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Effects of n–3 polyunsaturated fatty acids, rosiglitazone,
low caloric diet and environmental pollutants: obesity and
related disorders

Ph. D. Thesis

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Statement of authorship

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

In Prague …………….

<div align="right">Mgr. Ondřej Kuda</div>

Statement of co-authors

I certify that Ondřej Kuda substantially contributed to the formation of the papers used as a basis of this thesis, and that his participation specified at the end of the comments to each paper is correct.

In Prague …………………..

<div align="right">MUDr. Jan Kopecký, DrSc.</div>

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

A.   **<u>Kuda O</u>**, 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, 2009, in press (IF = 5.849)

B.   van Schothorst E, Flachs P, Franssen-van Hal N, **<u>Kuda O</u>**, Bunschoten A, Molthoff J, Vink C, Hooiveld G, Kopecky J, Keijer J

Induction of lipid oxidation by polyunsaturated fatty acids of marine origin in small intestine of mice fed a high-fat diet

BMC Genomics, in press (IF = 4.243)

C.   Polak J, Kovacova Z, Jacek M, Klimcakova E, Kovacikova M, Vitkova M, **<u>Kuda O</u>**, Sebela M, Samcova E, Stich V.

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

Clin Sci (Lond). 2007 Jun;112(11):557-65. (IF = 2.937)

D.   Mullerova D, Kopecky J, Matejkova D, Muller L, Rosmus J, Racek J, Sefrna F, Opatrna S, **<u>Kuda O</u>**, Matejovic M.

Negative association between plasma levels of adiponectin and polychlorinated biphenyl 153 in obese women under non-energy-restrictive regime.

Int J Obes (Lond). 2008 Dec;32(12):1875-8. Epub 2008 Sep 30. (IF = 4.704)

The above papers are included in full in this PhD thesis. For a list of all my published articles, see List of published papers (Section 5).

LIST OF ABBREVISTIONS

ALA	alpha linoleic acid
AMPK	AMP–activated protein kinase
CYP 2B	cytochrome P450, family 2, subfamily B
cHF	corn oil based high-fat diet
cHF+F or cHF–F1	corn oil based high-fat diet supplemented with fish oil
cHF–F2	cHF diet supplemented with high dose of fish oil
cHF+F+TZD	cHF+F diet supplemented with thiazolidinedione
cHF+TZD	cHF diet supplemented with thiazolidinedione
EPA	eicosapentaenoic acid (20:3 n–5)
DDT, DDE	dichloro-diphenyl-trichloroethane or –dichloroethane
DHA	docosahexaenoic acid (22:6 n–3)
IGT	impaired glucose tolerance
HMW	high molecular weight
LCD	low carolic diet
LMW	low molecular weight
LPL	lipoprotein lipase
MMW	medium molecular weight
OB	obese (group)
PEPCK	phosphoenolpyruvate carboxykinase
PCB	polychlorinated biphenyl
PGD2, PGJ2	prostaglandin D2 or J2
POP	persistent organic pollutant
PPAR	peroxisome proliferator–activated receptor
PPER	peroxisome proliferator response element
PUFA	polyunsaturated fatty acids
RXR	retinoid X receptor
sHF	semisynthetic high-fat diet
sHF–F2	sHF diet supplemented with high dose of fish oil
TG	triacylglycerols
TNF–α	tumor necrosis factor aplha
TZD	thiazolidinedione
VLDL–TG	very low-density lipoprotein – triglycerides
WAT	white adipose tissue
–KO	–knock out (genetic model)

CONTENTS

1. INTRODUCTION

1.1. Molecular and metabolic mechanisms linking obesity to insulin resistance

A side effect of contemporary human behaviour – overfeeding and lack of physical activity – is an excess of energy intake in humans. This unused energy is accumulated as body fat, which leads to higher incidence of obesity. Genetic predispositions and developed obesity band together could cause impairment of insulin signalling and loss of metabolic fuel homeostasis, which leads into outbreak of type 2 diabetes.

Main role of insulin is to stimulate glucose uptake in peripheral tissues, suppress hepatic glucose production and block release of fatty acids from adipose tissue. Thus, insufficient insulin secretion or action leads to metabolic complications, such as hyperglycaemia or dyslipidaemia, which further promote insulin resistance and cause serious tissue damage by subsequent mechanisms (see below). This state of long-term overloading of miscellaneous metabolic pathways can remain unchanged for relatively long time before full breakout of type 2 diabetes characterised as β-cells failure. This state of insulin resistance could be described as transition from obesity to type 2 diabetes.

Organs, which are affected by metabolic abnormalities, contribute to global mechanism of insulin resistance differently. The main impact could be overloading with toxic metabolite, induction of inflammation, disruption of secretory function or activation of anti-stress mechanisms.

1.1.1. Adipose tissue metabolic overload

Adipose tissue can store excess of dietary lipid up to the state of fullness, when additional lipids have to be stored in other organs and tissues. This lipid redistribution into tissues, which are not arranged to handle large portions of fatty acids, has deleterious effects on the respective function of target organ. Moreover, insulin resistant white adipose tissue (WAT) is not able to inhibit lipolysis and increased levels of glycerol and fatty acids in plasma further stimulate VLDL-TG secretion from the liver leading to hyperlipidaemia. Hypertrophy of adipose tissue, often accompanied with

hyperplasia, changes also endocrine function of adipose tissue, which is crucial for development of insulin resistance. Many adipose tissue peptide hormones, called adipokines or adipocytokines, are connected to inter-organ communication networks (see section 1.2.1). A potential role of an adipokine, adiponectin, in insulin sensitizing effect is widely investigated on miscellaneous models of obesity, insulin resistance and type 2 diabetes (see section 1.2.3).

Lipophilic character of adipose tissue provides a perfect repository for persistent organic pollutants (POP, see section 1.6). These harmful carcinogenic compounds could act as ligands for nuclear receptors and change expression profile of adipose tissue. When an obese contaminated organism loses weight, pollutants are washed up to the organism and deposit themselves into other lipid-rich tissue as brain or testes, where could again affect gene transcription. Strong positive correlations between contamination, obesity and insulin resistance were observed.

Low-grade and long-term inflammation is also involved in metabolic changes in obese rodents. Lipid overloading causes production of inflammatory mediators in the liver and adipose tissue, and contributes to insulin resistance and tissue stress. Adipose tissue is infiltrated by macrophages producing pro-inflammatory cytokines, which changes adipokine production of adipose tissue (see section 1.2.1).

1.1.2. Liver metabolic overload

The liver can store as well as produce lipid. Lipid-induced liver insulin resistance is thought to be a complex damage of lipid and glucose metabolism. Fatty acids are no longer directed to β-oxidation in mitochondria, but the excess is redirected into lipogenesis of cytosolic and membrane lipids, such as diacylglycerols, ceramides or triacylglycerols [1]. This surplus could interfere with a common cell signalling pathways using lipid signalling molecules (e.g. insulin signalling pathway) as well as elevate demands for triacylglycerol storage capacity [2].

Immoderate demands on endoplasmatic reticulum to synthetise phospholipids, ceramides or other complex lipids could cause also incomplete protein folding and deficit in key lipid precursors [3]. Decrease of production of proteins predetermined for endocrine function could be important also in adipose tissue or β-cells.

Concerning hyperglycaemic condition of insulin resistant organism, malonyl-CoA, a product of glucose metabolism, could be the causal agent of lipid metabolic switch [4], which inhibits entry of acyl-CoA to the mitochondria through carnitine-palmitoyltransferase 1, and serves as a precursor for *de novo* lipogenesis. Together with decreasing ability of insulin to inhibit hepatic glucose production, these complex changes of liver metabolism lead to hepatic steatosis and associated damages [5;6]. This phenotype could be reversed also with low caloric diet and body weight loss, yet this intervention affects other organs and their mechanism as well.

1.1.3. Skeletal muscle metabolic overload

Skeletal muscle is arranged for fuel combustion of either fatty acid or glucose. Oversupply of fatty acids leads to increased concentrations of lipid signalling molecules, as in the liver, which negatively correlates with muscle insulin sensitivity. Ceramide and diacylglycerol levels seem to be the most important players [7;8]. Chronic exposure of skeletal muscle to lipids without corresponding effect of exercise enhances the β-oxidation capacity disproportionately to subsequent metabolic pathways, which produces incompletely oxidised acyl-CoAs and overloads electron transport chain [9;10]. Muscle affected by this pressure is not able to perform successful switch from lipid to carbohydrate metabolism when the composition of diet changes. This state is called metabolic inflexibility [10]. Glucose uptake and prospective synthesis of glycogen is decreased and impaired [11] together with decreasing muscle insulin sensitivity and skeletal muscle remains fixed to lipid processing.

Together, partial contribution of each involved tissue and its respective damage could result in insulin resistance, where lipid overload, broken inter-tissue communication, intercellular stress or inflammation induces β-cell failure and type 2 diabetes.

1.1.4. Currently used therapies in type 2 diabetic patients

Insulin resistance and type 2 diabetes could be counteracted with several different therapies. The first and the most natural treatment is a change of life-style – an appropriate dietary regime with adequate physical activity. Subsequent choice is an

antidiabetic medication. There are two most commonly used drugs for the treatment of diabetic patients: metformin and thiazolidinediones (TZD). Metformin suppresses hepatic glucose production, stimulate glycolysis and fatty acid oxidation, and thus decreases blood glucose and lipid levels. TZD improve insulin sensitivity and induce redistribution of lipid from muscle and liver to adipose tissue (see section 1.5), but some adverse side-effects are associated with this therapy. Naturally occurring n–3 polyunsaturated fatty acids (PUFA), proving several beneficial effects on metabolic syndrome associated disorders, could be used as a supplementary therapy (see section 1.4).

