized macrophage phenotypes, the most frequently described are "classical activated" macrophages, also known as M1, producing pro-inflammatory cytokines and "alternatively activated" macrophages, known as M2, secreting anti-inflammatory cytokines. Besides the production of cytokines, they differ in function, expression of cell surface markers and production of chemokines (Table 3) [295, 297, 298]. M1 macrophage polarization is induced by pro-inflammatory signals and realizes a pro-inflammatory response in the tissue accompanied by increased reactivity to FA and LPS, while M2 macrophage polarization is mediated by anti-inflammatory stimuli and participates in an immunosuppressive response and tissue remodelling [295]. Therefore, it is important to know not only the total number of macrophages in the tissue, but also their phenotype in relation to pathobiological disorder. Two hypothesis have been suggested about changes of ATM phenotypes during obesity, either it is due to shift in ATM polarization or switch in phenotype of the same cell. In mice using macrophage pulse-labelling studies it has been revealed that macrophages resident in AT, positive for cell surface markers F4/80+ and CD11b+ display anti-inflammatory behaviour, while new recruited macrophages during high fat diet characterized as F4/80+, CD11b+ and CD11c+ positive cells are more pro-inflammatory [299, 300]. Regarding these results, the ratio M1/M2 phenotypes in AT during obesity appears to be changed in favour of pro-inflammatory M1 subtypes which confirms the hypothesis about a shift in macrophage polarization. However, information about ATM phenotype in humans is not so clear. Recent cross-sectional studies have characterized that human ATM display "an intermediate" phenotype able to express both pro- and anti-inflammatory genes [280, 284]. It suggests that human ATM are not strictly polarized in either M1 or M2 and could be modulated in relation to stimuli from microenvironment (e.g. PPARγ is a mediator of M2 polarization [301, 302]). *In vitro* experiments described that macrophages are able to repolarise after differentiation and change their expression profile [303]. With respect to characterization of ATM phenotype, there are some discrepancies between mice and humans. In contrast to the mice studies, human ATM do not express F4/80 and inducibile nitric oxide synthase (iNOS) which are typical

markers for M1 phenotype in mice [134, 280, 304] and are characterized by expression of cell surface markers CD14+/CD206+ [280, 284]. Reflection of ATM phenotype to weight loss has recently been demonstrated in study of Aron-Wisnewsky using immunohistochemistry analysis [305], in which the ratio M1/M2 phenotypes changed in behalf of the M2 subtype 3 months after gastric surgery. However, it is not known how moderate weight loss could influence the ATM phenotype in human.

Moreover, the exact role of macrophages in AT is also not well-known. In vitro studies reported that ATM participate in tissue remodelling and angiogenesis via the stimulation of endothelial cells migration into AT which in turn build a capillary network. In addition to these effects, they also modulate the proliferation of preadipocytes and inhibit adipocyte differentiation [280, 306-308]. In respect to these findings, it is hypothesized that ATM affect AT plasticity and reduce the accumulation of FA into AT that indirectly participate in ectopic accumulation of fat in the liver, muscle and pancreas leading to lipotoxicity and IR [266]. Varma et al. (2009) observed that macrophage infiltration into intermuscular AT in the skeletal muscle was associated with BMI and IR complications [309]. Contrary to the skeletal muscle, the liver is not infiltrated by new recruited macrophages [26, 203]. On the other hand, resident macrophages in the liver, i.e. Kupffer cells are capable of an inflammatory activation related to fatty liver diseases [258].

To summarzite, ATM play a key role in the development of obesity-induced inflammation. However, their biology in AT and stimuli for their recruitment into AT remain to be analyzed, mainly in humans. Regarding the distinct phenotype of ATM, it seems to be promising strategy to regulate the inflammation in AT. Nevertheless, it is still not well-known the ATM characterization in human in relation to different physiological status.

	Classically activated as "M1"	Alternatively activated as "M2"
Function	Antibacterial, defence against pathogen, removal of necrotic cells	Immunosupresive, Tissue repair, remodelling, Removal of apoptotic cells
Cytokines	TNF- α, IL-6, IL-12	IL-10, Il-1Ra
Chemokines	MIP-1α	AMAC-1
Cell surface markers	FcγRI (CD64) FcγRII (CD32) FcγRIII (CD16)	Mannose receptor CD206 CD163 (RM3/1) Scavenger receptor I
Killer molecules	iNOS	Arginase
Activating signals	LPS, IFNγ, TNF-α	IL-4, IL-10, glucocorticoid

Table 3 Characterization of classically and alternatively activated macrophages in mice (adapted from Goerdt et al 1999, Immunity 10: 137-142 and Mosser et al 2003, Journal of Leukocyte Biology 73: 209-212)[297, 298]

1.4. DIFFERENT ADIPOSE TISSUE DEPOTS IN RELATION TO OBESITY-RELATED COMPLICATIONS

The impact of body fat distribution in relation to obesity-related complications was first time highlighted by Jean Vague in 1947, who declared an important role of central (android) obesity [310]. This finding has been further confirmed by many etiological studies associating central obesity with metabolic complications, such as insulin resistance, type 2 diabetes, cardiovascular diseases and atherosclerosis [311-313]. In addition, dysfunction of fat distribution, lipodystrophy is also accompanied by complications of insulin resistance and lipotoxicity because of inappropriate function of the AT to produce adipokines and store TAG [314].

The total body fat is usually subdivided into two main categories i.e. subcutaneous and internal AT that are further specified according to their anatomical location. However, this classification seems to be confusing, when the physiological function of particular fat depots is also considered [315]. Subcutaneous AT (SAT) consists of truncal, abdominal, gluteo-femoral and mammary fat depots. Internal fat includes visceral (VAT) and non-visceral AT. A special category of VAT represents

ectopic AT localized in non-fat tissues, such as muscle, liver, pancreas, heart. SAT is responsible for 80% of the total body fat mass compared to 20% of internal AT [316]. With respect to fat classification in relation to trunk location, there are often used categories the lower-body fat (SAT mainly gluteo-femoral depot in lower body region) and the upper-body fat (SAT and VAT in upper body region) [311]. Generally, the fat mass accounts for 10-20% of the total body weight in lean males and for 20-30% in females. In obesity, increased TAG accumulation leads to fat mass expansion, including VAT in the abdomen, which has been considered as a main contributor to central (abdominal) obesity and represents a risk factor for the development of most metabolic and cardiovascular diseases. A greater VAT accumulation has been observed in men than women [317]. Moreover, an ectopic accumulation of TAG in the liver and muscle increases the lipotoxic effect of fat and induces IR. Contrary to VAT, the gluteo-femoral depot seems to have protective effect against metabolic disorders [318]. Recent findings have demonstrated that gluteal AT is "metabolically silent" in terms of FA metabolism compared to abdominal SAT [319].

One possible explanation for the association of VAT with metabolic disorders could be the hypothesis, known as "portal theory". It explains the contribution of intra-abdominal fat to the onset of insulin resistance via an increased release of FA and other secretory molecules through the portal vein directly into liver [320-322]. It has been reported that VAT is more lipolytically active (after stimulation with catecholamines) and less sensitive to antilipolytic effect of insulin than SAT. Contrary to the higher lipolytic activity in VAT, the expression profile of some lipolysis-related genes (lipases) is not uniform in each study. Furthermore, VAT also differs from abdominal SAT in terms of morphology, production of adipokines, as well as macrophage-induced inflammation [313, 323, 324]. VAT compared with SAT is more cellular, vascular, contains more immune cells, has less preadipocyte differentiating activity, a greater capacity to take up glucose and produces more inflammatory molecules. However, there are also some discrepancies in these findings. In some studies, no significant distinction was observed between these two depots [26, 284, 325, 326] or the results regarding some adipokines (e.g. visfatin, adiponectin, the chemokines necessary for the migration of immune cells into the tissue) were not consistent [198, 325]. Regarding the size of VAT adipocytes, it has been determined that VAT fat cells are smaller than SAT which is paradoxical in relation to the greater production of adipokines by VAT [21, 311, 317, 327, 328]. It could be explained by the VAT response to lipolytic signals that induce an

enhanced metabolic activity of this depot. The differences between SAT and VAT with respect to adipokines are summarized in Table 4, which I discussed in this work. All these facts about the greater metabolic and secretory activity of VAT suggest its key role in the pathophysiology of weight gain.

According to the current knowledge in the literature, the mechanism how VAT contributes to the development of metabolic disorders, it is not completely clear. Further studies are necessary for understanding the regulatory machinery in regional fat depots. Despite of a small area of VAT in proportion to total body mass it is abnormally active and may be used as a predictor of a higher risk for metabolic complications.

	Subcutaneous	**Visceral**
Morphology		
Size of adipocytes [317, 327, 328]	Larger	Smaller
Adipogenesis [329, 330]	↑	↓
Lipolysis		
HSL [331, 332]	↑↓	↑↓
LPL [331, 333]	↑↓	↑↓
β-adrenoreceptors [334-336]	↓	↑
α -adrenoreceptors [335, 336]	↑	↓
Adipokines		
Leptin [337, 338]	↑	↓
Adiponectin [339, 340]	↓↑	↑↓
TNF-α [326]	↔	↔
Visfatin [102, 219, 220, 257]	↓↔	↑↔
RBP4 [243]	↓	↑
Chemokines [198, 325]	↔↓	↔ ↑
Macrophage-induced inflammation [23, 26, 284, 341]	↓↔	↑ ↔

Table 4 Comparison of subcutaneous and visceral adipose tissue in relation to the expression of adipokines, bioactive molecules related to the lipolytic activity and macrophage-induced inflammation.
(↑ higher levels, ↓ lower levels, ↔ no differences)

1.5. OBESITY MANAGMENT (TREATMENT OF OBESITY)

Obesity is a multifaceted disorder, which represents a high risk for many metabolic and cardiovascular diseases associated with an increased burden on the health care system and consequently affects the quality of life. Hence, it needs appropriate obesity management to solve this world-wide problem that is not an easy task. It is a big challenge for lifelong period. The main goal of obesity management is to efficiently reduce the body weight, preferentially intra-abdominal AT leading to a decreased risk of mortality associated with health complications [342].

The typical strategies for obesity treatment are divided into 3 categories: non-pharmacological (diet and increased physical activity), pharmacological (anti-obesity drug treatment) and surgical (e.g. gastric banding). The non-pharmacological therapy is accompanied by cognitive behavioural intervention and psychological support [342]. The two last strategies are used in case of inefficient results with non-pharmacological treatment in morbidly obese, and some cases, obese patients. For overweight and obese patients, lifestyle modifications, such as changed dietary habits and increased physical activity, are primarily recommended to achieve weight loss. It has been revealed that combination of dietary intervention (DI) and exercise training reached greater improvement of the clinical parameters than each intervention alone [343, 344]. It is not necessary to achieve a normal or ideal weight that is rarely gained in practise. The results from epidemiological studies have demonstrated that the modest weight loss around 5-10% of the initial body weight is in most cases efficient to bring the appropriate health benefit [8, 345, 346].

Today's diet is shifted towards the intake of energy-dense foods high in fat and sugar, but low in vitamins, minerals and fibers. From the dietary macronutrient, glucose and fat serve as major sources of energy for the body; secondly are the proteins, which have, however, the highest satiety feature and could decrease food intake through stimulation of the central nervous system [347]. In respect to glucose postprandial kinetics, the carbohydrate-containing food is categorized according to the glycaemic index (GI) [348] into food with low GI inducing low postprandial glycaemia and prolong the feeling of satiety and food with high GI causing a rapid glucose release into the circulation leading to an increased food appetite. Therefore, in design of DI it may be relevant to avoid the consumption of food with a high GI and keeping balanced nutrient distribution during all day. However, the components of the ideal diet are still a matter of discussion. The several types of DIs used in the obesity management are

shown in Table 5. Among short-term DIs we distinguish very-low calorie diet (VLCD), low calorie diet (LCD) and hypocaloric balanced diet. VLCD is usually recommended for patients with BMI> 35, but it could also be included in multi-phase programs followed by a weight maintenance period for reaching a greater weight reduction. LCDs are subdivided according to the macronutrient content into low-fat or low-carbohydrate (also called as Atkins diet). They have been involved in many clinical studies [122, 349-355]. In addition, it should be mentioned that the type of dietary fat (saturated, monounsaturated, polyunsaturated (PUFA)) also influence a risk of metabolic and cardiovascular diseases. A positive effect of n-3 or n-6 PUFA consumption on health benefit has been shown [356-358]. Further, the diet with a higher content of fibers and low fat was efficient in weight loss and the improvement of metabolic complications [359]. However, no definite conclusion has been achieved about which type of diet is the most appropriate in obesity management. Most studies concluded that the total energy intake, but not the macronutrient component determines the beneficial effect (improvement of insulin sensitivity, decrease of inflammatory and cardiovascular risk) of low energy diets during short-term period [122, 350, 351, 353, 354].

Regarding the responsiveness of different fat depots to lifestyle modifications, there are some weight loss inducing programs that preferentially reduce VAT depot, such as exercise, caloric restriction [360, 361]. There is no specific borderline defined for the necessary amount of reduction in visceral depot that is needed to obtain favourable metabolic changes. However, it is known that most of VAT is reduced during the first 2 weeks of caloric restriction [362]. It has been reported that VAT is more sensitive to weight reduction than SAT [363].

Besides weight loss, another important goal of obesity management is to maintain weight loss and prevent the weight regain. Less than 10% of those who underwent DI could sustain their weight loss [32]. Recently, it has been suggested that repeated periods of weight gain and weight loss known as weight cycling or the “yo-yo” effect could evoke a more hypoxic condition in the AT leading to enhanced pro-inflammatory response. Weight cycling is often the consequence after a DI program with more difficult period to reduce body weight again [364]. Additionally, it is hypothesized that weight cycling might have higher impact on mortality than keeping the same body weight [365, 366]. Hence, a long-term DI (at least 6 months) including a weight maintenance period should be more prospective for patients to adapt to a new physiological condition and avoid their previous dieting habits. The clinical studies

including weight maintenance period, known as multi-phase DI are very rare in the literature [367-369]. Long-term follow up are recommended for improving metabolic parameters [370].

In each DI there are some non-responders, who are "resistant" to the caloric restriction program and keep their body weight. Therefore, the development of "a personalized diet" individually tailored for every patient would be more efficient.

Dietary intervention	**Comments**
VLCD (Very Low Calorie Diet)	~ 3300 kJ/day; liquid diet replacing normal food and supplying all essential nutrients
LCD (Low Calorie Diet)	~ 3300-5000 kJ/day
Hypocaloric balanced diets / Balanced deficit diets	≥ 5000-6300 kJ/day
Multi-phase dietary interventions	combine different types of dietary interventions, often include weight maintenance phase

Table 5 Commonly used dietary interventions in obesity management (adapted from Tsigos et. al. 2008, Obesity Facts 1: 106-116 and categories of International Diabetes Federation 2004)[371].

CHAPTER 2

AIMS OF THE THESIS

Obesity is characterized as a low-grade inflammation in the body accompanied by insulin resistant state in sensitive tissues and represents a risk factor for the development of many non-communicable diseases, such as type 2 diabetes or atherosclerosis. However, the causes of their development are still not clarified and attract attention of researchers. There exist many hypotheses about induction of insulin resistance. There is a complex network of orchestrated changes involved in the initiation of insulin resistance in obese AT, such as activation of inflammatory signalling pathways inducing increased secretion of pro-inflammatory molecules produced by different cells, enhanced accumulation of macrophages, as well as elevated release of FA by adipocytes into the circulation. All of these factors might be potential culprits of obesity-induced inflammation and insulin resistance.

Recently, novel adipokines, visfatin and RBP4 have been discovered to be associated with a pathophysiological function of AT in obesity. However, their role in the development of obesity-linked comorbidities in humans is poorly understood. Furthermore, little is known about a modulation of their expression profile in AT during longitudinal interventional studies in humans. Additionally, macrophages due to their secretory activity seem to be a crucial player in obesity-induced inflammatory machinery leading to insulin resistance. The cross-sectional studies in human have revealed their abundant number in AT of obese patients compared to lean, while their content declined after rapid weight reduction in morbidly obese patients. However, the effect of moderate weight loss as a result of long-term DI on ATM accumulation has not been investigated yet. It is known that DI in most cases leads to the improvement of clinical parameters. Nevertheless, molecular mechanisms of adaptations proceeded in AT during dietary-induced weight loss wait for unveiling.

A few of these tasks have been solved in this thesis. The goals of this work are subdivided into two parts according to objects, which are investigated in particular papers:

Part 1 Role of novel adipokines in the development of obesity and insulin resistance.

▪ To investigate the role of novel adipokines, visfatin and RBP4, in relation to weight loss-induced changes of metabolic variables in obese women undergoing a short-term or multi-phase DI (Paper 1 and 2).

▪ To study and compare the expression of novel adipokine RBP4 in different AT depots (SAT and VAT) in relation to insulin resistance in patients with or without metabolic syndrome (Paper 3).

Part 2 Role of tissue macrophages in obesity-induced inflammation and insulin resistance.

▪ To investigate the effect of diet-induced weight loss on AT composition, particularly the content of macrophage population in SAT of obese women (Paper 4).

CHAPTER 3

RESULTS

3.1 LIST OF ORIGINAL PAPERS

PART 1

Paper 1: Kovacikova M., Vitkova M. , Klimcakova E., Polak J., Hejnova J., Bajzova M., Kovacova Z., Viguerie N., Langin D., Stich V.

Visfatin expression in subcutaneous adipose tissue of premenopausal women: relation to hormones and weight reduction.

Eur J Clin Invest. 2008 Jul;38(7):516-22.

Paper 2: Vitkova M, Klimcakova E, **Kovacikova M**, Valle C, Moro C, Polak J, Hanacek J, Capel F, Viguerie N, Richterova B, Bajzova M, Hejnova J, Stich V, Langin D.

Plasma levels and adipose tissue messenger ribonucleic acid expression of retinol-binding protein 4 are reduced during calorie restriction in obese subjects but are not related to diet-induced changes in insulin sensitivity.

J Clin Endocrinol Metab. 2007 Jun;92(6):2330-5. Epub 2007 Apr 3.

Paper 3: Bajzova M*, **Kovacikova M***, Vitkova M, Klimcakova E, Polak J, Kovacova Z, Viguerie N, Vedral T, Mikulasek L, Sramkova P, Srp A, Hejnova J, Langin D, Stich V.

Retinol -binding protein 4 expression in visceral and subcutaneous fat in human obesity.

Physiol Res. 2008;57(6):927-34. Epub 2007 Nov 30, * equally contributed to the work

PART 2

Paper 4: Kovacikova M, Sengenes C, Kovacova Z, Siklova-Vítkova M, Klimcakova E, Polak J, Rossmeislova L, Bajzova M, Hejnova J, Hnevkovska Z, Bouloumié A, Langin D, Stich V.

Dietary intervention-induced weight loss decreases macrophage content in adipose tissue of obese women.

IJO, In press, accepted on 6th May 2010

3.2 COMMENTS ON THE ORIGINAL PAPERS

PART 1

AT represents the largest endocrine organ in the body and secretes a number of bioactive substances, which influence the physiological function of AT and other non-adipose organs. Most of them are associated with obesity-related comorbidities. It has been documented in many epidemiological studies that lifestyle modifications, including dietary interventions and regular physical activity, ammeliorate the clinical parameters "hand in hand" with the modulation of expression profile of inflammatory proteins like cytokines, chemokines and adipokines. Additionally, the contribution of different fat depots to the circulating levels of these molecules in obesity disturbances is important issue to examine. However, a lot of recently discovered molecules released from AT into the circulation have missing information about their role in the onset of obesity and the associated complications, such as insulin resistance and type 2 diabetes. Therefore, in the first part of this work I would like to comment on the studies, which are focused on the investigation of novel adipokines, visfatin and RBP4 in relation to dietary-induced metabolic changes and body fat distribution.

In the first paper we examined the effect of a short-term DI (3 months) on visfatin mRNA in SAT and plasma levels in a group of overweight/obese pre-menopausal women together with its potential regulatory factors. 47 subjects participated in this study. They were further subdivided into the particular groups according to BMI as lean, overweight and obese women. Subsequently, overweight and obese patients underwent 3 months dietary program. We started to investigate the effect of moderate weight loss induced by longitudinal study on visfatin expression (mRNA in SAT and plasma levels), since no clinical study had published the results solving this issue. Nevertheless, Haider et al. [226] has noted that weight reduction after gastric banding surgery in morbidly obese subjects decreases visfatin plasma levels.

In our study, 3 month hypocaloric diet led to the improvement of most clinical parameters, including BMI, waist circumference, fat mass, insulin sensitivity determined by HOMA-IR index. Regarding the hormone and adipokine plasma levels, insulin, free testosterone, leptin and TNF-α significantly decreased as a result of diet; however their values did not reach levels similar to lean subjects. mRNA levels of visfatin in SAT obtained by needle biopsy increased in response to weight loss induced by 3 months' intervention, while visfatin plasma levels had a tendency to decrease after

diet in a group of overweight/obese subjects. Moreover, mRNA levels of TNF-α did not change after the diet whereas its mRNA levels were surprisingly higher in lean controls compared to overweight/obese subjects.

In addition, we investigated the possible regulatory factors, including insulin, testosterone, and TNF-α in relation to visfatin expression. We found negative correlation between visfatin mRNA levels and circulating levels of free testosterone and the positive correlation with TNF-α mRNA levels in group with a wide range of BMI. In respect to anthropometric parameters, we determined negative correlation between visfatin mRNA levels and BMI.

To sum up, we presented here that moderate weight loss induced by 3 month hypocaloric diet led to increase of visfatin mRNA levels in SAT of overweight/obese women. Based on correlation analyses, we determined the relationship between visfatin mRNA levels and TNF-α expression and circulating free testosterone levels. Furthermore, visfatin expression was changed dependent on BMI. Thus, we could hypothesize that visfatin and TNF-α expression in SAT are co-regulated; however the exact molecular mechanism occurring in tissue is not clear.

In the second paper we investigated the effect of a long-term DI including weight maintenance period on RBP4 mRNA and plasma levels in a group of obese pre-menopausal women in relation to insulin resistance. In this study we recruited 24 obese women, who underwent a 6 month multi-phase DI consists of particular dietary phases: 4 weeks of VLCD, followed by 2 weeks of LCD and subsequently 3-4 months of WM phase. The patients were examined at the beginning and the end of each successive phase. This approach was novel, since most of studies researched this bioactive molecule in relation to obesity-related complications in cross-sectional design or in animal models [103, 236, 237].

In our longitudinal study, we demostrated besides the improvement of anthropometric parameters, including weight, BMI, and waist circumference, also positive effect on plasma levels of triglycerides, insulin, leptin, glycerol and cortisol in each phase of DI. Insulin sensitivity was measured in this study by the euglycaemic hyperinsulinemic clamp, which is considered as a “gold standard” technique among the methods used for the assessment of insulin sensitivity. Moreover, we examined the glucose disposal rate during a whole dietary program, which increased after VLCD and remained elevated till the end of DI. Regarding the adiponectin plasma levels measured

in this study, we did not find significant changes during the DI. In respect to RBP4 expression in AT, we confirmed that the primary source of its production is mature adipocytes and its expression increases during the differentiation of adipocytes. During a weight loss program, mRNA levels of RBP4 in SAT obtained by needle biopsy rapidly decreased after VLCD and increased in subsequent dietary phases. Nevertheless, RBP4 mRNA levels at the end of a whole DI did not reach higher values than the levels at the beginning. The similar gene expression profile was observed for GLUT4, whose activity is associated with RBP4. In plasma, we determined significant decrease of RBP4 after VLCD with consequent increase at the end of DI in group of obese women; however RBP4 plasma levels were lower at the end of diet compared to basal levels. Additionally, plasma RBP4 levels and glucose disposal rate had different profile in response to DI in particular phases, but we did not find any correlation between these values. Unexpectedly, we did not observe significant difference between RBP4 plasma levels in lean and obese women.

Taken together, we determined that RBP4 is predominantly produced by mature adipocytes compared to the SVF cells in AT and its mRNA and plasma levels were diminished during energy restriction phase, probably due to changed adipocyte metabolism. However, RBP4 decline was not correlated with the improvement of insulin sensitivity in obese subjects. Therefore, we suggest that RBP4 mirrors adipocyte function in different nutritional status of body, but it cannot be considered as a marker of insulin resistance.

In the third paper we examined RBP4 mRNA levels in the paired samples of SAT and VAT in a group of 59 women with a wide range of BMI (20 to 49 kg/m^2) in relation to insulin resistance. We focused on this objective because of the missing information about RBP4 production by different AT depots in the literature at that time.

In our cross-sectional study, the entire group of patients was subdivided into non-obese and obese women, whereas the obese subjects were further classified according to the IDF criteria [372] as the subjects with or without metabolic syndrome (MS). We found significant differences in clinical parameters between these three investigated subgroups, including BMI, waist, visceral fat mass, blood glucose and insulin levels, as well as glucose disposal rate. The insulin sensitivity was measured by the euglycemic-hyperinsulinemic clamp and the relative ratio between VAT and SAT area was calculated according to the results evaluated by computer tomography scans.

Notably, VAT ratio to SAT area was higher in a group of obese women with MS than in a group of obese without MS. We observed the same results for triglycerides. However, there was no difference between obese and lean subjects in these terms. Regarding the RBP4 mRNA levels in fat depots, we determined higher gene expression of RBP4 in SAT than VAT together with GLUT4 and leptin as a "standard adipokine" produced by AT in each subgroup, as well as in the entire group. On the other hand, we did not find any differences in plasma RBP4 levels between the investigated subgroups. Also we did not observe any correlation between plasma and mRNA levels of RBP4 in any fat depot, independently on the group of subjects. Moreover, we did not reveal any association between RBP4 levels (mRNA or plasma) and adiposity. However, in the entire group we found positive correlation between RBP4 plasma levels and triglycerides, as well as blood glucose. Additionally, mRNA levels of RBP4 in both fat depots positively correlated with GLUT4 mRNA levels in VAT.

To summarize, our cross-sectional study determined that RBP4 gene expression was higher in SAT than VAT together with other insulin signalling-associated genes like GLUT4 and leptin, independently on BMI and obesity-related complication, i.e. the presence of MS. According to these results, we cannot suggest that RBP4 production is in direct association with metabolic comorbidities induced by obesity.

PART 2

According to a huge body of evidence accumulated during more than last decade, obesity is characterized as a "low-grade inflammation" not only due to an increased secretion of pro-inflammatory molecules, but also because of the increased accumulation of immune cells in AT, such as macrophages, lymphocytes and neutrophils. Hovewer, their nature and role in AT is still not clear. The findings from animal and *in vitro* studies suggest a possible crosstalk between immune and metabolic cells in insulin-sensitive tissues, which triggers the production of many bioactive molecules finally inducing the systemic insulin resistance and other metabolic and cardiovascular disorders. Many researchers try to identify the missing knowledge on the vicious cycle between macrophages and adipocytes and disclose the first culprit in this complicated machinery in AT. Various approaches how to clarify these tasks were developed (e.g. *in vitro* co-culture systems, immunophenotyping of cells, target silencing of genes in particular cell populations etc.). One of them is the characterization of AT composition, i.e. particular cell populations in different dietary

conditions. It is a topic of second part of the thesis, which is focused on the determination of ATM content using flow cytometry method during a multi-phase DI and observation how the content of these immune cells reflect metabolic changes due to lifestyle modification.

In the fourth paper we investigated the effect of long-term DI including weight maintenance period on the ATM content in SAT of obese women in relation to metabolic parameters and plasma levels of specific inflammatory molecules. In addition, we aimed to characterize ATM phenotype in different nutritional status of the body. In this longitudinal study we enrolled 27 pre-menopausal women, who underwent the same 6 month multi-phase DI as that described in our previous clinical study investigating mRNA levels of RBP4 (see page 59) and consisting of two dietary periods: VLCD and weight stabilisation phase (WS) composed of LCD and WM phase.

For characterization of ATM in biopsy samples, we used flow cytometry method, which was previously applied only in the cross-sectional studies on AT samples obtained from plastic surgery [23, 28, 218, 280]. Because this method has not been established in needle biopsy samples with a small amount of tissue yet, we performed the preliminary study in a group of 16 women. In the SVF cells obtained from SAT, we characterized the content of monocyte/macrophage population by combination of cell surface markers CD45+/14+. With respect to DI, CD45+/14+ content in SAT changed only at the end of DI (WS), but not after VLCD (energy restriction). To confirm the flow cytometry results, we performed gene expression analyses of specific macrophage markers, such as CD14, CD68, CD163 and LYVE-1, whose expression profile supported the results from cytometric method. According to these findings, we performed another clinical study with the same design of DI and with newly recruited patients (a group of 11 women). In this subgroup of patients we determined a macrophage population using a wider panel of markers: CD45, CD14 and additional markers CD206 for characterization of tissue macrophages and CD16 to distinguish between two subtypes of ATM. During a multi-phase DI, the ATM content determined as CD45+/14+/206+ decreased only at the end of WS, at the end of the whole intervention, but not after VLCD phase. In respect to ATM phenotype identified according to expression of CD16 marker, we observed similar response to DI in both ATM subtypes CD45+/14+/206+/16+ and CD45+/14+/206+/16-, i.e. no change after

VLCD and reduction after WS. However, the relative ratio between these two subtypes did not change during the whole DI.

Use of needle biopsy samples for flow cytometry method has some limitations because of bleeding accompanied the process of AT gain as we revealed in the preliminary study. Despite the proper wash with saline, we detected a high content of granulocytes as a result of blood contamination in AT samples. On the other hand, their content did not change during the whole DI. In addition, we did not find any correlation between granulocyte and macrophage content in biopsy samples. Therefore, we could exclude the possible influence on the interpretation of results from flow cytometry. To clarify the characterization of ATM, in the following clinical study we used CD206, specific cell surface marker for tissue macrophages, which eliminates possible contribution of circulating monocytes in the proportion of macrophage population expressed by cytometric analysis.

With respect to monocyte/macrophage dynamic during dietary intervention, we analyzed plasma levels of selected chemokines and cytokine associated with macrophage function, such as MCP-1, CXCL5 and TNF-α. We observed the diet-induced decrease of chemokines MCP-1 and CXCL5, whereas TNF-α plasma levels did not alter during the whole protocol.

Regarding the statistical analyses of clinical parameters, we pooled both investigated subgroups into one group of 27 subjects because they underwent the same type of DI and moreover they were not statistically different in measured parameters at the baseline. From anthropometric and metabolic variables BMI, fat mass, waist circumference, cholesterol, triglycerides, insulin and HOMA-IR improved during a whole DI, whereas the marked diminution of these variables was reached after VLCD. In this study we measured insulin senstivity using HOMA-IR index. With respect to correlation analyses, we did not find any relationship between the diet-induced changes in metabolic parameters and plasma levels of bioactive molecules and ATM content. This lack of significancy could be explained by a small number of subjects enrolled in this study or the participations of other insulin-sensitive tissues on metabolic changes in the body.

Taken together, we demonstrated that a 6 month multi-phase DI, which induced beneficial metabolic effects, led also to a diminution of ATM content in SAT of obese women. However, the reduction of ATM content was reached only at the end of the whole DI and was not accompanied with any changes in ATM phenotype. Due to

different profile of metabolic changes and macrophage content during the long-term DI, we could suggest that immune system needs longer time for adaptation to a new metabolic homeostasis in the body.

Paper 1

Kovacikova M., Vitkova M. , Klimcakova E., Polak J., Hejnova J., Bajzova M., Kovacova Z., Viguerie N., Langin D., Stich V.

Visfatin expression in subcutaneous adipose tissue of premenopausal women: relation to hormones and weight reduction.

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ORIGINAL ARTICLE

Visfatin expression in subcutaneous adipose tissue of pre-menopausal women: relation to hormones and weight reduction

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ABSTRACT

Background A novel adipokine, visfatin, was found to be related to adiposity in humans and regulated by a number of hormonal signals. The aim of this study was to investigate the relationships of visfatin expression in adipose tissue with potential regulatory factors such as insulin, testosterone and tumor necrosis factor-α (TNF-α) and to elucidate the effect of a diet induced weight reduction on adipose tissue mRNA expression and plasma levels of visfatin.

Materials and methods Biopsies of subcutaneous abdominal adipose tissue (SCAAT) and plasma samples were obtained at the beginning of the study from 47 pre-menopausal women (age 38·7 ± 1·7 years, body mass index (BMI) 27·9 ± 1·4 kg m^{-2}), consisting of 15 lean, 16 overweight and 16 obese subjects. The subgroup of 32 overweight/obese women (age 42·1 ± 1·9 years, BMI 31·2 ± 0·9 kg m^{-2}) underwent a 12 week hypocaloric weight reducing diet and samples were obtained at the end of the diet. Biopsy samples were analysed for visfatin and TNF-α mRNA levels and plasma was analysed for relevant metabolites and hormones.

Results In the group of 47 subjects visfatin mRNA expression in SCAAT was negatively correlated with plasma free testosterone ($r = –0.363$, $P < 0.05$) and BMI ($r = –0.558$, $P < 0.01$) and positively associated with adipose tissue TNF-α mRNA expression ($r = 0.688$, $P < 0.01$). The diet resulted in the reduction of body weight and in the decrease of plasma insulin, free testosterone and TNF-α levels. In the group of overweight/obese subjects visfatin mRNA in SCAAT increased after the diet and the diet induced increase was positively correlated with the magnitude of body weight loss.

Conclusion Visfatin mRNA expression in SCAAT is associated with TNF-α expression, plasma free testosterone and BMI in pre-menopausal women. A weight reducing hypocaloric diet results in the increase of visfatin mRNA in SCAAT.

Keywords Adipose tissue, mRNA expression, obesity, TNF-α, visfatin, weight reduction.

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Introduction

Visfatin, a novel adipokine, previously known as pre-B-cell colony-enhancing factor (PBEF), has been suggested to play a role in the regulation of glucose homeostasis [1–3]. In the original study of Fukuhara [1] that was recently withdrawn, visfatin was suggested to exert insulin mimetic effects in various insulin sensitive tissues such as liver, muscle, and fat. These results were not confirmed in a recent study [3]. However, the findings of the latter study suggest a role for visfatin in the regulation of glucose stimulated insulin secretion in pancreatic islets.

As a result of previous studies, it has been suggested that visfatin may modify insulin sensitivity and speculations about the possible use of visfatin in the treatment of insulin resistance have been raised.

In humans, plasma visfatin has been reported to be higher in obese subjects compared to lean subjects [4–7]. However, the relationship between body mass index (BMI) and plasma visfatin is not straightforward, as the association between obesity and elevated plasma visfatin levels has not been confirmed by other studies [8,9].

