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Analýza mechanizmů spojených s benefičním účinkem různých lipidových forem Omega-3 polynenasycených mastných kyselin z mořských zdrojů na metabolizmus.

The analysis of mechanisms associated with beneficial metabolic effects of marine Omega-3 polyunsaturated fatty acids in different lipid forms.

Dizertační práce / PhD thesis

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I hereby declare that I am the sole author of this thesis, which I completed under the supervision of Martin Rossmeisl, MD, PhD. I have fully acknowledged and referenced the literature and work of the others, whether published or unpublished. Neither this thesis nor its part has been presented for another degree or diploma at this or any other university.

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Statement of coauthors

I hereby certify that MSc. Jana Pavlišová substantially contributed to the formation of the research papers used as a basis of this thesis, and that she correctly specified her personal contribution to the work in the text of this thesis.

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Abstract

Obesity, one of the most serious health problems of the 21st century, often occurs as a result of an imbalance between energy intake and energy expenditure. Dietary lipids play an important role in the development of obesity, partly because they represent the richest source of energy amongst all macronutrients. It is, however, not only the amount of consumed lipids, but also the composition of fatty acids, which strongly influences health effects of a particular diet. Saturated fatty acids (**SFA**) are generally considered as unhealthy due to their pro-inflammatory and lipotoxic properties, while monounsaturated fatty acids (**MUFA**) and polyunsaturated fatty acids (**PUFA**) represent a healthier alternative, as they are more readily oxidized and do not disrupt biochemical properties of cellular membranes. Amongst PUFA, PUFA of *n-3* series (**Omega-3**) represent an utterly unique class of lipids that have been documented to protect against cardiovascular disease and dyslipidemia in men and improve insulin sensitivity and glucose tolerance primarily in animal models of obesity.

Some molecular mechanisms of Omega-3 action have been already uncovered, such as the modification of biological membranes composition, activation of various transcription factors and membrane receptors, and their role as precursors for the synthesis of bioactive lipid mediators with anti-inflammatory and pro-resolving properties. In this thesis, we explored novel mechanisms and systems, which may be influenced by long-term dietary supplementation of Omega-3 to male C57BL/6 mice with obesity induced by a high-fat diet rich in PUFA of *n-6* series (**Omega-6**). Furthermore, we compared the effects of Omega-3 administered either in the form of triacylglycerols (**Omega-3 TAG**) or marine Omega-3 phospholipids (**Omega-3 PL**); the latter form of Omega-3 has been recently shown to induce more potent and consistent metabolic effects, especially on glucose metabolism and liver fat accumulation (i.e. hepatic steatosis), and, therefore, could represent a better alternative regarding the prevention and potentially also the treatment of obesity-linked metabolic diseases.

In the first project (**Publication A**), we analyzed the effects of Omega-3 TAG depending on the type of other lipids in the diet, i.e. the diets with the prevalence of either Omega-6 (**cHF diet**) or SFA and MUFA (**LHF diet**). Without Omega-3 supplementation, the two diets *per se* did not differ in their impact on body weight, the amount of body fat (adiposity) or insulin sensitivity examined by the hyperinsulinemic-euglycemic clamp technique. It could be due to the protective up-regulation of the enzyme stearoyl-CoA desaturase 1 (**SCD1**) that converts potentially harmful SFA contained in the LHF diet into less toxic MUFA. The accumulation of MUFA, however, might be connected to more pronounced hepatic steatosis, which was typical for LHF-fed animals. Omega-3 supplementation ameliorated hepatic steatosis by repressing lipogenesis and promoting fat oxidation, and these effects were independent of other lipids in the diet. On the contrary, white adipose tissue (**WAT**) inflammation and glucose tolerance were beneficially affected by Omega-3 only when supplemented within the cHF background, while these parameters tended to deteriorate further when Omega-3 were present in the LHF diet. The results of this project suggested that

the supplementation of Omega-3 on SFA-rich dietary background could be problematic, since under these conditions Omega-3 supplementation interferes with the protective mechanism based on the increased activity of SCD-1 in response to LHF feeding.

The second project (**Publication B**) was based on the observation that the long-term supplementation of Omega-3 TAG in dietary obese mice leads to increased plasma insulin levels following glucose administration, but only when glucose is applied orally; it was predicted that increased activity of the incretin system could be involved. We were unable to prove that Omega-3 supplementation increased the activity of the incretin system, as neither an increased secretion of glucagon-like peptide-1 (**GLP-1**), decreased activity of the degrading enzyme dipeptidyl peptidase 4 (**DPP-4**), nor changes in sensitivity towards glucose-dependent insulinotropic polypeptide (**GIP**) were observed following Omega-3 intake. However, Omega-3 supplementation normalized hypersecretion of GIP and increased concentrations of GIP receptors in WAT, which was otherwise observed in obese mice. Thus, the above effects of Omega-3 on GIP may represent a novel pathway by which these fatty acids could affect adiposity.

Finally, in **Publication C** and **Publication D**, we focused on the long-term dietary supplementation with Omega-3 PL derived from either herring meal or Krill oil. We showed that Omega-3 PL were able to efficiently reduce hepatic steatosis and normalize dyslipidemia by a complex and integrated inhibition of *de novo* lipogenesis and cholesterol biosynthesis together with stimulation of the oxidation of fatty acids, observed at the level of hepatic gene expression, while the presence of both Omega-3 and their lipid carrier, i.e. phospholipids, namely phosphatidylcholine (**PC**), are necessary to achieve the maximum effect. Furthermore, by using the hyperinsulinemic-euglycemic clamps in obese mice, we showed that Omega-3 PL ameliorated obesity-linked glucose intolerance and whole-body insulin resistance, mainly due to the beneficial effects on hepatic and muscle insulin sensitivity, while this effect was superior to Omega-3 TAG administered at a comparable dose.

Abstrakt

Obezita, jakožto jeden z nejzávažnějších zdravotních problémů 21. století, vzniká často v důsledku nerovnováhy mezi příjmem a výdejem energie. Příjem tuků pak hraje v rozvoji obezity důležitou roli částečně proto, že tuky jsou ve srovnání s ostatními makronutrienty nejkoncentrovanějším zdrojem energie. Zdravotní dopady konkrétní diety však nezáleží pouze na absolutním množství přijatých tuků. Důležitá je také kompozice mastných kyselin, přičemž nasycené mastné kyseliny (**SFA**) jsou kvůli svým prozánětlivým a lipotoxickým účinkům obecně považovány za méně zdravé, zatímco mononenasycené (**MUFA**) a polynenasycené mastné kyseliny (**PUFA**) představují zdravější alternativu, protože jsou v organizmu pohotově oxidovány a nenarušují fyziologické vlastnosti buněčných membrán. Zcela unikátní třídu lipidů pak představují *n-3* polynenasycené mastné kyseliny (**Omega-3**), jejichž příjem v potravě působí u lidí jako ochrana proti rozvoji kardiovaskulárního nemocnění a dyslipidemie, zatímco u zvířecích modelů obezity zlepšuje, kromě výše zmíněných parametrů, také citlivost k inzulínů a glukózovou toleranci.

Bylo již popsáno mnoho molekulárních mechanizmů, jakými mohou Omega-3 působit na metabolizmus. Omega-3 mohou modifikovat biochemické složení buněčných membrán, působit jako ligandy různých transkripčních faktorů či membránových receptorů, nebo sloužit jako prekurzory v syntéze bioaktivních lipidových molekul s protizánětlivými a rezolučními vlastnostmi. V této práci se zabýváme novými molekulárními mechanizmy a systémy, které mohou být ovlivněny dlouhodobým příjmem Omega-3 u samců myšího kmene C57BL/6 s obezitou indukovanou příjmem vysokotukové potravy bohaté na *n-6* polynenasycené mastné kyseliny (**Omega-6**). Dále jsme porovnávali účinky Omega-3 podávaných ve formě triacylglycerolů (**Omega-3 TAG**) a fosfolipidů (**Omega-3 PL**), přičemž Omega-3 PL vykazují v mnoha současných studiích silnější a reprodukovatelnější metabolické účinky především na glukózovou homeostázu a akumulaci tuku v játrech (jaterní steatózu) a mohly by tak představovat lepší alternativu podání Omega-3 za účelem prevence a potenciálně také léčby metabolických poruch spojených s obezitou.

V rámci prvního projektu (**Publikace A**) jsme analyzovali metabolické účinky Omega-3 TAG v závislosti na chemickém složení ostatních tuků v potravě, konkrétně na pozadí diety **cHF**, bohaté na Omega-6, a diety **LHF**, která obsahovala především SFA a MUFA. Podávání těchto dvou vysokotukových diet jako takových indukovalo u myší C57BL/6 srovnatelný nárůst tělesné hmotnosti a tukové masy (adipozity). Také narušení citlivosti k inzulínu, měřené metodou hyperinzulinemického-euglykemického zámku, se ukázalo být srovnatelné, což mohlo souviset s protektivním navýšením aktivity stearoyl-CoA desaturázy 1 (**SCD-1**), enzymu, který přetváří potenciálně lipotoxické SFA, obsažené v dietě LHF, na méně škodlivé MUFA. Akumulace MUFA však může být spojena s rozvojem silné jaterní steatózy, která byla typická právě pro myši krmené dietou LHF. Suplementace Omega-3 rozvoj jaterní steatózy potlačila prostřednictvím inhibice lipogeneze a stimulace oxidace mastných kyselin, přičemž tento efekt byl zcela nezávislý na složení ostatních tuků v dietě. Oproti tomu chronický zánět bílé tukové tkáně a glukózová intolerance byly suplementací Omega-3 pozitivně ovlivněny pouze

na pozadí diety cHF, zatímco na pozadí diety LHF se tyto metabolické problémy působením Omega-3 spíše prohlubovaly. Výsledky tohoto projektu naznačují, že suplementace Omega-3 na pozadí diety bohaté na SFA může být problematická, jelikož aktivita Omega-3 může interferovat s protektivní aktivitou SCD-1.