1.1.5. AMP–activated protein kinase

The AMP-activated protein kinase (AMPK) system is a key player in regulating energy balance at both the cellular and whole-body levels, placing it at centre stage in studies of obesity, diabetes and the metabolic syndrome [12]. AMPK is activated in response to changes of ATP/AMP ratio, exercise (muscle contraction), nutrient deprivation, n–3 PUFA or by hormones, e.g. leptin and adiponectin (reviewed in [13]). This "master switch" attenuates ATP–consuming metabolic pathways and activates ATP–producing processes, e.g. repress gluconeogenesis, lipolysis, cholesterol synthesis and supports glucose uptake, fatty acid oxidation through reduction of malonyl-CoA levels etc. TZD [14] and metformin [15] as well as n–3 PUFA [16] are able to switch AMPK on, which suggests a strategy for the treatment of obesity.

1.2. Endocrine function of adipose tissue

1.2.1. Storage and endocrine function of white adipose tissue

White adipose tissue used to be considered as the storage site for excess of dietary energy in form of triacyglycerols organized in a single lipid droplet inside adipocyte. Fatty acid released from blood lipoprotein particles by lipoprotein lipase are transferred into the adipose cell and stored. As long as the organism has enough energy to maintain common processes, pathways of lipogenesis and reesterification are active in adipose tissue. When energy is needed, triacylglycerols can be quickly hydrolysed by a series of

specific lipases, fatty acids exported to other tissues to be oxidised in mitochondria and remaining glycerol exported to the liver to participate in glucose metabolism.

Adipose tissue, in response to e.g. hyperplasia, could be infiltrated by macrophages, which degrade dead adipocytes and produce pro-inflammatory cytokines [17-19]. Macrophages surrounding individual dead adipocyte forms aggregates, which can be visualized by immunodetection as crown–like structure, a marker of WAT inflammation.

Besides this conventional point of view, WAT also secretes a large variety of peptides and cytokines, known as adipocytokines or adipokines [20], as well as "active" lipid metabolites, known as lipokines [21], serving as endo-, auto-, or paracrine signals. These messengers affect insulin sensitivity of skeletal muscle or liver, modulate their energy metabolism, are involved in neural control of feeding behaviour or manifestation of inflammation.

1.2.2. Leptin

The first characterised adipokine was leptin [22]. Leptin serves as a signal of energy sufficiency and of the amount of fat. Leptin plasma levels highly correlate with adipose tissue mass and provide this information to leptin-sensitive neurons in hypothalamus to decrease food intake [23;24]. Leptin receptors are present also in various tissues, where leptin mediated signal induce PPAR–α (peroxisome proliferator–activated receptor) driven β-oxidation and uncoupling of mitochondria [25]. High level of plasma leptin associated with impaired leptin signalling cascade, called leptin resistance, could be found in obese organism [26].

1.2.3. Adiponectin

Adiponectin is a messenger improving insulin sensitivity and changing energy metabolism of target tissues [27]. Transcription of AdipoQ gene is controlled also by PPAR–γ [28] and could be induced by insulin or TZD [29]. It is produced as 30 kDa protein unit consisting of 244 amino acids, which is able to form several multimeric structures – it can act as monomer, trimer, hexamer or multimer (12-18 units), usually referred as LMW (low molecular weight, trimer), MMW (medium molecular weight,

hexamer) or HMW (high molecular weight, ten or more units) [30]. These forms differ in an ability to activate response in different tissues [31;32]. HMW form delivers the signal for glucose and lipid utilization to the liver and skeletal muscle [33], whereas LMW and MMW were detected also in the brain stimulating mechanisms increasing food intake and decreasing energy expenditure [34]. In addition, adiponectin complexes could be cleaved into stem (N–terminal) and globular (C–terminal) parts showing different potency to pass the signal.

Two adiponectin cell-surface receptors – AdipoR1 and AdipoR2 – were discovered [35]. Their transcription is regulated by PPAR and liver X receptor ligands. AdipoR1 is expressed ubiquitously, including liver and skeletal muscle, while AdipoR2 is expressed mainly in the liver. HMW and MMW forms interact preferentially with AdipoR2, whereas AdipoR1 prefer MMW, LMW or globular adiponectin [34]. Overall, there are several combinations of tissue-specific receptor expression and affinities for various adiponectin forms.
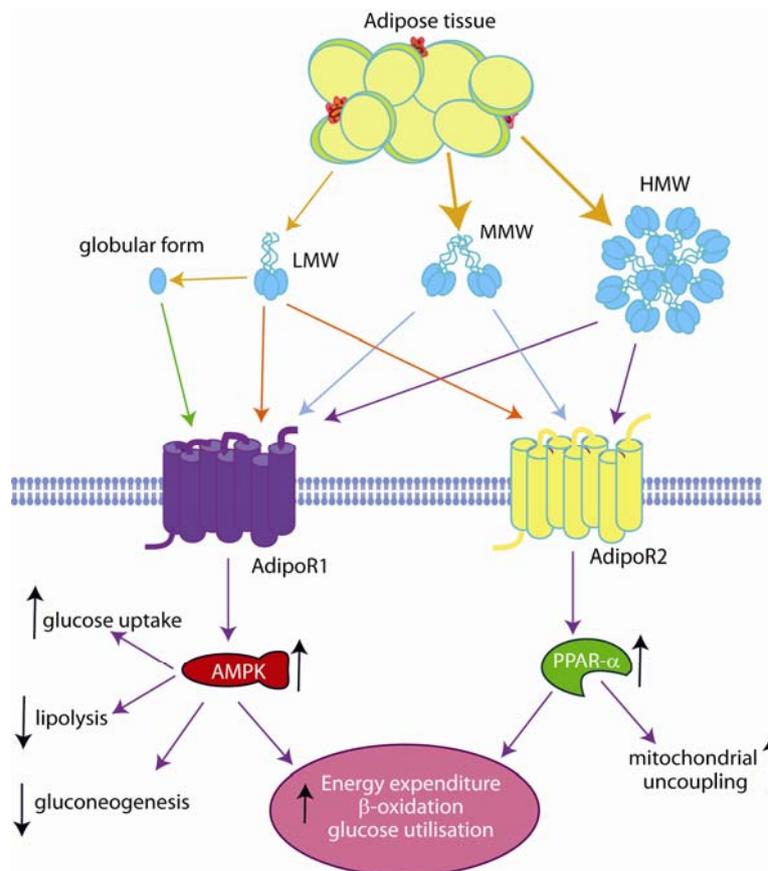

Figure 1.1 Adiponectin signalling

Diverse effects are mediated via adiponectin. Adiponectin induces AMPK phosphorylation leading to fatty acid oxidation in the liver and skeletal muscle [33], increases PPAR–γ transcription in adipose tissue, interacts with insulin signalling pathway facilitating glucose uptake in muscle and liver, and ameliorate pro-inflammatory effect of tumor necrosis factor-α [30]. Activation of PPAR–α target genes, such as acyl-CoA oxidase and uncoupling protein 2 was observed [36;37].

Plasma adiponectin levels are inversely correlated with adiposity and HMW form is positively associated with insulin sensitivity [38]. This suggests the importance of both total adiponectin levels and its HMW form to be induced by suitable therapy.

1.2.4. Central action of leptin and adiponectin

In addition to peripheral actions of leptin and adiponectin, their important role in the central nervous system was reported. Leptin activate its leptin receptor in the hypothalamus, which results into activation of a series of downstream kinases leading to increased metabolic rate and decreased food intake [39]. Leptin also inhibit AMPK in the hypothalamus [34].

Adiponectin was detected in cerebrospinal fluid, where was transported from the circulation [40;41]. Of note, only LMW and MMW adiponectin forms, and not HMW form, were observed. Furthermore, AdipoR1 and AdipoR2 expression, comparable to those in the liver, was found in hypothalamus [35]. This suggests the specific role of LMW and MMW adiponectin forms. Adiponectin activates AMPK in the hypothalamus, especially via AdipoR1 [34], increases food intake and decreases energy expenditure.