Studies by Haider *et al.* [7] and Manco *et al.* [10] revealed a reduction of plasma visfatin concentrations after weight loss in morbidly obese patients. Although visfatin has been believed

Table 1 Clinical characteristics of controls ($n = 15$), the group of overweight/obese pre-menopausal women ($n = 32$), the subgroup of overweight ($n = 16$) and obese subjects ($n = 16$) before and after diet induced weight loss

		Overweight		Obese		Overweight/Obese	
	Controls	Before diet	After diet	Before diet	After diet	Before diet	After diet
Weight (kg)	58·6 ± 1·4	77·5 ± 1·4†††	71·1 ± 1·3***	95·6 ± 3·8‡‡‡	88·0 ± 3·8***	86·6 ± 2·6	79·5 ± 2·5***
BMI (kg/m^2)	20·9 ± 0·5	27·4 ± 0·4†††	25·1 ± 0·4***	35·0 ± 1·3‡‡‡	32·3 ± 1·3***	31·2 ± 0·9	28·7 ± 0·9***
Fat (kg)	13·9 ± 1·2	29·1 ± 1·0†††	24·1 ± 1·0***	42·1 ± 2·6‡‡‡	36·7 ± 2·8***	35·6 ± 1·8	30·4 ± 1·9***
Waist circumference (cm)	72·4 ± 1·4	88·5 ± 1·8†††	81·2 ± 1·5***	103·6 ± 2·7‡‡‡	94·2 ± 2·5***	96·1 ± 2·1	87·7 ± 1·8***
Waist-to-hip ratio	0·8 ± 0·0	0·8 ± 0·0†	0·8 ± 0·0**	0·8 ± 0·0‡‡‡	0·8 ± 0·0**	0·8 ± 0·0	0·8 ± 0·0***
Systolic blood pressure (mm Hg)	113·9 ± 3·8	121 ± 5·2	117·5 ± 5·6	124·9 ± 1·9‡‡	123·4 ± 1·7	122·9 ± 2·7	120·4 ± 2·9
Diastolic blood pressure (mm Hg)	69·2 ± 2·2	75·6 ± 2·9	74·4 ± 3·0	76·8 ± 1·7‡‡	76·9 ± 1·9	76·2 ± 1·7	75·6 ± 2·7
Cholesterol (mmol L^{-1})	4·7 ± 0·2	6·1 ± 0·3†††	5·6 ± 0·3	5·5 ± 0·2‡‡‡	5·2 ± 0·2**	5·8 ± 0·2	5·4 ± 0·2**
Triglycerides (mmol L^{-1})	0·9 ± 0·1	1·3 ± 0·2†	1·1 ± 0·2	1·4 ± 0·1‡‡‡	1·1 ± 0·1**	1·3 ± 0·1	1·1 ± 0·1***
HDL-C (mmol L^{-1})	1·7 ± 0·1	1·7 ± 0·1	1·5 ± 0·1**	1·6 ± 0·1	1·4 ± 0·1**	1·6 ± 0·1	1·5 ± 0·1***
Glucose (mmol L^{-1})	4·5 ± 0·2	5·0 ± 0·2	4·9 ± 0·1	5·4 ± 0·1‡‡‡	5·0 ± 0·1**	5·2 ± 0·1	4·9 ± 0·1**
HOMA-IR	0·9 ± 0·1	1·9 ± 0·3†††	1·6 ± 0·3*	2·5 ± 0·3‡‡‡	1·7 ± 0·3**	2·2 ± 0·2	1·6 ± 0·2***

Data are presented as mean ± SEM.
*$P < 0·05$, **$P < 0·01$, ***$P < 0·001$: before vs. after diet; †$P < 0·05$, ††$P < 0·01$, †††$P < 0·001$: overweight before diet vs. controls; ‡‡$P < 0·01$, ‡‡‡$P < 0·001$: obese before diet vs. controls (BMI, body mass index; HDL-C, HDL-cholesterol; HOMA-IR, homeostasis model assessment of the insulin resistance index).

to be an insulin mimetic adipokine, several studies found no correlation between plasma levels or gene expression in adipose tissue and indices of insulin sensitivity [4,9].

Although visfatin was originally identified as a protein, produced mainly by visceral adipose tissue [1], higher expression of visfatin in visceral fat was not confirmed in a subsequent study [4]. Visfatin gene expression in visceral adipose tissue has been shown to increase with BMI [4,9], while the expression in subcutaneous adipose tissue was demonstrated to be either associated negatively [9] or uncorrelated [4] with BMI.

Factors regulating the visfatin expression in adipose tissue remain to be identified. Visfatin mRNA expression has been shown to be down regulated by cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in *in vitro* studies on 3T3-L1 adipocytes [11,12]. On the other hand, in human adipose tissue explants from the visceral depot, TNF-α induced an increase of visfatin mRNA levels [13]. In a recently published study, visfatin mRNA expression in 3T3-L1 pre-adipocytes was down regulated by insulin, testosterone, and palmitate and up regulated by dexamethasone [14].

Our aim was to investigate, using a clinical human study, associations between the candidate regulatory factors and visfatin mRNA expression in adipose tissue. In a group of pre-menopausal women including lean, overweight and obese subjects we investigated the relationship between visfatin mRNA expression in subcutaneous abdominal adipose tissue (SCAAT) and plasma levels of relevant candidate hormones. Additionally, in a subgroup of obese and overweight subjects, we studied the effect of diet induced weight reduction on visfatin expression in SCAAT.

Subjects and methods

Subjects: 47 lean ($n = 15$), overweight ($n = 16$) and obese ($n = 16$) pre-menopausal women (age 38·7 ± 1·7 years, range 25–57 years, BMI 27·9 ± 1·4 kg m^{-2}, range 17·3–50·5 kg m^{-2}), complete clinical characteristics of each subgroup of subjects are given in (Table 1) were recruited for the study. All subjects were drug free and did not suffer from any disease except for obesity. Their body weight had been stable for the three months prior to the entry examination. A written informed consent to participate in the study was obtained from each subject before the study began. The study was performed according to the Declaration of Helsinki and approved by the Ethical Committee of the Third Faculty of Medicine, Charles University, Prague.

Clinical protocol: The subjects were investigated at 8 a.m., after fasting overnight. Body height and weight were measured along with waist and hip circumferences. Body composition was assessed using multifrequency bioimpedance (Bodystat, Quad scan 4000, Isle of Man, UK.). Coefficients of variation (CV) of fat mass, fat free mass and impedance were 1·7%, 0·8% and 1·5%, respectively. Blood was collected into 50 μL of an anticoagulant and antioxidant cocktail (Immunotech SA, Marseille, France) and immediately centrifuged (1000 r.p.m., 4 °C). The plasma was stored at −80 °C until analysis. Thereafter needle biopsy

(diameter of needle 12 g) of subcutaneous adipose tissue was carried out in the abdominal region (10 cm laterally from umbilicus) under local anesthesia (1% Xylocain) as previously described [15]. The tissue was frozen immediately and stored at –80 °C until subsequent analysis.

A subgroup of 32 subjects (clinical characteristics are given in Table 1), who were evaluated as being either overweight (BMI > 25 kg m^{-2}) or obese (BMI > 30 kg m^{-2}), agreed to commit to a 12 week weight reducing hypocaloric diet and the above mentioned examinations including blood sampling and biopsy of subcutaneous abdominal adipose tissue were repeated at the end of the diet programme.

Dietary intervention: The diet was designed to provide 600 kcal/day less than the individually calculated energy requirements, which were based on the subject's calculated pre-treatment resting metabolic rate multiplied by a coefficient of correction for their physical activity level (1·3). The value chosen was based on a sedentary life style. The diet was designed to provide 25–30% of kcals from fat, 55–60% of kcals from carbohydrates, and 10–15% of kcals from proteins. The patients were monitored by dieticians and weighed weekly.

Quantification of visfatin expression by quantitative RT-PCR: Biopsy samples of abdominal SCAAT (about 1 g) were used for mRNA extraction. Total RNA extraction and reverse transcription were performed as previously described [15]. Real time quantitative PCR (qPCR) was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). A set of primers were designed for TNF-α using software Primer Express 1·5 (Applied Biosystems) and used at a final concentration of 900 nmol L–1 with SYBR-Green based chemistry PCR mix (Applied Biosystems, Foster City, CA, USA). To ensure that genomic DNA was not amplified, qPCR was performed on reverse transcription reactions without the addition of reverse transcriptase. Primers and a TaqMan probe for visfatin/PBEF were obtained from Applied Biosystems. 18S ribosomal RNA (Ribosomal RNA Control TaqMan assay kit, Applied Biosystems, Foster City, CA, USA) was used as a control to normalize gene expression.

Determination of plasma levels: Visfatin plasma levels were measured using an Enzyme Linked Immuno Sorbent Assay kit (ELISA) (Phoenix Pharm., Belmont, CA, USA). Assay sensitivity was 2 ng mL–1 and the intra-assay CV was 6·3%. Plasma concentrations of TNF-α were measured with a commercially available ultra sensitive ELISA kit (Biosource, Camarillo, CA, USA) and the intra-assay CV was 9·3%. Plasma glucose was determined using the glucose oxidase technique (Beckman Instruments, Fullerton, CA). Plasma concentrations of candidate hormones were determined using commercial radioimmunoassay kits. Homeostasis model assessment of the insulin resistance index (HOMA-IR) was calculated as follows: (fasting insulin in mU/mL) × (fasting glucose in mmol/L)/22·5.

Statistical analysis: Statistical analysis was performed using SPSS 12·0 for Windows (SPSS Inc., Chicago, IL, USA). Correlations were analysed using Pearson's correlation coefficient. Stepwise linear regression analysis was used to find potential dependence between variables. Before these statistical analyses, non-normally distributed parameters were logarithmically transformed. The effect of weight loss was tested using non-parametric Wilcoxon test for paired observations. Data are presented as mean ± SEM. Differences at the level of $P < 0{\cdot}05$ were considered statistically significant.

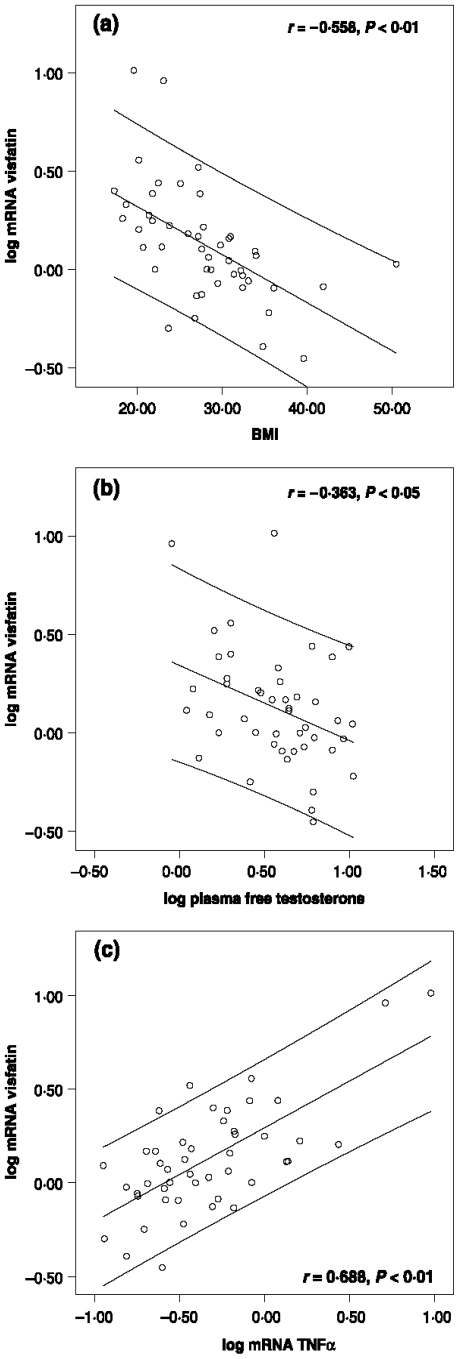
Results

Visfatin mRNA levels in SCAAT in relation to anthropometric variables. Negative correlations between visfatin mRNA levels and BMI were found ($r = -0{\cdot}558$, $P < 0{\cdot}01$) (Fig. 1a) for the entire group of 47 subjects. Visfatin mRNA levels also correlated negatively with body fat mass and with waist circumference ($r = -0{\cdot}560$ and $r = -0{\cdot}511$, respectively, both $P < 0{\cdot}01$). The above mentioned correlations did not exist in any of the subgroups. The negative association between visfatin mRNA expression and BMI was further supported by the finding of higher visfatin expression in lean subjects compared to overweight or obese (Table 3). As to plasma visfatin, no correlations with BMI or other anthropometric indices were found. No correlation between visfatin mRNA levels and plasma levels of visfatin was found in either group.

Visfatin mRNA levels in SCAAT in relation to hormonal variables. In the entire group a negative correlation between visfatin mRNA levels and plasma free testosterone ($r = -0{\cdot}363$, $P < 0{\cdot}05$) (Fig. 1b) was found. Visfatin mRNA expression correlated positively with TNF-α mRNA expression in SCAAT (Fig. 1c) ($r = 0{\cdot}688$, $P < 0{\cdot}01$). The latter correlation was found to be significant in the subgroup of lean ($P < 0{\cdot}05$) and borderline significant ($P = 0{\cdot}07$) in obese/overweight subjects. As to plasma visfatin, a negative correlation between plasma visfatin and plasma free testosterone was found in the subgroup of obese women ($r = -0{\cdot}788$, $P < 0{\cdot}01$). No other correlations of plasma visfatin with any of the hormonal variables were found.

Model comprising BMI,TNF-α expression in SCAAT and plasma free testosterone as independent and visfatin adipose tissue mRNA expression as dependent variables. In a model comprising the above mentioned variables, a stepwise linear regression analysis showed TNF-α mRNA expression in SCAAT and BMI to be principal predictors of visfatin mRNA expression (beta coefficient = 0·549, $P < 0{\cdot}01$ and –0·280, $P < 0{\cdot}05$, respectively).

Effect of diet induced weight reduction on clinical characteristics of obese and overweight subjects (Table 1). The dietary intervention resulted in decreases of body weight and BMI by 8%, body fat mass by 16% and waist circumference by 9% in the entire group of 32 overweight/obese women as well as in subgroups of overweight and obese women, respectively (Table 1). Blood lipid levels were also reduced as a result of the diet. The diet induced



weight reduction resulted in the decrease in insulin resistance as assessed by HOMA-IR 22%, $P < 0{\cdot}01$) in all the groups.

Effect of diet induced weight reduction on plasma hormone levels (Table 2). The diet intervention led to a reduction in plasma insulin, free testosterone and leptin levels in the entire group of overweight/obese women as well as in respective subgroups (except for insulin). However, levels of insulin, free testosterone and leptin remained higher in both subgroups when compared to lean subjects.

Effect of diet induced weight reduction on visfatin and TNF-α mRNA expression in SCAAT and on plasma levels of visfatin and TNF-α (Table 3). The 12 week hypocaloric weight reducing diet caused an increase of visfatin mRNA levels ($P < 0{\cdot}05$) and tended to decrease visfatin plasma levels in the entire overweight/obese group ($P < 0{\cdot}1$). The increase in visfatin gene expression did not reach significance in the subgroups of overweight or obese women, respectively (Table 3). mRNA levels of TNF-α in SCAAT were not changed after the diet while plasma TNF-α levels decreased ($P < 0{\cdot}05$). When the whole group of 32 overweight/obese women was stratified according to the magnitude of weight loss the diet induced increase of visfatin mRNA expression in SCAAT was higher in the group of responders with higher weight loss after the diet (data not shown). No such relationship was found for plasma visfatin. The relationship between the weight loss and the diet induced increase of visfatin mRNA expression was further supported by the finding of a positive correlation between these two variables in the whole overweight/obese group ($r = 0{\cdot}427$, $P < 0{\cdot}05$) and the overweight subgroup. The interrelationship between TNF-α and visfatin mRNA levels was supported by the finding of a positive correlation between the diet induced changes of the expression of the two molecules in SCAAT in the whole overweight/obese group.

Discussion

Visfatin expression has been reported to be related to adiposity and to be regulated by several hormonal signals known to be altered by obesity. In order to further investigate the relationship between visfatin expression in adipose tissue and obesity and obesity related hormones in humans, we measured mRNA levels of visfatin in subcutaneous abdominal adipose tissue (SCAAT). Our analyses were taken from a group of pre-menopausal women with a wide range of adiposity at baseline. Additional measurements were performed in a subgroup of overweight/obese women, after the diet induced weight reduction.

Figure 1 Correlation between visfatin mRNA expression in subcutaneous abdominal adipose tissue (SCAAT) and BMI (a), plasma free testosterone levels (b) and TNF-α mRNA expression in SCAAT (c). Data were log transformed to achieve normal distributions.

Table 2 Plasma hormonal levels of controls ($n = 15$), the group of overweight/obese pre-menopausal women ($n = 32$), the subgroup of obese ($n = 16$) and overweight subjects ($n = 16$) before and after diet induced weight loss

		Overweight		Obese		Overweight/Obese	
	Controls	**Before diet**	**After diet**	**Before diet**	**After diet**	**Before diet**	**After diet**
Insulin (mU L^{-1})	4·2 ± 0·5	8·7 ± 1·1†††	7·2 ± 1·2	10·1 ± 1·1	7·4 ± 1·0**	9·4 ± 0·8	7·3 ± 0·8***
Cortisol (nmol L^{-1})	413·7 ± 51·4	457·5 ± 46·1	478·9 ± 62·0	412·1 ± 49·3	404·6 ± 36·1	434·0 ± 33·5	440·5 ± 35·6
SHBG (nmol L^{-1})	95·9 ± 15·4	59·4 ± 8·3†	80·1 ± 11·7**	60·7 ± 9·7	71·0 ± 9·9*	60·1 ± 6·3	75·5 ± 7·6***
Free testosterone (nmol L^{-1})	2·6 ± 0·4	4·8 ± 0·6††	3·3 ± 0·5*	5·8 ± 0·7‡‡‡	4·7 ± 0·7**	5·3 ± 0·5	4·2 ± 0·5**
Total testosterone (nmol L^{-1})	1·5 ± 0·2	1·6 ± 0·3	1·2 ± 0·2	1·9 ± 0·3	1·9 ± 0·3	1·8 ± 0·2	1·6 ± 0·2
Leptin (ng mL^{-1})	11·9 ± 1·8	25·4 ± 2·5††	13·5 ± 1·9**	47·9 ± 3·1‡‡‡	26·6 ± 4·0***	39·2 ± 2·8	21·5 ± 2·6***

Data are presented as mean ± SEM.
*$P < 0·05$, **$P < 0·01$, ***$P < 0·001$: before vs. after diet, †$P < 0·05$, ††$P < 0·01$, †††$P < 0·001$: overweight before diet vs. controls; ‡‡$P < 0·01$, ‡‡‡$P < 0·001$: obese before diet vs. controls (SHBG, sex hormone binding globulin).

The study found a marked positive correlation between visfatin mRNA expression and TNF-α mRNA expression in SCAAT and a negative correlation with respect to body mass index and to plasma free testosterone. In a model comprising these three variables, the TNF-α expression and BMI appeared to be the strongest predictors of visfatin mRNA expression in SCAAT. Diet induced weight reduction, associated with the reduction of plasma levels of free testosterone, insulin and TNF-α, resulted in an increase of visfatin expression in SCAAT.

The negative relationship between BMI and visfatin mRNA in SCAAT found in this study is in agreement with results reported previously for gluteal [9] and abdominal subcutaneous adipose tissue [16]. In the present study, the negative relationship between BMI and visfatin expression is further supported by the finding of an increase of visfatin expression after the weight reduction. On the other hand, in a study by Berndt *et al.* [4], no relationship was found between BMI and visfatin mRNA in SCAAT in either females or males, although their group of subjects showed a higher range of ages when compared with ours.

The negative correlation between visfatin mRNA and free testosterone found in this study at baseline deserves attention. In a recent study [14], visfatin expression in 3T3-L1 adipocytes and pre-adipocytes was found to be up regulated by dexamethasone and down regulated by insulin and TNF-α, and, in pre-adipocytes, by testosterone. Thus, the above mentioned correlation for free testosterone might suggest that this regulatory pathway could be functional in humans. Although several studies suggest a relationship between plasma insulin and visfatin plasma or mRNA levels in adipose tissue [2,14], we could not confirm an association between fasting plasma insulin and visfatin mRNA in this study.

In respect to TNF-α, this study demonstrated a close relationship between visfatin and TNF-α mRNA levels in SCAAT but not to plasma TNF-α values. This may reflect a 'paracrine-like' regulation of visfatin expression in SCAAT by TNF-α secreted locally from adipose tissue. In fact, it has been suggested that TNF-α is not released from adipose tissue into the circulation [17] therefore plasma TNF-α levels may not reflect the TNF-α secretion from adipose tissue. Thus, the mRNA expression of TNF-α in SCAAT may represent a more appropriate reflection of the local TNF-α production in adipose tissue. The results of this study may support the findings of Hector *et al.* [13] that described an up regulation of visfatin expression in human adipose tissue explants by TNF-α. Alternatively, they may reflect a common factor regulating both TNF-α and visfatin. Additionally, the positive correlation between visfatin and TNF-α mRNA was observed in the peripheral blood mononuclear cells of obese subjects [18]. The down regulation of visfatin expression by TNF-α, described in other studies [11,12,14], was found in 3T3-L1 adipocytes and, thus, cannot be straightforwardly extended to human adipose tissue.

Obviously, we must be cautious in expanding the results obtained in adipocytes *in vitro* to human studies. It should be pointed out that visfatin is expressed by both adipocytes and the stromal vascular fraction (SVF) with the expression being significantly higher in the SVF of subcutaneous adipose tissue [16] and in the macrophage enriched SVF of visceral adipose tissue [19]. Therefore, the mRNA levels in subcutaneous adipose tissue, as assessed in this study, may reflect mainly visfatin expression in the SVF. No data about the regulation of visfatin expression in the SVF are available and its regulation by hormonal or TNF-α signals might be different from adipocytes. In this study, measurement of mRNA levels in the SVF fraction of adipose tissue was not possible due to the limited amount of tissue obtained using current techniques for needle biopsies.

Next, we assessed the effect of diet induced weight reduction on visfatin mRNA expression in SCAAT. To our knowledge, no study published so far has investigated the impact of weight loss on visfatin expression in a longitudinal study. The weight reducing

Table 3 Visfatin and TNF-α plasma levels and relative mRNA levels in SCAAT of controls (n = 15), the group of overweight/obese pre-menopausal women (n = 32), the subgroup of obese (n = 16) and overweight subjects (n = 16) before and after diet induced weight loss

	Controls	Overweight		Mean Change (95% CI)	Obese		Mean Change (95% CI)	Overweight/Obese		Mean Change (95% CI)
		Before diet	After diet		Before diet	After diet		Before diet	After diet	
Plasma										
Visfatin (ng mL^{-1})	6·5 ± 1·1	13·02 ± 2·1††	7·5 ± 1·01*	7·8 (3·5; 12·1)	13·4 ± 2·5	9·8 ± 2·2	1·8 (–4·8; 8·4)	14·4 ± 2·7	8·5 ± 1·4	6·1 (–0·3; 12·5)
TNF-α (ng mL^{-1})	0·5 ± 0·1	0·6 ± 0·1	0·5 ± 0·04	0·1 (–0·1; 0·2)	0·7 ± 0·04‡	0·5 ± 0·03**	0·1 (0·1; 0·2)	0·7 ± 0·03	0·6 ± 0·02*	0·11 (0·05; 0·17)
mRNA										
Visfatin (AU)	1·9 ± 0·2	1·4 ± 0·2††	1·7 ± 0·3	–0·4 (0·3; –1·04)	0·9 ± 0·1‡‡‡	1·6 ± 0·3	–0·7 (0·6;–1·9)	1·2 ± 0·3	1·7 ± 0·4*	–0·5 (–0·9; –0·1)
TNF-α (AU)	1·05 ± 0·2	0·4 ± 0·01††	0·4 ± 0·1	–0·04 (–0·3; 0·2)	0·3 ± 0·0‡‡	0·4 ± 0·1	–0·1 (–0·2; 0·1)	0·3 ± 0·03	0·4 ± 0·1	–0·1 (–0·2; 0·5)

Data are presented as mean ± SEM; CI, confidence interval.
*P < 0·05, **P < 0·01, ***P < 0·001: before vs. after diet; ††P < 0·01, †††P < 0·001: overweight before diet vs. controls; ‡P < 0·05, ‡‡P < 0·01, ‡‡‡P < 0·001: obese before diet vs. controls
mRNA: relative amounts are expressed as arbitrary units (AU) obtained after normalization by the 18S ribosomal RNA subunit (× 10^{-4}) (TNF-α, tumour necrosis factor-α).

hypocaloric diet resulted in an increase of visfatin mRNA in SCAAT. Furthermore, there was a clear positive relationship between the magnitude of weight loss and the increase of visfatin mRNA levels. These findings are in line with the idea of increasing visfatin gene expression in SCAAT with decreasing BMI. Weight reduction was also associated with a decrease in plasma levels of several hormones which have been suggested as regulators of visfatin adipose tissue expression (i.e. free testosterone, insulin and TNF-α). This is further supported by the finding of positive correlations between the diet induced changes of visfatin mRNA and TNF-α mRNA. Surprisingly, the response of plasma visfatin to the diet was in the opposite direction (although the decrease was borderline significant) than gene expression in SCAAT. We may hypothesize that increased visfatin expression could be a possible 'compensatory response' to decreased plasma levels after dieting. Furthermore, it should be pointed out that the above mentioned results concern subcutaneous adipose tissue and, as shown in several studies [4,9,16], visfatin expression is regulated differentially in visceral fat. Moreover, adipose tissue is not the only source of circulating visfatin: visfatin is also secreted by other tissues such as skeletal muscle, liver, bone marrow and lymphocytes [20]. The effect of the hypocaloric diet on visfatin secretion in these tissues is not known.

Diet induced weight reduction was associated with a tendency to a reduction of plasma visfatin. This is partly in line with the results published so far describing either reduction of plasma visfatin [7,10] or an increase of visfatin [21] in morbidly obese women after a weight loss induced by gastroplasty. The subjects of our study were not, contrary to the studies cited above, morbidly obese and their weight loss was much lower. These may be possible reasons for the lack of significant diet induced changes in plasma visfatin observed in our study. In fact, even after diet induced weight reduction, the body mass index and the plasma levels of insulin or free testosterone in the overweight and obese women remained markedly elevated compared to the lean ones. It may be hypothesized that a greater weight reduction, e.g. like that observed after bariatric surgery in morbidly obese subjects [7,10] is necessary for a significant reduction of plasma visfatin.

Visfatin is suggested to play a role in the regulation of glucose homeostasis [3]. In this study, we found no direct relationship between HOMA-IR and visfatin mRNA in SCAAT or visfatin plasma levels. These findings correspond to the results in human studies by Berndt [4] and Pagano [9]. Contrary to that, Varma *et al.* [16] described an independent (from BMI) positive relationship between visfatin expression in SCAAT and insulin sensitivity. Our data do not support a major role for visfatin in the regulation of whole body insulin sensitivity in humans.

In conclusion, our study shows that visfatin mRNA expression in SCAAT is related to TNF-α expression and to plasma free testosterone in a group of pre-menopausal women including lean and overweight/obese subjects. Moreover, visfatin expression

is negatively related to body mass index and the diet induced weight reduction results in an increase of the visfatin expression in subcutaneous adipose tissue.

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References

1 Fukuhara A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K *et al.* Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. *Science* 2005;**307**:426–30.
2 Haider DG, Schaller G, Kapiotis S, Maier C, Luger A, Wolzt M. The release of the adipocytokine visfatin is regulated by glucose and insulin. *Diabetologia* 2006;**49**:1909–14.
3 Revollo JR, Korner A, Mills KF, Satoh A, Wang T, Garten A *et al.* Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. *Cell Metab* 2007;**6**:363–75.
4 Berndt J, Kloting N, Kralisch S, Kovacs P, Fasshauer M, Schon MR *et al.* Plasma visfatin concentrations and fat depot-specific mRNA expression in humans. *Diabetes* 2005;**54**:2911–6.
5 Chen MP, Chung FM, Chang DM, Tsai JC, Huang HF, Shin SJ *et al.* Elevated plasma level of visfatin/pre-B cell colony-enhancing factor in patients with type 2 diabetes mellitus. *J Clin Endocrinol Metab* 2006;**91**:295–9.
6 Filippatos TD, Derdemezis CS, Kiortsis DN, Tselepis AD, Elisaf MS. Increased plasma levels of visfatin/pre-B cell colony-enhancing factor in obese and overweight patients with metabolic syndrome. *J Endocrinol Invest* 2007;**30**:323–6.
7 Haider DG, Schindler K, Schaller G, Prager G, Wolzt M, Ludvik B. Increased plasma visfatin concentrations in morbidly obese subjects are reduced after gastric banding. *J Clin Endocrinol Metab* 2006;**91**:1578–81.
8 Chen CC, Li TC, Li CI, Liu CS, Lin WY *et al.* The relationship between visfatin levels and anthropometric and metabolic parameters: association with cholesterol levels in women. *Metabolism* 2007;**56**:1216–20.
9 Pagano C, Pilon C, Olivieri M, Mason P, Fabris R, Serra R *et al.* Reduced plasma visfatin/pre-B cell colony-enhancing factor in obesity is not related to insulin resistance in humans. *J Clin Endocrinol Metab* 2006;**91**:3165–70.
10 Manco M, Fernandez-Real JM, Equitani F, Vendrell J, Valera Mora ME, Nanni G *et al.* Effect of massive weight loss on inflammatory adipocytokines and the innate immune system in morbidly obese women. *J Clin Endocrinol Metab* 2007;**92**:483–90.
11 Kralisch S, Klein J, Lossner U, Bluher M, Paschke R, Stumvoll M *et al.* Hormonal regulation of the novel adipocytokine visfatin in 3T3-L1 adipocytes. *J Endocrinol* 2005;**185**:R1–8.
12 Kralisch S, Klein J, Lossner U, Bluher M, Paschke R, Stumvoll M *et al.* Interleukin-6 is a negative regulator of visfatin gene expression in 3T3-L1 adipocytes. *Am J Physiol Endocrinol Metab* 2005;**289**:E586–90.
13 Hector J, Schwarzloh B, Goehring J, Strate TG, Hess UF, Deuretzbacher G *et al.* TNF-alpha Alters Visfatin and Adiponectin Levels in Human Fat. *Horm Metab Res* 2007;**39**:250–5.
14 MacLaren R, Cui W, Cianflone K. Visfatin expression is hormonally regulated by metabolic and sex hormones in 3T3-L1 pre-adipocytes and adipocytes. *Diabetes Obes Metab* 2007;**9**:490–7.
15 Klimcakova E, Polak J, Moro C, Hejnova J, Majercik M, Viguerie N *et al.* Dynamic strength training improves insulin sensitivity without altering plasma levels and gene expression of adipokines in subcutaneous adipose tissue in obese men. *J Clin Endocrinol Metab* 2006;**91**:5107–12.
16 Varma V, Yao-Borengasser A, Rasouli N, Bodles AM, Phanavanh B, Lee MJ *et al.* Human visfatin expression: relationship to insulin sensitivity, intramyocellular lipids and inflammation. *J Clin Endocrinol Metab* 2007;**92**:666–72.
17 Mohamed-Ali V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS *et al.* Subcutaneous adipose tissue releases interleukin-6, but not tumour necrosis factor-alpha, in vivo. *J Clin Endocrinol Metab* 1997;**82**:4196–200.
18 Samara A, Pfister M, Marie B, Visvikis-Siest S. Visfatin, low-grade inflammation and BMI. *Clin Endocrinol (Oxf)* 2008.
19 Curat CA, Wegner V, Sengenes C, Miranville A, Tonus C, Busse R *et al.* Macrophages in human visceral adipose tissue: increased accumulation in obesity and a source of resistin and visfatin. *Diabetologia* 2006;**49**:744–7.
20 Sethi JK, Vidal-Puig A. Visfatin. The missing link between intra-abdominal obesity and diabetes? *Trends Mol Med* 2005;**11**:344–7.
21 Krzyzanowska K, Mittermayer F, Krugluger W, Kopp HP, Schernthaner G. Increase in visfatin after weight loss induced by gastroplastic surgery. *Obesity (Silver Spring)* 2006;**14**:1886–9.

Paper 2

Vitkova M, Klimcakova E, **Kovacikova M**, Valle C, Moro C, Polak J, Hanacek J, Capel F, Viguerie N, Richterova B, Bajzova M, Hejnova J, Stich V, Langin D.

Plasma levels and adipose tissue messenger ribonucleic acid expression of retinol-binding protein 4 are reduced during calorie restriction in obese subjects but are not related to diet-induced changes in insulin sensitivity.

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Plasma Levels and Adipose Tissue Messenger Ribonucleic Acid Expression of Retinol-Binding Protein 4 Are Reduced during Calorie Restriction in Obese Subjects but Are Not Related to Diet-Induced Changes in Insulin Sensitivity

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Context: Retinol-binding protein 4 (RBP4) may play a role in the development of insulin resistance.

Objective: We investigated whether RBP4 adipose tissue mRNA expression and plasma level are related to insulin sensitivity during a diet-induced weight loss.

Design, Setting, Patients, and Intervention: Obese women followed a dietary intervention composed of a 4-wk very low-calorie diet (VLCD), a 2-month low-calorie diet, and 3–4 months of a weight maintenance (WM) phase.

Main Outcome Measures: Clinical investigation was performed before and at the end of each phase. Insulin sensitivity was assessed with the euglycemic hyperinsulinemic clamp. Adipose tissue mRNA and plasma levels of RBP4 were determined using reverse transcription-quantitative PCR and ELISA, respectively.

Results: Weight and fat mass decreased during VLCD and were stabilized during WM. Glucose disposal rate increased during VLCD and remained elevated thereafter. Plasma levels of RBP4 decreased after VLCD and, although increasing at subsequent phases, remained lower than prediet values. Adipose tissue mRNA levels were diminished after VLCD, and increased during low-calorie diet and WM to reach basal values. Basal RBP4 levels or diet-induced variations of RBP4 were not different in lean women and two groups of obese women with high- and low-insulin sensitivity.

Conclusions: Severe calorie restriction promotes a reduction in adipose tissue and plasma levels of RBP4. The study does not bring evidence for a role for RBP4 in the regulation of diet-induced changes in insulin sensitivity. (***J Clin Endocrinol Metab* 92: 2330–2335, 2007**)

TYPE 2 DIABETES is characterized by insulin resistance and relative insulin deficiency. The resistance to insulin action occurs in multiple tissues, including the liver with an increase in glucose production and skeletal muscles with a decrease in glucose use. Insulin resistance independent of overt diabetes is an important causative factor of the metabolic syndrome and constitutes an important risk factor for cardiovascular disease. Obesity is one of the principal causes for insulin resistance and risk factors for type 2 diabetes. The excess of fat mass is associated with release of multiple molecules with paracrine or endocrine action by adipose tissue that may contribute to the development of insulin resistance (1). A recently characterized potential candidate is retinol-binding protein 4 (RBP4) (2). Adipose tissue RBP4 expression and circulating levels are increased in several mouse models of insulin resistance. Genetic knockout of the insulin-stimulated glucose transporter 4 (Glut4) selectively in adipocytes results in impaired whole-body insulin sensitivity (3). Because adipocytes contribute little to whole-body glucose disposal, the existence of a factor released by the adipocytes and acting on the liver and skeletal muscle was predicted. RBP4 has been identified as such a factor (2). Overexpression of RBP4 or injection of recombinant RBP4 induced insulin resistance in mice, whereas pharmacologically decreased serum levels of RBP4 improved insulin sensitivity in high-fat

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* M.V. and E.K. contributed equally to the work.

Abbreviations: BMI, Body mass index; Glut4, glucose transporter 4; LCD, low-calorie diet; PPAR, peroxisome proliferator-activated receptor; RBP4, retinol-binding protein 4; VLCD, very low-calorie diet; WM, weight maintenance.

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diet-fed mice. Indeed, RBP4 impairs insulin signaling in skeletal muscle and affects glucose output in the liver.

