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Amelioration of obesity –associated disorders by *n*-3 PUFA and oleuropein: adipocentric view

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Statement of authorship

I certify that the thesis represents valid work elaborated under the supervision of Pavel Flachs, RNDr, PhD, 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.

In Prague …….

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Statement of co-authors

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

In Prague ……..

RNDr. Pavel Flachs PhD

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CONTENTS

ABSTRAKT

Téměř miliarda a půl dospělých na celém světě trpí nadváhou nebo obezitou (WHO, 2008). Onemocnění spojená s obezitou, jako diabetes 2. typu, dyslipidemie či další nemoci, která se při souběžném výskytu alespoň dvou z nich označují za „metabolický syndrom“, vedou velmi často ke kardiovaskulárním chorobám a globálně jsou považovány za jeden z nejpalčivějších zdravotních problémů. Komplexní etiologie těchto onemocnění zahrnuje jak genetické tak i environmentální faktory, jež se různým způsobem projevují v nárůstu onemocnění u jednotlivých pacientů. Léčba metabolického syndromu vyžaduje strategie, které kombinují různé farmakologické přístupy s odlišnými účinky. Kromě nich je ovšem třeba změnit i životní styl. Změna životního stylu, podpořená vyšší fyzickou aktivitou, nižším celkovým energetickým příjmem a kladením důrazu na nutriční složení potravy, je přitom sama o sobě nejlepší prevencí metabolického syndromu.

U polynenasycených mastných kyselin (FA) s dlouhým řetězcem řady *n*- 3 PUFA z mořských ryb, zejména eikosapentaenové (EPA; 20:5 *n*- 3) a dokosahexaenové (DHA ; 22:6 *n*- 3) kyseliny, bylo prokázáno, že působí jako přírodní hypolipidemika, brání rozvoji inzulínové rezistence a hrají klíčovou roli v patofyziologii metabolického syndromu. Další studie prokázaly inverzní vztah mezi citlivostí na inzulín a stupněm nasycení přijímaných lipidů. Studie na myších imbredních kmenech, které mají dietou indukovanou obezitu (C57BL/6), nám dávají důležité poznatky o jejich vlivu na savčí tkáň a umožňují nám testovat potenciální terapeutické postupy. Dále nám dovolují rozšířit naše studie o možné terapeutické využití kombinací *n*- 3 PUFA s antidiabetiky používané v klinické praxi.

n-3 PUFA snižují chronický zánět, který vzniká při obezitě v bílé tukové tkáni. Ten se vyznačuje tím, že při něm dochází ke změně sekrece adipokinů, které přispívají k rozvoji inzulínové rezistence. Adipocyty a infiltrující makrofágy přispívají ke změně sekrečních vlastností WAT při obezitě, což vede k výrazným změnám ve struktuře buněk a složení tkáně. Protizánětlivé účinky *n*- 3 PUFA pravděpodobně závisí na tvorbě jejich metabolitů, a to jak v adipocytech tak i makrofázích. Tyto lipidové mediátory, jako jsou prostaglandiny, resolviny a

protektiny, pocházejí buď z cílené enzymatické syntézy nebo z neenzymatických oxidačních reakcí. Mohou pak působit jako ligandy pro receptory na povrchu buněk, nebo mohou ovlivňovat signální proteiny, včetně transkripčních faktorů PPARγ a NF-kB atd.

EPA a DHA mohou být vytvořeny v lidském těle při relativně nízké rychlosti z esenciální α - linolenové kyseliny (ALA , 18:3 *n*- 3). Vzhledem k nízké spotřebě mořských ryb, různé odborné společnosti na celém světě doporučují zvýšení příjmu EPA + DHA na 0,5-1,0 gramy/den, čehož by bylo možné dosáhnout konzumací dvou jídel tučných mořských ryb týdně, nebo prostřednictvím výživových doplňků (omega 3). Také u pacientů s diabetem se doporučuje zvýšit příjem *n*-3 PUFA.

Působení na transkripční faktor PPARγ se děje i prostřednictvím anti-diabetických léčiv z rodiny thizolidinediony (TZD), které aktivují tento jaderný faktor a zvyšují tak inzulínovou citlivost zvýšením lipidové absorpce a mitochondrialní β-oxidace v bílé tukové tkáni. K aktivaci mitochondriální biogeneze a β-oxidace v bílé tukové tkání dochází jak působením TZD, tak i působením *n*-3 PUFA. Děje se tak přes důležité jaderné transkripční faktory PPARα/γ a PPARγ koaktivátor, PGC-1α.

Polyphenoly, jako například nejznámější resveratrol z vinných hroznů či katechiny ze zeleného čaje, ovlivňují celou řadu biochemických reakcí. Resveratrol má kromě jiných pozitivních účinků na organismus příznivý účinek na celkový obsah tělesného tuku a na ukládání bílé tukové tkáně u myší. Oleuropein, polyphenol extrahovaný z listů nebo plodů olivovníku, je jednou z mnoha složek olivového oleje. Polyphenolům je připisován i příznivý vliv na tukovou tkáň. Je známo, že mají vliv již na diferenciaci tzv. pre-adipocytů do vyzrálých tukových buněk. Důležitým jaderným transkripčním faktorem, který řídí diferenciaci a lipidový metabolismus v adipocytech je PPARγ.

Podkladem pro tuto disertační práci jsou tři publikované články (A, B a C). Všechny tři práce se věnovaly problematice bílé tukové tkáně. V těchto pracích jsme ukázali, že bílá tuková tkáň je flexibilní orgán a *n*-3 PUFAs, rostlinné polyphenoly, kalorická restrikce a TZD mají významnou úlohu v její biologii.

Tato disertační práce ukazuje, že EPA a DHA hrají důležitou roli v lipidové a glukózové homeostázi. Obohacení potravy o *n*-3 PUFAs, zvláště pak v kombinaci s antidiabetickými léčivy

převážně s rosiglitazonem či pioglitazonem nebo kalorickou restrikcí, by se mohlo stát důležitou součástí léčby a prevence onemocnění, která jsou spojená s obezitou.

Obecným cílem našeho výzkumu je napomoci léčbě obezity a souvisejících onemocnění podáváním *n*-3 PUFA mastných kyselin mořského původu v kombinaci s dalšími nefarmakologickými přístupy, zejména mírnou kalorickou restrikcí nebo užíváním rostlinných derivátů.

ABSTRACT

Globally 1, 5 billion adults are overweight or obese according to WHO. Obesity-associated diseases, namely type 2 diabetes, dyslipidemia and other morbidities clustered in the 'metabolic syndrome' predispose individuals to cardiovascular disease and represent a significant health problem around the world. Complex etiology of these diseases involves both genetic and environmental factors with a different contribution to disease progression in various individuals. Treatment of metabolic syndrome requires strategies combining several pharmacological approaches with multiple mechanisms of action. Besides these interventions, lifestyle changes are inevitable, and they are extremely important for the prevention of metabolic syndrome. Thus, physical activity, total energy intake, macronutrient composition of the diet, as well as minor dietary constituents are all important.

Long-chain polyunsaturated fatty acids (FAs) of *n*-3 series (*n*-3 PUFA) from marine fishes, mainly eicosapentaenoic (EPA; 20:5 *n*-3) and docosahexaenoic (DHA; 22:6 *n*-3) acids, have been shown to act as natural hypolipidemics, while preventing development of insulin resistance, the key feature in the pathophysiology of metabolic syndrome. Other studies have demonstrated an inverse relationship between insulin sensitivity and the degree of saturation of parent lipids. Studies with an obesity-prone mouse model (C57BL/6) provide important knowledge regarding their effect on mammalian tissues and to test potential therapeutic interventions. Further, this model allows us to extend our studies on the therapeutic use of a combination of *n*-3 PUFA with antidiabetic drugs.

n-3 PUFA decrease age- or disease-related chronic inflammation, including obesity-associated low-grade inflammation of WAT characterized by altered secretion patterns of adipokines, which contributes to development of insulin resistance. Both adipocytes and infiltrating macrophages contribute to changing secretory properties of white adipose tissue during obesity, reflecting pronounced changes in tissue structure and cellular composition. The anti-inflammatory effects of *n*-3 PUFA probably depend on the formation of their metabolites, both in adipocytes and in macrophages. These lipid mediators originate from either targeted enzymatic synthesis or no*n*-enzymatic oxidative reactions, such as prostaglandins, resolvins and protectins. They can act as ligands for surface receptors or can interact with signaling proteins

including PPARγ and NF-κB. Namely resolvins and protectins have pro-resolving and anti-inflammatory effects.

EPA and DHA are essential fatty acids, which could be elongated in the human body at a relatively low rate from α-linolenic acid (ALA;18:3 *n*-3). Due to the low consumption of sea fish, various professional societies worldwide recommend in their guidelines to increase the intake of EPA+DHA to 0.5-1.0 g/day, which could be accomplished by two meals of fatty sea fish weekly, or by means of encapsulated (omega 3) nutritional supplements. Additionally, diabetic patients may be advised to increase their intake of *n*-3 PUFA.

Thizolidinediones (TZDs) are a class of anti-diabetic drugs that activate the nuclear transcription factor, PPARγ, pathway resulting in increased insulin sensitivity through increased lipid absorption and mitochondrial β-oxidation in white adipose tissue.
For instance, both TZDs and *n*-3 PUFA induce mitochondrial biogenesis and β-oxidation. Mitochondrial induction involves transcription factors PPARα/γ and PPARγ coactivator, PGC-1α.

Polyphenols, the most well known of which is resveratrol from grapes, or catechins from green tea, affect a wide variety of biochemical reactions. Oleuropein, a polyphenol extracted from olive tree leaves is one of many components of olive oil. Polyphenols are attributed to a beneficial effect on adipose tissue. It is known to have an effect on the differentiation of the already pre-adipocytes into mature adipocytes important nuclear transcription factor for lipogenesis and overall lipid metabolism is PPARγ.
This thesis is based on three published articles (A, B and C). In the publications, we have shown that white adipose tissue is a flexible body and polyphenols, *n*-3 PUFAs, TZDs play an important role in its biology.

This dissertation has shown that EPA and DHA play an important role in lipid and glucose homeostasis. Enrichment of food of *n*-3 PUFAs, especially in combination with TZDs or caloric restriction, could become an important part of prevention and treatment of comorbidities associated with obesity.

LIST OF OWN PUBLICATION

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

A. Kus V, Flachs P, Kuda O, Bardova K, Janovska P, **Svobodova M**, Jilkova ZM, Rossmeisl M, Wang-Sattler R, Yu Z, Illig T, Kopecky J. *Unmasking differential effects of rosiglitazone and pioglitazone in the combination treatment with n-3 fatty acids in mice fed a high-fat diet.* PLoS One. 2011;6(11), (IF= 3.73)

B. Flachs P, Rühl R, Hensler M, Janovska P, Zouhar P, Kus V, Macek Jilkova Z, Papp E, Kuda O, **Svobodova M**, Rossmeisl M, Tsenov G, Mohamed-Ali V, Kopecky J. *Synergistic induction of lipid catabolism and anti-inflammatory lipids in white fat of dietary obese mice in response to calorie restriction and n-3 fatty acids.* Diabetologia 54: 2626-38, 2011, (IF= 6.973).

C. **Svobodova M**, Andreadou I, Skaltsounis AL, Kopecky J, Flachs P. *Oleuropein as an inhibitor of peroxisome proliferator-activated receptor gamma.* Genes Nutr. 2014 Jan;9, (IF= 3.33)

The above papers are included in full in this PhD thesis (see ENCLOSURES).

LIST OF ABBREVIATIONS

15d-PGJ_2	15-deoxy-$\Delta^{12,14}$ - prostaglandin J_2
AA	arachidonic acid
AMPK	AMP–activated protein kinase
BAT	brown adipose tissue
cHF	corn oil based high-fat diet
cHF+CR	cHF diet in combination with 10% of calorie restriction
cHF+F	cHF diet supplemented with fish oil
cHF+F+CR	cHF+F diet in combination with 10% of calorie restriction
cHF+F+TZD	cHF+F diet supplemented with thiazolidinedione
cHF+TZD	cHF diet supplemented with thiazolidinedione
COX3	cytochrome c oxidase subunit III
CR	calorie restriction
DAG	diacylglycerol
DHA	docosahexaenoic acid (22:6 *n*-3)
EPA	eicosapentaenoic acid (20:5 *n*-3)
FA	fatty acid
GLUT4	glucose transporter 4
HMW	high-molecular weight
IL	interleukin
IRS	insulin receptor substrate
NEFA	non-esterified fatty acids
NFκB	nuclear factor NF-kappa-B
NRF-1	nuclear respiratory factor 1
PGC-1α	peroxisome proliferative activated receptor, gamma, coactivator 1α
PI3K	phosphatidylinositol 3-kinase
PPAR	peroxisome proliferator-activated receptor

PUFA	polyunsaturated fatty acid
RQ	respiratory quotient
SCD-1	stearoyl-Coenzyme A desaturase 1
SREBP1	sterol regulatory element binding protein 1
TG	triacylglycerol
TNFα	tumor necrosis factor-alpha
TZD	thiazolidinediones
UCP1	uncoupling protein 1
VLDL	very low-density lipoprotein
WAT	white adipose tissue

1 INTRODUCTION

1.1 White fat, obesity and metabolic syndrome

White adipose tissue (**WAT**) is crucial for storage of metabolic energy. Excessive accumulation of WAT underlies obesity, which represents an increasing health care problem. The obesity leads to various chronic morbidities, including type 2 diabetes, dyslipidaemia, and cardiovascular disease, together called as metabolic syndrome. Insulin resistance is a key event in the pathophysiology of metabolic syndrome that may be detected many years before the clinical onset of hyperglycaemia. Normally, insulin released from pancreatic β - cells acts through specific receptors in target tissues , i.e. in muscle and adipose tissue and hepatocytes, which produces metabolic response (insulin increases glycogen synthesis, and operates lipogennesis and proteoanabolic), and causes the transport of glucose transporters using GLUT- 4 to the cell interior (hypoglycaemic effect). Among secretion and insulin action was observed an inverse relationship, which has a hyperbolic course. This means that the increase in an insulin secretion is associated with a decrease in insulin sensitivity, and *vice versa*.

Definition of Metabolic syndrome

By Mayo Clinic Staff

Metabolic syndrome is a cluster of conditions — increased blood pressure, a high blood sugar level, excess body fat around the waist and abnormal cholesterol levels — that occur together, increasing your risk of heart disease, stroke and diabetes.

Having just one of these conditions doesn't mean you have metabolic syndrome. However, any of these conditions increase your risk of serious disease. If more than one of these conditions occur in combination, your risk is even greater.

If you have metabolic syndrome or any of the components of metabolic syndrome, aggressive lifestyle changes can delay or even prevent the development of serious health problems.

Therefore, the hypertrophic fat cells enhance release of fatty acids (**FAs**) consequently the increasing accumulation of lipids in the peripheral tissues (so-called lipotoxicity). In fact, hypertrophic adipocytes themselves become resistant to insulin, which results in lower clearance of plasma triacylglycerols (**TG**) and higher FAs release from the adipose tissue. In

addition to FAs, also various adipocyte-secreted proteins (adipokines), like leptin, adiponectin, tumour necrosis factor α (TNFα), interleukin-6 (IL-6), and visfatin modulate sensitivity of other tissues to insulin and may be involved in the induction of systemic insulin resistance (Tilg and Moschen, 2006) (see Table 1.). Through the secretion of adipokines, WAT is involved in the control of energy balance, body temperature, immune response, blood clotting, bone mass, and thyroid and reproductive functions, as well as some other functions (Flachs et al 2009).

Moreover, hypertrophic WAT secretes various pro-inflammatory cytokines, including TNF-α (tumour necrosis factor-α), IL (interleukin)-6, IL-1 and MCP-1 (monocyte chemoattractant protein-1), and also mediators of the clotting processes, such as PAI-1 (plasminogen activator inhibitor-1) and certain complement factors (Tilg and Moschen, 2006). In fact, systemic low-grade inflammation has been proposed to play an important role in the pathogenesis of obesity-related insulin resistance(Wellen and Hotamisligil, 2005). Thus, adipose tissue of obese individuals contains a large number of macrophages that represent an additional source of pro-inflammatory cytokines (Weisberg et al., 2003), including TNF-α and other insulin-resistance-promoting adipokines (Hotamisligil, 2006). MCP-1 has been identified as a potential factor contributing to macrophage infiltration in adipose tissue (Kanda et al., 2006). Importantly, part of the anti-inflammatory effects of TZDs could result from the activation of M2 macrophages, at least in peripheral blood (Bouhlel et al., 2007). This mechanism might also be important for the suppression of a low-grade inflammation in adipose tissue (Tilg and Moschen, 2006).(See Fig. 1.)

The role of WAT in storing and releasing lipids for oxidation by skeletal muscle and other tissues became so firmly established decades ago that a persistent lack of interest hindered the study of the extraordinarily dynamic behavior of adipocytes.

WAT is a type of connective tissue which plays an important role in the functioning of the body. It is not an inert cell mass contributing only to storage of fat but also functions as an endocrine organ, contributing to inflammation and the innate immune response.

Epidemiological studies have found that the accumulation of visceral fat associates with metabolic disease (i.e. insulin resistence, type 2 diabetes, dyslipidemia, hypertension, atherosclerosis, hepatic steatosis, and cancer) while the accumulation of subcutaneous WAT

associates with improved insulin sensitivity and low risk for developing type 2 diabetes (Carey et al., 1997; Meisinger et al., 2006).

Table 1. Adipokines. Pro-inflammatory adipokines or Anti-inflammatory adipokines. Adapted from (Ouchi et al., 2011)

Adipokine	Primary source(s)	Binding partner or receptor	Functions
Leptin	Adipocytes	Leptin receptor	Appetite control through the central nervous system (for additional information see chapter 1.1.3)
Adiponectin	Adipocytes	Adiponectin receptors 1 and 2, T-cadherin, calreticulin–CD91	Insulin sensitizer, anti-inflammatory (for additional information see chapter 1.1.4)
RBP4	Adipocytes, liver, macrophages	Retinol (vitamin A), transthyretin	Implicated in systemic insulin resistance
Lipocalin 2	Adipocytes, macrophages	Unknown	Promotes insulin resistance and inflammation through TNF secretion from adipocytes
ANGPTL2	Adipocytes, other cells	Unknown	Local and vascular inflammation
TNF	Adipocytes, stromal vascular fraction cells	TNF receptor	Inflammation, antagonism of insulin signalling
IL-6	Adipocytes, stromal vascular fraction cells, liver, muscle	IL-6 receptor	Changes with source and target tissue
IL-18	Stromal vascular fraction cells	IL-18 receptor, IL-18 binding protein	Broad-spectrum inflammation
CCL2	Adipocytes, stromal vascular fraction cells	CCR2	Monocyte recruitment

CXCL5	Stromal vascular fraction cells (macrophages)	CXCR2	Antagonism of insulin signalling through the JAK–STAT pathway
Visfatin (NAMPT)	Adipocytes, macrophages, other cells	Unknown	Monocyte chemotactic activity
Resistin	Adipocytes (rodent), peripheral blood mononuclear cells (human),	Unknown	Promotes insulin resistance and inflammation through IL-6 and TNF secretion from macrophages
SFRP5	Adipocytes	WNT5a	Suppression of pro-inflammatory WNT signalling

ANGPTL2, angiopoietin-like protein 2; CCL2, CC-chemokine ligand 2; CXCL5, CXC-chemokine ligand 5; IL, interleukin; JAK, Janus kinase; NAMPT, nicotinamide phosphoribosyltransferase; RBP4, retinol-binding protein 4; SFRP5, secreted frizzled-related protein 5; STAT, signal transducer and activator of transcription; TNF, tumour necrosis factor.

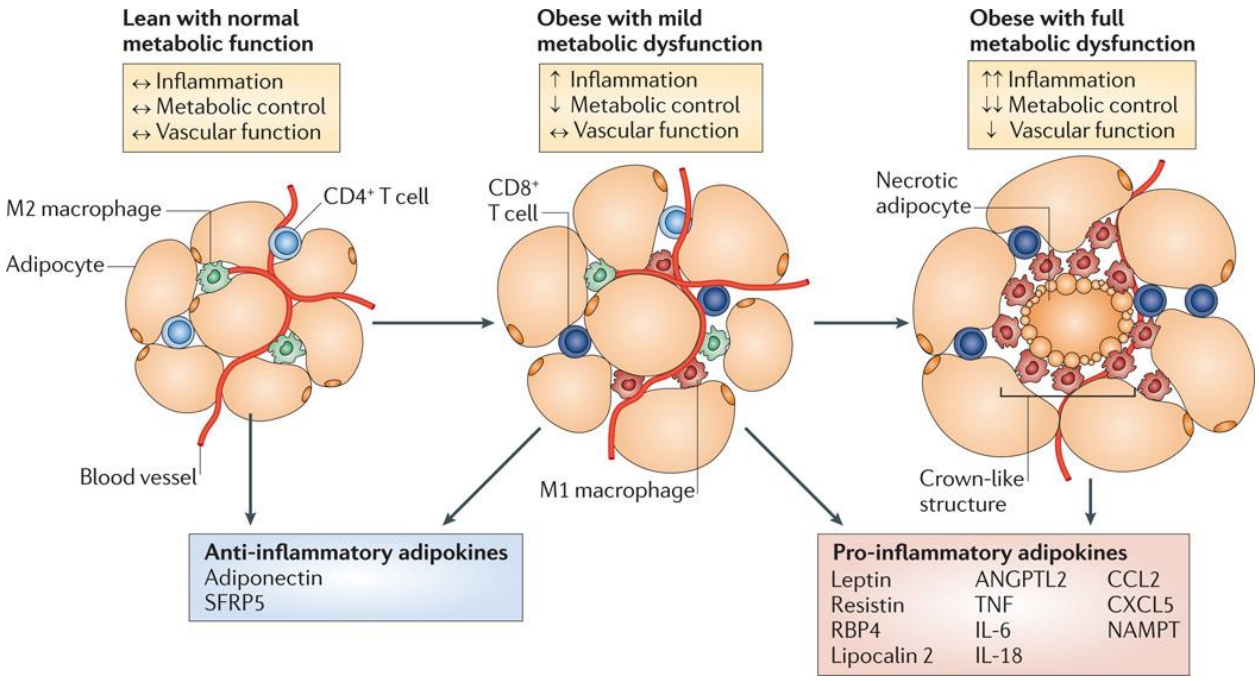

Fig.1. Adipose tissue can be described by at least three structural and functional classifications: lean with normal metabolic function, obese with mild metabolic dysfunction and obese with full metabolic

dysfunction. As obesity develops, adipocytes undergo hypertrophy owing to increased triglyceride storage. With limited obesity, it is likely that the tissue retains relatively normal metabolic function and has low levels of immune cell activation and sufficient vascular function. However, qualitative changes in the expanding adipose tissue can promote the transition to a metabolically dysfunctional phenotype. Macrophages in lean adipose tissue express markers of an M2 or alternatively activated state, whereas obesity leads to the recruitment and accumulation of M1 or classically activated macrophages, as well as T cells, in adipose tissue. Anti-inflammatory adipokines, including adiponectin and secreted frizzled-related protein 5 (SFRP5), are preferentially produced by lean adipose tissue. In states of obesity, adipose tissue generates large amounts of pro-inflammatory factors, including leptin, resistin, retinol-binding protein 4 (RBP4), lipocalin 2, angiopoietin-like protein 2 (ANGPTL2), tumour necrosis factor (TNF), interleukin-6 (IL-6), IL-18, CC-chemokine ligand 2 (CCL2), CXC-chemokine ligand 5 (CXCL5) and nicotinamide phosphoribosyltransferase (NAMPT). Obese individuals with adipose tissue in a metabolically intermediate state have improved metabolic parameters, diminished inflammatory marker expression and better vascular function compared with individuals that have metabolically dysfunctional adipose tissue. Metabolically dysfunctional adipose tissue can be associated with higher levels of adipocyte necrosis, and M1 macrophages are arranged around these dead cells in crown-like structures. Re-print from (Ouchi et al., 2011).

1.1.1 Brown adipose tissue

In contrast to WAT brown adipose tissue (**BAT**) is an organ of regulatory non-shivering thermogenesis, mediated by uncoupling protein-1 (**UCP-1**) in mitochondria, which are abundant in multilocular brown adipocytes (Cannon and Nedergaard, 2004). In contrast, large unilocular adipocytes of WAT, filled with TG and equipped with a small cytosolic compartment, serve as an energy storage device (Flachs et al 2009).

Brown adipocytes contain multiple smaller (multilocular) lipid droplets, are rich in mitochondria, and reside in depots that are highly innervated and vascularized. Brown adipocytes are specialized for thermogenesis. Brown adipocytes express almost all the genes that are expressed in white adipocytes, but they also express some distinct gene UCP1, which allows energy to be dissipated as heat without generating ATP. Most brown adipose tissue in rodents is located to the interscapular region. Humans have large depots of brown adipose tissue in infancy, but only small amounts that are dispersed throughout depots of white adipose tissue persist in adults (Nicholls, 1983).

1.1.2 Lipid metabolism in adipocyte

The central node of adipocyte metabolism is the complex and finely tuned system of lipolysis/re-esterification. A small shift in the balance among activities of the major metabolic pathways contributing to TAG/FA cycle, i.e. lipolysis of TAG and re-esterification of FA in cytoplasmic lipid droplet, as well as in associated metabolic fluxes such as mitochondrial β-oxidation, cytoplasmic de-novo FA synthesis and glyceroneogenesis could largely affect WAT functions. Notably, activity of TAG/FA cycle also plays a crucial role in transformation of white to brown adipocyte (Czech et al., 2013). The key molecular players that could participate in regulation of TAG/FA cycle are (see Fig. 2): protein kinase B (PKB), protein kinase A (PKA), protein kinase G (PKG), AMP-activated protein kinase (AMPK), pyruvate dehydrogenase kinase 4 (PDK4), p38α mitogen-activated protein kinase (p38 MAPK), peroxisome proliferator-activated receptors α/γ (PPARα/γ), the PPARγ coactivators α/β (PGC-1α/β), ChREBP and sterol regulatory element-binding protein 1c (SREBP-1c).

