Charles University in Prague

Faculty of Science

Department of Genetics and Microbiology

Academy of Sciences of the Czech Republic

Institute of Physiology, v.v.i

Synopsis of Ph.D. Thesis

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

---

Michaela Svobodová

Praha 2015

Doktorské studijní programy v biomedicine

Univerzita Karlova v Praze

a Akademie věd České republiky

Program: Molekulární a buněčná biologie, genetika a virologie

Předseda oborové rady: prof. RNDr. Stanislav Zadražil, DrSc.

Školicí pracoviště: Fyziologický ústav AV ČR, v.v.i.

Autor: Michaela Svobodová

Školitel: RNDr. Pavel Flachs, Ph.D.

S disertací je možno se seznámit v příslušných knihovnách
Přírodovědecké fakulty Univerzity Karlovy v Praze.

LIST OF ABBREVIATIONS

15d-PGJ_2	15-deoxy-$\Delta^{12,14}$ - prostaglandin J_2
AA	arachidonic acid
AMPK	AMP–activated protein kinase
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
CR	calorie restriction
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

Abstract

Globally 1, 5 billion adults are overweight or obese according to WHO. Obesity-associated diseases, namely type 2 diabetes, dyslipidaemia and other morbidities clustered in the 'metabolic syndrome' predispose individuals to cardiovascular disease and represent a significant health problem around the world. Complex aetiology 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 hypolipidaemics, 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.

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 hyperglycemia. 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 lipogenesis and proteoanabolic), and causes the transport of glucose transporters using GLUT- 4 to the cell interior (hypoglycemic 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*.

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, tumor 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 (27). 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 (5).

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.

1.2 Adiponectin

Adiponectin is a protein produced mainly mature adipocytes. Adiponectin levels in the plasma and adipose tissue are decreased in obese individuals compared with lean individuals. Adiponectin significantly regulates the metabolism of carbohydrates and lipids, increased utilization and transport glucose and free fatty acids into muscle, liver and fat cells.

1.3 Transcription factor PPARγ

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

The key role in the differentiation process is played by transcription factors from the **PPAR** and C/EBP (CCAAT/enhancer-binding protein)

families (2). Among adipogenic transcription factors PPAR γ stands out as a key regulator obligate for *in vitro* as well as *in vivo* development of adipocytes (17).

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) (26). 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 (23,25). 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 (24).

1.4 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 (16). 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 (20). Adipokines such as adiponectin and leptin are also potent activators of AMPK (8).

1.5 Beneficial effects n-3 LC PUFA

Marine fish oils, namely long-chain (LC) polyunsaturated fatty acids (PUFA) of n-3 series (omega-3), such as docosahexaenoic acid (DHA; 22:6n-3), eicosapentaenoic acid (EPA; 20:5n-3) and docosapentaenoic acid (DPA, 22:5n-3) act as natural hypolipidaemic and anti-inflammatory agents and enhance various factors of the metabolic syndrome (5, 6, 13).

Dietary intake of n-3 LC-PUFA is associated with a variety of cellular responses including changes in gene expression and FA composition of plasma membrane phospholipids, which could affect the synthesis of eicosanoids as well as the fluidity of biological membranes (12). Concerning of n-3 LC-PUFA in the context of obesity-associated insulin resistance, several potential mechanisms have been suggested:

a) Limitation of lipotoxicity in insulin-sensitive tissues
b) Elimination of inflammatory response in obese adipose tissue
c) Beneficial changes in the secretory profile of adipose tissue-derived hormones adipokines, namely adiponectin

d) Formation of biologically active lipid mediators, i.e. resolvins and protectins
e) Activation of AMP-activated protein kinase (AMPK)

1.6 Thiazolidinediones

Thiazolidinediones (TZDs) such as rosiglitazone and pioglitazone are among the preferred pharmacological agents in the treatment of insulin resistance in diabetic patients.

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 (11).

However, TZDs are also associated with unwanted side-effects, such as oedema and weight gain (21), increased risk of heart failure (15), and bone loss (10).

1.7 Oleuropein

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.

In vitro, oleuropein reduces the expression of PPARγ, inhibits adipogenesis and enhances osteoblastogenesis in stem cells derived from human bone marrow (19). Oleuropein acts on 3T3-L1 cells to reduce preadipocyte differentiation and lipid accumulation and thus regulate the size of fat cells (4).

1.8 Caloric restriction

Calorie restriction (CR) is an essential component in the treatment of obesity and associated diseases (18).

