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Integrating role of adipose tissue secretory functions in response to dietary and pharmacological treatments

Ph. D. Thesis

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Statement of authorship

I certify that the thesis represents valid work elaborated under the supervision of Jan Kopecký, MD, DSc, and that neither this manuscript nor one with substantially similar content under my authorship has been submitted in support of an application for any other academical degree. My participation in the published papers is specified at the end of the comments to each paper.

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Jan Kopecký, MD, DSc

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This thesis is based on the following papers, referred to by their capital letters in the text as indicated here:

A. **Macek Jilkova Z**, Pavelka S, Flachs P, Hensler M, Kus V, Kopecky J. Modulation of type I iodothyronine 5'-deiodinase activity in white adipose tissue by nutrition: possible involvement of leptin. *Physiol Research (in press)*, (IF = 1.739)

B. Kus V, Prazak T, Brauner P, Hensler M, Kuda O, Flachs P, Janovska P, Medrikova D, Rossmeisl M, **Jilkova Z**, Stefl B, Pastalkova E, Drahota Z, Houstek J, Kopecky J. Induction of muscle thermogenesis by high-fat diet in mice: association with obesity-resistance. *Am J Physiol Endocrinol Metab* 295: E356-E367, 2008, (IF = 4,129)

C. Kuda O, Jelenik T, **Jilkova Z**, Flachs P, Rossmeisl M, Hensler M, Kazdova L, Ogston N, Baranowski M, Gorski J, Janovska P, Polak J, Mohamed-Ali V, Burcelin R, Cinti S, Bryhn M, Kopecky J. n–3 fatty acids and rosiglitazone improve insulin sensitivity through additive stimulatory effects on muscle glycogen synthesis in mice fed high-fat diet. *Diabetologia* 52: 941-951, 2009, (IF = 6.328)

D. Rossmeisl M, Jelenik T, **Jilkova Z**, Slamova K, Kus V, Hensler M, Medrikova D, Povysil C, Flachs P, Mohamed-Ali V, Bryhn M, Berge K, Holmeide K, Kopecky J. DHA-derivatives in the prevention and reversal of obesity and glucose intolerance in mice. *Obesity* 17: 1023–1031, 2009, (IF = 2.798)

The above papers are included in full in this PhD thesis. For the complete list of my published articles, see List of publications (Section 7).

LIST OF ABBREVIATIONS

AICAR	aminoimidazole carboxamide ribonucleotide
AMPK	AMP–activated protein kinase
ATM	adipose tissue macrophage
cHF	corn oil-based composite high-fat diet
cHF+F	cHF diet supplemented with fish oil
cHF+F+TZD	cHF+F diet supplemented with thiazolidinedione
cHF+TZD	cHF diet supplemented with thiazolidinedione
CLS	crown-like structures
D1	type I iodothyronine 5'-deiodinase
D2	type II iodothyronine 5'-deiodinase
D3	type III 5-deiodinase
DAB	3,3′-diaminobenzidine
DHA	docosahexaenoic acid (22:6 n–3)
EPA	eicosapentaenoic acid (20:3 n–5)
HMW	high molecular weight form of adiponectin
IGT	impaired glucose tolerance
IL	interleukin
LMW	low molecular weight form of adiponectin
MCP-1	monocyte chemoattractant protein-1
MMW	medium molecular weight form of adiponectin
PAI-1	plasminogen activator inhibitor-1
PBS	p hosphate buffered saline
PPAR	peroxisome proliferator–activated receptor
PUFA	polyunsaturated fatty acids
T_3	3, 5, 3′, - triiodothyronine
T_4	thyroxine
TH	thyroid hormones
TG	triacylglycerols
TNF	tumor necrosis factor
TZD	thiazolidinediones
UCP-1	uncoupling protein - 1

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1 INTRODUCTION

1.1 Energy balance and body weight homeostasis

Life exists in a flux of energy transformations that are governed by the laws of thermodynamics. Energy can neither be created nor destroyed but can be transformed only from one form to another. Biological systems depend on the transformation of chemical energy in other forms of energy to perform chemical, mechanical or electrical work. Principles of energy balance are embodied in the following equation:

energy intake = energy expenditure + Δ energy stores

In the state of energy balance energy expenditure is equal to energy intake, according to the first law of thermodynamics. When the intake and expenditure of energy are not equal, then a change in body energy content will occur. Negative energy balance results in a degradation of energy stores while positive energy balance results in an increase in energy stores that leads to the obesity. In other words, the increased prevalence of obesity results mostly from overeating (excess of energy intake) and/or a lack of physical activity (decrease of energy expenditure).

1.1.1 Energy intake

The control of energy intake is a very complex process that depends on the ability of the brain to receive and integrate a wide range of signals indicating the nutritional state and the energy level of the organism and to produce appropriate responses. This signals come from a variety of peripheral organs, including adipose tissue, gastrointestinal tract, pancreas, liver and perhaps also skeletal muscle. One of the most important signals of adiposity is a circulating protein leptin (see 1.2.2.1 Leptin).

1.1.2 Energy expenditure

There are three components of energy expenditure: a) the energy spent for basal metabolism, b) the energy spent on physical activity and c) energy expenditure spent in response to variety of stimuli, including food, cold, stress, some type of drug treatment and others, referred to as adaptive thermogenesis (2).

Thermogenesis stimulated by cold exposure can be divided in two types: shivering and non-shivering thermogenesis. Shivering thermogenesis is a well-known mechanism protecting an organism against acute cold exposure. Activation of muscle contraction is mediated by induction of posterior hypothalamus. While no work is performed, heat is produced. However, after longer periods of shivering, muscle fatigue occurs. Non-shivering thermogenesis is an acclimatization mechanism to low ambient temperatures, leading to increased energy expenditure upon cold exposure. Diet-induced thermogenesis increases the energy expenditure in response to food intake (3). Recently, it has been shown that cold-induced non-shivering thermogenesis and diet-induced adaptive termogenesis is likely to share the same regulatory mechanism (4).

The most frequently studied mechanism of non-shivering thermogenesis is mitochondrial uncoupling in brown adipose tissue, which is considered to play a major role in the thermogenic response to both cold and food exposure. Uncoupling process is mediated by uncoupling protein 1 (UCP-1), a unique mitochondrial inner-membrane protein specifically expressed in brown adipocytes. UCP-1 causes a reflux of protons into mitochondrial matrix, by-passing the ATP synthase, and instead of using the energy stored in the proton gradient to produce ATP, heat is dissipated (5). For a long time, it was assumed that mitochondrial uncoupling is present only in small mammals whereas in adult humans is negligible. However, several studies published in recent years suggest that mitochondrial uncoupling in brown adipose tissue in adult humans can be physiologically significant (2).

Part of adaptive thermogenesis might be explained by a mechanism observed in the skeletal muscle, where leptin stimulates lipid oxidation, causing an increase in energy expenditure (see 1.2.2.1 Leptin). Another eligible mechanism for adaptive

thermogenesis could be also calcium cycling, as seen in some fish living in cold environment (e.g. tuna) (2).

1.1.2.1 Thyroid hormones and energy expenditure

It has long been accepted that thyroid hormones (TH) are important determinants of overall energy expenditure and of the basal metabolic rate (for review see (6)). Indeed, regulating thermogenesis is one of the major tasks of TH (7). Generally, in some tissues TH increase ATP turnover by stimulating its utilization and reduce the thermodynamic efficiency of ATP synthesis. Well described is the role of TH in non-shivering thermogenesis in brown adipose tissue, in which non-shivering thermogenesis is stimulated by the secretion of norepinephrine from the sympathetic nerves under the control of hypothalamus. Mediated by beta-3 noradrenergic receptor and in the presence of 3, 5, 3′, - triiodothyronine (T_3), norepinephrine promotes the synthesis of UCP-1, leading to dissipation of energy as heat (see above). The supply of adequate amounts of T_3 is ensured by the cold-induced enhancement of the type II iodothyronine 5’-deiodinase (D2), which deiodinates thyroxine (T_4) to T_3. The absence of T_3 blocks UCP1 synthesis, leading to hypothermia (8). However, the mechanism by which that TH determine the metabolic rate in other tissues is not yet fully explained.

1.1.3 Energy stores – obesity

Energy is stored in the body in the form of triacylglycerols (TG) and glycogen. Glycogen is mainly stored in the liver and skeletal muscle. Fat cells represent the normal site of fat storage that the body uses to balance out short-term fluctuations in energy intake and expenditure. A medical condition in which the excess of body fat has accumulated is called obesity and it has an adverse effect on health, leading to reduced life expectancy. The incidence of obesity has increased dramatically during recent decades (9). Adipocytes and adipose tissue dysfunction belong to the primary defects in obesity (see Inflammation of adipose tissue and insulin resistance in obesity 1.2.3). Moreover, when storage of dietary lipids in adipose tissue is exceeded, additional lipids have to be stored in other organs and tissues. Ectopic fat accumulation and lipid redistribution into tissues, which are not arranged to handle large portions of fatty acids,

have destructive effects on the appropriate function of target organ and are strongly linked to the development of insulin resistance, type 2 diabetes, fatty liver disease, hypertension, dyslipidemia and atherosclerosis (10).

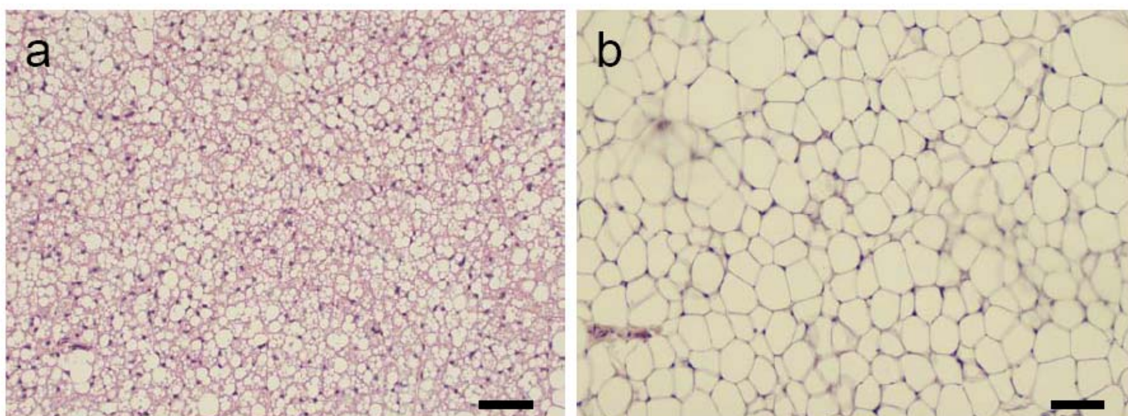
1.2 Central role of adipose tissue

Adipose tissue has been considered for years to be a simple lipid reservoir. The increasing knowledge, concerning the role of adipose tissue in energy balance and in the pathophysiology of metabolic disorders, have focused attention to this tissue.

1.2.1 Biology of adipose tissue

Adipose tissue is a complex, essential, and highly active metabolic and endocrine organ that is found in mammals in two different forms: white adipose tissue and brown adipose tissue. These tissues are characterized by different anatomic location, morphological structures, functions, and regulation. Both types of adipose tissues are able to store energy as TG, but whereas white fat releases this energy in the form of free fatty acids according to the needs of the organism, brown fat can convert it into a heat.

Figure 1 Mouse brown adipose tissue (a) and white adipose tissue (b), *bar = 100 µm*



1.2.1.1 Brown adipose tissue

Brown adipose tissue has a characteristic brown colour, originating mostly from its plentiful vascularization and high content of mitochondria and cytochromes. Brown adipose tissue is present mostly in newborn and hibernating mammals. Brown fat cells are multilocular, which means that these cells have considerable cytoplasm, with lipid droplets scattered throughout. Brown adipose tissue thermogenic function is assumed by the numerous mitochondria and by the presence of mitochondrial protein UCP-1, specifically expressed in brown adipocytes. UCP-1 is able to uncouple the oxidative phosphorylation in mitochondria, and thereby enables heat production, which is the principle of non-shivering thermogenesis (see 1.1.2 Energy expenditure)

1.2.1.2 White adipose tissue

White adipose tissue is specialized connective tissue that functions as the major storage site for fat in the form of TG. White adipocytes contain a large lipid droplet surrounded by a thin layer of cytoplasm and the nucleus is flattened and located on the periphery. Beside adipocytes, white adipose tissue contains connective tissue matrix, neural tissue, stromovascular cells, immune cells and preadipocytes (not yet filled with lipid) (11). Together these components function as an integrated unit. The largest fat depots are located subcutaneously and in the abdominal region. Visceral and subcutaneous adipose tissues depots display different metabolic properties, manifested by different expression level of genes involved in fat cell metabolism (12), and in the secretion of adipose tissue-derived factors that could be involved in some pathologies in both rodents and humans (13). White adipose tissue amount to 20-30 % of normal body weight, but may vary from a few up to 70 % (9).