RBP4 has been used clinically as a rapid turnover protein for assessing the short-term fluctuation of nutritional states. RBP4 is the only specific transport protein for retinol (vitamin A) in the circulation (4). It is produced by hepatocytes, which are believed to contribute to a large part of circulating RBP4, although adipocytes have the second-highest expression level (5). Elevated RBP4 levels have been reported in subjects with insulin resistance and type 2 diabetes (6–9). Correlations have been observed between serum RBP4 levels and the magnitude of insulin resistance in different groups of subjects (8). However, the cross-sectional design of most of the clinical studies performed so far does not allow for a determination of a putative causal role of RBP4 in the pathogenesis of insulin resistance and type 2 diabetes in humans (10). Here, we studied RBP4 in obese subjects enrolled in a multiple-phase weight reduction program based on a 4-wk very low-calorie diet (VLCD), followed by a 2-month low-calorie diet (LCD), and 3–4 months of a weight maintenance (WM) phase. RBP4 mRNA expression in sc adipose tissue and plasma RBP4 levels were determined before and at the end of each phase, and related to anthropometric and biological parameters, including glucose disposal rate assessed by the euglycemic hyperinsulinemic clamp.

Subjects and Methods

In vitro adipose tissue studies

Subcutaneous abdominal adipose tissue obtained from overweight women undergoing plastic surgery was digested with collagenase. Mature adipocytes were separated from the stromavascular fraction by mild centrifugation. Isolation of different cell types (endothelial cells, preadipocytes, and macrophages) in the stromavascular fraction was performed using surface antigen-coupled magnetic microbeads (11). Human preadipocytes in primary culture were differentiated as described (12). At d 13, 60–80% of cells were differentiated into lipid droplet-containing adipocytes. For culture of human adipose tissue explants, sc abdominal adipose tissue was cut into small pieces weighing approximately 10 mg or less. After washing steps, explants were cultured in DMEM F12 medium (Cambrex Corp., East Rutherford, NJ) containing 33 μmo/liter biotin, 17 μmol/liter pantothenate, and 50 μg/ml gentamycin supplemented with 10% of fetal calf serum. Explants were preincubated overnight to allow for removal of soluble factors and cellular debris released by cells broken during the preparation of the small pieces of adipose tissue. On d 2, explants were washed three times with PBS. Explants (200 mg/ml) were then incubated for 24 h in fresh medium. Aliquots of the medium were stored at −80 C for protein measurements. These studies were in agreement with the French National Institute of Health and Medical Research (Inserm) and the Toulouse University Hospital ethics regulation.

Subjects

Participants in the study were recruited at the Third Faculty of Medicine of Charles University and at the Institute for Mother and Child Care in Prague, Czech Republic. The clinical investigation was performed at the Department of Sports Medicine of the Third Faculty of Medicine. A group of 24 obese premenopausal women was included in the study. Exclusion criteria were weight changes of more than 3 kg within the 3 months before the start of the study, hypertension, diabetes, or hyperlipidemia treated by drugs, drug-treated obesity, pregnancy, participation in other trials, and alcohol or drug abuse. A control group of 12 lean women [age 38 ± 10 yr; body mass index (BMI) 21 ± 2 kg/m^2] was also investigated. The studies were approved by the Ethical Committee of the Third Faculty of Medicine. Volunteers were informed on the study, and written consent was obtained before study participation.

Dietary intervention

During the first 4 wk of the dietary intervention program, the obese subjects received a 800 kcal/d VLCD (liquid formula diet; Redita, Promil, Czech Republic). During the next 2 months, a LCD was designed to provide 600 kcal/d 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. The final period was a WM phase of 3–4 months, during which the patients were instructed to keep on a weight-maintaining diet. Patients consulted a dietitian once a week during the first 3 months of the program and once a month during the WM phase. They provided a written 3-d dietary record at each dietary consultation.

Clinical investigation

A complete clinical investigation was realized before and at the end of each phase in the morning. Anthropometric and resting metabolic rate measurements were performed as previously reported (13). Body composition was determined with multifrequency bioimpedance (Bodystat QuadScan 4000; Bodystat Ltd., Isle of Man, British Isles). Blood samples were drawn from an indwelling catheter in the antecubital vein. Needle microbiopsy of sc adipose tissue was performed under local anesthesia (1% Xylocaine; AstraZeneca PLC, London, UK) from the abdominal region (14–20-cm lateral to the umbilicus) (14). The euglycemic hyperinsulinemic clamp was performed according to the DeFronzo method (15). Priming plus continuous infusion of crystalline human insulin (Actrapid Human; Novo, A/S, Bagsvaerd, Denmark), 40 mU/m^2 body area·min, was given for 210 min. Euglycemia (the fasting blood glucose concentration) was maintained by a variable 20% glucose infusion. The infusion rate was determined by measuring arterialized plasma glucose every 5 min (Beckman Glucose Analyzer; Beckman Coulter, Inc., Fullerton, CA). Glucose consumption was calculated from the exogenous glucose infusion rates during the last 30 min of the clamp and corrected for kilogram of body weight (mg·min^{-1}·kg^{-1}) or kilogram of fat-free mass (mg·min^{-1}·kg^{-1} fat-free mass).

RNA analysis

Total RNA was extracted from adipose tissue biopsy samples, explants, and cells using the RNeasy Mini kit (QIAGEN, Inc., Valencia, CA). RT was performed with 500-ng total RNA using random hexamers (Promega Corp., Madison, WI) and Superscript II Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA). Real-time quantitative PCR was performed with TaqMan probe-based gene expression assays for RBP4, Glut4, peroxisome proliferator-activated receptor (PPAR)γ, and CD68, and a SYBR Green-based assay for adiponectin using ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). An 18S ribosomal RNA was used as control to normalize gene expression (Ribosomal RNA Control TaqMan Assay kit; Applied Biosystems). Each sample was performed in duplicate, and 10-ng cDNA was used as a template for real-time PCR. When the difference between the duplicates was above 0.5 Ct (threshold cycle), real-time PCR was performed again. Results are expressed as $2^{-\Delta Ct}$ values.

Determination of culture medium and plasma levels

RBP4 levels were measured using an ELISA kit (Immundiagnostik AG, Bensheim, Germany). Plasma samples were diluted so that the absorbance was in the middle of the range of linearity for the assay. Within-run coefficient of variation for RBP4 was 2.7%. Plasma glucose was determined by the glucose hexokinase technique (Konelab 60i; Labsystems CLD, Konelab, Finland). Plasma insulin was measured using chemiluminescent immunometric assay (Immulite 2000 Insulin; DPC Czech sro, Brno, Czech Republic). Nonesterified fatty acid levels were determined using an enzymatic procedure (Wako; Unipath Ltd., Bedford, UK). Leptin and adiponectin levels were determined using ELISA kits (BioVendor Laboratory Medicine, Brno, Czech Republic). Plasma β-hydroxybutyrate was measured by an enzymatic "Liqui-Color" kit (Stanbio Laboratory, Boerne, TX). Plasma levels of other parameters were determined using standard clinical biochemistry methods. To determine cell damage in adipose tissue explant culture, mea-

surement of adenylate kinase activity was performed in the culture medium using a bioluminescent assay (Cambrex, Corp.).

Statistical analysis

Data were analyzed using the SPSS software (SPSS, Inc., Chicago, IL). Nonparametric Wilcoxon signed rank or Mann-Whitney *U* tests were used for comparison of paired and unpaired values, respectively. Correlations were analyzed by Spearman's nonparametric test. The level of significance was set at $P < 0.05$.

Results

Expression of RBP4 in human adipose tissue

Comparison of mRNA expression in mature adipocytes and stromavascular cells from human sc adipose tissue revealed that RBP4 is expressed almost exclusively in mature adipocytes (Fig. 1A). As a control of cell isolation, Glut4 and adiponectin mRNA were detected mostly in adipocytes and CD68 mRNA, mostly in the stromavascular fraction. Direct detection on isolated endothelial cells, macrophages, and preadipocyte-like cells confirmed that the level of RBP4 expression was very low compared with that in mature adipocytes (data not shown). RBP4 gene expression was strongly induced during the conversion of human preadipocytes into adipocytes along with markers of adipocyte differentiation, such as PPARγ and adiponectin (Fig. 1B). We also wished to determine whether RBP4 was produced by human sc adipose tissue. Primary culture of adipose tissue explants showed that the production rate of RBP4 *in vitro* was comparable to that of adiponectin, a factor secreted at high levels by adipocytes (Fig. 1C). The release of adenylate kinase from the explants was very low, indicating that cell damage was limited in our culture conditions (data not shown).

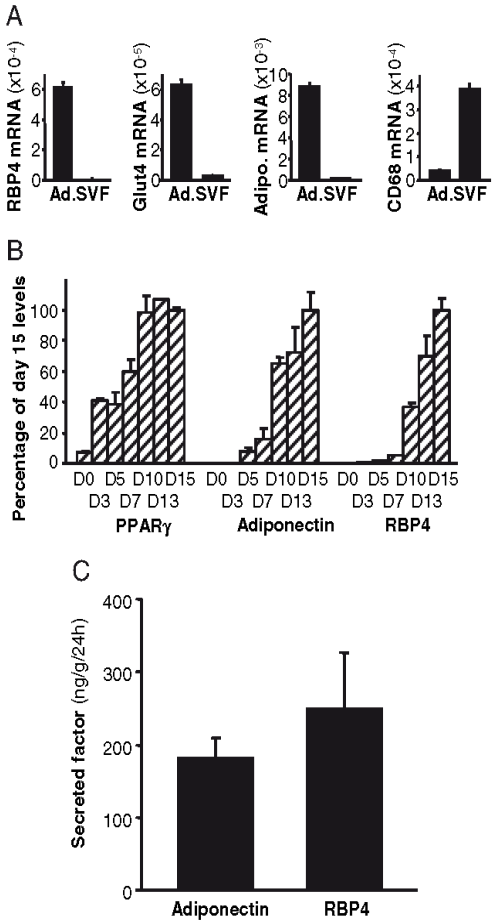

FIG. 1. RBP4 expression and production in human sc adipose tissue. A, RBP4, Glut4, adiponectin (Adipo), and CD68 mRNA expression was determined in mature adipocytes (Ad) and stromavascular cells (SVF) (n = 6). B, Time course of RBP4, adiponectin, and PPARγ mRNA expression during differentiation of human preadipocytes into adipocytes from d 0 (D0) to d 15 (D15) (n = 4). C, Production of RBP4 and adiponectin by human adipose tissue explants (n = 6). The levels of secreted products were normalized to the mass of adipose tissue. Values are means ± SEM.

Clinical parameters of obese subjects during a weight reduction program

Obese women followed a 6-month weight reduction program composed of three successive phases: a 4-wk 800 kcal/d VLCD, a 2-month LCD with 600 kcal less than the calculated daily energy requirements, and a 3–4 month WM diet. Anthropometric and plasma parameters were determined before and at the end of each dietary phase (Table 1). The subjects' body weight and BMI decreased during the VLCD and LCD phases. The body weight was stabilized during WM. The loss of weight was chiefly due to a decrease in fat mass. Evolution of waist circumference followed the same pattern as body weight. Regarding plasma parameters, there was a decrease in insulin, leptin, triglyceride, glycerol, and cortisol levels at all the time points. Glycemia was decreased only after VLCD, and the nonesterified fatty acid level was decreased at the end of the protocol. Circulating β-hydroxybutyrate was elevated during VLCD and then decreased during subsequent phases to reach basal levels at the end of the program. Plasma adiponectin level was not changed during the dietary intervention. To evaluate insulin sensitivity, euglycemic hyperinsulinemic clamps were performed at each phase (Table 1). The glucose disposal rate increased during VLCD, and remained elevated throughout the phases of LCD and WM.

Adipose tissue mRNA expression of RBP4 and Glut4 during a weight reduction program

Subcutaneous abdominal adipose tissue biopsies were performed before and at the end of each dietary phase. RBP4 mRNA expression decreased during VLCD (Fig. 2A). During LCD, there was an increase in RBP4 mRNA levels ($P < 0.01$) so that at the end of the LCD phase and at the end of WM, RBP4 mRNA values were not different from basal values. The profile of Glut4 mRNA expression was similar to that of RBP4 (Fig. 2B).

Plasma levels of RBP4 during a weight reduction program

Plasma RBP4 levels were decreased during VLCD (Fig. 2C). The LCD and WM phases were characterized by a gradual increase in RBP4 levels. At the end of the dietary intervention, plasma RBP4 levels were higher than VLCD values ($P < 0.01$).

TABLE 1. Clinical parameters of 24 obese women before and at the end of different phases of a weight reduction program

Parameter	Basal	VLCD 4 wk	LCD 8 wk	WM 12–16 wk
Weight (kg)	97 ± 16	90 ± 15[c]	87 ± 15[c]	87 ± 15[c]
BMI (kg/m^2)	35 ± 5	33 ± 5[c]	32 ± 4[c]	32 ± 5[c]
Fat mass (kg)	40 ± 12	35 ± 11[c]	31 ± 9[c]	32 ± 11[c]
Fat-free mass (kg)	58 ± 6	55 ± 7[b]	56 ± 8[b]	55 ± 7[c]
Waist (cm)	104 ± 13	99 ± 13[c]	96 ± 13[c]	96 ± 13[c]
Glucose (mmol/liter)	5.6 ± 0.4	5.3 ± 0.6[a]	5.4 ± 0.7	5.3 ± 0.8
Insulin (mU/liter)	13.6 ± 8.3	6.8 ± 3.7[c]	5.8 ± 2.6[c]	6.8 ± 2.8[c]
Glycerol (μmol/liter)	217 ± 77	160 ± 39[b]	145 ± 59[c]	150 ± 43[c]
Nonesterified fatty acid (μmol/liter)	693 ± 144	741 ± 126	589 ± 170[a]	564 ± 171[a]
β-hydroxybutyrate (mmol/liter)	0.16 ± 0.09	0.65 ± 0.36[c]	0.27 ± 0.16[c]	0.19 ± 0.17
Total cholesterol (mmol/liter)	4.82 ± 0.66	3.90 ± 0.76[c]	4.29 ± 0.69[c]	4.56 ± 0.66[a]
Triglycerides (mmol/liter)	1.43 ± 0.69	1.06 ± 0.35[c]	1.06 ± 0.37[b]	1.03 ± 0.29[b]
Leptin (ng/ml)	41 ± 15	20 ± 13[c]	24 ± 14[c]	27 ± 15[c]
Adiponectin (μg/ml)	8.4 ± 3.9	8.4 ± 3.3	8.3 ± 3.3	9.2 ± 3.6
Cortisol (nmol/liter)	205 ± 96	157 ± 67[b]	146 ± 61[b]	177 ± 102[a]
Glucose disposal rate (mg/kg·min)	2.98 ± 1.56	3.61 ± 1.65[b]	4.05 ± 1.66[c]	4.19 ± 1.74[c]
Glucose disposal rate (mg/kg fat-free mass·min)	4.88 ± 2.29	5.75 ± 2.43[a]	6.15 ± 2.31[c]	6.48 ± 2.51[c]

Values are means ± SD.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$ compared to basal values.

Nevertheless, throughout the dietary protocol, plasma RBP4 levels remained lower than the levels at the beginning of the program. The evolution of RBP4 levels was different from that of the glucose disposal rate that shows steadily higher values at the different time points of the dietary intervention than at the beginning of the program (Fig. 2D).

Relationship between plasma RBP4 levels and insulin resistance

No correlations were found between RBP4 level and glucose disposal rate before the diet ($r = -0.31$; $P > 0.1$). Similarly, no correlations were found between the diet-induced changes of RBP4 and glucose disposal rate when considering either the VLCD phase or the whole dietary program ($r = -0.22$, $P > 0.3$; $r = -0.14$, $P > 0.5$, respectively). A similar conclusion was reached when the glucose disposal rate was corrected for fat-free mass. To investigate whether the lack of relationship between RBP4 and glucose disposal rate is dependent on the level of baseline insulin sensitivity, the 24 obese subjects were stratified into two groups according to prediet glucose disposal rate (Table 2). No differences in either basal plasma RBP4 levels or in the diet-induced decreases of plasma levels were observed between the two groups. Similarly, if the subjects were stratified into two groups according to the magnitude of the changes in glucose disposal rate during VLCD and the entire program, there was no difference in the diet-induced variation of plasma RBP4 levels between the groups (data not shown). No correlations were found between plasma RBP4 and plasma triglyceride, high-density lipoprotein cholesterol, or waist circumference when considering the diet-induced responses of the variables (data not shown). To investigate further the relationship between plasma RBP4 levels and insulin resistance, a group of control lean women (BMI 21 ± 2 kg/m^2) was investigated and compared with the obese group. As expected, the glucose disposal rate was higher in the lean group than in the obese group (6.43 ± 1.61 *vs.* 2.98 ± 1.56 mg/kg/min, $P < 0.001$; and 8.27 ± 1.73 *vs.* 4.88 ± 2.29 mg/kg fat free mass/min, $P < 0.001$). However, plasma RBP4 levels did not differ between the two groups (26.8 ± 8.4 *vs.* 27.4 ± 7.4 mg/ml; $P > 0.8$).

Discussion

This study shows that RBP4 is strongly expressed in human adipocytes, as shown earlier in rat adipocytes (5). Negligible expression was detected in the stromavascular fraction of adipose tissue, as recently reported (16). During adipogenesis of human preadipocytes, there was a very strong induction of RBP4, which is typical of a marker of adipocyte differentiation (17). RBP4 is steadily secreted by human adipose tissue, as shown in experiments on adipose tissue explants. Therefore, RBP4 expression profile in human adipose tissue is similar to the profile described in rodents.

Studies in mice suggest that RBP4 is a factor produced by adipose tissue that induces insulin resistance in the liver and skeletal muscle (2). Plasma RBP4 was reported to be elevated in subjects with insulin resistance and type 2 diabetes, although the relationship between RBP4 and insulin resistance was not found in other cross-sectional studies on subjects with normal glucose tolerance or mildly insulin-resistant obese patients (6–8, 16, 18). Similarly, we did not find differences in plasma RBP4 levels between lean and obese women despite a lower glucose disposal rate in the latter group. To gain further insight into the relationship between insulin sensitivity and RBP4, we investigated, in a prospective study, nondiabetic obese women during different time points of a multiple-phase weight reduction program. Insulin sensitivity was assessed by the gold standard technique, the euglycemic hyperinsulinemic clamp. The subjects had a marked decrease in body weight and fat mass during VLCD, a further moderate diminution during LCD and a stabilization during the WM phase. The glucose disposal rate was increased during VLCD and remained elevated during the subsequent phases. The plasma levels of RBP4 were markedly diminished during VLCD and subsequently increased during the later phases while remaining lower than basal

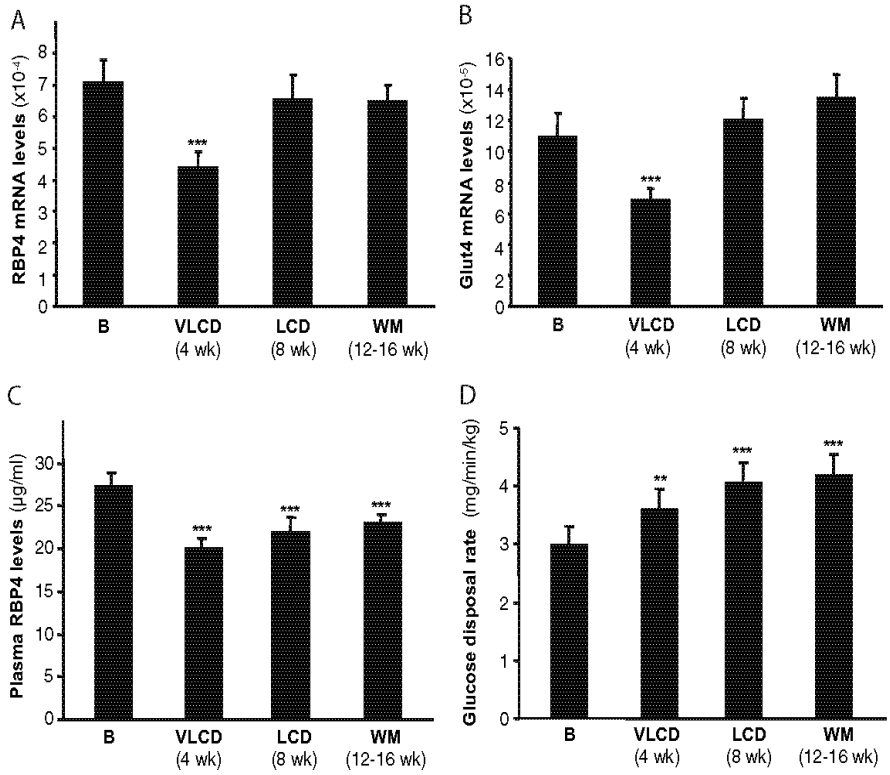

FIG. 2. Subcutaneous adipose tissue mRNA expression of RBP4 and Glut4, plasma level of RBP4, and glucose disposal rate during a weight reduction program in obese women (n = 24). A, Adipose tissue RBP4 mRNA levels. B, Glut4 mRNA levels. C, RBP4 plasma levels. D, Glucose disposal rate. Values are means ± SEM. **, $P < 0.01$. ***, $P < 0.001$ compared with basal values. B, Basal conditions; LCD, end of the LCD; VLCD, end of the VLCD; WM, end of the WM phase.

values. Therefore, the profile of the plasma RBP4 time course was not superimposable to the variations in insulin sensitivity indices. Moreover, no correlations were found between RBP4 and glucose disposal rate. It was pointed out that the variability of the RBP4 response to an intervention might be influenced by the baseline insulin sensitivity of subjects (19). However, stratification of the population of obese women into a high and low-insulin sensitivity group was associated neither with a significant difference in plasma RBP4 levels nor with different diet-induced responses of plasma levels of RBP4. When considering stratification according to the variations in insulin sensitivity induced by the dietary program, a similar conclusion was reached. Therefore, this kinetic study reveals that RBP4 is regulated by calorie restriction and weight loss but is not associated with insulin sensitivity in this population.

TABLE 2. Diet-induced changes in plasma levels of RBP4 in two subgroups with high and low initial insulin sensitivity

	Obese subjects with low-insulin sensitivity (n = 12)	Obese subjects with high-insulin sensitivity (n = 12)
Glucose disposal rate (mg/min/kg)	1.77 ± 0.12	4.18 ± 0.35[a]
Basal plasma RBP4 levels (µg/ml)	29 ± 3	26 ± 1
Decrease of plasma RBP4 levels during VLCD (%)	23 ± 8	26 ± 4
Decrease of plasma RBP4 levels during WM (%)	17 ± 5	13 ± 4

Values are means ± SEM.
[a] $P < 0.001$.

Plasma RBP4 levels are increased in mice with genetic ablation of Glut4 in adipocytes (2). Glut4 mRNA and protein levels are reduced in fat cells in several insulin-resistant states and in type 2 diabetes (20). In nonobese subjects with at least one first-degree relative with type 2 diabetes, a negative correlation was found between adipocyte Glut4 and plasma RBP4 levels (8). During the dietary intervention, the profile of variation of Glut4 mRNA levels was quite similar to that of RBP4 mRNA. Accordingly, a positive relationship between adipose tissue RBP4 and Glut4 gene expression was reported in adipose tissue from obese subjects (16). Therefore, the expected inverse relationship between adipose tissue Glut4 and RBP4 mRNA is not found in obese subjects.

Concentrations of serum proteins are used in assessing the

clinical status of patients with moderate-to-severe malnutrition. RBP4 is a clinically useful marker because it has a short half-life. In that respect, the decrease of plasma RBP4 levels during VLCD is in full agreement with earlier studies (21, 22). Moreover, this study shows that RBP4 gene expression in adipose tissue is subject to regulation by VLCD. Comparison of the evolution of adipose tissue mRNA and plasma levels suggests that the decrease of RBP4 levels during severe calorie restriction may be at least partly due to decreased adipocyte production. The down-regulation during VLCD may be related to ketosis because plasma β-hydroxybutyrate levels were increased specifically during this period. During LCD and WM, adipose tissue RBP4 mRNA levels returned to basal values, whereas the increase in plasma levels was more moderate and did not reach the prediet levels. Therefore, it may be hypothesized that, during these phases, the lower RBP4 levels reflect the reduction in fat mass. The magnitude of the decrease in fat mass during LCD and WM (22–23%) is indeed coherent with the decrease in RBP4 levels (15–20%). Another possibility is that the reduced plasma levels observed during long-term weight loss result from altered production by the liver, the probable major source of RBP4 in humans, but to date evidence is lacking for such a regulation (23). Finally, it may be noted that the pattern of changes in RBP4 and leptin levels were similar, raising the possibility of coregulatory mechanisms between the two adipokines.

To conclude, although RBP4 adipose tissue gene expression and plasma levels are reduced during severe calorie restriction, no relationship was observed between RBP4 and the improvement of insulin sensitivity induced during a weight reduction program in obese women. RBP4 is a marker of nutritional deficit but does not appear as a marker of insulin resistance during dietary intervention.

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References

1. Arner P 2003 The adipocyte in insulin resistance: key molecules and the impact of the thiazolidinediones. Trends Endocrinol Metab 14:137–145
2. Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, Kotani K, Quadro L, Kahn BB 2005 Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. Nature 436:356–362
3. Abel ED, Peroni O, Kim JK, Kim Y-B, Boss O, Hadro E, Minnemann T, Shulman GI, Kahn BB 2001 Adipose-selective targeting of the Glut4 gene impairs insulin action in muscle and liver. Nature 409:729–733
4. Quadro L, Blaner WS, Salchow DJ, Vogel S, Piantedosi R, Gouras P, Freeman S, Cosma MP, Colantuoni V, Gottesman ME 1999 Impaired retinal function and vitamin A availability in mice lacking retinol-binding protein. EMBO J 18:4633–4644
5. Tsutsumi C, Okuno M, Tannous L, Piantedosi R, Allan M, Witt SGD, Blaner WS 1992 Retinoids and retinoid-binding protein expression in rat adipocytes. J Biol Chem 267:1805–1810
6. Basualdo CG, Wein EE, Basu TK 1997 Vitamin A (retinol) status of first nation adults with non-insulin-dependent diabetes mellitus. J Am Coll Nutr 16:39–45
7. Abahusain MA, Wright J, Dickerson JW, de Vol EB 1999 Retinol, alpha-tocopherol and carotenoids in diabetes. Eur J Clin Nutr 53:630–635
8. Graham TE, Yang Q, Bluher M, Hammarstedt A, Ciaraldi TP, Henry RR, Wason CJ, Oberbach A, Jansson PA, Smith U, Kahn BB 2006 Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. N Engl J Med 354:2552–2563
9. Cho YM, Youn BS, Lee H, Lee N, Min SS, Kwak SH, Lee HK, Park KS 2006 Plasma retinol-binding protein-4 concentrations are elevated in human subjects with impaired glucose tolerance and type 2 diabetes. Diabetes Care 29:2457–2461
10. Polonsky KS 2006 Retinol-binding protein 4, insulin resistance, and type 2 diabetes. N Engl J Med 354:2596–2598
11. Curat CA, Miranville A, Sengenes C, Diehl M, Tonus C, Busse R, Bouloumie A 2004 From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes. Diabetes 53:1285–1292
12. Tiraby C, Tavernier G, Lefort C, Larrouy D, Bouillaud F, Ricquier D, Langin D 2003 Acquirement of brown fat cell features by human white adipocytes. J Biol Chem 278:33370–33376
13. Petersen M, Taylor MA, Saris WH, Verdich C, Toubro S, MacDonald I, Rossner S, Stich V, Guy-Grand B, Langin D, Martinez JA, Perdersen O, Holst C, Sorensen TI, Astrup A 2006 Randomised, multi-center trial of two hypoenergetic diets in obese subjects: high- versus low-fat content. Int J Obes 30:552–560
14. Klimcakova E, Polak J, Moro C, Hejnova J, Majercik M, Viguerie N, Berlan M, Langin D, Stich V 2006 Dynamic strength training improves insulin sensitivity without altering plasma levels and gene expression of adipokines in subcutaneous adipose tissue in obese men. J Clin Endocrinol Metab 91:5107–5112
15. DeFronzo RA, Tobin JD, Andres R 1979 Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Physiol 237:E214–E223
16. Janke J, Engeli S, Boschmann M, Adams F, Bohnke J, Luft FC, Sharma AM, Jordan J 2006 Retinol-binding protein 4 in human obesity. Diabetes 55:2805–2810
17. Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM 2000 Transcriptional regulation of adipogenesis. Genes Dev 14:1293–1307
18. Takashima N, Tomoike H, Iwai N 2006 Retinol-binding protein 4 and insulin resistance. N Engl J Med 355:1392
19. Oh J 2006 Retinol-binding protein 4 and insulin resistance. N Engl J Med 355:1394
20. Shepherd PR, Kahn BB 1999 Glucose transporters and insulin action–implications for insulin resistance and diabetes mellitus. N Engl J Med 341:248–257
21. Shetty PS, Watrasiewicz KE, Jung RT, James WP 1979 Rapid-turnover transport proteins: an index of subclinical protein-energy malnutrition. Lancet 2:230–232
22. Scalfi L, Laviano A, Reed LA, Borrelli R, Contaldo F 1990 Albumin and labile-protein serum concentrations during very-low-calorie diets with different compositions. Am J Clin Nutr 51:338–342
23. Blaner WS 1989 Retinol-binding protein: the serum transport protein for vitamin A. Endocr Rev 10:308–316

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Paper 3

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Retinol-Binding Protein 4 Expression in Visceral and Subcutaneous Fat in Human Obesity

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Summary

Retinol binding protein 4 (RBP4) is a novel adipokine which might be involved in the development of insulin resistance. The aim of the study was to investigate the expression of RBP4 mRNA in subcutaneous and visceral fat depots and the relationship between RBP4 plasma and mRNA levels relative to indices of adiposity and insulin resistance. In 59 Caucasian women (BMI 20 to 49 kg/m^2) paired samples of subcutaneous and visceral fat were obtained for RBP4, leptin and GLUT 4 mRNA analysis using reverse transcription-quantitative PCR. Euglycemic hyperinsulinemic clamp and computed tomography scans were performed. RBP4 mRNA levels as well as GLUT 4 mRNA and leptin mRNA levels were lower ($P<0.001$, $P<0.01$ and $P<0.001$, respectively) in visceral compared to subcutaneous fat. No differences were found in RBP4 mRNA expression in the two fat depots or in RBP4 plasma levels between subgroups of non-obese subjects (n=26), obese subjects without metabolic syndrome (n=17) and with metabolic syndrome (n=16). No correlations between RBP4 mRNA or plasma levels relative to adiposity, glucose disposal rate and GLUT 4 mRNA expression in adipose tissue were found. There was a weak positive correlation between plasma RBP4 and plasma triglycerides ($r = 0.30$, $p<0.05$) and between plasma RBP4 and blood glucose ($r = 0.26$, $p<0.05$). Regardless of the state of adiposity or insulin resistance, RBP4 expression in humans was lower in visceral than in subcutaneous fat. We found no direct relationship between either RBP4 mRNA or its plasma levels and the adiposity or insulin resistance.

Key words

Obesity • Insulin resistance • Visceral and subcutaneous adipose tissue • Retinol-binding protein 4

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Introduction

Obesity is one of the principal causes of insulin resistance and a significant risk factor for type 2 diabetes. Excess adipose tissue is associated with the release of several molecules having paracrine and/or endocrine activity capable of contributing to the development of insulin resistance. A recently characterized potential candidate is retinol binding protein 4 (RBP4) (Yang *et al.* 2005). Adipose tissue RBP4 mRNA expression and circulating plasma levels have been shown to be increased in several mouse models of insulin resistance.

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Overexpression of RBP4 or injection of recombinant RBP4 induces insulin resistance in mice, whereas pharmacologically decreased serum levels of RBP4 improved insulin sensitivity in mice maintained on high fat diets (Yang *et al.* 2005). Selective genetic knockout of the insulin-stimulated glucose transporter (GLUT 4) in adipocytes results in impaired whole-body insulin sensitivity, an effect thought to be mediated by RBP4 (Abel *et al.* 2001). In humans, elevated RBP4 levels have been reported in subjects with insulin resistance and type 2 diabetes (Yang *et al.* 2005, Cho *et al.* 2006, Graham *et al.* 2006). RBP4 is produced by hepatocytes but it is also produced in rat (Tsutsumi *et al.* 1992) and human (Janke *et al.* 2006) adipocytes. In a previous study we have shown that RPB4 is secreted by human adipose tissue explants and that it is expressed almost exclusively in mature adipocytes (the expression in stromavascular fraction of adipose tissue being negligible) (Vitkova *et al.* 2007).

Recently, mRNA expression of RBP4 has been found to be down-regulated in subcutaneous adipose tissue of obese women (Janke *et al.* 2006) and up-regulated in a group of ten women with polycystic ovary syndrome when compared to lean controls (Tan *et al.* 2007). Moreover, during a multiple phase dietary intervention, changes in RBP4 mRNA were not related to the improvement in insulin sensitivity (Janke *et al.* 2006). No relationship between plasma RBP4 levels and RBP4 mRNA expression in subcutaneous adipose tissue has been found (Janke *et al.* 2006). These findings might suggest a possible role of visceral fat depot in the RBP4 production. Therefore, we investigated RBP4 mRNA expression in paired samples of subcutaneous and visceral adipose tissue in a cohort of patients with a wide range of BMI and visceral fat mass, as evaluated by computed tomography, and diverse insulin sensitivity. This cohort was also used to study the relationship between plasma RBP4 and indices of adiposity and between plasma RBP4 and insulin resistance.

Methods

Subjects

Fifty-nine Caucasian women (age 21 to 66 years, BMI 19.6 to 48.5 kg/m^2) scheduled to have abdominal surgery (laparoscopic or laparotomic cholecystectomy, hysterectomy and gastric banding) were recruited for the study in collaboration with the Departments of Surgery and Gynecology at Královské Vinohrady Faculty Hospital in Prague. Subjects with following conditions were excluded from the study: malignancy, inflammatory conditions (based on clinical and laboratory findings), congestive heart failure, known coronary heart disease, known endocrinopathies, chronic liver or kidney disease and psychiatric disorders. All the subjects were weight-stable, i.e. their body weight fluctuations were less than 2 % during the preceding 3 months. The study was approved by the Ethical Committee of the Third Faculty of Medicine, Charles University, Prague. A written informed consent was obtained from each subject before the start of the study.

Study protocol

Prior to their surgery (7-14 days), each participant was examined after an overnight fast at 8.00 h.

Anthropometric measurements

Body height, weight and waist and hip circumference were measured and body composition was evaluated using bioelectrical impedance (QuadScan 4000, Bodystat, Douglas, British Isles). Visceral fat area and the relative ratio of intra-abdominal visceral fat to the subcutaneous fat area were calculated using computed tomography scans at the level L4-5 as previously described (Fujioka *et al.* 1987).

Euglycemic-hyperinsulinemic clamp

Insulin sensitivity was assessed using euglycemic-hyperinsulinemic clamp performed according to DeFronzo *et al.* (1979). The dose of insulin was 40 mU/min/m^2 body surface. The rate of glucose disposal was defined as the glucose infusion rate during the stable period of 30 min during the second hour of the clamp and was related to body weight (M – mg/min/kg). Blood samples for determination of baseline values were obtained before the clamp.

Adipose tissue samples

During the scheduled surgical procedure, paired samples of visceral (omental) and subcutaneous adipose tissue were obtained and processed immediately.

Analytical methods

Blood glucose, triglycerides, total and HDL cholesterol and plasma insulin were measured using standard procedures. Plasma RBP 4 concentrations were determined using sandwich ELISA (Immunodiagnostik AG, Behsheim, Germany). This kit is identical in

protocol and reagent composition with the ELISA kit from ALPCO Diagnostics (USA) tested by Graham *et al.* (2007). Plasma samples were diluted so that the absorbance was in the middle of the range of linearity for the assay (intraassay CV was 5 % and interassay CV was 9 %, within-run CV for RBP-4 was 2.7 %).