Cílem druhého projektu (**Publikace B**) bylo prověřit hypotézu, podle které vede dlouhodobá suplementace Omega-3 ke zvýšení aktivity inkretinového systému. Tato hypotéza vychází z pozorování, že myši C57BL/6, suplementované Omega-3, mají v odpovědi na podání glukózy výrazně zvýšenou hladinu inzulínu v krvi, ovšem pouze v případě, že je glukóza podána orálně. Tato hypotéza nebyla potvrzena, jelikož suplementace Omega-3 nevedla na našem modelu k navýšení sekrece **GLP-1** (glucagon-like peptide-1), k potlačení aktivity degradačního enzymu dipeptidylpeptidázy 4 (**DPP-4**), ani ke změnám v citlivosti organismu k hormonu **GIP** (glucose-dependent insulinotropic polypeptide). Nicméně hypersekrece GIP a zvýšená koncentrace GIP receptorů v bílé tukové tkáni, typické pro obézní myši, byly vlivem podávání Omega-3 částečně normalizovány, přičemž tento účinek by mohl představovat nový mechanismus působení Omega-3 na adipozitu.

Konečně, v **Publikacích C** a **D** jsme se zaměřili na metabolické účinky dlouhodobé suplementace Omega-3 PL izolovaných z masa sleďů, případně obsažených v oleji z mořského krilu. Ukázali jsme, že při podávání Omega-3 PL dochází ke komplexní regulaci de novo lipogeneze, biosyntézy cholesterolu a oxidace mastných kyselin na úrovni genové exprese v játrech, což ve výsledku vede k účinné redukci jaterní steatózy a potlačení dyslipidemie. K dosažení maximálního účinku je přitom potřeba přítomnosti obou složek molekul Omega-3 PL, tedy Omega-3 jako takových a jejich fosfolipidového nosiče, jmenovitě fosfatidylcholinu. Použitím metody hyperinzulinemického-euglykemického zámku jsme také ukázali, že dlouhodobé podávání Omega-3 PL vedlo k oslabení celotělové glukózové intolerance a inzulínové rezistence u obézních myší, přičemž tento efekt byl spojen především s posílením svalové a jaterní citlivosti k inzulínu a výrazně převyšoval efekt srovnatelné dávky Omega-3 ve formě triacylglycerolů.

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List of abbreviations

2-AG	2-arachidonoylglycerol
AA	Arachidonic acid (C20:4 *n-6*)
AEA	Arachidonoyl-ethanolamine (Anandamide)
ALA	α-linolenic acid (C18:3 *n-3*)
AMPK	AMP-activated protein kinase
Apo	Apolipoprotein
AUC	Area under the curve
BA	Bile acid
BMI	Body mass index
CLS	Crown-like structures
COX	Cyclooxygenase
CVD	Cardiovascular disease
DAG	Diacylglycerol
DGAT	Diacylglycerol acyltransferase
DHA	Docosahexaenoic acid (C22:6 *n-3*)
DPP-4	Dipeptidyl peptidase 4
EC	Endocannabinoids
EPA	Eicosapentaenoic acid (C20:5 *n-3*)
ER	Endoplasmic reticulum
eWAT	Epididymal white adipose tissue
FA	Fatty acid
FAS	Fatty acid synthase
FBG	Fasting blood glucose
GIP	Glucose-dependent insulinotropic polypeptide
GIPR	GIP receptor
GIR	Glucose infusion rate
GIT	Gastrointestinal tract
GLP-1	Glucagon-like protein 1
GLP-1R	GLP-1 receptor
GLUT-4	Glucose transporter type 4
GSIS	Glucose-stimulated insulin secretion
GTO	Glucose turn-over
HDL	High-density lipoprotein
HGP	Hepatic glucose production
HOMA-IR	Homeostatic model assessment of insulin resistance
i.p. GTT	Intraperitoneal glucose tolerance test
IL	Interleukin
IR	Insulin resistance
IRS-1	Insulin receptor substrate-1
KO	Krill oil
LA	Linoleic acid (C18:2 *n-6*)
LDL	Low-density lipoprotein
LOX	Lipoxygenase
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MCP-1	Monoacylglycerol acyltransferase

MS	Metabolic syndrome
MUFA	Monounsaturated fatty acids
mWAT	Mesenteric white adipose tissue
NEFA	Non-esterified fatty acid
oGTT	Oral glucose tolerance test
Omega-3	Polyunsaturated fatty acids of *n-3* series
Omega-3 PL	Omega-3 in a form of phospholipids
Omega-3 TAG	Omega-3 in a form of triacylglycerols
Omega-6	Polyunsaturated fatty acids of *n-6* series
PA	Palmitic acid (C16:0)
PC	Phosphatidylcholine
PC1/3	Prohormone convertase 1/3
PC2	Prohormone convertase 2
PDH	Pyruvate dehydrogenase
PFK	Phosphofructokinase
PI3K	Phosphatidylinositide-3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PL	Phospholipid
PLA2	Phospholipase A2
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acids
qPCR	Quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species
SCD-1	Stearoyl-CoA desaturase-1
SFA	Saturated fatty acids
SGLT2	Sodium-glucose cotransporter 2
SREBP-1	Sterol regulatory element-binding protein 1
sWAT	Subcutaneous white adipose tissue
T2DM	Type 2 diabetes mellitus
TAG	Triacylglycerols
TNFα	Tumor necrosis factor α
TZD	Thiazolidinedione
VLDL	Very low-density lipoprotein
WAT	White adipose tissue
WHO	World health organization

1. Introduction

1.1. Obesity, insulin resistance, and metabolic syndrome

At present, adult and even childhood obesity are so common that people scarcely recognize that it is more than an aesthetic problem. The most widely used diagnostic marker of obesity is the body mass index (**BMI**; body weight (kg)/height (m^2)); when it exceeds the value 25 it is classified as overweight, while the value 30 means the state of obesity. According to the latest Global status report by the World Health Organization (**WHO**), the average worldwide prevalence of overweight and obesity is ~40 % and ~13 %, respectively, with the obesity rate ranging from modest 5 % in Asian countries to alarming 30 % in most developed countries, including USA, Great Britain, and central European region (**Fig.1**)[1]. It is well established that obesity strongly increases likelihood of many life-threatening diseases, such as type 2 diabetes (**T2DM**), hypertension, coronary heart disease, stroke, certain types of cancer, obstructive sleep apnea, and osteoarthritis. The global prevalence of diabetes is ~9 %, while T2DM accounts for more than 90 % cases[1]. As the number of obese people has doubled from 1980 and the rise still has not stopped, global authorities have already recognized so called epidemics of obesity as one of the greatest threats of the 21st century.

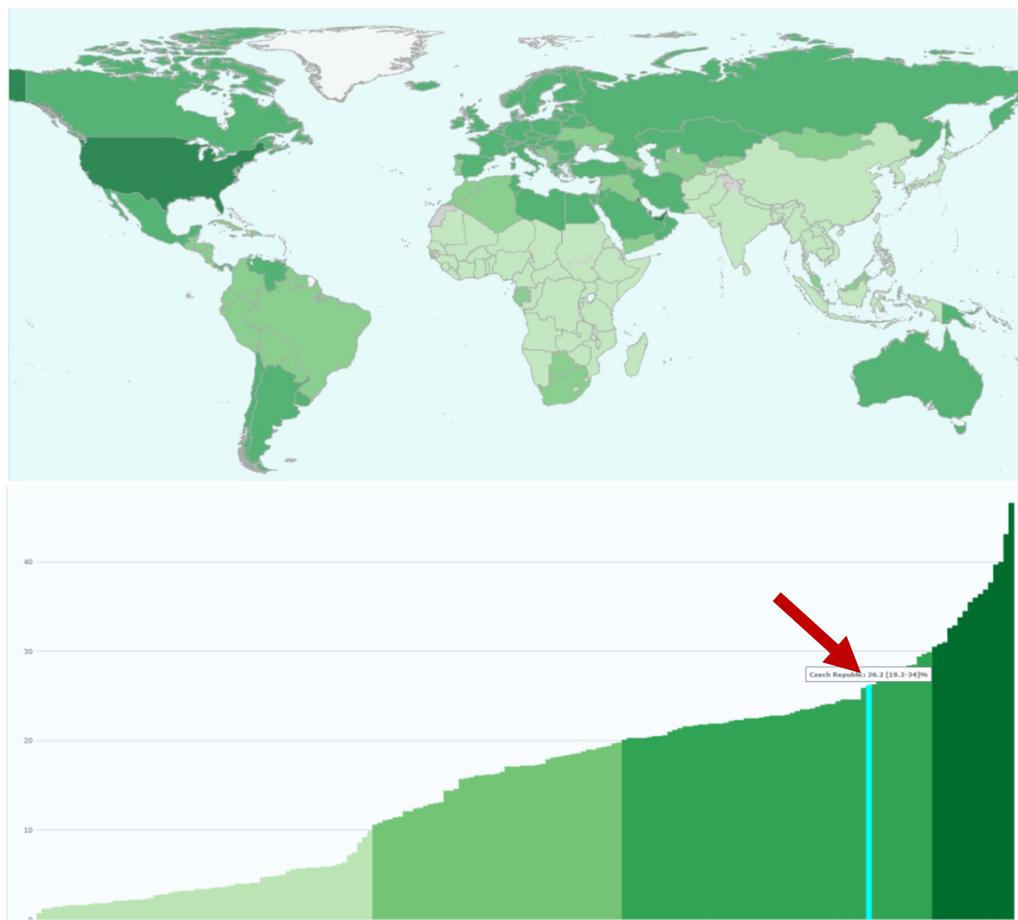


Fig. 1 Global prevalence of obesity

(BMI ≥ 30), edited "print screen" from WHO interactive map, www.who.org, 2014

Scale bar on lower graph: 0 - 40 %, Red arrow: Czech Republic with 26.2 % of obese citizens.