White adipose tissue can expand by increasing intracellular lipid accumulation leading to greater adipocyte size (hypertrophy) and/or by increasing the numbers of adipocytes (hyperplasia). Adipocyte hypertrophy, evident in obese subjects, was originally considered to be the only way whereby adipose tissue mass expands. However, adipocytes hyperplasia (adipogenesis), which occurs most significantly in response to energy imbalance in young age, is now recognized to contribute to the

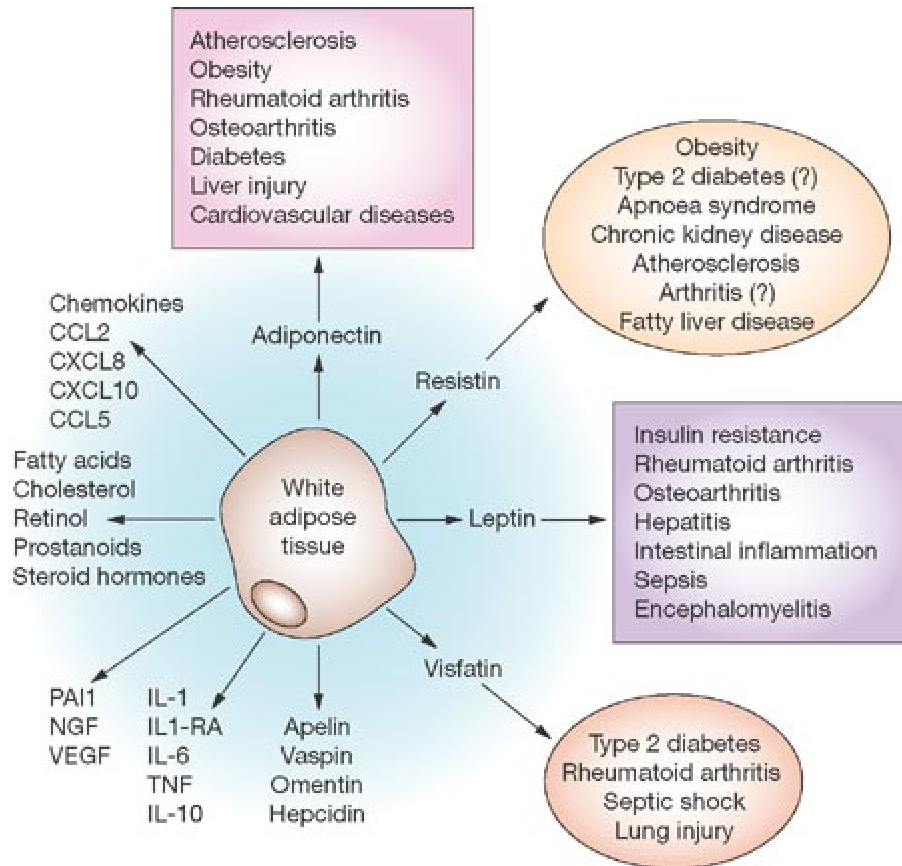
increased adipose tissue mass in obesity. Moreover, it was discovered recently that approximately 10 % of fat cells are renewed annually (14). Furthermore, there are also differences between adipose tissue depots. Whereas visceral fat mass increase primarily due to hypertrophy, subcutaneous fat exhibits both adipocyte hypertrophy as well as hyperplasia.

1.2.2 Endocrine function of adipose tissue

Adipose tissue plays a critical role in energy homeostasis by mobilizing TG reserves in a process of lipolysis in order to provide fatty acids. Fatty acids are important oxidative fuels for other tissues during times of energy deprivation such as fasting and exercise. During the lipolytic process, TG can be quickly hydrolysed by a series of specific lipases and free fatty acids are released into the circulation to be taken up by other tissues. Lipolysis is under tight regulation by insulin and catecholamines. Higher concentration of circulating fatty acids and TG are associated with lipid accumulation in multiple tissues, including liver and muscle, which leads to insulin resistance.

Besides this point of view, white adipose tissue also secretes a large variety of bioactive molecules, known as adipokines with endo-, auto-, or paracrine signal functions (15).

Figure 2 The multiple functions of white adipose tissue include the synthesis and secretion of adipokines, and the uptake, storage and synthesis of lipids.



Abbreviations: CCL, CC-chemokine ligand (CCL); CXCL, CXC-chemokine ligand (CXCL); IL, interleukin; IL1-RA, interleukin-1-receptor antagonist; NGF, nerve growth factor; PAI1, plasminogen activator inhibitor 1; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor (adapted from (1).

1.2.2.1 Leptin

The first characterised adipokine, leptin (from the Greek *leptos*, meaning "thin"), was discovered in 1994 (16). Identification of this hormone, mainly produced by adipocytes, has uncovered a new endocrine system of body weight regulation. Leptin circulates in plasma of mammals, and its concentration increases with accretion of adipose tissue mass and decreases when adipose tissue is reduced (17). Plasma leptin concentrations

correlate with subcutaneous fat volume (18) and body mass index, and diet induced weight loss decreases plasma leptin concentrations (19).Thereby leptin serves as a signal of energy sufficiency and of the amount of fat and provides this information to leptin-sensitive neurons in hypothalamus to regulate food intake (for review see (20)).

Moreover, leptin acts also directly in peripheral tissues. In the skeletal muscle, leptin selectively stimulates phosphorylation and activation of the alpha 2 catalytic subunit of AMP-activated protein kinase (α2 AMPK), which subsequently suppresses the activity of acetyl-CoA carboxylase and thereby stimulates fatty acid oxidation (21). In brown adipose tissue, leptin increases thermogenesis by increasing UCP-1 and lipoprotein lipase gene expression (22). On the whole, leptin serves as a hormonal signal in negative feedback loop that maintains homeostatic control of adipose tissue by modulating the activity of neural circuits that regulate food intake and energy expenditure. Leptin resistance is typical for a majority of obese subjects and is defined as decreased sensitivity to the anorexigenic effects of leptin, associated with impaired leptin signalling cascade (23).

Mice with mutation in the gene encoding leptin (*ob/ob* mice) (16) or the gene encoding the leptin receptor (*db/db* mice) (24) have obese phenotypes and they are used in many studies as models of obesity and type II diabetes. Injection of leptin into *ob/ob* mice reduces body fat content but *db/db* mice are leptin-resistant (19). In accord with previous observations, transgenic mice over-expressing leptin (*LepTg* mice) exhibit substantial reduction of adipose tissue (25).

1.2.2.2 Adiponectin

Adiponectin is a protein messenger, synthesized mainly by adipocytes, which improves insulin sensitivity and changes energy metabolism of target tissues (26). Adiponectin circulates in plasma and has a wide range of biological effects. Transcription of adiponectin gene is controlled by the transcription factor PPAR gamma and could be induced by insulin or thiazolidinediones (TZD) (27). It is produced as 30 kDa protein unit, consisting of 244 amino acids, which is able to form several multimeric structures. Adiponectin can act as monomer, trimer, hexamer or multimer (12-18 units), usually referred to as low-molecular weight trimer (LMW), medium-molecular weight hexamer

(MMW) or high-molecular weight (HMW) adiponectin with ten or more units (28). Serum adiponectin levels are decreased in obese subjects, particularly in individuals with visceral obesity and insulin resistance (29). Adiponectin seems to have an anti-inflammatory effect through inhibition of tumor necrosis factor-α (TNF-α) expression and reduction of its effects on the endothelial inflammatory response or through induction of the anti-inflammatory cytokines, such as interleukin-10 (IL-10), produced by immune cells (30). Moreover, adiponectin itself may be anti-atherosclerotic, as it acts as an endogenous antithrombotic factor and inhibits macrophage activation and foam cell accumulation (30).

1.2.2.3 Tumor necrosis factor-α

TNF-α is a pro-inflammatory cytokine, involved in apoptosis, cytotoxicity and inflammation. TNF-α stimulates the production of other cytokines, such as IL-1 and IL-6. Recently, number of studies supported the thesis of a crucial role for TNF-α in obesity-related insulin resistance (for review see (15;31;32)). Produced by macrophages within adipose tissue and by adipocytes themselves, TNF-α inhibits lipogenesis and stimulates lipolysis(33). With relation to adipocytes, it acts directly in insulin dependent processes, including homeostasis of carbohydrate and lipid metabolism. TNF-α expression and secretion are increased in obesity and correlate positively with body mass index and adipocyte volume (17;34).

1.2.2.4 Resistin

This hormone was first described in 2001, when a relationship was demonstrated between resistin and insulin resistance induced by obesity. Resistin is expressed within adipocytes of rodents but in human is also expressed in multiple other tissues (35). Resistin is a protein with pro-inflammatory properties and its levels are generally elevated with obesity (36).

1.2.2.5 Interleukin - 6

IL-6 is another pro-inflammatory adipokine with signaling potential, produced mainly by adipocytes, but also by immune cells, endothelial cells, fibroblasts, and myocytes (37). It has been demonstrated that adipose tissue is able to secrete a large amount of IL-6, with an increased production in obese subjects (38). There is a positive correlation between circulating IL-6 levels and insulin resistance (39) and with increased risk of coronary artery diseases (40). Weight loss decreases IL-6 concentrations (41).

1.2.2.6 Other adipokines

Many other adipokines were described:

Visfatin: Visfatin is insulinomimetic adipokine predominantly produced by visceral fat;

Interleukin-8: IL-8 has been implicated in the atherosclerotic process and involved in complications related to obesity (42).

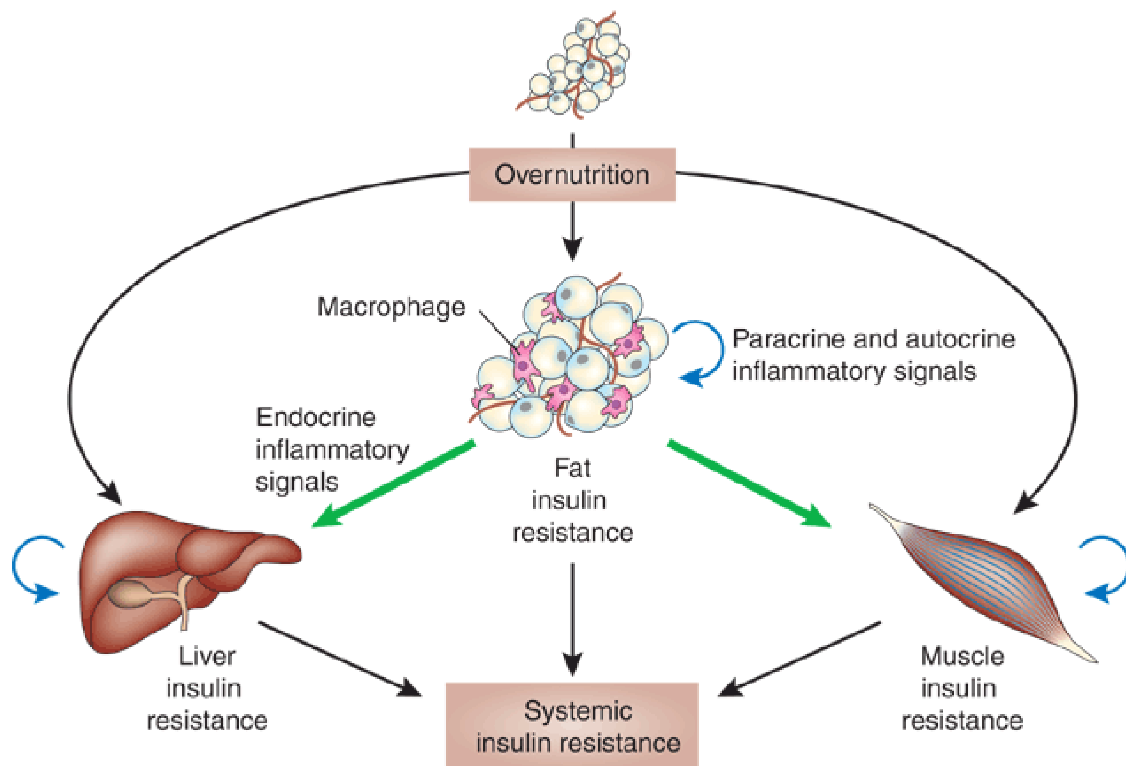
Monocyte chemoattractant protein-1 (MCP-1): Is thought to be the major chemotactic factor for monocytes. MCP-1 is produced by a variety of cells, including adipocytes (43), and its secretion by adipose tissue is correlated with specific macrophage markers and adiposity. MCP-1 release is higher in visceral fat depot compared to subcutaneous depot (44).

Plasminogen activator inhibitor-1 (PAI-1): PAI-1 provokes formation of thrombi. High plasma PAI-1 levels have been considered a risk factor for coronary heart disease in diabetic patients (15).

1.2.3 Inflammation of adipose tissue and insulin resistance in obesity

Obesity induces a state of low-grade inflammation that is associated with the development of type 2 diabetes (45). Hypertrophy of adipocytes leads to adipose tissue macrophage (ATM) infiltration, observed primarily in visceral white adipose tissue (46), possibly as a response to the hypoxia. ATM content positively correlates to amount of visceral fat and adipocytes size (47). Increased infiltration of adipose tissue with immune cells could lead to insulin resistance via increased levels of circulating cytokines, such as IL-6 and TNF-α, which contributes to systemically decreased insulin sensitivity via endocrine signalling (32).

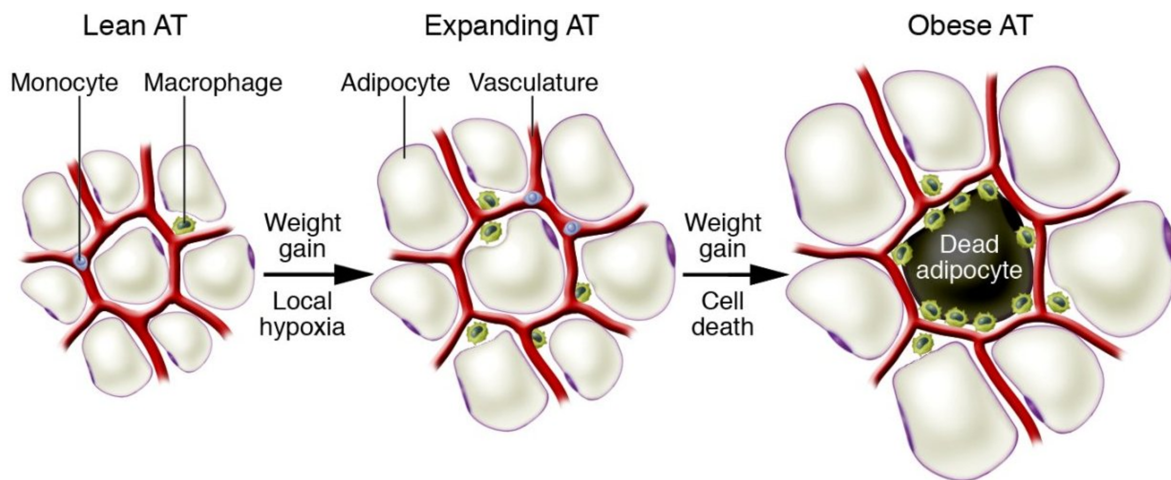
Figure 3 The development of systemic insulin resistance in obesity-induced inflammation.



(adapted from (48))

Histological analyses reveal that the vast majority (over 90 %) of macrophages of white adipose tissue form structures that are denominated crown-like structures (CLS) (49). In rodent, immunohistochemistry detection of macrophages is performed with antibodies to widely used markers for macrophages F4/80 or MAC-2/Galectin-3 (Figure 5) (50).