RNA analysis

Adipose tissue samples were washed, homogenized in RLT lysis buffer (Qiagen, Courtaboeuf, France) and stored at −80 °C until analyzed. Total RNA extraction was done with a RNeasy Mini kit (Qiagen, Courtaboeuf, France). Reverse transcription was performed with 1 μg of total RNA using random hexamers as primers and Superscript II reverse transcriptase (Invitrogen, Cergy Pontoise, France). Real-time quantitative PCR (qPCR) was performed with a Taqman probe-based RBP4 gene expression assay using a ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers and TaqMan probes for RBP4, leptin and GLUT4 were obtained from Applied Biosystems. Each sample was measured in duplicate and 10 ng of cDNA was used as a template for real-time qPCR. If the difference between duplicate samples exceeded 0.5 Ct, then qPCR was repeated. 18S ribosomal RNA was used as a control to normalize gene expression (Ribosomal RNA Control TaqMan Assay kit, Applied Biosystems). Results are expressed as $2^{-\Delta Ct}$ values.

Statistical analysis

The data were analyzed using SPSS 13.0 statistical software. The data are reported as means ± SEM. Before statistical analysis, non-normally distributed parameters were logarithmically transformed. Differences between visceral and subcutaneous fat were assessed using the Student t-test. A one way ANOVA was used for group comparisons with a Bonferoni post hoc analysis. The Pearson's correlation coefficient was calculated to quantify univariate associations.

Results

Stratification of the entire study group

The entire group of subjects consisted of 26 non-obese and 33 obese subjects. The obese group was further stratified according to the presence (n=17) or absence (n=16) of the metabolic syndrome evaluated according to the International Diabetes Federation criteria (Alberti *et al.* 2005). Relevant anthropometric and metabolic characteristics of the three groups are given in Table 1. Obese subjects with metabolic syndrome had a higher relative amount of visceral fat (when related to the subcutaneous depot) and a higher plasma triglycerides concentration compared with obese without metabolic syndrome. In fact, for these two variables, the latter group did not differ from non-obese subjects. The glucose disposal rate was lower in obese patients than in lean subjects.

RBP 4 expression in visceral and subcutaneous fat

RBP4 mRNA levels in visceral adipose tissue were markedly lower than those in the subcutaneous adipose tissue in each of the three subgroups (Table 2) as well as for the entire group (2.0±0.2 vs. 8.0±0.7 arbitrary units, $p<0.001$). There were no differences in RBP4 mRNA levels in either visceral or subcutaneous fat between the three groups. For the entire group, there was a significant correlation between RBP4 mRNA levels in visceral and subcutaneous fat ($r = 0.347$, $p<0.05$).

GLUT 4 and leptin expression in visceral and subcutaneous fat

In the three subgroups, leptin mRNA expression was lower in visceral compared to subcutaneous fat. GLUT 4 mRNA levels were lower in visceral fat, the difference, however, being significant only in obese subjects without metabolic syndrome (Table 2).

Plasma RBP4 concentrations

No differences in plasma RBP4 concentrations were seen between the three subgroups (Table 1). Additionally, no significant correlations between the plasma RBP4 levels and either visceral or subcutaneous mRNA levels were found for the entire group or each of the subgroups (data not shown).

Relationship between RBP4 indices and anthropometric or metabolic variables

For the entire study group, none of the RBP4 indices, i.e. plasma RBP4, subcutaneous mRNA and visceral mRNA levels, correlated with BMI, fat mass, waist circumference or with the CT-evaluated area of visceral or subcutaneous fat (Table 3). Similarly, none of the RBP4 indices correlated with glucose disposal rate, fasting plasma insulin, plasma LDL or HDL cholesterol. However, plasma RBP4 showed a weak correlation with plasma triglycerides and blood glucose (Fig. 1).

Table 1. Anthropometric and metabolic characteristics in non-obese patients (nonOB) and obese patients without (OB) and with (MS) metabolic syndrome.

Group	nonOB (n = 26)	OB (n = 17)	MS (n = 16)
Age (years)	40.2 ± 2.3	41.6 ± 2.4	49.4 ± 2.7
BMI (kg/m^2)	24.5 ± 0.73	37.1 ± 1.4 [a]	33.9 ± 1.2 [b]
Waist (cm)	83.3 ± 2.1	108.8 ± 2.7 [a]	107.3 ± 2.6 [b]
Fat mass (%)	30.2 ± 1.38	45.3 ± 1.16 [a]	41.9 ± 1.39 [b]
Visceral fat (cm^2)	74.1 ± 10.1	121.4 ± 9.8 [a]	156.2 ± 12.8 [b]
Subcutaneous fat (cm^2)	250.2 ± 25.3	510.5 ± 30.3 [a]	409.2 ± 29.9 [b,c]
Visceral /subcutaneous fat	0.28 ± 0.11	0.24 ± 0.07	0.42 ± 0.18 [b,c]
HDL-cholesterol (mmol/l)	1.48 ± 0.06	1.40 ± 0.07	1.25 ± 0.05 [b]
LDL-cholesterol (mmol/l)	3.13 ± 0.16	2.85 ± 0.27	3.0 ± 0.33
Triglycerides (mmol/l)	1.01 ± 0.12	1.22 ± 0.09	2.36 ± 0.36 [b,c]
Blood glucose (mmol/l)	4.78 ± 0.08	5.38 ± 0.12 [a]	6.22 ± 0.44 [b]
Insulin (mmol/l)	5.88 ± 0.54	10.6 ± 1.7 [a]	13.3 ± 1.5 [b]
Glucose disposal rate - M (mg.kg^{-1}.min^{-1})	6.17 ± 0.44	3.80 ± 0.34 [a]	2.81 ± 0.33 [b]
RBP4 plasma (mg/l)	27.8 ± 1.5	26.0 ± 1.6	29.2 ± 1.8

Values are means ± S.E.M. Groups are compared by one way ANOVA with Bonferroni´s multiple t-test post-hoc analysis: [a] $p<0.05$ OB vs. nonOB, [b] $p<0.05$ MS vs. nonOB, [c] $p<0.05$ MS vs. OB.

Table 2. Relationships of RBP4, GLUT 4 and leptin mRNA expression in visceral (VAT) and subcutaneous (SCAT) adipose tissue in non-obese (nonOB), obese without (OB) and obese with (MS) metabolic syndrome.

Group	nonOB (n = 26)		OB (n = 17)		MS (n = 16)	
	VAT	SCAT	VAT	SCAT	VAT	SCAT
mRNA RBP4	2.8 ± 0.4 [d]	8.9 ± 1.3	2.5 ±0.3 [d]	9.7 ± 1.3	1.8 ± 0.3 [d]	5.7 ± 0.9
mRNA GLUT 4	5.4 ± 1.07	7.7 ± 1.1	2.9 ± 0.5 [e]	6.0 ± 1.1	2.7 ± 0.8	3.4 ± 0.8
mRNA leptin	8.7 ± 1.9 [d]	30.4 ± 3.8	9.7 ± 1.5 [d]	65.1 ± 9.7	25.1± 6.3 [e]	43.6 ± 7.7

Values are means ± S.E.M. mRNA are expressed in arbitrary units (AU) x 10^{-4}. [d] mRNA expression in visceral vs. subcutaneous adipose tissue, $p<0.001$, [e] mRNA expression in visceral vs. subcutaneous adipose tissue, $p<0.05$

Relationship between RBP4 and GLUT 4 expressions

In the entire study group, GLUT 4 mRNA levels in visceral fat correlated with RBP4 mRNA levels in visceral as well as subcutaneous fat ($r = 0.447$, $p<0.01$) while no such correlations were found for GLUT4 in subcutaneous fat.

Discussion

Retinol binding protein 4 has been proposed as an adipokine involved in the regulation of systemic glucose metabolism and pathogenesis of insulin resistance (Yang *et al.* 2005, Graham *et al.* 2006, Cho *et al.* 2006, Janke *et al.* 2006). The expression of RBP4 has been demonstrated in subcutaneous and, recently, in visceral adipose tissue in humans (Tan *et al.* 2007). In our previous study (Vitkova *et al.* 2007) it was shown that 1) RBP4 is expressed mainly in adipocytes and the expression in stromavascular fraction of adipose tissue is negligible and 2) RBP4 is secreted from human adipose tissue explants. In the present study, we demonstrated that, in a group of women with a wide range of adiposity and insulin resistance, RBP4 expression in visceral fat was markedly lower than in subcutaneous fat. This finding was independent of BMI, amount of visceral or subcutaneous fat or the presence of metabolic syndrome in the examined subjects. In fact, no differences between the expression of RBP4 in either visceral or subcutaneous fat were found between the three subgroups stratified according to adiposity and presence of indices of

Table 3. Correlations of mRNA expression in subcutaneous (SCAT) and visceral (VAT) fat and of plasma RBP4 levels with selected anthropometric and metabolic variables in the entire group of subjects (n=59).

	logRBP4 plasma	mRNA RBP4 SCAT	mRNA RBP4 VAT
BMI	0.01	0.037	-0.081
Waist (cm)	0.039	0.076	-0.124
Fat mass (kg)	0.039	-0.048	-0.048
Visceral fat (cm^2)	0.116	0.056	-0.005
Subcutaneous fat (cm^2)	-0.01	-0.036	0.154
Triglycerides (mmol/l)	0.298 [a]	0.015	-0.166
HDL-cholesterol (mmol/l)	-0.106	0.024	-0.056
Blood glucose	0.261 [a]	0.021	0.025
Glucose disposal rate	-0.074	0.1	0.084

Data are Pearson´s correlation coefficient r, [a] $p<0.05$

metabolic syndrome. Our results suggest that, in humans, visceral adipose tissue is not significantly involved in RBP4-mediated effects on carbohydrate metabolism and /or insulin action or, alternatively, RBP4 does not appear to be a link between the visceral fat and insulin resistance.

Although RBP4 is expressed predominantly in mature adipocytes, the differences in gene expression between the two fat depots might be influenced by a higher macrophages infiltration in visceral compared with subcutaneous depots (Cancello *et al.* 2006). It may be hypothesized that the higher down-regulation of RBP4 in visceral fat by some of the macrophage-derived signals, such as TNF-α (Sell *et al.* 2007), might contribute to the lower RBP-4 expression in visceral fat. Alternatively, the effect of a higher „dilution" of adipocytes due to higher proportion of non-fat cells in visceral fat could be considered.

According to recent results showing the relationship between gene expression in adipocytes and adipose cell size (Skurk *et al.* 2007) it could be speculated that the difference in RBP4 expression between the visceral and subcutaneous fat depots might be associated with differences in adipocyte size in the two depots. The adipose cell size was not measured in the present study, although recent studies did not find major differences in

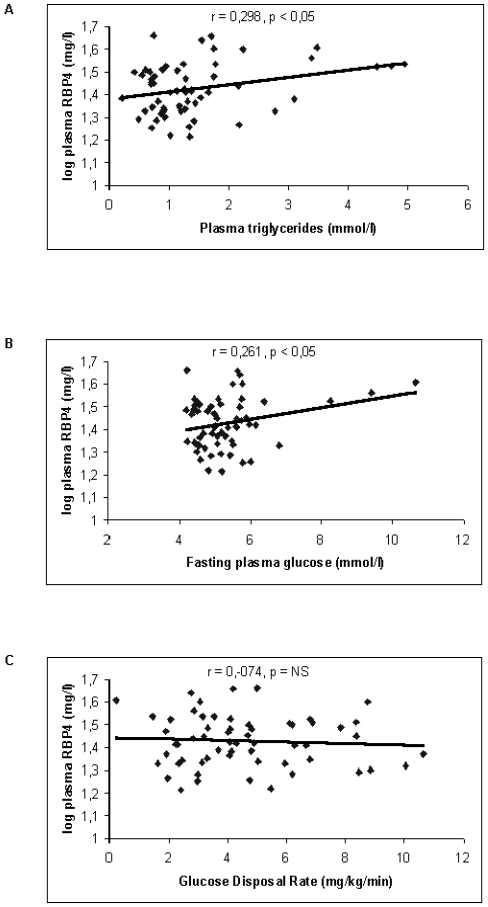

Fig. 1. Correlation between plasma RBP4 concentration (data were log transformed to achieve normal distribution), plasma triglycerides (A), fasting plasma glucose (B) and glucose disposal rate (C) in the entire group of subjects.

the size of adipocytes from visceral and subcutaneous depots (Winkler *et al.* 2003, Garaulet *et al.* 2006).

Subsequently, we investigated the relationship between the RBP4 mRNA levels in the two fat depots relative to indices of adiposity, including the absolute and relative amount of visceral fat, the magnitude of insulin resistance assessed by euglycemic hyperinsulinemic clamp and other obesity-related metabolic abnormalities (Table 3). No correlations between RBP4 mRNA in the two fat depots and the indices of adiposity, plasma lipids, blood glucose or glucose disposal rate during the clamp were found for the entire group nor for any of the three subgroups. In contrast to our results, Janke *et al.* (2006) found a down-regulation of RBP4 mRNA in subcutaneous fat in obese women, whereas Tan *et al.*

(2007) found an up-regulation of RBP4 mRNA expression in subcutaneous as well as in visceral fat depots in obese insulin-resistant women with polycystic ovary syndrome (PCOS). The differences in the patterns of age, degree of adiposity, body fat distribution, magnitude of insulin sensitivity and in androgenic status (in the study of Tan *et al.* 2007) could be possible reasons for the discrepancies. In fact, an up-regulation of RBP4 mRNA expression in human adipose tissue explants by 17-beta estradiol and testosterone was demonstrated (Tan *et al.* 2007).

In the present study, similarly as RBP4, markedly higher values of leptin mRNA levels in subcutaneous compared with visceral fat were found in the three subgroups. For leptin, the most solid data in respect with regional differences in expression are available and the latter finding is in agreement with previous studies (Masuzaki *et al.* 1995, Montague *et al.* 1997).

Animal studies suggested a reciprocal relationship between RBP4 and GLUT 4 expression in adipose tissue. In contrast to the studies of Janke *et al.* (2006) or Tan *et al.* (2007) no significant correlation was observed between GLUT4 and RBP4 mRNA in subcutaneous adipose tissue ($r = 0.078$, NS) while a positive correlation was found in visceral fat ($r = 0.447$, $p<0.01$).

Therefore, in spite of obvious limitations of clinical correlative studies, these results suggest that the regulation of RBP4 in humans differs from that in rodents. This is further supported by a recent study (Sell *et al.* 2007) that reported a positive relationship between RBP4 and GLUT 4 production in adipocytes derived from human mammary adipose tissue.

Recently, Klotig *et al.* (2007) found higher RBP4 mRNA levels in visceral fat compared with subcutaneous fat. Moreover, the authors found a negative correlation between GLUT4 and RBP4 expression in visceral but not in subcutaneous fat. The reasons of the above mentioned discrepancy between our study and that of Klotig *et al.* (2007) are difficult to discern, the differences in age range do not appear to be a probable cause. We replicated our RBP4 mRNA and leptin mRNA analysis and confirmed the relationship of leptin mRNA in visceral compared with subcutaneous fat. Our results are in agreement with several previous studies and this supports the reliability of mRNA analysis in the present study. When analyzing the relationships between plasma RBP4 and indices of adiposity, insulin resistance and metabolic syndrome, we found no differences in plasma RBP4 between the three subgroups (Table 1). Moreover, no significant correlations were found for the entire group or for the subgroups between plasma RBP4 and indices of adiposity, insulin resistance and metabolic syndrome with the exception of a weak positive correlation relative to plasma triglycerides and to blood glucose, which was in agreement with reported results (Erikstrup *et al.* 2006, Takashima *et al.* 2006). The absence of a relationship of plasma RBP4 levels in respect to the magnitude of insulin sensitivity is in agreement with several previous studies (Erikstrup *et al.* 2006, Janke *et al.* 2006, Broch *et al.* 2007, Tan *et al.* 2007) but in contrast to others (Cho *et al.* 2006, Graham *et al.* 2006, Balagopal *et al.* 2007, Haider *et al.* 2007, Lee *et al.* 2007). It has to be noted that, in the majority of those studies, insulin sensitivity was not – in contrast to the present study – assessed by euglycemic hyperinsulinemic clamp. In the present study we used a sandwich ELISA assay, which was identical to that showing a high correlation with insulin sensitivity (Graham *et al.* 2007).

In conclusion, our study demonstrates that RBP4 mRNA expression in visceral fat is low compared to subcutaneous fat regardless of the state of adiposity and the presence/absence of metabolic syndrome. Thus, RBP4 does not appear to be a potential link between visceral adiposity and obesity-related metabolic abnormalities. Additionally, our study failed to confirm a direct relationship between RBP4 adipose tissue expression or RBP4 plasma levels relative to adiposity or insulin sensitivity.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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References

ABEL ED, PERONI O, KIM JK, KIM Y-B, BOSS O, HADRO E, MINNEMANN T, SHULMAN GI, KAHN BB: Adipose-selective targeting of the Glut 4 gene impairs insulin action in muscle and liver. *Nature* **409**: 729-733, 2001.

ALBERTI KG, ZIMMET P, SHAW J: The metabolic syndrome – a new worldwide definition. *Lancet* **366**: 1059-1062, 2005.

BALAGOPAL P, GRAHAM TE, KAHN BB, ALTOMARE A, FUNANAGE V, GEORGE D: Reduction of elevated serum retinol binding protein in obese children by lifestyle intervention: association with subclinical inflammation. *J Clin Endocrinol Metab* **92**: 1971-1974, 2007.

BROCH M, VENDRELL J, RICART W, RICHART C, FERNANDEZ-REAL JM: Circulating retinol-binding protein-4, insulin sensitivity, insulin secretion and insulin disposition index in obese and nonobese subjects. *Diabetes Care* **30**: 1802-1806, 2007.

CANCELLO R, TORDJMAN J, PITOU C, GUILHEM G, BOUILLOTY JL, HUGOL D, COUSSIEU C, BASDEVNATY A, BARY HA, BEDOSSA P, GUERRE-MILLO M, CLEMENT K: Increased infiltration of macrophages in omental adipose tissue is associated with marked hepatic lesions in morbid human obesity. *Diabetes* **55**: 1554-1561, 2006.

CHO YM, YOUNG BS, LEE H, LEE N, MIN SS, KWAK SH, LEE HK, PARK KS: Plasma retinol-binding protein-4 concentrations are elevated in human subjects with impaired glucose tolerance and type 2 diabetes. *Diabetes Care* **29**: 2457-2461, 2006.

DEFRONZO RA, TOBIN JD, ANDERS R: Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol* **237**: E214-E223, 1979.

ERIKSTRUP CH., MORTENSEN OH, PEDERSEN BK: Retinol-binding protein 4 and insulin resistance. *N Engl J Med* **355**: 1393-1394, 2006.

FUJIOKA S, MATSUZAWA Y, TOKUNAGA K, TARUI S: Contribution of intra-abdominal fat accumulation to the impairment of glucose and lipid metabolism in human obesity. *Metabolism* **36**: 54-59, 1987.

GARAULET M, MORANTE-HERNANDEZ JJ, LUJAN J, TEBAR FJ, ZAMORA S: Relationship between fat cell size and number and fatty acid composition in adipose tissue from different fat depots in overweight/obese humans. *Int J Obes* **30**: 899-905, 2006.

GRAHAM TE, YANG Q, BLUHER M, HAMMARSTEDT A, CIARALDI TP, HENRY RR, WASON CJ, OBERBACH A, JANSSON PA, SMITH U, KAHN BB: Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. *N Engl J Med* **354**: 2552-2563, 2006.

GRAHAM TE, WASON CJ, BLUHER M, KAHN BB: Shortcomings in methodology complicate measurements of serum retinol binding protein (RBP4) in insulin-resistant human subjects. *Diabetologia* **50**: 814-823, 2007.

HAIDER DG, SCHINDLER K, PRAGER G, BOHDJALIAN A, LUGER A, WOLZT M, LUDVIK B: Serum retinol-binding protein 4 is reduced after weight loss in morbidly obese subjects. *J Clin Endocrinol Metab*. **92**: 1168-1171, 2007.

JANKE J, ENGELI S, BOSCHMANN M, ADAMS F, BOHNKE J, LUFT FC, SHARMA AM, JORDAN J: Retinol-binding protein 4 in human obesity. *Diabetes* **55**: 2805-2810, 2006.

KLOTIG N, GRAHAM TE, BERNDT J, KRALISCH S, KOVACS P, WASON CHJ, FASSHAUER M, SCHON M, STUMVOLL M, BLUHER M, KAHN B: Serum retinol-binding protein is more highly expressed in visceral than in subcutaneous adipose tissue and is a marker of intra-abdominal fat mass. *Cell Metab* **6**: 79-87, 2007.

LEE DC, LEE JW, IM JA: Association of serum retinol binding protein 4 and insulin resistance in apparently healthy adolescents. *Metabolism* **56**: 327-331, 2007.

MASUZAKI H, OGAWA Y, ISSE N, SATOH N, OKAZAKI T, SHIGEMOTO M, MORI K, TAMURA N, HOSODA K, YOSHIMASA Y *et al.:* Human obese gene expression. Adipocyte-specific expression and regional differences in the adipose tissue. *Diabetes* **44**: 855-858, 1995.

MONTAGUE CT, PRINS JB, SANDERS L, DIGBY JE, O'RAHILLY S: Depot- and sex-specific differences in human leptin mRNA expression: implication for the control of regional fat distribution. *Diabetes* **46**: 342-347, 1997.

SELL H, ECKEL J: Regulation of retinol binding protein 4 production in primary human adipocytes by adiponectin, troglitazone and TNF-α. *Diabetologia* **10**: 2221-2223, 2007.

SKURK T, HUBER-ALBERTI C, HERDER C, HAUNER H: Relationship between adipocyte size and adipokine expression and secretion. *J Clin Endocrinol Metab* **92**: 1023-1033, 2007.

TAKASHIMA N, TOMOIKE H, IWAI N: Retinol-binding protein 4 and insulin resistance. *N Engl J Med* **355**: 1392, 2006.

TAN BK, CHEN J, LEHNERT H, KENNEDY R, RANDEVA HS: Raised serum, adipocyte and adipose tissue retinol-binding protein 4 in overweight women with polycystic ovary syndrome: effects of gonadal and adrenal steroids. *J Clin Endocrinol Metab* **92**: 2764-2772, 2007.

TSUTSUMI C, OKUNO M, TANNOUS L, PIANTEDOSI R, ALLAN M, WITT SGD: Retinoids and retinoid-binding protein expression in rat adipocytes. *J Biol Chem* **267**:1805-1810, 1992.

VITKOVA M, KLIMCAKOVA E, KOVACIKOVA M, VALLE C, MORO C, POLAK J, HANACEK J, CAPEL F, VIGUERIE N, RICHTEROVA B, BAJZOVA M, HEJNOVA J, STICH V, LANGIN D: Plasma levels and adipose tissue mRNA expression of retinol-binding protein 4 are reduced during calorie restriction in obese subjects but are not related to diet-induced changes in insulin sensitivity. *J Clin Endocrinol Metab* 92: 2330-2335, 2007.

WINKLER G, KISS S, KESZTHELYI L, SAPI Z, ORY I, SALAMON F, KOVACS M, VARGHA P, SZEKERES O, SPEER G, KARADI I, SIKTER M, KASZAS E, DWORAK O, GERO G, CSEH K: Expression of tumor necrosis factor (TNF)-α protein in the subcutaneous and visceral adipose tissue in correlation with adipocyte cell volume, serum TNF-α, soluble serum TNF-receptor-2 concentrations and C-peptide level. *Eur J Endocrinol* **149**: 129-135, 2003.

YANG Q, GRAHAM TE, MODY N, PREITNER F, PERONI OD, ZABOLOTNY JM, KOTANI K, OUADRO L, KAHN BB: Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature* **436**: 356-362, 2005.

Paper 4

Kovacikova M, Sengenes C, Kovacova Z, Siklova-Vítkova M, Klimcakova E, Polak J, Rossmeislova L, Bajzova M, Hejnova J, Hnevkovska Z, Bouloumié A, Langin D, Stich V.

Dietary intervention-induced weight loss decreases macrophage content in adipose tissue of obese women.

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Dietary intervention-induced weight loss decreases macrophage content in adipose tissue of obese women

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Running title: Human adipose tissue macrophages during diet

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ABSTRACT

Objective: Accumulation of adipose tissue macrophages (ATM) is observed in obesity and may participate in the development of insulin resistance and obesity-related complications. The aim of our study was to investigate the effect of long term dietary intervention on ATM content in human adipose tissue.

Design: We performed a multi-phase longitudinal study.

Subjects and measurements: 27 obese pre-menopausal women (age 39 ± 2 years, BMI 33.7 ± 0.5 kg/m^2) underwent a 6-month dietary intervention consisting of two periods: 4 weeks of very low calorie diet (VLCD) followed by weight stabilization composed of 2 months of low calorie diet and 3-4 months of weight maintenance diet. At baseline and at the end of each dietary period, samples of subcutaneous adipose tissue (SAT) were obtained by needle biopsy and blood samples were drawn. ATMs were determined by flow cytometry using combinations of cell surface markers. Selected cytokine and chemokine plasma levels were measured by ELISA. In addition, in a subgroup of 16 subjects, gene expression profiling of macrophage markers in SAT was performed by real-time PCR.

Results: Dietary intervention led to a significant decrease of body weight, plasma insulin and C-reactive protein levels. After VLCD, ATM content defined by CD45+/14+/206+ did not change while it decreased at the end of the intervention. This decrease was associated with a down-regulation of macrophage marker mRNA levels (CD14, CD163, CD68, LYVE-1) and plasma levels of MCP-1 and CXCL5. During the whole dietary intervention, the proportion of two ATM subpopulations distinguished by the CD16 marker was not changed.

Conclusion: A 6-month weight-reducing dietary intervention, but not VLCD, promotes a decrease of the number of the whole ATM population with no change in the relative distribution of ATM subsets.

Keywords: obesity, subcutaneous adipose tissue, macrophages, dietary intervention, weight maintenance, obese women

INTRODUCTION

Obesity is a major health problem of the 21st century characterized by increased metabolic and cardiovascular complications. Its prevalence is reaching epidemic rate. During the last decade, systemic low-grade inflammation and increased accumulation of immune cells into adipose tissue (AT) have been associated with an excess of fat mass[1-6]. Inflammation results in changes of the secretory pattern of AT. The excess of fat mass is accompanied by elevated levels of pro-inflammatory cytokines (e.g. TNFα, IL-6), chemokines (e.g. IL-8, MCP-1) and acute phase proteins (e.g. SAA)[1, 7-9]. The increased production of these bioactive molecules could be linked to the altered cell composition in AT of obese subjects.

AT is a heterogeneous organ composed of mature adipocytes and stromavascular fraction (SVF) including mostly preadipocytes, endothelial cells, fibroblasts, macrophages and lymphocytes. Each cell type may contribute to cytokine secretion from AT. Much attention has been paid to adipose tissue macrophages (ATM) that have been considered as one of the main sources of circulating pro-inflammatory molecules[10-13]. Increased accumulation of ATM surrounding dead or dying adipocytes was observed in obese patients and correlated with adiposity[5, 14-16]. Genetic manipulation in rodents revealed that diminished content of ATM led to improved insulin sensitivity and decreased expression of inflammatory genes[17, 18]. There is growing evidence that ATM participate in an activation of inflammatory signalling pathways through Toll-like receptor and JNK-dependent pathways and lead to development of insulin resistance[19, 20]. Moreover, ATM-induced inflammation has been

associated with other co-morbidities such as cardiovascular diseases, liver steatosis or type 2 diabetes[14, 21-23].

Macrophages represent various cell populations with marked plasticity that mirror different functions depending on the stimuli that come from tissue microenvironment[24-26]. In mouse models, it has been reported that ATM have reparative (remodelling) function with anti-inflammatory phenotype (known as M2-alternatively activated macrophages) whereas newly recruited macrophages activated during obesity are mainly pro-inflammatory (known as M1- classically activated macrophages)[27, 28]. In humans, ATMs express both M1- and M2-related markers revealing an intermediate phenotype with inflammatory and anti-inflammatory capacities[10, 29, 30]. Little is known on the stimuli which influence AT inflammation and phenotype of immune cells. One of the possible factors altering immune system could be variation in nutritional status. Up to date no clinical study has investigated the effect of the diet-induced weight loss on ATM. Therefore, the aim of our study was to investigate the effect of weight reduction induced by long term dietary intervention on macrophage content and phenotype in subcutaneous adipose tissue (SAT) of obese women.

MATERIALS AND METHODS

Subjects

Twenty-seven obese pre-menopausal women participated in the study. A subgroup of 16 subjects (subgroup 1; age 39 ± 2 years, BMI 33.3 ± 0.5 kg/m^2) was enrolled in a preliminary study aimed at establishing flow cytometry analysis in needle biopsy samples of AT. After the initial study, another subgroup of 11 subjects (subgroup 2; age 39 ± 2 years, BMI 34.2 ± 0.9 kg/m^2) was enrolled in an identical protocol in which flow cytometry analysis was performed again with a deeper characterization of ATM. All

subjects were drug-free and did not suffer from any disease except for obesity. Their body weight had been stable for 3 months prior to the entry examination. A written informed consent to participate in the study was obtained from each subject 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).

Dietary intervention and clinical investigation

The two subgroups of patients underwent the same dietary protocol. During the first four weeks of the dietary intervention program, the subjects received a very-low calorie diet (VLCD) of 800 kcal/day (liquid formula diet, Redita, Promil). Then they underwent a weight stabilisation phase (WS) that consisted of a 2 months` low-calorie diet (LCD) and 3-4 months` weight maintenance diet (WM). 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. During WM the patients were instructed to keep on the weight-maintaining diet. Patients had regular consultations with a dietician during the whole dietary intervention and each time they provided a written 3 days´ dietary record. A complete clinical investigation was realized before and at the end of VLCD and WS phases in the morning in the fasting state. Body height and weight were measured along with waist and hip circumferences. Body composition was assessed using multi-frequency bioimpedance (Bodystat, Quad scan 4000, Isle of Man, British Isles). Coefficients of variation of fat mass, fat free mass and impedance were 1.7%, 0.8% and 1.5%, respectively. 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 was stored at -80°C until analysis. Thereafter needle

biopsy (diameter of needle 12G) of SAT was carried out in the abdominal region (10 cm laterally from umbilicus) under local anesthesia (1% Xylocain) as previously described[31]. In subgroup 1, one part of SAT (around 0.5g) was immediately frozen at -80°C until subsequent gene expression analysis and the other part of SAT (around 2g) was directly used for isolation of SVF cells. In subgroup 2, SAT was processed immediately to obtain SVF for flow cytometry.

Isolation of stromavascular fraction cells

SAT was washed with saline and digested with type I collagenase (SERVA, Heidelberg, Germany) for 1h in 37°C water shaking bath and subsequently centrifuged and filtered through 100-, 70-, 40-μm sieves to isolate SVF cells.

Flow cytometry analysis

Isolated SVF cells were used for flow cytometry analyses[24, 32]. In subgroup 1, cells were fixed in CellFix (BD Bioscience, Bedford, MA) (10min at 4°C) and after proper washing with PBS kept at 4°C until immunofluorescence analysis. In subgroup 2, fresh cells were labelled and analysed subsequently after isolation. They were resuspended in 100μl PBS solution containing 0.5% BSA and 2mmol/l EDTA and incubated with fluorescence-labelled monoclonal antibodies (FITC-conjugated antibody CD16; PE-conjugated antibody CD14; PerCP-conjugated antibodies CD45 and APC-conjugated antibodies CD206 and CD44) or the appropriate isotype controls (BD Bioscience, Bedford, MA) for 30min at 4°C according to protocol of Curat et al.[24]. After washing with PBS, the labelled cells were analyzed by flow cytometry using FACSCalibur flow cytometer and CellQuest Pro Software (BD Biosciences, NJ, USA). The number of ATM populations was expressed in percentage (subgroup1) or per g of AT after

determination of the total number of cells in SVF (subgroup2). To determine the effect of fixation, freshly isolated and fixed cells were tested for comparison of flow cytometry results. Fixation of the samples was associated with the modulation of the fluorescence intensity but not with the percentage of fluorescent cells. No change in number of SVF cells per g of AT was observed at the end of VLCD or WM when compared to pre-diet condition.

Because bleeding accompanied needle biopsy, we checked for blood contamination in AT samples. Cells positive for both CD44 and CD45 and with high granularity (high side scatter), hallmark of granulocytes, were identified in the SAT biopsy samples. Of note, granulocytes represented a substantial fraction of cells (around 25%), but its content remained constant during dietary intervention (24.2 ± 2.7% vs. 22.9 ± 2.6%). If blood contamination explained the changes in macrophage content, one could expect a parallel variation in macrophage and granulocyte numbers. However, we found no correlation between granulocyte number and macrophage content. In addition, the number of CD45/14/206- monocytes originating from the circulation was not changed during the dietary intervention. Hence, we could not find evidence of the influence of blood contaminating cells on the interpretation of flow cytometry results.

Quantification of macrophage markers expression by quantitative real time PCR (RT-qPCR)

In subgroup 1, about 0.5g of AT was used for mRNA extraction. Total RNA extraction and reverse transcription (RT-PCR) were performed as previously described[31]. 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).

A set of primers was designed for CD163 and LYVE-1 using software Primer Express 1.5 (Applied Biosystems) and used at a final concentration of 300 nmol/l with SYBR-Green based chemistry PCR mix (Applied Biosystems, Foster City, CA, USA). Primers and TaqMan probes for CD14 and CD68 were obtained from Applied Biosystems. 18S ribosomal RNA (Ribosomal RNA Control TaqMan Assay kit, Applied Biosystems, Foster City, CA, USA) was used as an endogenous control to normalize gene expression. Results are presented as $2^{\Delta Ct}$ values.

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). Circulating levels of selected bioactive molecules were measured by commercial ELISA kits: TNF-α (ultrasensitive, Invitrogen, Camarillo, CA), CXCL5/ENA-78 (Duoset, R&D Systems, Minneapolis, MN) and MCP-1 (Duoset, eBioscience, San Diego, CA). Homeostasis model assessment of the insulin resistance index (HOMA-IR) was calculated as follows: (fasting insulin (mU/l) x fasting glucose (mmol/l)) / 22.5. Serum C-reactive peptide levels were analyzed by immunoturbidimetry using ultrasensitive kit (Orion-Diagnostica). Plasma levels of other parameters were determined using standard clinical biochemistry methods.

Statistical analyses

Statistical analysis was performed using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL). The effect of weight loss on clinical data, gene expression and flow cytometry data was tested using non parametric Wilcoxon test for paired observations. Comparison of baseline clinical data between the two interventional subgroups was performed by

Mann-Whitney U test for unpaired values. Correlations between clinical data and flow cytometry data was analyzed using Spearman correlation coefficient. Data are presented as mean ± SEM. Differences at the level of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of dietary intervention on anthropometric and metabolic parameters in obese women

In the entire group of subjects (n = 27), obesity-related anthropometric parameters (weight, BMI, waist circumference) decreased after VLCD and, further on, at the end of WS (Table 1). Insulin resistance, as assessed by HOMA-IR, was lower at the end of both phases than at baseline. Plasma cholesterol, plasma triglycerides, plasma insulin as well as C-reactive protein levels were lower after VLCD and WS than at baseline. However, most clinical parameters showed main variations during VLCD. Statistical comparison by non parametric Mann-Whitney test revealed no difference between the two subgroups of subjects (data not shown).