Besides several congenital metabolic disorders, obesity is most often a result of an imbalance between energy intake and expenditure, sustained by a sedentary lifestyle, stress, and improper dietary habits of the current population. It is characterized by an excessive storage of lipids, either in the subcutaneous or abdominal adipose tissue depots, or even ectopically in non-adipose organs such as the liver, heart, and skeletal muscle. Both adipose and non-adipose cells overloaded with lipids are prone to specific changes in paracrine and endocrine signaling, which are often connected to the development of a chronic low-grade inflammation and together with an increase in systemic levels of non-esterified fatty acids (NEFA) lead to the development of insulin resistance (IR), i.e. the state when the disruption of insulin signaling pathways prevents insulin from effectively regulating glucose homeostasis and other metabolic processes (for details see Section 1.2). Hypertrophied adipocytes from abdominal regions exert increased lipolytic activity[2,3], which is relatively resistant towards the anti-lipolytic effects of insulin[4–6], and they are also more prone towards changes in signaling; this is the reason why the so called abdominal (central) obesity, often seen in men and postmenopausal women, presents a higher metabolic risk than obesity connected with an increased growth of peripheral subcutaneous adipose tissue (**Fig.2**)[7].

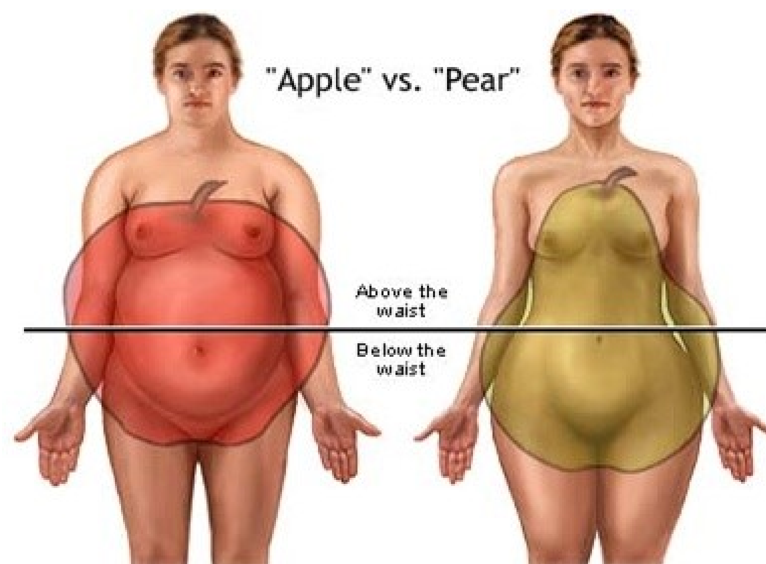


Fig. 2 Central vs. peripheral obesity
Central obesity (marked as the "apple type"), also known as abdominal, or android obesity, is characterized by higher waist/hip circumference ratio, as the majority of fat is stored inside the visceral cavity. It is more frequent in men; however, menopause, some treatments, stress, and hormonal disruptions induce a shift towards android obesity in women as well.
Figure source: http://www.adameducation.com

Obesity, especially the central type, and IR are the driving force for development of the whole cluster of metabolic disorders collectively known as the metabolic syndrome (MS). This syndrome was first defined as the "Syndrome X" by prof. Gerald Raeven in 1988[8]. There are four major core components of MS: (i) central obesity, (ii) insulin resistance, (iii) dyslipidemia, and (iv) hypertension[9].

A widely used definition of MS for use in clinical practice according to International Diabetes Federation stands as follows[9]:

For patients to be defined as having MS, they must have central obesity (based on a measurement of waist circumference with ethnicity specific values) or BMI > 30, plus any two of the following four factors:

- raised triacylglycerols (**TAG**; >150 mg/dl), or specific treatment for this abnormality in lipid metabolism;
- reduced high-density lipoprotein (**HDL**) cholesterol (i.e. <40 mg/dl and <50 mg/dl in males and females, respectively), or specific treatment for this abnormality in lipid metabolism;
- raised blood pressure (systolic >130 mm Hg or diastolic >85 mm Hg), or treatment of previously diagnosed hypertension;
- raised fasting blood glucose (>100 mg/dl), or previously diagnosed T2DM.

As the clustering of metabolic abnormalities represents a significantly higher risk of T2DM and cardiovascular disease (**CVD**) development than any of these symptoms alone, the diagnosis of MS represents an important step regarding the initiation of an early-stage prevention, which promises better results and is more cost-effective.

1.2. Molecular mechanisms behind insulin resistance

Insulin resistance manifests mainly in metabolically active organs such as white adipose tissue (**WAT**), skeletal muscle, liver, and pancreatic β-cells. It is widely accepted that IR does not primarily develop on the systemic level, but rather gradually spreads from one system to another. The epicenter of IR development is still a subject of discussion; however, WAT is a likely candidate, as it is the first site affected by obesity[10].

Primarily recognized physiological role of WAT was the regulation of whole-body lipid metabolism by storing dietary fatty acids (**FA**) in the form of TAG in the post-absorptive state and releasing NEFA in the process of lipolysis in the fasted state. NEFA have been documented to be lipotoxic, if they are not neutralized by binding to Coenzyme-A, proteins, or glycerol[10]. Moreover, lipids stored ectopically in the cells of non-adipose organs could be a source of metabolites that can have lipotoxic properties, while the long-term deposition of lipids in non-adipose tissues coincides with an organ damage[11]. Thus, the ability of WAT to react flexibly to the changes in lipid supply and demand plays an important role not only in energy metabolism, but also in preventing lipotoxicity.

With the discovery of WAT-produced hormones such as leptin (in 1994) and adiponectin (in 1995), another physiological role of WAT started to emerge[12]. Nowadays, WAT is considered to be an important endocrine organ that secrets adipocyte-specific hormones and

cytokines (collectively termed “adipokines”), which regulate processes such as whole-body energy metabolism, immune response, and insulin sensitivity.

In the state of severe obesity, when the volume of hypertrophied adipocytes increases substantially due to the excessive storage of lipids, both the flexible distribution of lipids and the signaling function become disrupted, which ultimately leads to the development of a chronic low-grade inflammation and insulin resistance.

1.2.1. Fatty acids and lipotoxicity

Physiological release of NEFA from WAT, i.e. the process of lipolysis, occurs in a highly regulated manner during fasting and after exercise, when lipids represent the main fuel for the skeletal muscle, heart, kidney, and the liver. Under these conditions glucose becomes a scarce source, since it has to be reserved for the needs of the central nervous system. On the contrary, when glucose levels are high and glucose oxidation should be prioritized over lipids, FA need to be safely stored in WAT in order to prevent lipotoxicity and oxidative damage resulting from the mitochondrial substrate overload and reactive oxygen species (**ROS**) production, which occurs in obesity. Such substrate channeling is regulated at the hormonal level, e.g. by the ratio of insulin and glucagon; however, multiple non-hormonal mechanisms also exist, by which NEFA, glucose, and various products of their oxidation inhibit utilization of the opposing substrate[13,14]. This cyclic regulation, by which glucose regulates the release of FA from WAT, while NEFA orchestrate the substrate selection in skeletal muscle, was firstly described in 1963 by Philip Randle and his colleagues[14] and is, therefore, recognized as the Randle´s cycle (**Fig.3**).

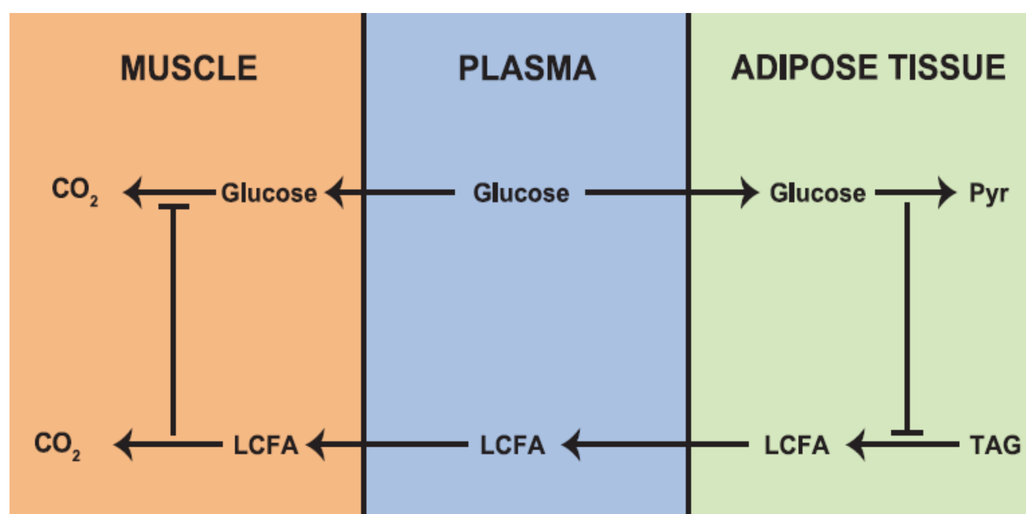


Fig. 3 Randle´s cycle

Randle´s cycle, or the glucose-fatty acid cycle, describes the regulation of substrate channeling, by which glucose and fatty acids compete for shared catabolic apparatus of mitochondrial enzymes. While glucose uses both hormonal and non-hormonal mechanisms to regulate NEFA release from adipose tissue, NEFA are the leading substrate which regulates the substrate selection in skeletal muscle by inhibiting glucose uptake and oxidation. Figure source: Hue et al., Am J Physiol Endocrinol Metab, 2009

Principles of the Randel´s cycle describe how FA induce short-term, regulated glucose intolerance and IR under physiological conditions, for example in the fasting state, after a high-fat meal, or in pregnancy, when physiological IR ensures adequate supply of glucose to the fetus, while mother's organism switches to the oxidation of lipids[10,15]. Randle was, however, also the first one to propose that the same mechanisms can induce long-term insulin resistance in obesity and T2DM[14], which were both shown to be associated with a chronic elevation in the concentration of circulating NEFA[16–18].