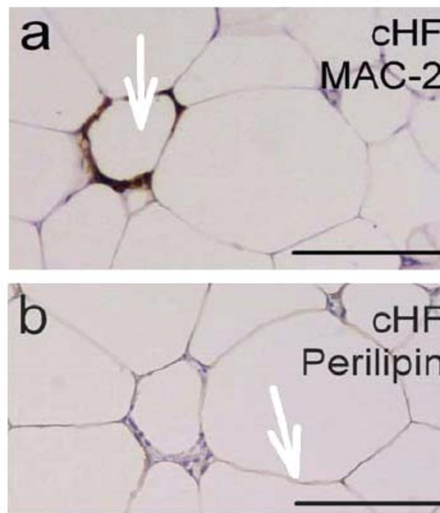
Figure 4 Adipose tissue expansion



Adipose tissue expansion during weight gain leads to recruitment of macrophages through a variety of signals, which may include local hypoxia. These macrophages predominantly localize around dead adipocytes (adapted from (51)).

The macrophage infiltration of adipose tissue in obese subjects is tightly linked to adipocytes death. By immunohistochemistry was demonstrated that CLS dead adipocytes surrounded by ATM are perilipin (the marker of viable adipocytes) negative, while all viable adipocytes not surrounded by ATM are perilipin positive (Figure 5). The most consistent residual part of the dead adipocyte is the free lipid droplet that must be reabsorbed by the organism. Thus the residual cell debris and free lipid droplet could act as a foreign body requiring a chronic and persistent activity of macrophages (49).

Figure 5 Crown-like structure



Crown-like structure: detailed view of adipose tissue from mice with visualized (**a**) MAC-2 immunoreactive macrophages and (**b**) Perilipin. *bar = 100 µm*

Inflammation of adipose tissue could be presented partially as a compensatory mechanism for increased adipose tissue turnover in obese states, which might protect obese individuals against deleterious effects of fat accumulation by promotion of adipocyte death and angiogenesis. On the other hand it was demonstrated that amelioration of inflammatory state in white adipose tissue leads to improvement of the obesity-related pathologies, in particular to improvement of insulin sensitivity (50;52). For instance, a weight loss induced by negative energy balance reduces macrophage activation and infiltration in adipose tissue. Moreover, the systemic inflammation is improved by reduction of inflammatory cytokines and chemokines produced by adipose tissue (53), leading to improved insulin sensitivity.

1.2.4 The amelioration of inflammatory state in white adipose tissue

1.2.4.1 Thiazolidinediones

Antidiabetic drugs of the TZD class are potent and selective activators of PPAR gamma, known as one of the key regulators of glucose homeostasis, able to promote adipocyte differentiation and lipid storage. PPAR gamma determines the differentiation process of preadipocytes into mature adipocytes and could also induce apoptosis of mature adipocytes. TZD are used in the treatment of type 2 diabetes, since these drugs improve insulin sensitivity and reduce hyperinsulinemia, hepatic steatosis, and inflammation (52).

Both in vitro and in vivo studies provide evidence that TZD have anti-inflammatory properties (54). TZD inhibit macrophage activation and decrease inflammatory cytokine expression and release in macrophage and monocyte. Treatment with TZD reduces secretion of resistin, IL-6, MCP-1 and TNF-α while increasing the insulin-sensitizing hormone adiponectin(44;55). Furthermore, it has been shown that the HMW/total adiponectin and HMW/LMW ratios are plausible indicators of TZD–induced changes in insulin sensitivity (27). These results suggest that TZD have anti-inflammatory properties improving the low-grade inflammatory state observed in obesity.

1.2.4.2 n–3 polyunsaturated fatty acids

Polyunsaturated fatty acids of n–3 (ω−3) family (n-3 PUFA) are important essential fatty acids, naturally occurring for example in sea fish. n–3 PUFA are components of phospholipids in cellular membranes, while also acting as regulatory ligands in gene transcription, where the effects of n–3 PUFA are mostly mediated by PPAR alpha (56).

Table 1 Nomenclature of selected n–3 polyunsaturated fatty acids

Trivial name	Abbreviation	Carboxyl reference
Eicosapentaenoic acid	EPA	C 20:5 (Δ5,8,11,14,17)
Docosahexaenoic acid	DHA	C 22:6 (Δ4,8,12,15,19)

Dietary n-3 PUFA seem to postpone diabetes development (57) and have considerable effect on gene expression in a variety of tissues, including adipose tissue, where they regulate the expression of genes involved in adipocyte differentiation and lipid metabolism (58). Hypolipidaemic and anti-obesogenic effects of n–3 PUFA probably depend on the in situ suppression of lipogenesis and increase of fatty acid oxidation in several tissues (59). This metabolic switch might reduce accumulation of toxic fatty acid-derivatives and thereby protect the insulin signaling in the liver and skeletal muscle (60). Furthermore, n-3 PUFA prevent high-fat diet-induced matrix remodeling, adipocyte enlargement in adipose tissue of obese subjects (61) and adipose tissue inflammation (50;62). In addition, n-3 PUFA exert immunomodulatory effects by induction of adiponectin (63) and by a decrease in the production of classic inflammatory mediators such as arachidonic acid-derived eicosanoids and inflammatory cytokines (64).

Besides acting directly as regulatory ligands, n–3 PUFA act also through their biologically active lipid mediators, resolvins, docosatrienes, and protectins as general classes, since each possesses unique chemical structures that are features of the new chemical classes and are biosynthesized by new pathways(65-67).

1.2.5 Thyroid hormones metabolism in white adipose tissue

Thyroid hormones (TH) play the major role in development of many tissues as well as in the regulation of many physiological processes. White adipose tissue represents an important target for TH, (68-70). Multiple biological effects of TH depend on intracellular levels of T_3, which binds to thyroid hormone receptor and is mostly generated in peripheral tissues by outer–ring deiodination of T_4. Type I and type II

iodothyronine 5’-deiodinase (D1 and D2, respectively) could catalyse the reaction. D1 exerts on relatively broad substrate specificity, while also catalysing inner-ring deiodination of T_4 to produce reverse T_3, an inactive form of TH, as well as deiodination of other TH derivatives (71). D2 also catalyses conversion of T_3 into 3,3’-diiodothyronine (T_2). Type III iodothyronine 5-deiodinase (D3), catalyses inner-ring deiodination of T_4 and T_3, to produce reverse T_3 and T_2. D1 is mainly present in the liver, kidneys, thyroid gland, and pituitary. Due to its high activity, the hepatic D1 is traditionally regarded as being an important source of circulating T_3, while D2, which is mainly present in the brain, brown fat, placenta, pituitary, and muscle, is essential to the local generation of T_3 in the tissues (72). In brown fat, D2 activity is required for tissue differentiation and thermogenic function (68). In white adipose tissue, T_3 regulates both lipolysis and lipogenesis by induction of the key lipogenic enzymes such as ACC, malic enzyme and fatty acid synthase (69). D3 has been suggested to stimulate the proliferation of white fat cells, while D2 could be linked to the differentiation programme of adipocytes, as revealed by in vitro experiments (68). Furthermore, thyroid hormone action is mediated by the family of thyroid hormone receptors. Recent finding suggests that thyroid hormone receptor TRα1 could contribute to subcutaneous adipose tissue expandability in obese subjects (73). However, the metabolism and biological role of TH in white adipose tissue is only poorly described.

2 METHODS

All methods and techniques, commonly used in our laboratory, including the characterization of used animals and treatments, are described in the selected publications (see Section 9). Since, the light microscopy, immunohistochemical analysis and morphometry analysis of adipose tissue have been recently introduced to our laboratory by the author of this PhD thesis, these methods will be described in detail.

2.1 Light microscopy and immunohistochemical analysis

Light microscopy allows studying the organization of adipocytes and other cell types present in adipose tissue, as well as providing data on the functional state of the tissue, particularly the size of adipocytes.

Sample collection and fixation of adipose tissue can be considered the most important processing step. Because of the unique texture and a high lipid content of adipose tissue, mechanical compression and deformation can cause irreversible damage to the tissue. Samples of white adipose tissue with optimal size of about 1 cm^2 are fixed in 4% formaldehyde for period of 12 to 24 h. After the fixation, samples are dehydrated and embedded in paraffin. The samples of adipose tissue are then cut into 3-5 µm sections.

2.1.1 Morphometry analysis of adipose tissue

The utilization of morphometric techniques for the study of adipose tissue has become an indispensable tool for the correct interpretation of morphological changes. The sections of adipose tissue are stained by hematoxylin, a dye staining the nucleus, and then with eosin, which stains acid cytoplasm. Six to eight digital images per tissue section are captured using Olympus AX70 light microscope and a DP 70 camera (Olympus, Tokyo, Japan). Adipocyte morphometry is performed using the image analysis system with an automated measurement of cells area, such as Lucia IMAGE 4.81 or NIS-Elements AR 3.0 (Laboratory Imaging, Prague, Czech Republic).

2.1.2 Immunohistochemistry of adipose tissue

Immunohistochemistry of adipose tissue was used in our laboratory to detect a macrophage marker, MAC-2/galectin-3. After de-paraffination in xylene, sections are gradually rehydrated in ethanol (briefly: Xylen 1x/15 min, Xylen 2x/15 min, Absolute ethanol 4x/ 10 min, 75 % ethanol 1x/10 min, 50 % ethanol 1x/10 min, Distilled water 1x/5 min). 1% hydrogen peroxide in methanol/30 min is used to block endogenous peroxidase activity. Slides are washed in phosphate buffered saline (PBS). To reduce non-specific background staining, normal serum (obtained from the same animal that produced the secondary antibody; in this case normal horse serum,Vector S-2000) is diluted 1:50 in PBS and used for the incubation of sections for 30 min at room temperature in a humid chamber. A primary antibody is diluted 1:3000 (for purified monoclonal Anti-MAC-2/galectin-3, Cedarlane CL8942AP) and slides are incubated overnight at 4 °C in a humid chamber. Samples are then washed in PBS (2x/15min), incubated with secondary antibody diluted in PBS 1:200 (Horse Anti-mouse IgG biotin, Vector BA-2000) and washed in PBS (2x/15min). Avidin biotinylated enzyme complex is established by "ABC" technique, which creates a biotin link between the horseradish peroxidase enzyme and the secondary antibody reagent (74) during incubation for 30 min at room temperature in a humid chamber. Samples are washed in PBS (2x/15min) and histoenzymatic visualisation of peroxidase is made by 3,3′-diaminobenzidine (DAB) tablets. Samples are then washed in tap water, weakly stained with hematoxylin, and slides with tissue sections are mounted in a mounting medium for microscopy (Eukitt or other). Six to eight digital images per tissue section are captured using Olympus AX70 light microscope and a DP 70 camera (Olympus, Tokyo, Japan). Density of crown-like structures (CLS), formed by MAC-2-positive macrophages surrounding dead adipocytes, could be then calculated as a number of CLS per particular area divided by number of adipocytes per the same particular area.

3 AIMS OF THE THESIS

The general goal of this thesis was to deepen the knowledge of adipose tissue biology and to understand the integrating role of adipose tissue in modulation of lipid metabolism and adiposity by leptin and thyroid hormones (see the specific aims 1 and 2, below). Reflecting the prominent role of adipose tissue secretory function in the etiopathogenesis of the insulin resistance, and in the regulation of whole-body inflammatory response by adipokines, a part of the work was focused on the improvement of strategies for the prevention and treatment of inflammation associated with obesity, while focusing on the beneficial effects of a specific composition of dietary lipids combined with and anti-diabetic drugs TZD (see the specific aims 3 and 4, below). All the experiments were performed in mice.

The specific aims of this thesis were:

1. to characterise metabolism of thyroid hormones in white adipose tissue, especially its modulation in response to physiological changes of fat mass, and stimulation of D1 activity by leptin;

2. to characterise plasma levels of leptin and thyroid hormones in response to a high-fat diet, using a model of obesity-resistant (A/J) and obesity-prone (B6) strains of mice;

3. to evaluate possible additive beneficial effects of the combination treatment by n–3 PUFA and rosiglitazone on adipose tissue inflammatory state and insulinemia in the prevention and reversal of diet-induced obesity; and

4. to investigate the effect of DHA-derivatives on adipose tissue biology and inflammation in the prevention and reversal of diet-induced obesity.

4 RESULTS TO SELECTED PUBLICATIONS

4.1 Publication A: Deiodinase 1 in white adipose tissue

Modulation of type I iodothyronine 5'-deiodinase activity in white adipose tissue by nutrition: possible involvement of leptin

Our objective was to describe possible changes in the activities of TH-metabolising enzymes in white adipose tissue, and the role of TH metabolism in adipose tissue during obesogenic treatment, caloric restriction and, in response to leptin treatment in mice.

First, an "**obesogenic treatment**" was performed to characterise effect of adipose tissue expansion on metabolism of TH in white adipose tissue.

Male mice born and maintained at 30 °C were weaned at four weeks of age to either the low-fat (LF) or high-fat (HF) diet. The LF diet contained 25 %, 9 %, and 66 % calories in the form of protein, fat, and carbohydrate, respectively. The HF diet, proven to be obesogenic in C57BL/6J mice, contained 15 %, 59 %, and 26 % calories in the form of protein, fat, and carbohydrate. All the analyses were performed two or eight weeks after the weaning.

After eight weeks of dietary treatment, only body weight gain but not body weight was significantly higher in the HF-diet group. However, already after two weeks, the weights of both epididymal (visceral) and dorsolumbar (subcutaneous) fat depots were significantly higher in the HF-diet group, as compared with the LF diet-fed mice. As revealed by histological analysis, the size of adipocytes in both white fat depots also increased in response to the HF diet. Plasma concentration of leptin increased significantly after two weeks of HF feeding, and after eight weeks, leptin levels were even more profoundly elevated. While there were no differences between the LF and HF groups in plasma levels of free T_4 and T_3 levels, the total levels of these hormones were significantly increased after two weeks of HF diet-feeding. However, after eight weeks, only total T_3 remained increased (Table 2).