Monocyte/macrophage content and gene expression in subcutaneous adipose tissue of obese women during a dietary weight loss program

As this work was the first attempt to perform flow cytometry analysis on needle SAT biopsy samples we performed a preliminary study in the subgroup 1. Monocyte/macrophage content in SVF was identified by CD45 (common leukocyte antigen) and CD14 (co-receptor of TLR4) markers. We found no change in percentage of CD45+/14+ cells after VLCD. However, when compared the baseline to WS and VLCD to WS values, we observed diet-induced decrease of this cell population in SAT

(Fig. 1A). To confirm flow cytometry results, we determined gene expression of macrophage markers CD14, CD68, CD163, and LYVE-1 by real-time PCR. The profile of the diet-induced response of macrophage marker mRNA levels was similar to that of macrophage content (Fig. 1B). The pattern of change in percentage of CD45+/14+ cells was similar in the subgroup 2, i.e. no change after VLCD and decrease after WS ($p<0.05$, data not shown).

Effect of dietary intervention on the phenotype of adipose tissue macrophages.

In subgroup 2, adaptations of ATM during dietary intervention were investigated using a wider panel of macrophage CD markers. As the combination of CD45 and CD14 markers identifies not only population of tissue macrophages, but also blood monocytes, we used additional markers to specifically characterize ATM. The triple positive population identified by CD45, CD14 and CD206 marker (scavenger mannose receptor) corresponds to tissue macrophages.

In the first phase of the dietary intervention, after VLCD, we did not observe any change in the number of CD45+/14+/206+ ATMs (Fig. 2A). However, at the end of the dietary intervention, after WS, the number of ATMs was decreased (by 43%). When the phenotype of ATM was specifically investigated using the discriminating marker CD16 (immunoglobulin G Fcγ receptor III), the relative ATM subpopulation cell content characterized by the expression of CD45+/14+/206+/16+ and CD45+/14+/206+/16- was not altered after VLCD, but decreased at the end of the dietary program (by 43% and 42%, respectively) (Fig 2B, C). The content of both ATM subpopulations did not differ at the beginning of diet. As the profile of changes was similar for the both populations, we did not observe diet-induced changes in the ratio between the two subtypes of ATM.

Effect of dietary intervention on plasma levels of MCP-1, CXCL5 and TNF-α.

We further investigated plasma levels of selected chemokines and cytokine related to macrophage function, such as MCP-1, CXCL5 and TNF-α in the entire group of 27 obese women (Table 1). The pattern of MCP-1 plasma level was similar to that of monocyte/macrophage content during dietary intervention, i.e. no alteration after VLCD and decrease after WS. The circulating level of CXCL5 was already reduced after VLCD and remained decreased at the end of the whole protocol. TNF-α plasma levels did not show changes during the dietary intervention.

Relationship between ATM content and clinical parameters during dietary intervention.

In subgroup 2 of 11 obese women, no correlations were found between basal levels of ATM content characterized as CD45+/14+/206+, clinical parameters and cytokine and chemokines plasma levels. The same findings were observed between the dietary intervention-induced changes of ATM content and these parameters. In addition, in the entire group of 27 obese women no correlation was found between diet-induced alterations of the content of CD45+/14+ cells and circulating levels of investigated molecules CXCL5, MCP-1 and TNF-α.

DISCUSSION

In the present study, we investigated the effect of different phases of a weight-reducing dietary intervention on ATM content and phenotype. During the first 4 weeks of VLCD, there was no change in ATM content and phenotype determined by flow cytometry analysis, in line with a lack of variation of macrophage marker mRNA levels. After WS, at the end of the 6-month dietary intervention, we observed a significant decrease of the whole ATM population and macrophage marker gene expression with no alteration in

the relative proportion of two ATM subpopulations distinguished by expression of the CD16 marker.

Limited data are available on the regulation of ATM content in humans. Previous studies revealed an increase of ATM in SAT during obesity[10, 24, 30]. For the first time, we used flow cytometry approach to study ATM during several phases of a dietary intervention. Multicolour flow cytometry analysis allowed quantitative assessment and phenotypic characterization of ATM on a limited amount of AT obtained by needle biopsy, a mandatory condition for longitudinal studies. Moreover, compared to immunohistochemistry, flow cytometry analysis allows more accurate characterization (i.e. immunophenotypization) of particular cell populations because of the use of a combination of specific markers and the analysis of a larger number of cells. However, the procedure presents several limitations. One is blood contamination causing infiltration of granulocytes into the sample. However, their content did not change during dietary intervention, as well as the content of CD45/14/206- cells which might be considered as monocytes coming from the circulation. Furthermore, we used in our immunofluorescent analysis specific marker for tissue macrophages, i.e. CD206 which is not expressed on circulating monocytes [10] (also our own experience) to determine the whole population of ATM. The regulation of ATM content in SAT was similar in two independent groups of subjects and in accordance with mRNA measurement of specific macrophage markers.

The data obtained here are in agreement with a recently reported whole genome transcriptomic analysis of AT gene expression during a long term dietary intervention with similar design[33]. Indeed, in the VLCD phase, there was an increase or no change of inflammation- and macrophage- related genes. At the end of the whole dietary program, there was a coordinated down-regulation of these genes including CD14, CD68 and

CD163 as shown in the present study. Our results are also in agreement with the study of Cancello et al.[1] who observed reduction in macrophage recruitment in SAT of morbidly obese subjects 3 months after bypass surgery determined by immunohistochemistry using CD68 and HAM56 marker and gene expression profiling. The current study brings new information about the dynamics of ATM content characterized by combination of more macrophage markers during dietary intervention in moderate obese subjects and, importantly, at multiple dietary time points.

We also observed diet-induced changes in circulating levels of the monocyte/macrophage chemoattractant protein MCP-1 and a recently published chemokine produced by ATM, CXCL5[34]. The alteration of MCP-1 levels mirrored the changes in content of the CD45+/14+ cells whereas CXCL5 already decreased after VLCD as reported by Chavey et al.[34]. Nevertheless, we did not find correlation between these chemokines and ATM content. The lack of relation may be explained by the fact that these molecules are secreted into the circulation by various cells in different tissues[35, 36]. Regarding the plasma levels of TNF-α, we did not find any changes in response to the weight reduction protocol. Similar conclusions were reached in several clinical studies with different lengths and types of diets recently reviewed by Klimcakova et al.[37]. Moreover, Mohamed-Ali et al.[38] showed that AT is a poor contributor to circulating levels of TNF-α. The lack of correlation between ATM content and other clinical parameters may have several origins. First, the number of subjects (n=11) enrolled in our longitudinal study aimed at ATM content investigation was limited. Second, most changes in metabolic parameters of obese subjects, i.e. CRP, insulin, HOMA-IR, triglycerides occurred during VLCD whereas ATM content and gene expression decreased during WS. Finally, other insulin-sensitive tissues, such as liver and skeletal muscle, have probably been affected by the dietary intervention. It has

indeed been shown that macrophages resident in these tissues (such as Kupffer cells in the liver) could be activated and induce worsening of insulin sensitivity in obese patients[39, 40].

It is known that the nature of macrophages is heterogeneous and their phenotype can be modified in various conditions[26, 41, 42]. It is therefore important to determine not only the total number of macrophages but also their phenotype in AT. From a wide range of macrophage polarization states, the most frequently described have been M1 as ”classically activated” releasing pro-inflammatory cytokines and M2 as “alternatively activated” producing anti-inflammatory cytokines[29, 43-45]. However, the reliable markers for M1 and M2 characterization in human AT are still not clearly defined. Most results come from *in vitro* or animal experiments and only limited number of human studies solves this problem. In our study, we characterized the phenotype of ATMs using cell surface markers CD45, CD14, CD206 and the discriminating marker CD16. The CD206 marker is sometimes reported as an anti-inflammatory marker. However, in recently published studies [10, 30] it has been used for characterization of ATMs with mixed phenotype. Therefore in this context it cannot be considered as an M2 marker. The CD16 marker has been used to show the existence of two major subsets of monocyte/macrophage subpopulations with distinct roles in target tissues[10, 46-48]. Both, CD45+/14+/206+/16+ and CD45+/14+/206+/16-, subtypes of ATM did not change after VLCD and decreased after WS. The ratio between the two cell types was not modified after WS. These data suggest a decrease in total macrophage content rather than a change in macrophage phenotype after WS. In line, our previous study showed a down-regulation of both pro- (e.g. human leukocyte antigen-D region (HLA-DRA), osteopontin (SPP1)) and anti-inflammatory (e.g. IL-10) genes[33]. These findings favor “the balanced macrophage activation hypothesis” which explains macrophage activation

as a complex of cyclic processes to keep balance between pro- and anti-inflammatory states in inflamed tissue[49]. In the mouse, high fat diet induces a recruitment of ATM with M1 properties and no change in M2 macrophages[27, 28]. As the ratio of ATM subpopulations did not change after WM in our study, one can hypothesize that dietary-induced weight loss does not mirror dietary-induced weight gain or that species differences account for the discrepancy. Both assertions may prove correct. VLCD induced a substantial decrease in body weight; however, the number of ATM was not decreased. The differences between human and mouse macrophages including ATM is well documented[10, 29, 50]. A recent study investigating human ATM phenotype by immunohistochemistry analysis reported that the M1/M2 ratio shifted in favor of M2 status 3 months after gastric surgery[51]. This study investigated class III morbidly obese subjects and used single markers to calculate numbers of M1 and M2 macrophages whereas we studied class I obese women following a 6 month dietary intervention and used combination of cell surface markers specific for macrophages. Human ATMs appear to have predominantly an anti-inflammatory M2-like phenotype (reparative/remodeling activity), but they also produce pro-inflammatory cytokines[10, 30]. Depending on microenvironmental stimuli they could change their functional state. One of the possible factors could be PPARγ inducing M2 polarization of ATM activated through PUFA action or hypoxia as a signal for activation of inflammatory and ER stress-associated pathways [52-54].

In conclusion, our study provides evidence that ATM content in moderately obese women is not modified after the short term severe calorie restriction phase of a dietary weight-reducing program but is substantially decreased after a 6-month period including weight maintenance. The ATM diminution is not accompanied by a change in macrophage phenotype. Further studies are needed to identify the determinants of ATM

dynamics during weight reduction and to find other specific markers for the identification of macrophage activation.

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References

1. Cancello R, Henegar C, Viguerie N, Taleb S, Poitou C, Rouault C *et al.* Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. *Diabetes* 2005; **54**(8)**:** 2277-86.
2. Duffaut C, Zakaroff-Girard A, Bourlier V, Decaunes P, Maumus M, Chiotasso P *et al.* Interplay Between Human Adipocytes and T Lymphocytes in Obesity. CCL20 as an Adipochemokine and T Lymphocytes as Lipogenic Modulators. *Arterioscler Thromb Vasc Biol* 2009.
3. Elgazar-Carmon V, Rudich A, Hadad N, Levy R. Neutrophils transiently infiltrate intra-abdominal fat early in the course of high-fat feeding. *J Lipid Res* 2008; **49**(9)**:** 1894-903.
4. Kintscher U, Hartge M, Hess K, Foryst-Ludwig A, Clemenz M, Wabitsch M *et al.* T-lymphocyte infiltration in visceral adipose tissue: a primary event in adipose tissue inflammation and the development of obesity-mediated insulin resistance. *Arterioscler Thromb Vasc Biol* 2008; **28**(7)**:** 1304-10.
5. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 2003; **112**(12)**:** 1796-808.
6. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ *et al.* Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 2003; **112**(12)**:** 1821-30.
7. Heilbronn LK, Campbell LV. Adipose tissue macrophages, low grade inflammation and insulin resistance in human obesity. *Curr Pharm Des* 2008; **14**(12)**:** 1225-30.
8. Hotamisligil GS. Inflammation and metabolic disorders. *Nature* 2006; **444**(7121)**:** 860-7.
9. Poitou C, Coussieu C, Rouault C, Coupaye M, Cancello R, Bedel JF *et al.* Serum amyloid A: a marker of adiposity-induced low-grade inflammation but not of metabolic status. *Obesity (Silver Spring)* 2006; **14**(2)**:** 309-18.
10. Bourlier V, Zakaroff-Girard A, Miranville A, De Barros S, Maumus M, Sengenes C *et al.* Remodeling phenotype of human subcutaneous adipose tissue macrophages. *Circulation* 2008; **117**(6)**:** 806-15.
11. Fain JN, Madan AK, Hiler ML, Cheema P, Bahouth SW. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology* 2004; **145**(5)**:** 2273-82.
12. Maury E, Ehala-Aleksejev K, Guiot Y, Detry R, Vandenhooft A, Brichard SM. Adipokines oversecreted by omental adipose tissue in human obesity. *Am J Physiol Endocrinol Metab* 2007; **293**(3)**:** E656-65.
13. Shoelson SE, Herrero L, Naaz A. Obesity, inflammation, and insulin resistance. *Gastroenterology* 2007; **132**(6)**:** 2169-80.
14. Cancello R, Tordjman J, Poitou C, Guilhem G, Bouillot JL, Hugol D *et al.* Increased infiltration of macrophages in omental adipose tissue is associated with marked hepatic lesions in morbid human obesity. *Diabetes* 2006; **55**(6)**:** 1554-61.
15. Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E *et al.* Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res* 2005; **46**(11)**:** 2347-55.

16. Ortega Martinez de Victoria E, Xu X, Koska J, Francisco AM, Scalise M, Ferrante AW, Jr. *et al.* Macrophage content in subcutaneous adipose tissue: associations with adiposity, age, inflammatory markers, and whole-body insulin action in healthy Pima Indians. *Diabetes* 2009; **58**(2)**:** 385-93.
17. Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R *et al.* MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest* 2006; **116**(6)**:** 1494-505.
18. Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K *et al.* CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *J Clin Invest* 2006; **116**(1)**:** 115-24.
19. Lumeng CN, Deyoung SM, Saltiel AR. Macrophages block insulin action in adipocytes by altering expression of signaling and glucose transport proteins. *Am J Physiol Endocrinol Metab* 2007; **292**(1)**:** E166-74.
20. Nguyen MT, Favelyukis S, Nguyen AK, Reichart D, Scott PA, Jenn A *et al.* A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways. *J Biol Chem* 2007; **282**(48)**:** 35279-92.
21. Apovian CM, Bigornia S, Mott M, Meyers MR, Ulloor J, Gagua M *et al.* Adipose macrophage infiltration is associated with insulin resistance and vascular endothelial dysfunction in obese subjects. *Arterioscler Thromb Vasc Biol* 2008; **28**(9)**:** 1654-9.
22. Cipolletta C, Ryan KE, Hanna EV, Trimble ER. Activation of peripheral blood CD14+ monocytes occurs in diabetes. *Diabetes* 2005; **54**(9)**:** 2779-86.
23. Tordjman J, Poitou C, Hugol D, Bouillot JL, Basdevant A, Bedossa P *et al.* Association between omental adipose tissue macrophages and liver histopathology in morbid obesity: influence of glycemic status. *J Hepatol* 2009; **51**(2)**:** 354-62.
24. Curat CA, Miranville A, Sengenes C, Diehl M, Tonus C, Busse R *et al.* From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes. *Diabetes* 2004; **53**(5)**:** 1285-92.
25. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 2008; **8**(12)**:** 958-69.
26. Stout RD, Suttles J. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J Leukoc Biol* 2004; **76**(3)**:** 509-13.
27. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* 2007; **117**(1)**:** 175-84.
28. Lumeng CN, DelProposto JB, Westcott DJ, Saltiel AR. Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes. *Diabetes* 2008; **57**(12)**:** 3239-46.
29. Bourlier V, Bouloumie A. Role of macrophage tissue infiltration in obesity and insulin resistance. *Diabetes Metab* 2009.
30. Zeyda M, Farmer D, Todoric J, Aszmann O, Speiser M, Gyori G *et al.* Human adipose tissue macrophages are of an anti-inflammatory phenotype but capable of excessive pro-inflammatory mediator production. *Int J Obes (Lond)* 2007; **31**(9)**:** 1420-8.
31. Klimcakova E, Polak J, Moro C, Hejnova J, Majercik M, Viguerie N *et al.* Dynamic strength training improves insulin sensitivity without altering plasma levels and gene expression of adipokines in subcutaneous adipose tissue in obese men. *J Clin Endocrinol Metab* 2006; **91**(12)**:** 5107-12.

32. Miranville A, Heeschen C, Sengenes C, Curat CA, Busse R, Bouloumie A. Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. *Circulation* 2004; **110**(3)**:** 349-55.
33. Capel F, Klimcakova E, Viguerie N, Roussel B, Vitkova M, Kovacikova M *et al.* Macrophages and adipocytes in human obesity: adipose tissue gene expression and insulin sensitivity during calorie restriction and weight stabilization. *Diabetes* 2009; **58**(7)**:** 1558-67.
34. Chavey C, Lazennec G, Lagarrigue S, Clape C, Iankova I, Teyssier J *et al.* CXC ligand 5 is an adipose-tissue derived factor that links obesity to insulin resistance. *Cell Metab* 2009; **9**(4)**:** 339-49.
35. Conti P, DiGioacchino M. MCP-1 and RANTES are mediators of acute and chronic inflammation. *Allergy Asthma Proc* 2001; **22**(3)**:** 133-7.
36. Walz A, Schmutz P, Mueller C, Schnyder-Candrian S. Regulation and function of the CXC chemokine ENA-78 in monocytes and its role in disease. *J Leukoc Biol* 1997; **62**(5)**:** 604-11.
37. Klimcakova E, Kovacikova M, Stich V, Langin D. Adipokines and dietary interventions in human obesity. *Obes Rev*.
38. Mohamed-Ali V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS *et al.* Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. *J Clin Endocrinol Metab* 1997; **82**(12)**:** 4196-200.
39. Arkan MC, Hevener AL, Greten FR, Maeda S, Li ZW, Long JM *et al.* IKK-beta links inflammation to obesity-induced insulin resistance. *Nat Med* 2005; **11**(2)**:** 191-8.
40. Varma V, Yao-Borengasser A, Rasouli N, Nolen GT, Phanavanh B, Starks T *et al.* Muscle inflammatory response and insulin resistance: synergistic interaction between macrophages and fatty acids leads to impaired insulin action. *Am J Physiol Endocrinol Metab* 2009; **296**(6)**:** E1300-10.
41. Suganami T, Nishida J, Ogawa Y. A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor alpha. *Arterioscler Thromb Vasc Biol* 2005; **25**(10)**:** 2062-8.
42. Surmi BK, Hasty AH. Macrophage infiltration into adipose tissue: initiation, propagation and remodeling. *Future Lipidol* 2008; **3**(5)**:** 545-556.
43. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol* 2003; **3**(1)**:** 23-35.
44. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* 2004; **25**(12)**:** 677-86.
45. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. *Front Biosci* 2008; **13:** 453-61.
6. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 2003; **19**(1)**:** 71-82.
47. Rogacev KS, Ulrich C, Blomer L, Hornof F, Oster K, Ziegelin M *et al.* Monocyte heterogeneity in obesity and subclinical atherosclerosis. *Eur Heart J* 2009.
48. Ziegler-Heitbrock L. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. *J Leukoc Biol* 2007; **81**(3)**:** 584-92.
49. McGrath MS, Kodelja V. Balanced macrophage activation hypothesis: a biological model for development of drugs targeted at macrophage functional states. *Pathobiology* 1999; **67**(5-6)**:** 277-81.

50. Zeyda M, Stulnig TM. Adipose tissue macrophages. *Immunol Lett* 2007; **112**(2)**:** 61-7.
51. Aron-Wisnewsky J, Tordjman J, Poitou C, Darakhshan F, Hugol D, Basdevant A *et al.* Human Adipose Tissue Macrophages: M1 and M2 Cell Surface Markers in Subcutaneous and Omental Depots and after Weight Loss. *J Clin Endocrinol Metab* 2009.
52. Bouhlel MA, Derudas B, Rigamonti E, Dievart R, Brozek J, Haulon S *et al.* PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell Metab* 2007; **6**(2)**:** 137-43.
53. Deguchi JO, Yamazaki H, Aikawa E, Aikawa M. Chronic hypoxia activates the Akt and beta-catenin pathways in human macrophages. *Arterioscler Thromb Vasc Biol* 2009; **29**(10)**:** 1664-70.
54. Odegaard JI, Ricardo-Gonzalez RR, Goforth MH, Morel CR, Subramanian V, Mukundan L *et al.* Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. *Nature* 2007; **447**(7148)**:** 1116-20.

Table 1. Clinical and biochemical characteristics of obese subjects during dietary intervention (n=27).

	Basal	After VLCD	After WS
Weight (kg)	93.6 ± 1.7	85.9±1.6$^{\#\#\#}$	81.1±1.8*** $^{+++}$
BMI (kg/m^2)	33.7±0.5	30.9±0.4$^{\#\#\#}$	29.2±0.6*** $^{+++}$
Fat mass (kg)	38.6±1.1	33.7±1.0$^{\#\#\#}$	29.2±1.3*** $^{+++}$
Fat free mass (kg)	54.9±0.9	52.2±0.9$^{\#\#\#}$	51.9±0.9***
Waist circumference (cm)	101.7±1.3	93.5±1.4$^{\#\#\#}$	88.5±1.6*** $^{+++}$
Waist-to-hip ratio	0.9±0.0	0.8±0.0$^{\#\#\#}$	0.8±0.0***
Systolic blood pressure (mm Hg)	116.4±2.4	115.1±2.1	113.5±2.2
Diastolic blood pressure (mm Hg)	75.0±1.7	71.2±1.9$^{\#}$	72.5±1.6
Cholesterol (mmol/L)	5.2±0.2	3.9±0.2$^{\#\#\#}$	4.8±0.1** $^{+++}$
Triglycerides (mmol/L)	1.3±0.1	1.1±0.1$^{\#}$	1.1±0.1*
HDL-C (mmol/L)	1.5±0.1	1.2±0.1$^{\#\#\#}$	1.5±0.1^{+++}
Glucose (mmol/L)	5.2±0.1	4.9±0.1$^{\#\#}$	5.1±0.1^{+}
Insulin (mU/L)	10.2±1.1	6.4±0.7$^{\#\#\#}$	6.7±0.6***
HOMA-IR	2.4±0.3	1.4±0.2$^{\#\#\#}$	1.6±0.2***
CRP (mg/L)	3.8±0.6	2.2±0.4$^{\#}$	2.1±0.4**
MCP-1 (ng/ml)	124.3 ± 10.6	119.6 ±12.4	101.2 ±12.9**
CXCL5 (ng/ml)	214.9 ± 45.0	118.6 ± 21.6$^{\#}$	123.8 ± 22.5*
TNF-α (ng/ml)	1.4 ± 0.1	1.6 ± 0.1	1.6 ± 0.1

Data are presented as mean ± SEM.

$^{\#}$ p<0.05, $^{\#\#\#}$ p<0.001: basal vs VLCD

*p<0.05, ** p<0.01, *** p<0.001: basal vs WS

+ p<0.05, +++ p< 0.001: VLCD vs WS

BMI: body mass index; HDL-C: HDL cholesterol; HOMA-IR: homeostasis model assessment of the insulin resistance index; CRP: C-reactive protein; MCP-1: monocyte-chemoattractant protein-1; CXCL5: chemokine (C-X-C motif) ligand 5; TNF-α: tumor necrosis factor alpha; VLCD: very low calorie diet; WS: weight stabilization

Figure legends

Figure 1. Effect of dietary intervention on monocyte/macrophage content and gene expression in subcutaneous adipose tissue in subgroup 1 of obese women (n=16). (A) Multicolor flow cytometric analysis of monocyte/macrophage population characterized as CD45+/CD14+ cells, * $p<0.05$ basal vs. WS; ++ $p< 0.01$: VLCD vs WS (B) Gene expression of macrophage specific markers, ** $p<0.01$ basal vs. WS; + $p<0.05$, ++ $p< 0.01$, +++ $p< 0.001$: VLCD vs WS. Data are presented as mean fold change ± SEM;
VLCD: very low calorie diet; WS: weight stabilization; LYVE-1: Lymphatic Vessel Endothelial Receptor-1.

Figure 2. Effect of dietary intervention on adipose tissue macrophage content evaluated by flow cytometry based on the fluorescence-labeled antibodies CD45, CD14, CD206, and CD16 in subgroup 2 of obese women (n=11). (A) Number of CD45+/14+/206+ ATM population (B) Number of CD45+/14+/206+/16+ ATM subset. (C) Number of CD45+/14+/206+/16- ATM subset. Data are presented as mean number of cells per g of AT ± SEM; * $p<0.05$, ** $p<0.01$: basal vs WS.
VLCD: very low calorie diet; WS: weight stabilization.

Figure 1

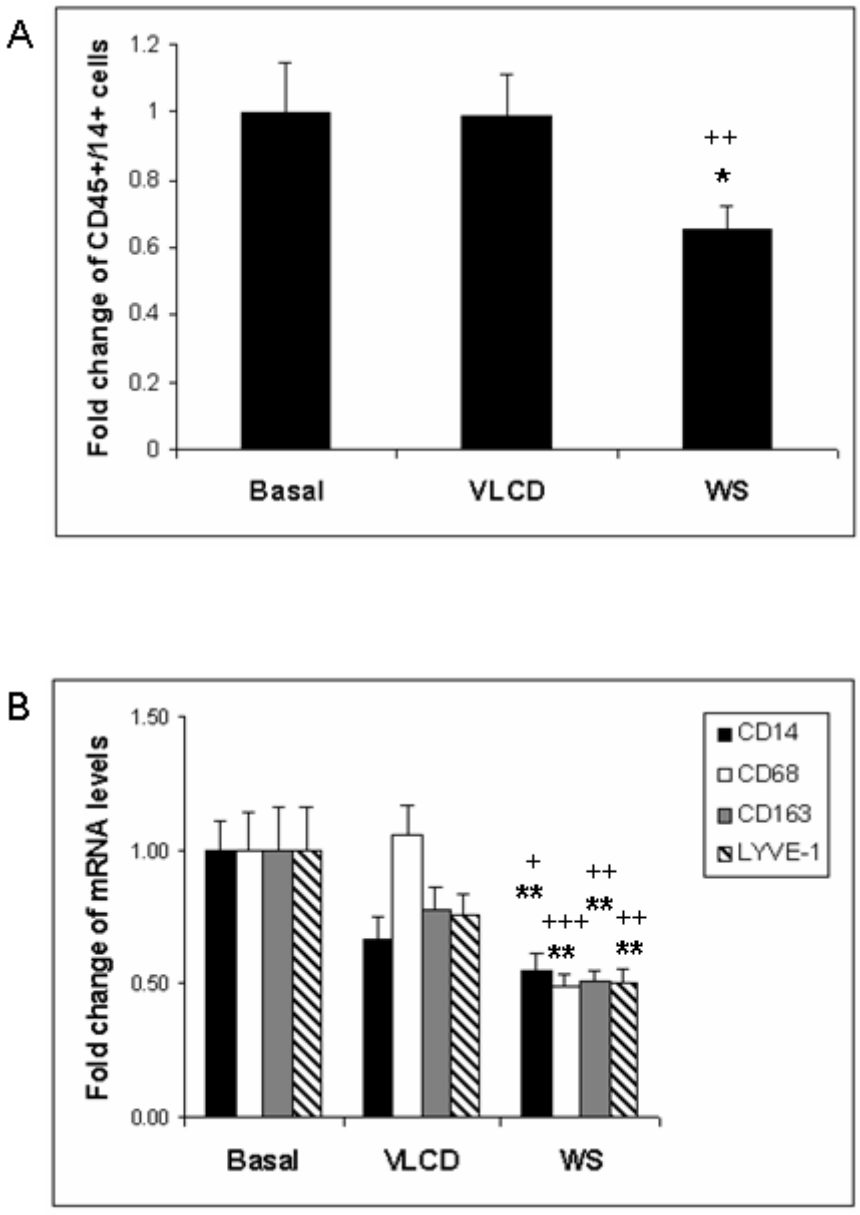
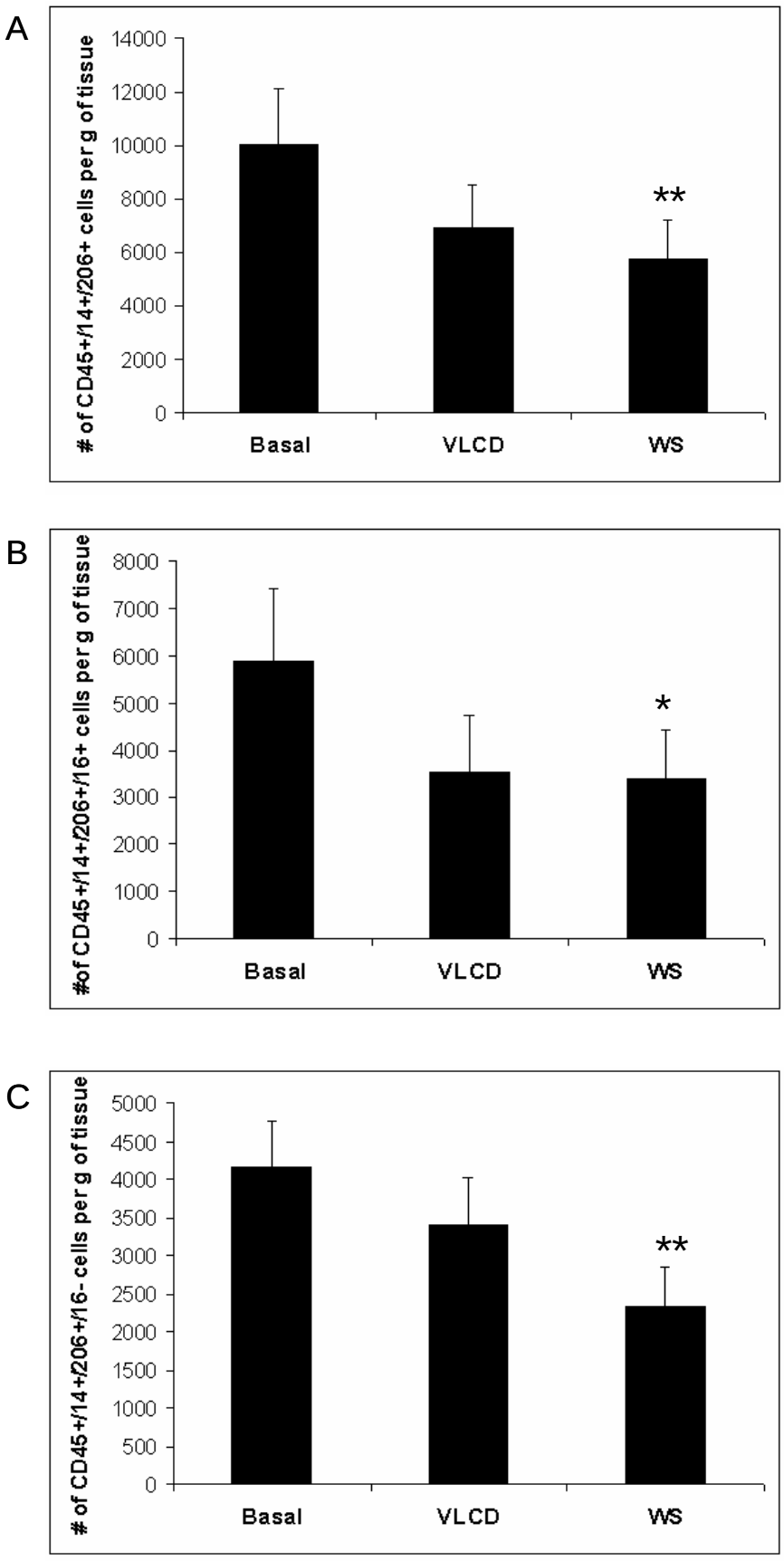

A
Fold change of CD45+/14+ cells
1.2
1
0.8
0.6
0.4
0.2
0
++
*
Basal
VLCD
WS
B
Fold change of mRNA levels
1.50
1.00
0.50
0.00
CD14
CD68
CD163
LYVE-1
+
**
++
**
+++
**
++
**
Basal
VLCD
WS

Figure 2


A
# of CD45+/14+/206+ cells per g of tissue
14000
12000
10000
8000
6000
4000
2000
0
**
Basal
VLCD
WS
B
#of CD45+/14+/206+/16+ cells per g of tissue
8000
7000
6000
5000
4000
3000
2000
1000
0
*
Basal
VLCD
WS
C
# of CD45+/14+/206+/16- cells per g of tissue
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
**
Basal
VLCD
WS

CHAPTER 4

DISCUSSION AND CONCLUSIONS

There is no doubt that obesity represents one of the biggest threats of health in this century with rapidly increased prevalence from year to year. Moreover, it is associated with a large number of non-communicable disorders, which worsen the lifespan. One of the accompanied phenomenons in obesity is a chronic, low-grade inflammation, which activates on the one hand immune cells and on the other hand worsens or inhibits the function of metabolic cells in insulin-sensitive organs. This intensive dialogue between two types of cells in metabolic disorder is not accidental. AT depots are vascularized by a dense network of capillaries and connected with lymph nodes, which provide influx of immune cells into the tissue and vice versa AT serves as a source of lipids for immune cells activation [373]. According to experimental findings, adipokines, FFA, or immune cells have been nominated among the possible culprits of AT inflammation. They could spin "the spiral of signalling cascades" inducing insulin resistance and defect of innate immunity. For reversion of these complications, there are recommended the non-pharmacological approaches at the beginning of obesity treatment, such as lifestyle modifications including DI and physical activity which could help in the improvement of metabolic, as well as biochemical parameters.

In our clinical studies, we tried to contribute to better understanding the molecular basis of beneficial effect of DI. We investigated the role of novel adipokines, visfatin and RBP4, as well as ATM in obesity-induced inflammation and insulin resistance and how they reflect to the dietary-induced changes.

PART 1

In previous studies visfatin and RBP4 have been shown to influence glucose homeostasis and participate in obesity-induced comorbidities, such as insulin intolerance and type 2 diabetes [103, 216, 236]. Regarding the visfatin response to a short-term DI, we demonstrated that visfatin mRNA levels in SAT increased dependent on the magnitude of weight loss, whereas its plasma levels did not change (had

tendency to decrease) after 3 months of DI. Until we published our findings, no study had presented results about mRNA levels of visfatin in relation to longitudinal intervention and only the cross-sectional studies, *in vitro* experiments, or studies examined plasma levels of visfatin were performed [220, 224, 225, 228, 229, 232]. To better understand the regulation of visfatin expression in AT during lifestyle modification, we investigated different bioactive molecules TNF-α, free testosterone, and insulin as possible regulatory factors influenced its transcriptional levels according to the knowledge obtained from *in vitro* studies [228-230, 232]. Based on the correlation analyses, we found negative association between visfatin mRNA levels and free testosterone and BMI and a positive relationship between visfatin and TNF-α mRNA expression. Furthermore, in a model comprising the above mentioned factors, we determined TNF-α mRNA levels and BMI as the strongest predictors of visfatin expression in SAT. We reported that transcriptional levels of TNF-α were higher in lean than in overweight/obese subjects. This controversial finding is difficult to explain. Nevertheless, the studies investigated mRNA levels of TNF-α are not consistent. Bastard et al. [123] has published that TNF-α mRNA levels significant increase after VLCD program. Moreover, Koistinen et al. [374] did not find any difference in TNF-α mRNA expression between lean and obese subjects. In our study, the similar expression profiles of both target adipokines, visfatin and TNF-α suppose a possible paracrine effect of TNF-α in AT, which was already proven in *in vitro* study by Hector et al. [229]. It may also reflect their regulation at the transcriptional level by a common factor in SAT. This response of adipokine expression to a short-term nutritional restriction suggests that these signalling molecules could be involved in adipocyte metabolism, which is dynamically changed during DI. Moreover, *in vitro* experiments and animal studies have revealed a role of TNF-α in regulation of lipolysis [177, 375], whereas the role of visfatin in this process has not been reported yet. On the other hand, recent study of Revollo et al. [216] showed that visfatin plays a key role in the regulation of insulin secretion by β-cells. However, in our study, we did not find any correlation between visfatin expression and insulin levels. In respect to circulating levels of visfatin, we must be cautious in the interpretation of the DI effect on its plasma levels because this adipokine is besides AT also produced by other tissues, such as the skeletal muscle, lymphocytes, macrophages, liver and bone marrow [217-219]. Moreover, its mRNA expression is differently regulated in SAT and VAT [219, 220, 224]. According to the presented results of our study, it is not clear whether visfatin acts as a pro- or anti-

inflammatory molecule and what kind of molecular pathways it activates. On the other hand, we demostrated here for the first time the response of visfatin expression to a short-term DI. Nevertheless, further investigations are needed to clarify its physiological function and participation in obesity-induced complications.