The obesity-associated chronic increase in plasma NEFA concentrations results mainly from a rise in FA turnover in hypertrophied adipocytes[2,10,19,20]. Once elevated, FA antagonize the insulin-mediated anti-lipolytic actions, which promotes further increase in circulating NEFA concentrations[10]. Elevated NEFA levels represent a plausible primary cause of obesity-associated IR, as the dynamic changes in their concentrations have been shown to correlate with the changes in the degree of IR[21,22]. For example, decreasing NEFA levels in healthy as well as obese and T2DM subjects by the administration of long-lasting anti-lipolytic agent Acipimox leads to a proportional increase in insulin-mediated glucose uptake. It is, however, important to note that in T2DM subjects Acipimox could not restore full capacity of glucose uptake suggesting that NEFA represent one but not the only cause of IR in these patients[23].

Randle et al. hypothesized, that NEFA reduce net glucose utilization mainly by inhibiting glycolytic enzymes, namely pyruvate dehydrogenase (PDH) and phosphofructokinase (PFK), as summarized in **Fig.4**. Data obtained from nuclear magnetic resonance, however, showed that quantitatively more important is the inhibition of cellular glucose uptake, as no metabolites of the glycolytic pathway such as glucose-6-phosphate or pyruvate accumulate in the cells in the conditions of lipid-induced suppression of glucose utilization[24,25].

It is known that FA inhibit glucose uptake by interfering with the downstream insulin signaling, i.e. by inducing IR. Elevated NEFA concentrations coincide with a decrease in tyrosine phosphorylation of insulin receptor substrate 1 (**IRS-1**) and blunted activity of phosphoinositide-3-kinase (**PI3K**), both of which are the key members of the insulin signaling pathway[24,26]. It was shown that the increase in intracellular FA leads to increased formation of membrane-bound diacylglycerols (**DAG**), which subsequently activate atypical protein kinases C, namely PKCθ[26,27] and PKCε[27] in rodents, or PKCβII and PKCδ in humans[28]. These serine-kinases subsequently phosphorylate serine residues on IRS-1, which interferes with the tyrosine-kinase activity of insulin receptor. Serine phosphorylation of IRS-1 thus results in blunted tyrosine phosphorylation of the same substrate, which is critical for signal transduction to other members of the insulin signaling cascade, including PI3K[26,29]. The need for the formation of FA metabolites rather than direct involvement of FA themselves explains, why IR develops several hours after lipid administration, while NEFA enter the cells already after several minutes[29].

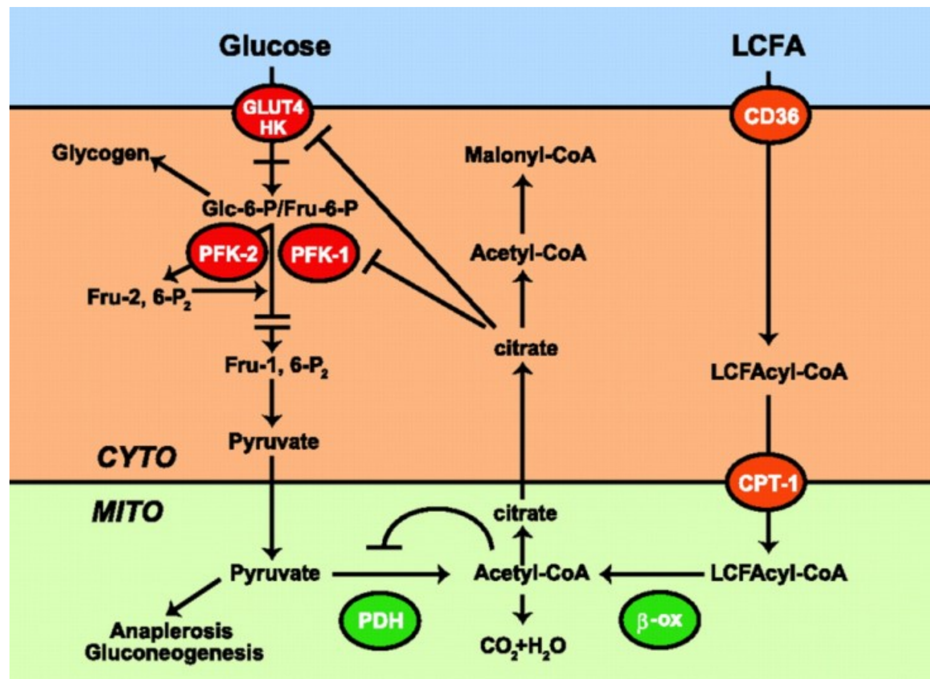


Fig. 4 Enzymatic interactions between FA and glucose oxidation pathways
Various products of FA oxidation have been shown to inhibit key glycolytic enzymes, thus preventing an overload of mitochondrial oxidative enzymes and ROS production. Figure source: Hue et al., Am J Physiol Endocrinol Metab, 2009

It is, however, important to note, that even this almost paradigmatic hypothesis faces its challenges, nowadays, and that many publications contradict the fact that circulating NEFA concentrations are increased in obesity[30], and that NEFA represent the primary cause of IR. These publications, as well as the meta-analyses of human studies regarding the physiological and pathophysiological concentrations of circulating NEFA, are summarized in the review article by Karpe et al. from 2011[31].

1.2.2. Ceramides

Ceramides represent another class of lipids, which has been repeatedly shown to convey the toxic effects of lipid overload by inducing IR, apoptosis, and mitochondrial damage[32]. Ceramides are the intermediates in the synthesis of complex membrane sphingolipids and as such may be produced by the hydrolysis of sphingolipids, as well as synthesized *de novo* via the condensation of palmitate (C16:0) and serine by serine palmitoyltransferase[33]. They also serve as intracellular signaling molecules that play an important role in apoptosis and the response to various stressful stimuli[32].

Increased concentration of ceramides in circulation and in the skeletal muscle was shown to coincide with obesity, IR and apoptosis induced by lipid overload[34–36]. Some scientists consider *de novo* synthesized ceramides to be the main factor in the induction of IR and T2DM, as genetic or pharmaceutical inhibition of ceramide synthesis prevented IR and T2DM

development in several experimental models such as Zucker Diabetic Fatty rats[37] or rats treated by corticosteroids[38]; corticosteroids were also shown to induce IR[39,40] by promoting ceramide synthesis *de novo*[38].

The rise in ceramides may be induced simply by nutrient (i.e. palmitate and serine) oversupply[33]; however, regulation of ceramide synthesis and degradation at the level of gene expression and/or enzyme activity is also important. The latter was shown to be mediated by hormones, intestinal microbiota[41], inflammation[42,43], and NEFA themselves[44–46], showing that ceramides represent a link between most factors that are inducing IR and other metabolic disorders.

Ceramides induce IR by interfering with the translocation and phosphorylation of Akt/protein kinase B (**PKB**)[47], the key mediator of insulin signal transduction, and by stimulating the activity of protein phosphatase 2A (**PP2A**)[48] and protein kinase C-ζ (**PKCζ**)[49,50]. They have also been shown to activate a nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3 (**NRLP3**) inflammasome, a transient protein structure applied in recognizing non-microbial stress-related stimuli which is strongly involved in the induction of obesity-related WAT inflammation[43].

1.2.3. Chronic low-grade inflammation of adipose tissue

Inflammation is a physiological response to cellular stress. As the potential for cellular hyperplasia of WAT (multiplication and maturation of new adipocytes) is limited, lipid-overloaded adipocytes have to increase their storage capacity by pathological growth, i.e. hypertrophy. Hypertrophied adipocytes have to endure various stress stimuli, including hypoxia, endoplasmic reticulum stress, lipotoxicity, increased ROS production resulting from overloaded or deregulated metabolic pathways, and necrosis. Therefore, hypertrophied adipocytes and immune cells residing within WAT switch intercellular signalization to a pro-inflammatory mode, thus calling for revascularization, tissue rebuild, and clearance of dying cells.

Physiological short-term inflammation is a highly regulated process that possesses intrinsic mechanisms to be resolved and finalized. On the contrary, inflammation induced by a mild chronic stimulus, for example in obesity, progresses on a subclinical level for a very long periods (months and years) while inducing processes that are localized and beneficial in the short term but deleterious when induced chronically and systemically[51]. Such type of inflammation is called "low-grade chronic inflammation", or "metabolic inflammation".

As is summarized in **Fig.5**, The secretory profile of a healthy "lean" WAT is characterized mainly by adipokines with anti-inflammatory properties, such as adiponectin, transforming growth factor β (**TGFβ**), interleukin (**IL**)-10, IL-4, IL-13, IL-1 receptor antagonist (**IL-1Ra**), or apelin. On the contrary, obesity and IR has been shown to be associated with increased levels

of pro-inflammatory adipokines such as tumour necrosis factor α (**TNFα**), leptin, visfatin, resistin, angiotensin II, and plasminogen activator inhibitor 1 (**PAI1**)[52].