Table 2 Growth characteristics, adiposity, plasma hormones levels and D1 activity during obesogenic treatment

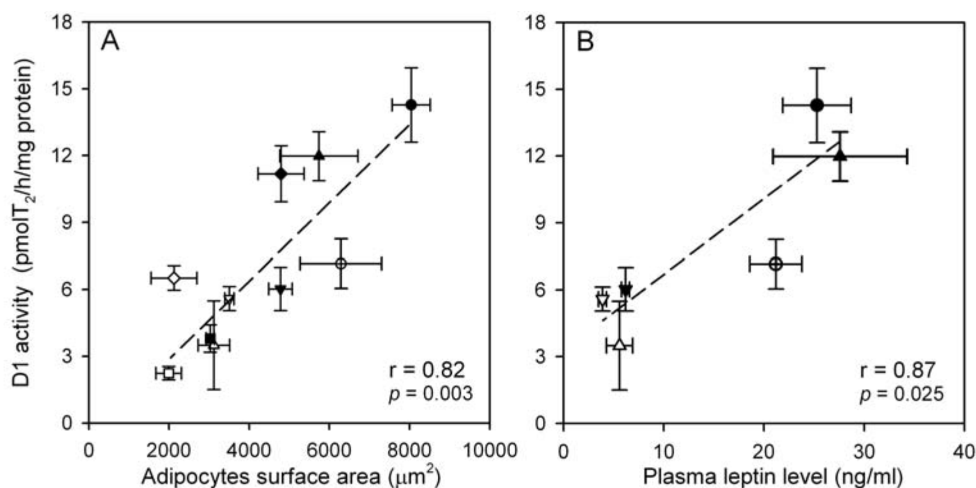
	2 weeks		8 weeks	
	LF	**HF**	**LF**	**HF**
Body weight (g)				
Initial	17.5 ± 0.7	17.3 ± 0.5	17.8 ± 0.8	18.0 ± 0.9
Final	20.8 ± 0.3	20.7 ± 0.4	26.8 ± 1.0	31.2 ± 2.3
Gain	3.3 ± 0.4	3.4 ± 0.6	9.0 ± 0.7	13.2 ± 1.5*
Weight of fat depots (mg)				
EPI	242 ± 13	366 ± 22**	447 ± 63	1311 ± 320**
DL	143 ± 4	200 ± 7**	180 ± 13	442 ± 80**
BAT	105 ± 5	71 ± 3**	154 ± 8	137 ± 15
Adipocytes surface area (μm^2)				
EPI	3505 ± 115	4779 ± 292*	3120 ± 392	5739 ± 973*
DL	1993 ± 317	3029 ± 105*	2126 ± 572	4794 ± 575*
Plasma levels				
Leptin (ng/ml)	3.9 ± 0.4	6.2 ± 0.4**	5.6 ± 1.3	27.6 ± 6.7**
total T_4 (nmol/l)	36.9 ± 0.8	41.6 ± 1.1**	62.8 ± 4.0	62.9 ± 1.4
total T_3 (nmol/l)	0.96 ± 0.03	1.30 ± 0.03**	0.71 ± 0.05	1.07 ± 0.05**
free T_4 (pmol/l)	13.8 ± 0.6	13.2 ± 0.9	12.2 ± 0.8	11.3 ± 0.6
free T_3 (pmol/l)	5.23 ± 0.54	4.90 ± 0.40	4.40 ± 0.55	4.21 ± 0.58
D1 activity (pmol T_2/h/mg protein)				
EPI	5.6 ± 0.5	6.0 ± 1.0	3.5 ± 1.9	12.0 ± 1.1*
DL	2.2 ± 0.3	3.8 ± 0.6*	6.5± 0.5	11.2 ± 1.3*
BAT	2.0 ± 0.3	2.1 ± 0.3	0.9 ± 0.2	0.8 ± 0.3
Liver	1288 ± 87	2178 ± 153**	877 ± 144	2364 ± 444*

Mice weaned at four weeks after birth onto low-fat (LF) or a high-fat (HF) diet were analysed after two (n = 18) and eight weeks (n = 7-9), following the obesogenic treatment protocol. Mice were born and maintained at 30°C. *EPI* - epididymal white fat, *DL* - dorsolumbar white fat, *BAT* - interscapular brown fat. The morphometry data are based on >2400 cells taken randomly from six different areas per animal (n = 3-4). Activity of D1 (pmol T_2/h/mg protein) was evaluated after two (n = 14-17) and eight (n = 3-8) weeks. Data are means ± SE. * p<0.05; **p<0.005 for the effect of diet.

Specific activity of D1 increased in both white fat depots in response to the HF diet, the strongest effect (~3.4-fold induction) being on epididymal fat after eight weeks. On the other hand, the dietary treatment had no effect on specific D1 activity in interscapular brown fat (Table 2). In the liver, specific D1 activity was several orders of magnitude higher than in adipose tissue and it was stimulated by the HF diet (Table 2).

Specific activity of D1 in white adipose tissue increased together with the size of adipocytes (Figure 6 A), as well as with the plasma leptin level (Figure 6B), suggesting correlative relations. Compared with D1, specific activities of both D2 and D3 in white fat depots were ~50- (epididymal fat) to ~100-fold (dorsolumbar fat) lower and did not change in response to the HF diet (not shown).

Figure 6 Correlation of D1 activity and size of adipocytes and plasma leptin levels



A. Correlation of the white adipose tissue D1 activity (two weeks, n = 14-17; eight weeks n = 3-8) and surface area of adipocytes (n = 3-4). Epididymal fat at two (triangle down) and eight weeks (triangle up), and dorsolumbar fat at two (square) and eight weeks (diamond) from mice fed HF (black) or LF diet (white), following the obesogenic treatment protocol; epididymal fat from the HF-AL-mice (black circle) and HF-CR-mice (crossed circle), following the caloric restriction treatment protocol. **B.** Correlation of the white adipose tissue D1 activity (two weeks, n = 14-17; eight weeks n = 3-8) and plasma leptin levels (n = 7-8). Epididymal fat at two (triangle down) and eight weeks (triangle up) from mice fed HF (black) or LF diet (white), following the obesogenic treatment protocol; epididymal fat from the HF-AL-mice (black

circle) and HF-CR-mice (crossed circle), following the caloric restriction treatment protocol. Data are means ± SE; for original data, see Table2 and Table 3.

Second, **caloric restriction** was applied in adult mice fed the HF diet to find out whether the HF diet-induced elevation of D1 activity in white adipose tissue could be reversed when fat accumulation is compromised without changing the diet composition.

Male mice born and maintained at 22 °C were fed the LF diet after weaning and then fed the HF diet for seven weeks, beginning at the age of three month. During the last five weeks of the HF-feeding, one group of mice was fed ad libitum (HF-AL), while the other group of mice was subjected to 10% caloric restriction (HF-CR) compared with the HF-AL mice.

Compared with ad libitum fed HF-AL mice, a five-week-caloric restriction in HF-CR mice resulted in a reduction in body weight gain, a decrease in the weight of adipose tissue depots, and the reversal of adipocytes hypertrophy in epididymal fat pad (Table 3). In association with the changes in white fat content, plasma leptin levels and leptin gene expression in epididymal fat were significantly reduced by caloric restriction. The HF-CR mice had relatively low plasma levels of free T_3 (Table 3).

Compared with the HF-AL mice, the HF-CR mice exhibited ~2.0-fold lower specific activity of D1 in epididymal adipose tissue. Expression of the SCD-1 gene, a marker of the leptin metabolic effect, in epididymal adipose tissue increased in response to caloric restriction (Table 3). In contrast to adipose tissue, specific D1 activity in the liver was not significantly affected by caloric restriction (Table 3).

Table 3 Growth characteristics, adiposity, plasma hormones levels and D1 activity during caloric restriction

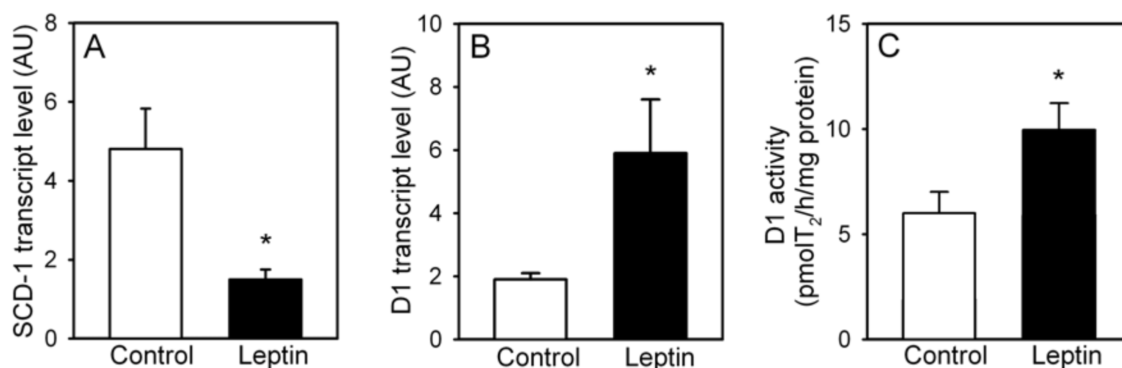
	HF-AL	HF-CR
Body weight (g)		
Initial	28.3 ± 0.6	28.2 ± 0.6
Final	35.9 ± 0.9	30.8 ± 0.5**
Gain	7.6 ± 0.4	2.6 ± 0.6**
Weight of fat depots (mg)		
EPI	1913 ± 136	1402 ± 72**
DL	679 ± 45	475 ± 23**
BAT	192 ± 10	139 ± 5**
Adipocytes surface area (μm^2)		
EPI	8038 ± 475	6289 ± 1015
Plasma levels		
Leptin (ng/ml)	25.3 ± 3.4	21.2 ± 2.6*
total T_4 (nmol/l)	45.0 ± 4.7	54.6 ± 6.5
total T_3 (nmol/l)	1.75 ± 0.08	1.75 ± 0.07
free T_4 (pmol/l)	11.4 ± 0.7	11.6 ± 0.7
free T_3 (pmol/l)	3.46 ± 0.18	2.65 ± 0.09**
D1 activity (pmol T_2/h/mg protein)		
EPI	14.3 ± 1.6	7.2 ± 1.1**
Liver	2566 ± 281	1946 ± 202
Transcript levels of the genes in EPI (AU)		
Leptin	6.1 ± 0.4	2.9 ± 0.4**
SCD-1	0.39 ± 0.06	0.75 ± 0.03**

Mice were born and maintained at 22 ^oC and weaned onto LF diet at four weeks of age. From the age of three months, all mice were fed HF diet for seven weeks, following the caloric restriction protocol. During the last five weeks of feeding on the HF diet, some mice were fed ad libitum (HF-AL) while some animals were calorie restricted by 10% (HF-CR). *EPI* - epididymal white fat, *DL* - dorsolumbar white fat, *BAT* -

interscapular brown fat. Data are means ± SE (n = 11-12). * p<0.05; **p<0.005 for the effect of diet. The morphometry data are based on >2400 cells taken randomly from six different areas per animal (n = 4).

Third, **leptin treatment** was used to reveal whether the D1 activity in white fat could be increased by leptin secreted from hypertrophic adipocytes. To verify this hypothesis, mice weaned to the LF diet were subcutaneously injected with three doses (3 mg/kg) of recombinant mouse leptin or saline. D1 gene transcript levels and D1 activity were evaluated in epididymal fat 16 hours after the last leptin injection. As expected, the expression of the SCD-1 gene was substantially, suppressed by leptin.

Figure 7 Effect of leptin on gene expression (A, B) **and D1 activity** (C) **in epididymal fat.**



At two weeks after weaning to LF diet, mice were injected with three doses (3mg/kg) of leptin for three days and epididymal adipose tissue was dissected 16 hours after the last injection. Data are means ± SE (n = 4-5). *p<0.05 for the effect of leptin vs. saline-injected mice.

Our results demonstrate for the first time changes in D1 activity in white adipose tissue under conditions of changing adiposity, and a stimulatory effect of leptin on D1 activity in white adipose tissue. We suggest that D1 has a functional role in white adipose tissue with D1 possibly being involved in the control of adipose tissue metabolism and/or accumulation of the tissue.

My contributions to this work were management and coordination of the in vivo and ex vivo experiments, light microscopy, morphometric analysis as well as biochemical characterisation of the models.

4.2 Publication B: Leptin and muscle thermogenesis

Induction of muscle thermogenesis by high-fat diet in mice: association with obesity-resistance

The aim of this study was to reveal whether muscle non-shivering thermogenesis could be stimulated by a HF diet, especially in obesity resistant A/J mice compared with obesity-prone C57BL/6J (B/6J) mice.

Experiments were performed on male mice born and maintained at 30 °C. Four-week-old mice were randomly weaned onto LF or HF diet for 2 wk. At weaning, as well as at the time of sacrifice, mice of both strains had similar body weights, independent of the type of diet. Accordingly, body weight gains during the 2-week post-weaning period and caloric intake were similar in all animal subgroups (Table 4). Weight of white fat depots (subcutaneous and epididymal) was increased by HF diet in both strains. As expected, HF diet strongly increased leptin levels in A/J mice, while no significant induction of leptin was detected in B/6J mice (Table 4). In contrast, plasma levels of both T_4 and T_3 were not affected by HF diet in A/J mice, but they were increased in B/6J mice, indicating the stimulatory effect of HF diet on thyroid function in B/6J but not in A/J mice (Table 4).

In the A/J LF mice, cold exposure (4°C) resulted in hypothermia, whereas the A/J HF, B/6J LF, and B/6J HF mice were cold tolerant. Cold sensitivity of the A/J LF mice was associated with a relatively low whole body energy expenditure under resting conditions, which was normalized by the HF diet. In both strains, the HF diet induced UCP-1-mediated thermogenesis, with a stronger induction in A/J mice. Only in A/J mice: 1) the HF diet augmented activation of whole body lipid oxidation by cold; and 2) at 30°C, oxygen consumption, total content, and phosphorylation of AMPK, and AICAR [aminoimidazole carboxamide ribonucleotide (AICAR), a pharmacological activator of AMPK] -stimulated palmitate oxidation in soleus muscle was increased by the HF diet in parallel with significantly increased leptinemia (for data see publication B). Gene

expression data in soleus muscle of the A/J HF mice indicated a shift from carbohydrate to fatty acid oxidation (for data see publication B).