Our next study dedicated to RBP4 expression in relation to a multi-phase DI, showed significant reduction of RBP4 mRNA in SAT and plasma levels after energy restriction in VLCD. This diminution remained decreased only for plasma values at the end of the whole DI, after WM phase. To our knowledge, no study published so far has investigated this adipokine in a long-term DI (6 months), including WM phase in relation to insulin sensitivity. To determine the cellular source of RBP4 in AT, we examined RBP4 mRNA levels in different cell fractions in SAT. We revealed that RBP4 is mainly expressed by the adipocytes compared to the SVF cells and thus besides adiponectin and leptin belongs to "the true adipokines" exclusively secreted by the fat cells. However, the main source of RBP4 production in the body is the liver [238]. Hence, we did not find any correlation between mRNA in SAT and plasma levels of RBP4 in our clinical study. It could be explained by the fact that only 20% of circulating RBP4 is produced by adipocytes. According to findings in animal studies, it has been suggested that RBP4 production by AT influences glucose homeostasis and insulin sensitivity in the liver and skeletal muscle [103, 236]. In human cross-sectional studies, RBP4 circulating levels have been documented to be higher and associated with insulin resistance in type 2 diabetes patients or insulin-resistant subjects. Thus, it could be hypothesized that RBP4 plasma levels could be used as a quantitative marker for obesity complications related to insulin resistance, such as diabetes or atherosclerosis. However, this evidence was not confirmed in other clinical studies investigated subjects with normal glucose tolerance or obese insulin-resistant patients [237, 244, 376]. We also did not find any correlation between RBP4 plasma levels and glucose disposal rate or insulin sensitivity measured by the euglycemic hyperinsulinemic clamp. Moreover, RBP4 in plasma did not differ between lean and obese subjects in our clinical study. Regarding the measurement of RBP4 circulating levels, it has been recently published that the existing differences among results could be caused by the various methods with different sensitivity applied for the assessment of this adipokine in the blood [377]. We used for RBP4 analysis the commercial sandwich ELISA kit, which has been shown in the above mentioned methodological paper as an assay with a high inverse correlation

index in relation to insulin sensitivity. In spite of this fact, we did not receive similar results. It could be explained by different variations in profile of circulating RBP4 and the indices of insulin sensitivity during the whole DI. Moreover, when we subdivided all obese patients into the groups with low and high insulin sensitivity, we also did not obtain any significant association between these variables.

In previous studies investigating obese or diabetic patients, RBP4 production has been associated with the down-regulation of GLUT4 mRNA [103, 243]. This finding indicated that RBP4 negatively regulates glucose metabolism in insulin resistant state. However, it has not been confirmed in the study by Janke et al.[237] and likewise in our study, in which we observed similar response of GLUT4 and RBP4 mRNA levels to lifestyle modification, i.e. significant decrease after VLCD and subsequently increased to basal values at the end of the whole DI. To conclude these results, we can suggest that RBP4 acts as a "nutrient sensor" in adipocyte, whose activity is regulated by adipocyte metabolism influenced by energy restriction in VLCD, but not by insulin resistant state of the body. In addition, RBP4 has a rapid turn-over, what is a useful feature for clinical marker in monitoring the pathophysiological status of patients [378].

According to these interesting findings, we spread our scope of RBP4 investigation from longitudinal to cross-sectional study, in which we determined mRNA expression of RBP4 in different AT depots in relation to metabolic complications. We showed that SAT exhibited higher RBP4 mRNA levels than VAT, independently of BMI and the presence/absence of MS in the investigated patients. Moreover, we did not find any variations in RBP4 plasma levels between subjects with different state of adiposity and presence of MS, as well as any correlation with insulin resistance. Thus we confirmed the findings from our previous longitudinal study. However, our results are not in agreement with the outcomes from the study of Kloting et al.[243] who has reported greater mRNA expression of RBP4 in VAT compared to SAT, as well as higher RBP4 plasma levels in patients with type 2 diabetes or impaired glucose tolerance. Additionally, positive correlation between RBP4 circulating levels and mRNA in VAT and negative relationship between RBP4 and GLUT4 mRNA levels in VAT has been found. Contrary to this, we documented positive association between these two variables. The discrepancies found in both studies could be caused by the differences in analytical methods. Kloting et al. [243] used different housekeeping gene 36B4 (known as acidic ribosomal phosphoprotein PO) for the normalisation of RBP4 gene expression in paired AT samples, whereas we applied 18S ribosomal RNA. To

increase the reliability of our results, we performed mRNA expression of leptin, which is considered as a typical adipokine more expressed by SAT [337]. With respect to the measurements of RBP4 plasma concentrations, we applied the sandwich commercial ELISA assay (the same as in our previous study) compared to western blotting used in the study of Kloting [243]. According to our results, it seems that RBP4 does not play a key role in the systemic insulin resistance and visceral obesity; however its function is dependent on nutritional state of the body, but the exact mechanism is not clear.

AT as an endocrine organ produces a lot of bioactive molecules with different functions. Hence, the investigation of only single or a few molecules in relation to these comorbidities represents a limiting factor in our and also other clinical studies because these disturbances are evoked by a set of mechanisms that we cannot reveal at the level of one adipokine and conclude any clear outcomes. For future analyses, it would be prospective to study the global network between the functions of multiple signal molecules cooperated in the triggering of inflammatory machinery realized in AT during obesity. For instance, it has already been reported that leptin and adiponectin participate in the regulation of glucose metabolism via the activation of AMPK. Moreover, visfatin and RBP4 have also been associated with the regulation of glucose homeostasis (insulin-induced glucose transport); however there is missing knowledge about their interaction in this process. The novel high-troughput technologies, i.e. functional genomic approaches (e.g. trasncriptomics, proteomics, metabolomics, nutrigenomics etc.) could bring the necessary information.

PART 2

The rising number of evidence about an important role of immune cells in the implication of obesity and its related comorbidities attracted attention of many researchers, who started to study the changes in AT composition during different conditions. Most of findings have been reported from animal, *in vitro* or human cross-sectional studies focusing mainly on monocyte/macrophage population presented in AT and its activation in obesity [23, 26, 76, 218, 257, 284, 299, 300]. However, to our knowledge, no study so far has investigated the effect of moderate weight loss induced by long-term DI (6 months) including WM phase on ATM population (regarding the content and phenotype), which was analysed by flow cytometry in SAT of obese subjects in relation to metabolic changes accompanied the whole dietary program. We observed that the ATM content characterized as CD45+/14+/206+ did not change after

the period of energy restriction (VLCD), whereas at the end of DI, after WS the number of these cells significantly decreased. These results have also been confirmed at the transcriptional level of macrophage markers and plasma levels of chemokines. Regarding the ATM phenotype, we did not observe any variation in the relative ratio between two ATM subpopulations distinguished by the expression of cell surface marker CD16.

The results presented in this work are consistent with our previous clinical study of Capel et al. [379] who observed similar dynamics in gene expression profile of macrophage-related genes in SAT during multi-phase DI, i.e. no change (or increase) after short-term VLCD and a decrease at the end of the whole 6 month DI. Interestingly, contrary to this profile, adipocyte-related genes showed an inverse pattern of gene expression with marked decrease after VLCD. With respect to clinical parameters determined in our current work, the improvement of most of them e.g. BMI, fat mass, cholesterol, plasma glucose, insulin senstivity, CRP was reached after first weeks of energy restriction (VLCD), while the improvement of inflammatory response occurred after longer period, at the end of WS. These findings suggest a possible interplay between immune system and metabolism. One can hypothesize that the severe calorie restriction during VLCD affects primarily the fat cells and diminishes fat metabolism. On the other hand, VLCD represents a kind of metabolic stress for the organism which keeps immune cells in the activated state. They need longer period for adaptation to a new metabolic state of organism. However, there are raising questions how these systemic changes are regulated in various nutritional states. It has been suggested that cells possess “some sensors for caloric perturbations” [266]. Obesity is accompanied by many cellular changes realized at organelle levels like a mitochondria-induced oxidative stress or ER stress leading to the activation of inflammatory signalling pathways producing cytokines. Therefore, it is important to take into account also these signalling cascades affected during metabolic changes in cells.

To find some indirect functional effects of monocyte/macrophage dynamic in response to DI, we investigated circulating levels of chemokines MCP-1 and CXCL5. MCP-1 showed similar pattern of dietary-induced changes as alteration of ATM content. Plasma level of CXCL5 already decreased after VLCD and remained decreased at the end of the whole dietary intervention as it was reported in the study of Chavey et al. [212] However, in this multi-phase DI we observed no change of TNF-α plasma level in contrast to results of our previous study with a short-term DI. This non-uniform

response of TNF-α to nutritional intervention has already been reported in several longitudinal studies with different types and lenghts of diet summarized in the review of Klimcakova et al.[112]. It could be explained by the fact that AT contributes to a circulating level of TNF-α only marginally [190] and its change in plasma might not dirrectly mirror the dietary-induced changes of TNF-α secretion from AT.

Regarding the methodological aspects of our longitudinal study, we applied for the first time the method of flow cytometry for immunophenotyping and measurement of kinetic of the SVF cells, i.e. monocytes/macrophages during different phases of DI. Moreover, this approach was performed on a small amount of SAT obtained from needle biopsies. Until this investigation, flow cytometry has only been used in the cross-sectional studies on AT samples gained from plastic surgery [23, 218, 280, 284]. It has some advantages when compared with other methods, such as immunohistochemistry or gene expression investigated only single molecule, flow cytometry provides the analysis using multiple parameters (e.g. the size and granularity of cells, fluorescent-labelled cell surface or intracellular markers) for specific characterization of particular cell populations. On the other hand, cytometric application on needle biopsy samples brings some struggles. One of the main problems accompanied this method is blood contamination expressed by a high content of granulocytes. However, we observed in our study neither the changes in their content nor the correlation between granulocyte and macrophage number during the whole DI, so we could exclude possible influence on the interpretation of our results from flow cytometry. In addition, we included in our cytometric analysis specific marker for tissue macrophages CD206, which is not expressed on circulating monocytes [280] (also our own experience).

With respect to macrophage phenotype, it is known that macrophages represent heterogenous population of cells with high plasticity modulating their phenotype according to stimuli coming from microenvironment [296]. After publishing the paper of Lumeng et al. [299] about presence of different ATM phenotypes classified as M1 (pro-infammatory) and M2 (anti-inflammatory) in the AT and their changes during high-fat diet in mice, it became “a hot topic” to study not only the total content of these immune cells in AT, but also their phenotype in relation to various obesity-linked disorders and conditions in humans. However, there is a problem with the classification of M1 and M2 phenotype in human AT because the reliable markers are still missing. Most of findings come from animal or *in vitro* studies. In literature there is only the study of Zeyda et al. [284] and Bourlier et al.[280], which solve this issue. Based on

their findings, we used in our study for the characterization of ATM phenotypes the combination of cell surface markers CD45, CD14, CD206 and discriminating factor CD16. However, we found neither the differences between the content of particular ATM subtypes nor changes in their relative proportion in the whole ATM population during the whole DI. Contrary to our results, the recent study of Aron-Wisnewsky et al. [305] based on immunohistochemical analysis has demonstrated that 3 months diet after gastric banding led to a shift of the M1/M2 ratio towards the M2 phenotype in morbidly obese patients. This discrepancy could be explained by different degree of obesity in investigated subjects of both studies, as well as different method for identification of ATM phenotype (the advantages of flow cytometry compared to immunohistochemistry are mentioned above). Our data incline rather to a theory about the "balanced macrophage activation", which explains a set of molecular processes to keep a homeostasis between the pro- and anti-inflammatory states of macrophages in the inflamed tissue; however this balance could be impaired under the pathophysiological conditon [380]. Moreover, in the cross-sectional studies human ATM have been classified as cells with anti-inflammatory M2-like remodeling phenotype, which are also able to produce pro-inflammatory cytokines [280, 284]. According to stimuli coming from surrounding cells they could change their functional status, e.g. PUFA stimulation of PPARγ leads to M2 polarization [302].

However, in which proportion ATM are "good or bad guys" in tissue and what their exact role in AT is, it still remains as an unanswered question. They have been described as the cells accumulated around dying adipocytes and built so-called "crown-like structures" (CLS), but the phenotype of ATM in this structure is not known [256, 381]. We also confirmed the presence of ATM by immunohistochemical detection showing CLS in the AT (positive signal for CD68, CD163 marker) at the beginning and after VLCD phase, but not at the end of DI that can otherwise reflect the changes of inflammatory state in AT during long-term period (unpublished data).

It seems that primary role of ATM in the inflamed AT is to capture the lipid droplets released from dying adipocytes. It has been revealed that macrophages participate in the cholesterol efflux and lipid metabolism [265], which is a sharing characteristic with adipocytes and could have a crucial impact on the ATM activation and phenotype. Perhaps, the study of lipid metabolism in macrophages in relation to their functional changes could bring new insights into the classification of ATM phenotypes. Besides AT, also other metabolic organs like the liver and skeletal muscle

are infiltrated by immune cells, whose content and activation in this regions has not been analysed in detail [258, 309]. Finding the reliable markers for the characterization of macrophage phenotypes in particular organs could be prospective and serve as a tool in diagnostic prediction of various obesity-linked complications. The M1/M2 polarization was studied so far in *in vitro* and animal models but studying this process *in vivo* is difficult as there are a lot of steps in isolation of ATM that could activate these cells and influence the results but till now we do not have another method how to analyze this activation state in organism.

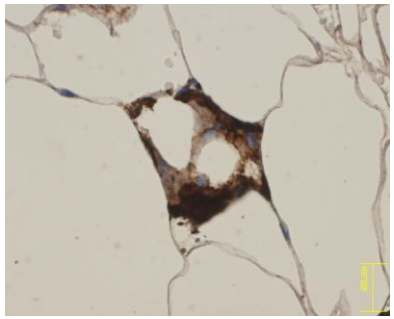
When we compared our results with data from already existing studies, we have found some discrepancies, which hampered to find clear conclusions in respect to particular molecules investigating in these works. They could be caused by the differences in the design of experimental studies (e.g. gender, age, size of investigated subjects, different method used for measurements of bioactive molecules, degree of obesity, type and lenght of investigation, magnitude of weight loss etc.). In addition, our clinical studies miss some functional experiments, which could verify our findings coming from association analyses. However, the amount of tissue gained from needle biopsies is limited for a performance of different types of experiments. Therefore, we also could not measure the size of adipocytes that has been recently published as a key factor associated with nutritional changes and secretion of adipokines [290]. It is well-known that AT is not the only tissue participating in glucose metabolism, but there are also other insulin-sensitive tissues like the skeletal muscle or liver which contribute to metabolic homeostasis in the body. However, in our clinical studies we were limited in availability of these tissues to study. The "complete picture" about the responsiveness of "these trio players" to different interventions could shed a light on missing information about how these organs are connected via signalling molecules and influence insulin activity. Nevertheless, these mentioned limitations would be inspirative for the set up of further clinical studies.

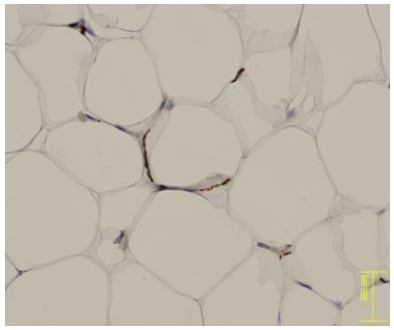
To summarize our findings discussed in this work and depicted in Figure 12, we observed that both types of DI (short-term and multi-phase) led to significant weight loss (8% and 13%, respectively), improvement of insulin sensitivity and lipid profile in plasma of obese patients. Moreover, we documented that leptin as a bioactive molecule measured in both DIs declined its circulating level after dietary-induced weight loss.

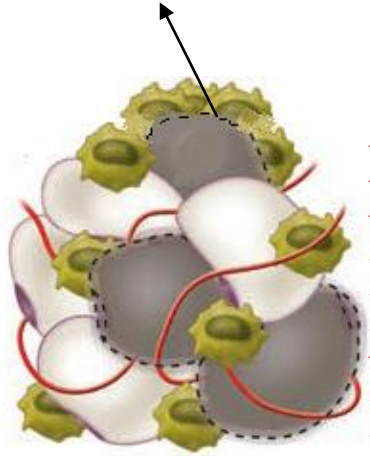
However, visfatin plasma levels after the short-term DI and total adiponectin (APM) plasma levels during the multi-phase DI did not change. We observed significant decrease of RBP4 plasma levels during the whole long-term DI; however the main decline was reached after VLCD. Plasma levels of chemokines MCP-1 and CXCL5 also reduced in response to long-term dietary intervention. However, TNF-α did not change equally in both types of weight-reducing programs.

Therefore, we can hypothesize that only leptin uniformly reflects to different nutritional interventions induced weight loss accompanied by the changes in metabolic parameters. Thus, it could be a good marker of effective weight reduction in all clinical studies. Further, we observed significant reduction of the whole ATM population without any changes in the relative proportion of particular ATM subtypes at the end of the whole dietary program. These results were also confirmed at the transcriptional level of macrophage specific markers and plasma levels of investigating chemokines. On the other hand, genes related to adipocyte metabolism like GLUT4 and RBP4 decreased during first dietary period, after VLCD and subsequently increased to almost basal values at the end of DI (except of visfatin, whose mRNA levels increased after weight loss). These conclusive findings confirm previously reported results about opposite regulation of metabolic and inflammatory signalling pathways during nutritional intervention and highlight the importance of WM phase in the design of DI because of its positive effect on a decrease of inflammatory response in AT.

Figure 12. Summary of our results from the clinical studies shown in this work. Effect of a short-term and multi-phase DI on mRNA and plasma levels of target bioactive molecules and ATM in relation to diet-induced weight loss.







↔ ATM content
↔ ATM markers mRNA
↔ TNF-α, MCP-1 plasma
↓ CXCL5 plasma
↓ RBP4 mRNA,plasma
↓ GLUT4 mRNA
↔ APM plasma
↓ leptin plasma
↓ HOMA-IR
↓ TAG

↔ TNF-α mRNA
↓ TNF-α plasma

↑ visfatin mRNA
↔ visfatin plasma
↓ leptin plasma
↓ HOMA-IR
↓ TAG

↓ ATM content
↔ ATM phenotype (M1/M2)
↓ ATM markers mRNA
↔ TNF-α plasma
↓ MCP-1, CXCL5 plasma
↔ RBP4 mRNA,
↓ RBP4 plasma
↔ GLUT4 mRNA
↔ APM plasma
↓ leptin plasma
↓ HOMA-IR
↓ TAG

Basal VLCD LCD WM

3 months

6 months

Dietary-induced weight loss

→ Multi-phase DI (6 months)

→ Short-term DI (3 months)

CHAPTER 5

FUTURE PERSPECTIVES

Our results raised new questions and objectives for further studies.

First, the increased mRNA expression of visfatin in response to a weight loss program in our study promotes a speculation about its possible anti-inflammatory function. However, it is still not clear how this molecule acts, if it has its own receptor and what kind of signalling pathways it triggers during an inflammatory condition related to obesity. Therefore, it may be advisable to perform *in vitro* experiments investigating the function of visfatin via silencing its expression and vice versa via its overexpression in the target cells like adipocytes and macrophages.

Furthermore, the expected implication of RBP4 in the development of insulin resistance has not been confirmed by our studies, while this molecule sensitively reacted to energy restriction and positively correlated with GLUT4 expression. Hence, *in vitro* studies on the signalling pathways involved in RBP4 activation under different conditions (e.g. higher glucose, FFA or insulin concentration) in adipocytes would represent an exiting avenue for further research.

Our and other clinical studies have investigated the consequences of obesity. However, it would be beneficial to reveal the signs of initiated pathophysiological changes of AT at the beginning, before the onset of obesity-related complications. One of the possible approaches could be the characterization of the ATM activation state. With respect to macrophage phenotype, it is necessary to find reliable markers for identification of particular ATM subpopulations, which could be used in the diagnosis of metabolic complications. One possibility could be brought by studies on markers such as LXR, SRA, CD36 that are involved in the lipid metabolism of macrophages and might affect the macrophage phenotype in the AT and other metabolic tissues, which are also affected by a positive energy balance.

Future studies should address these points and achieve findings with a possible application in clinical practice of obesity managment.

ANNEXE

Adipokines and dietary interventions in human obesity.
Klimcakova E, Kovacikova M, Langin D, Stich V.
Obes Rev. **2010** Jan 6. [Epub ahead of print]

Effect of hyperinsulinemia and very-low-calorie diet on interstitial cytokine levels in subcutaneous adipose tissue of obese women.
Siklova-Vitkova M Phd, Polak J, Klimcakova E, Vrzalova J, Hejnova J, Kovacikova M, Kovacova Z, Bajzova M, Rossmeislova L, Hnevkovska Z, Langin D, Stich V
Am J Physiol Endocrinol Metab. **2009** Sep 1. [Epub ahead of print]

Macrophages and adipocytes in human obesity: adipose tissue gene expression and insulin sensitivity during calorie restriction and weight stabilization.
Capel F, Klimcáková E, Viguerie N, Roussel B, Vítková M, Kováciková M, Polák J, Kovácová Z, Galitzky J, Maoret JJ, Hanácek J, Pers TH, Bouloumié A, Stich V, Langin D.
Diabetes. **2009** Jul;58(7):1558-67. Epub 2009 Apr 28.

Secretion of adiponectin multimeric complexes from adipose tissue explants is not modified by very low calorie diet.
Kovacova Z, Vitkova M, Kovacikova M, Klimcakova E, Bajzova M, Hnevkovska Z, Rossmeislova L, Stich V, Langin D, Polak J.
Eur J Endocrinol. **2009** Apr;160(4):585-92. Epub 2009 Jan 20.

Effect of hypocaloric diet-induced weight loss in obese women on plasma apelin and adipose tissue expression of apelin and APJ.
Castan-Laurell I, Vitkova M, Daviaud D, Dray C, Kovacikova M, Kovacova Z, Hejnova J, Stich V, Valet P
European Journal of Endocrinology, **2008** Apr 7

The atrial natriuretic peptide- and catecholamine-induced lipolysis and expression of related genes in adipose tissue in hypothyroid and hyperthyroid patients.

Polak J, Moro C, Klimcakova E, Kovacikova M, Bajzova M, Vitkova M, Kovacova Z, Sotornik R, Berlan M, Viguerie N, Langin D, Stich V.

Am J Physiol Endocrinol Metab. **2007** Jul;293(1):E246-51. Epub 2007 Mar 27.

An increase in plasma adiponectin multimeric complexes follows hypocaloric diet-induced weight loss in obese and overweight pre-menopausal women.

Polak J, Kovacova Z, Jacek M, Klimcakova E, Kovacikova M, Vitkova M, Kuda O, Sebela M, Samcova E, Stich V.

Clin Sci (Lond). **2007** Jun;112(11):557-65.

SUMMARY

Obesity, mainly characterized by an abnormal accumulation of fat mass in the body, represents a high risk for the development of metabolic and cardiovascular diseases, such as insulin resistance, type 2 diabetes or atherosclerosis. A huge amount of studies have shown that the obese state is accompanied by an increased secretion of pro-inflammatory cytokines, chemokines and acute phase proteins and by an elevated recruitment of immune cells into the AT. Hence, obesity is now considered as "a low-grade inflammation" persisting over a longer period that causes dysfunction of the AT and leads to release of a number of bioactive molecules with a different role in the pathophysiology of insulin-sensitive tissues. Moreover, macrophage-induced disturbances in the AT during obesity seem to be one of the possible triggers in this inflammatory machinery and therefore attract the attention of many research groups. One of the approaches how to improve the obesity-related pathological conditions is the lifestyle modification including physical activity and DI.

Therefore, the general goal of this work was to investigate the molecular adaptations of human AT in relation to DIs with respect to its secretory activity as well as cellular composition focused on macrophages population. Specifically, we studied the role of novel adipokines, such as visfatin and RBP4, related to insulin resistance and AT metabolism. Furthermore, we wanted to characterize the effect of the dietary-induced changes on the content of ATM together with metabolic amelioration.

In our studies, we found that lifestyle modifications had a beneficial effect on metabolic and biochemical parameters depending on the duration and type of DI. Regarding both of the investigated adipokines, visfatin and RBP4, we revealed modifications at the transcriptional and cirulating levels during DI. However, we did not find any association with the pattern of evolution of insulin resistance. Our findings do not support a clear hypothesis on the role of these adipokines in the diet-induced improvement of insulin sensitivity and other obesity-related metabolic disturbances. In respect to the changes of ATM content during long-term DI, we demonstrated using flow cytometry that the AT composition was changed at the end of the whole dietary intervention (i.e. after WM phase). This diet-induced AT remodelling was expressed by a decrease of ATM number without any effect on their

phenotype. Nevertheless, most of metabolic parameters (including insulin sensitivity) improved already after VLCD. These results suggest that changes in immune response last longer than alteration in metabolic pathways. Therefore, WM phase constitutes an important phase in the adaptation to a new metabolic balance.

In conclusion, our results showed that the diet-induced changes led to different alterations in the expression of metabolic and inflammatory genes together with the modulation of the immune cells content in AT. However, these findings raise further questions in terms of understanding the processes occuring in obesity and create the aims for future studies that would focus on the identification of potential factors responsible for the molecular modulation in AT (e.g. ER stress, hypoxia, FA overload etc.) and activation of signalling pathways overlapping these “running circles”.

SÚHRN

Obezita, charakterizovaná hlavne abnormálym hromadením tukovej hmoty v tele predstavuje vysoké riziko vzniku metabolických a kardiovaskulárnych ochorení ako je inzulínová rezistencia, diabetes 2. typu alebo ateroskleróza. Na základe väčšieho počtu štúdii bolo preukázané, že obézny stav je doprevádzaný zvýšenou sekréciou pro-zápalových cytokínov, chemokínov alebo proteínov akútnej fázy a zvýšeným atrahovaním imunitných buniek do tukového tkaniva. Preto je obezita v súčasnej dobe považovaná za pro-zápalový stav organizmu pretrvávajúci dlhšie obdobie, ktoré navodzuje dysfunkciu tukového tkaniva a vedie k produkcii veľkého množstva bioaktivných molekúl s rôznou úlohou v patofyziológii inzulín-senzitívnych tkanív. Okrem toho makrofágmi indukované poruchy v tukovom tkanive počas obezity sa zdajú byť jedným z možných stimulov v tejto „zápalovej mašinérii“ a práve preto pútajú pozornosť viacerých výskumných tímov. Jedným z možných prístupov ako zlepšiť následky navodené obezitou je zmena životného štýlu zahrňujúca pohybovú aktivitu a dietnu intervenciu.

Preto hlavným cieľom tejto práce bolo sledovať molekulárne adaptácie ľudského tukového tkaniva počas dietných intervencii so zameraním sa na jeho sekrečnú aktivitu ako aj bunkové zloženie, hlavne na populáciu makrofágov. Konkrétne sme študovali úlohu novoobjavených adipokínov visfatínu a RBP4 vo vzťahu k inzulínovej rezistencii a metabolizmu tukového tkaniva. Ďalej sme chceli charaktetrizovať vplyv zmien navodených dietou na zastúpenie tkanivových makrofágov s náväznosťou na metabolické zlepšenie organizmu.

Z našich štúdii sme zistili, že zmena životného štýlu mala pozitívny efekt na metabolické a biochemické parametre v závislosti na dĺžke a type dietnej intervencie. U oboch vyšetrovaných adipokínov sme zistili modifikácie na úrovni mRNA a plazmatických hladín počas diety. Avšak nenašli sme žiadnu asociáciu s inzulínovou rezistenciou. Naše výsledky nepodporujú hypotézu o úlohe týchto adipokínov pri zlepšení inzulínovej senzitivity a iných metabolických komplikáciach vplyvom dietnej intervencie. Čo sa týka počtu tkanivových makrofágov počas dietnej intervencie, sme zistili pomocou prietokovej cytometrie, že bunkové zloženie tukového tkaniva sa zmenilo v dôsledku úbytku váhy (na konci celej dietnej intervencie, vo fáze udržania váhy). Táto dietou navodená remodelácia

tukového tkaniva bola vyjadrená znížením počtu makrofágov bez vplyvu na ich fenotyp. Na rozdiel od toho, väčšina metabolických parametrov vrátane inzulínovej senzitivity sa zlepšila už po VLCD. Tieto výsledky nasvedčujú o tom, že zmeny v imunitnej odpovedi potrebujú viac času na adaptáciu než metabolické procesy. Preto fáza udržania váhy tvorí dôležitú periodu pri adaptácii na novú metabolickú rovnováhu.

Na záver, naše výsledky preukazujú, že dietou navodené zmeny viedli k rôznym modifikáciam expresie génov súvisejúcich s metabolizmom a imunitnou odpoveďou organizmu v súlade so zmenami počtu imunitných buniek, t.j. makrofágov zastúpených v tukovom tkanive. Avšak tieto zistenia vedú k ďalším otázkam týkajúcich sa pochopenia procesov uskutočňujúcich sa pri obezite a tvoria podklad pre naväzujúce štúdie so zameraním sa na identifikovanie možných faktorov zodpovedných za molekulárne modulácie v tukovom tkanive (napr. ER stres, hypoxia, zvýšené hladiny mastných kyselín atď.) a aktivácii signálnych dráh, ktoré sú zahrnuté v týchto prebiehajúcich dejoch počas obezity.

REFERENCES

1 Hainer V, Toplak H and Mitrakou A. Treatment modalities of obesity: what fits whom? *Diabetes Care* 2008;31 Suppl 2:S269-77.

2 WHO. The challenge of obesity prevalence in the WHO European Region. 2005.

3 Kopelman PG. Obesity as a medical problem. *Nature* 2000;404:635-43.

4 WHO. Obesity:Preventing and managing the global epidemic. Report of a WHO consultation. Geneva *WHO Technical Report series 894* 2000.

5 Flegal KM, Graubard BI, Williamson DF and Gail MH. Cause-specific excess deaths associated with underweight, overweight, and obesity. *Jama* 2007;298:2028-37.

6 Orpana HM, Berthelot JM, Kaplan MS, Feeny DH, McFarland B and Ross NA. BMI and mortality: results from a national longitudinal study of Canadian adults. *Obesity (Silver Spring)*;18:214-8.

7 Barker DJ, Osmond C, Golding J, Kuh D and Wadsworth ME. Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *Bmj* 1989;298:564-7.

8 Weinstock RS, Dai H and Wadden TA. Diet and exercise in the treatment of obesity: effects of 3 interventions on insulin resistance. *Arch Intern Med* 1998;158:2477-83.

9 Cole TJ, Bellizzi MC, Flegal KM and Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. *Bmj* 2000;320:1240-3.

10 WHO CE. Appropriate body-mass index for Asian populations and its implications for policy and intervention strategies. *Lancet* 2004;363:157-63.

11 Zhu S, Wang Z, Heshka S, Heo M, Faith MS and Heymsfield SB. Waist circumference and obesity-associated risk factors among whites in the third National Health and Nutrition Examination Survey: clinical action thresholds. *Am J Clin Nutr* 2002;76:743-9.

12 Maes HH, Neale MC and Eaves LJ. Genetic and environmental factors in relative body weight and human adiposity. *Behav Genet* 1997;27:325-51.

13 Stunkard AJ, Harris JR, Pedersen NL and McClearn GE. The body-mass index of twins who have been reared apart. *N Engl J Med* 1990;322:1483-7.

14 Barsh GS, Farooqi IS and O'Rahilly S. Genetics of body-weight regulation. *Nature* 2000;404:644-51.

15 Neel JV. Diabetes mellitus: a "thrifty" genotype rendered detrimental by "progress"? *Am J Hum Genet* 1962;14:353-62.

16 Farooqi I. Genetics of Obesity in humans. *Endocrine Reviews* 2006;27:710-8.

17 Elliott RM and Johnson IT. Nutrigenomic approaches for obesity research. *Obes Rev* 2007;8 Suppl 1:77-81.

18 Nedergaard J, Bengtsson T and Cannon B. Unexpected evidence for active brown adipose tissue in adult humans. *Am J Physiol Endocrinol Metab* 2007;293:E444-52.

19 Langin D. Adipose tissue: Development, Anatomy and Functions. 2008:79-108.

20 Fain JN, Madan AK, Hiler ML, Cheema P and Bahouth SW. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology* 2004;145:2273-82.

21 Maury E, Ehala-Aleksejev K, Guiot Y, Detry R, Vandenhooft A and Brichard SM. Adipokines oversecreted by omental adipose tissue in human obesity. *Am J Physiol Endocrinol Metab* 2007;293:E656-65.
22 Cancello R, Henegar C, Viguerie N, Taleb S, Poitou C, Rouault C *et al.* Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. *Diabetes* 2005;54:2277-86.
23 Curat CA, Miranville A, Sengenes C, Diehl M, Tonus C, Busse R *et al.* From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes. *Diabetes* 2004;53:1285-92.
24 Duffaut C, Zakaroff-Girard A, Bourlier V, Decaunes P, Maumus M, Chiotasso P *et al.* Interplay between human adipocytes and T lymphocytes in obesity: CCL20 as an adipochemokine and T lymphocytes as lipogenic modulators. *Arterioscler Thromb Vasc Biol* 2009;29:1608-14.
25 Elgazar-Carmon V, Rudich A, Hadad N and Levy R. Neutrophils transiently infiltrate intra-abdominal fat early in the course of high-fat feeding. *J Lipid Res* 2008;49:1894-903.
26 Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL and Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 2003;112:1796-808.
27 Sengenes C, Miranville A, Maumus M, de Barros S, Busse R and Bouloumie A. Chemotaxis and differentiation of human adipose tissue CD34+/CD31- progenitor cells: role of stromal derived factor-1 released by adipose tissue capillary endothelial cells. *Stem Cells* 2007;25:2269-76.
28 Miranville A, Heeschen C, Sengenes C, Curat CA, Busse R and Bouloumie A. Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. *Circulation* 2004;110:349-55.
29 Sengenes C, Lolmede K, Zakaroff-Girard A, Busse R and Bouloumie A. Preadipocytes in the human subcutaneous adipose tissue display distinct features from the adult mesenchymal and hematopoietic stem cells. *J Cell Physiol* 2005;205:114-22.
30 Maumus M, Sengenes C, Decaunes P, Zakaroff-Girard A, Bourlier V, Lafontan M *et al.* Evidence of in situ proliferation of adult adipose tissue-derived progenitor cells: influence of fat mass microenvironment and growth. *J Clin Endocrinol Metab* 2008;93:4098-106.
31 Bjorntorp P. Size, number and function of adipose tissue cells in human obesity. *Horm Metab Res* 1974;Suppl 4:77-83.
32 Hausman DB, DiGirolamo M, Bartness TJ, Hausman GJ and Martin RJ. The biology of white adipocyte proliferation. *Obes Rev* 2001;2:239-54.
33 Schaffler A, Scholmerich J and Buchler C. Mechanisms of disease: adipocytokines and visceral adipose tissue--emerging role in nonalcoholic fatty liver disease. *Nat Clin Pract Gastroenterol Hepatol* 2005;2:273-80.
34 Ahmadian M, Wang Y and Sul HS. Lipolysis in adipocytes. *Int J Biochem Cell Biol* 2009.
35 Frayn KN. Adipose tissue as a buffer for daily lipid flux. *Diabetologia* 2002;45:1201-10.
36 Lafontan M and Langin D. Lipolysis and lipid mobilization in human adipose tissue. *Prog Lipid Res* 2009;48:275-97.
37 Swierczynski J, Goyke E, Wach L, Pankiewicz A, Kochan Z, Adamonis W *et al.* Comparative study of the lipogenic potential of human and rat adipose tissue. *Metabolism* 2000;49:594-9.