Altogether, adiponectin and leptin could inversely regulate food intake and energy expenditure through the hypothalamus [42]. Adiponectin or leptin could activate or deactivate AMPK in the hypothalamus in relation to decrease or increase of a fat mass. While only LMW and MMW adiponectin forms are able to enter cerebrospinal fluid and activate AMPK, HMW form could be the crucial messenger for AMPK activation in the liver and skeletal muscle. In obesity, HMW adiponectin levels are reduced, whereas MMW and LMW levels remain unchanged, thus able to activate AMPK in the hypothalamus independently of AMPK activation in peripheral tissues. Of

note, TZD stimulated induction of almost strictly HMW form of adiponectin could couple the effect on the hypothalamus and other tissues.
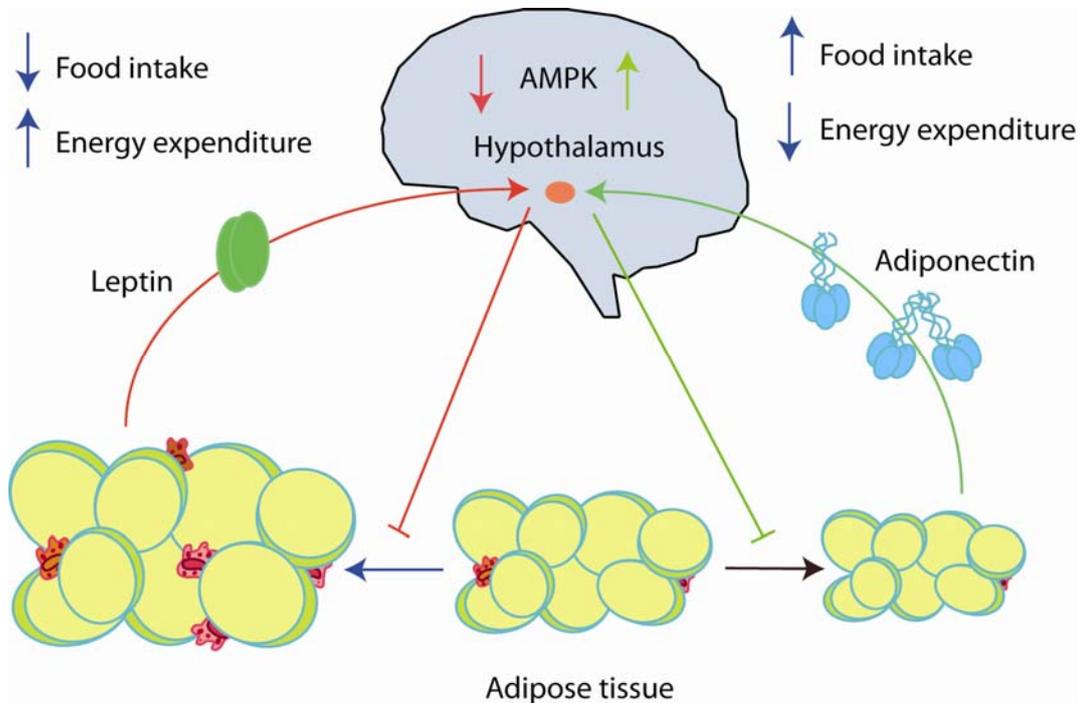

Figure 1.2 Leptin and adiponectin impact on brain (adapted from [42])

Several other adipokines (e.g. visfatin, retinol–binding–protein 4, resistin) are secreted by adipose tissue under specific circumstances. As no other adipokines are directly related to this work, full overview can be found in the review [43].

1.3. Peroxisome proliferators-activated receptors

Peroxisome proliferators-activated receptors (PPARs), belonging to the superfamily of nuclear receptors, play a key role in transcription of genes involved in lipid and glucose metabolism. There are three different PPAR subtypes in mouse genome, designated as PPAR–α, PPAR–β (also referred as PPAR–δ), and PPAR–γ. Each of them has specific role and tissue localization. While PPAR–α is mainly expressed in the liver and regulates fatty acid oxidation, PPAR–β is expressed ubiquitously at moderate levels and is responsible for fuel utilization in extrahepatic tissues and support preadipocyte

differentiation, and PPAR–γ is expressed in adipose tissue where controls both energy balance and lipid metabolism.

1.3.1. PPAR gene, protein, and transcription mechanism

The PPAR subfamily members are encoded by different genes, and PPAR–γ has two isoforms: PPAR–γ1 and PPAR–γ2 coming from alternate gene splicing. Little is known about regulation of PPAR gene expression.

PPAR protein consists of several distinct domains. Highly conserved DNA-binding domain at the N-terminal region binds through two zinc fingers to specific DNA sequences called PPREs (peroxisome proliferator hormone response elements). Ligand-binding domain at the C-terminal region is responsible for ligand-dependent activation. The two main domains are connected by short flexible hinge region [44].

PPARs form heterodimers with retinoid X receptor α (RXRα) and bind to the PPREs, which are present in the promoters of PPAR–regulated genes. PPAR/RXRα complex is silenced by corepressor, until ligand binding lowers the mobility of the complex and facilitate interactions with cofactors and coactivators [44-46]. This leads to the activation of the target gene and transcription via RNA Pol II iniciation complex is initiated.

Endogenous ligands for the PPARs include free fatty acids [47] and their metabolites eicosanoids [48;49]. Artificial ligands can be classified according to the PPAR subtype. PPAR–α is the main target of fibrate drugs and PPAR–γ is the main target of thiazolidinediones [50]. Several experimental drugs affecting PPAR–β or PPAR–α/γ as dual agonists are being investigated.
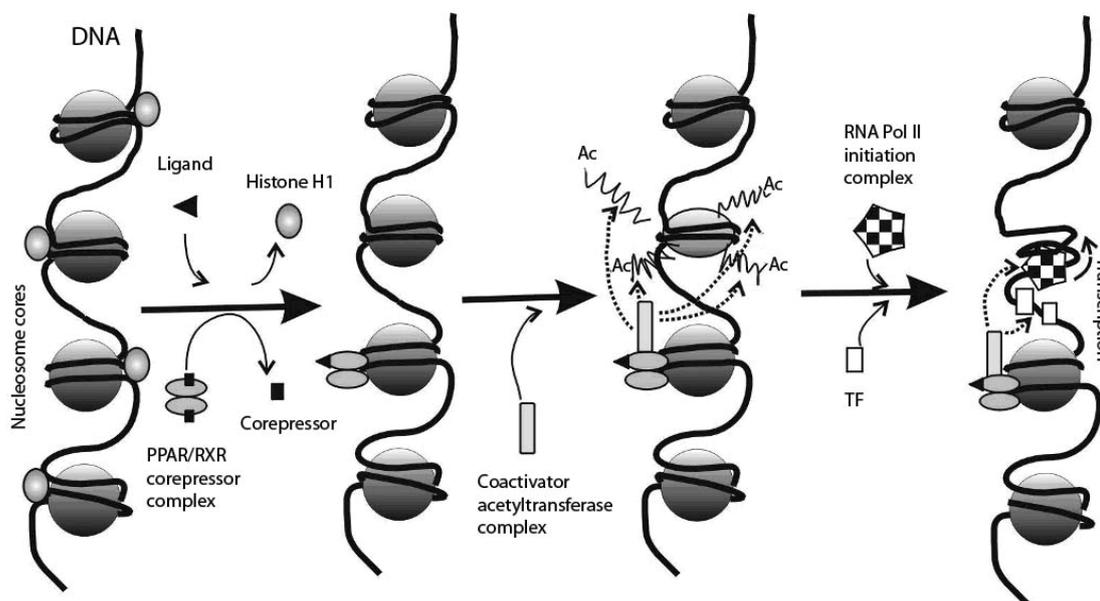
Figure 1.3 – Transcriptional activity of PPARs, adapted from [44]

1.3.2. PPAR–γ and its role in adipogenesis

Adipogenesis refers to the differentiation process of preadipocyte into mature adipocyte. Preadipocyte can differentiate into white fat or brown fat cell, respectively. During the differentiation into white adipocyte, expression of genes involved in lipid metabolism is induced. PPAR–γ is important regulator of transcription for many proteins, which are specific for adipose tissue, e.g. aP2, PEPCK, or LPL [44]. Mutations in PPAR gene are associated with aberrant adipose tissue metabolism of lipodystrophy.

Apart from adipocyte differentiation, PPAR–γ could also induce apoptosis of mature adipocyte. This effect was reported in the study, where PPAR–γ ligand troglitazone induced formation of new small adipocytes, while large adipocytes commited apoptosis [51;52].

PPAR–γ is also connected with the maintenance of glucose homeostasis. Apart from lipodystrophy, PPAR–γ defect results in diabetes, insulin resistance, or hypertension. TZD, known as strong PPAR–γ ligands, are able to revert insulin resistance and are used in human medicine for diabetic patients [53]. However, the mechanistic of improved insulin sensitivity due to the TZD treatment is still unknown.

It is widely accepted that WAT is the main target of TZD and that defect of PPAR–γ in WAT affects lipid and glucose metabolism in the liver and adipose tissue.

1.3.3. PPAR–α and its role in lipid metabolism

Nomenclature of PPAR came from its ability to promote proliferation of peroxisomes and induction of β-oxidation of long chain fatty acids [37]. Enhanced transcription of genes which are necessary for microsomal and peroxisomal fatty acid oxidation was described by [54] and PPAR–α was determined to be a key regulator of lipid oxidation. PPAR–α expression is induced during preadipocyte differentiation and PPAR–α KO mice have larger adipose stores [55].

Activation of PPAR–α in the liver stimulates lipid oxidation and reduces plasma triacylglycerols in rodents [56], thus decreases VLDL-TG production and limits triacylglycerol supply for adipose tissue. PPAR–α could be activated through endogenous ligands including variety of saturated and unsaturated long-chain fatty acids [47], or by its exogenous ligands - fibrates, which are used as hypolipidemic agents in humans (e.g. fenofibrate, bezafibrate) [57]. Of note, PUFA, such as DHA, are more potent in PPAR–α activation compared with other fatty acids [48].

PPAR–α induction could also affect whole-body insulin sensitivity through modulation of adipose tissue secretory function: decrease of leptin levels and body weight [58], increase of plasma adiponectin levels [59].