Thus, PKB (also known as Akt) is a main target for signals that activate phosphoinositide 3-kinase, such as insulin and growth factors. In adipocytes, PKB is required for the insulin-induced translocation of glucose transporter 4 (GLUT4) to the plasma membrane as well as for the effects of insulin on lipid metabolism. Insulin promotes lipid storage by increasing FA uptake, activating de-novo FA synthesis and counteracting catecholamine-induced lipolysis (Yen et al., 2008).

PKA (also known as cAMP-dependent protein kinase) is essential in β-adrenergic stimulation by catecholamines, which leads to increase in cyclic AMP (cAMP) and PKA activation, resulting in phosphorylation of perilipin 1 and HSL. Perilipin-1 phosphorylation facilitates adipose triglyceride lipase (ATGL) activation and the translocation of phosphorylated/activated HSL from the cytoplasm to lipid droplet surface.

PKG activity is dependent on cytoplasmic level of cyclic GMP (cGMP). Cardiac hormones/natriuretic peptides ANP and BNP bind to the natriuretic peptide receptor A (NPRA) to activate of the guanylyl cyclase domain to convert GTP to cGMP, with subsequent activation of PKG, which than phosphorylates perilipin 1 and HSL to enhance lipolysis, similarly as PKA (Herman et al., 2012).

AMPK acts as the key regulator of metabolic pathways in adipocytes. A reduction in the energy charge of the cell (increased AMP/ATP ratio) or increased calcium levels inside the cell evoke activation of AMPK, leading to suppression of ATP-consuming processes (e.g. de-novo FA synthesis) and stimulation of biochemical pathways that promote generation of ATP (e.g. β-oxidation and glucose uptake). Moreover AMPK could stimulate uptake of FA by increasing intravascular lipoprotein lipase (LPL) activity and by modulating intracellular lipolysis of TAG via a complex mechanism (Fig. 2.); (Kusminski and Scherer, 2012). In the long- term, activation of AMPK reinforces these effects through phosphorylation of transcription factors and co-activators that regulate gene expression.

PDK4 is involved in control of fuel partitioning. Mitochondrial pyruvate dehydrogenase (PDH) catalyses the irreversible decarboxylation of pyruvate to acetyl-CoA and CO2 and serves as a metabolic switch between glucose and FA utilization. Its phosphorylation/inhibition by pyruvate PDK4 enables pyruvate to be used for glyceroneogenesis (Naukkarinen et al., 2014). A group of p38 MAPKs are stress-activated protein kinases that respond to cellular stress and cytokines, with roles related to inflammation. In adipocytes, as shown in Fig. 2, p38 MAPK is activated by a cascade downstream of both PKA and PKG (Herman et al., 2012). p38 MAPK then phosphorylates PGC-1α, which boosts its co-activator function as well as phosphorylating activating transcription factor 2 (ATF2/CREBP2) in order to drive expression of PGC-1α itself. p38 MAPK family is involved in the adrenergically-mediated induction of both, PDK4 in WAT (Diraison et al., 2002) and BAT-thermogenic programme (Herman et al., 2012).

PPARs serve as ligand-dependent transcription factors (nuclear receptors), which control expression of genes that function in lipid and carbohydrate metabolism. Various endogenous compounds have been identified as their ligands, including FAs, oxylipins, endocannabinoids and phospholipids (see Fig. 2.); (Bordicchia et al., 2012). PPARγ is the key regulator of adipocyte differentiation as well as lipogenesis in WAT (upstream from both ChREBP and SREBP-1c (Yen et al., 2008).

Dynamic control of biogenesis and respiratory function of mitochondria largely depends on the PGC-1 family of transcriptional regulators (PGC-1α/β); (Cadoudal et al., 2008). These

coactivators can interact with, and regulate the activities of PPARs, ATFs, nuclear respiratory factors and others transcription factors.

ChREBP (also known as MLXIPL) is a transcription factor activated by glucose that is highly expressed in hepatocytes, pancreatic β-cells, myocytes, as well as in brown and white adipocytes. ChREBP target genes are involved in the pathways of glucose and lipid metabolism (see Fig. 2.). ChREBP is inactivated by phosphorylation by both PKA and AMPK. In WAT, glucose-mediated activation of canonical ChREBPα isoform induces expression of ChREBPβ, as an important step in the induction of lipogenesis and increase in insulin sensitivity via adipocyte-derived signalling molecules (Nye et al., 2008). Notably, levels of ChREBP are much higher in BAT than in WAT, which is consistent with the rate of de-novo FA synthesis (Wan et al., 2010).

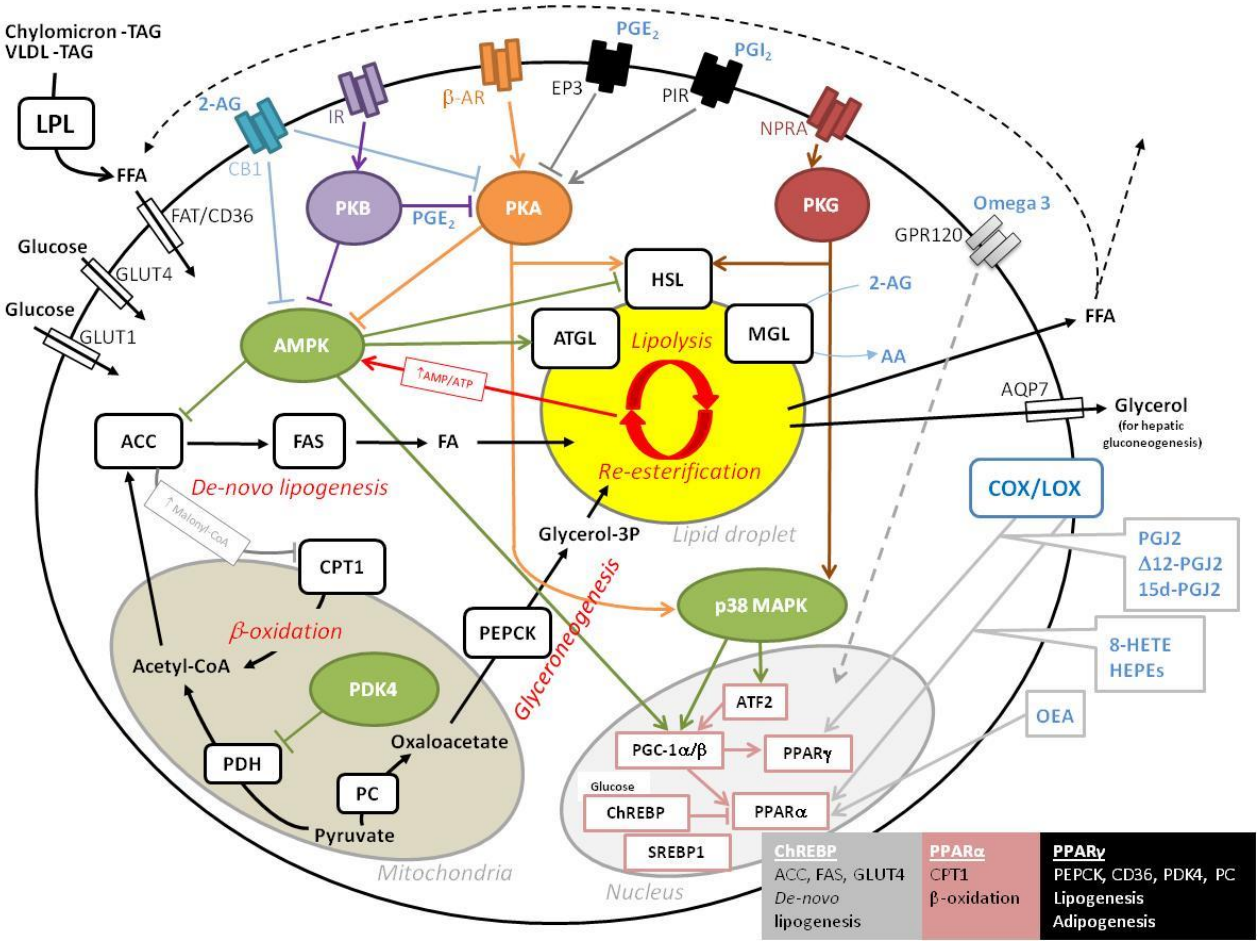

Fig. 2. - Pathways engaged in regulation of adipocyte metabolism. As explained in the main text, the central node of adipocyte metabolism is lipolysis of TAG and re-esterification of FA in cytoplasmic lipid droplet (i.e. TAG/FA cycle). The associated metabolic pathways are de-novo lipogenesis, glyceroneogenesis via phosphoenolpyruvate carboxykinase (PEPCK), and β-oxidation of FA in mitochondria. The induction of TAG/FA [FA liberated from TAG during lipolysis may be released from adipocyte and taken back by FA translocase CD36 (CD36/FAT)] requires ATP, leading to lowering of cellular energy status (an increased AMP/ATP ratio) and activation of AMPK. The AMPK activity compromised by PKB and at the early stages of adrenergic stimulation, also by PKA. AMPK phosphorylates acetyl-CoA carboxylase (ACC), which leads to a decrease in de-novo lipogenesis and to decrease in cellular malonyl-CoA. Malonyl Co-A is an inhibitor of carnitine palmitoyltransferase I (CPT-1), which catalyzes the rate-limiting step in β-oxidation. In longer term, the activation of AMPK could also decrease glucose oxidation by increasing expression of pyruvate dehydrogenase (PDK) 4 by modulation of PGC-1/PPARγ transcriptional activity. Both, PKA and PKG induce lipolysis by direct phosphorylation of HSL and through phosphorylation of perilipin 1 also activate ATGL. PKA and PKG can phosphorylate/activate p38 MAPK. Thus, in response to β-agonist or natriuretic peptides, p38 MAPK phosphorylates the transcriptional regulators ATF2 and PGC-1α, which leading to the induction of expression of PPARα and PPARγ target genes (pink box and black box on the bottom). PKA- but not PKG-

mediated lipolysis is inhibited by insulin (25). Endogenous PPARs ligands are depicting in rectangular callouts on the right. 2-AG, 2-arachidonoyl glycerol; AA, arachidonic acid; AQP7, aquaporin 7; β-AR, β-adrenergic receptor; COX, cyclooxygenase; CB1, cannabinoid receptor type 1; EP3, prostaglandin E receptor 3; FAS, fatty acid synthase; FFA, free fatty acids; GPR120, G protein-coupled receptor 120; GLUT-*, glucose transporter; IR, insulin receptor; LOX, lipoxygenase; MGL, monoacylglycerol lipase; PC, pyruvate carboxylase; PIR, prostaglandin I2 receptor; OEA, oleoylethanolamide; PG*, prostaglandin; VLDL, very-low-density lipoprotein. Reprint from (Masoodi et al., 2014).

1.1.3 Leptin

The discovery of leptin has led to a new era in nutritional biology. Leptin was discovered in mice in 1994 by Jeffrey M. Friedman. Leptin was the first identified adipocytokine, its primary structure is composed of 167 amino acids, and it is primarily expressed in adipose tissue. However, leptin has also been detected in many other tissues, including the placenta, mammary glands, breast milk, testes, ovaries, endometrium, stomach, hypothalamus, and pituitary gland (Al-Suhaimi and Shehzad, 2013). The adipokine leptin is the product of the obese gene (ob; also known as Lep), which was identified in ob/ob mice by positional cloning. Leptin regulates feeding behaviour through the central nervous system. Mice that lack leptin (ob/ob mice) show hyperphagia (abnormally increased feeding), obesity and insulin resistance, and the administration of leptin to ob/ob mice reverses these changes (Ouchi et al., 2011).

A higher amount of leptin is secreted by subcutaneous adipocytes than by the visceral adipocytes. The serum concentration of leptin is predominantly defined by body fat mass (Al-Suhaimi and Shehzad, 2013).

1.1.4 Adiponectin

Adiponectin is a protein produced mainly mature adipocytes and in human plasma is present a large amount approximately 0.01% all plasma proteins (Arita et al., 1999). Adiponectin levels in the plasma and adipose tissue are decreased in obese individuals compared with lean individuals. Consistent with this, the production of adiponectin by adipocytes is inhibited by pro-inflammatory factors, such as TNF and IL-6, as well as by hypoxia and oxidative stress (Ouchi et al., 2011). Adiponectin significantly regulates the metabolism of carbohydrates and

lipids, increased utilization and transport glucose and free fatty acids into muscle, liver and fat cells. In hepatocytes the effect of adiponectin is suppressed gluconeogenesis. These metabolic and insulin-sensitizing effects are mediated activation of the enzyme AMPK (see also chapter 1.3), which represents a kind of central cellular energy crossover. A decrease in cellular ATP (immediately utilization energy substrate) AMPK activated and influence numerous cellular signaling pathways including expression of the appropriate genes mechanisms are triggered leading to the restoration of energy balance in the cell, e.g., oxidation intracellular stored lipid and glucose (Yamauchi et al., 2002).

Adiponectin plasma concentration is a clinically relevant marker comprised of multiple isoforms that may be utilized in a pharmacological context. A group of antidiabetic agents known collectively as thiazolidinediones, (e.g., rosiglitazone) simultaneously decrease insulin resistance significantly while increasing the level of total plasma adiponectin and high-molecular weight isoforms (Pajvani et al., 2004). PPARγ agonists promote adipocyte differentiation, and adiponectin secretion is stimulated in adipocytes by the activation of PPARγ (Ouchi et al., 2011).

1.2 Peroxisome proliferator-activated receptors

PPAR (peroxisome proliferator-activated receptors) belong to the nuclear receptor superfamily of ligand-activated transcription factors. Currently, three PPAR receptors are commonly described: PPARα, PPARβ/δ and PPARγ (Memon et al., 2000).

PPARα is expressed mainly in liver, muscle, kidney (Memon et al., 2000; Su et al., 1998). PPARα participates in the uptake and oxidation of fatty acids and also to regulate the metabolism of lipoproteins (Meertens et al., 1998; Motojima et al., 1998).

PPARβ/δ was found in many tissues but the highest expression is in gut, kidney and heart. It plays a role in the development, epidermal proliferation and embryo implantation (Haluzík and Haluzík, 2006).

PPARγ influences the storage of fatty acids in the adipose tissue. With the C/EBP transcription factors, PPARγ is part of the adipocyte differentiation program that induces the maturation of pre-adipocytes into fat cells. Most of the PPARγ target genes in adipose tissue

are directly implicated in lipogenic pathways, including lipoprotein lipase (LPL), adipocyte fatty acid binding protein (A-FABP or aP2), acyl-CoA synthase and fatty acid transport protein (FATP) (Memon et al., 2000). The PPARγ protein exists in two isoforms that are expressed from the same gene by utilizing distinct promoters. PPARγ2 differs from PPARγ1 by the presence of an additional stretch of 30 amino acid residues in the ligand-independent domain at the N-terminal end resulting in a higher transcriptional activity compared to PPARγ1 (Werman et al., 1997; Zhu et al., 1995). The two PPARγ isoforms also show a distinct expression pattern: PPARγ1 is abundantly expressed in adipose tissue, large intestine, and hematopoietic cells, and to a lower degree in kidney, liver, muscles, pancreas, and small intestine. PPARγ2 is restricted to white and brown adipose tissue under physiological conditions (Vidal-Puig et al., 1997).

1.3 AMP-activated protein kinase

AMP-activated protein kinase (**AMPK**) has been proposed to function as a 'fuel gauge' to monitor cellular energy status in response to nutritional environmental variations. AMPK is a key regulator of cellular and whole-body energy homeostasis that co-ordinates metabolic pathways in order to balance nutrient supply with energy demand. Activation of AMPK protects cells from physiological and pathological stresses that lower cellular energy charge (increase the AMP/ATP ratio) including nutrient starvation, hypoxia/ischaemia and exercise (Oakhill et al., 2009). Glucose uptake as well as fatty acid oxidation is stimulated by AMPK. Contrarily, lipogenesis, cholesterol synthesis and gluconeogenesis are inhibited by AMPK. AMPK functions as a heterotrimeric complex consisting of a catalytic (α) and regulatory (β and γ) subunits. *n*-3 PUFA activates the AMPK and plays that an important role in the regulation of lipid and glucose metabolism (Suchankova et al., 2005). Adipokines such as adiponectin and leptin are also potent activators of AMPK (Hardie, 2008).

The link between adiponectin and AMPK seems to be especially important concerning on the stimulatory effects of *n*-3 PUFA on adiponectin expression/secretion in adipose tissue (Flachs et al., 2006). This suggests that *n*-3 PUFA could modulate some of their metabolic effects through adiponectin-dependent activation of AMPK in the liver and/or skeletal muscle. The importance of a possible link between adipose tissue-derived adiponectin (stimulated by *n*-

3 PUFA) and activation of AMPK in target tissues is further supported by transgenic mice (α2AMPK-KO mice) with inactivated α2 catalytic subunit of AMPK, the major AMPK catalytic subunit in the liver, which demonstrate impaired insulin sensitivity (Viollet et al., 2003) and hypertrophy of adipose tissue (Villena et al., 2004).

The AMPK is an evolutionarily conserved sensor of cellular energy status, which integrates nutritional and hormonal signals in peripheral tissues and the hypothalamus. In response to a rise in the cellular AMP/ATP ratio, AMPK becomes activated through phosphorylation of its catalytic α-subunit by upstream kinases. Activated AMPK then phosphorylates and inactivates a number of enzymes involved in biosynthetic pathways (e.g. FA synthesis, protein synthesis), thus preventing further ATP utilization, while stimulating FA oxidation and mitochondrial biogenesis (Kahn et al., 2005). Activators of AMPK include antidiabetic drugs like metformin and thiazolidinediones (Fryer et al., 2002) as well as adipokines adiponectin (Yamauchi et al., 2002) and leptin (Minokoshi et al., 2002). The activation of AMPK seems to be important for the stimulatory effect of adiponectin on FA oxidation and glucose transport in skeletal muscle, while in the liver AMPK is necessary for the inhibitory effect of adiponectin on PEPCK and glucose 6-phosphatase gene expression as well as on hepatic glucose production (Yamauchi et al., 2002). In addition, AMPK, independently of its kinase activity, co-activates PPAR-α (Bronner et al., 2004). The effect of *n*-3 PUFA on liver lipid accumulation is mediated by adiponectin-AMPK axes as shown in our laboratory (Jelenik et al., 2010).

The AMPK activator, 5-aminoimidazole-4-carboxamide ribonucleuside (AICAR), blocks the expression of the lipogenic genes and transcription factors PPARγ and C/EBPβ if added at the early phase of differentiation of 3T3-L1 adipocytes (Habinowski and Witters, 2001).

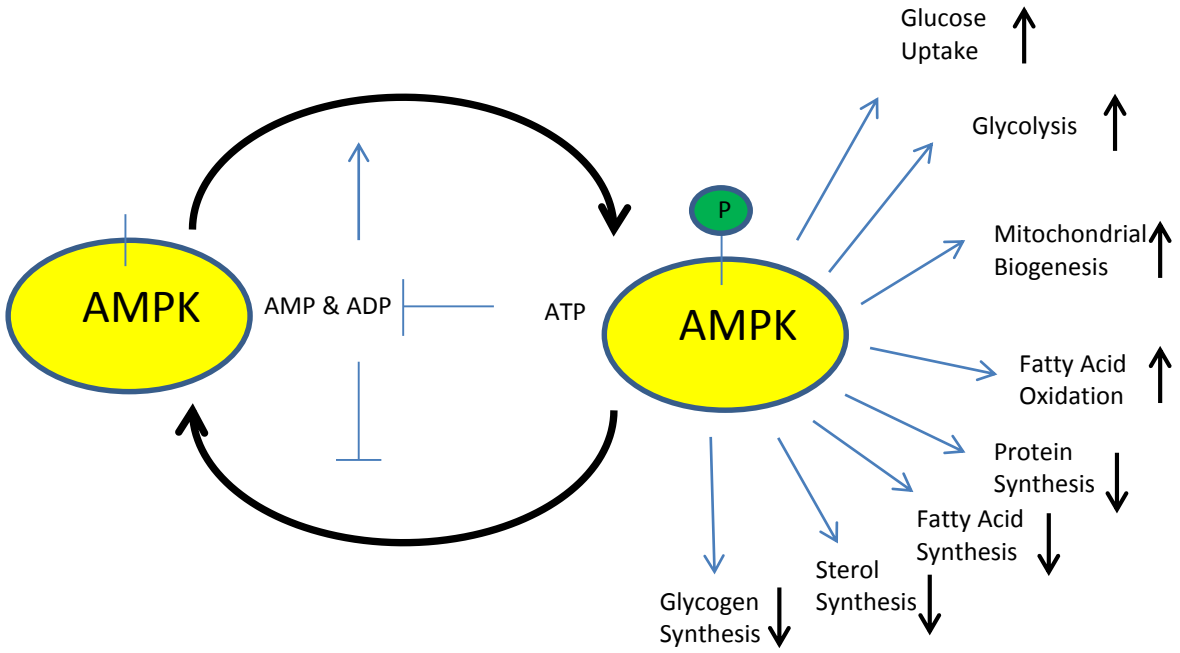

Fig.3. Activated AMPK and its control on metabolism. Activated AMPK mediates its control on metabolism by phosphorylation of many downstream targets, resulting in restored energy homeostasis. Adapted from (Srivastava et al., 2012)

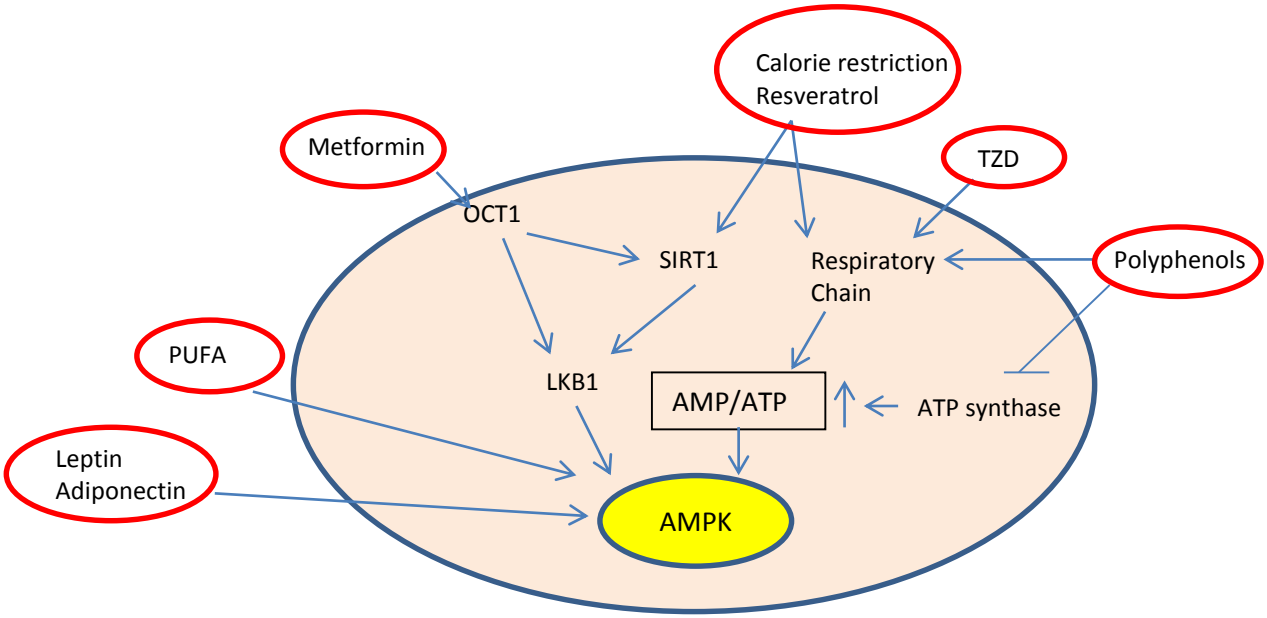

Fig.4. Many activators, such as resveratrol and TZDs, inhibit mitochondrial ATP production and thus activate AMPK indirectly by increasing the cellular AMP : ATP ratio. Resveratrol also acts via SIRT1-mediated mechanism to influence LKB1 and, eventually, phosphorylation of AMPK. The anti-diabetic drugs metformin require the transporter OCT1 to enter cells but also work through modulation of AMP : ATP ratio. Adipokines, leptin, and adiponectin activate AMPK by influencing α subunit indirectly. Adapted from (Srivastava et al., 2012).

1.4 Adipocyte differentiation

In humans, preadipocytes begin to differentiate into adipose tissue during late embryonic development, with a majority of the differentiation occurring shortly after birth (Burdi et al., 1985). *In vitro* study of preadipocyte differentiation is problematic. First, it is difficult to isolate preadipocytes from other fibroblast-like cells. Second, large amounts of fat tissue are required because preadipocytes constitute only a small percentage of total fat tissue. In addition, primary cultures have a limited life span in culture. Nevertheless, the primary culture is widely used. Besides preadipocyte differentiation has been studied by using *in vitro* models of adipogenesis as well. A cell line derived from cloning is homogenous in cell stage of differentiation. This allows for a definitive response to treatments. In addition, these cells can be passaged indefinitely, which provides a consistent source of preadipocytes for experimentation. Adipocyte precursor cell lines can be segregated into two classes, i.e., pluripotent fibroblasts and unipotent preadipocytes. The pluripotent fibroblasts (10T1/2, Balb/c 3T3, 1246, RCJ3.1 and CHEF/18 fibroblasts) have the ability to be converted into several cell types. The second class of culture cells, the unipotent preadipocytes (3T3-L1, 3T3-F422A, 1246, Ob1771, TA1 and 30A5), have undergone determination and can either remain as preadipocytes or undergo conversion to adipose tissue. They are ideal for studying the molecular events responsible for the conversion of preadipocytes into adipocytes. The 3T3-L1 and 3T3-F422A culture lines, derived from disaggregated Swiss 3T3 mouse embryos (Green and Kehinde, 1975) are the most widely used culture models.

The adipogenic conversion of most mouse and human preadipocyte cell lines, including the murine 3T3-L1 cell line, requires stimulation with a pharmacological cocktail. This cocktail consists of the synthetic glucocorticoid dexamethasone, the cAMP elevating agent 1-methyl-3-

isobutyl xanthine (MIX), and pharmacological doses of insulin, which activates both the insulin receptor and the insulin-like growth factor receptor (IGF-R) (Green and Kehinde, 1975). In addition, efficient differentiation of many primary fibroblastic precursors requires supplementation with high affinity agonists of the peroxisome proliferator activated receptor γ (PPAR γ) (Petrovic et al., 2010). (See chapter 1.2)

Adipocytes are derived from mesenchymal stem cells (MESCs), common precursors for adipocytes, osteoblasts, myocytes and chondrocytes.