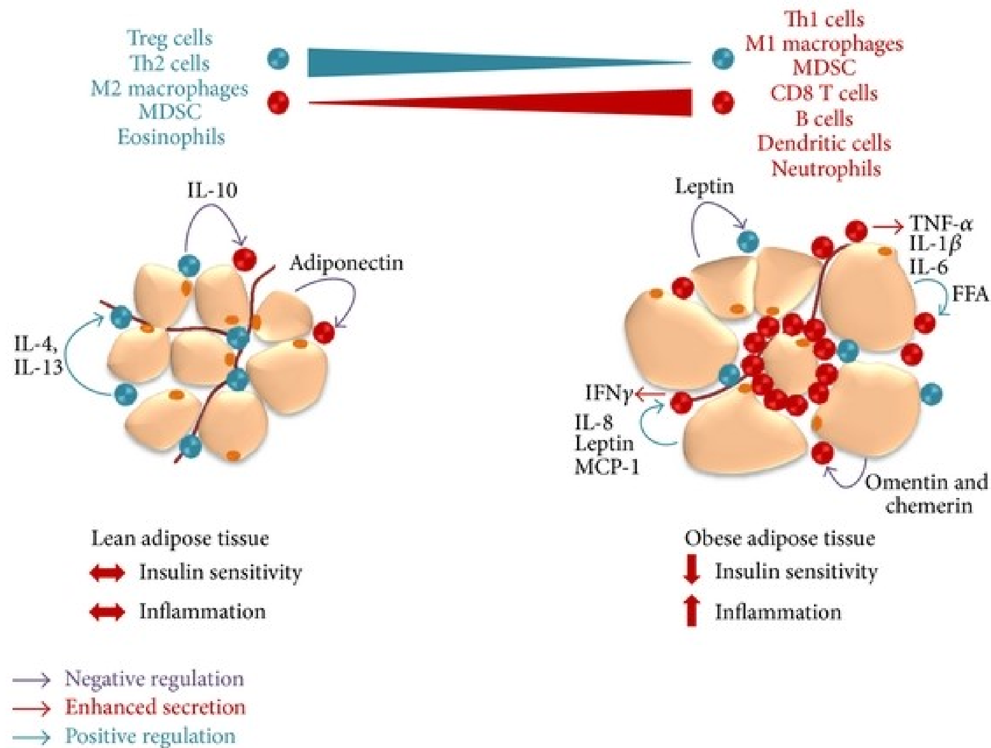


Fig. 5 Lean vs. obese adipose tissue

WAT consists not only of adipocytes, but also of stromal-vascular cells, which contain, among others, complex variety of endothelial and immune cells. In WAT of lean individuals, the anti-inflammatory and regulatory immune cells (blue dots) prevail over the pro-inflammatory immune cells (red dots). Signaling molecules such as adiponectin and interleukin (IL)-4, IL-13, and IL-10 help maintain insulin sensitivity and down-regulate pro-inflammatory responses, while the opposite is true for signaling molecules typical of the obese state, in which hormones and cytokines such as leptin, TNFα, IL-1β, IL-6, and IL-8, MCP-1, and others prevail. Figure source: Makki et al., ISRN Inflamm, 2013

A shift from lean towards obese phenotype also brings changes to the population of immune cells. In a simplified view, resident macrophages in WAT of lean subjects exert predominantly the anti-inflammatory and protective phenotype (i.e. M2 macrophages), which is changed to a more pro-inflammatory phenotype (i.e. M1 macrophages) as obesity progresses[53]. Chemotactic molecules such as chemokine (C-C motif) ligand 2, also called monocyte chemotactic protein-1 (**MCP1**), are secreted by hypertrophied adipocytes, thus promoting tissue infiltration of blood monocytes that mature and polarize towards M1 phenotype[54]; other pro-inflammatory immune cells such as CD8- and CD4-positive T-cells, neutrofils, dendritic cells, and mast cells are present as well. In contrast, the number of anti-inflammatory immune cells such as M2 macrophages and T-regulatory lymphocytes declines (for overview see **Fig.5**; reviewed in[55,56]). Clusters of M1 macrophages may be observed on histological samples of "obese" WAT surrounding dead adipocytes and forming well known "crown-like structures" (**CLS**; see **Fig.6**)[57].

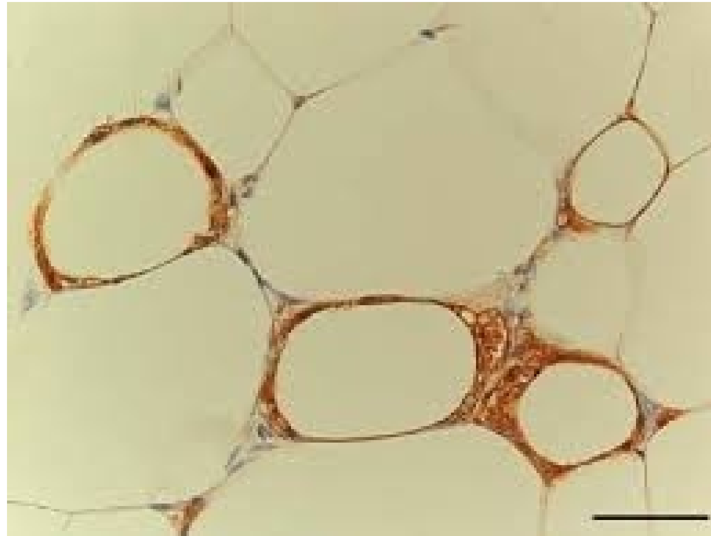


Fig. 6 Crown-like structures

Histological section of "obese" WAT showing clusters of macrophages surrounding dead adipocytes, which form the so-called crown-like structures (CLS). The number of CLS is increased in WAT of obese subjects. M1-polarized macrophages were visualized using the antibody against a macrophage-specific marker Galectin-3 (also called Mac-2), which mediates macrophage phagocytic and pro-inflammatory responses. Scale bar: 50 µm. Figure source: Murano et al., J Lipid Res, 2008

The causal link between WAT inflammation and IR progression has been firmly established. Pro-inflammatory adipokines, as well as cytokines produced solely by WAT-resident immune cells, have been repeatedly shown to induce IR and cell death both directly by interfering with the insulin signaling pathway and indirectly by promoting secretion of more pro-inflammatory cytokines and altering lipid and glucose metabolism. Precise molecular mechanisms that mediate insulin-desensitizing properties of many cytokines are not yet fully understood[52]. Some plausible mechanisms may be demonstrated with the example of TNFα, the most studied and firstly recognized pro-inflammatory adipokine involved in the initiation and progression of IR[58]. TNFα interferes with insulin signaling by inducing serine phosphorylation of IRS-1 via the activation of inhibitor κB kinase-β (**IKK-β**)[59], thus blunting the tyrosine phosphorylation, which is necessary for the transduction of insulin signal[60]. TNFα further inhibits gene expression of lipoprotein lipase (**LPL**)[61], stimulates lipolysis, and decreases protein levels of IRS-1, glucose transporter type 4 (**GLUT4**), peroxisome proliferator-activated receptor (**PPAR**)γ, perilipin, and adiponectin in adipocytes[62,63]. Taken together, these changes lead to a decrease in glucose uptake and lipid storage in adipocytes, while plasma glucose, very low-density lipoprotein (**VLDL**) particles, and plasma NEFA levels rise, which is beneficial in a short term, as increased concentration of energy substrates in the blood ensures proper support for energy-demanding immune processes; however, in the long run, increased blood glucose levels (i.e. hyperglycemia) and NEFA levels further promote the development of IR and glucotoxicity.

Other examples of cytokines that are known to rise in obesity and directly interfere with insulin signaling are leptin, resistin, IL-7, retinol-binding protein 4 (**RBP4**), and visfatin, while the uncovered molecular mechanisms of their actions are reviewed elsewhere[52,64].

1.3. Incretin system and its deregulation in obesity and type 2 diabetes

Incretins are peptide hormones secreted from the intestinal endocrine (enterocrine) cells in response to the ingestion of nutrients. They significantly potentiate endogenous secretion of insulin, enhance viability of pancreatic β-cells, and exert many other biological effects, which cooperatively prepare the organism to maximize the utilization of ingested meal and prevent big oscillations of glycemia[65]. Discovery of the incretin system was triggered by the observation, that a single dose of glucose given orally induces up to 70 % stronger release of insulin than the isoglycemic glucose infusion applied intravenously[66,67]. Currently, the two most studied incretins are glucagon-like peptide 1 (**GLP-1**) and glucose-dependent insulinotropic polypeptide (**GIP**); the latter is also known as gastric inhibitory peptide due to its firstly discovered ability to inhibit gastric acid secretion in non-physiological concentrations[68].

1.3.1. Secretion and interaction with receptors

Both incretins are synthesized by the post-translational splicing of their prohormone precursors; proGIP and preproglucagon for GIP and GLP-1, respectively. ProGIP is cleaved by prohormone convertase 1/3 (**PC1/3**), yielding GIP, as the only recognized bioactive product[69]. Synthesis of GLP-1 is also mediated by PC1/3, which cleaves preproglucagon to produce GLP-1, and other bioactive molecules including glicentin, oxyntomodulin, intervening peptide-1, and GLP-2[65,70,71]. Preproglucagon is also expressed in pancreatic α-cells, where it serves for the production of glucagon by specific cleavage with prohormone convertase 2 (**PC2**)[72].

The main sites of incretin secretion are the small and large intestine. GIP is secreted from the enterocrine K-cells, which are most abundant in proximal parts of the small intestine (duodenum > jejunum > ileum), while GLP-1 is secreted from the L-cells, which are dispersed throughout the intestinal tract in an opposite gradient (colon + ileum > jejunum > duodenum). Both GIP and GLP-1 secretion can be stimulated by direct nutrient interaction with membrane receptors on the surface of enterocrine cells, with the strongest response to glucose (especially for GLP-1), amino acids, and fats (mainly for GIP); however, as the early phase of GIP as well as GLP-1 release occurs just several minutes after food ingestion, other neural and endocrine mechanisms are likely to be involved in the secretion of these incretins[73,74].