Calorie restriction-signaling mutually interacts with AMPK, the sensor of energy state and the key regulator of fuels partitioning (22). Moreover, calorie restriction induces mitochondrial biogenesis in various tissues (7), with a relatively strong effect in white WAT (9, 14). The induction of mitochondria involves transcription factors PPARα/γ and PPARγ coactivator, PGC-1α (7, 14).

2. METHODS

Animals and treatments
Glucose homeostasis
Murine 3T3-L1 cells/adipocytes
Isolation, plating and culture condition of primary adipocytes
Cell viability assays
Trypan Blue assay
WST-1 assay
Oil red-O staining
Reporter-gene assay
Isolation RNA and RT – PCR
Quantitative real time PCR
The rate of fatty acid oxidation in isolated hepatocytes
Statistical analysis

3. 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 anti-diabetic drugs from TZD family and 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*.

4. RESULTS OF PUBLICATIONS

Publication A

Unasking 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. (2011) PLOS ONE Volume: 6 Issue: 11 (IF: 4.092; citations: 13)*

Fatty acids of marine origin, i.e. docosahexaenoic and eicosapentaenoic acid (DHA and EPA, respectively) act as hypolipidemics, but they do not improve glycemic control in diabetic patients. Thiazolidinediones (TZDs), like rosiglitazone and pioglitazone, i.e. specific activators of peroxisome proliferator-activated receptor-gamma improve whole-body insulin sensitivity. It was shown that a combination treatment using a DHA and EPA concentrate (DHA/EPA) and rosiglitazone provided, by complementary mechanisms, additive beneficial effects on dyslipidemia and impaired glucose tolerance (IGT) in obese mice. Aim of the project was to further study mechanism of action of TZDs, namely of roziglitazone and pioglitazone.

Male C57BL/6 mice were fed high-fat diet. The effects of DHA/EPA (replacing 15% dietary lipids), rosiglitazone (10 mg/kg diet), or pioglitazone (50 mg/kg), or combination of both DHA/EPA and rosiglitazone or pioglitazone on body weight, adiposity, metabolic markers and adiponectin in plasma, liver and muscle triacylglycerol accumulation analyzed. Intraperitoneal glucose tolerance test was used to characterize the changes of glucose homeostasis. Metabolic

flexibility was tested between two limited condition – fasted and re-fed status.
DHA/EPA and TZDs exerted additive effects in prevention of obesity, dyslipidemia, and IGT, while suppressing hepatic triacylglycerol accumulation and inducing adiponectin. The treatment also improved metabolic flexibility. In conclusion both type of TZD could be used in combination with DHA/EPA as complementary therapies to counteract dyslipidemia and insulin resistance. The combination treatment may reduce dose requirements and hence the incidence of adverse side-effects of the thiazolidinedione therapy.

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. (2011) DIABETOLOGIA Volume: 54 Issue: 10 Pages: 2626-2638 (IF: 6,814; citations: 28)*

Calorie restriction (CR) is an essential component in the treatment of obesity and associated diseases. n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA) act as natural hypolipidemics, reduce risk of cardiovascular disease and could prevent development of obesity and insulin resistance. We aimed to characterize the efficacy and underlying mechanisms of the

combination treatment with n-3 LC-PUFA and mild CR in the prevention of obesity and associated disorders in mice.
Male C57BL/6J mice were habituated to a corn oil-based high-fat diet (cHF) for 2 weeks and then randomly assigned to various dietary treatments for 5 weeks or 15 weeks: (i) cHF, ad libitum; (ii) cHF+F, cHF diet with n-3
LC-PUFA concentrate (EPAX 1050 TG) replacing 15% of dietary lipids, ad libitum; (iii) cHF+CR, cHF diet reduced by 10% as compared with the ad libitum cHF-fed mice; and (iv) cHF+F+CR.
Compared with the cHF diet, the combination treatment (cHF+F+CR) most efficiently reduced body weight gain and the weight of both abdominal and subcutaneous white adipose tissue (WAT) depots, while it also improved insulin sensitivity (HOMA index) and glucose tolerance (OGTT). Ectopic lipid accumulation in the liver and skeletal muscle was also markedly decreased by the combination treatment. Moreover, the infiltration of WAT by macrophages, indicating a chronic low-grade inflammation of the tissue, was prevented by the cHF+F+CR treatment. Specifically in abdominal WAT, the conversion of PUFA to anti-inflammatory lipid mediators was additively induced by CR and n-3 LC-PUFA. For instance, the levels of 15-deoxy-prostaglandin J2 and neuroprotectin D1 were increased 6.6-fold and 35-fold, respectively, by the cHF+F+CR treatment as compared with the cHF mice. Furthermore, gene expression analysis revealed synergistic activation of PPARa/PGC-1 *a*
signalling pathways by the cHF+F+CR treatment in abdominal WAT, but not in the liver, muscle or brown fat. The above effects of the combination treatment correlated with the rate of palmitate oxidation and mitochondrial respiratory capacity in WAT. Thus, the maximal rate of palmitate oxidation measured in epididymal WAT ex