38 Hajri T and Abumrad NA. Fatty acid transport across membranes: relevance to nutrition and metabolic pathology. *Annu Rev Nutr* 2002;22:383-415.
39 Chmurzynska A. The multigene family of fatty acid-binding proteins (FABPs): function, structure and polymorphism. *J Appl Genet* 2006;47:39-48.
40 Gargiulo CE, Stuhlsatz-Krouper SM and Schaffer JE. Localization of adipocyte long-chain fatty acyl-CoA synthetase at the plasma membrane. *J Lipid Res* 1999;40:881-92.
41 Jenkins CM, Mancuso DJ, Yan W, Sims HF, Gibson B and Gross RW. Identification, cloning, expression, and purification of three novel human calcium-independent phospholipase A2 family members possessing triacylglycerol lipase and acylglycerol transacylase activities. *J Biol Chem* 2004;279:48968-75.
42 Villena JA, Roy S, Sarkadi-Nagy E, Kim KH and Sul HS. Desnutrin, an adipocyte gene encoding a novel patatin domain-containing protein, is induced by fasting and glucocorticoids: ectopic expression of desnutrin increases triglyceride hydrolysis. *J Biol Chem* 2004;279:47066-75.
43 Zimmermann R, Strauss JG, Haemmerle G, Schoiswohl G, Birner-Gruenberger R, Riederer M *et al.* Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. *Science* 2004;306:1383-6.
44 Sengenes C, Berlan M, De Glisezinski I, Lafontan M and Galitzky J. Natriuretic peptides: a new lipolytic pathway in human adipocytes. *Faseb J* 2000;14:1345-51.
45 Moller N and Jorgensen JO. Effects of growth hormone on glucose, lipid, and protein metabolism in human subjects. *Endocr Rev* 2009;30:152-77.
46 Hauner H, Petruschke T, Russ M, Rohrig K and Eckel J. Effects of tumour necrosis factor alpha (TNF alpha) on glucose transport and lipid metabolism of newly-differentiated human fat cells in cell culture. *Diabetologia* 1995;38:764-71.
47 Lafontan M and Berlan M. Fat cell alpha 2-adrenoceptors: the regulation of fat cell function and lipolysis. *Endocr Rev* 1995;16:716-38.
48 Richelsen B. Prostaglandin E2 action and binding in human adipocytes: effects of sex, age, and obesity. *Metabolism* 1988;37:268-75.
49 Kaartinen JM, Hreniuk SP, Martin LF, Ranta S, LaNoue KF and Ohisalo JJ. Attenuated adenosine-sensitivity and decreased adenosine-receptor number in adipocyte plasma membranes in human obesity. *Biochem J* 1991;279 (Pt 1):17-22.
50 Gaudiot N, Jaubert AM, Charbonnier E, Sabourault D, Lacasa D, Giudicelli Y *et al.* Modulation of white adipose tissue lipolysis by nitric oxide. *J Biol Chem* 1998;273:13475-81.
51 Brasaemle DL. Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. *J Lipid Res* 2007;48:2547-59.
52 Wolins NE, Rubin B and Brasaemle DL. TIP47 associates with lipid droplets. *J Biol Chem* 2001;276:5101-8.
53 Parton RG and Simons K. The multiple faces of caveolae. *Nat Rev Mol Cell Biol* 2007;8:185-94.
54 Kishida K, Kuriyama H, Funahashi T, Shimomura I, Kihara S, Ouchi N *et al.* Aquaporin adipose, a putative glycerol channel in adipocytes. *J Biol Chem* 2000;275:20896-902.
55 Nordstrom EA, Ryden M, Backlund EC, Dahlman I, Kaaman M, Blomqvist L *et al.* A human-specific role of cell death-inducing DFFA (DNA fragmentation factor-alpha)-like effector A (CIDEA) in adipocyte lipolysis and obesity. *Diabetes* 2005;54:1726-34.

56 Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM and Puigserver P. Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. *Nature* 2005;434:113-8.
57 Randle PJ, Garland PB, Hales CN and Newsholme EA. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* 1963;1:785-9.
58 Jaworski K, Ahmadian M, Duncan RE, Sarkadi-Nagy E, Varady KA, Hellerstein MK *et al.* AdPLA ablation increases lipolysis and prevents obesity induced by high-fat feeding or leptin deficiency. *Nat Med* 2009;15:159-68.
59 Unger JW, Moss AM and Livingston JN. Immunohistochemical localization of insulin receptors and phosphotyrosine in the brainstem of the adult rat. *Neuroscience* 1991;42:853-61.
60 Goldfine ID and Williams JA. Receptors for insulin and CCK in the acinar pancreas: relationship to hormone action. *Int Rev Cytol* 1983;85:1-38.
61 Whitsett JA, Johnson CL and Hawkins K. Differences in localization of insulin receptors and adenylate cyclase in the human placenta. *Am J Obstet Gynecol* 1979;133:204-7.
62 Cai D, Dhe-Paganon S, Melendez PA, Lee J and Shoelson SE. Two new substrates in insulin signaling, IRS5/DOK4 and IRS6/DOK5. *J Biol Chem* 2003;278:25323-30.
63 Fantin VR, Sparling JD, Slot JW, Keller SR, Lienhard GE and Lavan BE. Characterization of insulin receptor substrate 4 in human embryonic kidney 293 cells. *J Biol Chem* 1998;273:10726-32.
64 Lavan BE, Lane WS and Lienhard GE. The 60-kDa phosphotyrosine protein in insulin-treated adipocytes is a new member of the insulin receptor substrate family. *J Biol Chem* 1997;272:11439-43.
65 Patti ME, Sun XJ, Bruening JC, Araki E, Lipes MA, White MF *et al.* 4PS/insulin receptor substrate (IRS)-2 is the alternative substrate of the insulin receptor in IRS-1-deficient mice. *J Biol Chem* 1995;270:24670-3.
66 Sun XJ, Rothenberg P, Kahn CR, Backer JM, Araki E, Wilden PA *et al.* Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature* 1991;352:73-7.
67 Kido Y, Burks DJ, Withers D, Bruning JC, Kahn CR, White MF *et al.* Tissue-specific insulin resistance in mice with mutations in the insulin receptor, IRS-1, and IRS-2. *J Clin Invest* 2000;105:199-205.
68 Muoio DM and Newgard CB. Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. *Nat Rev Mol Cell Biol* 2008;9:193-205.
69 Rother KI, Imai Y, Caruso M, Beguinot F, Formisano P and Accili D. Evidence that IRS-2 phosphorylation is required for insulin action in hepatocytes. *J Biol Chem* 1998;273:17491-7.
70 Avruch J. Insulin signal transduction through protein kinase cascades. *Mol Cell Biochem* 1998;182:31-48.
71 Taniguchi CM, Emanuelli B and Kahn CR. Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol* 2006;7:85-96.
72 Gosmanov AR, Umpierrez GE, Karabell AH, Cuervo R and Thomason DB. Impaired expression and insulin-stimulated phosphorylation of Akt-2 in muscle of obese patients with atypical diabetes. *Am J Physiol Endocrinol Metab* 2004;287:E8-E15.
73 Werner ED, Lee J, Hansen L, Yuan M and Shoelson SE. Insulin resistance due to phosphorylation of insulin receptor substrate-1 at serine 302. *J Biol Chem* 2004;279:35298-305.

74 Ueki K, Kondo T and Kahn CR. Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms. *Mol Cell Biol* 2004;24:5434-46.
75 Kanety H, Feinstein R, Papa MZ, Hemi R and Karasik A. Tumor necrosis factor alpha-induced phosphorylation of insulin receptor substrate-1 (IRS-1). Possible mechanism for suppression of insulin-stimulated tyrosine phosphorylation of IRS-1. *J Biol Chem* 1995;270:23780-4.
76 Suganami T, Tanimoto-Koyama K, Nishida J, Itoh M, Yuan X, Mizuarai S *et al.* Role of the Toll-like receptor 4/NF-kappaB pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. *Arterioscler Thromb Vasc Biol* 2007;27:84-91.
77 Zick Y. Ser/Thr phosphorylation of IRS proteins: a molecular basis for insulin resistance. *Sci STKE* 2005;2005:pe4.
78 Trayhurn P, Wang B and Wood IS. Hypoxia and the endocrine and signalling role of white adipose tissue. *Arch Physiol Biochem* 2008;114:267-76.
79 Gregor MG and Hotamisligil GS. Adipocyte stress: The endoplasmic reticulum and metabolic disease. *J Lipid Res* 2007.
80 Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E *et al.* Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* 2004;306:457-61.
81 Richardson DW and Vinik AI. Metabolic implications of obesity: before and after gastric bypass. *Gastroenterol Clin North Am* 2005;34:9-24.
82 Sparks LM, Xie H, Koza RA, Mynatt R, Hulver MW, Bray GA *et al.* A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle. *Diabetes* 2005;54:1926-33.
83 Kido Y, Nakae J and Accili D. Clinical review 125: The insulin receptor and its cellular targets. *J Clin Endocrinol Metab* 2001;86:972-9.
84 Chen H. Cellular inflammatory responses: novel insights for obesity and insulin resistance. *Pharmacol Res* 2006;53:469-77.
85 Cook KS, Min HY, Johnson D, Chaplinsky RJ, Flier JS, Hunt CR *et al.* Adipsin: a circulating serine protease homolog secreted by adipose tissue and sciatic nerve. *Science* 1987;237:402-5.
86 Zhang Y, Proenca R, Maffei M, Barone M, Leopold L and Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994;372:425-32.
87 Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T *et al.* Effects of the obese gene product on body weight regulation in ob/ob mice. *Science* 1995;269:540-3.
88 Funahashi T, Nakamura T, Shimomura I, Maeda K, Kuriyama H, Takahashi M *et al.* Role of adipocytokines on the pathogenesis of atherosclerosis in visceral obesity. *Intern Med* 1999;38:202-6.
89 Trayhurn P and Wood IS. Adipokines: inflammation and the pleiotropic role of white adipose tissue. *Br J Nutr* 2004;92:347-55.
90 Hauner H. Secretory factors from human adipose tissue and their functional role. *Proc Nutr Soc* 2005;64:163-9.
91 Juge-Aubry CE, Henrichot E and Meier CA. Adipose tissue: a regulator of inflammation. *Best Pract Res Clin Endocrinol Metab* 2005;19:547-66.
92 Bastard JP, Jardel C, Bruckert E, Blondy P, Capeau J, Laville M *et al.* Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. *J Clin Endocrinol Metab* 2000;85:3338-42.

93 Jager J, Gremeaux T, Cormont M, Le Marchand-Brustel Y and Tanti JF. Interleukin-1beta-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression. *Endocrinology* 2007;148:241-51.
94 Manigrasso MR, Ferroni P, Santilli F, Taraborelli T, Guagnano MT, Michetti N *et al.* Association between circulating adiponectin and interleukin-10 levels in android obesity: effects of weight loss. *J Clin Endocrinol Metab* 2005;90:5876-9.
95 Matsuki T, Horai R, Sudo K and Iwakura Y. IL-1 plays an important role in lipid metabolism by regulating insulin levels under physiological conditions. *J Exp Med* 2003;198:877-88.
96 Rocha VZ, Folco EJ, Sukhova G, Shimizu K, Gotsman I, Vernon AH *et al.* Interferon-gamma, a Th1 cytokine, regulates fat inflammation: a role for adaptive immunity in obesity. *Circ Res* 2008;103:467-76.
97 Hotamisligil GS, Shargill NS and Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 1993;259:87-91.
98 Dahlman I, Kaaman M, Olsson T, Tan GD, Bickerton AS, Wahlen K *et al.* A unique role of monocyte chemoattractant protein 1 among chemokines in adipose tissue of obese subjects. *J Clin Endocrinol Metab* 2005;90:5834-40.
99 Kim CS, Park HS, Kawada T, Kim JH, Lim D, Hubbard NE *et al.* Circulating levels of MCP-1 and IL-8 are elevated in human obese subjects and associated with obesity-related parameters. *Int J Obes (Lond)* 2006;30:1347-55.
100 Straczkowski M, Dzienis-Straczkowska S, Stepien A, Kowalska I, Szelachowska M and Kinalska I. Plasma interleukin-8 concentrations are increased in obese subjects and related to fat mass and tumor necrosis factor-alpha system. *J Clin Endocrinol Metab* 2002;87:4602-6.
101 Dray C, Knauf C, Daviaud D, Waget A, Boucher J, Buleon M *et al.* Apelin stimulates glucose utilization in normal and obese insulin-resistant mice. *Cell Metab* 2008;8:437-45.
102 Fukuhara A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K *et al.* Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. *Science* 2005;307:426-30.
103 Graham TE, Yang Q, Bluher M, Hammarstedt A, Ciaraldi TP, Henry RR *et al.* Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. *N Engl J Med* 2006;354:2552-63.
104 McTernan CL, McTernan PG, Harte AL, Levick PL, Barnett AH and Kumar S. Resistin, central obesity, and type 2 diabetes. *Lancet* 2002;359:46-7.
105 Chiellini C, Santini F, Marsili A, Berti P, Bertacca A, Pelosini C *et al.* Serum haptoglobin: a novel marker of adiposity in humans. *J Clin Endocrinol Metab* 2004;89:2678-83.
106 Poitou C, Viguerie N, Cancello R, De Matteis R, Cinti S, Stich V *et al.* Serum amyloid A: production by human white adipocyte and regulation by obesity and nutrition. *Diabetologia* 2005;48:519-28.
107 Yudkin JS, Stehouwer CD, Emeis JJ and Coppack SW. C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? *Arterioscler Thromb Vasc Biol* 1999;19:972-8.
108 Bekri S, Gual P, Anty R, Luciani N, Dahman M, Ramesh B *et al.* Increased adipose tissue expression of hepcidin in severe obesity is independent from diabetes and NASH. *Gastroenterology* 2006;131:788-96.

109 Kloting N, Berndt J, Kralisch S, Kovacs P, Fasshauer M, Schon MR *et al.* Vaspin gene expression in human adipose tissue: association with obesity and type 2 diabetes. *Biochem Biophys Res Commun* 2006;339:430-6.
110 Yang RZ, Lee MJ, Hu H, Pray J, Wu HB, Hansen BC *et al.* Identification of omentin as a novel depot-specific adipokine in human adipose tissue: possible role in modulating insulin action. *Am J Physiol Endocrinol Metab* 2006;290:E1253-61.
111 Trayhurn P. Endocrine and signalling role of adipose tissue: new perspectives on fat. *Acta Physiol Scand* 2005;184:285-93.
112 Klimcakova E, Kovacikova M, Stich V and Langin D. Adipokines and dietary interventions in human obesity. *Obes Rev*.
113 Brzozowski T, Konturek PC, Konturek SJ, Brzozowska I and Pawlik T. Role of prostaglandins in gastroprotection and gastric adaptation. *J Physiol Pharmacol* 2005;56 Suppl 5:33-55.
114 Nawrot-Porabka K, Jaworek J, Leja-Szpak A, Palonek M, Szklarczyk J, Konturek SJ *et al.* Leptin is able to stimulate pancreatic enzyme secretion via activation of duodeno-pancreatic reflex and CCK release. *J Physiol Pharmacol* 2004;55 Suppl 2:47-57.
115 Hoggard N, Mercer JG, Rayner DV, Moar K, Trayhurn P and Williams LM. Localization of leptin receptor mRNA splice variants in murine peripheral tissues by RT-PCR and in situ hybridization. *Biochem Biophys Res Commun* 1997;232:383-7.
116 Lollmann B, Gruninger S, Stricker-Krongrad A and Chiesi M. Detection and quantification of the leptin receptor splice variants Ob-Ra, b, and, e in different mouse tissues. *Biochem Biophys Res Commun* 1997;238:648-52.
117 Ceddia RB. Direct metabolic regulation in skeletal muscle and fat tissue by leptin: implications for glucose and fatty acids homeostasis. *Int J Obes (Lond)* 2005;29:1175-83.
118 Siegrist-Kaiser CA, Pauli V, Juge-Aubry CE, Boss O, Pernin A, Chin WW *et al.* Direct effects of leptin on brown and white adipose tissue. *J Clin Invest* 1997;100:2858-64.
119 Friedman JM. Leptin and the regulation of body weight. *Harvey Lect* 1999;95:107-36.
120 Martin LJ, Mahaney MC, Almasy L, MacCluer JW, Blangero J, Jaquish CE *et al.* Leptin's sexual dimorphism results from genotype by sex interactions mediated by testosterone. *Obes Res* 2002;10:14-21.
121 Miller GD, Frost R and Olive J. Relation of plasma leptin concentrations to sex, body fat, dietary intake, and peak oxygen uptake in young adult women and men. *Nutrition* 2001;17:105-11.
122 Arvidsson E, Viguerie N, Andersson I, Verdich C, Langin D and Arner P. Effects of different hypocaloric diets on protein secretion from adipose tissue of obese women. *Diabetes* 2004;53:1966-71.
123 Bastard JP, Hainque B, Dusserre E, Bruckert E, Robin D, Vallier P *et al.* Peroxisome proliferator activated receptor-gamma, leptin and tumor necrosis factor-alpha mRNA expression during very low calorie diet in subcutaneous adipose tissue in obese women. *Diabetes Metab Res Rev* 1999;15:92-8.
124 de Luis DA, Sagrado MG, Conde R, Aller R and Izaola O. Changes of ghrelin and leptin in response to hypocaloric diet in obese patients. *Nutrition* 2008;24:162-6.
125 Garaulet M, Viguerie N, Porubsky S, Klimcakova E, Clement K, Langin D *et al.* Adiponectin gene expression and plasma values in obese women during very-low-calorie diet. Relationship with cardiovascular risk factors and insulin resistance. *J Clin Endocrinol Metab* 2004;89:756-60.

126 Williams KV, Mullen M, Lang W, Considine RV and Wing RR. Weight loss and leptin changes in individuals with type 2 diabetes. *Obes Res* 1999;7:155-63.
127 Clement K, Vaisse C, Lahlou N, Cabrol S, Pelloux V, Cassuto D *et al.* A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature* 1998;392:398-401.
128 Licinio J, Caglayan S, Ozata M, Yildiz BO, de Miranda PB, O'Kirwan F *et al.* Phenotypic effects of leptin replacement on morbid obesity, diabetes mellitus, hypogonadism, and behavior in leptin-deficient adults. *Proc Natl Acad Sci U S A* 2004;101:4531-6.
129 Munzberg H, Bjornholm M, Bates SH and Myers MG, Jr. Leptin receptor action and mechanisms of leptin resistance. *Cell Mol Life Sci* 2005;62:642-52.
130 Howard JK and Flier JS. Attenuation of leptin and insulin signaling by SOCS proteins. *Trends Endocrinol Metab* 2006;17:365-71.
131 Fantuzzi G and Faggioni R. Leptin in the regulation of immunity, inflammation, and hematopoiesis. *J Leukoc Biol* 2000;68:437-46.
132 La Cava A and Matarese G. The weight of leptin in immunity. *Nat Rev Immunol* 2004;4:371-9.
133 Faggioni R, Feingold KR and Grunfeld C. Leptin regulation of the immune response and the immunodeficiency of malnutrition. *Faseb J* 2001;15:2565-71.
134 Bourlier V and Bouloumie A. Role of macrophage tissue infiltration in obesity and insulin resistance. *Diabetes Metab* 2009;35:251-60.
135 Hu E, Liang P and Spiegelman BM. AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J Biol Chem* 1996;271:10697-703.
136 Maeda K, Okubo K, Shimomura I, Funahashi T, Matsuzawa Y and Matsubara K. cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (AdiPose Most abundant Gene transcript 1). *Biochem Biophys Res Commun* 1996;221:286-9.
137 Scherer PE, Williams S, Fogliano M, Baldini G and Lodish HF. A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem* 1995;270:26746-9.
138 Shapiro L and Scherer PE. The crystal structure of a complement-1q family protein suggests an evolutionary link to tumor necrosis factor. *Curr Biol* 1998;8:335-8.
139 Delaigle AM, Jonas JC, Bauche IB, Cornu O and Brichard SM. Induction of adiponectin in skeletal muscle by inflammatory cytokines: in vivo and in vitro studies. *Endocrinology* 2004;145:5589-97.
140 Pineiro R, Iglesias MJ, Gallego R, Raghay K, Eiras S, Rubio J *et al.* Adiponectin is synthesized and secreted by human and murine cardiomyocytes. *FEBS Lett* 2005;579:5163-9.
141 Wolf AM, Wolf D, Avila MA, Moschen AR, Berasain C, Enrich B *et al.* Up-regulation of the anti-inflammatory adipokine adiponectin in acute liver failure in mice. *J Hepatol* 2006;44:537-43.
142 Wang Y, Lam KS, Chan L, Chan KW, Lam JB, Lam MC *et al.* Post-translational modifications of the four conserved lysine residues within the collagenous domain of adiponectin are required for the formation of its high molecular weight oligomeric complex. *J Biol Chem* 2006;281:16391-400.
143 Lara-Castro C, Luo N, Wallace P, Klein RL and Garvey WT. Adiponectin multimeric complexes and the metabolic syndrome trait cluster. *Diabetes* 2006;55:249-59.
144 Pajvani UB, Du X, Combs TP, Berg AH, Rajala MW, Schulthess T *et al.* Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin. Implications fpr metabolic regulation and bioactivity. *J Biol Chem* 2003;278:9073-85.

145 Waki H, Yamauchi T, Kamon J, Kita S, Ito Y, Hada Y *et al.* Generation of globular fragment of adiponectin by leukocyte elastase secreted by monocytic cell line THP-1. *Endocrinology* 2005;146:790-6.
146 Fisher FF, Trujillo ME, Hanif W, Barnett AH, McTernan PG, Scherer PE *et al.* Serum high molecular weight complex of adiponectin correlates better with glucose tolerance than total serum adiponectin in Indo-Asian males. *Diabetologia* 2005;48:1084-7.
147 Hara K, Horikoshi M, Yamauchi T, Yago H, Miyazaki O, Ebinuma H *et al.* Measurement of the high-molecular weight form of adiponectin in plasma is useful for the prediction of insulin resistance and metabolic syndrome. *Diabetes Care* 2006;29:1357-62.
148 Pajvani UB, Hawkins M, Combs TP, Rajala MW, Doebber T, Berger JP *et al.* Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione-mediated improvement in insulin sensitivity. *J Biol Chem* 2004;279:12152-62.
149 Bobbert T, Rochlitz H, Wegewitz U, Akpulat S, Mai K, Weickert MO *et al.* Changes of adiponectin oligomer composition by moderate weight reduction. *Diabetes* 2005;54:2712-9.
150 Karbowska J and Kochan Z. Role of adiponectin in the regulation of carbohydrate and lipid metabolism. *J Physiol Pharmacol* 2006;57 Suppl 6:103-13.
151 Matsubara M, Maruoka S and Katayose S. Inverse relationship between plasma adiponectin and leptin concentrations in normal-weight and obese women. *Eur J Endocrinol* 2002;147:173-80.
152 Ryo M, Nakamura T, Kihara S, Kumada M, Shibazaki S, Takahashi M *et al.* Adiponectin as a biomarker of the metabolic syndrome. *Circ J* 2004;68:975-81.
153 Bruun JM, Lihn AS, Verdich C, Pedersen SB, Toubro S, Astrup A *et al.* Regulation of adiponectin by adipose tissue-derived cytokines: in vivo and in vitro investigations in humans. *Am J Physiol Endocrinol Metab* 2003;285:E527-33.
154 Fasshauer M, Kralisch S, Klier M, Lossner U, Bluher M, Klein J *et al.* Adiponectin gene expression and secretion is inhibited by interleukin-6 in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* 2003;301:1045-50.
155 Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H *et al.* Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat Med* 2002;8:731-7.
156 Kovacova Z, Vitkova M, Kovacikova M, Klimcakova E, Bajzova M, Hnevkovska Z *et al.* Secretion of adiponectin multimeric complexes from adipose tissue explants is not modified by very low calorie diet. *Eur J Endocrinol* 2009;160:585-92.
157 Kishida K, Nagaretani H, Kondo H, Kobayashi H, Tanaka S, Maeda N *et al.* Disturbed secretion of mutant adiponectin associated with the metabolic syndrome. *Biochem Biophys Res Commun* 2003;306:286-92.
158 Waki H, Yamauchi T, Kamon J, Ito Y, Uchida S, Kita S *et al.* Impaired multimerization of human adiponectin mutants associated with diabetes. Molecular structure and multimer formation of adiponectin. *J Biol Chem* 2003;278:40352-63.
159 Chow WS, Cheung BM, Tso AW, Xu A, Wat NM, Fong CH *et al.* Hypoadiponectinemia as a predictor for the development of hypertension: a 5-year prospective study. *Hypertension* 2007;49:1455-61.
160 Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE *et al.* Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *J Clin Endocrinol Metab* 2001;86:1930-5.

161 Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S *et al.* Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* 2003;423:762-9.
162 Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K and Tobe K. Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *J Clin Invest* 2006;116:1784-92.
163 Hug C, Wang J, Ahmad NS, Bogan JS, Tsao TS and Lodish HF. T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin. *Proc Natl Acad Sci U S A* 2004;101:10308-13.
164 Mao X, Kikani CK, Riojas RA, Langlais P, Wang L, Ramos FJ *et al.* APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function. *Nat Cell Biol* 2006;8:516-23.
165 Zhu W, Cheng KK, Vanhoutte PM, Lam KS and Xu A. Vascular effects of adiponectin: molecular mechanisms and potential therapeutic intervention. *Clin Sci (Lond)* 2008;114:361-74.
166 Ouchi N, Kihara S, Arita Y, Nishida M, Matsuyama A, Okamoto Y *et al.* Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. *Circulation* 2001;103:1057-63.
167 Yokota T, Oritani K, Takahashi I, Ishikawa J, Matsuyama A, Ouchi N *et al.* Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood* 2000;96:1723-32.
168 Kim JY, van de Wall E, Laplante M, Azzara A, Trujillo ME, Hofmann SM *et al.* Obesity-associated improvements in metabolic profile through expansion of adipose tissue. *J Clin Invest* 2007;117:2621-37.
169 Torti FM, Dieckmann B, Beutler B, Cerami A and Ringold GM. A macrophage factor inhibits adipocyte gene expression: an in vitro model of cachexia. *Science* 1985;229:867-9.
170 Zechner R, Newman TC, Sherry B, Cerami A and Breslow JL. Recombinant human cachectin/tumor necrosis factor but not interleukin-1 alpha downregulates lipoprotein lipase gene expression at the transcriptional level in mouse 3T3-L1 adipocytes. *Mol Cell Biol* 1988;8:2394-401.
171 Kriegler M, Perez C, DeFay K, Albert I and Lu SD. A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. *Cell* 1988;53:45-53.
172 Grell M. Tumor necrosis factor (TNF) receptors in cellular signaling of soluble and membrane-expressed TNF. *J Inflamm* 1995;47:8-17.
173 Aggarwal BB. TNFalpha. *Cytokine Reference* 2000:413-34.
174 Abel ED, Peroni O, Kim JK, Kim YB, Boss O, Hadro E *et al.* Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. *Nature* 2001;409:729-33.
175 Prins JB, Niesler CU, Winterford CM, Bright NA, Siddle K, O'Rahilly S *et al.* Tumor necrosis factor-alpha induces apoptosis of human adipose cells. *Diabetes* 1997;46:1939-44.
176 Xu H, Sethi JK and Hotamisligil GS. Transmembrane tumor necrosis factor (TNF)-alpha inhibits adipocyte differentiation by selectively activating TNF receptor 1. *J Biol Chem* 1999;274:26287-95.

177 Emanuelli B, Peraldi P, Filloux C, Chavey C, Freidinger K, Hilton DJ *et al.* SOCS-3 inhibits insulin signaling and is up-regulated in response to tumor necrosis factor-alpha in the adipose tissue of obese mice. *J Biol Chem* 2001;276:47944-9.
178 Gao Z, Hwang D, Bataille F, Lefevre M, York D, Quon MJ *et al.* Serine phosphorylation of insulin receptor substrate 1 by inhibitor kappa B kinase complex. *J Biol Chem* 2002;277:48115-21.
179 Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF and Spiegelman BM. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science* 1996;271:665-8.
180 Rui L, Yuan M, Frantz D, Shoelson S and White MF. SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. *J Biol Chem* 2002;277:42394-8.
181 Jain RG, Phelps KD and Pekala PH. Tumor necrosis factor-alpha initiated signal transduction in 3T3-L1 adipocytes. *J Cell Physiol* 1999;179:58-66.
182 Kim KY, Kim JK, Jeon JH, Yoon SR, Choi I and Yang Y. c-Jun N-terminal kinase is involved in the suppression of adiponectin expression by TNF-alpha in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* 2005;327:460-7.
183 Ruan H, Hacohen N, Golub TR, Van Parijs L and Lodish HF. Tumor necrosis factor-alpha suppresses adipocyte-specific genes and activates expression of preadipocyte genes in 3T3-L1 adipocytes: nuclear factor-kappaB activation by TNF-alpha is obligatory. *Diabetes* 2002;51:1319-36.
184 Uysal KT, Wiesbrock SM, Marino MW and Hotamisligil GS. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature* 1997;389:610-4.
185 Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R and Simsolo RB. The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. *J Clin Invest* 1995;95:2111-9.
186 Hotamisligil GS, Arner P, Atkinson RL and Spiegelman BM. Differential regulation of the p80 tumor necrosis factor receptor in human obesity and insulin resistance. *Diabetes* 1997;46:451-5.
187 Hotamisligil GS, Arner P, Caro JF, Atkinson RL and Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest* 1995;95:2409-15.
188 Dandona P, Weinstock R, Thusu K, Abdel-Rahman E, Aljada A and Wadden T. Tumor necrosis factor-alpha in sera of obese patients: fall with weight loss. *J Clin Endocrinol Metab* 1998;83:2907-10.
189 Jellema A, Plat J and Mensink RP. Weight reduction, but not a moderate intake of fish oil, lowers concentrations of inflammatory markers and PAI-1 antigen in obese men during the fasting and postprandial state. *Eur J Clin Invest* 2004;34:766-73.
190 Mohamed-Ali V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS *et al.* Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. *J Clin Endocrinol Metab* 1997;82:4196-200.
191 Bernstein LE, Berry J, Kim S, Canavan B and Grinspoon SK. Effects of etanercept in patients with the metabolic syndrome. *Arch Intern Med* 2006;166:902-8.
192 Dominguez H, Storgaard H, Rask-Madsen C, Steffen Hermann T, Ihlemann N, Baunbjerg Nielsen D *et al.* Metabolic and vascular effects of tumor necrosis factor-alpha blockade with etanercept in obese patients with type 2 diabetes. *J Vasc Res* 2005;42:517-25.

193 Wang B and Trayhurn P. Acute and prolonged effects of TNF-alpha on the expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture. *Pflugers Arch* 2006;452:418-27.
194 Van Coillie E, Van Damme J and Opdenakker G. The MCP/eotaxin subfamily of CC chemokines. *Cytokine Growth Factor Rev* 1999;10:61-86.
195 Melgarejo E, Medina MA, Sanchez-Jimenez F and Urdiales JL. Monocyte chemoattractant protein-1: a key mediator in inflammatory processes. *Int J Biochem Cell Biol* 2009;41:998-1001.
196 Conti P and DiGioacchino M. MCP-1 and RANTES are mediators of acute and chronic inflammation. *Allergy Asthma Proc* 2001;22:133-7.
197 Christiansen T, Richelsen B and Bruun JM. Monocyte chemoattractant protein-1 is produced in isolated adipocytes, associated with adiposity and reduced after weight loss in morbid obese subjects. *Int J Obes (Lond)* 2005;29:146-50.
198 Bruun JM, Lihn AS, Pedersen SB and Richelsen B. Monocyte chemoattractant protein-1 release is higher in visceral than subcutaneous human adipose tissue (AT): implication of macrophages resident in the AT. *J Clin Endocrinol Metab* 2005;90:2282-9.
199 Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R *et al.* MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest* 2006;116:1494-505.
200 Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K *et al.* CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *J Clin Invest* 2006;116:115-24.
201 Inouye KE, Shi H, Howard JK, Daly CH, Lord GM, Rollins BJ *et al.* Absence of CC chemokine ligand 2 does not limit obesity-associated infiltration of macrophages into adipose tissue. *Diabetes* 2007;56:2242-50.
202 Kiefer FW, Zeyda M, Todoric J, Huber J, Geyeregger R, Weichhart T *et al.* Osteopontin expression in human and murine obesity: extensive local up-regulation in adipose tissue but minimal systemic alterations. *Endocrinology* 2008;149:1350-7.
203 Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ *et al.* Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 2003;112:1821-30.
204 Gerhardt CC, Romero IA, Cancello R, Camoin L and Strosberg AD. Chemokines control fat accumulation and leptin secretion by cultured human adipocytes. *Mol Cell Endocrinol* 2001;175:81-92.
205 Sartipy P and Loskutoff DJ. Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proc Natl Acad Sci U S A* 2003;100:7265-70.
206 Kumar SN and Boss JM. Site A of the MCP-1 distal regulatory region functions as a transcriptional modulator through the transcription factor NF1. *Mol Immunol* 2000;37:623-32.
207 Fain JN and Madan AK. Regulation of monocyte chemoattractant protein 1 (MCP-1) release by explants of human visceral adipose tissue. *Int J Obes (Lond)* 2005;29:1299-307.
208 Boisvert WA. Modulation of atherogenesis by chemokines. *Trends Cardiovasc Med* 2004;14:161-5.
209 Kim MP, Wahl LM, Yanek LR, Becker DM and Becker LC. A monocyte chemoattractant protein-1 gene polymorphism is associated with occult ischemia in a high-risk asymptomatic population. *Atherosclerosis* 2007;193:366-72.