Additional GIP-producing cells are localized in pancreatic α-cells, where PC2 rather than PC1/3 cleaves the proGIP precursor to yield functional GIP hormone[75]. GLP-1 can be also produced by pancreatic α-cells, as IL-6 was found to stimulate PC1/3 expression in α-cells. Physiologically, IL-6 levels rise during the exercise; therefore, GLP-1 production in pancreas enhances insulin response after exercise in order to maximize glucose uptake into skeletal muscle. However, chronically elevated IL-6 levels in obesity could then contribute

to the compensatory increase in basal as well as stimulated insulin secretion in subjects with IR[76].

After entering the circulation, both incretins are rapidly deactivated by the proteolytic activity of dipeptidyl peptidase 4 (**DPP-4**). This peptidase, also marked as T-cell antigen CD26, is ubiquitous and occurs in a soluble circulating form as well as bound to the membrane of numerous cells, including endothelial cells, adipocytes, hepatocytes, immune cells, and many others[77]. DPP-4 cleaves the N-terminal dipeptide with a specific amino acid sequence from many proteins including incretins, thus transforming active GLP-1(7-36) and GIP(1-42) into their inactive forms, i.e. GLP-1(9-36) and GIP(3-42)[78]. The biological half-life is ~5 min for active GIP and only ~1-2 min for active GLP-1, thus suggesting that GIP can act as an endocrine factor, while GLP-1 executes its effects mainly on the paracrine level or as a peripheral neuromodulator[65].

GIP and GLP-1 execute their biological functions by binding to G-protein-coupled membrane receptors GIP-receptor (**GIPR**) and GLP-1 receptor (**GLP-1R**), respectively.

1.3.2. Biological actions of GLP-1 and GIP

Both GLP-1 and GIP play a crucial role in maintaining fasting as well as postprandial glucose homeostasis, as was shown in animals treated with GLP-1R antagonist exendin(9-39) and GIPR peptide antagonists or antisera, as well as in transgenic mice lacking GLP-1R or GIPR. These experiments showed that the blockage of GLP-1R signaling leads to an increase in fasting as well as postprandial glycemia[79], decreased glucose clearance and acceleration of gastric emptying[80], while GIP seems to play a dominant role in regulating glycemia in the postprandial state[81]. While GIP executes most of its functions as an endocrine factor, many functions of GLP-1 are mediated by the central nervous system, which communicates with the intestines via GLP-1R expressed on the *nervus vagus* terminals in the portal vein[82]. Thus, vagal innervation mediates, for example, the GLP-1-induced inhibition of food intake and gastric emptying and stimulation of peripheral glucose clearance[83]. Another topic is the effect of GLP-1 on hepatic glucose and lipid metabolism. GLP-1R agonists were shown to decrease hepatic glucose production[84] and ameliorate hepatic steatosis[85]; however, it is not clear whether these effects are direct or mediated by CNS, as the evidence regarding the presence of GLP-1R on hepatocyte membranes remain controversial[83].

The most well-known but still incompletely understood biological effect of incretins is the potentiation of glucose-stimulated insulin secretion (**GSIS**), i.e. the incretin effect. Both GIP and GLP-1 increase the secretion of insulin in a strictly glucose-dependent manner by binding to G-protein-coupled receptors on the membrane of β-cells of pancreatic islets. The signaling events that ultimately lead to release of insulin include a rise in the intracellular cAMP levels and protein kinase A (**PKA**) activity, activation of specific guanidine nucleotide exchange factor Epac2, an interaction with sulphonylurea receptor subunit of K_{ATP} channel, and finally a rise in intracellular Ca^{2+} [83,86] (for details on the insulin secretion pathway see

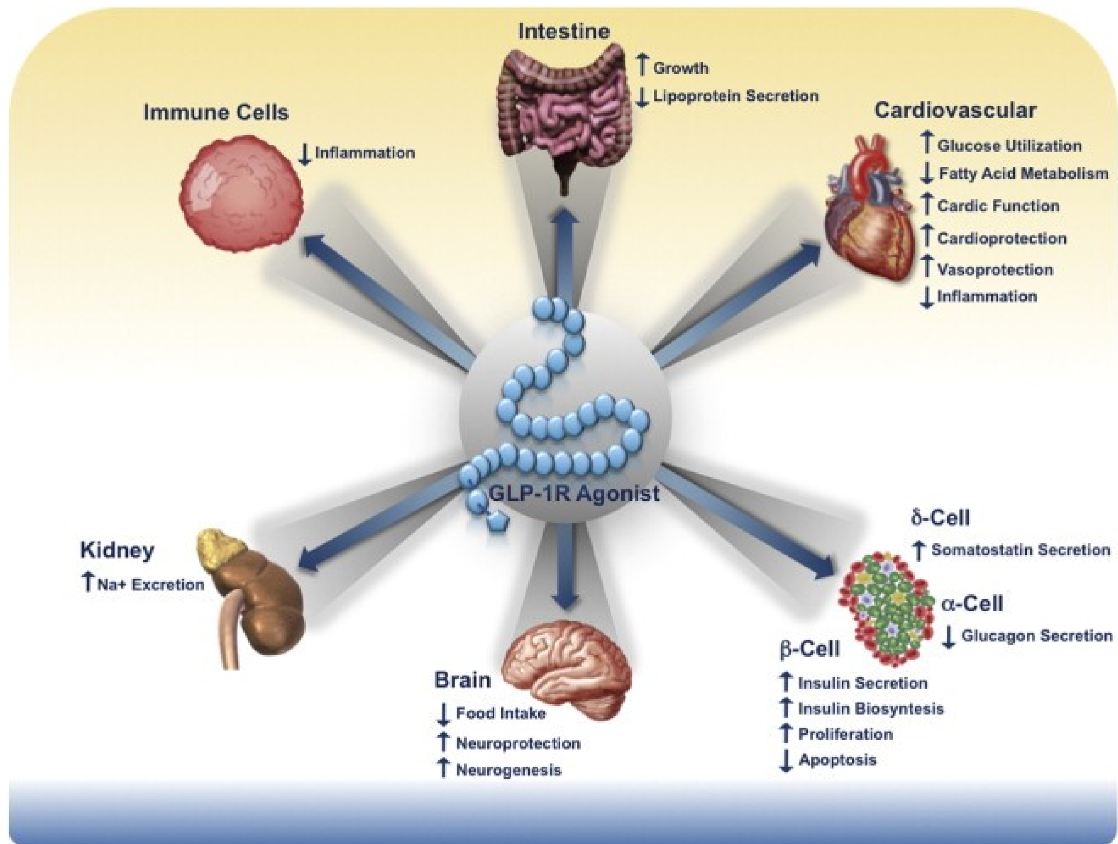
section 1.2.1). Apart from stimulating exocytosis of insulin granules, GIP and GLP-1 also augment gene expression and mRNA stability of proinsulin, the peptide precursor of insulin, and increase the β-cell mass by preventing apoptosis and, in case of GLP-1, also by stimulating proliferation and maturation of new β-cells[65]. Moreover, GLP-1 contributes to the maintenance of glucose homeostasis by the glucose-dependent inhibition of glucagon secretion[87] and restoration of glucose sensitivity in β-cells[88].

Both incretins also exert many extra-pancreatic functions that are summarized in **Fig.7**. Concerning possible interaction between the incretin system and polyunsaturated FA (**PUFA**) of *n-3* series (**Omega-3**), some attention must be paid to the adipogenic properties of GIP. Unlike GLP-1, GIP release is strongly stimulated by dietary FA[89,90]. GIPR is profusely expressed on the membranes of mature adipocytes[91] and its expression correlates positively with adipocyte maturation, adipogenesis, and the activity of the transcription factor PPARγ[92]. Adipose GIPR activation has been linked to several anabolic processes within WAT, including the stimulation of glucose uptake[93], increased expression of genes for LPL and fatty acid synthase (**FAS**)[94], and reduction in glucagon-induced lipolysis[95], indicating a role of GIPR in the accumulation of lipids in adipocytes.

Overactivation of GIPR has been linked to excessive lipid accumulation, and inhibition of GIP/GIPR signalization has been suggested as a potential treatment for obesity[96–98]. However, GIPR activation promotes adipogenesis while protecting WAT against inflammation and lipotoxicity; for example, increased concentrations of GIP in DPP-4-defficient rodents leads to the growth of abdominal WAT depots and visceral adipocytes, which is, however, associated with seemingly paradoxical improvements in glucose homeostasis[99].

1.3.3. Deregulation of the incretin system in obesity and type 2 diabetes

Loss of the incretin effect, i.e. a significant decrease in the early-phase insulin secretion, is an early specific marker of T2DM (reviewed in[100]). Various disruptions of the incretin system have been reported in case of obesity and pre-diabetic state; however, the exact nature of these disruptions is hard to decipher, as various studies reported GIP and GLP-1 levels to be unchanged, decreased, or in case of GIP also increased in obesity and T2DM, perhaps due to the cross-reactivity of applied assays[100]. However, GLP-1 analogues are capable of increasing insulin secretion and restoring glucose homeostasis in obese and pre-diabetic subjects[101], while even supraphysiological doses of GIP had no effect on insulin secretion[102,103], thus indicating that the loss of incretin effect is due to a decrease in active GLP-1 and/or the development of GIP resistance[104].