vivo was 2-fold higher in the cHF+F+CR mice as compared with the cHF mice (60 .01 ± 8.04 vs. 24.73 ± 5.67 pmol 02/s/mg DNA; p = 0.012), when measured in the presence of FCCP, an uncoupler of oxidative phosphorylation.
We show that n-3 LC-PUFA augment the anti-inflammatory and metabolic effects of CR through the induction of mitochondrial biogenesis and fatty acid oxidation in WAT, while inducing tissue-specific changes in the production of anti-inflammatory lipid mediators. Thus, this study reveals a new mechanism of anti-inflammatory and CR.

Publication C

Oleuropein as an inhibitor of peroxisome proliferator-activated receptor gamma. Svobodova M, Andreadou I, Skaltsounis AL, Kopecky J, Flachs P. *(2014) GENES AND NUTRITION Volume: 9 Issue:1 (IF: 3,149; citations: 0)*

Oleuropein, which is a major phenolic compound found in olive leaves and oil, exerts numerous beneficial effects including antioxidant, anti-inflammatory, anti-atherogenic and anti-cancer activities. Furthermore, oleuropein suppresses the adipocyte differentiation *in vitro.* We aimed to further characterize molecular mechanisms underlying anti-adipogenic effects of oleuropein.
3T3-L1 cells and mouse primary culture of differentiated adipocytes from subcutaneous fat were used. The differentiation medium present during the first 48h contained 10% FCS, 2uM dexamethasone and 100 nM BRL and 5ug/ml insulin which is added for all

differentiation time. After 2 days differentiation the medium was replaced with DMEM containing 10% FCS and 5ug/ml insulin. Cell viability/proliferation was analyzed using Trypan blue and WST-1 assay, and triglycerides were stained with Oil Red O. Gene expression was analyzed by qRT-PCR. Cell based gene reporter assays for PPARα, PPARδ/β and PPARγ (GeneBLAzer, Invitrogen, USA) were used to study direct effects on transcriptional activity of peroxisome proliferator-activated receptors (PPARs).
Results: Oleuropein (>100 μM) decreased viability of proliferating preadipocytes and didn't exerted cytotoxic effects in post confluent cells after induction of differentiation (up to 400 μM). Oleuropein dose-dependently (10 - 400 μM) inhibited adipocyte differentiation in both experimental models and suppressed gene expression of PPARγ, C/EBP1α, SREBP-1c and FAS. PPARα and PPARδ/β activity were not affected by oleuropein (10-200 μM). Contrary, PPARγ transcription activity was diminished by oleuropein (>100 μM).
Oleuropein suppresses both preadipocyte proliferation and adipocyte differentiation *in vitro*. Moreover, our data suggest, that oleuropein exerts anti-adipogenic effect through direct inhibition of PPARγ activity.

5. DISCUSION

Our experiments on dietary obese mice suggest that also *n*-3 LC-PUFA and TZDs may elicit beneficial additive effects with respect to treatment of impaired glucose tolerance and dyslipidaemia in diabetic patients. Thus in mice, only rosiglitazone but not pioglitazone in the combination with *n*-3 LC-PUFA prevented

accretion of body fat, in correlation with the inducibility of fatty acid β-oxidation. However, even in the absence of any effect on body weight in combination with anti-diabetic drugs, the combination treatment unmasked stronger effect of pioglitazone on glucose homeostasis, triglyceridaemia and hepatic steatosis, depending probably on the induction of adiponectin. Importantly, total cholesterol levels in plasma were strongly decreased in response to both TZDs in their combinations with n-3 LC-PUFA.