1.4. n–3 polyunsaturated fatty acids

1.4.1. Nomenclature

PUFA of n–3 (ω−3) family are important essential fatty acids (Table 1.1). Human or rodent organism is not able to produce n–3 PUFA *de novo*, and thus is dependent on the composition of food lipids. Dietary n–3 α–linolenic acid serves as a precursor, which is elongated and desaturated to from longer and more desaturated fatty acids, such as EPA or DHA. However excessive amounts of ALA slow down the formation of EPA and DHA, already inefficient, that's why increased dietary uptake of EPA and DHA is

preferred. Main utilization for n–3 PUFA are structural function in cellular membranes or acting as regulatory ligands in gene transcription.

Table 1.1 Nomenclature of selected n–3 polyunsaturated fatty acids

Trivial name	Abbreviation	Systematic name	Carboxyl reference
α–Linoleic acid	ALA	(Z,Z,Z)-9,12,15-octadecatrienoic acid	C 18:3 (Δ9,12,15)
Eicosapentaenoic acid	EPA	(5Z,8Z,11Z,14Z,17Z)-icosa-5,8,11,14,17-pentaenoic acid	C 20:5 (Δ5,8,11,14,17)
Docosahexaenoic acid	DHA	(4Z,7Z,10Z,13Z,16Z,19Z)-docosa-4,7,10,13,16,19-hexaenoic acid	C 22:6 (Δ4,8,12,15,19)

1.4.2. Biological effects and mechanisms

Naturally occurring n–3 long-chain PUFA, which are abundant in sea fish, act as hypolipidaemics, reduce cardiac events and decrease progression of atherosclerosis [60]. Therefore, n–3 PUFA are now regarded as healthy constituents of diets for diabetic patients [61;62]. Several studies in obese humans even demonstrated reduction of adiposity after n–3 PUFA supplementation [63;64]. However, in diabetic patients n–3 PUFA appear to have little effect on glycaemic control [63;65;66]. In rodents fed a high-fat diet, n–3 PUFA efficiently prevent development of obesity [67-69] as well as impaired glucose tolerance (IGT; refs. [70;71]). Low–grade WAT inflammation could be also prevented [72].

Whole-body hypolipidaemic and anti-obesity effects of n–3 PUFA probably depend on the in situ suppression of lipogenesis and increase of fatty acid oxidation in several tissues [69;73;74]. This metabolic switch might reduce accumulation of toxic fatty acid-derivatives, while protecting the insulin signalling in liver and muscle [70;71].

The effects of n–3 PUFA are largely mediated by PPAR–α and PPAR–β/δ [75]. However, PPAR–γ, liver X receptor α, hepatic nuclear factor-4, sterol regulatory element binding protein-1, are also involved [71;73;76]. Results from our laboratory [68;69] have demonstrated that substitution of only 15 % of dietary lipids in high-fat diet (mainly corn oil) by EPA/DHA concentrate (EPAX 1050TG) prevented fat accumulation in abdominal fat depots in mice. In cell culture experiments, DHA

inhibited differentiation of preadipocytes and even induce their apoptosis [77]. Fish oil also induces adiponectin [78] and influence also other adipokines [79;80].

Besides acting directly as regulatory ligands, n–3 PUFA act also through their active metabolites, eicosanoids, and other lipid molecules [81]. Adipocytes produce series-2 eicosanoids from arachidonic acid and higher availability of n–3 PUFA shift the production of eiconanoids in favour of series-3 and -5. This could be crucial for the prevention of WAT inflammation. Production of prostaglandin of the 2-series, PGD2, known PPAR–γ ligand promoting adipocyte differentiation; is reduced after n–3 PUFA administration [82], which supports the idea of n–3 PUFA ability to decrease accumulation of fat stores. Also other prostaglandins, such as 15d-PGJ2 and Δ12-PGJ2, [47;49] as well as oxidised fatty acids [83] activate PPAR–γ.

1.5. Thiazolidinediones

1.5.1. Nomenclature

The medication class of thiazolidinedione represents a group of chemical compounds, which share the thiazolidinedione core and differ in the side-chain arms (Table 1.2).

Table 1.2 Thiazolidinedione drugs, adapted from [84]

Trivial name	Rosiglitazone	Pioglitazone	Troglitazone
Systematic name	5-[4-(2-[methyl(pyridin-2-yl)amino]ethoxy)benzyl]thiazolidine-2,4-dione	5-(4-[2-(5-ethylpyridin-2-yl)ethoxy]benzyl)thiazolidine-2,4-dione	5-(4-[[(6-hydroxy-2,5,7,8-tetramethylchroman-2-yl)methoxy]benzyl)thiazolidine-2,4-dione
Structure			
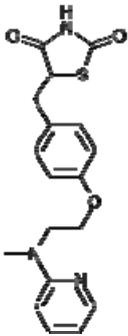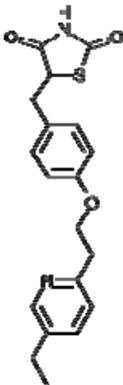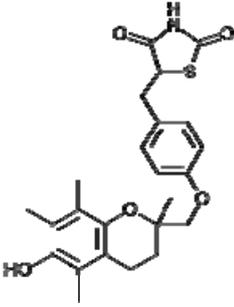
These compounds are used as medicinal drugs for therapy of type 2 diabetes mellitus and related diseases as potent PPAR–γ ligands [53]. Rosiglitazone (product Avandia) and pioglitazone (product Actos) are two representatives allowed for human therapy. Troglitazone had to be withdrawn from the market because of the increased incidence of drug-induced hepatitis.

Variety of new compounds sharing the thiazolidinedione core with alternative side chains able to activate PPAR–α are being prepared and tested, e.g. aleglitazar, muraglitazar, tesaglitazar (dual/pan-PPAR agonists).

1.5.2. Mechanism of TZD action

These compounds are likely to improve glycemic control, mostly by repartitioning fat away from skeletal muscle [85] and hence, augmenting insulin action in skeletal muscle adipose tissue, and liver [72;86-90]. TZD could also prevent inflammation [91]. TZD like rosiglitazone and pioglitazone, are also associated with unwanted side-effects, such as oedema, weight gain [92], a possible risk of heart failure [93], and bone loss [94]. Widely used dose of rosiglitazone promoted obesity, as observed in most studies in mice (e.g. ref. [72;88]). In humans, rosiglitazone therapy reduced the abdominal and increased subcutaneous fat weight [92].

TZD are potent PPAR–γ ligands and could activate many PPAR–dependent genes in different tissues. Although the main effect of these ligands seems to be preserved throughout the TZD drugs, discrepancy in the effect of different TZD medicinals on gene expression [95-97] suggest existence of complicated regulatory mechanism.

Adipocyte secretory function could be modulated by TZD. Treatment with TZD [76;86] has previously been shown to induce adiponectin in mice or prevent TNF-α induced insulin resistance [98;99]. Moreover, the promoter of adiponectin gene contains a PPRE [28] and TZD increase the production of adiponectin [29]. It has been shown that the HMW/total adiponectin and HMW/LMW ratios are plausible indicators of TZD–induced changes in insulin sensitivity [100].

1.6. Persistent organic pollutants

There are many organic compounds released by industry into the biosphere, which are not biologically degradable and pollute the environment. These environmental pollutants are resistant to biological and chemical degradation, cycle through the food chain and thanks to their lipophilic character could accumulate in fat-rich tissues such as brain, brown adipose tissue or WAT. Many of them were banned from using in the last century, but durable residues could be still detected in the biosphere. Beside the variety of carcinogenic effects, there are indications that also tissue sensitivity to insulin could be modulated by persistent organic pollutants.

1.6.1. POP classification

POP could be divided into five main groups [101]: (i) organochlorine pesticides; (ii) polychlorinated biphenyls (PCB); (iii) dioxins; (iv) polybrominated flame retardants; and (v) other pollutants e.g. phtalates. Organochlorine pesticides, especially 2,2-bis-(4-chlorophenyl)-1,1-dichloroethene (p,p'-DDE), 2,2-bis-(4-chlorophenyl)-1,1,1-trichloroethane (p,p'-DDT) and hexachlorbenzene, could be detected in human WAT at high concentrations. Polychlorinated biphenyl congeners, e.g. 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153), have carcinogenic effects and affect chromatin integrity. Dioxin contamination is connected with cancer as well as with diabetes and cardiovascular diseases.

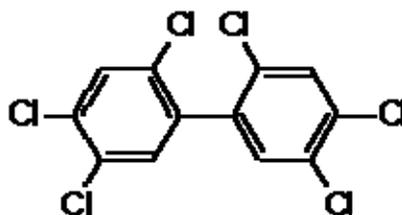Figure 1.4 PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl)

1.6.2. Mechanism of POP action

In adipose tissue, POP show endocrine disruption potency through mimicking or antagonising estrogen- or androgen-mediated processes, interaction with PPAR–γ or aryl hydrocarbon receptor, and thus modulate metabolism of adipose tissue and its endorine functions [101]. Negative association between POP and adiponectin, was found with

tetrachlorodibenzo-*p*-dioxin [102] or PCB 77 [103], both POP having dioxin-like properties while interacting with the aryl hydrocarbon receptor (AhR). However, PCB 153 displays little or no binding affinity for AhR, and PCB 153 is considered to be a partial androgen antagonist [104]. Similar to phenobarbital, PCB 153 interacts with cytochrome P450, the CYP 1A, and especially with CYP 2B enzymes. Yoshinari at al. [105] also demonstrated that in white adipose tissue of rats, phenobarbital induced CYP 2B enzymes, and hence, the same effect could be expected in human adipose tissue. Interestingly, xenobiotic-mediated increase of CYP 2B in rat hepatocytes is augmented by insulin [106] and expression of CYP 2B is increased in diabetic patients. Tetrachlorodibenzo-p-dioxin (Agent Orange) could induce low-grade inflammation of adipose tissue in mice [107] and also downregulation of adiponectin.