Adipocyte differentiation involves a temporally regulated set of gene-expression events (Fig.5.).

The key role in the differentiation process is played by transcription factors from the PPAR and C/EBP (CCAAT/enhancer-binding protein) families (Cho et al., 2008). In experiments using cell culture, DHA inhibited adipocyte differentiation and induced apoptosis in post-confluent preadipocytes (Kim et al., 2006). Among adipogenic transcription factors PPAR γ stands out as a key regulator obligate for in vitro as well as in vivo development of adipocytes. PPARγ is a member of the nuclear-receptor superfamily and is necessary and sufficient for adipogenesis (Rosen and Spiegelman, 2000). The expression of PPARγ is sufficient to induce adipocyte differentiation in fibroblasts (Tontonoz et al., 1994) and no factor has been discovered that promotes adipogenesis in the absence of PPARγ. *In vivo* studies in differentiated adipocytes with an inducible knockout of Pparg lead to adipocyte death followed by generation of new adipocytes (Imai et al., 2004).

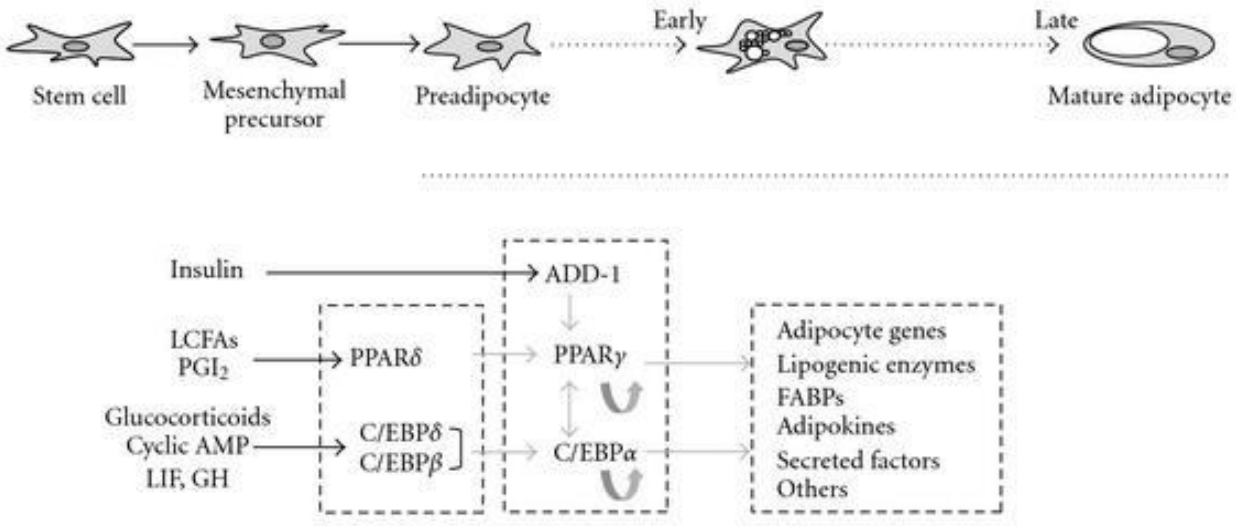

Fig.5. Adipocyte differentiation (adipogenesis) and transcriptional events in adipogenesis. A pluripotent stem cell precursor gives rise to a multipotential mesenchymal precursor cell with a potential to differentiate into an adipocyte. The preadipocyte enters the adipogenesis stage via environmental and gene expression signals. In an early stage of adipogenesis, major transcriptional factors such as PPARγ and C/EBPα are expressed, and these factors strongly regulate the expressions of adipogenesis-related genes. The adipocyte secretes various factors, including adipokines, and the secreted factors play an important role in glucose and lipid metabolism, immune system, appetite regulation, and vascular disease. LCFAs: long-chain fatty acids; PGI_2: prostacyclin; LIF: leukemia inhibitory factor; GH: growth hormone; ADD-1: adipocyte determination and differentiation factor-1; FABPs: fatty acid-binding proteins. (Cho et al., 2008)

1.5 Beneficial effects *n*-3 PUFA

Marine fish oils, namely long-chain (LC) polyunsaturated fatty acids (PUFA) of *n*-3 series (omega-3), such as docosahexaenoic acid (DHA; 22:6*n*-3), eicosapentaenoic acid (EPA; 20:5*n*-3) and docosapentaenoic acid (DPA, 22:5*n*-3) act as natural hypolipidemic and anti-inflammatory agents and enhance various factors of the metabolic syndrome (Flachs et al., 2009; Flachs et al., 2014; Mozaffarian et al., 2013).

Table 2. Nomenclature of omega-3.

Name		Abbreviation		
Trivial	Chemical	Carboxyl-reference	Omega reference	Other
Linolenic acid	9,12,15 - octadecenoic acid	18:3 D9,12,15	18:3*n*-3 or 18:3 ω3	ALA
Eicosapentaenoic acid *Icosapentaenoic acid or Timnodonic acid*	5,8,11,14,17- eicosapentaenoic acid	20:5 D5,8,11,14,17	20:5*n*-3 or 20:5 ω3	EPA
Docosahexaenoic acid	4,8,12,15,19- docosahexaenoic acid	22:6 D4,8,12,15,19	22:6*n*-3 or 22:6 ω3	DHA

Dietary intake of *n*-3 PUFA is associated with a variety of cellular responses including changes in gene expression and FAs composition of plasma membrane phospholipids, which affects the synthesis of eicosanoids, signaling molecules made by oxidation of 20-carbon fatty acid (see chapter 1.5.4), as well as the fluidity of biological membranes (Lombardo and Chicco, 2006).

Concerning of *n*-3 PUFA in the context of obesity-associated insulin resistance, several potential mechanisms have been suggested:

1. Limitation of lipotoxicity in insulin-sensitive tissues
2. Elimination of inflammatory response in obese adipose tissue
3. Beneficial changes in the secretory profile of adipose tissue-derived hormones adipokines, namely adiponectin
4. Formation of biologically active lipid mediators, i.e. resolvins and protectins (See Fig.6)
5. Activation of AMP-activated protein kinase (AMPK) (See chapter 1.3)

1.5.1 Limitation of lipotoxicity in insulin-sensitive tissues

Intracellular accumulation of FA metabolites (e.g. diacylglycerol, fatty acyl-CoAs, ceramides) in non-adipose tissues, as found in obesity, is known to inhibit insulin signaling and glucose uptake

(Muoio and Newgard, 2008). Dietary *n*-3 PUFA down-regulate the expression of lipogenic genes while up-regulating genes involved in FA catabolism (Clarke, 2001). These effects are largely mediated by peroxisome proliferator-activated receptors (PPARs), with PPAR-α and PPAR-δ (-β) representing the main targets for *n*-3 PUFA (Forman et al., 1997); however, other factors are also involved, namely sterol regulatory element-binding protein-1c [SREBP-1c; (Teran-Garcia et al., 2007)] and carbohydrate-responsive element-binding protein [ChREBP; (Dentin et al., 2005)]. The inhibitory effects of *n*-3 PUFA on lipogenic genes such as fatty acid synthase (FAS) and stearoyl-CoA desaturase-1 (Scd-1) seems to be especially important, since decreased Scd-1 has been shown to protect against obesity and hepatic steatosis (Cohen et al., 2002), and inhibition of Scd-1 by adipose tissue-derived lipokines such as palmitoleate (16:1*n*-7) has beneficial effects on hepatic insulin sensitivity (Cao et al., 2008). Neschen et al. have recently shown important roles for PPAR-α and diacylglycerol in the maintenance of hepatic insulin sensitivity and the regulation of hepatic glucose production by n-3 PUFA (Neschen et al., 2007). *n-3* PUFA induce mitochondrial biogenesis and oxidative capacity in adipose tissue of mice by up-regulating PPAR-γ coactivator-1α (PGC-1α) and nuclear respiratory factor-1, respectively, thus possibly preventing the exposure of non-adipose tissues to excess FA released from fat depots (Flachs et al., 2005).

Dietary *n*-3 PUFA down-regulate lipogenic genes within the liver and adipose tissue, while up-regulating the expression of genes involved in lipid oxidation (Raclot and Oudart, 1999), resulting in the induction of FA oxidation in mitochondria and peroxisomes. These effects are largely mediated by transcription factors peroxisome proliferator-activated receptors (PPARs). In adipose tissue, ligand binding activates PPARγ, which results in the expression of genes involved in adipocyte differentiation and genes involved in lipogenesis and lipid transport. However, FA oxidation in adipocytes can be also induced through PPARα and PPARδ (Forman, Chen, and Evans 1997;Luquet et al. 2005).

Besides their interactions with PPARs, *n*-3 PUFA also affect adipose tissue through the formation of eicosanoids and other biologically active lipids, acting as pro- and anti-inflammatory regulators. In response to high fat diets, tissues mainly produce the pro-inflammatory lipid mediators, while converting arachidonic acid into series 2 prostaglandins, thromboxanes and

leukotrienes. On the other hand, when significant amounts of EPA and DHA are given, the less inflammatory series 3 prostaglandins and thromboxanes are formed. In this context, EPA and DHA could also give rise to anti-inflammatory and pro-resolving mediatore molecules such as lipoxins and resolvins (Serhan, 2005; Smith, 2005)). Indeed, *n*-3 PUFA could reduce adipose tissue inflammation in genetically obese mice (Todoric et al., 2006).

1.5.2 Elimination of inflammatory response in obese adipose tissue

Adipose tissue in obesity is characterized by inflammatory changes and hypertrophic adipocytes, secreting various inflammatory cytokines (Tilg and Moschen, 2006). It has been shown that adipose tissue inflammation and macrophage infiltration induced by high-fat feeding could be reduced by *n*-3 PUFA in genetically diabetic mice (Todoric et al., 2006) as well as in C57BL/6 mice with dietary obesity (Kuda et al., 2009). Thus, limitation of adipose tissue inflammation could be an important mechanism in the beneficial effects of *n*-3 PUFA on obesity-associated insulin resistance.

1.5.3 Beneficial changes in the secretory profile of adipose tissue-derived hormones adipokines, namely adiponectin

n-3 PUFA, administered in the context of either high-fat (Flachs et al., 2005; Neschen et al., 2006) or sucrose-rich (Rossi et al., 2005) diet, can stimulate secretion of insulin-sensitizing hormone adiponectin in rodents (see chapter 1.1.4). Since obesity-associated insulin resistance is closely related to low plasma adiponectin (Abbasi et al., 2004), increased adiponectin levels by *n*-3 PUFA should have beneficial metabolic effects.

1.5.4 Formation of biologically active lipid mediators, i.e. resolvins and protectins

n-3 PUFA decrease the production of classic inflammatory mediators such as arachidonic acid-derived eicosanoids and inflammatory cytokines (Calder, 2006). In addition, EPA and DHA have been shown to serve as substrates for the conversion to a series of lipid mediators designated resolvins and protectins, which mediate the beneficial effects of *n*-3 PUFA on adipose tissue and protect the liver from steatosis (González-Périz et al., 2009).

The formation of eicosanoids starts with release of the parent fatty acid from the membrane by the action of phospholipase enzymes. The released fatty acid then acts as a substrate for a number of cyclooxygenase, lipoxygenase enzymes or spontaneous formation - autooxidation. Conversion by cyclooxygenase give rise to eicosanoids called prostaglandins and tromboxanes. The fatty acids profile in the membrane of cells is important for which prostaglandins, leukotrienes or tromboxanes will be produced. Arachidonic acid is given rise to series 2 prostaglandins and tromboxanes and series 4 leukotrienes, which are pro-inflammatory in character. In contrast, EPA and DHA is a precursor to the series 3 prostaglandins and tromboxanes and series 5 leukotrienes, which have anti-inflammatory or pro-resolving character (see Fig.6).

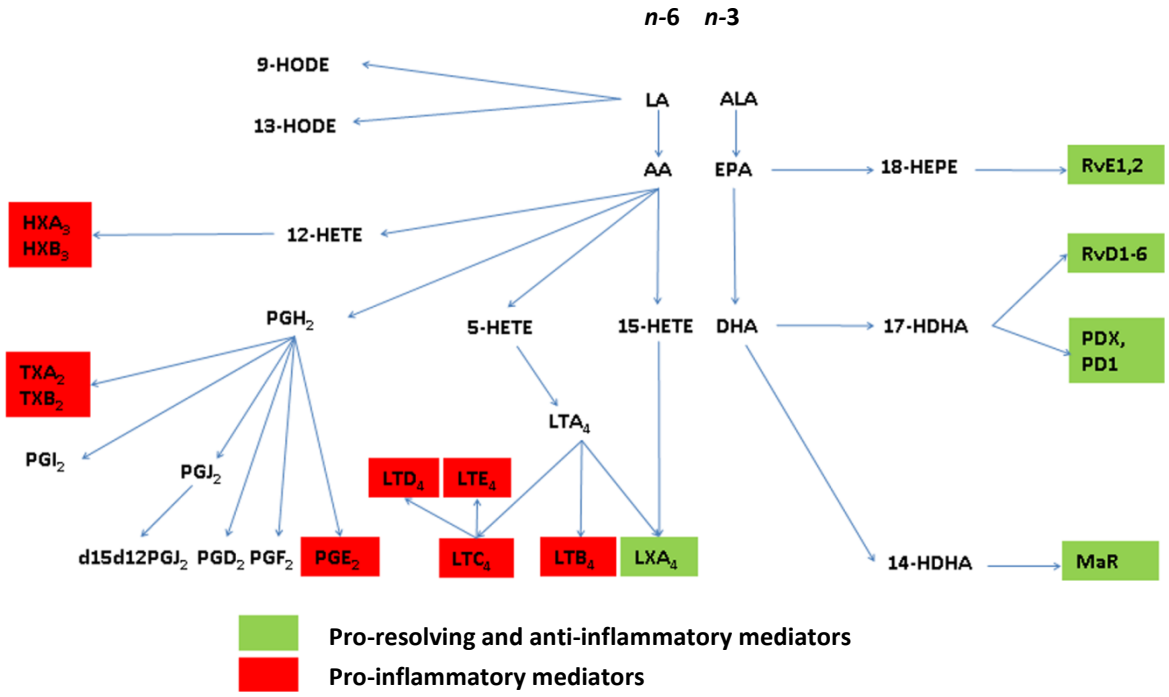

Fig.6. PUFA-metabolites and lipid mediators originating from the n6- and n3-polyunsaturated fatty acids (PUFAs). In addition, bioactive pro-resolving as well as pro-inflammatory eicosanoids and docosanoids were marked in red or green, respectively. Abbreviations: AA: arachidonic acid; ALA: alpha-linolenic; DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid; HEPE: hydroxyeicosapentaenoic acid; HETE: hydroxyeicosatetraenoic acid; HDHA: hydroxydocosahexaenoic acid¸ LA: linoleic acid; LT: leukotriene; LX: lipoxin, HODE: hydroxyoctadecadienoic acid; HX: hepoxilin; MaR: maresin; PD: protectin; PG: prostaglandin; Rv: resolvin; TX: thromboxane. Re-print from (Elabdeen et al., 2013).

1.6 Synthesis of *n*-6 and *n*-3 PUFA

n-3 and *n-6* LC-PUFAs are synthesized from linoleic acid (LA, 18:2*n*-6) and α-linolenic acid (ALA; 18:3n−3), respectively. Humans can obtain LA and ALA only through their diets, because the required Δ^{12}- and Δ^{15}-desaturase enzymes for *de novo* synthesis from stearic acid are not available. Furthermore, conversion of dietary ALA into EPA is limited, with a reported maximum of 17% (Harnack et al., 2009); however, this does not necessarily mean it is insufficient. For example, plant based diets without foods rich in EPA or DHA, but contain high amounts of ingested ALA, are not deficient in EPA or DHA (Cunnane et al., 1993; Freese and Mutanen, 1997). Because the efficacy of n−3 long-chain polyunsaturated fatty acid (LC-PUFA) synthesis decreases down the cascade of ALA conversion, DHA synthesis from ALA is even more restricted than that of EPA (Brenna, 2002; Sinclair et al., 2002). High levels of linoleic acid (LA; 18:2n−6) contribute to reduced EPA synthesis because of the competition between ALA and LA for common desaturation and elongation enzymes (Brenna, 2002; Sinclair et al., 2002). Hence, conversion of ALA into the n−3 PUFAs might be changed by increasing ALA intake or by decreasing LA intake ((Burdge and Wootton, 2003; Pawlosky et al., 2003).

PUFA are usually stored in the cellular membranes and surface layer intracellular lipid droplets. Their position within the phospholipid is mainly sn-2. The bioavailability of dietary DHA and especially EPA had better efficiency when the omega-3 was supplemented in phospholipids in mice (Rossmeisl et al., 2012) or in humans (Schuchardt et al., 2011), than the omega-3 marine fish oil was in TG nor ethyl-esters (Neubronner et al., 2011) as demonstrated in healthy volunteers.

1.6.1 Sources

The structure of *n*-3 FAs differs depending on the source. These variations impact the bioavailability and bioactivity of these fatty acids. There has been much confusion regarding the differences between these *n*-3 FAs based on source.

1.6.1.1 Plant sources of n-3 FAs

Alpha-linolenic acid (ALA) is an 18 carbon omega-3 fatty acid derived from plants (Crawford et al., 2000). This is the predominant *n*-3 FA derived from plants, and is an essential fatty acid in

the human diet (Bjerve et al., 1987). There has been confusion regarding the essentiality of *n*-3 FAs. Long chain omega-3 highly unsaturated fatty acids, which come from fish and other animals sources and will be discussed further below, are not actually essential fatty acids, since they can be synthesized from ALA in humans (Burdge and Calder, 2005). ALA is found in seeds, nuts, and plants. Seed oils are the richest source of ALA, such as flaxseed oil (AKA linseed oil), canola oil (AKA rapeseed oil), and soybean oil (AKA soya) (Voskuil et al., 1996). Other sources of ALA include chia seeds, walnuts, soybeans, and hemp. In vegetables, purlsane has the highest abundance of ALA relative to other plants, such as watercress and mint.

1.6.1.2 Animal sources of n-3 FAs

Grass fed animals included cattle and sheep contain *n*-3 FAs that are obtained from their diet (McAfee et al., 2011). Grass fed cows yield milk that is also enriched in *n*-3 FAs (Hebeisen et al., 1993). However, fish, are the most popular candidate for *n*-3 FAs from a meat source. The type of *n*-3 FAs in fish are different than plants, and are predominantly eicosaspentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are longer and more highly unsaturated than ALA. Both EPA and DHA can be synthesized from ALA. EPA is a 20 carbon fatty acid, with 5 double bonds, and DHA is 22 carbons long with 6 double bonds. In both cases, they are denoted as omega-3 fatty acids because their first double bond originates at the omega-3 position.

1.6.1.3 Other sources of n-3 FAs

While consuming *n*-3 FAs from food may be the best route of exposure for maximal fat absorption through the small intestine (Jeanes et al., 2004), fish oil supplements are a very popular source of *n*-3 FAs, as well. Fish oil supplements are rich in EPA and DHA(Tatarczyk et al., 2007). The relative abundance of each of these fatty acids depends on the brand and specific fish from which the supplement is derived (Tatarczyk et al., 2007). Krill oil supplements are also increasing in popularity with comparable efficacy (Ulven et al., 2011), as concerns regarding contamination of fish oil have become of concern (Suominen et al., 2011). Other sources of *n*-3 FAs include fortified foods such as eggs (Yalcin and Unal, 2010), pastas, yogurt (McCowen et al., 2010), and bread (Yep et al., 2002).

1.7 Thiazolidinediones

Thiazolidinediones (TZDs) such as rosiglitazone and pioglitazone are among the widely used pharmacological agents in the treatment of insulin resistance in diabetic patients. These compounds are likely to improve glycaemic control mostly by repartitioning fat away from skeletal muscle (Kim et al., 2003), while augmenting insulin action in liver, adipose tissue and skeletal muscle (Kim et al., 2004; LeBrasseur et al., 2006; Wilson-Fritch et al., 2004). Numerous clinical studies have demonstrated that TZDs, at doses eliciting maximum stimulation of insulin sensitivity, reduce accumulation of hepatic lipids (steatosis), which is frequently associated with systemic insulin resistance, and can be also used for the treatment of nonalcoholic steatohepatitis (Ratziu et al., 2010) and alcoholic fatty liver disease (Shen et al., 2010). Moreover, a reduction in plasma non-esterified fatty acid (NEFA) levels is a consistent observation across many clinical trials involving TZDs (Kubota et al., 2006). Treatment with TZDs reduces adipocyte hypertrophy and low-grade inflammation of adipose tissue in obesity (Kubota et al., 2006; Kubota et al., 1999), while inducing secretion of insulin-sensitizing hormone adiponectin (Kubota et al., 2006). The induction of adiponectin is probably responsible in large for the insulin-sensitizing effects of TZDs. However, TZDs are also associated with unwanted side-effects, such as oedema and weight gain (Yang and Smith, 2007), increased risk of heart failure (Nissen and Wolski, 2007), and bone loss (Lazarenko et al., 2007). Therefore, novel treatment strategies are required, which would allow for the use of sub-optimal doses of TZDs in combinations with other pharmacological or dietary treatments. Rosiglitazone was banned in Europe in 2010 and in the USA was put under selling restrictions due to the analyses suggesting an increased risk of cardiovascular events. Pioglitazone was put under selling restrictions in Germany and France due to raising the risk of bladder cancer, but in other countries all the world is still used.

Two major intracellular regulatory mechanisms are involved in the action of TZDs: (i) direct binding of TZDs to nuclear peroxisome proliferator activated receptor-γ (PPARγ), leading to the activation of a transcriptional program of adipocyte differentiation in adipose tissue, with a much less understood consequences in other tissues; and (ii) rapid stimulation of AMP-activated protein kinase (AMPK) in liver, skeletal muscle and other tissues (LeBrasseur et al., 2006). AMPK controls metabolic fluxes in response to changing cellular energy levels, namely the partitioning between

lipid oxidation and lipogenesis (Kahn et al., 2005; Zhang et al., 2009). Interestingly, the AMPK cascade has emerged as a major signalling system mediating the adiponectin action in the liver. Phosphorylation of PGC-1α by AMPK leads to induction of genes encoding fatty acid oxidation enzymes (Shen et al., 2010). Simultaneously, AMPK directly inhibits activity of the enzyme acetyl-CoA carboxylase (ACC), thus decreasing levels of malonyl-CoA. Since this key lipogenic intermediate inhibits mitochondrial carnitine palmitoyltransferase 1 and thus inhibits fatty acid β-oxidation (McGarry et al., 1978), reductions in malonyl-CoA levels in response to AMPK activation leads to the inhibition of lipogenesis and stimulation of mitochondrial fatty acid oxidation. AMPK also stimulates the uptake and utilization of glucose in various tissues (Barnes and Zierath, 2005; Carling, 2004). In adipocytes, AMPK inhibits lipolysis in response to the TZD treatment (Bourron et al., 2010).

1.8 Polyphenols

Plant-derived polyphenols, secondary plant products are abundant micronutrients in our diet, and evidence for their role in the prevention of degenerative diseases such as cancer, cardiovascular diseases and others including obesity. The health effects of polyphenols depend on the amount consumed and on their bioavailability. The main sources of phenolic compounds are fruits, vegetables and beverages. In nature, phenolics are usually found conjugated to sugars and organic acids and can be classified into two major types: flavonoid and non-flavonoid phenolics (Landete, 2012). The flavonoids themselves are divided into six subclasses as a function of the type of heterocycle involved: flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavonos (*Gharras*, 2009). Flavonoids and other plant phenolics, such as phenolic acids, stilbenes, tannins and lignins are especially common in flowering tissues, leaves and woody parts. They are important for the plant for the growing and the defense against infection and injury (Kähkönen et al., 1999).

Saura-Calixto et al. estimated the mean daily intake of polyphenols in the Spanish diet between 2.5 and 3 g/day and in the USA in 1976 it was calculated between 1 and 1.1 g/day of polyphenols. The amount of the compounds is higher than other known antioxidant examples vitamin C, E or carotenoids.

The biological effects of polyphenols have been studied for decades. Polyphenols are molecules that have been shown to slow or prevent the oxidation of other molecules. They promote antioxidant effects (Lopez-Vélez et al., 2003, Franke let al., 1993). Polyphenols gives rise to anti-inflammatory activity because inhibit arachidonic acid pathway (Santangelo et al., 2007), Kang et al., 2009). The effects of polyphenols on adipose tissue was observed in catechins from green tea (Lin et al., 2005), resveratrol from red grapes (Floyd et al., 2008) and curcumin from curcuma (Ejaz et al., 2009). Polyphenols are extensively metabolized both in various tissues and by the gut microbiota (Manach et al., 2004, Walle et al., 2004).

1.8.1 Resveratrol

Resveratrol—3,40,5-trihydroxy-trans-stilbene (MW: 228.2)—is a natural non-flavonoid polyphenol compound containing a stilbene structure similar to that of estrogen diethylstilbestrol (see Fig. 7.). It is a fat-soluble compound existing in cis, trans-, and piceid isomeric forms (Carrizzo et al., 2013).

Resveratrol is considered a nutraceutical present in grapes, peanuts, pine trees, cassia and other plants, and many food products (Ramprasath and Jones, 2010). In wine, the concentration of resveratrol varies: red wines contain between 0.2 and 5.8 mg/L, depending upon the grape variety, whereas white wines contain ~0.68 mg/L (Romero-Pérez et al., 1999). Several studies have attributed the biochemical action of resveratrol to stimulate the activity of SIRT1 (Alcaín and Villalba, 2009) and consequently is activated by AMPK (see chapter 1.3) (Srivastava et al., 2012).