Dietary n-3 LC PUFA augments the anti-obesity effects of mild calorie restriction while improving lipid metabolism and glucose homeostasis. These effects are probably reflected by the large synergistic induction of mitochondrial FA oxidation in WAT, linked to a suppression of low-grade inflammation of this tissue. The synergistic induction of specific anti-inflammatory lipid mediators, namely 15d-PGJ2 (the most potent endogenous activator of PPARγ;, the LC n-6 PUFA metabolite), and protectin D1, the LC n-3 PUFA metabolite, may underlie both the anti-inflammatory and metabolic effects of the combination treatment in WAT. Further exploration of the strategy to target WAT by combining two complementary and physiological approaches, i.e. dietary intake of LC n-3 PUFA and mild restriction of energy intake, may be valuable for the prevention and treatment of metabolic syndrome.

Oleuropein suppresses adipocyte differentiation inhibiting both expression and activity of PPARγ. As also revealed in recent clinical trial (3), dietary supplementation by polyphenols from olive leaf, and namely oleuropein, may be used as part of novel therapeutic strategies for prevention and treatment of obesity and insulin

resistance. But for combination with anti-diabetic drugs due to suppressing the transcription factor PPARγ is probably not suitable.

Table 1: Summary of effects on growth characteristics and metabolic features of mice

		n-3 LC PUFA + TZDs (vs. cHF diet)	n-3 LC 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,N.D. not determined.

Table 2: Gene expression

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

	SCD1, FAS	SCD1 increased	
	Fatty acid oxidation: CPT-1, PPARα	increased	no effect
	Mitochondrial biogenesis: PGC-1α	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-1α Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, PPARα Peroxisome proliferator-activated receptor alpha, SCD1 Stearoyl-CoA desaturase

6. CONCLUSIONS

1. Our results reveal differential effects of rosiglitazone and pioglitazone, unmasked in the combination treatment with *n*-3 LC-PUFA, and support the notion that *n*-3 LC-PUFA could be used as add-on treatment to TZDs in order to improve diabetic patient's therapy.

2. The dietary LC 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.

3. Oleuropein *in vitro* exerts anti-adipogenic effect through inhibition of both expression and activity of PPARγ.

7. References

1. **Cannon B and Nedergaard J.** Brown adipose tissue: function and physiological significance. *Physiol Rev* 84: 277-359, 2004.

2. **Cho MC, Lee K, Paik SG, and Yoon DY.** Peroxisome Proliferators-Activated Receptor (PPAR) Modulators and Metabolic Disorders. *PPAR Res* 2008: 679137, 2008.

3. **de Bock M, Derraik JG, Brennan CM, Biggs JB, Morgan PE, Hodgkinson SC, Hofman PL, and Cutfield WS.** Olive (Olea europaea L.) leaf polyphenols improve insulin sensitivity in middle-aged overweight men: a randomized, placebo-controlled, crossover trial. *PLoS One* 8: e57622, 2013.

4. **Drira R, Chen S, and Sakamoto K.** Oleuropein and hydroxytyrosol inhibit adipocyte differentiation in 3 T3-L1 cells. *LIFE Sci* 89: 708-716, 2011.

5. **Flachs P, Rossmeisl M, Bryhn M, and Kopecky J.** Cellular and molecular effects of n-3 polyunsaturated fatty acids on adipose tissue biology and metabolism. *Clin Sci (Lond)* 116: 1-16, 2009.

6. **Flachs P, Rossmeisl M, and Kopecky J.** The effect of n-3 fatty acids on glucose homeostasis and insulin sensitivity. *Physiol Res* 63 Suppl 1: S93-118, 2014.

7. **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, and 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: 2626-2638, 2011.

8. **Hardie DG.** AMPK: a key regulator of energy balance in the single cell and the whole organism. *Int J Obes (Lond)* 32 Suppl 4: S7-12, 2008.

9. **Higami Y, Pugh TD, Page GP, Allison DB, Prolla TA, and Weindruch R.** Adipose tissue energy metabolism: altered gene expression profile of mice subjected to long-term caloric restriction. *FASEB J* 18: 415-417, 2004.

10. **Lazarenko OP, Rzonca SO, Hogue WR, Swain FL, Suva LJ, and Lecka-Czernik B.** Rosiglitazone induces decreases in bone mass and strength that are reminiscent of aged bone. *Endocrinology* 148: 2669-2680, 2007.

11. **LeBrasseur NK, Kelly M, Tsao TS, Farmer SR, Saha AK, Ruderman NB, and Tomas E.** Thiazolidinediones can rapidly activate AMP-activated protein kinase in mammalian tissues. *Am J Physiol Endocrinol Metab* 291: E175-181, 2006.