2. SPECIFIC AIMS OF WORK

The general and long-reaching goal of this thesis was to improve strategies for prevention and treatment of obesity and associated diseases (i.e. metabolic syndrome), namely type 2 diabetes, while focusing on beneficial effects of specific composition of dietary lipids, anti-diabetic drugs TZD and caloric restriction (see the specific aims 1, 2 and 3, below). Reflecting the prominent role of adipose tissue secretory function in the etiopathogenesis of the metabolic syndrome, part of the efforts was focused on adiponectin (see the specific aims 3 and 4, below).

The specific aims of this thesis were:

1. to evaluate possible additive beneficial effects of the combination treatments by n–3 PUFA and rosiglitazone in the model of diet-induced obesity in mice,

2. to investigate the effect of n–3 PUFA supplementation on lipid oxidation in small intestine of mice fed high-fat diet,

3. to characterise changes in both plasma adiponectin levels and distribution of adiponectin multimeric forms after low caloric diet in obese patients,

4. to evaluate a possible effect of the contamination of organism by persistent organic pollutant PCB 153 on plasma levels of adiponectin in obese patients.

3. SUMMARY TO SELECTED PUBLICATIONS

3.1. Publication A: n–3 PUFA 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

We hypothesised that partially overlapping mechanisms of action of n–3 PUFA and TZD, namely the activation of metabolic switch by n–3 PUFA and the induction of lipid repartitioning by TZD, could have synergistic effects in a combination treatment, leading to an improvement of glycaemic profile so far not described for these strategies when considered separately. We, therefore, evaluated the effects of: (1) partial replacement of dietary lipids by n–3 PUFA; (2) a low non obesogenic dose of rosiglitazone; and (3) a combination of both on whole-body variables of glucose and lipid metabolism in mice fed high-fat diet. To further elucidate the effects of the treatments, a detailed analysis of liver and muscle metabolism was also performed.

First, a 'prevention study' was performed to characterise the effects of n–3 PUFA, rosiglitazone and their combination, on developing obesity and IGT in mice fed high-fat diet. At 3 months of age, male C57BL/6N mice 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: (1) 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; (2) cHF diet supplemented with rosiglitazone (cHF+TZD) (10 mg/kg diet); and (3) cHF diet supplemented with EPA, DHA and rosiglitazone (cHF+F+TZD). Some mice were maintained on the standard chow diet. Various analyses were performed at 5 to 20 weeks after initiation of treatment.

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, prior to the subsequent 8-week-long treatment as above; i.e. with cHF, cHF+F, cHF+TZD and cHF+F+TZD, respectively.

Body weight was increased by cHF diet with significant differences between chow and cHF becoming apparent at 4 weeks (Fig. 3.1a). The combination treatment, cHF+F+TZD, reduced body weight gain significantly (Fig. 3.1a). Body composition was analysed and total weight of eviscerated carcass and total lipid content reflected changes

in body weight. Histological analysis combined with immunodetection of macrophages revealed cHF diet-induced hypertrophy of adipocytes in epididymal fat, accompanied by a low-grade inflammation of adipose tissue. 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.
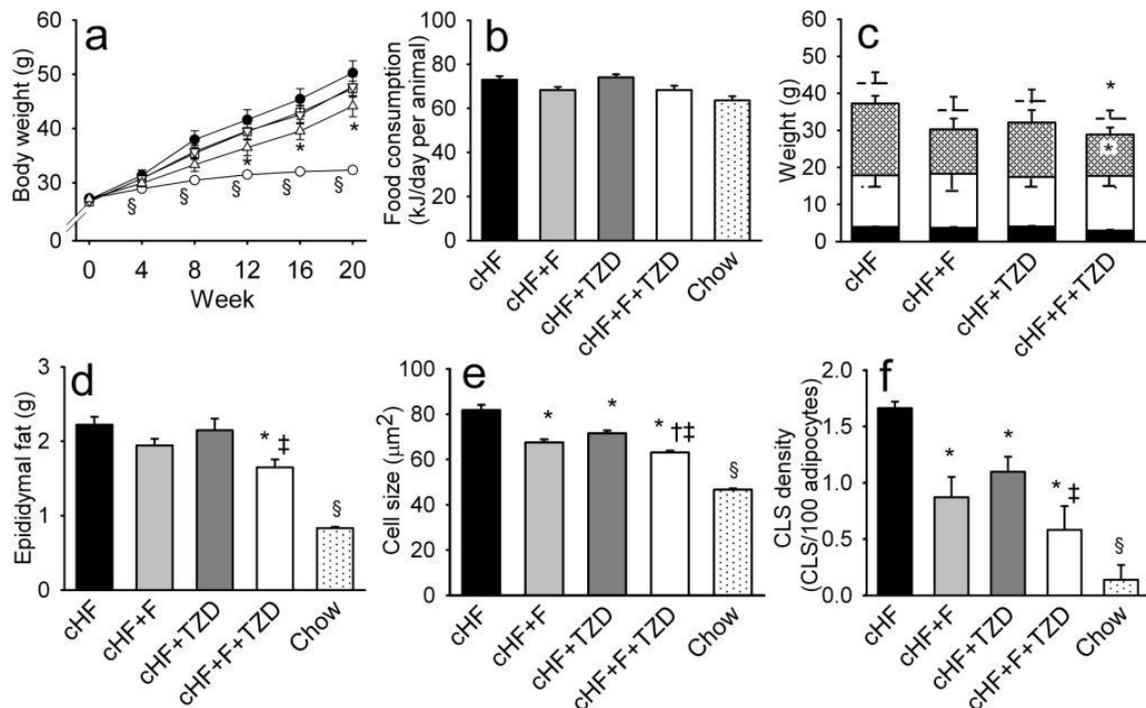

Figure 3.1 Characterisation of the model

Body weight (**a**), food consumption (**b**), body composition and size of adipocytes (**c–e**), and macrophage infiltration of adipose tissue (**f**). 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** Mean food consumption during 20-week treatment (*n*=8). **c** Body composition at 15 weeks. Bar height, weight of eviscerated carcass; black section, protein; white + black sections, lean body mass; crossed section, fat; dashed horizontal lines above bars, body weight of mice before killing. Error bars, SE (*n*=5–6). **d** Epididymal fat at 8 weeks, weight of fat depot. **e** Size of adipocytes. **f** 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. **d–f**) Data are means±SE (*n*=7–8). *$p \leq 0.05$ for difference from cHF; †$p \leq 0.05$ for difference from cHF+F; ‡$p \leq 0.05$ for difference from cHF+TZD (ANOVA); §$p \leq 0.05$ for difference from cHF (*t* test)

Compared with standard chow, the cHF diet induced accumulation of triacylglycerols in liver and gastrocnemius. Compared with cHF, none of the treatments significantly affected the triacylglycerol accumulation, except for cHF+TZD, which increased liver triacylglycerols at 8 weeks. The cHF+F+TZD diet also increased liver

triacylglycerol content at 8 weeks. The cHF+F+TZD diet significantly lowered ceramide content in the soleus muscle as compared with cHF-fed mice after 20 weeks.

Plasma triacylglycerol and NEFA levels were significantly suppressed by cHF+F+TZD. Cholesterol levels were reduced at 8 but not at 20 weeks. We also investigated the effect of 8 weeks of treatment on liver VLDL-triacylglycerol synthesis. All the treatments significantly decreased the rate of VLDL-triacylglycerol synthesis, with the strongest reduction by cHF+F+TZD (Figure 2 of Publication A). This confirms the hypolipidemic effect of the combination.

Insulinaemia increased between 8 and 20 weeks of high-fat feeding, suggesting development of insulin resistance. The rise in insulin levels was prevented to a similar extent by cHF+TZD and cHF+F+TZD diets, while the cHF+F diet exhibited a significant but smaller effect.

Both at 8 and 20 weeks, the cHF+F and cHF+TZD diets increased total immunoreactive adiponectin in plasma. An even stronger induction was observed with a combination of the two treatments (Figure 3.2). Multimeric adiponectin complexes in plasma were also analysed. Although the ratio between high molecular weight (HMW) and total adiponectin was similar in the cHF- and chow-fed mice, it was increased by all the other treatments, with the highest additive effect observed in cHF+F+TZD-fed animals, irrespective of treatment duration.