Resveratrol can mimic calorie restriction (CR) (see chapter 1.9) in obese persons (Olholm et al., 2010; Timmers et al., 2011), which results in improved insulin resistance, decreased levels of blood glucose, TG and cytokines as well as increased intramyocellular lipid levels, decreased intrahepatic lipid content and decreased systolic blood pressure. These resveratrol-induced beneficial health effects are also thought to be mediated by SIRT1 activation (Howitz et al., 2003). This suggests that CR and resveratrol may exert their beneficial effects on adipocytes through a common mechanism. The increasing of the SIRT1 was shown in plant-derived

resveratrol in vitro cultured mature human Simpson–Golabi–Behmel syndrome (SGBS) adipocytes (Renes et al., 2014).

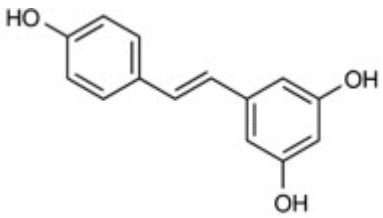

Fig. 7. Structure of resveratrol.

1.8.2 Oleuropein

The traditional Mediterranean diet is a prototype of a healthy diet and is associated with a low risk of cardiovascular disease (Keys et al., 1986). The Mediterranean diet is characterized by a high intake of vegetables, legumes, fruits, cereals, fish and the key features of a traditional Mediterranean diet are the use of olive oil as the principle component of fat. Olive oil is a source of at least 30 phenolic compounds, and particularly, extra virgin olive oil contains considerable amounts of phenolic compounds, e.g. hydroxytyrosol and oleuropein (see Fig. 8), which are responsible for its peculiar taste and high stability. Oleuropein concentration varies with cultivar and climate and is several times higher in the olive leaf than the oil (Omar, 2010). Beneficial effects of olive leaf extract have been described *in vivo*, including a reduction in adiposity and hyperlipidemia in diet-induced obese rats(Oi-Kano et al., 2008), improvement of alloxan induced diabetes in rabbits (Al-Azzawie and Alhamdani, 2006b) and improvement of insulin sensitivity in overweight humans (de Bock et al., 2013b). Purified oleuropein considerably reduces the infarct size in both normal and hypercholesterolemic rabbits subjected to ischemia/reperfusion, protects the reperfused myocardium from the oxidative damage, and decreases total cholesterol and triglyceride levels (Andreadou et al., 2006b). The anti-carcinoma effect has been observed in MCF-7 breast cancer cells (Sirianni et al., 2010). *In vitro*, oleuropein reduces the expression of PPARγ, inhibits adipogenesis and enhances osteoblastogenesis in stem cells derived from human bone marrow (Santiago-Mora et al.,

2011). Oleuropein (in concentration higher than 100 µM) acts on 3T3-L1 cells to reduce preadipocyte differentiation and lipid accumulation and thus regulate the size of fat cells. Also the expression of key transcriptional modulator of adipocyte differentiation genes PPARγ, CCAAT/enhancer binding protein α (C/EBP1α), and sterol regulatory element binding transcription factor 1c (SREBP-1c) and their downstream target genes were suppressed by oleuropein in a dose dependent manner during the differentiation process (Drira et al., 2011).

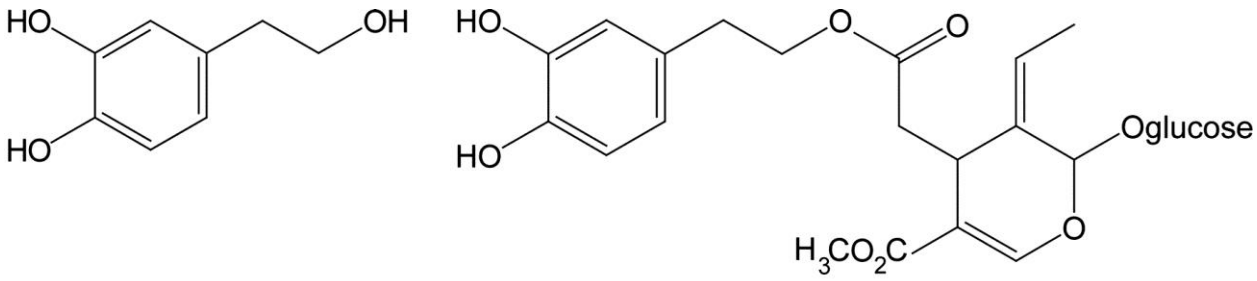

Fig. 8. Structure of hydroxytyrosol and oleuropein

1.9 Caloric restriction

Calorie restriction (CR) is an essential component in the treatment of obesity and associated diseases (Sacks et al., 2009). In fact, calorie restriction and metabolic syndrome are possibly opposite extremes of the same metabolic spectrum (Guarente, 2006).

Calorie restriction-signaling mutually interacts with AMPK (see chapter 1.3), the sensor of energy state and the key regulator of fuels partitioning (Zhang et al., 2009). Moreover, calorie restriction induces mitochondrial biogenesis in various tissues (Flachs et al., 2011), with a relatively strong effect in white WAT (Higami et al., 2004; Nisoli et al., 2005). The induction of mitochondria involves transcription factors PPARα/γ and PPARγ coactivator PGC-1α (Flachs et al., 2011; Nisoli et al., 2005). The important role of WAT in energy homeostasis is underscored by the findings that WAT is one of the key organs being affected by calorie restriction (CR), the most effective strategy to prolong a healthy life at least in several animal species (Nisoli et al., 2005). Metabolism and secretary functions of WAT are also markedly modulated by *n*-3 long-chain polyunsaturated fatty acids (*n*-3 PUFA) of marine origin, namely eicosapentaenoic acid

(EPA; 20:5 *n*-3) and docosahexaenoic acid (DHA; 22:6 *n*-3), which exert numerous beneficial effects on health, including improvements in lipid metabolism and prevention of obesity and diabetes (Flachs et al., 2009). The metabolic changes induced by both CR and *n*-3 PUFA include induction of mitochondrial biogenesis and lipid catabolism in WAT (Flachs et al., 2005; Nisoli et al., 2005). Moreover, nitric oxide (NO) generated by eNOS increases mitochondrial biogenesis and enhances respiration and ATP content in various mammalian cells (Nisoli et al., 2005). Thus, CR was unable to induce significant mitochondrial biogenesis in a number of tissue of eNOS-/- mice, including WAT, suggesting that both energy expenditure and oxidative metabolism are partly NO-dependent (Nisoli et al., 2005).

In mice and lower organisms, calorie restriction prolongs lifespan while inducing sirtuins modifying gene expression by deacetylating several transcription factors (Guarente, 2006). The increasing evidence suggesting that SIRT1, the mammalian ortholog of the SIR2 gene mediates the life-extending effect of CR in yeast (Anderson et al., 2003; Lin et al., 2000), is a key regulator of cell defenses and survival in mammals in response to stress (Luo et al., 2001). The enhanced expression of SIRT1 by CR is consistent with a potential increase in lifespan. This transcription factor may be an evolutionary ancient biological stress response that slows aging, promoting the mobilization of fat into the blood from WAT stores, the down-regulation of adipogenesis, and the long-term survival of irreplaceable cells (Luo et al., 2001; Nisoli et al., 2005; Picard et al., 2004). Resveratrol can mimic CR in obese persons (Olholm et al., 2010; Timmers et al., 2011) (see chapter 1.8.1).

2 AIMS OF THE THESIS

The general goal of the study is to improve the strategy for obesity treatment and prevention using *n*-3 fatty acids of marine origin in combination with other natural approaches, namely mild calorie restriction or the plant-derived polyphenols in dietary obese mice.

Specific aims:

1. To study whether pioglitazone, a TZD-drug with partially diverse biological effects, approved for the treatment of diabetic patients until recently, could elicit the additive beneficial effects when combined with n-3 PUFA in mice fed obesogenic high-fat diet. Main focus of the experiments was to characterize the effects on body weight gain, as well as metabolic flexibility and glucose homeostasis, including the underlying mechanisms. The involvement of adiponectin, one of the major adipokines, was also investigated.

2. To verify a hypothesis whether combined treatment using *n*-3 PUFA and calorie restriction could induce additive beneficial metabolic effects in mice fed high-fat diet. Special focus was to characterize the involvement of WAT in the whole body responses to the combination treatment.

3. To characterize the molecular mechanism of the action of oleuropein, polyphenols extracted from olive leaves, using cell line 3T3-L1 and SVF isolated from gonadal and dorsolumbar adipose tissue of mice and differentiated *in vitro*.

3 MATERIALS and METODS

3.1 Materials

Instruments

AIDA 3,28 software	Raytest, Germany
Centrifuge	Hettich
Digital camera Penguin 600CL	Pixera, USA
Glucometer	LifeScan, USA
Lasergene 8 software DNASTAR	NanoDrop, USA
LightCycler 480 II instrument	ROCHE, Switzerland
LightCycler 480 Syber Green I Master kit	ROCHE, Germany
Microplate reader absorbance	Wallac Victor 1420, Perkin-Elmer, USA
Microscope Nicon TE 300	Japan
spektrofotometr NanoDrop	DNASTAR, Inc., USA
TECAN Safire2	Tecan Group Ltd., Switzerland
Vi-CELL XR analyzer	Beckman Counter, USA

Kits

GeneBLAzer® PPARα UAS-bla HEK293T, PPAR β/δ HEK 293T DA, and PPARγ 293H DA,	Life Technologies Corporation, USA
Cell Proliferation reagent WST-1	ROCHE, Germany

Diets for animal

standardní laboratorní dieta, ST-1 (ST dieta)	*Velaz, ČR*
vysokotuková laboratorní dieta (HF dieta)	dieta byla připravena ve Fyziologickém ústavu AV ČR
product EPAX 1050 TG; EPAX a.s.,	Lysaker, Norway
rosiglitazone	Avandia; GlaxoSmithKline, USA
pioglitazone	Actos; Takeda, Japan

Chemicals

14C palmitate	ParkinElmer, USA
2-izopropanol	*Penta, CR*
5x pufr (Reverse Transcriptase)	*Invitrogen, Gibco BRL, Germany*

AICAR	Sigma Aldrich, Co., USA
Albumin Serva	*Lachema,CR*
Ascorbic acid	Sigma Aldrich, Co., USA
BRL 49653	Cayman CHEMICAL, Cayman Europe, Estonia
$CaCl_2$	*Fluka, USA*
Calf serum	Sigma Aldrich, Co., USA
Catalase	Sigma Aldrich, Co., USA
Chloroform	*Penta,CR*
Collagenase	*Sigma, USA*
Diethyleter	*Lachema,CR*
Dimethyl sufoxide	Sigma Aldrich, Co., USA
DMEM	LONZA, Belgium
D-MEM F12	LONZA, Belgium
dNTP (10mM)	*Invitrogen, Gibco BRL, Germany*
DTT (0.1M) (1,4-dithio-L-threitol)	S*igma, USA*
EDTA	*Sigma ,USA*
Etanol	*Penta,CR*
Ethidium bromid	S*igma,USA*
FBS	LONZA, Belgium
gentamycin	LONZA, Belgium
Glucose	P*enta, CR*
Insulin	Sigma Aldrich, Co., USA
KCl	*Penta,CR*
KH_2PO_4	*Lachema,CR*
M199	LONZA, Belgium
$MgSO_4.7H_2O$	Sigma Aldrich, Co., USA
M-MLVreverzní transkriptáza (200 U/µl)	*Invitrogen, Gibco BRL, Germany*
NaCl	*Merck,Germany*
$NaHCO_2$	Sigma Aldrich, Co., USA
oligo T (100 M)	*Invitrogen, Gibco BRL, Germany*
Oligo-dT primers	Promega
penicilin + streptomycin	LONZA, Belgium
pneumoxid (95%O_2,5%CO_2)	*Linde,CR*
primers for PCR	*Invitrogen, Gibco BRL, Germany*
Reverse Transcriptase	Invitrogen
Rneasy® Lipid Tissue Mini kit	*Qiagen, Germany*
T0070907	Cayman CHEMICAL, Cayman Europe, Estonia
TRI Reagent	Sigma Aldrich, Co., USA
TRIzol	*Invitrogen, Vinco BRL, Německo*

Trypan Blue dye	Sigma Aldrich, Co., USA
Trypsin	LONZA, Belgium
Type II collagenase	Sigma Aldrich, Co., USA

3.2 Animal experiments

Mice C57BL/6 is the most widely used inbred strain. It is commonly used as a general purpose strain and background strain for the generation of congenics carrying both spontaneous and induced mutations. The mice fed a high-fat diet develop obesity, mild to moderate hyperglycemia, and hyperinsulinemia.

Male C57BL/6N mice (Charles River Laboratories, Sulzfeld, Germany) and male mice (C57BL/6J; Jackson Laboratory, ME, USA) were maintained at 22°C on 12-h light-dark cycle (light from 6.00 a.m.) with free access to water and; extruded Ssniff R/M-H diet; Ssniff Spezialdieten GmbH, Soest, Germany).

The animal experiments were specifically approved by the Animal Care and Use Committee of the Institute of Physiology Academy of Sciences of the Czech Republic v.v.i. (Approval Number: 172/2009) and conducted under the guidelines.

Diet composition (See also Fig.1)

Standart low fat diet **(Chow)** - lipid content ~3.4% wt/wt
High fat **(cHF)** diet - lipid content ~35% wt/wt, mainly corn oil
High fat + *n*-3 PUFA **(cHF+F)** - diet supplemented with *n*-3 PUFA which replaced 15% wt/wt of dietary lipids concentrate (46% DHA, 14% EPA, wt/wt, as TG)
High fat diet + rosiglitazone **(cHF+ROSI)** - diet supplemented with 10 mg rosiglitazone/kg diet
High diet + pioglitazone **(cHF+PIO)** - diet supplemented with 50 mg pioglitazone/kg diet High fat + *n*-3 PUFA + rosiglitazone **(cHF+F+ROSI)** - diet supplemented with both *n*-3 PUFA concentrate and rosiglitazone
High fat + *n*-3 PUFA + pioglitazone **(cHF+F+PIO)** - diet supplemented with both *n*-3 PUFA concentrate and pioglitazone
High fat + restriction of energy intake **(cHF + CR)** - the ration was reduced by 10% wt/wt compared with mice fed ad libitum with the same type of diet

High fat +*n*-3 PUFA + restriction of energy intake **(cHF+F+CR) -** diet supplemented with both *n*-3 PUFA concentrate and the ration was reduced by 10% wt/wt compared with mice fed ad libitum with the same type of diet.

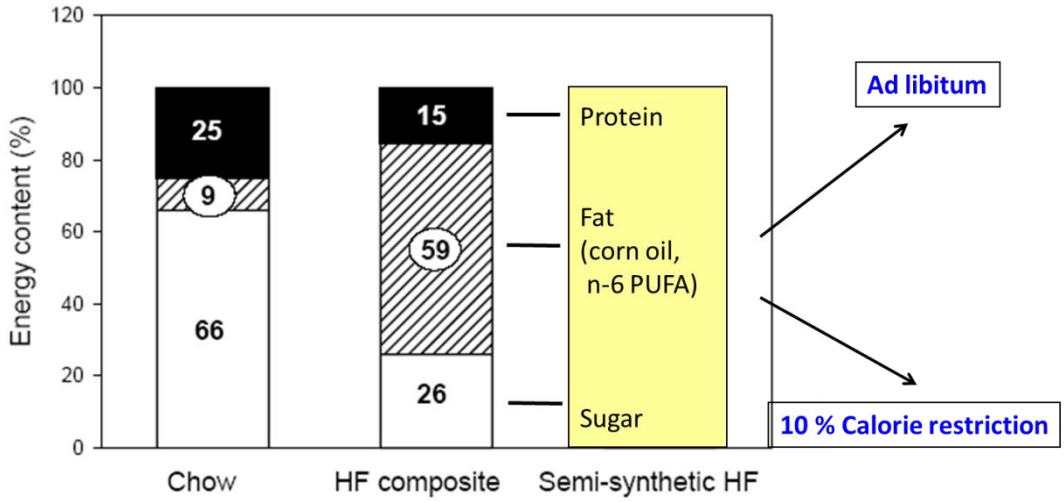

Fig.1. Composition of diets

During the treatment lasting for 8 weeks in publication A and 5 weeks (or 15 weeks) in publication B, fresh portion of food was distributed daily and food consumption and body weights were recorded once a week. Eventually, to analyse all the animals under identical nutritional conditions, mice were fasted during the day (between 8.00 a.m. and 6.00 p.m.), and then allowed free access to feed during the night and in the morning until the time of killing the animals under pentobarbital anaesthesia (between 9.00 and 11.00 am). Liver and gastrocnemius muscle were dissected and EDTA-plasma was isolated and stored for further analyses. To characterize the effect of the treatment on insulin sensitivity in obese mice, a separate experiment was performed, in which all the animals were fed cHF diet between 3 and 7 months of age, and then singly caged animals were randomly assigned (n = 8).

Glucose homeostasis

Intraperitoneal glucose tolerance test was performed in overnight fasted mice (food was removed between 5:30 p.m. and 8:30 a.m., i.e the time of the start of the test by the injection

of D-glucose (1 g/kg body weight), in which glycaemia was assessed using tail bleeds just before the injection (fasting blood glucose at the baseline), and during 180 min after the injection using glucometers. Insulin levels were also determined at the baseline and 30 min after the glucose injection. HOMA index was calculated by the following formula: FASTED plasma insulin (mU/l) x FASTED plasma glucose (mmol/l) / 22.5.

Collection of plasma samples during FASTED to RE-FED transition

When indicated EDTA-plasma was collected using tail bleeding and adapted procedure described by Viollet et al (Viollet et al., 2003). Before the bleeding, one half of mice within each experimental group was either (i) fasted for 14 hours (food was removed between 8:00 am and 10:00 pm, while mice were kept in a clean new cage), or (ii) fasted for 10 hours (between 8:00 am and 6:00 pm, similarly as above) and allowed free access to food for the following 3 hours. In each mouse, plasma glucose, NEFA, TG and insulin were assessed in both FASTED and RE-FED state, while altering the above protocols during two subsequent days. HOMA index was calculated by the following formula: FASTED plasma insulin (mU/l) x FASTED plasma glucose (mmol/l) / 22.5.

3.3 Cell cultures and related techniques

Cell Cultures

3T3-L1 is a cell line derived from (mouse) 3T3 cells that is used in biological research on adipose tissue. 3T3-L1 cells have a fibroblast-like morphology, but, under appropriate conditions, the cells differentiate into an adipocyte-like phenotype. 3T3-L1 cells of the adipocyte morphology increase the synthesis and accumulation of triglycerides. These cells are also sensitive to lipogenic and lipolytic hormones and drugs, including epinephrine, isoproterenol, and insulin. 3T3-L1 cells are extensively used to study adipogenesis.

Cells were plated and grown to 2 days post confluence in DMEM supplemented by 10% calf serum and penicillin/streptomycin in a humidified atmosphere of 10 % CO_2 at 37°C. The cells were induced to differentiate using medium containing 10% fetal bovine serum, 2 µM

dexamethasone, 0.1 µM rosiglitazone (BRL 49653, in dimethyl sulfoxide), and 5µg/ml insulin for 48 hours. Afterwards the cells were maintained in DMEM containing 10% fetal bovine serum and 5µg/ml insulin.

Isolation, plating and culture condition of adipose precursor cells/primary SVF cultures

Male C57BL/6 mice were used for the preparation of primary SVF cultures. Adipose depots (subcutaneous dorsolumbar and abdominal gonadal) were dissect under sterile conditions, minced with scissors and digested using collagenase (3 mg/ml, type II collagenase; Sigma-Aldrich, Co., USA; Cat. # C-6885) in modified Krebs-Ringer bicarbonate buffer (118.5 mM NaCl, 4.8 mM KCl, 2.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.1 mM $MgSO_4.7H_2O$, 25 mM $NaHCO_2$, 5 mM glucose and 4% (w/v) bovine serum albumin fraction V; pH 7.4). The cell suspension was filtered through 250 µm-nylon mesh, floating mature adipocytes were discarded and the infranatant (SVF) was filtered again through 25 µm-nylon mesh. The filtered infranatant was centrifuged at 700 x g for 10 minutes. The pellet was resuspended in D-MEM F12 (containing 5µg/ml insulin and gentamycin) and centrifuged again. The cells were resuspended in medium with 10 % fetal bovine serum, counted in Bürker chamber and plated at a density of 2,000 cells/cm^2. Cells were grown at 37 °C in atmosphere of 5% CO2. The medium (D-MEM F12 containing 10 % fetal bovine serum, 5 µg/ml insulin and gentamycin) was changed two times during a week. After 5-7 days, rosiglitazone was added to the dishes at final concentration of 100 nM. The cells were kept for 7 days until full adipocyte differentiation.

Isolation and culture of adult primary mouse hepatocytes

Mouse was narcotized and the abdomen was opened. The liver was perfused with 50ml of Hepes buffer +50 µl EDTA 0,5M into v. cava inferior. Sever the portal vein and immediately increase pump flow rate to approx. 5ml/min And after washing the liver was digested with Hepes buffer (+ CaCl2 375 mg/ml + collagenase 0,4 mg/ml - filtered on 0,2 µm) for 15 min. The liver was placed into PBS and the tissue was disintegrated by slow shaking. Cell suspension was washed three times in PBS and then put in 10mL of plating medium M199 + BSA (7,5%), FBS (10%), Insulin (100u/ml), T3 (1mM), Dexamethason (10mg/ml), Penicillin-Streptomycin (100x).

The viability of the hepatocytes was determined according to the Trypan Blue exclusion. After adherence, the medium was changed.

The rate of fatty acid oxidation in isolated hepatocytes

Male C57BL/6 mice were used for the preparation of primary hepatocytes culture (see above). Palmitate oxidation was assesed using [1-14C] palmitate (PerkinElmer, USA). Cells were incubated for 45 min at 37°C in 800 ul of a medium (M199) supplemented with 1% BSA, 50 µM cold palmitate and [1-14C] palmitate (0,38 µCi/reaction) with or without 1 mM AICAR. The reactions were terminated by aspiration of the media; cells were washed by PBS and then incubated in 800 ul of 5 % perchloric acid for 15 min at room temperature. Palmitate oxidation was determined by measuring production of 14C labeled acid-soluble metabolites (ASM), a measure of tricarboxylic acid cycle intermediates and acetyl esters. The ASM were assessed in supernatants of the acid precipitate. Identical incubations in parallel wells without radioactivity were conducted to determine protein concentrations.

Cell viability assays

The viability of 3T3-L1 cells was tested with or without catalase (100 units/ml catalase from bovine liver; Sigma-Aldrich, Co., USA,).

Trypan Blue assay

Cells were detached using Trypsin–EDTA solution in phosphate buffered saline (pH 7.4) for 10 min at room temperature, immediately stained with 0.5% Trypan blue dye (Sigma-Aldrich, Co., USA) and the cell number was evaluated using a Vi-CELL XR analyzer (Beckman Coulter, USA). Three samples were used for each experimental group, and 50 measurements were performed on each sample (i.e., 150 measurements in total).

WST-1 assay Cells were cultivated with in microplates (96 wells) in a final volume of 100µl/well culture medium. Cell Proliferation Reagent WST-1 (10 µl/well; Roche Diagnostics GmbH, Germany) was added and the cells were incubated for 4 hours. Then the formazan product was quantified at 420 – 480 nm with a microplate reader absorbance reader (Wallac Victor 1420, Perkin-Elmer, USA).

Oil red-O staining

After differentiation, 3T3-L1 cells or primary SVF cultures were washed twice with phosphate buffered saline (pH 7.4), fixed with 4% paraformaldehyde at room temperature for 12h, and then were washed twice with sterile water and once with 60% isopropanol. Subsequently, cell were stained with 5g/L Oil red-O (in 60% isopropanol) at room temperature for 2h and washed with 60% isopropanol and sterile water. Pictures were taken using an inverted microscope (Nicon TE 300, Nicon, Japan) equipped with digital camera (Penguin 600 CL, Pixera, USA). The plates with stained cells were measured by multiwell plate reader (Wallac Victor 1420, Perki*n*-Elmer, USA) at the absorbance 490-nm. Alternatively, captured pictures were analyzed using AIDA 3.28 software (Raytest, Germany).

Reporter-gene assay in vitro

Cellular activation of PPAR nuclear receptors was assessed in reporter gene assays according to the manufacturer's protocol (GeneBLAzer® PPARα UAS-bla HEK293T, PPAR β/δ HEK 293T DA, and PPARγ 293H DA, Life Technologies Corporation, USA). In brief, cells were stably expressing GAL4-specific PPAR ligand-binding domain fusion protein and UAS-β-lactamase reporter gene. Cells were incubated with indicated concentrations of compounds. Cells were incubated with testing compound or antagonist for 30 minutes before adding agonist and then incubated in a humid 37°C/5% CO_2 incubator for 16 hours. Fluorescence intensity at 460 and 530 nm emission following excitation at 406 nm was measured using a TECAN Safire2 (Tecan Group Ltd., Switzerland) with optimal gain settings determined by the instrument. After subtraction of fluorescence background from cell-free wells, the ratio of fluorescence intensity at 460 *versus* 530 nm (designated as 460:530 nm) was calculated. T0070907 (Cayman CHEMICAL, Cayman Europe, Estonia) was used as specific PPARγ inhibitor (Lee et al.).

3.4 Gene expression

RNA was always isolated from tissue or cell samples by TriReagent solution (MRC). Total RNA from the tissue samples was extracted by standard phenol/chloroform extraction and ethanol precipitation. RNA was stored at -80°C. RNA was checked by NanoDrop 1000 (Thermo Scientific). One thousand nanograms of total RNA was transcribed to cDNA using M-MLV Reverse Transcriptase (Invitrogen) and Oligo-dT primers (Promega). RT-minus controls were always prepared (no reverse transcriptase added). cDNA was stored at -20°C until analysis by real-time PCR, but at most for 2 weeks.

Quantitative real- time PCR

Total RNA isolated using TRI Reagent (Sigma-Aldrich, Co., USA). Levels of various transcripts were evaluated using LightCycler 480 II instrument (Roche Diagnostic Ltd., Switzerland) and LightCycler 480 SYBR Green I Master kit (Roche Diagnostic Ltd., Germany). PCR condition were 95°C for 5 min and 45 cycles of 95°C for 10 s, 55-60°C for 10 s and 72°C for 20 s. Specificity of the amplified PCR product was assessed by performing a melting curve analysis. Lasergene 11 software (DNASTAR, Inc., USA) was used to design primers. To correct for inter sample variation; levels of the transcript were normalized using geometrical mean of two reference genes - *Cyphβ* and *Eef2*.