Table 4 Growth characteristics and plasma hormone levels

	B/6J		A/J	
	LF	HF	LF	HF
BW (g)	18.6 ± 0.3	18.4 ± 0.5	17.0 ± 0.7	18.4 ± 0.7
BWG (g)	5.63 ± 0.39	4.91 ± 0.20	4.71 ± 0.50	5.31 ± 0.28
FC (kcal/day)	7.74 ± 0.8	7.78 ± 0.4	8.06 ± 0.7	8.11 ± 0.4
Weight of fat depots (mg)				
BAT	90 ± 3	57 ± 3*	69 ± 3†	61 ± 3*
DL	152 ± 4	180 ± 10*	167 ± 9	252 ± 14*†
EPI	160 ± 8	239 ± 21*	163 ± 14	292 ± 26*
Plasma levels				
Leptin (ng/ml)	4.48 ± 0.38	5.21 ± 0.54	3.35 ± 0.34†	9.42 ± 1.15*†
T4 (nmol/L)	37 ± 2	46 ± 2*	45 ± 1†	45 ± 3
T3 (nmol/L)	1.5 ± 0.2	1.9 ± 0.2*	1.9 ± 0.2†	1.9 ± 0.2

Six-week-old mice reared at 30 ^{o}C and weaned at 4 weeks after birth onto LF or HF diets were analyzed. Mean body weight at the time of weaning was similar in all subgroups of mice (12.3 – 13.5 g). BW, body weight at 6 weeks of age; BWG, gain of body weight during a period of 2 weeks after weaning; FC, mean food consumption measured at day 2, 4, 9, and 13 after weaning; BAT, interscapular brown fat; DL, dorsolumbar white fat; EPI, epididymal white fat. Data are means ± S.E. (n = 11-14). *Significant effect of diet; †significant effect of genotype.

Our results suggest that muscle non-shivering thermogenesis and lipid oxidation can be stimulated by HF diet in the obesity-resistant phenotype of A/J mice. Furthermore they indicate that a HF diet could induce non-shivering thermogenesis in oxidative muscle, possibly via the leptin-AMPK axis. Thus, white adipose tissue, by secreting leptin, played an integrating role in the adaptive induction of thermogenesis in response to increased consumption of dietary fat, and also in protection against development of obesity.

My main contribution to this work was the determination of plasma hormones levels and measurements of oxygen consumption in skeletal muscles.

4.3 Publication C: n–3 fatty acids and rosiglitazone in combination

n–3 fatty acids and rosiglitazone improve insulin sensitivity through additive stimulatory effects on muscle glycogen synthesis in mice fed high-fat diet

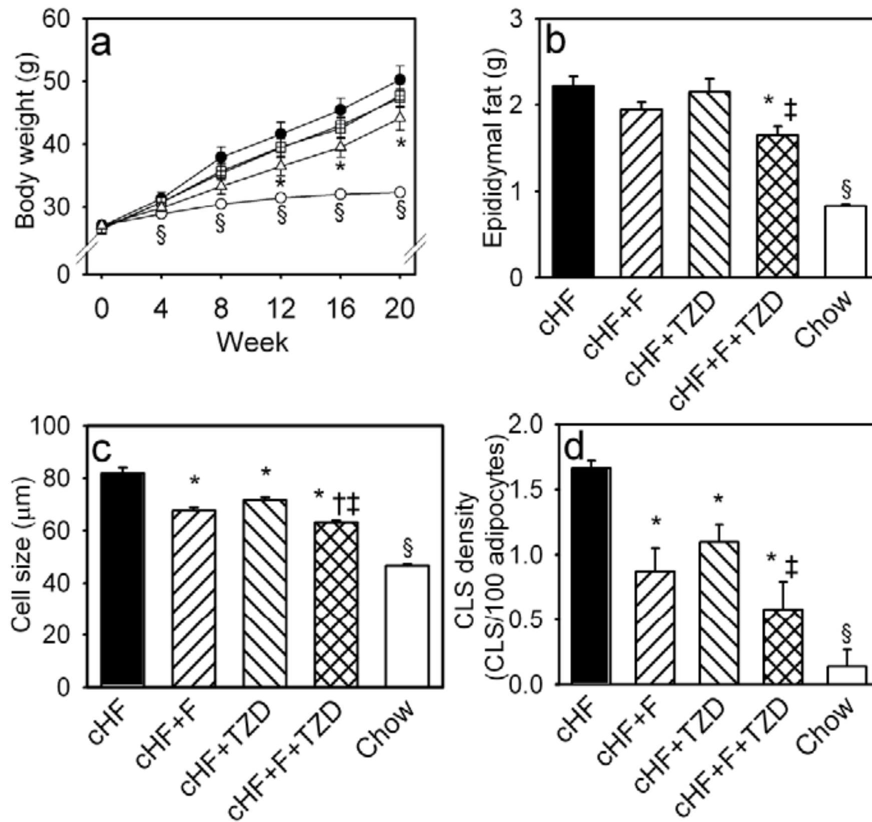
Based on preliminary results we hypothesized that the partially overlapping mechanisms of action of n–3 PUFA and TZD could have additive or synergistic effects in a combination treatment leading to an improvement of lipid and glucose homeostasis in obesity.

In initial analysis, a "**prevention study**" was performed to characterise the effects of n–3 PUFA, rosiglitazone and their combination, on developing obesity and impaired glucose tolerance (IGT) in mice fed high-fat diet. After weaning, male C57BL/6N mice were fed standard chow diet. At 3 months of age they were randomly assigned to a corn oil-based high-fat diet (cHF; lipid content ~35.2% wt/wt, mainly corn oil) or to the following treatments: (i) cHF diet supplemented with EPA and DHA (cHF+F) as concentrate of n–3 PUFA (46% DHA, 14% EPA; 1050TG; EPAX, Lysaker, Norway) replacing 15% of dietary lipids; (ii) cHF diet supplemented with rosiglitazone (cHF+TZD) (10 mg/kg diet); and (iii) cHF diet supplemented with EPA, DHA and rosiglitazone (cHF+F+TZD). One group of mice was maintained on the standard chow diet. Various analyses were performed at different time points between 5 to 20 weeks after initiation of treatments.

cHF diet increased body weight with significant differences between combination treatment, cHF+F+TZD, and cHF becoming apparent at 12 weeks (Figure 8 a). The size of adipocytes was modulated in accord to reduced weight of epididymal fat tissue after combination treatment (Figure 8 b-c). Histological analysis combined with immunodetection of macrophages revealed cHF diet-induced hypertrophy of adipocytes in epididymal fat (Figure 8 c), accompanied by increased content of macrophages aggregated in crown-like structures surrounding individual adipocytes (Figure 8 d), indicating induction of low-grade inflammation of adipose tissue in cHF-mice. The

induction of adipocyte hypertrophy and macrophage infiltration was significantly counteracted by cHF+F and cHF+TZD diets, with cHF+F+TZD diet exerting the strongest effect.

Figure 8 Characterisation of the model – "*prevention study*"



Body weight (**a**), weight of epididymal fat (**b**), size of adipocytes (**c**), and macrophage infiltration of adipose tissue (**d**). Three-month-old mice were placed on cHF diet or various cHF-based diets (cHF+F, cHF+TZD and cHF+F+TZD), or maintained on a chow diet; this treatment lasted for up to 20 weeks. **a** Body weights during 20-week treatment by cHF (black circles), cHF+F (white squares), cHF+TZD (crossed squares), cHF+F+TZD (white triangles) or chow (white circles) diet (*n*=16). **b** Epididymal fat at 8 weeks, weight of fat depot. **c** Size of adipocytes. **d** Relative count of crown-like structures (CLS), formed by MAC-2-positive macrophages surrounding adipocytes. The morphometry data are based on measurements of more than 1,000 cells taken randomly from six different areas per animal (*n*=3-4); (**a–b**) (*n*=7–8). Data are means±SE $*p \leq 0.05$ for difference from cHF; $^{\dagger}p \leq 0.05$ for difference from cHF+F; $^{\ddagger}p \leq 0.05$ for difference from cHF+TZD (ANOVA); $^{\S}p \leq 0.05$ for difference from cHF (*t* test).

Insulinemia increased between 8 and 20 weeks of high-fat feeding, suggesting development of insulin resistance (Table 5). The rise in insulin levels was prevented to a similar extent by cHF+TZD and cHF+F+TZD, while cHF+F exhibited also a significant but smaller effect. Both at 8 and 20 weeks, cHF+F and cHF+TZD increased total immunoreactive adiponectin in plasma. Even a stronger induction was observed by a combination of the two treatments (Table 5). Multimeric adiponectin complexes in plasma were also analyzed. The ratio between high molecular weight (HMW) and total adiponectin was increased by all the treatments with the highest additive effect observed in case of cHF+F+TZD (Table 5). Induction of HMW adiponectin, reduction of fat cell hypertrophy and decrease of adipose tissue inflammation provided the evidence of amelioration of high-fat diet-induced inflammation and might contribute to the beneficial effects on glucose homeostasis.

Table 5 Prevention of hyperinsulinemia and induction of adiponectin

	cHF	cHF+F	cHF+TZD	cHF+F+TZD
8 weeks				
Insulin (ng/ml)	2.2 ± 0.4	1.4 ± 0.2	1.8 ± 0.2	1.1 ± 0.3[a]
Adiponectin (µg/ml)	8.6 ± 0.4	11.0 ± 0.5	10.3 ± 0.2	12.3 ± 0.7[a]
HMW/total	0.27 ± 0.02	0.30 ± 0.03	0.35 ± 0.02[a]	0.41 ± 0.01[abc]
20 weeks				
Insulin (ng/ml)	7.5 ± 1.1	4.2 ± 0.8[a]	2.4 ± 0.3[ab]	1.8 ± 0.2[ab]
Adiponectin (µg/ml)	5.3 ± 0.3	8.2 ± 0.6[a]	8.6 ± 0.4[a]	10.1 ± 0.4[abc]
HMW/total	0.33 ± 0.03	0.46 ± 0.01[a]	0.49 ± 0.02[a]	0.55 ± 0.02[ab]

Three-month-old mice were placed on various diets and insulin and adiponectin were measured in plasma of mice killed in ad libitum fed state after 8 or 20 weeks following the initiation of treatments. HMW/total; ratio between concentration of high molecular weight and total adiponectin. Data are means ± SE (n=8). [a]Significantly different from cHF; [b]significantly different from cHF+F; [c]significantly different from cHF+TZD (ANOVA).

Other effects of treatment on metabolic markers as well as liver and muscle gene expression and metabolism were analysed. Euglycaemic–hyperinsulinaemic clamps

were used to characterize the changes in insulin sensitivity (for data see publication C). Dyslipidaemia and insulin resistance were improved after treatment, while suppressing hepatic lipogenesis and decreasing muscle ceramide concentration. The improvement in glucose tolerance reflected a synergistic stimulatory effect of the combined treatment on muscle glycogen synthesis and its sensitivity to insulin.

Second, in a "**reversal study**", obesity and IGT were induced by feeding male C57BL/6J mice the cHF diet between 3 and 7 months of age. Then, they were randomly assigned to eight-week-long treatment as above; i.e. with cHF, cHF+F, cHF+TZD and cHF+F+TZD, respectively. Compared with cHF, body weight gain was suppressed by cHF+F, while cHF+TZD significantly decreased plasma insulin levels. Only cHF+F+TZD affected all the parameters studied, showing additive effects of DHA/EPA and rosiglitazone in the reversal of obesity. The combination treatment also reversed dyslipidaemia and IGT (for data see publication C).

Table 6 Reversal of obesity, dislipidemia, and impaired glucose homeostasis

	cHF	cHF+F	cHF+TZD	cHF+F+TZD
Body weight				
Initial (g)	43.2 ± 2.4	43.3 ± 2.2	45.0 ± 2.0	44.9 ±1.7
Final (g)	47.3 ± 2.4	43.0 ± 2.2	47.5 ± 1.7	41.8 ± 1.3
Gain (g)	4.1 ± 1.0	-0.3 ± 1.0[a]	2.5 ± 1.1	-3.1 ± 1.8[abc]
Plasma insulin				
Insulin (ng/ml)	3.40 ± 0.36	2.37 ± 0.53	1.90 ± 0.49[a]	1.43 ± 0.22[a]

To induce obesity and IGT, mice were fed cHF diet between 3 and 7 months of age, since then the mice were treated by different diets for 8 more weeks. Body weight was recorded just before and at the end of the 8-week-treatment. After the treatment, plasma insulin was measured in *ad libitum* fed mice. Data are means ± SE (n=8). [a]Significantly different from cHF; [b]significantly different from cHF+F; [c]significantly different from cHF+TZD (ANOVA).

In conclusion, the combined treatment using n–3 PUFA and a low-dose rosiglitazone generated additive effects in the prevention as well as reversal of adipose

tissue hypertrophy induced by dietary fat. n–3 PUFA and rosiglitazone can be therefore used as complementary therapies to counteract dyslipidaemia and insulin resistance. The combination treatment may reduce dose requirements and hence the incidence of adverse side effects of thiazolidinedione therapy. The beneficial effects of the combination treatment were in large based on the amelioration of adipose tissue inflammation associated with obesity and modulation of the pattern of secretion of adiopokines.

My contributions to this work were light microscopy, morphometric analysis and MAC-2 immunohistochemical analysis of adipose tissue as well as determination of plasma insulin levels.