1. HMW native marker
2. cHF
3. cHF
4. cHF+F
5. cHF+F
6. cHF+TZD
7. cHF+TZD
8. cHF+F+TZD
9. cHF+F+TZD
10. Ssniff
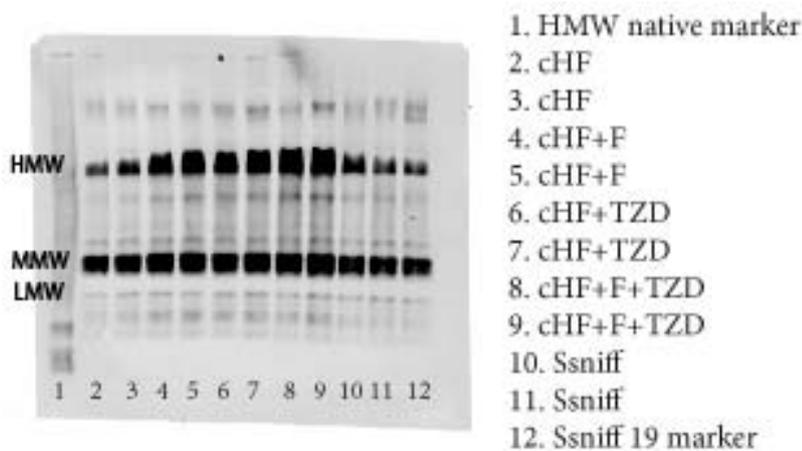11. Ssniff
12. Ssniff 19 marker

Figure 3.2 Representative immunoblot of adiponectin multimeric forms

To characterise insulin sensitivity and glycaemic control, a glucose tolerance test was performed at 8 weeks. Both fasted glycaemia and area under the glycaemic curve of mice on cHF+F+TZD diet displayed the largest improvements.

At 8 weeks, a euglycaemic–hyperinsulinaemic clamp was also performed to evaluate precisely the changes in whole-body insulin. In the hyperinsulinaemic conditions, the amount of exogenous glucose required to maintain euglycaemia, i.e. the glucose infusion rate, was highest in cHF+F+TZD group representing the strongest effect among all the treatments. Hepatic glucose production in the hyperinsulinaemic conditions was decreased in the cHF+F mice to a significantly lower level than in the other groups, suggesting that DHA/EPA improves hepatic insulin sensitivity. The cHF+F+TZD and cHF+TZD (but not cHF+F) treatments significantly improved whole-body glucose turnover, with cHF+F+TZD showing the most dramatic effect. Whole-body glycogen synthesis was strongly stimulated by cHF+F+TZD (Figure 3.3). To confirm these results, measurements were also performed *ex vivo*, in dissected diaphragms with or without insulin stimulation. Compared with chow, cHF reduced the rate of basal (~2.5-fold) and insulin-stimulated (~2.8-fold) glycogen synthesis. This deleterious effect of cHF feeding was completely prevented by cHF+F+TZD (Figure 3.3). Neither cHF+F nor cHF+TZD had any significant effect on basal or insulin-stimulated glycogen synthesis. Improvements in insulin-stimulated glycogen synthesis also correlated with the induction of Akt/PKB phosphorylation at Ser473 in soleus muscle of mice fed cHF+F+TZD.
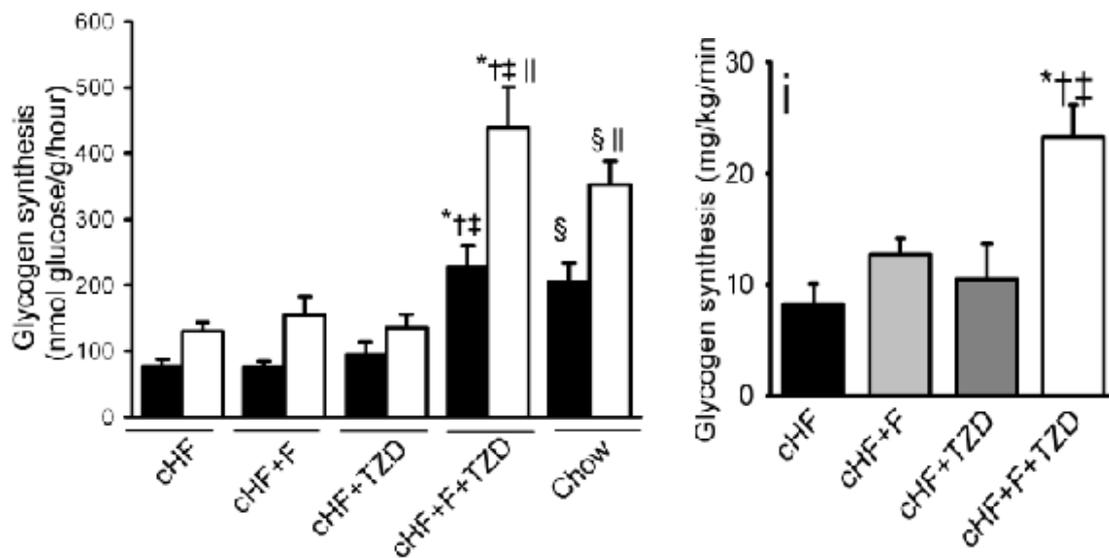
Figure 3.3 Glycogen synthesis *ex vivo* (left) and *in vivo* (right)

LEFT: Glycogen synthesis in diaphragm muscle. At 5 weeks after initiation of treatment as described, left and right hemidiaphragms of mice were dissected and incubated with [U-^{14}C]glucose in the absence (black bars) or presence (white bars) of 250 μU/ml insulin to measure the rate of glycogen synthesis. Data are means±SE (n=7–8). RIGHT: Glycogen synthesis during euglycaemic–hyperinsulinaemic clamp. Data are means±SE (n=6–9). *$p \le 0.05$ for difference from cHF; †$p \le 0.05$ for difference from cHF+F; ‡$p \le 0.05$ for difference from cHF+TZD (ANOVA); ‖$p \le 0.05$ for difference from non-insulin-stimulated value within the diet (ANOVA); §$p \le 0.05$ for difference from cHF (t test)

Analysis of gene expression from gastrocnemius, revealed a trend for induction of lipid catabolism–related genes by the combination treatment, supporting the activation of a switch augmenting lipid over glucose catabolism. Analysis of hepatic gene expression revealed a downregulation of gluconeogenic genes by cHF+F+TZD, while upregulation of lipid catabolism–related genes was observed.

The effects of cHF+F and cHF+TZD and their combination were also studied in obese mice with IGT. Compared with cHF-fed animals, body weight gain, triacylglycerols and NEFA levels were suppressed by cHF+F, while cHF+TZD decreased plasma triacylglycerols and insulin levels. Glucose tolerance was markedly improved by cHF+F+TZD, showing additive effects of DHA/EPA and rosiglitazone in the reversal of obesity, while decreasing plasma NEFA and cholesterol levels.

In conclusion, an original combined treatment using n–3 PUFA with low-dose rosiglitazone generated additive effects in the prevention as well as reversal of adipose tissue hypertrophy, hyperlipidaemia and impaired glycaemic control in mice fed an

obesogenic diet. Multiple mechanisms underlined the beneficial whole-body effects of the combination treatment with a prominent synergistic stimulation of insulin-sensitive muscle glycogen synthesis. The combined use of n–3 PUFA and TZD thus represents a potential strategy for treatment of type 2 diabetes and other obesity-associated metabolic disorders. The inclusion of n–3 PUFA in treatment with TZD may reduce the dose requirement and the incidence of adverse side effects associated with the TZD-based therapy.

My contributions to this work were management and coordination of the experiments, in vivo and ex vivo experiments, and phenotypical and biochemical characterisation of the model, thus I performed or participated in all experiments except for clamp study (TJ and MR), immunohistology (ZJ) and gene expression (MH and PF).

3.2. Publication B: n–3 PUFA and intestinal β–oxidation

Induction of lipid oxidation by polyunsaturated fatty acids of marine origin in small intestine of mice fed a high-fat diet

To elucidate molecular effects of n–3 PUFA *in vivo*, several gene expression analyses have been undertaken in animal models. The majority of those studies focused on liver and WAT, which is not surprising given the fact that these are considered the main target organs in a dietary intervention with fatty acids. Since the intestine contributes to a significant extend to the resting metabolic rate and daily energy expenditure, it is of relevance to also understand the effects on this organ.

We hypothesized that, using long-term dietary intervention studies, dietary fatty acid composition may modulate gene expression and lipid metabolism in the intestine, and that especially EPA and DHA may stimulate expression of genes involved in lipid catabolism. To examine this, we performed gene expression analysis of the mouse small intestine and colon, using whole genome oligonucleotide arrays and validation experiments using quantitative real time PCR (qRT-PCR), in addition to functional intestinal fatty acid beta oxidation measurement.

Two experiments on C57BL/6J mice were performed. Mice in the pilot study were maintained for 4 weeks on semisynthetic high-fat diets (sHF) differing in the composition of n–3 PUFA [68]. After this intervention, part of small intestine and colon were isolated from each mouse and samples of epithelial cells were stored for subsequent extraction of RNA. Obtained samples were pooled per tissue and per diet, hybridized on Affymetrix GeneChip mouse arrays and carefully analysed (see Methods of Publication B).

The second experiment was focused on functional analysis of lipid oxidation in small intestine. Animal were fed for 6 weeks high–fat diet and high–fat diet with 15 or 44 % of lipids replaced by EPAX 1050TG (cHF-F1 and cHF-F2, respectively). These diets are identical with the diets used in Publication A. At the end of this experiment, intestinal tissue was isolated for measurement of fatty acid β–oxidation and gene expression analysis by quantitative RT-PCR.