12. **Lombardo YB and Chicco AG.** Effects of dietary polyunsaturated n-3 fatty acids on dyslipidemia and insulin resistance in rodents and humans. A review. *J Nutr Biochem* 17: 1-13, 2006.

13. **Mozaffarian D, Lemaitre RN, King IB, Song X, Huang H, Sacks FM, Rimm EB, Wang M, and Siscovick DS.** Plasma phospholipid long-chain ω-3 fatty acids and total and cause-specific mortality in older adults: a cohort study. *Ann Intern Med* 158: 515-525, 2013.

14. **Nisoli E, Tonello C, Cardile A, Cozzi V, Bracale R, Tedesco L, Falcone S, Valerio A, Cantoni O, Clementi E, Moncada S, and Carruba MO.** Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. *Science* 310: 314-317, 2005.

15. **Nissen SE and Wolski K.** Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes. *N Engl J Med* 356: 2457-2471, 2007.

16. **Oakhill JS, Scott JW, and Kemp BE.** Structure and function of AMP-activated protein kinase. *Acta Physiol (Oxf)* 196: 3-14, 2009.

17. **Rosen ED and Spiegelman BM.** Molecular regulation of adipogenesis. *Annu Rev Cell Dev Biol* 16: 145-171, 2000.

18. **Sacks FM, Bray GA, Carey VJ, Smith SR, Ryan DH, Anton SD, McManus K, Champagne CM, Bishop LM, Laranjo N, Leboff MS, Rood JC, de Jonge L, Greenway FL, Loria CM, Obarzanek E, and Williamson DA.** Comparison of weight-loss diets with different compositions of fat, protein, and carbohydrates. *N Engl J Med* 360: 859-873, 2009.

19. **Santiago-Mora R, Casado-Díaz A, De Castro MD, and Quesada-Gómez JM.** Oleuropein enhances osteoblastogenesis and inhibits adipogenesis: the effect on differentiation in stem cells derived from bone marrow. *Osteoporos Int* 22: 675-684, 2011.

20. **Suchankova G, Tekle M, Saha AK, Ruderman NB, Clarke SD, and Gettys TW.** Dietary polyunsaturated fatty acids enhance hepatic AMP-activated protein kinase activity in rats. *Biochem Biophys Res Commun* 326: 851-858, 2005.

21. **Yang X and Smith U.** Adipose tissue distribution and risk of metabolic disease: does thiazolidinedione-induced adipose tissue redistribution provide a clue to the answer? *Diabetologia* 50: 1127-1139, 2007.

22. **Zhang BB, Zhou G, and Li C.** AMPK: an emerging drug target for diabetes and the metabolic syndrome. *Cell Metab* 9: 407-416, 2009.

23. **Zhu, Y., C. Qi, J. R. Korenberg, X. N. Chen, D. Noya, M. S. Rao, and J. K. Reddy**, 1995, Structural organization of mouse peroxisome proliferator-activated receptor gamma (mPPAR gamma) gene:

alternative promoter use and different splicing yield two mPPAR gamma isoforms: Proc Natl Acad Sci U S A, v. 92, p. 7921-5.

24. **Vidal-Puig, A. J., R. V. Considine, M. Jimenez-Liñan, A. Werman, W. J. Pories, J. F. Caro, and J. S. Flier**, 1997, Peroxisome proliferator-activated receptor gene expression in human tissues. Effects of obesity, weight loss, and regulation by insulin and glucocorticoids: J Clin Invest, v. 99, p. 2416-22.

25. **Werman, A., A. Hollenberg, G. Solanes, C. Bjorbaek, A. J. Vidal-Puig, and J. S. Flier**, 1997, Ligand-independent activation domain in the N terminus of peroxisome proliferator-activated receptor gamma (PPARgamma). Differential activity of PPARgamma1 and -2 isoforms and influence of insulin: J Biol Chem, v. 272, p. 20230-5.

26. **Memon, R. A., L. H. Tecott, K. Nonogaki, A. Beigneux, A. H. Moser, C. Grunfeld, and K. R. Feingold**, 2000, Up-regulation of peroxisome proliferator-activated receptors (PPAR-alpha) and PPAR-gamma messenger ribonucleic acid expression in the liver in murine obesity: troglitazone induces expression of PPAR-gamma-responsive adipose tissue-specific genes in the liver of obese diabetic mice: Endocrinology, v. 141, p. 4021-31.

27. **Tilg, H., and A. R. Moschen**, 2006, Adipocytokines: mediators linking adipose tissue, inflammation and immunity: Nat Rev Immunol, v. 6, p. 772-83.