Table 1. List of using primers

Gene	Code GenBank	Sequence
Eef-2	13629	CCCAAAGGATGCGCTCTCGTT TGCGGTGTCTGTAGTGGCTTGATT
Cyclophilin β	19035	ACTACGGGCCTGGCTGGGTGAG TGCCGGAGTCGACAATGATGA
Pgc-1α	19017	TGCGGTGTCTGTAGTGGCTTGATT CCCAAAGGATGCGCTCTCGTT
Pparγ	19016	GCCTTGCTGTGGGGATGTCTC CCTCGCCTTGGCTTTGGTCAG
Fas	14104	GGCTGCCTCCGTGGACCTTATC GTCTAGCCCTCCCGTACACTCACTCGT

C/Ebpα	12606	CGCTGGCCGGCCTCTTCCCCTACC GCCCCGCAGCGTGTCCAGTTCA
Sreb-1c	20787	GCTTCCGGCCTGCTATGA CTCGTGTGGCTCCGGGAC
Scd-1	20249	TAGCTTTGGGTGCCTTATCTCTTTC CTCTCCAGCCAGCCTCTTGACTATTC
Pparα	19013	TGCGCAGCTCGTACAGGTCATCAA CCCCCATTTCGGTAGCAGGTAGTCTTA
Cd-68	12514	CACTTCGGGCCATGTTTCTCTTG AGGGGCTGGTAGGTTGATTGTCGT
Gyk	14933	TCGTTCCAGCATTTTCAGGGTTAT TCAGGCATGGAGGGTTTCACTACT
Mcad	11364	TCGCCCCGGAATATGACAAAA GCCAAGGCCACCGCAACT
Lcad	11363	TGGCATCAACATCGCAGAGAAACA ACCGATACACTTGCCCGCCGTCAT
Vlcad	11370	CAGGGGTGGAGCGTGTGC CATTGCCCAGCCCAGTGAGTTCC
CoxIII	7668711	TCATCGTCTCGGAAGTATTTTT ATTAGTAGGGCTTGATTTATGTGG
Cpt1-α	12894	GCAGCTCGCACATTACAAGGACAT AGCCCCCGCCACAGGACACATAGT
Ucp1	22227	CACGGGGACCTACAATGCTTACAG GGCCGTCGGTCCTTCCTT

3.5 Statistical analysis

All values are presented as means ± SE. Logarithmic transformation was used to stabilize variance in cells when necessary. Data were evaluated by a one-way ANOVA with Holm-Sidak posthoc test using SigmnaStat 3.5 statistical software. Comparisons were judged to be significant at $p \leq 0.05$.

4 RESULTS

4.1.1 Publication A

Unmasking differential effects of rosiglitazone and pioglitazone in the combination treatment with *n*-3 fatty acids in mice fed a high-fat diet. *Kus V[1], Flachs P, Kuda O, Bardova K, Janovska P, Svobodova M, Jilkova ZM, Rossmeisl M, Wang-Sattler R, Yu Z, Illig T, Kopecky J.*

TZD anti-diabetic drug, namely rosiglitazone and pioglitazone, proved to be useful for the pharmacological treatment of hyperglycaemia in the patients, while used as an add-on treatment to metformin and other pharmaceuticals. Our previous study in dietary obese mice indicated additivity in preservation of insulin sensitivity and in amelioration of major metabolic syndrome phenotypes by the combination treatment using *n*-3 PUFA and rosiglitazone. The aim of this study was to investigate whether pioglitazone, a TZD-drug with partially diverse biological effects, approved for the treatment of diabetic patients until recently, could elicit the additive beneficial effects when combined with *n*-3 PUFA in mice fed obesogenic high-fat diet. Main focus of the experiments was to characterize the effects on body weight gain, as well as metabolic flexibility and glucose homeostasis, including the underlying mechanisms. The involvement of adiponectin, one of the major adipokines, was also investigated. We used an adult male mice (C57BL/6J) fed an obesogenic corn oil-based high-fat diet (cHF) for 8 weeks, or randomly assigned to various dietary treatments (i) cHF+F, cHF with *n*-3 PUFA concentrate replacing 15% of dietary lipids; (ii) cHF+ROSI, cHF with 10 mg rosiglitazone/kg diet; (iii) cHF+F+ROSI; (iv) cHF+PIO, cHF with 50 mg pioglitazone/kg diet; and (v) cHF+F+PIO, or chow-fed (see the schema Fig. 1). Treatments by cHF+F, cHF+ROSI or cHF+PIO tended to counteract the obesogenic effect of cHF diet. Importantly, cHF+F+ROSI treatment reduced significantly body weight gain (see Fig.2). In contrast, no effect of pioglitazone used as add-on treatment to *n*-3 PUFA (cHF+F+PIO) on body weight. None of the treatments affected food consumption (Table 1).

The changes in body weight could be explained by modulation of adiposity, as documented by weights of epididymal, mesenteric and subcutaneous fat depots, with only the subcutaneous fat reflecting the additive weight-reducing effects of the cHF+F+ROSI treatment

(Table 1). Accordingly, only cHF+F+ROSI but not cHF+F+PIO treatment decreased plasma levels of leptin, the major adipokine, the levels of which reflect the magnitude of fat accumulation (Table 1). None of the treatments affected muscle lipid content. While cHF+F or cHF+F+ROSI treatments had no significant effects on hepatic lipid content, hepatic lipid content was increased by cHF+ROSI treatment as compared with the cHF mice. In contrast, hepatic lipid content was not affected by cHF+PIO treatment, and cHF+F+PIO treatment even lowered hepatic lipids to the Chow mice levels (Table 1). Both TZDs increased hepatic expression of the gene for stearoyl-CoA desaturase-1 (SCD-1) that is essential for lipogenesis (Table 1).

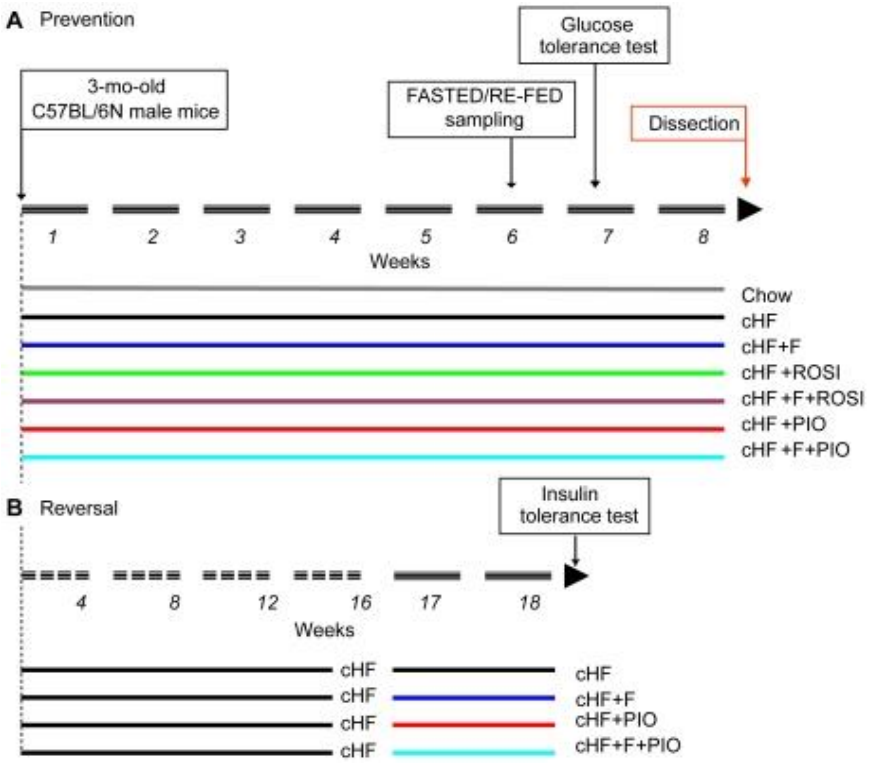

Fig.1. Overview of experimental setup.

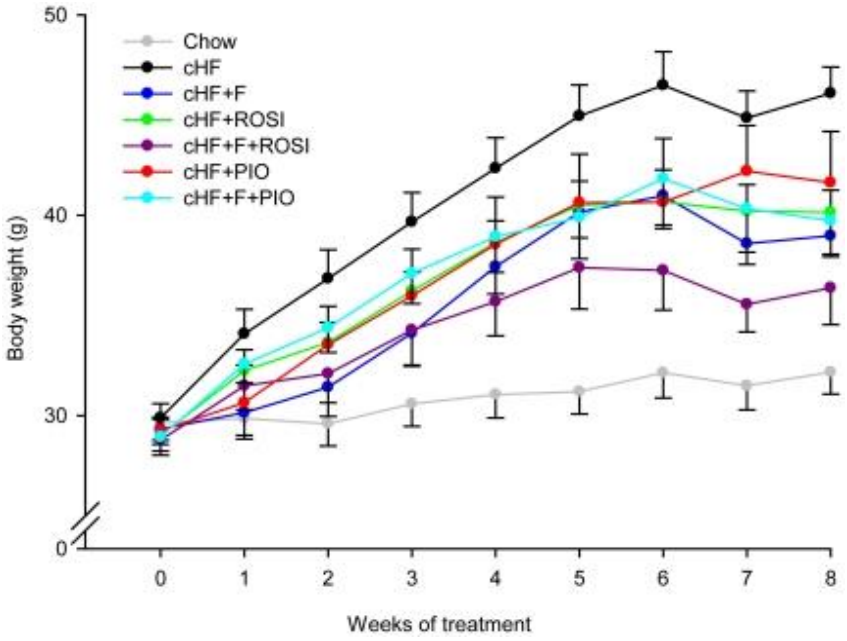

Fig.2. Body weights during differential dietary treatment. At 3 month of age, subgroups of mice were placed on cHF diet, or treated various cHF-based diets: cHF+F, cHF+ROSI, cHF+F+ROSI, cHF+PIO and cHF+F+PIO, or maintained on Chow diet for 8 weeks. Irregularities of the body weight caused by glucose tolerance test performed during week 7 (see Fig. 1) are apparent. Data are means ± SE (n = 7–8).

Table 1. Growth characteristics, adiposity and lipid accumulation.

	Chow	cHF	cHF+F	cHF+ROSI	cHF+F+ROSI	cHF+PIO	cHF+F+PIO
Body weight (g)							
Initial	29.6±0.9	29.9±0.7	29.3 0.8	29.0±0.9	28.8±0.8	29.3±0.6	28.9±0.7
Final	32.1±1.3	46.5±1.7[f]	41.0±1.6	40.7±1.6	37.3±1.9[a]	40.7±3.2	41.8±2.3
Body weight gain	2.1±0.6	16.0±1.2[f]	11.7±0.8	11.5±1.2	8.1±1.3[a]	11.3±2.9	12.1±1.7
FC (kJ/day/animal)	72±4	72±2	69±2	70±2	65±2	71±2	65±1
Feed efficiency (mg BW/kJ)	3.2±0.6	27.4±2.7[f]	17.8±2.6[a]	20.6±1.6	16.7±1.3[a]	20.4±1.8	23.7±1.7
Fat depots weights (mg)							
Epididymal fat	783±129	3205±243[f]	2336±86	2270±263	2238±287	2289±479	2196±187
Mesenteric fat	348±22	1237±168[f]	711±78[a]	840±82	656±105[a]	868±146	607±10[a]
Subcutaneous fat	240±37	968±61[f]	732±84[a]	586±4[a]	404±111[a,b]	636±106[a]	636±83[a]
Leptin (ng/ml)	8±2	72±5[f]	53±4	58±10	46±6[a]	72±10	61±8
Triacylglycerol content (mg/g)							
Liver	31±3	87±10[f]	51±5[c]	145±29[a,b,d]	75±18[c]	100±18	34±4[a,c,d,e]
Muscle	18±3	37±7[f]	30±7	34±2	35±6	29±6	39±4
Hepatic *Scd-1*	1.10±0.06	0.19±0.07[f]	0.09±0.04	0.54±0.05[a,b]	0.34±0.11	0.40±0.11[b]	0.16±0.05[c]

Three-month-old mice were placed on various diets and killed after 8 weeks of the dietary treatment as described in Methods. Body weight and food consumption (FC; recorded weekly) data are related to the initial 6 weeks of the treatment; due to irregularities of the body weight and FC data caused by glucose tolerance test performed during week 7, data from the last 2 weeks of the treatment could not be included (see Fig. 1). Fat depot weights and levels of plasma leptin were recorded after the killing. Expression of SCD-1 gene in the liver was evaluated using quantitative RT-PCR analysis and standardized using elongation factor 1α (Gene ID: 13627). Data are means±SE (n = 7-8).

[a]Significantly different from cHF.
[b]significantly different from cHF+F.
[c]significantly different from cHF+ROSI.
[d]significantly different from cHF+F+ROSI.
[e]significantly different from cHF+PIO (ANOVA).
[f]Significantly different from Chow (t-test).
doi:10.1371/journal.pone.0027126.t001

During week 6, plasma levels of NEFA, TG, and total cholesterol, and glycemia were assessed in FASTED and RE-FED state. (Fig.3) The cHF mice showed smaller changes in the levels of NEFA and glucose and even opposite changes in the levels of TG in response to the FASTED/RE-FED state as compared with the Chow mice. None of the treatments preserved the NEFA response, with the exception of the cHF+F+ROSI treatment, which tended to normalize it. In contrast to NEFA, the TG response was fully preserved by both combination treatments (cHF+F+ROSI and cHF+F+PIO). Single-type treatments (cHF+F, cHF+ROSI, and cHF+PIO) had no significant effect on the TG response. The glycaemic response tended to be preserved by all the treatments, with only marginal differences between them.

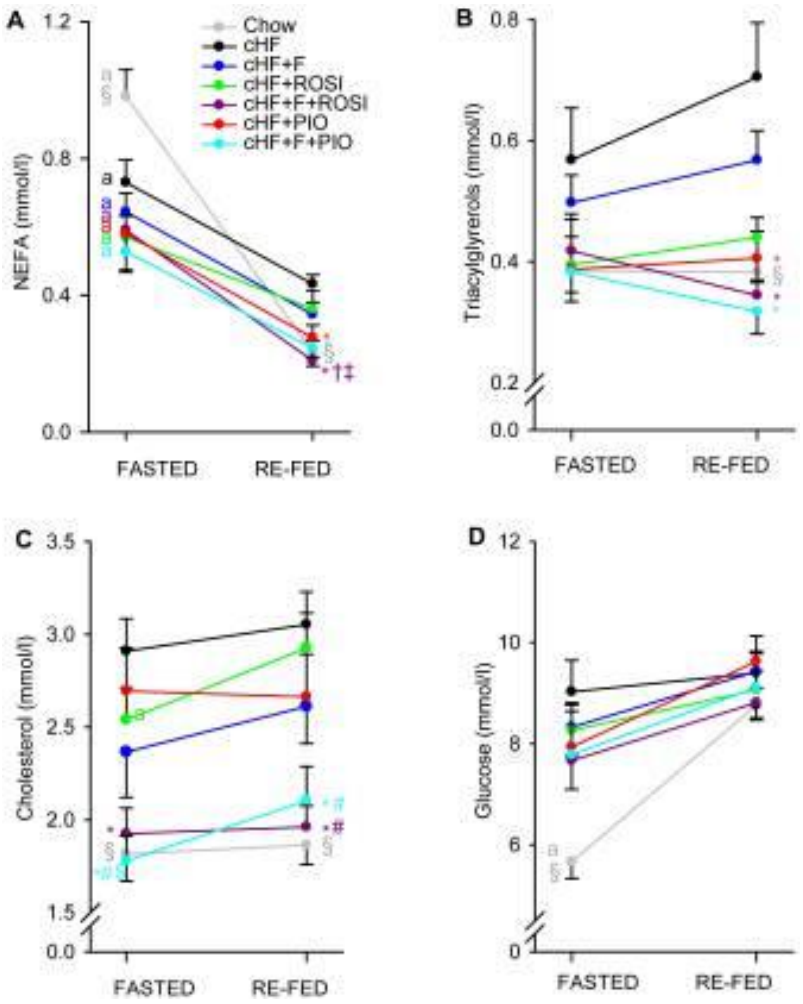

Fig. 3. Plasmatic lipids levels and glycaemia during FASTED/RE-FED transition.
At 3 month of age, subgroups of mice were placed on cHF diet, or treated various cHF-based diets. During week 6 of the treatment, plasma NEFA (**A**), TG (**B**) and total cholesterol (**C**) levels and blood glucose (**D**) were assessed in FASTED and in RE-FED state as described in Methods in mice fed by Chow or cHF diet, or treated by cHF+F, cHF+ROSI, cHF+F+ROSI, cHF+PIO, and cHF+F+PIO diet. Data are means±SE (**n** = 7–8). [a] significantly different between FASTED and RE-FED state; *significantly different from cHF; [†]significantly different from cHF+F; ‡significantly different from cHF+ROSI; [#]significantly different from cHF+F+ROSI (ANOVA).[§]Significantly different from cHF (t-test).

Glucose tolerance test was performed during week 7. (Fig.4). cHF-feeding impaired glucose tolerance (Fig. 4A,C), while the treatments provided a similar kind of protection as in the case of their effect on fasted glycaemia. In this case it was ~1.6-fold higher in the cHF mice than in the chow mice. This increase was fully prevented by the cHF+F treatment, as well as by the combination treatments (cHF+F+ROSI and cHF+F+PIO), while cHF+ROSI and cHF+PIO

treatments tended to had smaller effects. cHF-feeding impaired glucose tolerance, while the treatments provided a similar kind of protection as in the case of their effect on fasted glycaemia. The concentration of insulin was also measured at 0’ and 30’min (Fig. 4D) and how was expected the higher levels were detected in cHF mice. And how shown the HOMA Index that the combinations as well as rosiglitasone and pioglitazone with *n*-3 PUFA preserve the glucose homeostasis (Fig. 4E).

Plasma levels of total adiponectin one of the two major adipokines as well as of its biologically active high molecular weight (HMW) form increased in response to cHF+F treatment (Fig. 4F). Even a stronger induction was observed with both combination treatments, and it was significantly stronger in case of the cHF+F+PIO as compared with cHF+F+ROSI treatment. Single-type treatments using either rosiglitazone or pioglitazone had no effect (Fig. 4F). Thus, a remarkable synergism between *n*-3 PUFA and pioglitazone in the induction of adiponectin was found, resulting in ~2.3-fold and ~3.6-fold higher levels of total and HMW adiponectin, respectively, as compared to the cHF mice. These results verify to the key involvement of WAT in the effect of the combination treatment.

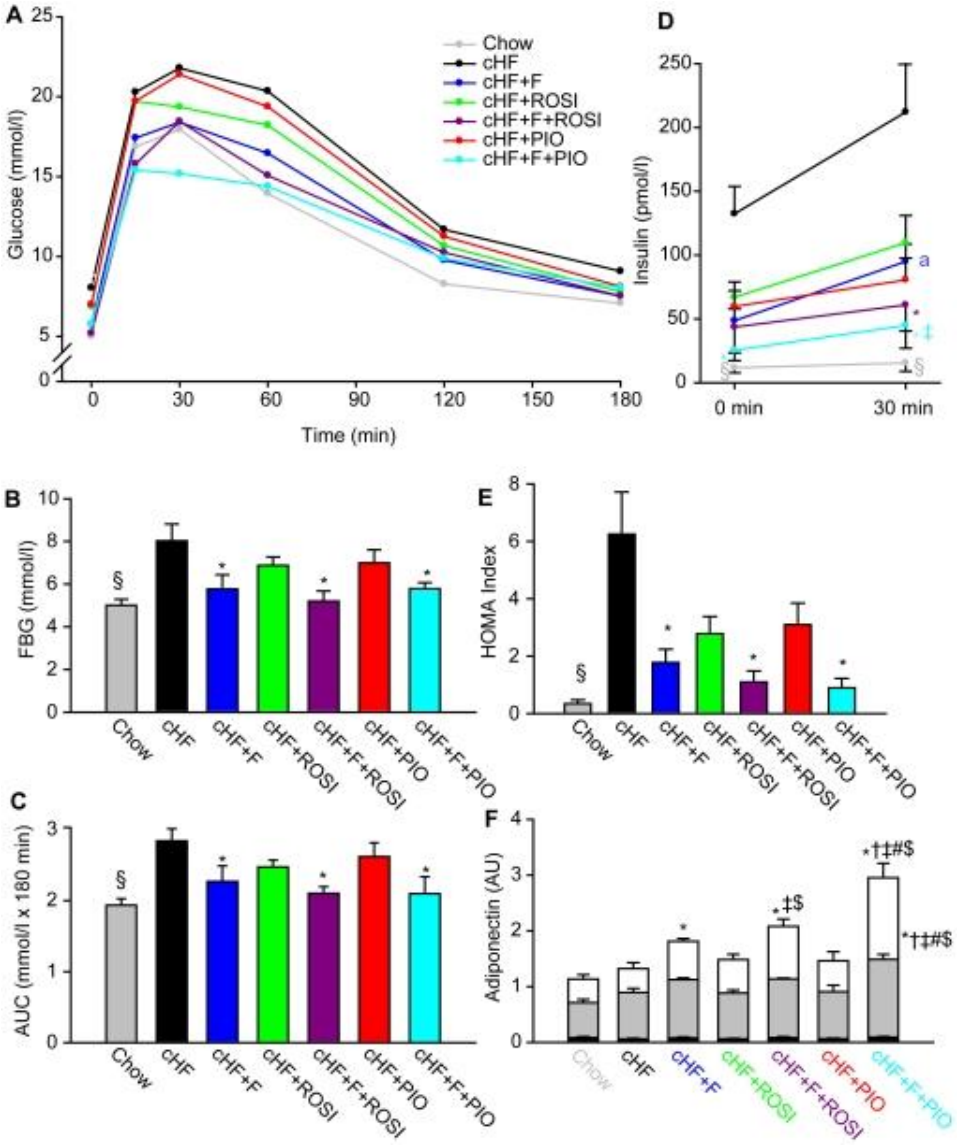

Fig.4. Preservation of glucose homeostasis during high-fat feeding by differential dietary treatment. At 3 month of age, subgroups of mice were placed on cHF diet, or treated various cHF-based diets. During week 7 of the treatment, glucose tolerance test was performed (**A–C**). **A.** Plasma glucose levels following i.p. glucose injection (time 0) to mice fed by Chow or cHF diet, or treated by cHF+F, cHF+ROSI, cHF+F+ROSI, cHF+PIO, and cHF+F+PIO diet. **B.** Fasting blood glucose levels at the baseline (time 0; see **A**). **C.**Total area under the glycaemic curve values as above (**A**). **D.** Insulin levels in plasma (at time 0 and at 30 min); lines and symbols as above (**A**). **E** HOMA index calculated from glucose and insulin plasma levels at time 0. **F.** Adiponectin levels in plasma at time 0; bar height, total immunoreactive adiponectin; black section, low molecular weight adiponectin; gray section, medium molecular weight adiponectin; white section, high molecular weight adiponectin; corresponding SE values are indicated. **B–F.** Data are means±SE (n = 7–8). [a] significantly different between 0 min and 30 min. *Significantly different from cHF; [†]significantly different from cHF+F; ‡significantly different from

cHF+ROSI; #significantly different from cHF+F+ROSI; $significantly different from cHF+PIO (ANOVA). §Significantly different from cHF (t-test). AUC, area under the curve; AU, arbitrary units; FBG, fasting blood glucose.

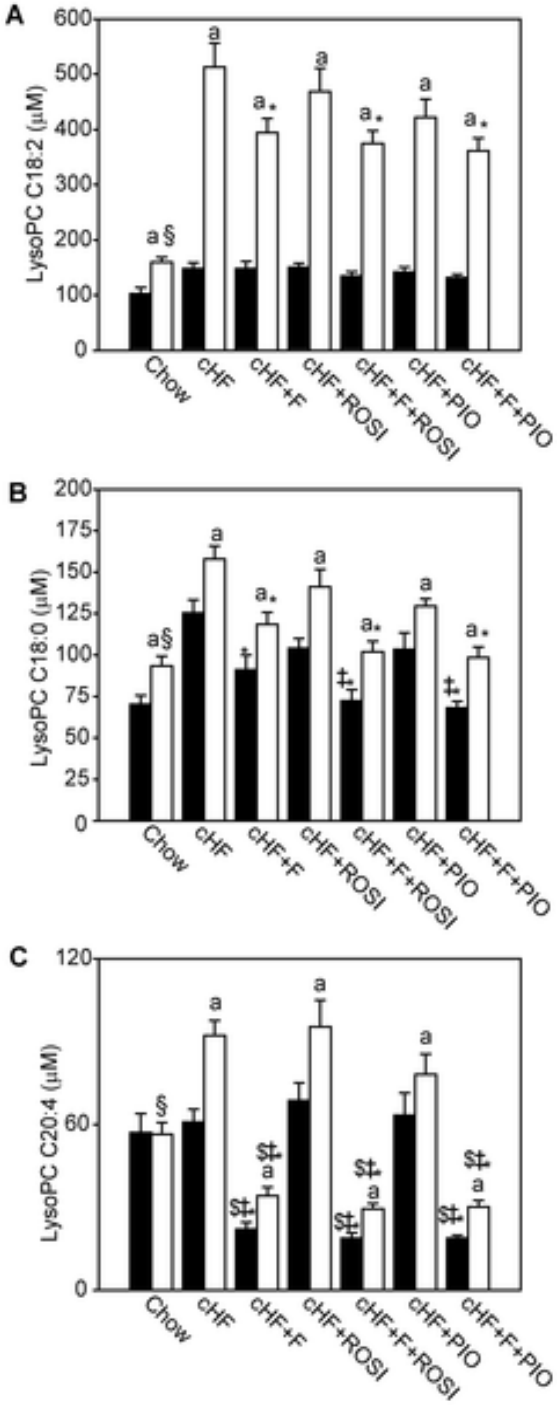

Fig. 6. Chemical concentrations of various lysophosphatidylcholines measured in plasma in the FASTED and RE-FED state in all groups of mice. At 3 month of age, subgroups of mice were placed on cHF diet, or treated various cHF-based diets. During week 6 of the treatment, targeted metabolomics analysis was performed. Changes in concentrations of selected analytes are shown (see text, see Fig. 7). A. linoleoyl lysophosphatidylcholine (C18:2). B. stearoyl lysophosphatidylcholine (C18:0). C. arachidonoyl lysophosphatidylcholine (C20:4). Data are means±SE (n = 7–8). a significantly different between FASTED (black bars) and RE-FED (white bars) state; *significantly different from cHF; ‡significantly different from cHF+ROSI; $significantly different from cHF+PIO (ANOVA). §Significantly different from cHF (t-test).