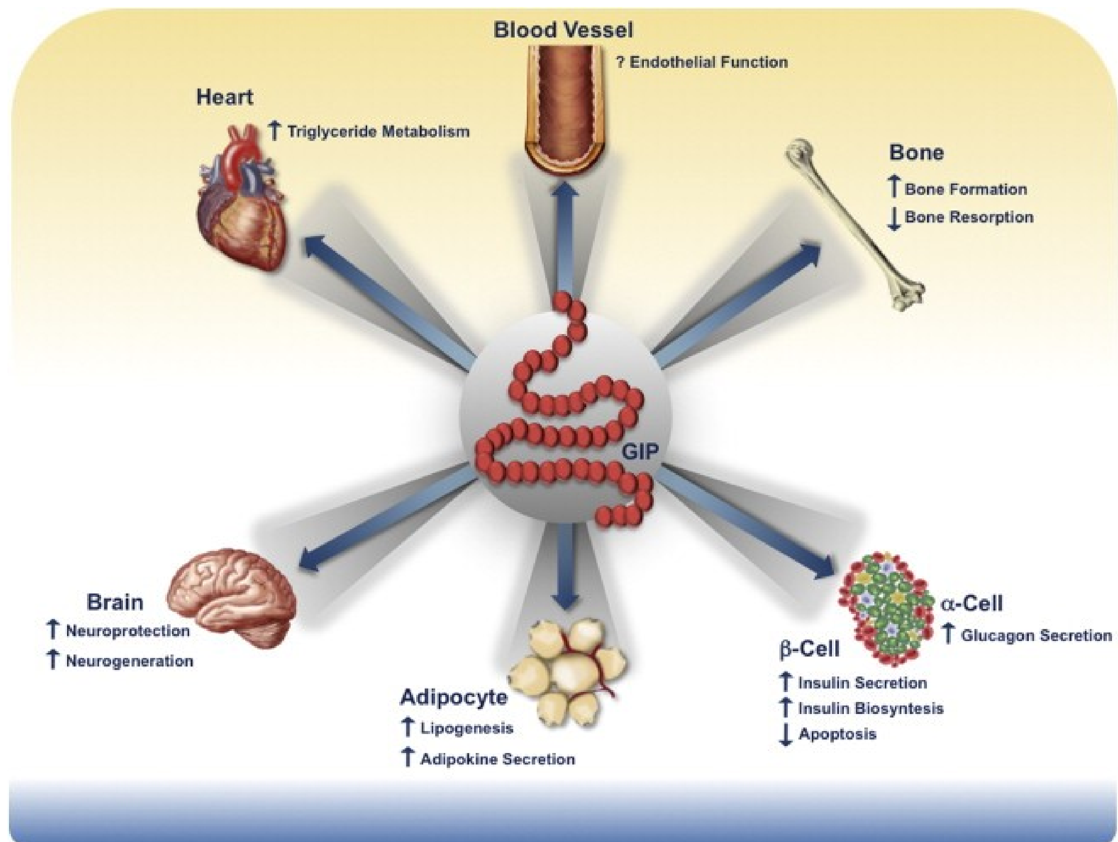


Fig. 7 Direct biological actions of GLP-1 (upper panel) and GIP (lower panel)

Figure source: Campbell and Drucker, Cell Metab, 2013

While the detection of a diminished activity of GLP-1 remains elusive, the concept of GIP resistance is relatively firmly established. Lack of GIP effect on insulin secretion coincides with a decrease in GIPR expression in pancreas, which seems to be induced by hyperglycemia and reversed by its normalization[105,106] and the activation of PPARα[107].

GIP resistance is, similarly to IR, linked to a chronic elevation in GIP secretion. As insulin inhibits GIP secretion in a negative feedback loop, which was shown to be blunted in hyperglycemic rodents[108], a development of selective IR in K-cells could be a cause for increased GIP secretion in obese subjects. Irwin et al. even showed that this effect is independent of elevated blood glucose levels[109]. Another possible mechanism for increased secretion of GIP is based on hyperplasia of intestinal K-cells observed in rats fed a high-fat diet[110].

Deregulation of the incretin system in obesity may also be linked to increased DPP-4 activity. DPP-4 has been recently shown to be an adipokine, the secretion of which rises mainly in hypertrophied adipocytes of visceral WAT. DPP-4 activity correlates with adiposity and other markers of MS, and its overactivation in obesity could result in faster degradation of incretins, manifesting as a decrease in the incretin effect[111].

In summary, obesity coincides with an increase in GIP levels, which can lead to the development of GIP resistance in the pancreas and the loss of GIP-induced incretin effect. In some cases, GLP-1-induced incretin effect is also diminished and can be reversed using exogenous GLP-1R agonists.

1.4. Treatment strategies for metabolic syndrome and type 2 diabetes

1.4.1. Pharmacological interventions

The primary goal of treating T2DM is to maintain good glycemic control, i.e. fasting blood glucose (**FBG**) < 5.5 mmol/l. Because there is usually some endogenous insulin in pre-diabetic state and early phases of T2DM, therapy is more focused on improving insulin sensitivity and decreasing lipotoxicity rather than on increasing insulin levels, as with type 1 diabetes mellitus[112].

Since most oral therapies gradually lose their effects, treatment usually progresses from oral monotherapy to the combination of two and more oral treatments with gradual dose increase. In case of β-cell dysfunction, oral therapies are supported by basal insulin or even multiple insulin injections per day. The combinatory approach not only improves glycemic control, but also enables to lower the dosage, thus reducing the incidence of adverse effects[112].

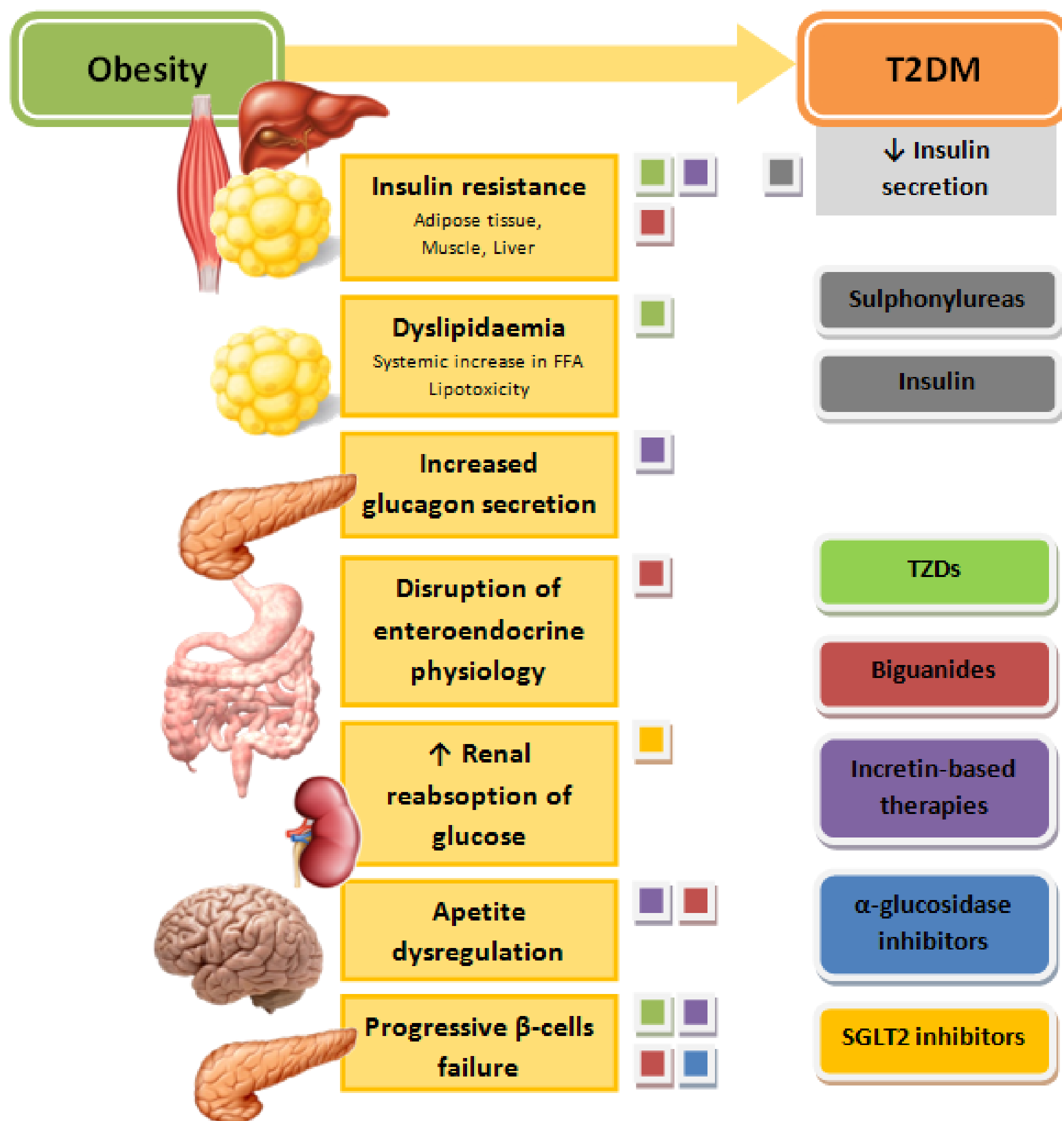


Fig. 8 Overview of T2DM therapy

Obesity predisposes to T2DM. Main factors (in yellow fields) contributing to the development of T2DM (according to DeFronzo lecture from 2009[113]), and T2DM medication classes (in colored fields). Small colored squares besides the main factors correspond to the medication classes of the same color. TZDs, thiazolidinediones; SGLT2, sodium-glucose cotransporter 2. Figure source: Pavlisova, 2016

Sulphonylureas, Thiazolidinediones, Dimethylbiguanides, and inhibitors of α-glucosidase and sodium-glucose cotransporter 2

Sulphonylureas were discovered in 1950s as the first drugs for the treatment of T2DM. The main mechanism of their action is to enhance endogenous secretion of insulin, although they have been shown to exert some direct extra-pancreatic insulin-mimicking properties, as well[114]. Physiological secretion of insulin from β-cells is linked to the process of glycolysis and an increase in intracellular ATP levels, followed by a closure of ATP-sensitive potassium channels Kir, changes in membrane potential, and finally the opening of voltage-dependent calcium channels, resulting in an increase in intracellular calcium and the release

of insulin-containing vesicles. In this context, sulphonylureas bind to a sulphonylurea receptor subunit of Kir, thus mimicking the effect of rising ATP and closing the potassium channels[115].