4.4 Publication D: Docosahexaenoic acid derivatives

DHA-derivatives in the prevention and reversal of obesity and glucose intolerance in mice

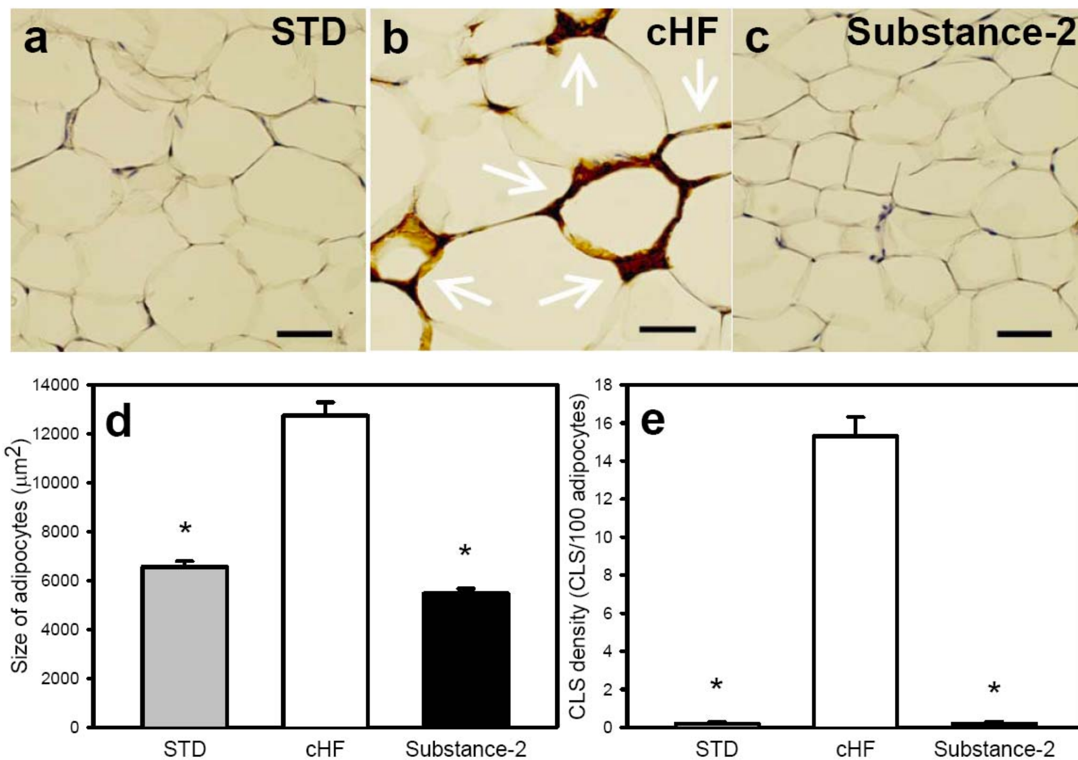
Our objective was to determine the efficacy of α-substituted DHA derivatives as lipid-lowering, antiobesity, and antidiabetic agents.

C57BL/6 mice were given a corn oil–based high-fat (35% weight/weight) diet (cHF), or cHF with 1.5% of lipids replaced with α-methyl DHA ethyl ester (Substance 1), α-ethyl DHA ethyl ester (Substance 2), α,α-di-methyl DHA ethyl ester (Substance 3), or α-thioethyl DHA ethyl ester (Substance 4) for four months. Compared to the cHF-fed mice, all DHA derivatives except Substance 3 reduced weight gain and Substance 2 exerted the strongest effects. Mice fed Substance 2 had a reduced food intake and reduced the weight of subcutaneous and epididymal white adipose tissue by 73 and 42 %, respectively, while the remaining DHA derivatives had less effect on adiposity.

In the "prevention study," histological analysis of epididymal white adipose tissue (Figure 9) revealed adipocyte hypertrophy in the cHF-fed mice, resulting in an approximately twofold increase in the mean cell size. This effect was completely prevented by Substance 2 (Figure 9d). Moreover, Substance 2 also completely prevented obesity-associated macrophage infiltration of white adipose tissue, as revealed by immunohistochemical detection of Mac-2 (Figure 9b; white arrows). Macrophages aggregate in crown-like structures surrounding individual adipocytes. While the density of crown-like structures was ~77-fold higher in cHF-fed compared with STD-fed mice, Substance 2 completely prevented this effect (Figure 9c,e). Moreover, in epididymal white adipose tissue Substance 2 reduced mRNA levels of CD68 and MCP-1, two factors closely linked to macrophage function, by 91 and 56 %, respectively. In the "reversal study," Substance 2 reduced the accumulation of macrophages in epididymal white adipose tissue by 65 % and expression of CD68 and MCP-1 by 32 and 50 %, respectively. Thus, Substance 2 completely prevents and even partially reverses

adipocyte hypertrophy and macrophage infiltration of white adipose tissue, induced by the obesogenic cHF diet. Given the strong effect of Substance 2 on adiposity and obesity-associated inflammation of white adipose tissue, two major adipokines, leptin and adiponectin, were evaluated after 2 months of treatment in the "prevention study." Compared with cHF-fed mice, plasma leptin levels were strongly reduced by Substance 2 (4.4 ± 0.3 vs. 86.0 ± 6.2 ng/ml; P < 0.00001) and reached the levels observed in the STD-fed mice (6.9 ± 0.9 ng/ml). Plasma adiponectin levels were also slightly reduced (Substance 2, 7.1 ± 0.6 vs. cHF, 9.3 ± 0.5; P < 0.05) and were similar to those observed in STD-fed mice (6.9 ± 0.4 ng/ml).

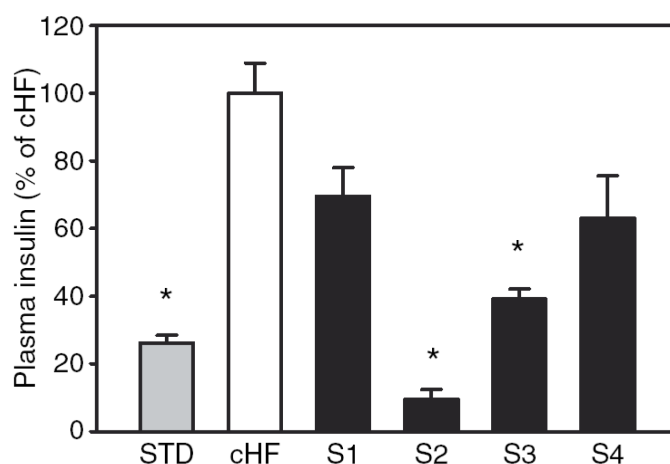
Figure 9 The effect of Substance-2 on adipose tissue morphology and macrophage infiltration in the „*prevention study*"



The amount of MAC-2 immunoreactive macrophages (brownish color) was analyzed in epididymal fat. Sections were counterstained with hematoxylin-eosin. (**a**) Mice fed a low-fat chow (STD) diet. (**b**) Composite high-fat (cHF) diet. (**c**) Substance-2**.** Arrows indicate crown-like structures (CLS) surrounding individual adipocytes, where the majority of macrophages are localized. Scale bars = 50 μm. (**d**) Size of adipocytes. (**e**) CLS density. The morphometry data are based on more than 1,000 cells taken randomly from 5 different areas per animal (*n* = 3). **P* < 0.05 vs. cHF diet (ANOVA).

Plasma markers of glucose and lipid metabolism, glucose tolerance, tissue lipid content, and gene regulation were also characterized. The cHF induced obesity, hyperlipidemia and hyperinsulinemia, while Substance 2 and Substance 3 exerted protective effects (Figure 10). Substance 2 also lowered plasma insulin in dietary obese mice in the "reversal study". Compared with cHF-fed mice, plasma insulin levels were reduced by Substance 2 (1.40 ± 0.33 vs. 4.34 ± 0.33 ng/ml; $P < 0.001$) similar to the levels observed in the STD-fed mice (1.12 ± 0.54ng/ml).

Figure 10 The effect of DHA-derivatives on plasma insulin in the „*prevention study*".



Mice were fed either a low-fat chow (STD), composite high-fat (cHF) diet, or cHF diet, in which 1.5% of lipids was replaced by various DHA-derivatives (S1, Substance-1; S2, Substance-2; S3, Substance-3; S4, Substance-4). Plasma insulin in *ad libitum* fed mice at the time of killing. Data are expressed as percentages of the control cHF diet and represent means ± s.e. (STD, n = 11; cHF, n = 7-13; Substances, n = 8). *P < 0.05 vs. cHF diet (ANOVA).

In summary, among the four DHA derivatives tested, Substance 2 (α-ethyl DHA ethyl ester) appeared to exhibit a similar range of beneficial effects on obesity and associated metabolic traits as naturally occurring n-3 PUFA, but with a higher efficacy. These effects reflect extremely efficient reduction of the obesity-associated inflammation of adipose tissue, suggesting important beneficial effect on the secretion of pro- and anti-inflammatory adipokines from the adipose tissue. Therefore, α-ethyl DHA

ethyl ester could qualify as a novel drug for the treatment of obesity, obesity associated low-grade inflammation, dyslipidemia, and insulin resistance.

My contributions to this work were light microscopy of adipose tissue and liver, morphometric analysis and MAC-2 immunohistochemical analysis of adipose tissue as well as determination of plasma insulin, leptin and adiponectin levels.

5 DISCUSSION

Obesity represents a predominant risk factor for the development of metabolic diseases like type 2 diabetes mellitus and it is characterized by adipocyte hypertrophy (75). The size of adipocytes influences the adipocytes biology and secretory functions of adipose tissue in general (17). Understanding the mechanisms regulating growth and secretory activity of adipose tissue is of paramount importance. Moreover, obesity represents a chronic subclinical inflammatory state linking obesity to insulin resistance (45;76) and hypertrophic adipocytes contribute to this phenomenon (77). An improvement of strategies used in the prevention and treatment of inflammation associated with obesity is therefore urgently needed.

The four studies described in this thesis address several topics related to adipose tissue biology, and thus contributing to the understanding of the integrating role of adipose tissue secretory functions in response to dietary and pharmacological treatments. The first study describes a possible role of adipose tissue TH metabolism in the modulation of its function under conditions of changing adiposity (Publication A). In experiments on mice, we have demonstrated for the first time that (i) the changes in the metabolism of TH in white adipose tissue were associated with physiological changes of fat mass and the size of adipocytes; (ii) only D1, but not D2 or D3, was involved in this process; and (iii) D1 activity in adipose tissue was stimulated by the administration of exogenous leptin. It is well established that leptin release from adipose tissue is positively associated with the size of adipocytes (78). Specific activity of D1 in white fat depots correlated with the size of adipocytes and leptin levels both during HF diet-induced fat expansion and during adipose tissue involution induced by a mild caloric restriction of the HF diet-fed mice. Moreover, the administration of exogenous leptin stimulated D1 activity in white fat. This observation was in accord with the leptin action in other tissues, such as the liver, thyroid gland, and pituitary (79-81). One possible explanation is that D1-generated T_3 could be involved in the induction of angiogenesis in white adipose tissue through the T_3-dependent activation of hypoxia-inducible factor-

1 controlling transcription of the angiogenic genes (82). However, a relatively strong response of adipose tissue D1 activity to leptin, which was associated with the down-regulation of SCD-1, suggests that T_3 formed by D1 may be also involved in the modulation of metabolism in white adipose tissue by leptin.

A unique role of leptin, secreted from adipose tissue, in the complex control of energy homeostasis of the organism is a subject of the second study (Publication B). We have demonstrated a profound difference between A/J and B/6J mice in their response to HF diet, which was previously explained by the strain-specific differences in the sensitivity to leptin with only A/J mice retaining leptin sensitivity of adipose tissue after several weeks of HF feeding (83). We also confirmed the previous results (84), indicating that the differential response to HF diet could be attributed to a relatively high induction of UCP1-mediated thermogenesis in brown fat in A/J mice. In addition, our study demonstrated a HF diet-induced increase of oxygen consumption in soleus muscle, specific for A/J mice, indicating the activation of non-shivering thermogenesis in this type of muscle. These results strongly suggest the involvement of the leptin-AMPK axis (85) in the induction of non-shivering thermogenesis in oxidative muscle by HF diet. Taken together white adipose tissue, by secreting leptin, plays an integrating role in the adaptive induction of thermogenesis in response to increased fat content in the diet and thereby contribute to cold resistance and obesity resistance in mice.

Based on the prominent role of adipose tissue secretory function in the development of insulin resistance associated with chronic subclinical inflammation in obesity, part of the work was also focused on the improvement of strategies for the prevention and treatment of adipose tissue inflammation. The observations that n-3 PUFA can prevent macrophage infiltration of adipose tissue (50) and reduce adiposity (86) are in accord with other beneficial effects of n-3 PUFA (87-89). TZD represent preferred therapeutic agents for insulin resistance in type 2 diabetic patients due to their ability to improve chronic inflammation associated with obesity (90). In experiments on mice, we have demonstrated that long-term treatment combining partial replacement of dietary lipids by the DHA/EPA concentrate and a low dose of rosiglitazone markedly and in an additive manner prevented development of dyslipidaemia and insulin resistance, reduced accumulation of body fat and adipocyte hypertrophy, and also

improved inflammation of adipose tissue (Publicatin C). It has been observed previously that the treatment by DHA/EPA (63) or rosiglitazone (91) induced adiponectin. Our current study showed for the first time that the combination treatment increased plasma adiponectin more potently than either treatment alone. The additive effects of DHA/EPA and rosiglitazone in the reduction of fat cell hypertrophy, induction of HMW adiponectin, and decrease of adipose tissue inflammation might contribute to the beneficial effects on glucose homeostasis. Moreover, the combination treatment may reduce dose requirements and therefore reduce the incidence of adverse side effects of TZD therapy (92;93)

The anti-inflammatory properties of EPA and DHA on adipose tissue are well described (50;94), however it has been shown recently that DHA appears to exhibit higher efficiency than EPA (95;96). Therefore we hypothesized, that DHA derivatives could exhibit a similar range of beneficial effects on obesity and associated metabolic traits as naturally occurring n-3 PUFA, but with a higher efficacy (Publication D). In fact, among the four chemical DHA derivatives tested in high-fat diet-fed mice, only the Substance 2 (α-ethyl DHA ethyl ester) completely prevented and partially reversed the development of obesity, fat accumulation, dyslipidemia and IGT. In agreement with the effect on body weight and adiposity, Substance 2 also profoundly affected white adipose tissue properties. Substance 2 reduced (i) tissue cellularity, (ii) the size of adipocytes, and (iii) macrophage infiltration of white adipose tissue. These effects reflect an extremely efficient reduction of the obesity-associated inflammation of adipose tissue, suggesting important beneficial effects on the secretion of pro- and anti-inflammatory adipokines from the tissue. Therefore, α-ethyl DHA ethyl ester could qualify as a novel drug for the treatment of obesity, obesity-associated low-grade inflammation, dyslipidemia, and insulin resistance.