As observed before, dietary intervention with n–3 PUFA resulted in anti-adipogenic and anti-diabetic effects. Newly, we compared, using whole genome

microarray analysis as initial step, the control sHF diet, which was rich in ALA and free of EPA or DHA, with the isocaloric sHF-F2 diet, in which 44 % of lipids were replaced by an EPAX 1050TG concentrate. Set of 110 unique genes was identified, where increased expression was observed for 80 genes, while 30 genes showed decreased expression (Table 1 of Publication B). Functional interpretation showed changes in pathways of fatty acid uptake, fatty acid oxidation, cholesterol and steroid hormon biosynthesis, amongst others. Detailed inspection of the expression data revealed that n–3 PUFA induced genes involved in β–oxidation, both in mitochondria and peroxisomes.

To investigate in more detail inter-individual variation in gene expression, we selected 6 genes representing the major pathways being influenced by the diet intervention (see above) and analysed their expression by qRT-PCR. Representative genes cover: fatty acid β–oxidation in peroxisomes (acetyl-CoA acyltransferase) and mitochondria (carnitine palmitoyltransferase 1 and acetyl-CoA carboxylase), the switch between glycolysis and fatty acid oxidation (pyruvate dehydrogenase kinase 4), biosynthesis of steroid hormones (3-beta-hydroxysteroid dehydrogenase), and biosynthesis of cholesterol (squalene epoxidase). For all genes the mean gene expression ratios were similar to the observed ratios in the microarray experiment using data from pooled samples. Moreover, gene expression changes using individual samples were statistically significant for all genes (Figure 1 and Table 3 of Publication B).

Analysis of colon samples did not revealed any effect of n–3 PUFA diet, which underscores the different function of the small intestine (mainly uptake and transfer of nutrients) and the colon (mainly water absorption).

To validate functionally increased intestinal fatty acid β-oxidation, as suggested by increased gene expression, the second animal experiment was performed and expression analysis of acetyl-CoA acyltransferase and carnitine palmitoyltransferase 1 independently confirmed the observed differences (Figure 3.4a,b), and moreover, showed a dose-response effect: increased gene expression upon increased n–3 PUFA content in the diets (Figure 3.4a,b). Furthermore, functional analysis showed indeed a dose-dependent increase in β–oxidation upon increased n–3 PUFA content in the diet compared to control diet (Figure 3.4c).
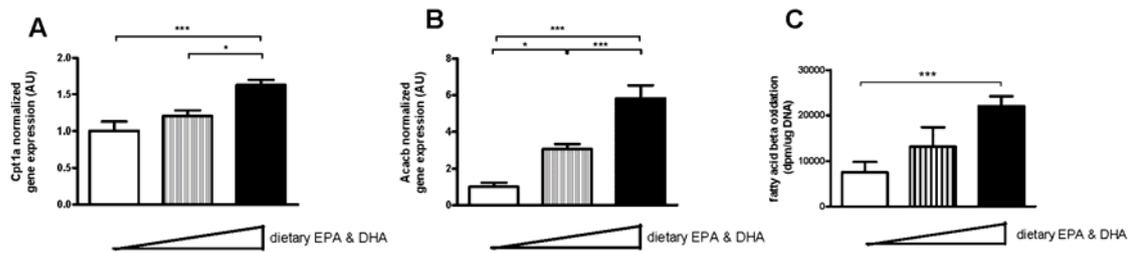
Figure 3.4 Dose dependent intestinal gene expression and β–oxidation

In a second animal experiment, intestinal gene expression analysis was used to confirm and biologically validate observations in a dose-dependent manner. Gene expression analysis was performed using qRT-PCR of small intestinal samples of individual wildtype mice that received the control diet (n=9, white bars), EPA&DHA diet (n=9, black bars), and intermediate levels of dietary EPA&DHA (n=9, hatched bars). The triangle shows increasing dietary EPA&DHA content. Gene expression levels were normalized using calnexin and averaged per group; the mean expression level of the control group was arbitrarily set at 1. Bars represent mean ± standard error. Genes shown are a: Cpt1a, and b: Acacb. Functional fatty acid beta oxidation was used for biological validation (c). Intestinal tissue fatty acid beta oxidation (n=7–9) is shown for mice that received control diet (white bars), EPA&DHA diet (black bars), and intermediate levels of dietary EPA-DHA (hatched bars). The triangle shows increasing dietary EPA&DHA content. Statistical significance was analyzed using one-way ANOVA and Tuckey's post hoc tests: * $p<0.05$, *** $p<0.01$. AU, arbitrary units.

In conclusion, we proved the involvement of small intestine in the complex changes of lipid metabolism exerted by long term dietary intake of EPA and DHA, namely by gene expression analysis and functional ex-vivo beta oxidation analysis. Furthermore, we show that these effects are regulated in a dose-dependent manner. In view of its large contribution to overall energy metabolism, modulation of gene expression and metabolism in the intestine by dietary lipids, and especially long-chain n–3 PUFA of marine origin, represents a promising target for the prevention of obesity and associated co-morbidities.

My main contributions to this work were management of the second animal study and measurement of the intestinal fatty acid oxidation.

3.3. Publication C: Adiponectin and diet induced weight loss

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

The aim of the present study was to investigate whether diet-induced changes in body weight and insulin sensitivity were associated with changes in the quantity of adiponectin multimeric complexes.

A total of 20 overweight or obese women (age, 39.4±9.5 years; body mass index, 32.2±6.4 kg/m^2) underwent 12 weeks of low caloric diet (600 kcal/day less than individually calculated energy requirements). Plasma samples were drawn before and after the study for biochemical analysis and western blot detection of adiponectin multimeric complexes.

The low-caloric diet resulted in a weight reduction (89.8±16.4 kg compared with 83.1±15.6 kg; $p<0.001$) and an improvement in whole-body insulin sensitivity, as measured by HOMA (homeostasis model assessment index; 1.9±0.8 compared with 1.5±0.7; $p=0.013$).

Total plasma adiponectin levels were measured using ELISA or determined by Western blotting as a sum of multimeric forms. As a result of LCD intervention, total adiponectin levels measured using ELISA increased by 36 %, whereas total adiponectin determined by Western blotting increase by 13.5 %. Result from ELISA reached borderline significance ($p=0.08$), while Western blotting results were statistically significant ($p=0.07$). Analysis of the distribution of adiponectin multimeric complexes revealed increases in the quantities of HMW, MMW and LMW by 5.5, 8.5 and 18.1 % respectively ($p<0.05$ for all of the forms). Ratio of HMW and total adiponectin, which is important for evaluation of potential site and quality of the impact, was reduced.

Comparison between total adiponectin levels in plasma and anthropometric or metabolic variables did not show any significant correlations, whereas HMW form was closely associated with fasting glucose levels and the MMW form was associated with HDL–cholesterol levels. Diet–induced changes in the HMW form negatively correlated with waist-to-hip ratio and percentage of fat mass at the end of the study.

Table 3.1 Adiponectin before and after low-caloric diet (adapted Table 2, Publication C)

	Before LCD	After LCD	*p* value
Total adiponectin			
by ELISA (µg/ml)	3.2 ± 1.7	4.4 ± 3.9	0.080
By Western blotting (QL)	575 ± 215	655 ± 219	0.007
HMW (QL)	84.9 ± 37.1	89.6 ± 37.9	0.008
MMW (QL)	133.9 ± 57.3	145.1 ± 55.2	0.045
LMW (QL)	356.2 ± 138.6	420.8 ± 145.2	0.003
HMW/total ratio	0.14 ± 0.04	0.13 ± 0.04	0.009
HMW/LMW ratio	0.55 ± 0.25	0.52 ± 0.25	0.026
HMW/MMW ratio	0.65 ± 0.25	0.63 ± 0.24	0.461

Adiponectin multimeric complexes and total plasma adiponectin before and after a 12-week LCD Values are means+−S.D., n =20. QL, quantity of light units normalized byreference to the QL of recombinant adiponectin MMW.

Our present finding of increased HMW and MMW forms afterweight loss is in agreement with earlier studies [108;109]. However, we have demonstrated for the first time that the LMW form also increased after dietary intervention. In fact, the LMW form was the isoform with the highest increase. The specific biological role and function of the LMW form relative to the other adiponectin multimeric complexes has not yet been established, so the interpretation of the increased LMW adiponectin remains open for discussion. Hypothetically, LMW increase could act as a message for the hypothalamus in response to the LCD and weight loss (see 1.2.4).

The HMW form has been suggested to be the most physiologically potent form of adiponectin and might be the form responsible for its beneficial insulin-sensitizing and anti-atherosclerotic effects [32;109]. It has been shown that the HMW/total adiponectin and HMW/LMW ratios are plausible indicators of TZD–induced changes in insulin sensitivity [32]. In the present study, we found an association between the HMW form and fasting glucose levels before the weight loss, which was compatible with the hypothesis of an important role of the HMW form in the regulation of insulin sensitivity under basal steady-state conditions. The HMW form increased by 5.5 % after weight loss, but no association with fasting glucose was observed at the end of the study. This small elevation in HMW adiponectin is probably of limited clinical significance, and other regulatory mechanisms possibly play more important roles in the control of fasting glucose following acute weight loss, i.e. changes in other plasma cytokines (interleukin-6, tumour necrosis factor and leptin) [110].