210 Walz A, Burgener R, Car B, Baggiolini M, Kunkel SL and Strieter RM. Structure and neutrophil-activating properties of a novel inflammatory peptide (ENA-78) with homology to interleukin 8. *J Exp Med* 1991;174:1355-62.
211 Walz A, Schmutz P, Mueller C and Schnyder-Candrian S. Regulation and function of the CXC chemokine ENA-78 in monocytes and its role in disease. *J Leukoc Biol* 1997;62:604-11.
212 Chavey C, Lazennec G, Lagarrigue S, Clape C, Iankova I, Teyssier J *et al.* CXC ligand 5 is an adipose-tissue derived factor that links obesity to insulin resistance. *Cell Metab* 2009;9:339-49.
213 Boisvert WA, Santiago R, Curtiss LK and Terkeltaub RA. A leukocyte homologue of the IL-8 receptor CXCR-2 mediates the accumulation of macrophages in atherosclerotic lesions of LDL receptor-deficient mice. *J Clin Invest* 1998;101:353-63.
214 Samal B, Sun Y, Stearns G, Xie C, Suggs S and McNiece I. Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor. *Mol Cell Biol* 1994;14:1431-7.
215 Fukuhara A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K *et al.* Retraction. *Science* 2007;318:565.
216 Revollo JR, Korner A, Mills KF, Satoh A, Wang T, Garten A *et al.* Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. *Cell Metab* 2007;6:363-75.
217 Sethi JK and Vidal-Puig A. Visfatin: the missing link between intra-abdominal obesity and diabetes? *Trends Mol Med* 2005;11:344-7.
218 Curat CA, Wegner V, Sengenes C, Miranville A, Tonus C, Busse R *et al.* Macrophages in human visceral adipose tissue: increased accumulation in obesity and a source of resistin and visfatin. *Diabetologia* 2006;49:744-7.
219 Varma V, Yao-Borengasser A, Rasouli N, Bodles AM, Phanavanh B, Lee MJ *et al.* Human visfatin expression: relationship to insulin sensitivity, intramyocellular lipids, and inflammation. *J Clin Endocrinol Metab* 2007;92:666-72.
220 Berndt J, Kloting N, Kralisch S, Kovacs P, Fasshauer M, Schon MR *et al.* Plasma visfatin concentrations and fat depot-specific mRNA expression in humans. *Diabetes* 2005;54:2911-6.
221 Chen MP, Chung FM, Chang DM, Tsai JC, Huang HF, Shin SJ *et al.* Elevated plasma level of visfatin/pre-B cell colony-enhancing factor in patients with type 2 diabetes mellitus. *J Clin Endocrinol Metab* 2006;91:295-9.
222 Filippatos TD, Derdemezis CS, Kiortsis DN, Tselepis AD and Elisaf MS. Increased plasma levels of visfatin/pre-B cell colony-enhancing factor in obese and overweight patients with metabolic syndrome. *J Endocrinol Invest* 2007;30:323-6.
223 Krzyzanowska K, Mittermayer F, Krugluger W, Kopp HP and Schernthaner G. Increase in visfatin after weight loss induced by gastroplastic surgery. *Obesity (Silver Spring)* 2006;14:1886-9.
224 Pagano C, Pilon C, Olivieri M, Mason P, Fabris R, Serra R *et al.* Reduced plasma visfatin/pre-B cell colony-enhancing factor in obesity is not related to insulin resistance in humans. *J Clin Endocrinol Metab* 2006;91:3165-70.
225 Haider DG, Pleiner J, Francesconi M, Wiesinger GF, Muller M and Wolzt M. Exercise training lowers plasma visfatin concentrations in patients with type 1 diabetes. *J Clin Endocrinol Metab* 2006;91:4702-4.
226 Haider DG, Schindler K, Schaller G, Prager G, Wolzt M and Ludvik B. Increased plasma visfatin concentrations in morbidly obese subjects are reduced after gastric banding. *J Clin Endocrinol Metab* 2006;91:1578-81.

227 Manco M, Fernandez-Real JM, Equitani F, Vendrell J, Valera Mora ME, Nanni G *et al.* Effect of massive weight loss on inflammatory adipocytokines and the innate immune system in morbidly obese women. *J Clin Endocrinol Metab* 2007;92:483-90.
228 Haider DG, Schaller G, Kapiotis S, Maier C, Luger A and Wolzt M. The release of the adipocytokine visfatin is regulated by glucose and insulin. *Diabetologia* 2006;49:1909-14.
229 Hector J, Schwarzloh B, Goehring J, Strate TG, Hess UF, Deuretzbacher G *et al.* TNF-alpha alters visfatin and adiponectin levels in human fat. *Horm Metab Res* 2007;39:250-5.
230 Kralisch S, Klein J, Lossner U, Bluher M, Paschke R, Stumvoll M *et al.* Hormonal regulation of the novel adipocytokine visfatin in 3T3-L1 adipocytes. *J Endocrinol* 2005;185:R1-8.
231 Kralisch S, Klein J, Lossner U, Bluher M, Paschke R, Stumvoll M *et al.* Interleukin-6 is a negative regulator of visfatin gene expression in 3T3-L1 adipocytes. *Am J Physiol Endocrinol Metab* 2005;289:E586-90.
232 MacLaren R, Cui W and Cianflone K. Visfatin expression is hormonally regulated by metabolic and sex hormones in 3T3-L1 pre-adipocytes and adipocytes. *Diabetes Obes Metab* 2007;9:490-7.
233 Moschen AR, Kaser A, Enrich B, Mosheimer B, Theurl M, Niederegger H *et al.* Visfatin, an adipocytokine with proinflammatory and immunomodulating properties. *J Immunol* 2007;178:1748-58.
234 Dahl TB, Yndestad A, Skjelland M, Oie E, Dahl A, Michelsen A *et al.* Increased expression of visfatin in macrophages of human unstable carotid and coronary atherosclerosis: possible role in inflammation and plaque destabilization. *Circulation* 2007;115:972-80.
235 Murphy KG and Bloom SR. Are all fats created equal? *Nat Med* 2006;12:32-3.
236 Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM *et al.* Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature* 2005;436:356-62.
237 Janke J, Engeli S, Boschmann M, Adams F, Bohnke J, Luft FC *et al.* Retinol-binding protein 4 in human obesity. *Diabetes* 2006;55:2805-10.
238 Tsutsumi C, Okuno M, Tannous L, Piantedosi R, Allan M, Goodman DS *et al.* Retinoids and retinoid-binding protein expression in rat adipocytes. *J Biol Chem* 1992;267:1805-10.
239 Scantlebury T, Maslowska M and Cianflone K. Chylomicron-specific enhancement of acylation stimulating protein and precursor protein C3 production in differentiated human adipocytes. *J Biol Chem* 1998;273:20903-9.
240 Ost A, Danielsson A, Liden M, Eriksson U, Nystrom FH and Stralfors P. Retinol-binding protein-4 attenuates insulin-induced phosphorylation of IRS1 and ERK1/2 in primary human adipocytes. *Faseb J* 2007;21:3696-704.
241 Cengiz C, Ardicoglu Y, Bulut S and Boyacioglu S. Serum retinol-binding protein 4 in patients with nonalcoholic fatty liver disease: does it have a significant impact on pathogenesis? *Eur J Gastroenterol Hepatol* 2009.
242 Cho YM, Youn BS, Lee H, Lee N, Min SS, Kwak SH *et al.* Plasma retinol-binding protein-4 concentrations are elevated in human subjects with impaired glucose tolerance and type 2 diabetes. *Diabetes Care* 2006;29:2457-61.
243 Kloting N, Graham TE, Berndt J, Kralisch S, Kovacs P, Wason CJ *et al.* Serum retinol-binding protein is more highly expressed in visceral than in subcutaneous adipose tissue and is a marker of intra-abdominal fat mass. *Cell Metab* 2007;6:79-87.

244 Silha JV, Nyomba BL, Leslie WD and Murphy LJ. Ethnicity, insulin resistance, and inflammatory adipokines in women at high and low risk for vascular disease. *Diabetes Care* 2007;30:286-91.
245 Stefan N, Hennige AM, Staiger H, Machann J, Schick F, Schleicher E *et al.* High circulating retinol-binding protein 4 is associated with elevated liver fat but not with total, subcutaneous, visceral, or intramyocellular fat in humans. *Diabetes Care* 2007;30:1173-8.
246 Broch M, Vendrell J, Ricart W, Richart C and Fernandez-Real JM. Circulating retinol-binding protein-4, insulin sensitivity, insulin secretion, and insulin disposition index in obese and nonobese subjects. *Diabetes Care* 2007;30:1802-6.
247 Promintzer M, Krebs M, Todoric J, Luger A, Bischof MG, Nowotny P *et al.* Insulin resistance is unrelated to circulating retinol binding protein and protein C inhibitor. *J Clin Endocrinol Metab* 2007;92:4306-12.
248 Ribel-Madsen R, Friedrichsen M, Vaag A and Poulsen P. Retinol-binding protein 4 in twins: regulatory mechanisms and impact of circulating and tissue expression levels on insulin secretion and action. *Diabetes* 2009;58:54-60.
249 Yao-Borengasser A, Varma V, Bodles AM, Rasouli N, Phanavanh B, Lee MJ *et al.* Retinol binding protein 4 expression in humans: relationship to insulin resistance, inflammation, and response to pioglitazone. *J Clin Endocrinol Metab* 2007;92:2590-7.
250 Balagopal P, Graham TE, Kahn BB, Altomare A, Funanage V and George D. Reduction of elevated serum retinol binding protein in obese children by lifestyle intervention: association with subclinical inflammation. *J Clin Endocrinol Metab* 2007;92:1971-4.
251 Haider DG, Schindler K, Prager G, Bohdjalian A, Luger A, Wolzt M *et al.* Serum retinol-binding protein 4 is reduced after weight loss in morbidly obese subjects. *J Clin Endocrinol Metab* 2007;92:1168-71.
252 Craig RL, Chu WS and Elbein SC. Retinol binding protein 4 as a candidate gene for type 2 diabetes and prediabetic intermediate traits. *Mol Genet Metab* 2007;90:338-44.
253 Kovacs P, Geyer M, Berndt J, Kloting N, Graham TE, Bottcher Y *et al.* Effects of genetic variation in the human retinol binding protein-4 gene (RBP4) on insulin resistance and fat depot-specific mRNA expression. *Diabetes* 2007;56:3095-100.
254 Tamori Y, Sakaue H and Kasuga M. RBP4, an unexpected adipokine. *Nat Med* 2006;12:30-1; discussion 1.
255 Hotamisligil GS. Inflammation and metabolic disorders. *Nature* 2006;444:860-7.
256 Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E *et al.* Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res* 2005;46:2347-55.
257 Nguyen MT, Favelyukis S, Nguyen AK, Reichart D, Scott PA, Jenn A *et al.* A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways. *J Biol Chem* 2007;282:35279-92.
258 Arkan MC, Hevener AL, Greten FR, Maeda S, Li ZW, Long JM *et al.* IKK-beta links inflammation to obesity-induced insulin resistance. *Nat Med* 2005;11:191-8.
259 Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J *et al.* Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med* 2005;11:183-90.
260 Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K *et al.* A central role for JNK in obesity and insulin resistance. *Nature* 2002;420:333-6.
261 D'Alessandris C, Lauro R, Presta I and Sesti G. C-reactive protein induces phosphorylation of insulin receptor substrate-1 on Ser307 and Ser 612 in L6

myocytes, thereby impairing the insulin signalling pathway that promotes glucose transport. *Diabetologia* 2007;50:840-9.

262 Sell H, Dietze-Schroeder D, Kaiser U and Eckel J. Monocyte chemotactic protein-1 is a potential player in the negative cross-talk between adipose tissue and skeletal muscle. *Endocrinology* 2006;147:2458-67.

263 Wellen KE and Hotamisligil GS. Inflammation, stress, and diabetes. *J Clin Invest* 2005;115:1111-9.

264 Bouloumie A, Sengenes C, Portolan G, Galitzky J and Lafontan M. Adipocyte produces matrix metalloproteinases 2 and 9: involvement in adipose differentiation. *Diabetes* 2001;50:2080-6.

265 Schmitz G and Grandl M. Lipid homeostasis in macrophages - implications for atherosclerosis. *Rev Physiol Biochem Pharmacol* 2008;160:93-125.

266 Schenk S, Saberi M and Olefsky JM. Insulin sensitivity: modulation by nutrients and inflammation. *J Clin Invest* 2008;118:2992-3002.

267 Karin M, Takahashi T, Kapahi P, Delhase M, Chen Y, Makris C *et al.* Oxidative stress and gene expression: the AP-1 and NF-kappaB connections. *Biofactors* 2001;15:87-9.

268 Summers SA. Ceramides in insulin resistance and lipotoxicity. *Prog Lipid Res* 2006;45:42-72.

269 Marciniak SJ and Ron D. Endoplasmic reticulum stress signaling in disease. *Physiol Rev* 2006;86:1133-49.

270 Morino K, Petersen KF and Shulman GI. Molecular mechanisms of insulin resistance in humans and their potential links with mitochondrial dysfunction. *Diabetes* 2006;55 Suppl 2:S9-S15.

271 Ron D and Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol* 2007;8:519-29.

272 Hosogai N, Fukuhara A, Oshima K, Miyata Y, Tanaka S, Segawa K *et al.* Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. *Diabetes* 2007;56:901-11.

273 Wang B, Wood IS and Trayhurn P. Dysregulation of the expression and secretion of inflammation-related adipokines by hypoxia in human adipocytes. *Pflugers Arch* 2007;455:479-92.

274 Ofei F, Hurel S, Newkirk J, Sopwith M and Taylor R. Effects of an engineered human anti-TNF-alpha antibody (CDP571) on insulin sensitivity and glycemic control in patients with NIDDM. *Diabetes* 1996;45:881-5.

275 Larsen CM, Faulenbach M, Vaag A, Volund A, Ehses JA, Seifert B *et al.* Interleukin-1-receptor antagonist in type 2 diabetes mellitus. *N Engl J Med* 2007;356:1517-26.

276 Shoelson SE, Herrero L and Naaz A. Obesity, inflammation, and insulin resistance. *Gastroenterology* 2007;132:2169-80.

277 Tilg H and Moschen AR. Inflammatory mechanisms in the regulation of insulin resistance. *Mol Med* 2008;14:222-31.

278 Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO *et al.* Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* 2006;313:1137-40.

279 Wellen KE and Hotamisligil GS. Obesity-induced inflammatory changes in adipose tissue. *J Clin Invest* 2003;112:1785-8.

280 Bourlier V, Zakaroff-Girard A, Miranville A, De Barros S, Maumus M, Sengenes C *et al.* Remodeling phenotype of human subcutaneous adipose tissue macrophages. *Circulation* 2008;117:806-15.

281 Cancello R, Tordjman J, Poitou C, Guilhem G, Bouillot JL, Hugol D *et al.* Increased infiltration of macrophages in omental adipose tissue is associated with marked hepatic lesions in morbid human obesity. *Diabetes* 2006;55:1554-61.
282 Lumeng CN, Deyoung SM, Bodzin JL and Saltiel AR. Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity. *Diabetes* 2007;56:16-23.
283 Strissel KJ, Stancheva Z, Miyoshi H, Perfield JW, 2nd, DeFuria J, Jick Z *et al.* Adipocyte death, adipose tissue remodeling, and obesity complications. *Diabetes* 2007;56:2910-8.
284 Zeyda M, Farmer D, Todoric J, Aszmann O, Speiser M, Gyori G *et al.* Human adipose tissue macrophages are of an anti-inflammatory phenotype but capable of excessive pro-inflammatory mediator production. *Int J Obes (Lond)* 2007;31:1420-8.
285 Solinas G, Vilcu C, Neels JG, Bandyopadhyay GK, Luo JL, Naugler W *et al.* JNK1 in hematopoietically derived cells contributes to diet-induced inflammation and insulin resistance without affecting obesity. *Cell Metab* 2007;6:386-97.
286 Charriere G, Cousin B, Arnaud E, Andre M, Bacou F, Penicaud L *et al.* Preadipocyte conversion to macrophage. Evidence of plasticity. *J Biol Chem* 2003;278:9850-5.
287 Arnold L, Henry A, Poron F, Baba-Amer Y, van Rooijen N, Plonquet A *et al.* Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J Exp Med* 2007;204:1057-69.
288 Heilbronn LK and Campbell LV. Adipose tissue macrophages, low grade inflammation and insulin resistance in human obesity. *Curr Pharm Des* 2008;14:1225-30.
289 Jernas M, Palming J, Sjoholm K, Jennische E, Svensson PA, Gabrielsson BG *et al.* Separation of human adipocytes by size: hypertrophic fat cells display distinct gene expression. *Faseb J* 2006;20:1540-2.
290 Skurk T, Alberti-Huber C, Herder C and Hauner H. Relationship between adipocyte size and adipokine expression and secretion. *J Clin Endocrinol Metab* 2007;92:1023-33.
291 Huang H, Park PH, McMullen MR and Nagy LE. Mechanisms for the anti-inflammatory effects of adiponectin in macrophages. *J Gastroenterol Hepatol* 2008;23 Suppl 1:S50-3.
292 Poitou C, Divoux A, Faty A, Tordjman J, Hugol D, Aissat A *et al.* Role of serum amyloid a in adipocyte-macrophage cross talk and adipocyte cholesterol efflux. *J Clin Endocrinol Metab* 2009;94:1810-7.
293 Nomiyama T, Perez-Tilve D, Ogawa D, Gizard F, Zhao Y, Heywood EB *et al.* Osteopontin mediates obesity-induced adipose tissue macrophage infiltration and insulin resistance in mice. *J Clin Invest* 2007;117:2877-88.
294 Clement K, Viguerie N, Poitou C, Carette C, Pelloux V, Curat CA *et al.* Weight loss regulates inflammation-related genes in white adipose tissue of obese subjects. *Faseb J* 2004;18:1657-69.
295 Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A and Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* 2004;25:677-86.
296 Stout RD and Suttles J. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J Leukoc Biol* 2004;76:509-13.
297 Goerdt S and Orfanos CE. Other functions, other genes: alternative activation of antigen-presenting cells. *Immunity* 1999;10:137-42.
298 Mosser DM. The many faces of macrophage activation. *J Leukoc Biol* 2003;73:209-12.

299 Lumeng CN, Bodzin JL and Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* 2007;117:175-84.
300 Lumeng CN, DelProposto JB, Westcott DJ and Saltiel AR. Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes. *Diabetes* 2008;57:3239-46.
301 Charo IF. Macrophage polarization and insulin resistance: PPARgamma in control. *Cell Metab* 2007;6:96-8.
302 Odegaard JI, Ricardo-Gonzalez RR, Goforth MH, Morel CR, Subramanian V, Mukundan L *et al.* Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. *Nature* 2007;447:1116-20.
303 Gratchev A, Kzhyshkowska J, Kothe K, Muller-Molinet I, Kannookadan S, Utikal J *et al.* Mphi1 and Mphi2 can be re-polarized by Th2 or Th1 cytokines, respectively, and respond to exogenous danger signals. *Immunobiology* 2006;211:473-86.
304 Schneemann M and Schoeden G. Macrophage biology and immunology: man is not a mouse. *J Leukoc Biol* 2007;81:579; discussion 80.
305 Aron-Wisnewsky J, Tordjman J, Poitou C, Darakhshan F, Hugol D, Basdevant A *et al.* Human adipose tissue macrophages: m1 and m2 cell surface markers in subcutaneous and omental depots and after weight loss. *J Clin Endocrinol Metab* 2009;94:4619-23.
306 Bouloumie A, Curat CA, Sengenes C, Lolmede K, Miranville A and Busse R. Role of macrophage tissue infiltration in metabolic diseases. *Curr Opin Clin Nutr Metab Care* 2005;8:347-54.
307 Constant VA, Gagnon A, Landry A and Sorisky A. Macrophage-conditioned medium inhibits the differentiation of 3T3-L1 and human abdominal preadipocytes. *Diabetologia* 2006;49:1402-11.
308 Lacasa D, Taleb S, Keophiphath M, Miranville A and Clement K. Macrophage-secreted factors impair human adipogenesis: involvement of proinflammatory state in preadipocytes. *Endocrinology* 2007;148:868-77.
309 Varma V, Yao-Borengasser A, Rasouli N, Nolen GT, Phanavanh B, Starks T *et al.* Muscle inflammatory response and insulin resistance: synergistic interaction between macrophages and fatty acids leads to impaired insulin action. *Am J Physiol Endocrinol Metab* 2009;296:E1300-10.
310 Vague J. [Not Available.]. *Presse Med* 1947;55:339.
311 Lafontan M and Berlan M. Do regional differences in adipocyte biology provide new pathophysiological insights? *Trends Pharmacol Sci* 2003;24:276-83.
312 Lemieux S and Despres JP. Metabolic complications of visceral obesity: contribution to the aetiology of type 2 diabetes and implications for prevention and treatment. *Diabete Metab* 1994;20:375-93.
313 Wajchenberg BL. Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. *Endocr Rev* 2000;21:697-738.
314 Garg A. Adipose tissue dysfunction in obesity and lipodystrophy. *Clin Cornerstone* 2006;8 Suppl 4:S7-S13.
315 Shen W, Wang Z, Punyanita M, Lei J, Sinav A, Kral JG *et al.* Adipose tissue quantification by imaging methods: a proposed classification. *Obes Res* 2003;11:5-16.
316 Abate N. Insulin resistance and obesity. The role of fat distribution pattern. *Diabetes Care* 1996;19:292-4.
317 Tchoukalova YD, Koutsari C, Karpyak MV, Votruba SB, Wendland E and Jensen MD. Subcutaneous adipocyte size and body fat distribution. *Am J Clin Nutr* 2008;87:56-63.

318 Yusuf S, Hawken S, Ounpuu S, Bautista L, Franzosi MG, Commerford P *et al.* Obesity and the risk of myocardial infarction in 27,000 participants from 52 countries: a case-control study. *Lancet* 2005;366:1640-9.
319 Tan GD, Goossens GH, Humphreys SM, Vidal H and Karpe F. Upper and lower body adipose tissue function: a direct comparison of fat mobilization in humans. *Obes Res* 2004;12:114-8.
320 Bjorntorp P. "Portal" adipose tissue as a generator of risk factors for cardiovascular disease and diabetes. *Arteriosclerosis* 1990;10:493-6.
321 Frayn KN. Visceral adiposity and lipid oxidation. *Am J Clin Nutr* 1996;63:977-8.
322 Kabir M, Catalano KJ, Ananthnarayan S, Kim SP, Van Citters GW, Dea MK *et al.* Molecular evidence supporting the portal theory: a causative link between visceral adiposity and hepatic insulin resistance. *Am J Physiol Endocrinol Metab* 2005;288:E454-61.
323 Fontana L, Eagon JC, Trujillo ME, Scherer PE and Klein S. Visceral fat adipokine secretion is associated with systemic inflammation in obese humans. *Diabetes* 2007;56:1010-3.
324 Linder K, Arner P, Flores-Morales A, Tollet-Egnell P and Norstedt G. Differentially expressed genes in visceral or subcutaneous adipose tissue of obese men and women. *J Lipid Res* 2004;45:148-54.
325 Huber J, Kiefer FW, Zeyda M, Ludvik B, Silberhumer GR, Prager G *et al.* CC chemokine and CC chemokine receptor profiles in visceral and subcutaneous adipose tissue are altered in human obesity. *J Clin Endocrinol Metab* 2008;93:3215-21.
326 Montague CT, Prins JB, Sanders L, Zhang J, Sewter CP, Digby J *et al.* Depot-related gene expression in human subcutaneous and omental adipocytes. *Diabetes* 1998;47:1384-91.
327 Hou XG, Moser S, Sarr MG, Thompson GB, Que FG and Jensen MD. Visceral and subcutaneous adipose tissue diacylglycerol acyltransferase activity in humans. *Obesity (Silver Spring)* 2009;17:1129-34.
328 Lundgren M, Svensson M, Lindmark S, Renstrom F, Ruge T and Eriksson JW. Fat cell enlargement is an independent marker of insulin resistance and 'hyperleptinaemia'. *Diabetologia* 2007;50:625-33.
329 Tchkonia T, Giorgadze N, Pirtskhalava T, Thomou T, DePonte M, Koo A *et al.* Fat depot-specific characteristics are retained in strains derived from single human preadipocytes. *Diabetes* 2006;55:2571-8.
330 Tchkonia T, Tchoukalova YD, Giorgadze N, Pirtskhalava T, Karagiannides I, Forse RA *et al.* Abundance of two human preadipocyte subtypes with distinct capacities for replication, adipogenesis, and apoptosis varies among fat depots. *Am J Physiol Endocrinol Metab* 2005;288:E267-77.
331 Berndt J, Kralisch S, Kloting N, Ruschke K, Kern M, Fasshauer M *et al.* Adipose triglyceride lipase gene expression in human visceral obesity. *Exp Clin Endocrinol Diabetes* 2008;116:203-10.
332 Reynisdottir S, Dauzats M, Thorne A and Langin D. Comparison of hormone-sensitive lipase activity in visceral and subcutaneous human adipose tissue. *J Clin Endocrinol Metab* 1997;82:4162-6.
333 Fried SK, Russell CD, Grauso NL and Brolin RE. Lipoprotein lipase regulation by insulin and glucocorticoid in subcutaneous and omental adipose tissues of obese women and men. *J Clin Invest* 1993;92:2191-8.
334 Arner P, Hellstrom L, Wahrenberg H and Bronnegard M. Beta-adrenoceptor expression in human fat cells from different regions. *J Clin Invest* 1990;86:1595-600.

335 Hellmer J, Marcus C, Sonnenfeld T and Arner P. Mechanisms for differences in lipolysis between human subcutaneous and omental fat cells. *J Clin Endocrinol Metab* 1992;75:15-20.
336 Mauriege P, Galitzky J, Berlan M and Lafontan M. Heterogeneous distribution of beta and alpha-2 adrenoceptor binding sites in human fat cells from various fat deposits: functional consequences. *Eur J Clin Invest* 1987;17:156-65.
337 Montague CT, Prins JB, Sanders L, Digby JE and O'Rahilly S. Depot- and sex-specific differences in human leptin mRNA expression: implications for the control of regional fat distribution. *Diabetes* 1997;46:342-7.
338 Van Harmelen V, Reynisdottir S, Eriksson P, Thorne A, Hoffstedt J, Lonnqvist F *et al.* Leptin secretion from subcutaneous and visceral adipose tissue in women. *Diabetes* 1998;47:913-7.
339 Lihn AS, Bruun JM, He G, Pedersen SB, Jensen PF and Richelsen B. Lower expression of adiponectin mRNA in visceral adipose tissue in lean and obese subjects. *Mol Cell Endocrinol* 2004;219:9-15.
340 Motoshima H, Wu X, Sinha MK, Hardy VE, Rosato EL, Barbot DJ *et al.* Differential regulation of adiponectin secretion from cultured human omental and subcutaneous adipocytes: effects of insulin and rosiglitazone. *J Clin Endocrinol Metab* 2002;87:5662-7.
341 Harman-Boehm I, Bluher M, Redel H, Sion-Vardy N, Ovadia S, Avinoach E *et al.* Macrophage infiltration into omental versus subcutaneous fat across different populations: effect of regional adiposity and the comorbidities of obesity. *J Clin Endocrinol Metab* 2007;92:2240-7.
342 Wilding JP. Treatment strategies for obesity. *Obes Rev* 2007;8 Suppl 1:137-44.
343 Bruun JM, Helge JW, Richelsen B and Stallknecht B. Diet and exercise reduce low-grade inflammation and macrophage infiltration in adipose tissue but not in skeletal muscle in severely obese subjects. *Am J Physiol Endocrinol Metab* 2006;290:E961-7.
344 Lopez-Fontana CM, Sanchez-Villegas A, Martinez-Gonzalez MA and Martinez JA. Daily physical activity and macronutrient distribution of low-calorie diets jointly affect body fat reduction in obese women. *Appl Physiol Nutr Metab* 2009;34:595-602.
345 Aucott L, Poobalan A, Smith WC, Avenell A, Jung R and Broom J. Effects of weight loss in overweight/obese individuals and long-term hypertension outcomes: a systematic review. *Hypertension* 2005;45:1035-41.
346 Williamson DF, Pamuk E, Thun M, Flanders D, Byers T and Heath C. Prospective study of intentional weight loss and mortality in never-smoking overweight US white women aged 40-64 years. *Am J Epidemiol* 1995;141:1128-41.
347 Hainer V. Etiopatogeneze obezity. *Zaklady klinicke obezitologie* 2004.
348 Jenkins DJ, Wolever TM, Taylor RH, Barker H, Fielden H, Baldwin JM *et al.* Glycemic index of foods: a physiological basis for carbohydrate exchange. *Am J Clin Nutr* 1981;34:362-6.
349 Bradley U, Spence M, Courtney CH, McKinley MC, Ennis CN, McCance DR *et al.* Low-fat versus low-carbohydrate weight reduction diets: effects on weight loss, insulin resistance, and cardiovascular risk: a randomized control trial. *Diabetes* 2009;58:2741-8.
350 Capel F, Viguerie N, Vega N, Dejean S, Arner P, Klimcakova E *et al.* Contribution of energy restriction and macronutrient composition to changes in adipose tissue gene expression during dietary weight-loss programs in obese women. *J Clin Endocrinol Metab* 2008;93:4315-22.

351 de Luis DA, Aller R, Izaola O, Gonzalez Sagrado M, Bellioo D and Conde R. Effects of a low-fat versus a low-carbohydrate diet on adipocytokines in obese adults. *Horm Res* 2007;67:296-300.
352 Lim SS, Noakes M, Keogh JB and Clifton PM. Long-term effects of a low carbohydrate, low fat or high unsaturated fat diet compared to a no-intervention control. *Nutr Metab Cardiovasc Dis* 2009.
353 O'Brien KD, Brehm BJ, Seeley RJ, Bean J, Wener MH, Daniels S *et al.* Diet-induced weight loss is associated with decreases in plasma serum amyloid a and C-reactive protein independent of dietary macronutrient composition in obese subjects. *J Clin Endocrinol Metab* 2005;90:2244-9.
354 Viguerie N, Vidal H, Arner P, Holst C, Verdich C, Avizou S *et al.* Adipose tissue gene expression in obese subjects during low-fat and high-fat hypocaloric diets. *Diabetologia* 2005;48:123-31.
355 Wylie-Rosett J and Davis NJ. Low-carbohydrate diets: an update on current research. *Curr Diab Rep* 2009;9:396-404.
356 Kunesova M, Braunerova R, Hlavaty P, Tvrzicka E, Stankova B, Skrha J *et al.* The influence of n-3 polyunsaturated fatty acids and very low calorie diet during a short-term weight reducing regimen on weight loss and serum fatty acid composition in severely obese women. *Physiol Res* 2006;55:63-72.
357 Mori T, Kondo H, Hase T, Tokimitsu I and Murase T. Dietary fish oil upregulates intestinal lipid metabolism and reduces body weight gain in C57BL/6J mice. *J Nutr* 2007;137:2629-34.
358 Ramel A, Martinez A, Kiely M, Morais G, Bandarra NM and Thorsdottir I. Beneficial effects of long-chain n-3 fatty acids included in an energy-restricted diet on insulin resistance in overweight and obese European young adults. *Diabetologia* 2008;51:1261-8.
359 Lindstrom J, Peltonen M, Eriksson JG, Louheranta A, Fogelholm M, Uusitupa M *et al.* High-fibre, low-fat diet predicts long-term weight loss and decreased type 2 diabetes risk: the Finnish Diabetes Prevention Study. *Diabetologia* 2006;49:912-20.
360 Shadid S and Jensen MD. Effects of pioglitazone versus diet and exercise on metabolic health and fat distribution in upper body obesity. *Diabetes Care* 2003;26:3148-52.
361 You T, Murphy KM, Lyles MF, Demons JL, Lenchik L and Nicklas BJ. Addition of aerobic exercise to dietary weight loss preferentially reduces abdominal adipocyte size. *Int J Obes (Lond)* 2006;30:1211-6.
362 Brochu M, Tchernof A, Turner AN, Ades PA and Poehlman ET. Is there a threshold of visceral fat loss that improves the metabolic profile in obese postmenopausal women? *Metabolism* 2003;52:599-604.
363 Bjorntorp P. *Regional Obesity*. Philadephia: JB Lippincott Co: 1992 pp. 79-86.
364 Strohacker K and McFarlin BK. Influence of obesity, physical inactivity, and weight cycling on chronic inflammation. *Front Biosci (Elite Ed)*;2:98-104.
365 Wannamethee SG, Shaper AG and Walker M. Weight change, weight fluctuation, and mortality. *Arch Intern Med* 2002;162:2575-80.
366 Williamson DF. "Weight cycling" and mortality: how do the epidemiologists explain the role of intentional weight loss? *J Am Coll Nutr* 1996;15:6-13.
367 Behre CJ, Gummesson A, Jernas M, Lystig TC, Fagerberg B, Carlsson B *et al.* Dissociation between adipose tissue expression and serum levels of adiponectin during and after diet-induced weight loss in obese subjects with and without the metabolic syndrome. *Metabolism* 2007;56:1022-8.

368 Bruun JM, Pedersen SB, Kristensen K and Richelsen B. Opposite regulation of interleukin-8 and tumor necrosis factor-alpha by weight loss. *Obes Res* 2002;10:499-506.
369 Lappalainen T, Kolehmainen M, Schwab U, Pulkkinen L, Laaksonen DE, Rauramaa R *et al.* Serum concentrations and expressions of serum amyloid A and leptin in adipose tissue are interrelated: the Genobin Study. *Eur J Endocrinol* 2008;158:333-41.
370 Brown T, Avenell A, Edmunds LD, Moore H, Whittaker V, Avery L *et al.* Systematic review of long-term lifestyle interventions to prevent weight gain and morbidity in adults. *Obes Rev* 2009;10:627-38.
371 Tsigos C, Hainer V, Basdevant A, Finer N, Fried M, Mathus-Vliegen E *et al.* Management of obesity in adults: European clinical practice guidelines. *Obes Facts* 2008;1:106-16.
372 Alberti KG, Zimmet P and Shaw J. The metabolic syndrome--a new worldwide definition. *Lancet* 2005;366:1059-62.
373 Knight SC. Specialized perinodal fat fuels and fashions immunity. *Immunity* 2008;28:135-8.
374 Koistinen HA, Bastard JP, Dusserre E, Ebeling P, Zegari N, Andreelli F *et al.* Subcutaneous adipose tissue expression of tumour necrosis factor-alpha is not associated with whole body insulin resistance in obese nondiabetic or in type-2 diabetic subjects. *Eur J Clin Invest* 2000;30:302-10.
375 Feingold KR, Doerrler W, Dinarello CA, Fiers W and Grunfeld C. Stimulation of lipolysis in cultured fat cells by tumor necrosis factor, interleukin-1, and the interferons is blocked by inhibition of prostaglandin synthesis. *Endocrinology* 1992;130:10-6.
376 Takashima N, Tomoike H and Iwai N. Retinol-binding protein 4 and insulin resistance. *N Engl J Med* 2006;355:1392; author reply 4-5.
377 Graham TE, Wason CJ, Bluher M and Kahn BB. Shortcomings in methodology complicate measurements of serum retinol binding protein (RBP4) in insulin-resistant human subjects. *Diabetologia* 2007;50:814-23.
378 Shetty PS, Watrasiewicz KE, Jung RT and James WP. Rapid-turnover transport proteins: an index of subclinical protein-energy malnutrition. *Lancet* 1979;2:230-2.
379 Capel F, Klimcakova E, Viguerie N, Roussel B, Vitkova M, Kovacikova M *et al.* Macrophages and adipocytes in human obesity: adipose tissue gene expression and insulin sensitivity during calorie restriction and weight stabilization. *Diabetes* 2009;58:1558-67.
380 McGrath MS and Kodelja V. Balanced macrophage activation hypothesis: a biological model for development of drugs targeted at macrophage functional states. *Pathobiology* 1999;67:277-81.
381 West M. Dead adipocytes and metabolic dysfunction: recent progress. *Curr Opin Endocrinol Diabetes Obes* 2009;16:178-82.