Results of all presented studies support the concept of an integrating role of adipose tissue secretory functions in the whole-body responses to dietary constituents as well as to pharmacological agents, which ameliorate obesity and associated insulin resistance.

6 CONCLUSIONS

Concerning the specific aims of the thesis, the following conclusions may be formulated:

1. For the first time, changes in D1 activity in white adipose tissue were demonstrated under conditions of changing adiposity, and a stimulatory effect of leptin on D1 activity in white adipose tissue was also found. We suggest that D1 has a functional role in white adipose tissue with D1 possibly being involved in the control of adipose tissue metabolism and/or accumulation of the tissue.

2. Differences in HF diet induced changes in plasma levels of leptin and thyroid hormones between obesity-resistant A/J and obesity-prone B6 strains of mice were found. Our results suggest a role for muscle non-shivering thermogenesis and lipid oxidation in the obesity-resistant phenotype of A/J mice and indicate that the HF diet could induce thermogenesis in oxidative muscle, possibly via the leptin-AMPK axis. Thus, white adipose tissue, by secreting leptin, plays an integrating role in the adaptive induction of thermogenesis in response to increased consumption of dietary fat, and also in the protection against development of obesity.

3. The combined treatment using n–3 PUFA and a low-dose rosiglitazone generated additive effects in the prevention as well as reversal of adipose tissue hypertrophy induced by HF diet. n–3 PUFA and rosiglitazone can be therefore used as complementary therapies to counteract dyslipidaemia and insulin resistance. The combination treatment may reduce dose requirements and hence the incidence of adverse side effects of thiazolidinedione therapy. The beneficial effects of the combination treatment were mostly based on the amelioration of adipose tissue inflammation associated with obesity and on the modulation of the secretion pattern of adipokines.

4. Among the four chemical DHA derivatives tested in high-fat diet-fed mice, Substance 2 (α-ethyl DHA ethyl ester) appeared to exhibit a similar range of beneficial effects on obesity and associated metabolic traits as naturally occurring n-3 PUFA, but with a much higher efficacy. These effects reflect extremely efficient reduction of the obesity-associated inflammation of adipose tissue, suggesting an important beneficial effect on the secretion of pro- and anti-inflammatory adipokines from the tissue. Therefore, α-ethyl DHA ethyl ester could qualify as a novel drug for the treatment of obesity, obesity-associated low-grade inflammation, dyslipidemia, and insulin resistance.

In conclusion, results of all four studies support the concept of the integrating role of adipose tissue in the whole-body responses to dietary constituents as well as pharmacological agents which ameliorate obesity and associated insulin resistance. Our results suggest that the integrating role strongly depends on a changing profile of adipokines secreted from the tissue.

7 LIST OF PUBLICATIONS

Published articles

- **Macek Jilkova Z**, Pavelka S, Flachs P, Hensler M, Kus V, Kopecky J. Modulation of type I iodothyronine 5'-deiodinase activity in white adipose tissue by nutrition: possible involvement of leptin. *Physiol. Research, in press,* (IF = 1.739)

- Kopecky J, Rossmeisl M, Flachs P, Kuda O, Brauner P, **Jilkova Z**, Stankova B, Tvrzicka E, Bryhn M. n-3 PUFA: bioavailability and modulation of adipose tissue function. *Proc Nutr Soc,* 2009, *in press,* (IF = 4.304)

- Kuda O, Jelenik T, **Jilkova Z**, Flachs Z, Rossmeisl M, Hensler M, Kazdova L, Ogston N, Baranowski M, Gorski J, Janovska P, Polak J, Mohamed-Ali V, Burcelin R, Cinti S, Bryhn M, Kopecky J. n–3 fatty acids and rosiglitazone improve insulin sensitivity through additive stimulatory effects on muscle glycogen synthesis in mice fed high-fat diet. *Diabetologia* 52: 941-951, 2009, (IF = 6.328)

- Rossmeisl M, Jelenik T, **Jilkova Z**, Slamova K, Kus V, Hensler M, Medrikova D, Povysil C, Flachs P, Mohamed-Ali V, Bryhn M, Berge K, Holmeide AK, Kopecky J. DHA-derivatives in the prevention and reversal of obesity and glucose intolerance in mice. *Obesity* 17: 1023–1031, 2009, (IF = 2.798)

- Kus V, Prazak T, Brauner P, Hensler M, Kuda O, Flachs P, Janovska P, Medrikova D, Rossmeisl M, **Jilkova Z**, Stefl B, Pastalkova E, Drahota Z, Houstek J, Kopecky J. Induction of muscle thermogenesis by high-fat diet in mice: association with obesity-resistance. *Am J Physiol Endocrinol Metab* 295: E356-E367, 2008, (IF = 4,129)

- Skladal P, **Jilkova Z**, Svoboda I, Kolar V. Investigation of osteoprotegerin interactions with ligands and antibodies using piezoelectric biosensors. *Biosens Bioelectron* 20: 2027-34, 2005, (IF = 5,330)

Manuscript in preparation

- Jelenik T, Rossmeisl M, Kuda O, **Macek Jilkova Z**, Medrikova D, Kus V, Hensler M, Janovska P, Miksik I, Baranowski M, Gorski , Jensen TE, Flachs P, Viollet B, Kopecky J. AMP-activated protein kinase α2 subunit is required for the preservation of hepatic insulin sensitivity by n-3 polyunsaturated fatty acids

8 REFERENCES

1. Lago,F, Dieguez,C, Gomez-Reino,J, Gualillo,O: Adipokines as emerging mediators of immune response and inflammation. *Nat.Clin.Pract.Rheumatol.* 3:716-724, 2007

2. Wijers,SL, Saris,WH, Marken Lichtenbelt,WD: Recent advances in adaptive thermogenesis: potential implications for the treatment of obesity. *Obes.Rev.* 10:218-226, 2009

3. Rothwell,NJ, Stock,MJ, Stribling,D: Diet-induced thermogenesis. *Pharmac.ther.* 17:251-268, 1982

4. Wijers,SL, Saris,WH, Marken Lichtenbelt,WD: Individual thermogenic responses to mild cold and overfeeding are closely related. *J.Clin.Endocrinol.Metab* 92:4299-4305, 2007

5. Rothwell,NJ, Stock,MJ: A role for brown adipose tissue in diet-induced thermogenesis. *Nature* 281:31-35, 1979

6. Silva,JE: Thermogenic mechanisms and their hormonal regulation. *Physiol Rev.* 86:435-464, 2006

7. Himms-Hagen J: Thyroid hormones and thermogenesis. In Mammalian Thermogenesis. Girardier L, Stock MJ, Eds. London, Chapman and Hall, 1983, p. 141-163

8. Carvalho,SD, Kimura,ET, Bianco,AC, Silva,JE: Central role of brown adipose tissue thyroxine 5'-deiodinase on thyroid hormone-dependent thermogenic response to cold. *Endocrinology* 128:2149-2159, 1991

9. Kopelman,PG: Obesity as a medical problem. *Nature* 404:635-643, 2000

10. Despres,JP, Lemieux,I: Abdominal obesity and metabolic syndrome. *Nature* 444:881-887, 2006

11. Cinti S: The adipose organ. Milano, Italy, Editrice Kurtis, 1999

12. Montague,CT, Prins,JB, Sanders,L, Zhang,J, Sewter,CP, Digby,J, Byrne,CD, O'Rahilly,S: Depot-related gene expression in human subcutaneous and omental adiipocytes. *Diabetes* 47:1384-1391, 1998

13. DiGirolamo,M, Fine,JB, Tagra,K, Rossmanith,R: Qualitative regional differences in adipose tissue growth and cellularity in male Wistar rats fed ad libitum. *Am.J.Physiol* 274:R1460-R1467, 1998

14. Spalding,KL, Arner,E, Westermark,PO, Bernard,S, Buchholz,BA, Bergmann,O, Blomqvist,L, Hoffstedt,J, Naslund,E, Britton,T, Concha,H, Hassan,M, Ryden,M, Frisen,J, Arner,P: Dynamics of fat cell turnover in humans. *Nature* 2008

15. Fonseca-Alaniz,MH, Takada,J, Alonso-Vale,MI, Lima,FB: Adipose tissue as an endocrine organ: from theory to practice. *J.Pediatr.(Rio J.)* 83:S192-S203, 2007

16. Zhang,Y, Proenca,R, Maffei,M, Barone,M, Leopold,L, Friedman,JM: Positional cloning of the mouse obese gene and its human homologue. *Nature* 372:425-432, 1994

17. Skurk,T, Alberti-Huber,C, Herder,C, Hauner,H: Relationship between adipocyte size and adipokine expression and secretion. *J.Clin.Endocrinol.Metab* 92:1023-1033, 2007

18. Staiger,H, Tschritter,O, Machann,J, Thamer,C, Fritsche,A, Maerker,E, Schick,F, Haring,HU, Stumvoll,M: Relationship of serum adiponectin and leptin concentrations with body fat distribution in humans. *Obesity Research* 11:368-372, 2003

19. Friedman,JM, Halaas,JL: Leptin and the regulation of body weight in mammals. *Nature* 395:763-770, 1998

20. Friedman,JM: Leptin at 14 y of age: an ongoing story. *Am.J.Clin.Nutr.* 89:973S-979S, 2009

21. Minokoshi,Y, Kim,YB, Peroni,OD, Fryer,LG, Muller,C, Carling,D, Kahn,BB: Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature* 415:339-343, 2002

22. Scarpace,PJ, Matheny,M, Pollock,BH, Tuemer,N: Leptin increases uncoupling protein expression and energy expenditure. *Am.J.Physiol.* 273:E226-E230, 1997

23. Scarpace,PJ, Zhang,Y: Elevated leptin: consequence or cause of obesity? *Front Biosci.* 12:3531-3544, 2007

24. Chen,H, Charlat,O, Tartaglia,LA, Woolf,EA, Weng,X, Ellis,SJ, Lakey,ND, Culpepper,J, Moore,KJ, Breitbart,RE, Duyk,GM, Tepper,RI, Morgenstern,JP: Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Cell* 84:491-495, 1996

25. Ke,Y, Qiu,J, Ogus,S, Shen,WJ, Kraemer,FB, Chehab,FF: Overexpression of leptin in transgenic mice leads to decreased basal lipolysis, PKA activity, and perilipin levels. *Biochem.Biophys.Res.Commun.* 312:1165-1170, 2003

26. Scherer,PE, Williams,S, Fogliano,M, Baldini,G, Lodish,HF: A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol.Chem.* 270:26746-26749, 1995

27. Pajvani,UB, Hawkins,M, Combs,TP, Rajala,MW, Doebber,T, Berger,JP, Wagner,JA, Wu,M, Knopps,A, Xiang,AH, Utzschneider,KM, Kahn,SE, Olefsky,JM, Buchanan,TA, Scherer,PE: Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione-mediated improvement in insulin sensitivity. *J.Biol.Chem.* 279:12152-12162, 2004

28. Kadowaki,T, Yamauchi,T, Kubota,N, Hara,K, Ueki,K, Tobe,K: Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *J Clin.Invest* 116:1784-1792, 2006

29. Arita,Y, Kihara,S, Ouchi,N, Takahashi,M, Maeda,K, Miyagawa,J, Hotta,K, Shimomura,I, Nakamura,T, Miyaoka,K, Kuriyama,H, Nishida,M, Yamashita,S, Okubo,K, Matsubara,K, Muraguchi,M, Ohmoto,Y, Funahashi,T, Matsuzawa,Y: Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem.Biophys.Res.Commun.* 257:79-83, 1999

30. Ouchi,N, Kihara,S, Funahashi,T, Matsuzawa,Y, Walsh,K: Obesity, adiponectin and vascular inflammatory disease. *Curr.Opin.Lipidol.* 14:561-566, 2003

31. Fantuzzi,G: Adipose tissue, adipokines, and inflammation. *Journal of Allergy and Clinical Immunology* 115:911-919, 2005

32. de Ferranti,S, Mozaffarian,D: The perfect storm: Obesity, adipocyte dysfunction, and metabolic consequences. *Clin.Chem.* 54:945-955, 2008

33. Galic,S, Oakhill,JS, Steinberg,GR: Adipose tissue as an endocrine organ. *Mol.Cell Endocrinol.* 2009

34. Gahceci,M, Gokalp,D, Bahceci,S, Tuzcu,A, Atmaca,S, Arikan,S: The correlation between adiposity and adiponectin, tumor necrosis factor alpha, interleukin-6 and high sensitivity C-reactive protein levels. Is adipocyte size associated with inflammation in adults? *J.Endocrinol.Invest.* 30:210-214, 2007

35. Steppan,CM, Bailey,ST, Bhat,S, Brown,EJ, Banerjee,RR, Wright,CM, Patel,HR, Ahima,RS, Lazar,MA: The hormone resistin links obesity to diabetes. *Nature* 409:307-312, 2001

36. Steppan,CM, Lazar,MA: Resistin and obesity-associated insulin resistance. *Trends Endocrinol.Metab* 13:18-23, 2002

37. Fernandez-Real,JM, Ricart,W: Insulin resistance and chronic cardiovascular inflammatory syndrome. *Endocr.Rev.* 24:278-301, 2003

38. MohamedAli,V, Goodrick,S, Rawesh,A, Yudkin,J, Coppack,S: Subcutaneous adipose tissue releases interleukin-6 but not tumour necrosis factor-alpha in vivo. *Diabetes* 46:215, 1997

39. Vozarova,B, Weyer,C, Hanson,K, Tataranni,PA, Bogardus,C, Pratley,RE: Circulating interleukin-6 in relation to adiposity, insulin action, and insulin secretion. *Obesity Research* 9:414-417, 2001

40. Fasshauer,M, Paschke,R: Regulation of adipocytokines and insulin resistance. *Diabetologia* 46:1594-1603, 2003

41. Wang,ZH, Masternak,MM, Al Regaiey,KA, Bartke,A: Adipocytokines and the regulation of lipid metabolism in growth hormone transgenic and calorie-restricted mice. *Endocrinology* 148:2845-2853, 2007

42. Bruun,JM, Pedersen,SB, Richelsen,B: Regulation of interleukin 8 production and gene expression in human adipose tissue in vitro. *J.Clin.Endocrinol.Metab* 86:1267-1273, 2001