Nevertheless, no significant association between total adiponectin or its multimeric complexes and insulin sensitivity, as evaluated using a euglycaemic hyperinsulinaemic clamp, was found in the study by Bobbert et al. [111]. No associations between the HOMA index and any of the adiponectin oligomeric complexes, ratios or total plasma adiponectin were observed in our present study either at baseline or with respect to the diet-induced changes. On the basis of these findings and on those by Bobbert et al. [111], it can be hypothesized that the abovementioned ratios and associations of HMW adiponectin with parameters of insulin sensitivity might be specific to TZD treatment and may play only a minor role in LCD–induced changes in insulin sensitivity. A study [112] showing that TZD treatment selectively stimulates the secretion of the HMW form in human adipocytes supports this hypothesis further.

The increase in all three types of adiponectin multimeric complexes in the presence of a non-significant change in total plasma adiponectin levels measured using ELISA is due to intrinsic differences between the two methods. ELISA provides a quantitative determination of actual plasma concentration, whereas Western blotting yields semi-quantitative data in the form of arbitrary units (quantity of light). Moreover, the difference could be due to different binding capacities of the respective clones of antibodies used in the ELISA and Western blotting.

In conclusion, diet-induced weight loss associated with insulin-sensitizing effects promotes an increase in the amount of HMW, MMW and LMW adiponectin multimeric complexes in plasma. No direct relationships between the diet-induced changes in individual adiponectin multimeric complexes and those of insulin sensitivity were found. Further studies elucidating the physiological relevance and function of adiponectin multimeric complexes with respect to obesity and insulin resistance are warranted.

My main contribution to this work was collaboration on western blot analysis of adiponectin multimeric complexes.

3.4. Publication D: Adiponectin and contamination with PCB 153

Negative association between plasma levels of adiponectin and polychlorinated biphenyl 153 in obese women under non-energy-restrictive regime.

Epidemiological studies have shown that some of the persistent organic pollutants, namely PCB 153 – one of 209 known polychlorinated biphenyl congeners – and p,p'-DDE, both highly concentrated POP in humans, determined in plasma, are associated with increased risk of type 2 diabetes [113;114]. PCB 153, used in technical application until the 1970s, is still present in food, as a main source of human exposition with a half life of 27.5 years [115].

The aim of this study was to reveal whether accumulation of the persistent organic pollutants is connected with obesity and insulin resistance. The study was designed as a longitudinal intervention trial, where group of 27 obese women was studied before (OB) and after (OB-LCD) a 3–months low-caloric-diet intervention (LCD; energy intake 5 MJ daily). As a control group, nine women without LCD intervention were used.

Anthropometry (body weight, height and BMI) and plasma analysis (adiponectin, C–reactive protein, interleukin–6, glucose and insulin) were performed in all the subjects. Simultaneously, plasma level concentration of seven various POP the most frequently found in adipose tissue (hexachlorocyklohexane β, hexachlorbenzene, p,p'-DDT, p,p'-DDE and polychlorinated biphenyls congeners: PCB 138, PCB 153, and PCB 180) using gas chromatography-mass spectrometry were determined at both time–point in experimental and control group.

Only levels of PCB 153, not any other measured pollutant, showed statistically significant correlation with plasma levels of adiponectin, and not with any other plasma markers of insulin resistance. The negative correlation between total adiponectin levels was confirmed by using a regression analysis. The parameters of the above described regression model equal r = 2.567, a = 9.2 (p < 0.003, R^2 = 0.332, see also Fig. 3.5). The negative correlation between plasma total adiponectin and CB 153 was not proved in C and disappeared in OB-LCD group, while BMI decreased significantly due to LCD (Table 1 of Publication D).
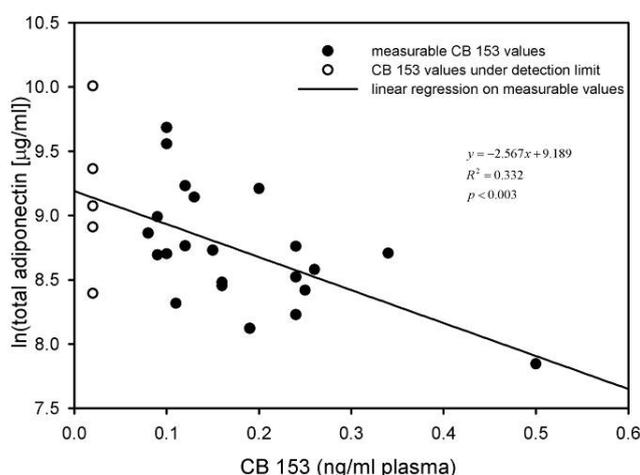
Figure 3.5 Negative correlation between plasma levels of adiponectin and PCB 153

Negative correlation between plasma levels of total immunoreactive adiponectin and PCB 153 in OB. The negative correlation between plasma total adiponectin and PCB 153 was not proved in C (for measurable data only: P<0.123, r=2.28, a=3.38, R^2=0.2692; for pooled data: P<0.337, r=-5.989, a=3.86, R^2=0.0268) and disappeared in OB-LCD, whereas BMI decreased significantly due to LCD (for measurable data only: P<0.026, r=9.921, a=3.56, R^2=0.4037; for pooled data: P<0.139, r=3.803, a=3.77, R^2=0.0782).

To further analyse the effect of PCB 153 on plasma adiponectin, analysis of distribution of multimeric forms was performed. Finding of the negative correlation between total adiponectin and PCB 153 levels in plasma in OB was further supported by a negative correlation between both high and medium molecular weight form of adiponectin and PCB 153 (p<0.01, and p < 0.05, respectively). Interpretation of adiponectin multimeric forms analysed in OB-LCD samples was not possible due to poor quality of plasma.

To take into account complex network of relations between various markers, monofactorial analysis was used to reveal any interesting correlations without success. However, using mathematical modelling of dependence of adiponectin on insulin and PCB 153 suggests that insulin affects the interaction between PCB 153 and adiponectin (see Results of Publication D).

Our results suggest suppression of adiponectin by PCB 153 in obese women under non–energy–restrictive regime, which may contribute to the association of PCB 153 (and other POP) with type 2 diabetes. Indeed, in support of this notion, a negative association between POP and adiponectin was found with tetrachlorodibenzo-p-dioxin [102] or PCB 77 [103]. PCB 153 interacts with cytochrome P450, the CYP 1A and

especially with CYP 2B enzymes, and interestingly, xenobiotic-mediated increases of CYP 2B in rat hepatocytes is augmented by insulin [106], and expression of CYP 2B is increased in diabetic patients. Furthermore, n–3 PUFA downregulate phenobarbital-induced CYP 2B expression [116], whereas they upregulate adiponectin and improve glucose homeostasis [78]. Therefore, a hypothesis may be tested, whether PCB 153 downregulates adiponectin through induction of CYP 2B, or whether adiponectin and CYP 2B represent two independent targets for PCB 153 in adipose tissue. Further studies should be performed to reveal (i) whether it was the negative energy balance or the new achieved lighter body weight in itself that resulted in the disappearance of negative correlation between adiponectin and PCB 153; and (ii) whether PCB 153 was not only a biomarker of body exposition to some other biological active pollutant, which has not been measured.

In conclusion, we found a negative association between plasma levels of PCB 153 and adiponectin, including its HMW and MMW forms, in obese women in non–diet–restrictive regime. The interaction between PCB 153, and possibly also other POP, and adiponectin may contribute to the relatively high risk of development of type 2 diabetes in humans exposed to environmental pollutants.

To further explore the link between adiponectin and PCB 153, series of experiments on mice was performed. Lean or obese mice were treated by oral gavage or intraperitoneal injection of PCB 153 (35 and 70 mg /kg body weight) or PCB 77 (50 mg/kg body weight) dissolved in corn oil and after 14 days plasma adiponectin concentration and distribution of multimeric forms were analysed. Unfortunately, contamination by PCBs resulted either to death and wasting away effects or to the state, when none change in adiponectin were observed. Using mouse model we were not able to mimics the long-term exposition of obese organism to POP (not shown).

My main contribution to this work was analysis of distribution of adiponectin multimeric forms, analysis of the clinical data, and complementary mouse study.

4. CONCLUSIONS

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

1. Additional beneficial effects of the combined treatment by n–3 PUFA of marine origin and anti-diabetic drug rosiglitazone on obesity and related disorders were proven in mice fed high-fed diet. In comparison with either n–3 PUFA or TZD effects alone, the combined treatment was more potent in amelioration of the following adverse phenotypes:

   a. body weight, body weight gain and body lipid content;
   b. dyslipidaemia;
   c. muscle insulin resistance demonstrated as enhanced muscle glycogen synthesis;
   d. obesity-associated inflammation of adipose tissue; and
   e. in addition, additive induction of adiponectin was found, especially its multimeric form, which induces insulin sensitivity.

   These results on mouse model suggest possible application for human medicine, where fish oil concentrates may enhance efficacy of the treatment by TZD, and reduce the risk of the adverse effect of the TZD-therapy.

2. Relatively specific induction of lipid oxidation by n–3 PUFA in the intestine was found, which may be important for e.g. anti-obesity and hypolipidemic effects of these lipids.

3. Changes in adiponectin concentration as well as distribution of its multimeric forms as a result of low caloric diet were found in human patients.

4. Association between lower levels of adiponectin as well as of its high molecular weight form in plasma and contamination by persistent organic pollutant PCB 153 was proven in obese patients.

5. LIST OF PUBLISHED PAPERS

Published articles

- **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
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  Rosiglitazone increases hepatic synthesis and plasma levels of palmitoleate in high-fat diet-fed mice

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