In order to characterise further differential effects of various treatments, plasma concentrations of 163 metabolites providing representative sets of amino acids, sugars, acylcarnitines and phospholipids were measured using flow injection analysis/thermospray mass spectrometry (**FIA-MS**) with Biocrates Absolute**IDQ**^{TM} targeted metabolomics technology. Plasma samples collected in both FASTED and RE-FED states during week 6 were analysed. Due to a relatively large error of their quantification, 27 metabolites were excluded from all the analyses described below.

Namely the contents of C18:2-, C18:0- and C20:4-lysophosphatidylcholine acyls, representing 3 out of 6 most abundant lysophosphatidylcholine acyl species present, were higher in the cHF mice than in the Chow mice, especially in the RE-FED state (Fig. 6A,B,C). This elevation was compromised by all the treatments, in accordance with the known association of the above lipids with obesity-induced low-grade systemic inflammation [51], [52], and suggesting amelioration of the inflammatory state by the treatments. The combination treatments tended to exert the most pronounced effects (Fig. 6A,B,C). The content of 20:4-lysophosphatidylcholine increased in response to RE-FED state in all mice fed cHF-based diets, but in the Chow mice. Its levels in the cHF+F, cHF+F+ROSI and cHF+F+PIO mice were very low (Fig. 6C).

For a more detailed characterization of the differential effects of rosiglitazone and pioglitazone on plasma metabolome was used PLS-DA analysis. The first (axis X) as well as the second (axis Y) PLS-DA component showed a weak separation between the cHF mice and the TZD-treated mice, however no separation between the cHF+ROSI and cHF+PIO mice could be observed (Fig. 7A), indicating similar changes in the concentrations of most of the measured metabolites during the FASTED/RE-FED transition, independent of the type of TZD. When the cHF, cHF+F+ROSI and cHF+F+PIO mice were analysed, the first PLS-DA component showed a strong separation between the cHF mice and mice subjected to the combination treatments. Importantly, in contrast to a lack of separation between the single-TZD-treatments, the second PLS-DA component showed a separation between two combination treatments (Fig. 7B). Therefore, these data were further analysed to identify metabolites discriminating between the cHF+F+ROSI and cHF+F+PIO mice.

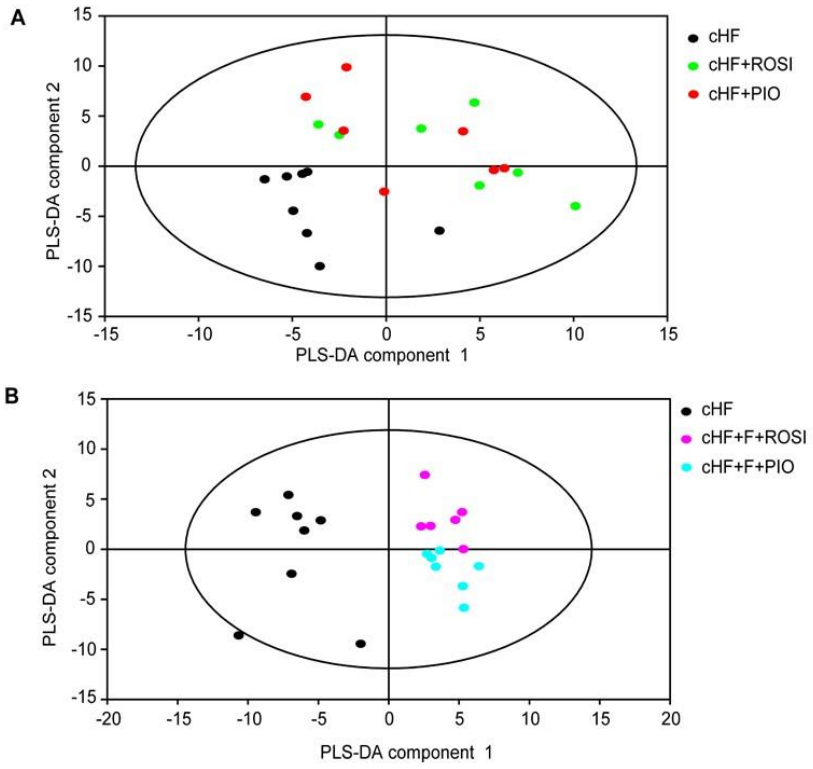

Fig. 7. Comparisons of the effects of treatment by TZD-containing diets on plasma metabolome. At 3 month of age, subgroups of mice were placed on cHF diet, or treated various cHF-based diets. During week 6 of the treatment, targeted metabolomics analysis was performed. In total, plasma concentrations of 163 metabolites were determined in both FASTED and RE-FED states during week 6 of the treatment using FIA-MS with the Biocrates AbsoluteIDQTM targeted metabolomics technology. After removal of unstable metabolites, 136 metabolites were included in a partial least squares-discriminant analysis (PLS-DA), using delta values (DV) calculated as a difference in the concentration. 2D-scatter plots of the first (axis X) and the second (axis Y) PLS-DA component are shown for selected groups of mice (n = 7–8). A. Mice fed cHF diet (black circles), or treated using cHF+ROSI (green circles) or cHF+PIO diet (red circles). B. Mice fed cHF diet (black circles), or treated using cHF+F+ROSI (violet circles), or cHF+F+PIO (cyan circles) diet. C. Contribution scores for the separation between the cHF+F+ROSI and cHF+F+PIO treatments (see B) for each metabolite are shown. Positive value of the score corresponds to a larger DV of the metabolite in the cHF+F+ROSI as compared with the cHF+F+PIO mice.

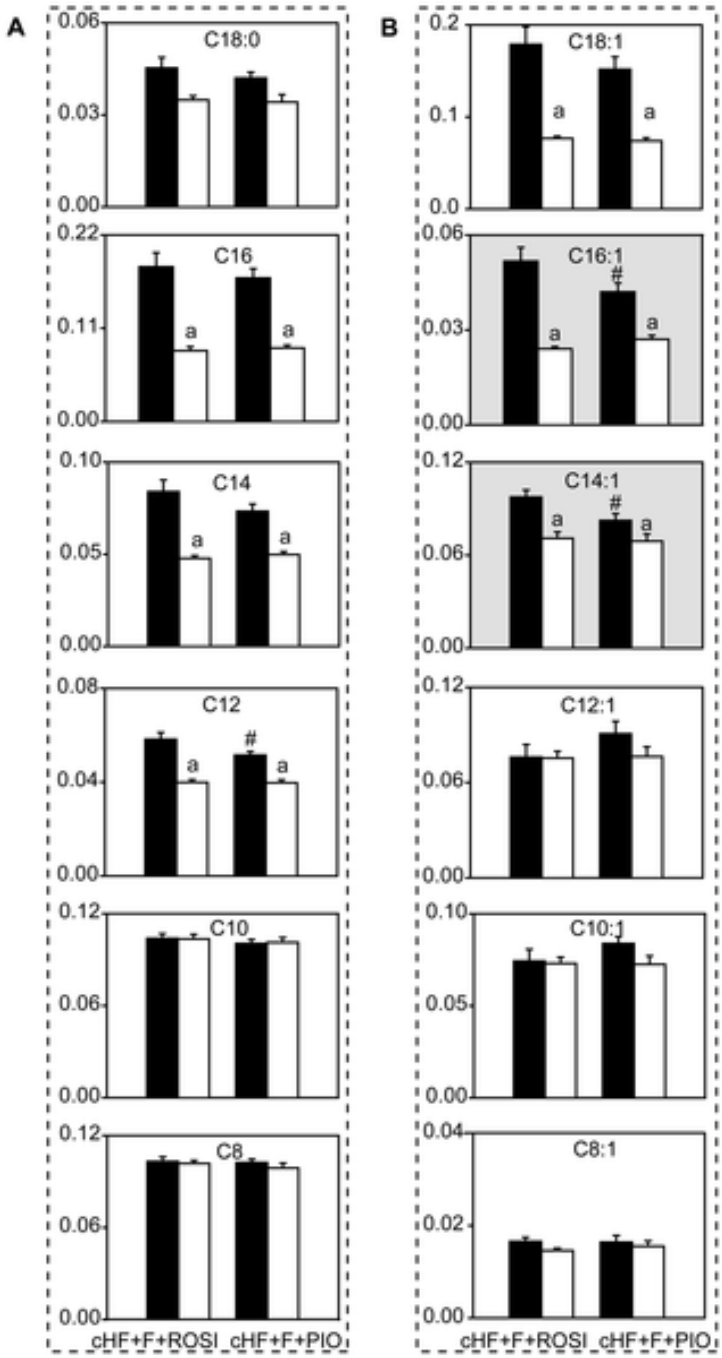

Figure 8. Concentrations of selected even-chain acylcarnitines measured in plasma in the FASTED and RE-FED state in the cHF+F+ROSI and cHF+F+PIO mice.

Data are means±SE (n = 7–8). A. Saturated acylcarnitines. B. Monounsaturated acylcarnitines. Individual acylcarnitines are denoted by their side chains. Axis Y, concentration of each carnitine (μM); gray background, acylcarnitines showing the largest contribution scores (>2) in the separation between the cHF+F+ROSI and cHF+F+PIO mice. a significant difference between FASTED (black bars) and RE-FED (white bars) state; #significantly different from cHF+F+ROSI (ANOVA).

Even side-chain species acylcarnitines in plasma, and especially those with longer chains, reflect activity of mitochondrial β-oxidation (Koves et.al 2008, Lehmann et al 2010). Acylcarnitines with saturated (Fig. 8A) and monounsaturated (Fig. 8B) even side-chains C12-C18 showed lower levels in RE-FED as compared with FASTED state, and the response to

FASTED/RE-FED transition of these lipids discriminated between cHF+F+ROSI and cHF+F+PIO treatments, while tetradecenoylcarnitine (C14:1) and palmitoleylcarnitine (C16:1) represented the major discriminating metabolites (Fig. 8B)

Plasma concentrations of 163 metabolites were evaluated using a targeted metabolomics approach. Both TZDs preserved glucose homeostasis and normal plasma lipid levels while inducing adiponectin, with pioglitazone showing better effectiveness. The beneficial effects of TZDs were further augmented by the combination treatments. cHF+F+ROSI but not cHF+F+PIO counteracted development of obesity, in correlation with inducibility of fatty acid β-oxidation, as revealed by the metabolomic analysis. By contrast, only cHF+F+PIO eliminated hepatic steatosis. Our results in dietary obese mice reveal differential effects of rosiglitazone and pioglitazone, unmasked in the combination treatment with *n*-3 PUFA, and support the notion that *n*-3 PUFA could be used as add-on treatment to TZDs in order to improve diabetic patient's therapy.

My main contributions to this work were handling of animals and performing GTT, collection of samples during the termination the experiment, RNA isolation from the tissues, reverse transcription and quantification of gene expression using qRT-PCR.

4.1.2 Publication B

Synergistic induction of lipid catabolism and anti-inflammatory lipids in white fat of dietary obese mice in response to calorie restriction and *n*-3 fatty acids. *Flachs P, Rühl R, Hensler M, Janovska P, Zouhar P, Kus V, Macek Jilkova Z, Papp E, Kuda O, Svobodova M, Rossmeisl M, Tsenov G, Mohamed-Ali V, Kopecky J.*

The aim of the this story was to verify a hypothesis whether combined treatment using *n*-3 PUFA and calorie restriction could induce additive beneficial metabolic effects in mice fed high-fat diet. Special focus was to characterize the involvement of WAT in the whole body responses to the combination treatment. Male mice C57BL/6J were fed by corn oil based high-fat diet (cHF; lipid content 35% wt/wt) for two weeks and then randomly assigned for 5 weeks or 15 weeks to various dietary treatments: (i) cHF, *ad libitum* (free access to food), (ii) cHF+F, *ad libitum*, which 15% of dietary lipids were replacing by *n*-3 PUFAs concentrate, (iii) cHF+CR, ratio of food was reduced by 10% compared with *ad libitum* fed mice on the same type of diet, (iv) cHF+F+CR, diet enriched by *n*-3 PUFAs and food reduced by 10%.

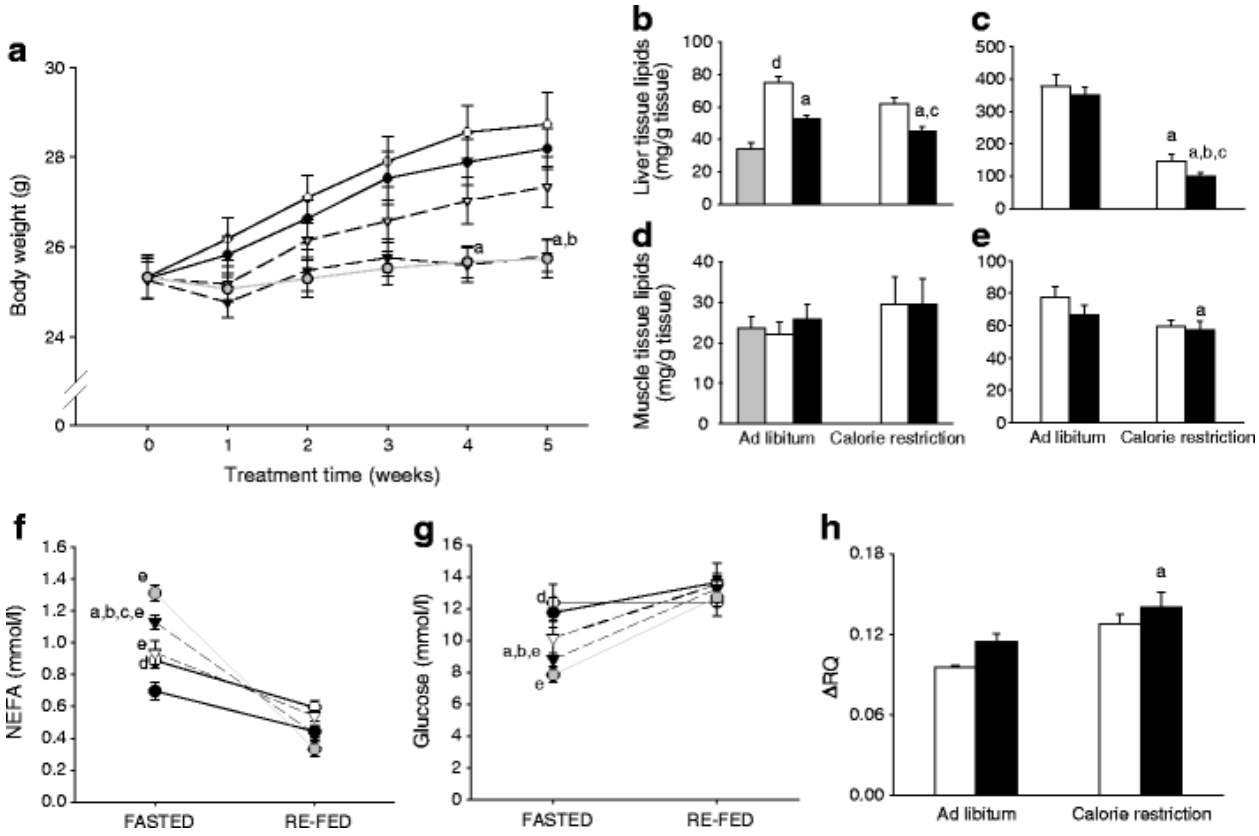

Fig. 1. Prevention of diet-induced obesity, hepatic steatosis, and metabolic inflexibility. a Body weight of mice; week 0, start of the differential dietary treatment. Results from a typical experiment (n = 10; see also Table 1). White circles, cHF; black circles, cHF + F; white triangles, cHF + CR; black triangles, cHF + F + CR; grey circles, Chow. Five independent short-term (5 weeks) and one long-term (15 weeks) experiments were performed with similar results (not shown; $p < 0.05$ vs. cHF for all the treatments). b–e Ectopic lipid accumulation in liver (b, c) and gastrocnemius muscle (d, e) after 5 weeks (b, d) or 15 weeks (c, e) of the treatment; n = 10. Data for the Chow-fed mice are shown only for week 5, but similar values at week 15 should be expected [16]. NEFA (f) and (g) glucose levels in plasma after 4 weeks of the treatment, measured in either the FASTED or RE-FED state (see ESM; n = 10). White circles, cHF; black circles, cHF + F; white triangles, cHF + CR; black triangles, cHF + F + CR; grey circles, Chow. h During the fourth week of the treatment, metabolic flexibility was assessed as a maximal induction in RQ values in response to a glucose load administered by intragastric gavage to animals fasted overnight (12 h) (n = 9–10). White bars, cHF or cHF + CR diet; black bars, cHF + F or cHF + F + CR. Data from an independent experiment using the Chow-fed mice showed $\Delta RQ = 0.15 \pm 0.01$ (n = 9). For details, see ESM Fig. 2 and ESM Table 1. Data are means ± SEM. a,b,cSignificant difference (ANOVA) compared with cHF, cHF + F, and cHF + CR, respectively; dsignificant difference (t test) compared with Chow-fed mice esignificant difference (repeated-measures ANOVA) between the FASTED and RE-FED states

Feeding mice cHF diet increased the body weight of the mice (Fig.1. a). The other combination treatment cHF+F and cHF+CR partly prevented the cHF-induced obesity, while combination treatment (cHF+F+CR) provided a full protection during the experiment. cHF diet induced accumulation of lipids in the liver and skeletal muscle (Fig. 1.b,c resp. d,e). In the liver, all the treatments decreased TG content at 5 weeks, with the strongest effect of cHF+F+CR diet in response to the longer treatment. After 15 weeks, body weights in mice fed different diets were as followed: cHF, 42.7 ± 1.2 g; cHF + F, 36.8 ± 1.9 g; cHF + CR, 34.7 ± 2.5 g; cHF + F + CR, 33.1 ± 0.8 g. In the skeletal muscle, the cHF-induced TG accumulation was significantly prevented only by the combination (cHF+F+CR) treatment lasting for 15 weeks. After 5 weeks, all treatments showed hypolipidemic effect, with the most pronounced effect in combination treatment. This effect was negatively correlated with the plasma level of β-hydroxybutyrate, the marker of lipid catabolism. Glucose levels in plasma were similar in all the groups. However, insulin levels were markedly decreased by cHF+CR treatment and even more by the cHF+F+CR treatment.

Combination treatment improved metabolic flexibility (which is associated with insulin sensitivity), based on both the response of plasma level of NEFA and glucose to FASTED to RE-FED transition and the glucose-induced increase in RQ (Fig. 1. F, g and h).

For better understanding, which way *n*-3 PUFAs and CR or both play role against development of obesity, genes involved in mitochondrial biogenesis and lipid catabolism in epididymal and subcutaneous adipose tissue, interscapular brown fat, liver and gastrocnemius muscle were measured (Fig.2.). When comparing gene expression from the tissues it is obvious that the largest changes occur in epididymal fat.

Gene expression analysis in epididymal fat showed a strong induction of mitochondrial biogenesis and increasing oxidative capacity of mitochondria by the combination. In contrast, no significant changes were observed in this cluster of genes in brown fat (Fig. 2c), liver (Fig. 2d) or skeletal muscle (Fig. 2e). Concerning the genes of β-oxidation such as very long chain, long chain and medium chain acyl-CoA dehydrogenases (*Vlcad, Lcad* and *Mcad*), additive induction of *Vlcad* and *Lcad* was noticed in the epididymal fat (Fig. 2a) and a slight increase was observed in response to cHF+F or cHF+F+CR treatments in the liver (Fig 2d).

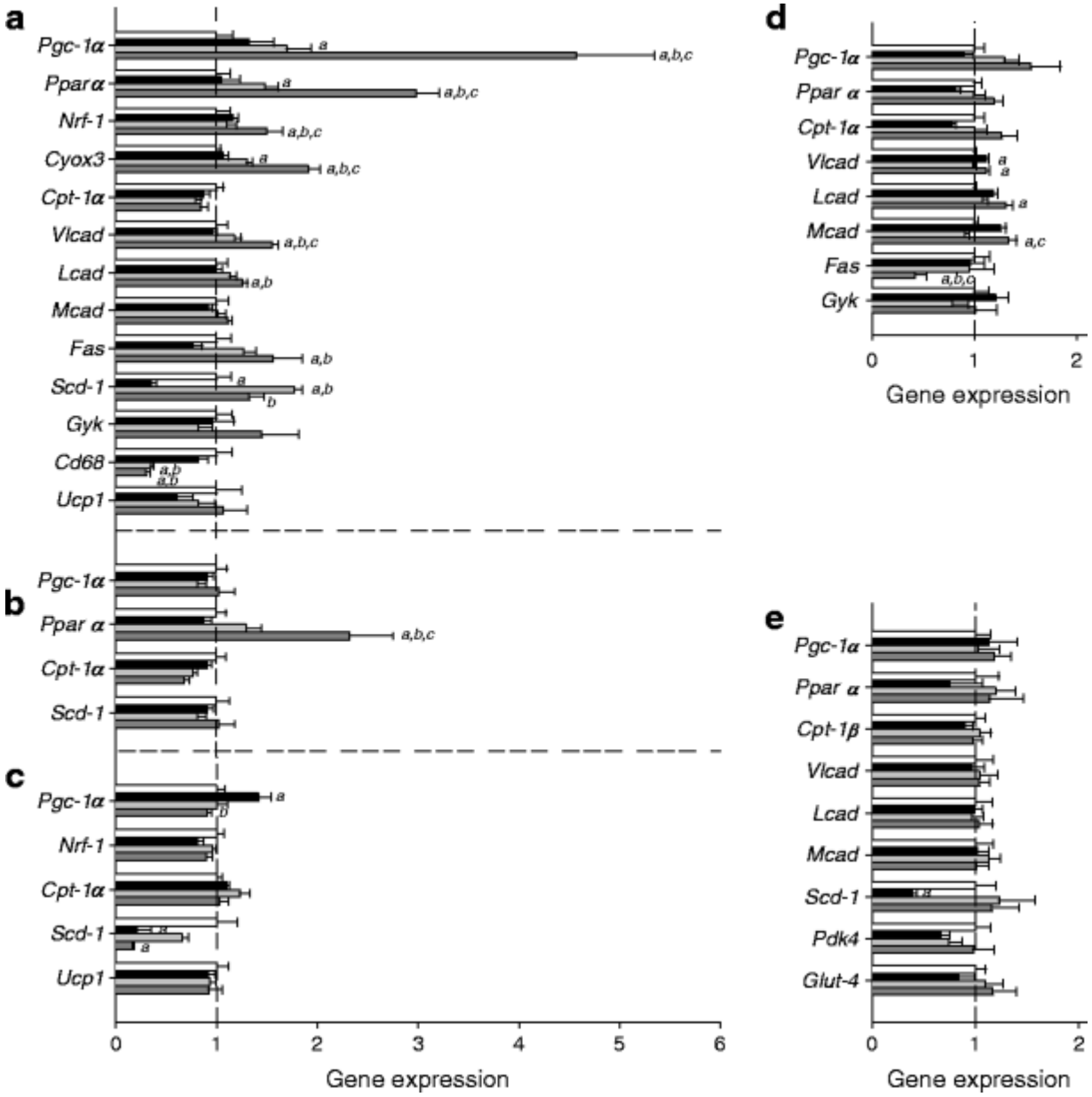

Fig. 2. Specific induction of mitochondrial genes in epididymal fat. Quantitative real-time RT-PCR data showing relative levels of gene expression (cHF = 1) in epididymal (a) and subcutaneous (b) WAT, interscapular brown fat (c), liver (d), and gastrocnemius muscle (e) after 5 weeks of the treatment. Data are means ± SEM; n = 10; white bars, cHF; black bars, cHF + F; light grey bars, cHF + CR; dark grey bars, cHF + F + CR; a,b,cSignificant difference (ANOVA) compared with cHF, cHF + F and cHF + CR, respectively. An independent experiment showed no difference in either Pgc-1α or Pparα expression in epididymal fat between the cHF and the Chow-fed mice (the cHF/Chow ratio of the Pgc-1α and Pparα transcript levels was 1.08 ± 0.05 and 1.04 ± 0.08, respectively; n = 6). All analyses were performed after 5 weeks of treatment

To confirm the synergistic induction of mitochondrial fatty acid oxidation by *n*-3 PUFAs and calorie restriction in epididymal fat, biochemical assessment was performed *ex-vivo* after 5 weeks of the dietary treatment. cHF diet stimulated radiolabeled palmitate oxidation in fragments of epididymal fat (~1.3-fold) compared with chow, and this activity was further significantly stimulated only by the combination treatment (~1.6-fold). Similar results have seen in isolated adipocytes from epididymal fat (Fig. 3a,b). Mitochondrial oxidative capacity was characterized in permeabilized adipocytes isolated from epididymal fat, using respirometry. Adipocytes from the cHF+F+CR mice exerted ~2-fold higher oxygen consumption compare to the cHF mice (Fig.3c,d,e)

To assess changes in lipid catabolism in the other tissues, palmitate oxidation was measured in isolated hepatocytes and in skeletal muscles, after 5-weeks treatment. Only combination treatment increased the hepatic fatty acid oxidation compared to the cHF mice, but it had no effect on fatty acid oxidation in either gastrocnemius or soleus muscle (Tab 1).

Tab 1. After 5 weeks of the treatment, oxidation of [1-^{14}C] palmitate was measured either in isolated hepatocytes, or in whole soleus muscle (oxidative type), or in a fragment of gastrocnemius muscle (mixed type). Data are expressed as means±SEM; *n*= 8-12; *a*- significant difference (ANOVA) compared to cHF. *- significant difference (*t*-test) compared to Chow. N.E. not estimated.