43. Wang,B, Jenkins,JR, Trayhurn,P: Expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture: integrated response to TNF-alpha. *Am.J.Physiol Endocrinol.Metab* 288:E731-E740, 2005

44. Bruun,JM, Lihn,AS, Pedersen,SB, 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* 90:2282-2289, 2005

45. Shoelson,SE, Lee,J, Goldfine,AB: Inflammation and insulin resistance. *J Clin.Invest* 116:1793-1801, 2006

46. Murano,I, Barbatelli,G, Parisani,V, Latini,C, Muzzonigro,G, Castellucci,M, Cinti,S: Dead adipocytes, detected as crown-like structures (CLS), are prevalent in visceral fat depots of genetically obese mice. *J Lipid Res.* 2008

47. Strissel,KJ, Stancheva,Z, Miyoshi,H, Perfield,JW, DeFuria,J, Jick,Z, Greenberg,AS, Obin,MS: Adipocyte death, adipose tissue remodeling, and obesity complications. *Diabetes* 56:2910-2918, 2007

48. de Luca,C, Olefsky,JM: Stressed out about obesity and insulin resistance. *Nat.Med.* 12:41-42, 2006

49. Cinti,S, Mitchell,G, Barbatelli,G, Murano,I, Ceresi,E, Faloia,E, Wang,S, Fortier,M, Greenberg,AS, Obin,MS: Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res.* 46:2347-2355, 2005

50. Todoric,J, Loffler,M, Huber,J, Bilban,M, Reimers,M, Kadl,A, Zeyda,M, Waldhausl,W, Stulnig,TM: Adipose tissue inflammation induced by high-fat diet in obese diabetic mice is prevented by n-3 polyunsaturated fatty acids. *Diabetologia* 49:2109-2119, 2006

51. Neels,JG, Olefsky,JM: Inflamed fat: what starts the fire? *J.Clin.Invest* 116:33-35, 2006

52. Tsuchida,A, Yamauchi,T, Takekawa,S, Hada,Y, Ito,Y, Maki,T, Kadowaki,T: Peroxisome proliferator-activated receptor (PPAR)alpha activation increases adiponectin receptors and reduces obesity-related inflammation in adipose tissue: comparison of activation of PPARalpha, PPARgamma, and their combination. *Diabetes* 54:3358-3370, 2005

53. Xydakis,AM, Case,CC, Jones,PH, Hoogeveen,RC, Liu,MY, Smith,EO, Nelson,KW, Ballantyne,CM: Adiponectin, inflammation, and the expression of the metabolic syndrome in obese individuals: the impact of rapid weight loss through caloric restriction. *J.Clin.Endocrinol.Metab* 89:2697-2703, 2004

54. Libby,P, Plutzky,J: Inflammation in diabetes mellitus: role of peroxisome proliferator-activated receptor-alpha and peroxisome proliferator-activated receptor-gamma agonists. *Am.J.Cardiol.* 99:27B-40B, 2007

55. Xu,H, Barnes,GT, Yang,Q, Tan,G, Yang,D, Chou,CJ, Sole,J, Nichols,A, Ross,JS, Tartaglia,LA, Chen,H: Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J.Clin.Invest* 112:1821-1830, 2003

56. Sampath,H, Ntambi,JM: Polyunsaturated fatty acid regulation of gene expression. *Nutr.Rev.* 62:333-339, 2004

57. Rivellese,AA, Lilli,S: Quality of dietary fatty acids, insulin sensitivity and type 2 diabetes. *Biomed.Pharmacother.* 57:84-87, 2003

58. Sessler,AM, Ntambi,JM: Polyunsaturated fatty acid regulation of gene expression. *J.Nutr.* 128:923-926, 1998

59. Flachs,P, Horakova,O, Brauner,P, Rossmeisl,M, Pecina,P, Franssen-van Hal,NL, Ruzickova,J, Sponarova,J, Drahota,Z, Vlcek,C, Keijer,J, Houstek,J, Kopecky,J: Polyunsaturated fatty acids of marine origin upregulate mitochondrial biogenesis and induce beta-oxidation in white fat. *Diabetologia* 48:2365-2375, 2005

60. Neschen,S, Morino,K, Dong,J, Wang-Fischer,Y, Cline,GW, Romanelli,AJ, Rossbacher,JC, Moore,IK, Regittnig,W, Munoz,DS, Kim,JH, Shulman,GI: N-3 Fatty Acids Preserve Insulin Sensitivity In Vivo in a PPAR{alpha}-Dependent Manner. *Diabetes* 56:1034-1041, 2007

61. Huber,J, Loffler,M, Bilban,M, Reimers,M, Kadl,A, Todoric,J, Zeyda,M, Geyeregger,R, Schreiner,M, Weichhart,T, Leitinger,N, Waldhausl,W, Stulnig,TM: Prevention of high-fat diet-induced adipose tissue remodeling in obese diabetic mice by n-3 polyunsaturated fatty acids. *Int.J Obes.(Lond)* 31:1004-1013, 2007

62. Kopecky,J, Rossmeisl,M, Flachs,P, Kuda,O, Brauner,P, Jilkova,Z, Stankova,B, Tvrzicka,E, Bryhn,M: n-3 PUFA: bioavailability and modulation of adipose tissue function. *Proc.Nutr.Soc.*1-9, 2009

63. Flachs,P, Mohamed-Ali,V, Horakova,O, Rossmeisl,M, Hosseinzadeh-Attar,MJ, Hensler,M, Ruzickova,J, Kopecky,J: Polyunsaturated fatty acids of marine origin induce adiponectin in mice fed high-fat diet. *Diabetologia* 49:394-397, 2006

64. Broughton,KS, Wade,JW: Total fat and (n-3):(n-6) fat ratios influence eicosanoid production in mice. *Journal of Nutrition* 132:88-94, 2002

65. Serhan,CN: Novel omega -- 3-derived local mediators in anti-inflammation and resolution. *Pharmacol.Ther.* 105:7-21, 2005

66. Gonzalez-Periz,A, Horrillo,R, Ferre,N, Gronert,K, Dong,B, Moran-Salvador,E, Titos,E, Martinez-Clemente,M, Lopez-Parra,M, Arroyo,V, Claria,J: Obesity-induced insulin resistance and hepatic steatosis are alleviated by {omega}-3 fatty acids: a role for resolvins and protectins. *FASEB J.* 2009

67. Galli,C, Calder,PC: Effects of Fat and Fatty Acid Intake on Inflammatory and Immune Responses: A Critical Review. *Annals of Nutrition and Metabolism* 55:123-139, 2009

68. Obregon,MJ: Thyroid hormone and adipocyte differentiation. *Thyroid* 18:185-195, 2008

69. Oppenheimer,JH, Schwartz,HL, Lane,JT, Thompson,MP: Functional relationship of thyroid hormone-induced lipogenesis, lipolysis, and thermogenesis in the rat. *J.Clin.Invest* 87:125-132, 1991

70. Yen,PM: Physiological and molecular basis of thyroid hormone action. *Physiol Rev.* 81:1097-1142, 2001

71. Galton,VA, Schneider,MJ, Clark,AS, St Germain,DL: Life without thyroxine to 3,5,3'-triiodothyronine conversion: studies in mice devoid of the 5'-deiodinases. *Endocrinology* 150:2957-2963, 2009

72. Pavelka,S, Kopecky,P, Bendlova,B, Stolba,P, Vitkova,I, Vobruba,V, Plavka,R, Houstek,J, Kopecky,J: Tissue metabolism and plasma levels of thyroid hormones in critically ill very premature infants. *Pediatr.Res.* 42:812-818, 1997

73. Ortega,FJ, Moreno-Navarrete,JM, Ribas,V, Esteve,E, Rodriguez-Hermosa,JI, Ruiz,B, Peral,B, Ricart,W, Zorzano,A, Fernandez-Real,JM: Subcutaneous Fat Shows Higher Thyroid Hormone Receptor-alpha1 Gene Expression Than Omental Fat. *Obesity.(Silver.Spring)* 2009

74. Hsu,SM, Raine,L, Fanger,H: A Comparative-Study of the Peroxidase-Antiperoxidase Method and An Avidin-Biotin Complex Method for Studying Polypeptide Hormones with Radioimmunoassay Antibodies. *American Journal of Clinical Pathology* 75:734-738, 1981

75. Hirsch,J, Batchelor,B: Adipose-Tissue Cellularity in Human Obesity. *Clinics in Endocrinology and Metabolism* 5:299-311, 1976

76. Perseghin,G, Petersen,K, Shulman,GI: Cellular mechanism of insulin resistance: potential links with inflammation. *Int.J.Obes.Relat Metab Disord.* 27 Suppl 3:S6-11, 2003

77. Jernas,M, Palming,J, Sjoholm,K, Jennische,E, Svensson,PA, Gabrielsson,BG, Levin,M, Sjogren,A, Rudemo,M, Lystig,TC, Carlsson,B, Carlsson,LMS, Lonn,M: Separation of human adipocytes by size: hypertrophic fat cells display distinct gene expression. *FASEB J.* 20:1540-+, 2006

78. Guo,KY, Halo,P, Leibel,RL, Zhang,YY: Effects of obesity on the relationship of leptin mRNA expression and adipocyte size in anatomically distinct fat depots in mice. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* 287:R112-R119, 2004

79. Araujo,RL, Andrade,BM, da Silva,ML, Ferreira,AC, Carvalho,DP: Tissue-specific deiodinase regulation during food restriction and low replacement dose of leptin in rats. *Am.J.Physiol Endocrinol.Metab* 296:E1157-E1163, 2009

80. Cabanelas,A, Lisboa,PC, Moura,EG, Pazos-Moura,CC: Acute effects of leptin on 5'-deiodinases are modulated by thyroid state of fed rats. *Horm.Metab Res.* 39:818-822, 2007

81. Cabanelas,A, Lisboa,PC, Moura,EG, Pazos-Moura,CC: Leptin acute modulation of the 5'-deiodinase activities in hypothalamus, pituitary and brown adipose tissue of fed rats. *Horm.Metab Res.* 38:481-485, 2006

82. Otto,T, Fandrey,J: Thyroid hormone induces hypoxia-inducible factor 1alpha gene expression through thyroid hormone receptor beta/retinoid x receptor alpha-

dependent activation of hepatic leukemia factor. *Endocrinology* 149:2241-2250, 2008

83. Prpic,V, Watson,PM, Frampton,IC, Sabol,MA, Jezek,GE, Gettys,TW: Differential mechanisms and development of leptin resistance in A/J versus C57BL/6J mice during diet-induced obesity. *Endocrinology* 144:1155-1163, 2003

84. Surwit,RS, Wang,S, Petro,AE, Sanchis,D, Raimbault,S, Ricquier,D, Collins,S: Diet-induced changes in uncoupling proteins in obesity-prone and obesity-resistant strains of mice. *Proc.Natl.Acad.Sci.U.S A.* 95:4061-4065, 1998

85. Solinas,G, Summermatter,S, Mainieri,D, Gubler,M, Pirola,L, Wymann,MP, Rusconi,S, Montani,JP, Seydoux,J, Dulloo,AG: The direct effect of leptin on skeletal muscle thermogenesis is mediated by substrate cycling between de novo lipogenesis and lipid oxidation. *FEBS Lett.* 577:539-544, 2004

86. Kabir,M, Skurnik,G, Naour,N, Pechtner,V, Meugnier,E, Rome,S, Quignard-Boulange,A, Vidal,H, Slama,G, Clement,K, Guerre-Millo,M, Rizkalla,SW: Treatment for 2 mo with n-3 polyunsaturated fatty acids reduces adiposity and some atherogenic factors but does not improve insulin sensitivity in women with type 2 diabetes: a randomized controlled study. *Am.J.Clin.Nutr.* 86:1670-1679, 2007

87. Lavie,CJ, Milani,RV, Mehra,MR, Ventura,HO: Omega-3 Polyunsaturated Fatty Acids and Cardiovascular Diseases. *Journal of the American College of Cardiology* 54:585-594, 2009

88. Fedor,D, Kelley,DS: Prevention off insulin resistance by n-3 polyunsaturated fatty acids. *Current Opinion in Clinical Nutrition and Metabolic Care* 12:138-146, 2009

89. Baker,PW, Gibbons,GF: Effect of dietary fish oil on the sensitivity of hepatic lipid metabolism to regulation by insulin. *J Lipid Res.* 41:719-726, 2000

90. Buckingham,RE: Thiazolidinediones: Pleiotropic drugs with potent anti-inflammatory properties for tissue protection. *Hepatology Research* 33:167-170, 2005

91. Pfutzner,A, Marx,N, Walcher,D, Lobig,M, Seidel,D, Forst,T: Impact of Rosiglitazone on Visfatin and Adiponectin Plasma Concentrations in Patients with Type 2 Diabetes and Coronary Artery Disease. *Clinical Laboratory* 54:237-241, 2008

92. Koistinen,HA, Galuska,D, Chibalin,AV, Yang,J, Zierath,JR, Holman,GD, Wallberg-Henriksson,H: 5-amino-imidazole carboxamide riboside increases glucose transport and cell-surface GLUT4 content in skeletal muscle from subjects with type 2 diabetes. *Diabetes* 52:1066-1072, 2003

93. Nissen,SE, Wolski,K: Effect of Rosiglitazone on the Risk of Myocardial Infarction and Death from Cardiovascular Causes. *N.Engl.J Med.* 356:2457-2471, 2007

94. Calder,PC: n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am.J.Clin.Nutr.* 83:1505S-1519S, 2006

95. Weldon,SM, Mullen,AC, Loscher,CE, Hurley,LA, Roche,HM: Docosahexaenoic acid induces an anti-inflammatory profile in lipopolysaccharide-stimulated human THP-1 macrophages more effectively than eicosapentaenoic acid. *Journal of Nutritional Biochemistry* 18:250-258, 2007

96. Yusof,HM, Miles,EA, Calder,P: Influence of very long-chain n-3 fatty acids on plasma markers of inflammation in middle-aged men. *Prostaglandins Leukotrienes and Essential Fatty Acids* 78:219-228, 2008

9 SELECTED PUBLICATIONS