	Chow	cHF	cHF+F	cHF+CR	cHF+F+CR
Hepatocytes					
FA oxidation (dpm/µg DNA)	17±3	31±2*	36±4	27±2	42±4[a]
Gastrocnemius muscle					
FA oxidation (dpm/mg tissue)	N.E.	42±5	N.E.	N.E.	44±4
Soleus muscle					
FA oxidation (dpm/mg tissue)	N.E.	123±12	N.E.	N.E.	140±10

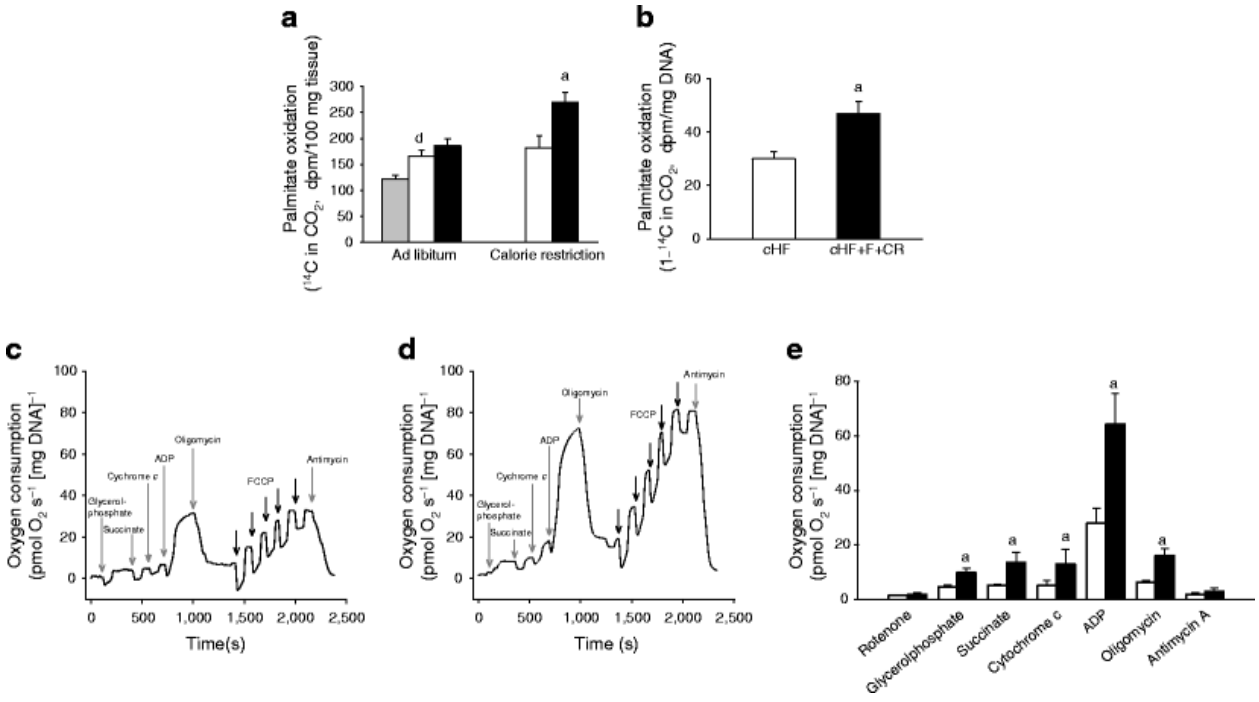

Fig. 3 Induction of fatty acid oxidation and mitochondrial oxidative capacity in epididymal fat. a Oxidation of [1- 14C]palmitate into CO2 by tissue fragments. Data are means ± SEM; n = 10. White bars, cHF or cHF + CR; black bars, cHF + F or cHF + F + CR; grey bars, Chow. aSignificant difference (ANOVA) compared with cHF; dsignificant difference (t test) compared with Chow-fed mice. b Oxidation of [1-14C]palmitate into CO2 by freshly isolated adipocytes. Data are means ± SEM; n = 8. aSignificant difference (t test). c, d, e Evaluation of mitochondrial respiratory capacity and oxidative phosphorylation in permeabilised adipocytes isolated from epididymal fat of the cHF and cHF + F + CR mice using multiple substrate-inhibitor titration respirometry. c, d Representative oxygen flux curves (c cHF; d cHF + F + CR). Adipocyte added in suspension (0.2 ml) to 2 ml incubation medium (10 mmol/l Tris-HCl, 80 mmol/l KCl, 3 mmol/l MgCl2, 5 mmol/l KH2PO4, 1 mmol/l EDTA and 1% wt/wt fatty-acid-free BSA, pH 7.4) were permeabilised with the addition of 3 µl digitonin (10 mg/ml DMSO), mitochondrial complex I was inhibited by 2 µl of 1 mmol/l rotenone, and respiration was stimulated by successive additions of: (1) 20 µl 1 mol/l glycerol 3-phosphate; (2) 20 µl 1 mol/l succinate; and (3) 10 µl 0.3 mol/l ADP (before the ADP addition, mitochondrial integrity was tested by addition of 10 µl of 4 mmol/l reduced cytochrome c). Subsequently, ATP synthase was inhibited by 2 µl oligomycin (4 mg/ml), respiration was re-activated by additions of uncoupler of oxidative phosphorylation (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; FCCP; 0.5 µl of 1 mmol/l FCCP at each addition), and finally, respiration was inhibited by 2 µl of 5 mmol/l antimycin A (inhibitor of complex III). e Averaged values from respirometry (n = 4; pooled samples from two animals). White bars, cHF; black bars, cHF + F + CR. Data are means ± SEM. aSignificant difference (paired t test) between the groups. All analyses were performed after 5 weeks of treatment

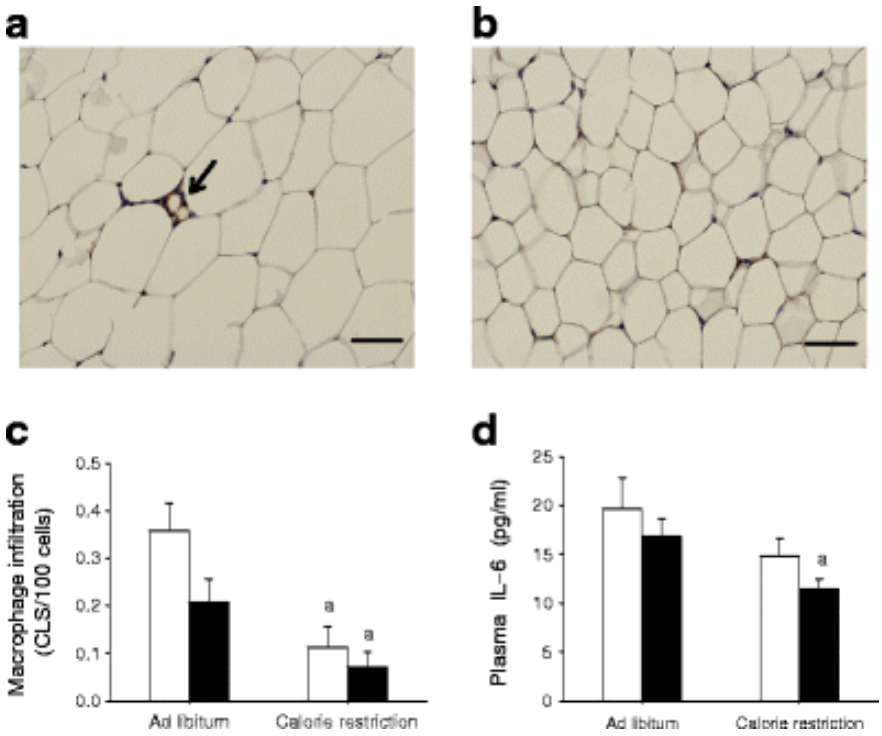

Fig. 4 Prevention of adipose tissue and systemic inflammation. Immunohistochemical analysis of epididymal fat of the cHF mice (a) and the cHF + F + CR mice (b) with visualised MAC-2 antigen (expressed on activated macrophages); arrow indicates aggregates of macrophages forming CLS, which surround dead adipocytes. c Relative count of CLS; n = 6. d Plasma level of IL-6, marker of systemic inflammatory status. Data are means ± SEM; n = 10; white bars, cHF or cHF + CR; black bars, cHF + F or cHF + F + CR; aSignificant difference (ANOVA) compared with the cHF mice. The morphometry data are based on more than 1,000 cells taken randomly from six different areas per animal. All analyses were performed after 5 weeks of the treatment

Obesity is associated with low-grade adipose tissue inflammation. Imunohistochemical analysis of epididymal fat after 5 weeks of various treatments revealed a reduction of content macrophages aggregated in crow*n*-like structures (CLS) surrounding death adipocytes compare with cHF. mRNA marker of macrophages CD68 was also decreased in the same pattern as CLS in the epididymal fat. Dietary *n*-3 PUFAs supplementation, CR or both inhibited formation of various pro-inflammatory eicosanoids in the adipose tissue and liver as well as induced the anti-inflammatory molecules (Fig.4).

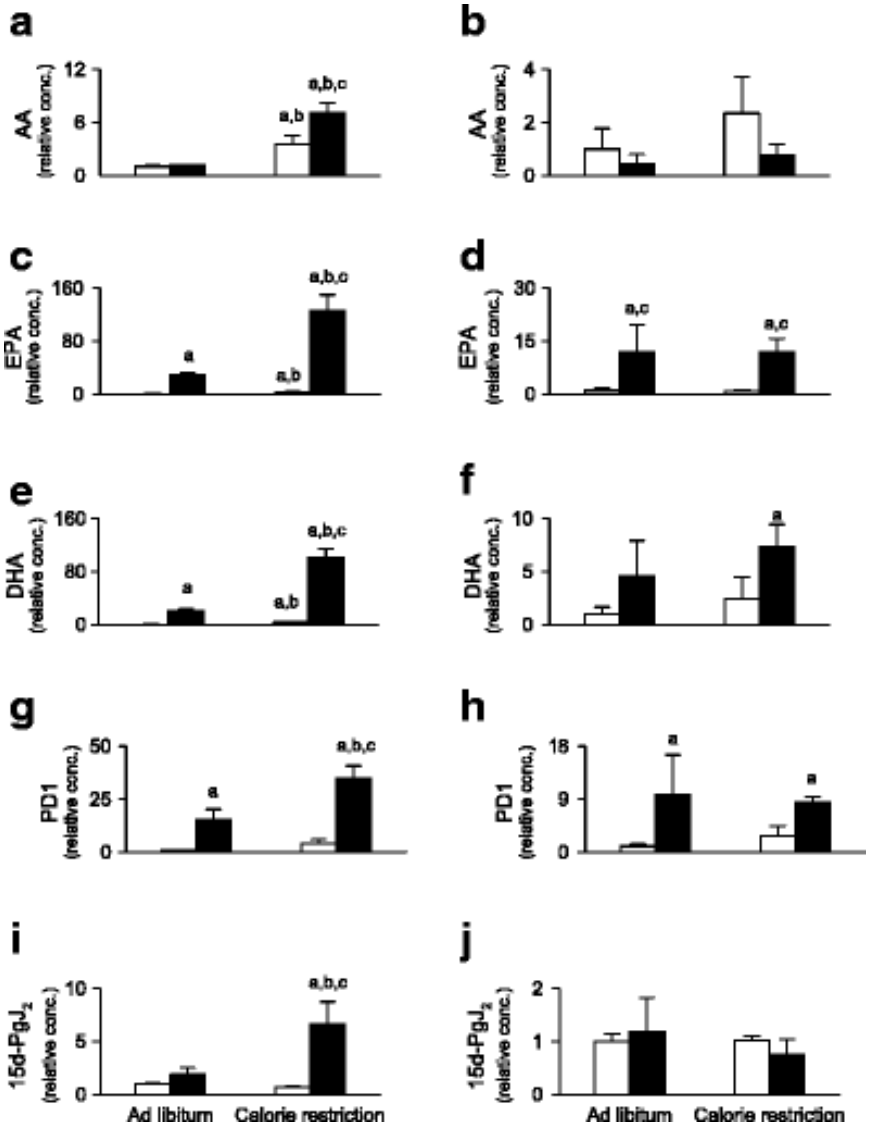

Fig. 5 Synergistic induction of anti-inflammatory lipid mediators in adipose tissue. a–j Selected results of lipidomic HPLC MS-MS analysis of epididymal fat (a, c, e, g, i) and liver (b, d, f, h, j) after 5 weeks of treatment. a, b AA; c, d EPA; e, f DHA; g, h PD1; i, j 15d-PgJ2. Data are expressed as relative concentration of individual lipids adjusted to wet weight (cHF = 1) and expressed as means ± SEM; n = 3. White bars, cHF or cHF + CR; black bars, cHF + F or cHF + F + CR. a,b,c Significant differences (ANOVA) compared with cHF, cHF + F, cHF + CR, respectively.

Lipidomic analysis was performed in epididymal fat and in the liver. In total, 24 lipid species were quantified. In WAT, but not in the liver, the first two PLS-DA components separated mice into four distinct groups corresponding to dietary treatments. Following contribution-score analysis, the most important lipids were identified for each of the intervention groups. As

shown in Fig. 5a,c,e the levels of AA, EPA and DHA in the free FA fraction of WAT were significantly higher in all treated groups compared with the cHF mice (except for AA in the ad libitum mice treated by n - 3 PUFA). The cHF + F + CR treatment exerted the most pronounced effect. In the liver, free EPA and DHA levels were significantly affected only by *n*-3 PUFA, independent of calorie restriction (Fig. 5d,f). In both tissues, the levels of primary monohydroxy metabolites derived from linoleic (HODE), AA (HETE), EPA (HEPE) and DHA (HDoHE) correlated with those of the corresponding FAs (Fig. 5). The lipoxygenase (LOX) products were the most abundant autacoids in both tissues of the cHF controls. Dietary supply of EPA and DHA triggered formation of *n*-3 PUFA-derived metabolites in both tissues. The WAT-specific liberation of FAs from membrane phospholipids in response to calorie restriction resulted in the formation of lipid mediators derived from both *n*-3 and *n*-6 PUFA (Fig. 5). Only in WAT, dietary *n*-3 PUFA combined with calorie restriction synergistically increased the levels of protectin D1 (PD1; Fig. 5g), a well described anti-inflammatory lipid mediator derived from DHA, or PD1 isomers such as PDX. In accordance with a previous finding, resolvin E1 (derived from EPA) was below the level of detection. Unexpectedly, 15-deoxy-Δ12,15-prostaglandin J2 (15d-PGJ2), an anti-inflammatory mediator and potent PPARγ agonist derived from AA, was also synergistically upregulated by the combination treatment specifically in WAT (Fig. 5i).

In summary, dietary *n*-3 PUFAs augment the anti-obesity effects of mild calorie restriction in mice while improving lipid metabolism and glucose homeostasis. These effects probably reflect in large synergistic induction of mitochondrial fatty acid oxidation in white adipose tissue, linked to a suppression of low-grade inflammation of this tissue.

My main contributions to this work were handling of animals and performing GTT, collection of samples during the termination the experiment, gene expression analysis in WAT, primary hepatocytes isolation and culturing and fatty acid oxidation in primery hepatocytes.

4.1.1 Publication C

Oleuropein as an inhibitor of peroxisome proliferator-activated receptor gamma. Svobodova M, Andreadou I, Skaltsounis AL, Kopecky J, Flachs P.

The aim of this study was to characterize the molecular mechanism of the action of oleuropein, polyphenols extracted from olive leaves, using cell line 3T3-L1 and SVF isolated from gonadal and dorsolumbar adipose tissue of mice and differentiated *in vitro*.

Exposure of proliferating 3T3-L1 cells to increasing concentrations of oleuropein (from 10 to 400 μM) for 24 h resulted in lower viability of the cells. When 100 units catalase/ml was added into medium for culturing for pre-confluent cells, the inhibitory effect was completely prevented (Fig. 1—upper panel). Interestingly, no cytotoxic effects were detected in post-confluent cells after induction of adipocyte differentiation (Fig. 1—lower panel). The results were confirmed by two independent approaches, trypan blue (Fig. 1) and WST-1 assays (data not shown).

Oleuropein (>100μM) decreased viability of proliferating preadipocytes and didn't exerted any cytotoxic effects in post-confluent cells after induction of differentiation. Oleuropein dose-dependently (>100μM) inhibited adipocyte differentiation and suppressed gene expression of PPARγ, C/EBP1α, SREBP-1c and FAS in both experimental models. PPARα and PPARδ/β activity were not affected by oleuropein. In contrary, PPARγ transcription activity was diminished by oleuropein (>100μM).

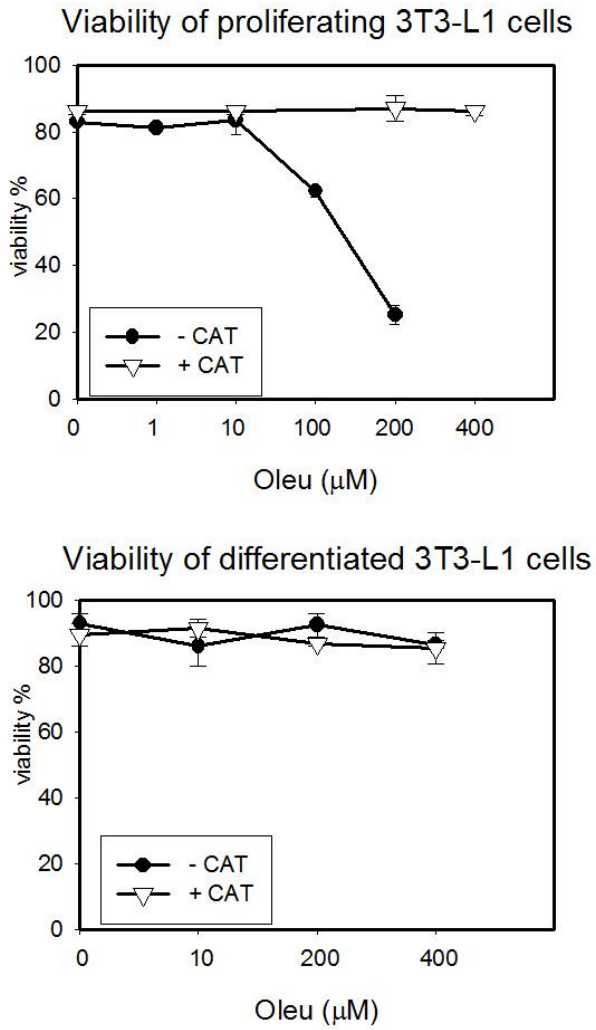

Fig. 1 Effect of oleuropein on viability of proliferating and differentiated 3T3-L1 cells. Cells were cultured in appropriate medium at 37 °C in a humidified 5 % CO2, in the absence or presence of oleuropein (Oleu—0, 1, 10, 100, 200 and 400 μM) and in the presence or absence of catalase (CAT; 100 units/ml)

We verified that oleuropein, added to the medium simultaneously with induction of differentiation by dexamethasone and rosiglitazone, significantly inhibited 3T3-L1 adipocytes differentiation. Moreover, we next examined the effect of oleuropein in primary SVF cultures of dorsolumbar and gonadal fat, physiologically more relevant model of adipocytes in vitro. (Fig.1) Oleuropein did not show any negative effect on the viability of cells induction of adipocytes

differentiation unlike proliferating cells. Oleuropein has cytotoxic effect on proliferating 3T3-L1, which is prevented by catalase (Fig. 2.).

To confirm that oleuropein inhibits the adipocyte differentiation pathway, we analyzed the effect of oleuropein on the expression of the genes for PPARγ, FAS, C/EBPα and SREBP-1c using qRT-PCR. In accordance with the results of others (Drira et al., 2011), oleuropein in a dose-dependent manner (starting at a 10 μM concentration) reduced expression of the genes for the transcription factors PPARγ and C/EBPα, and pro-lipogenic SREBP-1c and its target gene FAS (Fig. 3). The presence of catalase (100 units/ml) in the medium during cell differentiation resulted in a decrease of C/EBPα transcript level in the control (non-treated by oleuropein) 3T3-L1 adipocytes, while it had no effect on the expression of the other genes studied. Catalase diminished the effect of 400 μM oleuropein (the highest concentration studied) on C/EBPα and SREBP-1c expression (Fig. 3).

We used the cell-based reporter gene assay to test whether oleuropein directly affects the activity of nuclear receptors PPARα, PPARβ/δ and PPARγ. Oleuropein between 10 and 400 μM concentrations did not affect the activity PPARα or β/δ (see Fig. 4). Contrary, PPARγ activity, either basal or rosiglitazone activated, was inhibited (by 30–50 %) by oleuropein (200 μM; Fig. 5). The inhibitory effect of oleuropein was stronger than that of the specific inhibitor of PPARγ—T0070907 (Lee et al., 2002) at concentration of 0.1 μM.

Oleuropein suppresses both preadipocyte proliferation and adipocyte differentiation *in vitro*. Moreover, our data suggest oleuropein exerts anti-adipogenic effect through direct inhibition of PPARγ transcriptional activity.

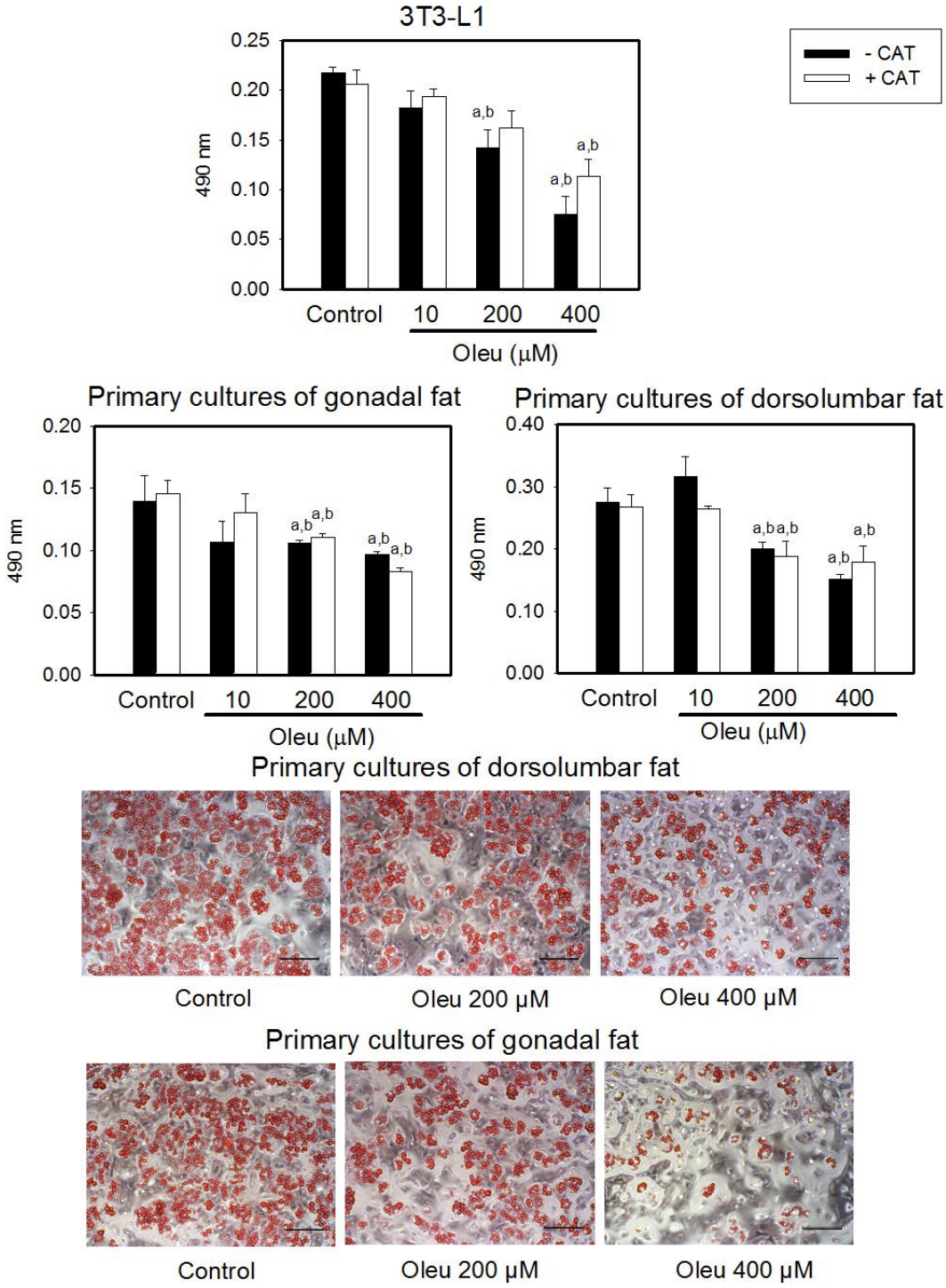

Fig. 2. Effect of oleuropein on differentiation of 3T3-L1 adipocytes and SVF cells isolated from dorsolumbar and gonadal adipose tissue of C57BL/6J mice in primary cultures. Cultured cells were exposed for 11 days to differentiation medium containing or not oleuropein (Control or Oleu—0, 10, 200

and 400 µM) in the absence or presence of catalase (CAT; 100 units/ml) at 37 °C in a humidified 5 % CO2, respectively, 10 % CO2. Cells were stained with Oil Red O. a,b Significantly different from Control without a or with b catalase, *significant effect of catalase (two-way ANOVA)

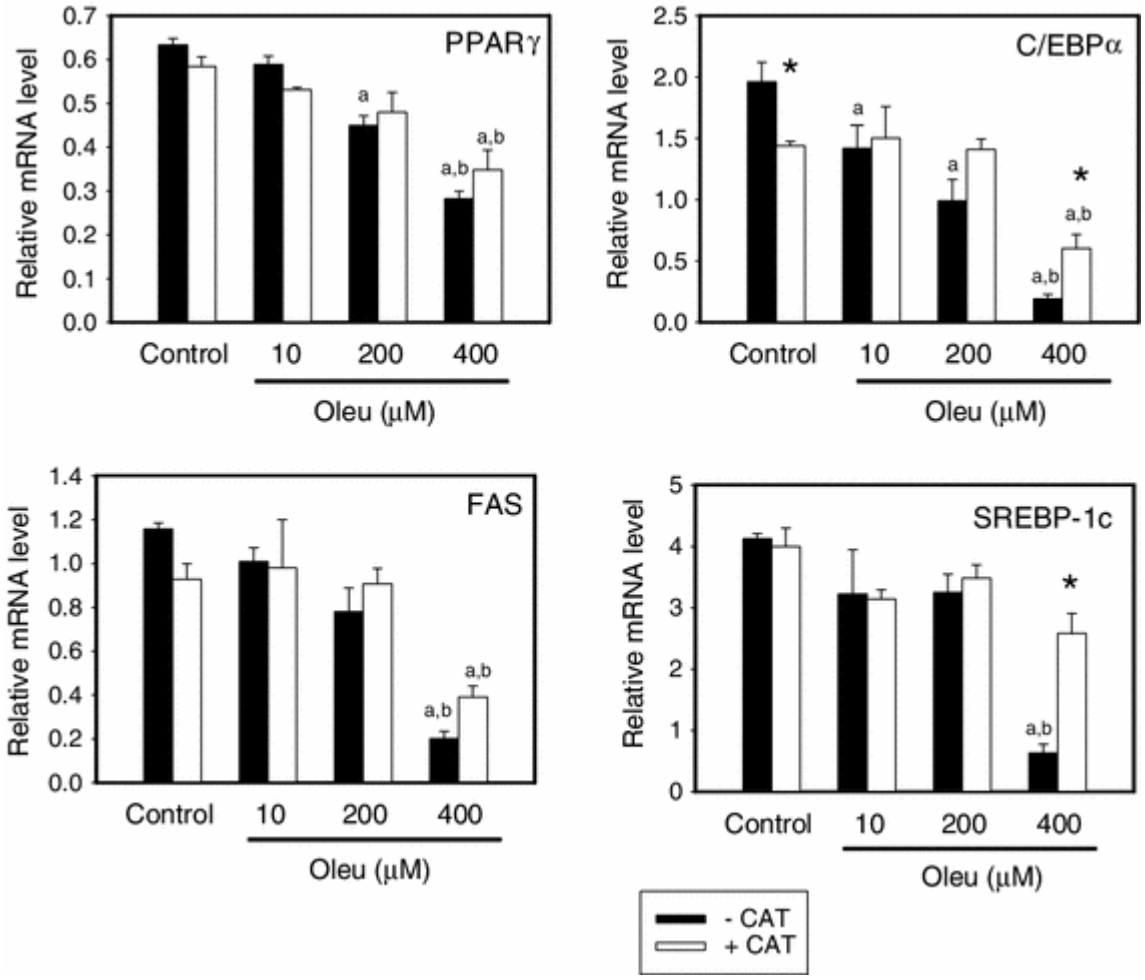

Fig. 3. Effect of oleuropein on expression of PPARγ, FAS, C/EBPα and SREBP-1c genes in 3T3-L1 adipocytes. Gene expression was evaluated in cells differentiated in vitro as described in legend to Fig. 2. The relative expression of the genes was quantified by qRT-PCR. a,b Significantly different from Control withouta or withb catalase, *significant effect of catalase (two-way ANOVA)

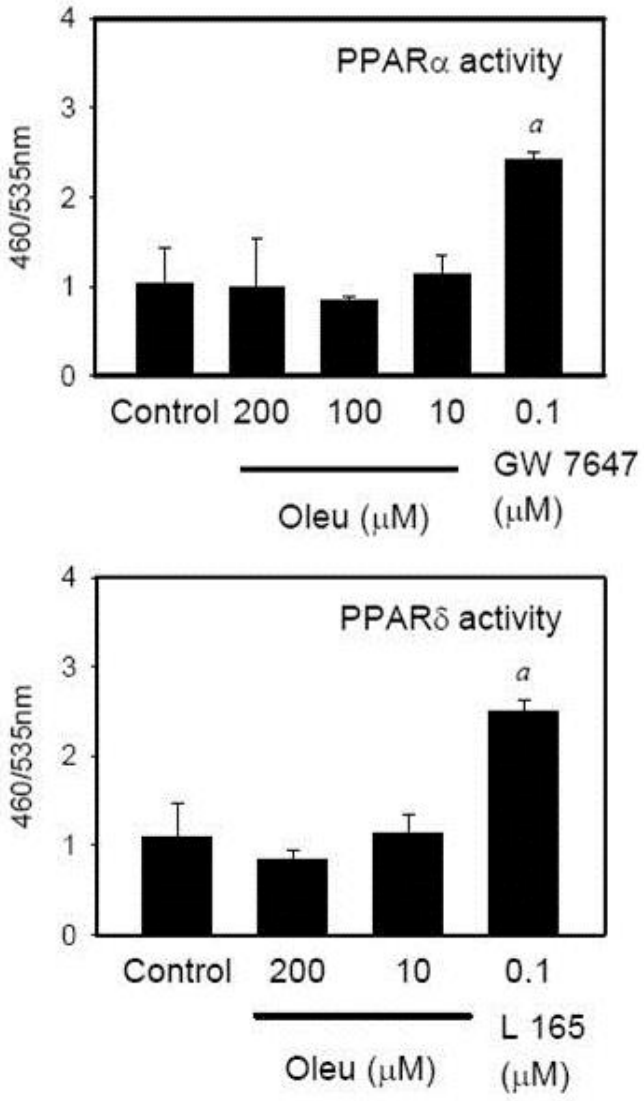

Fig. 4. Cell based PPARα (PPARδ) reporter-gene assay. Cells were incubated with oleuropein (Oleu) in concentration from 10 to 200μM at 37°C in a humidified 5% CO2 for 16 hours. GW 7647 (L 165) was used as an activator PPARα (PPARδ) in concentration 0.1 μM.
a - Significant difference (ANOVA) compared with control

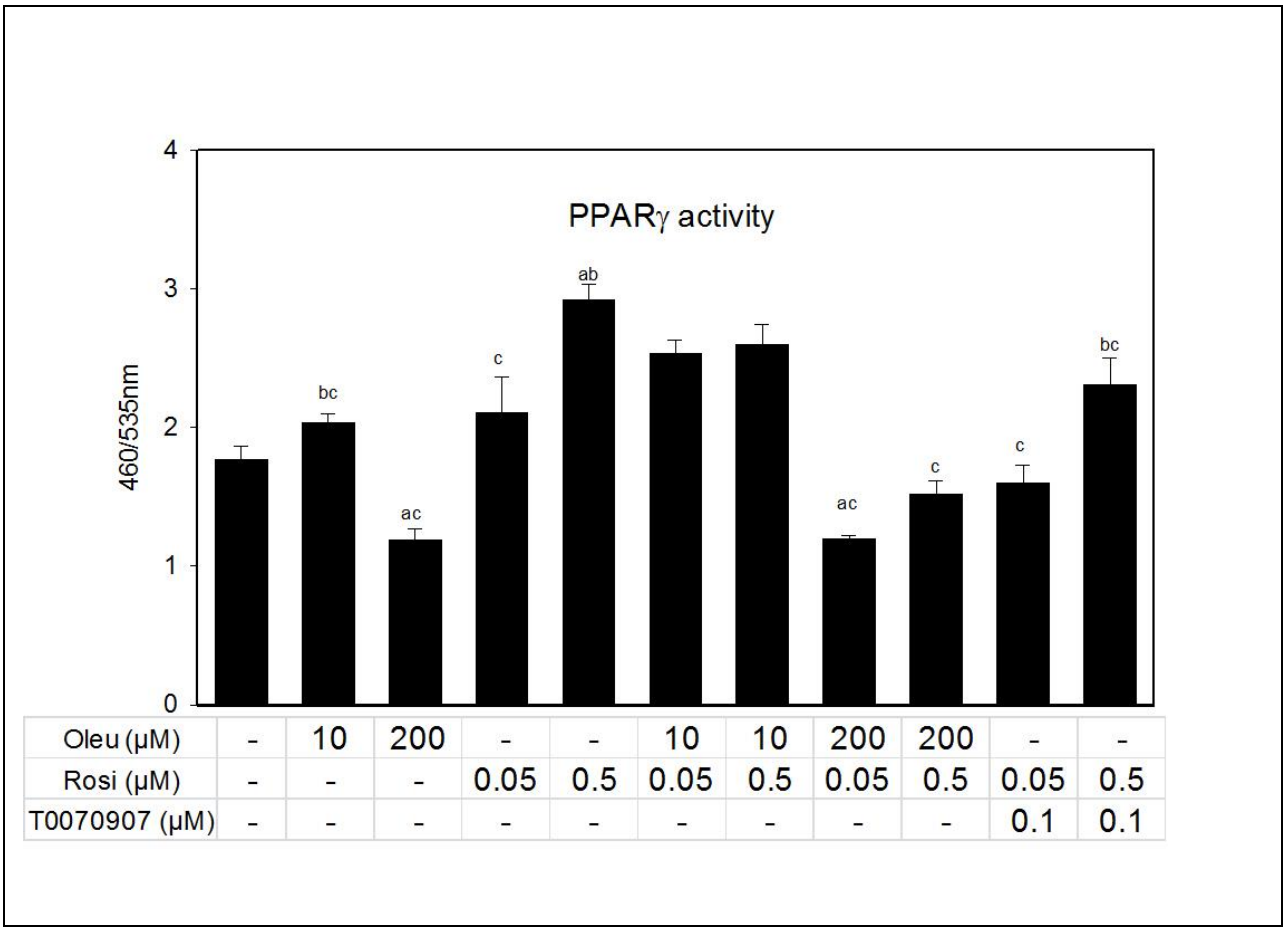

Fig. 5. Cell-based PPARγ reporter gene assay. Cells were incubated with oleuropein (Oleu) in concentration 10 and 200 µM. Rosiglitazone (Rosi) was used as an activator PPARγ in concentration 0.05 and 0.5 µM and T0070907 as specific inhibitor of PPARγ at concentration 0.1 µM. Cells were incubated with Oleu or T0070907 for 30 min before adding Rosi and then incubated at 37 °C in a humidified 5 % CO2 for 16 h. a,b,c Significant difference (one-way ANOVA) compared with control, Oleu 200 µM and Rosi 0.5 µM, respectively

My main contributions to this work were performing in vitro technics - isolation, plating and culture maintenance of adipose precursor cells/primary SVF cultures from gonadal and subcutaneous depot, cell line 3T3-L1, cell viability assays, Oil Red O staining, reporter gene assay, RNA isolation from the cell cultures, control of quality of isolated RNA, reverse transcription and quantification of gene expression using qRT-PCR and statistical analysis.

5 DISCUSSION

This thesis was focused on the analysis of the molecular mechanisms underling the beneficial effect of naturally occuring *n*-3 PUFA on glucose homeostasis and insulin sensitivity namely when *n*-3 PUFA were used in combination treatments with anti-diabetic drugs or calorie restriction. We sought to learn whether these treatments could provide additional benefit using the model of dietary obese and insulin resistant B6/J mice. (Publications A and B).Moreover, using an invitro system, we have characterized molecular mechanism action of oleuropein, i.e. plant-derived compound, plant polyphenols could mimic the calorie restriction and could help in prevention or treatment of obesity and associated disorders (Publication C). Modulation of WAT functions in responsive the above treatments represent an unifying theme of our studies.

5.1 The combination treatments comparison

Increased accumulation of body fat represents a common side effect of the TZD therapy. Diabetic patients treated with rosiglitasone enhanced accumulation of subcutaneous fat (Yang and Smith, 2007), as well as in mice treated by rosiglitasone using the doses much higher than in our study (Kim et al., 2004; Wilso*n*-Fritch et al., 2004). In contrast, our study in mice has shown (Publication A) that the combination treatment using relatively low doses of rosiglitasone and *n*-3 PUFA exerted surprising additive effects in the prevention of body weight gain, namely due to a reduced accumulation of subcutaneous fat. Nevertheless, cHF + *n*-3 PUFA or cHF + CR treatment partially prevented the cHF-induced obesity, while the combination treatment calorie restriction with *n*-3 PUFA provided full protection (Publication B, and Table below).

Impaired metabolic flexibility is linked to insulin resistance and these two defects are in the core of the development of metabolic syndrome. Our results from Publication B demonstrate an improvement of metabolic flexibility in mice by the combination treatment CR + *n*-3 PUFA , based on both the response of plasma levels of NEFA and glucose to FASTED to RE-FED transition and the glucose-induced increase in respiratory quotient (RQ) (see Table 1 in

Publication B). In Publication A the long-term treatment combining *n*-3 PUFA and either rosiglitasone or pioglitazone markedly improved systemic markers of lipid and glucose homeostasis in mice fed a high-fat diet. Moreover, the insulin tolerance test performed in dietary obese mice in Publication A suggested that the combined use of *n*-3 PUFA and pioglitazone could provide an additive benefit in reverting insulin resistance. These findings have implications for the treatment of insulin resistance in diabetic patients.

None of the above treatments preserved the NEFA response, with the exception of the cHF+F+ROSI treatment, which tended to normalize it. In contrast to NEFA, the TG response was fully preserved by both combination treatments (cHF+F+ROSI and cHF+F+PIO). Single-type treatments (cHF+F, cHF+ROSI, and cHF+PIO) had no significant effect on the TG response. The glycaemic response tended to be preserved by all the treatments, with only marginal differences between them. Moreover, plasma levels of acylcarnitines with long saturated and monounsaturated even side-chains (C12-C18) in response to re-feeding correlate with the activity of β-oxidation, mainly in skeletal muscle (Koves et al., 2008; Lehmann et al., 2010), and also in the liver (Hallows et al., 2011). While these are suppressed by insulin, thus reflecting the switch between fatty acid and carbohydrate catabolism (Mihalik et al., 2010; Zhao et al., 2009). The stronger suppression of plasma levels of the long-chain acylcarnitines in response to re-feeding in the cHF+F+ROSI as compared with the cHF+F+PIO mice suggest that the anti-obesity effect of the cHF+F+ROSI treatment reflects the relatively strong stimulation of β-oxidation. High-fat diet-induced insulin resistance in skeletal muscle is characterized by an impaired switching to carbohydrate oxidation during the FASTED/RE-FED transition, which is mirrored by smaller changes in the tissue levels of long-chain acylcarnitines (Koves et al., 2008). This improvement of metabolic flexibility correlated with changes in body weight, adiposity and glycaemic control in accordance with the notion that metabolic flexibility is closely associated with insulin sensitivity (Galgani et al., 2008; Storlien et al., 2004) and that impaired flexibility represents an early defect in the development of type 2 diabetes (Corpeleijn et al., 2008). Several studies in humans demonstrated that *n*-3 PUFA supplementation could help to reduce obesity (Kunesová et al., 2006).

The synergistic anti-steatotic effect of the cHF+F+PIO treatment is even more striking in the light of the opposite effects elicited by TZDs, concerning both hepatic steatosis and Scd-1

expression (see Table 1 and 2). cHF+F+PIO treatment resulted not only in the lowest plasma TG levels, but also fully prevented the cHF-induced hepatic steatosis, suggesting that the hypolipidaemic effect resulted from the modulation of hepatic lipid metabolism. *n*-3 PUFA in combination with CR resulted in more pronounced reduction of TG liver content than *n*-3 PUFA treatment alone (see Table 1). It is consistent with the depression of VLDL-TG formation, and the notion that the hepatic effects were mediated by the adiponectin-AMPK axis, as proposed for both TZDs (Nawrocki et al., 2006) and *n*-3 PUFA (Jelenik et al., 2010).

The gene expression analysis showed that the additive combination cHF + *n*-3 PUFA + CR strongly inducted PGC-1α, PPARα expression in epididymal adipose tissue, suggesting increased biogenesis and enhanced oxidative capacity of WAT mitochondria (see table 2). Analysis of gene expression from gastrocnemius, a mixed-fibre muscle, revealed a trend for induction of Pdk4, Cpt1a and Cpt1b by the combination treatment, supporting the notion that the combination treatment with TZDs induced a switch augmenting lipid over glucose catabolism. Interestingly, the cHF+TZD diet strongly induced expression of Scd1, while cHF+F and cHF+F+TZD treatments had an opposite effect (see Table 2. below).

In WAT, in contrast to the liver, the levels of EPA, DHA, AA and their active metabolites, including the anti-inflammatory molecules PD1/PDX and prostaglandin 15d-PGJ_2, were increased in a synergistic manner by the combination treatment, because dietary *n*-3 PUFA supplementation resulted in the inhibition of formation of various *n*-6 PUFA-derived pro-inflammatory eicosanoids in both tissues as well as in the induction of the anti-inflammatory molecules. The induction of prostaglandin 15d-PGJ2 was an unexpected finding as 15d-PGJ2 is derived from the metabolism of *n*-6 PUFA, and enzymatic formation of *n*-6 PUFA metabolites is, in general, inhibited upon *n*-3 PUFA supplementation. This suggests that dietary *n*-3 PUFA, especially in combination with calorie restriction, selectively activates formation of 15d-PGJ2 from prostaglandin D2 (PGD2), the major product of cyclooxygenase (COX) in many tissues (Scher and Pillinger, 2005), reflecting possibly the ability of EPA- and DHA-derived peroxyl radicals to favour the formation of 'less pro-inflammatory' peroxidation products derived form AA (Davis et al., 2006). Moreover, 15d-PGJ_2 is the most potent endogenous ligand for PPARγ (Kliewer et al., 1995). Thus, the activation of PPARγ via 15d-PGJ_2 binding could be responsible

for the additive effects of the combination treatment on adipose tissue energy and lipid metabolism. This idea is supported by the fact that rosiglitasone, a PPARγ ligand, increases PPARγ coactivator 1α (PGC-1α) production, mitochondrial mass, palmitate oxidation and mitochondrial uncoupling protein 1 (UCP1) in the adipose tissue of genetically obese ob/ob mice (Wilso*n*-Fritch et al., 2004).

It has been found that brown adipocyte-like cells originating from rosiglitasone-treated epididymal WAT cell precursors *in vivo* represent a new subtype of adipocytes, called 'brite' cells, which differ from classic white and brown adipocytes, but possess mitochondrial UCP1-mediated thermogenesis (Nedergaard and Cannon, 2010). Recent studies (Madsen et al., 2010) demonstrated the involvement of COX-2 in the induction of these fat-burning cells and the importance of the COX-2-mediated mechanisms for the resistance to dietary obesity in mice. Our results convincingly demonstrate a marked induction of mitochondrial oxidative capacity in permeabilised adipocytes isolated from epididymal fat of mice subjected to mild calorie restriction combined with *n*-3 PUFA intake. Moreover, changes in palmitate oxidation in both intact isolated adipocytes and in fat fragments obtained from the above mice document induction of energy expenditure by the combination treatment. Importantly, these changes occurred even in the absence of UCP1 induction but they were accompanied by the simultaneous activation of PPARα/PGC-1α and PPARγ signalling. Induction of futile substrate cycling in adipocytes by this mechanism (Langin, 2010) might explain the increased lipid catabolism in the absence of mitochondrial uncoupling. This idea is consistent with our current findings that the combination treatment increases capacity for de novo lipogenesis in epididymal fat, both at the level of gene expression and biochemical activity. Fat-depot-specific differences affecting the inducibility of energy-dissipating adipocytes should be explored further. In addition to the stimulation of lipid catabolism in WAT, FA oxidation in liver—but not in skeletal muscle—could contribute to the whole-body effects of the combination treatment. However treatments affecting adipose tissue by multiple mechanisms, such as combining *n*-3 PUFAs with either caloric restriction or antidiabetic/anti-obesity drugs has proved more effective than single treatments. These results are relevant regarding therapeutic strategies of human patients with obesity and related disease. (see (Flachs et al., 2013)

Table 1: Summary of the effects of the combination treatments using *n*-3 PUFA on growth and metabolic features of mice

		***n*-3 PUFA + TZDs (vs. cHF diet)**	***n*-3 PUFA + CR (vs. cHF diet)**
Energy balance	**Body weight gain**	decreased*	decreased*
	Food intake	no effect	no effect
Glucose homeostasis	**GTT (total AUC)**	decreased	decreased
	Fasting glucose	decreased	decreased*
	HOMA-index	decreased	decreased*
	Metabolic flexibility (INCA**)**	improved	improved*
Plasma (random fed)	**NEFA**	decreased*	no data
	TAG	decreased*	decreased*
	Cholesterol	decreased	no data
	Glucose	no data	no chase
	Adiponectin	increased (PIO)*, increased (ROSI)	increased*
	Leptin	decreased	decreased*
	Insulin	decreased*	Decreased
WAT			
Subcutaneus WAT	**Total mass**	decreased*	decreased*
	Adipocytes size	decreased*	decreased
	Insulin sensitivity	increased*	Increased
	Infammation (CLS)**	decreased	decreased
Epididymal WAT	**Total mass**	decreased	decreased*
	Insulin sensitivity	increased*	increased*
	Adipocytes size	decreased	decreased*
	Infammation (CLS)**	decreased	decreased*
Liver	**Weight**	decreased (PIO)*, no effect (ROSI)	decreased*
	Insulin sensitivity	increased	increased*
	Tissue lipid content	decreased (PIO)*, no change (ROSI)	decreased*
Muscle (gastrocnemius)	**Weight**	no effect	no effect
	Insulin sensitivity	increased	increased*
	Tissue lipid content	no effect	decreased ***
	Glycogen content	increased*	no data

* Additive effect of the combination treatment;**Number of CLS per 100 adipocytes; *** after 15 weeks of the treatment; *Abbreviations:* AUC, total area under the curve; CLS, crown like structure; GTT , glucose tolerance test; INCA, indirect calorimetry.

Table 2: Summary of the effect of the combination treatments using *n*-3 PUFA on gene expression

		n-3 PUFA + TZDs (vs. cHF diet)	n-3 PUFA + CR (vs. cHF diet)
Subcutaneus WAT	**Fatty acid sytnetsis: SCD1, FAS**	no effect	no effect
	Fatty acid oxidation: CPT-1, PPARa	no data	increased*
	Mitochondrial biogenesis: PGC-1a	no data	no effect
	Glyceroneogenesis: PEPCK, PDK4	increase	increase
Epididymal WAT	**Fatty acid sytnetsis: SCD1, FAS**	no effect	increased*
	Fatty acid oxidation: CPT-1, PPARa	no effect	no effect
	Mitochondrial biogenesis: PGC-1a	increased	big increased*
	Glyceroneogenesis: PEPCK, PDK4	increased	increased*
Liver	**Glucose metabolism: PDK4**	no effect	no effect
	Fatty acid sytnetsis: SCD1, FAS	FAS decreased, SCD1 increased	decreased*
	Fatty acid oxidation: CPT-1, PPARa	increased	no effect
	Mitochondrial biogenesis: PGC-1a	decreased*	no effect
Muscle (gastrocnemius)	**Glucose metabolism: PDK4**	no effect	no effect
	Fatty acid sytnetsis: SCD1	no effect	no effect
	Fatty acid oxidation: CPT-1	increased	no effect

CPT-1 Carnitine palmitoyltransferase I, PDK4 Pyruvate dehydrogenase lipoamide kinase isozyme 4, PEPCK Phosphoenolpyruvate carboxykinase, PGC-1a Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, PPARa Peroxisome proliferator-activated receptor alpha, SCD1 Stearoyl-CoA desaturase

5.2 The oleuropein effect

Oleuropein and other polyphenols from olive were studied, among others, for their ability to protect against various components of metabolic syndrome, e.g. obesity, dyslipidemia, type 2 diabetes, cardiac ischemia and other disorders, both in humans (de Bock et al., 2013a) and experimental animal models (Andreadou et al., 2006a), (Al-Azzawie and Alhamdani, 2006a),(Oi-Kano et al., 2008). Supplementation with olive leaf polyphenols (90% of oleuropein) for 12 weeks significantly improved insulin sensitivity and pancreatic β-cell secretory capacity in overweight middle-aged men at risk of developing the metabolic syndrome (de Bock et al.,

2013a). Importantly, the 15–20% improvement in insulin sensitivity observed with olive leaf polyphenols supplementation is comparable to those seen with medications commonly used to treat diabetes (de Bock et al., 2013a). For example, metformin (250 mg) improved insulin sensitivity by 17% in a group of sedentary overweight no*n*-diabetics (Ou et al., 2006). Another study demonstrated a 28% improvement in insulin sensitivity after treatment with 30 mg pioglitazone for 26 weeks (Miyazaki et al., 2002).

We focused on the molecular pathway engaged in the oleuropein effects the adipose tissue. Our current findings reveal that oleuropein suppressed not only the differentiation of 3T3-L1 cell (Drira et al., 2011), but also that of adipocytes in primary SVF cultures derived from either dorsolumbar or gonadal adipose tissue of the mice. SVF cultures with multiple cell types are more predictive of *in vivo* condition and can be obtained from various depots and following various *in vivo* treatments.

The important feature of the adipocyte differentiation is the increased expression of the PPARγ gene. In general, bioactive plant polyphenols, like as green tea catechins, resveratrol, curcumin and also oleuropein, are known to effect PPARγ expression. Accordingly, in the current study increasing concentration of oleuropein in the medium resulted in a remarkable reduction of mRNA levels for the key transcription factors and markers of adipogenesis, including PPARγ, C/EBP α, SREB-1c and FAS. Moreover, we found, using the gene-reporter assay, that oleuropein directly inhibits transcription activity of PPARγ. Contrary, we didn't find any effects on transcriptional activity of other members of PPAR nuclear receptor family - PPARα and PPAR β/δ.

The proof of the inhibition of PPARγ by oleuropein is new piece of evidence enlarging the knowledge on the molecular mechanisms of this polyphenol of action.

6 CONCLUSIONS

1. In mice fed obesogenic high-fat diet, treatment with *n*-3 PUFA in combination with either rosiglitazone or pioglitazone unmasked differential effects the two TZDs. Only the combined treatment using *n*-3 PUFA and rosiglitazone reduced body weight gain. Both types of the combined treatments exerted additive stimulatory effect on secretion of adiponectin from WAT and improved glucose homeostasis. These results support the notion that *n*-3 PUFA could be used as add-on treatment to TZDs in order to improve diabetic patient's therapy.
2. The dietary *n*-3 PUFAs augment the anti-obesity effects of mild calorie restriction in mice fed high fat diet while improving lipid metabolism and glucose homeostasis. These effects probably reflect in large synergistic induction of mitochondrial fatty acid oxidation in WAT, linked to a suppression of low-grade inflammation of this tissue. Increased intake of dietary *n*-3 PUFA could augment weight reduction in obese patients subjected to very low calorie diet.
3. Oleuropein *in vitro* exerts anti-adipogenic effect through inhibition of both expression and activity of PPARγ. The major transcription intracellular regulatory mechanism in fat cells. These results argue for further clinical studies focused on the anti-obesity effects of plant polyphenols, namely oleuropein.

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