Thiazolidinediones (TZD) prevent and treat T2DM by increasing insulin sensitivity. TZD were shown to act as PPARγ ligands, hereby promoting differentiation and maturation of new adipocytes (adipogenesis), thus enabling safe deposition of excess lipids within WAT[116]. By improving the metabolic function of WAT, TZD also normalize inflammation and lipid deposition within liver in patients with liver steatosis or steatohepatitis[117]. Their significant potency in maintaining good glycemic control is, however, outbalanced by potentially severe adverse effects including fluid retention and exacerbation of cardiovascular disease; therefore, some TZD (e.g. Troglitazone, Rosiglitazone) have been banned from market[118].

Metformin as the only dimethylbiguanide drug in use is the first-line medicine and the most frequently prescribed drug to treat T2DM. [119119] Metformin suppresses hepatic gluconeogenesis, which is inhibited by insulin in healthy individuals but overactive in the state of IR[120]. Inhibition of *de novo* gluconeogenesis by metformin is linked to the activation of AMP-activated protein kinase (AMPK); this enzyme is activated by increased levels of AMP in a situation of energy depletion and stimulates catabolic processes at the expense of energy consuming anabolic reactions. Metformin can stimulate AMPK by (i) activating its upstream kinase LKB1[121], (ii) promoting formation of the AMPK-αβγ heterotrimeric complex[122], and (iii) inhibiting mitochondrial respiratory chain complex 1[123] and AMP deaminase[124]; however, much larger doses of metformin would have to be applied to induce the inhibition of mitochondrial respiratory chain enzymes[119]. In the gut, metformin could beneficially alter the composition of gut microbiota[125] and activate AMPK within enterocytes, which could lead to a decrease in intestinal permeability for lipopolysaccharide (LPS) endotoxin[119]. Adverse effects of metformin are usually mild, however, impaired renal function can lead to accumulation of metformin in the body, which may induce severe lactic acidosis. Therefore, metformin treatment is contraindicated in conditions of moderate to severe renal impairment[127].

Inhibitors of α-glucosidases on the membrane of intestinal brush border cells affect the digestion of glucose-containing glycoproteins, oligosaccharides, and complex dietary sugars (e.g. starch, glycogen). As a result, absorption of dietary carbohydrates is decreased, thus preventing postprandial hyperglycemia in patients with IR and/or T2DM. The advantage of α-glucosidase inhibitors lies in the absence of systemic side-effects, as they are poorly absorbed and their activities are localized into the intestines. Mild side effects such as flatulence, abdominal pain, and diarrhea result from bacterial metabolism of undigested carbohydrates[128].

Inhibitors of sodium-glucose cotransporter 2 (SGLT2), also marked as gliflozins, decrease blood glucose levels by increasing excretion of glucose in urine (glycosuria). SGLT2, localized mainly in renal proximal tubules, utilizes gradient energy created by Na^+/K^+ ATPase on the basolateral membrane of renal tubules to reabsorb glucose and Na^+ ions from

glomerular filtrate. When hyperglycemia reaches a specific threshold, SGLT2 transporters reach their maximum capacity and glucosuria appears as a typical marker of diabetes[129]. Pharmacological inhibition of SGLT2 lowers this threshold and thus acts against a compensatory increase in SGLT2 concentration occurring in T2DM; as a result, the ability to sequestrate excessive glucose in urine is maintained[130]. Moreover, SGLT2 inhibition influences excretion of other ions such as Cl^-, thus increasing diuresis and lowering blood and glomerular pressure. SGLT2 inhibitors, therefore, treat T2DM and prevent CVD by normalizing glycemia and hypertension while preventing kidney damage[129].

Incretin-based therapy

As described in section 1.3, incretins play a crucial role in postprandial glucose metabolism by increasing GSIS in pancreatic β-cells, while promoting β-cells growth and viability at the same time[65]. [100]As one of the most effective treatment strategies in T2DM, incretin-based therapy involves either the inhibition of DPP-4 or activation of GLP-1R by synthetic agonists[104]. In the latter case, GLP-1R agonists such as exenatide (Exendin) and liraglutide have a long-lasting potency due to their resistance towards DPP-4 cleavage. Their use is associated with a minimal risk of hypoglycemia, as the incretin effect is glucose-dependent[131]. However, a disadvantage of GLP-1 mimetics is a need for daily applications using subcutaneous injections; on the other hand, very stable analogues are being developed to decrease a frequency of injections. Concerning DPP-4 inhibitors (also known as gliptins), they prolong biological half-life of endogenous incretins by long-term inhibition of this degrading enzyme. Unlike incretin mimetics, gliptins can be applied orally. Adverse effects of DPP-4 inhibitors are relatively mild, including digestion problems, headaches, and increased risk of respiratory infections[132].

1.4.2. Life-style modifications

While pharmacological interventions usually exert stronger effects than life-style modifications, they mainly aim for the single target within the complex regulatory network. On the contrary, life-style modifications influence the organism at the systemic level. As obesity and T2DM result mainly from an inappropriate dietary and exercise habits, life-style modifications should be considered an inseparable part of any treatment strategy. General approach in life-style modifications is an increase in physical activity and/or a change in dietary patterns, which results in a daily caloric deficit and enhanced nutrient intake[133]. Beneficial metabolic effects of exercise are well known (reviewed by Karstoft and Pedersen in 2016[134]), while dietary approaches may include a simple reduction of calorie intake, low-carbohydrate diets, and lowering of glycemic load[135]. However, apart from limiting the amount of calories, modifications in the composition of respective nutrients may also bring significant impact on health and metabolism (see below).

1.5. Metabolic effects of dietary lipids

With 37 kJ per 1 g, lipids represent the richest available source of energy. Various lipid molecules are also the main constituents of cellular membranes and serve as bioactive and signaling molecules. Biochemical properties of lipid molecules depend strongly on the length and saturation of FA that are esterified to various templates to form acylglycerols, phospholipids (**PL**), sphingolipids, cholesteryl-esters, and other complex lipid molecules. Dietary lipids are, therefore, not only a source of energy, but also important nutrients needed for the proper function of cellular membranes and signalization.

Dietary FA can be divided into several categories including saturated FA (**SFA**), monounsaturated FA (**MUFA**), and polyunsaturated FA (**PUFA**), which can be further divided PUFA of n-3 series (**Omega-3**) and PUFA of n-6 series (**Omega-6**). It has been shown, that not only the quantity of lipids, but also the composition of FA regarding the length and degree of unsaturation influence potential metabolic effects of a mixed diet[136,137]. The following chapters describe how these FA classes differ in their impact on health and metabolism.

1.5.1. Saturated fatty acids

The most abundant dietary SFA are palmitic (C16:0) and stearic acid (C18:0). As the absence of a double bond within the carbon chain increases melting temperature of lipid molecules, SFA are mostly solid at room temperature. Main dietary sources of SFA are lard, butter, cheese, and other dairy products, or some specific vegetable oils, such as palm oil or coconut oil[138]. Some human studies provide evidence that increased consumption of SFA supports the development of visceral obesity and has a detrimental impact on metabolism, mainly on the ectopic accumulation of liver fat, when compared to MUFA or PUFA[139–141]. These results are supported by animal studies, which also show a deterioration of insulin sensitivity and WAT inflammation in animals fed preferentially with SFA[142–144].

Multiple molecular mechanisms could explain why SFA affect the organism more detrimentally than other FA species. Palmitic acid (**PA**; C16:0) is considered to be a lipotoxic compound, as it serves as a substrate for the synthesis of lipotoxic ceramides (see section 1.2.2). Experiments in cell cultures have shown that treatment with PA but not MUFA or PUFA increased the production of ROS and cellular apoptosis[145–147]. As mentioned in the section 1.2.3, SFA, especially PA, are also proinflammatory compounds, as they serve as ligands of Toll-like receptor 4 (**TLR4**)[148,149], and the SFA treatment increases the production of proinflammatory cytokines *in vitro*[150]. The rate of mitochondrial β-oxidation of SFA is relatively low and further decreases with the increasing length of the carbon chain[151]. The incorporation of SFA into nascent TAG appears to be limited[147,152], which may cause an accumulation of the intermediates of TAG synthesis, including DAG, which are considered lipotoxic (see section 1.2.1). However, dietary SFA are effectively incorporated into membrane PL, thereby reducing the fluidity of cell membranes and affecting the function of transmembrane proteins. Increased accumulation of SFA in the membrane of endoplasmic

reticulum (**ER**) was linked to ER stress and unfolded-protein stress response, which may lead to apoptosis[144,153,154].

Based on the above-described evidence, it is reasonable to consider SFA as potentially harmful. This was also reflected by WHO that incorporated the preferential consumption of "healthy" PUFA at the expense of SFA into their recommendations for a healthy diet[155].

1.5.2. Monounsaturated fatty acids

MUFA contain one double bond, which makes MUFA molecules bent at one place and mostly liquid at room temperature. The most common dietary MUFA are the palmitoleic (C16:1 *n-7*) and oleic (C18:1 *n-9*) acids. A particularly high content of oleic acid may be found in olive oil, meats (especially pork and beef), or nuts (macadamia, hazelnuts); however, the content of oleic acid in fatty foods is generally high[138].

Metabolic effects of dietary MUFA have been often investigated in comparison with SFA. While the effects of SFA were mostly deleterious (see above), those of MUFA were rather neutral or even protective, which was true for cell culture experiments[145,156,157], as well as for animal[158,159] and human studies[139,160]. When combined with SFA treatment, MUFA even managed to protect the cells against SFA-induced lipotoxicity[152,156,157,161].

These phenomena may be explained by several possible molecular mechanisms (**Fig. 9**). Firstly, FA in general serve as ligands for intracellular PPAR transcription factors. After binding the ligand, PPAR translocate into the nucleus, where they heterodimerize with a retinoid X receptor and attach themselves to the PPAR response element within the DNA molecule in order to regulate transcription of various PPAR-responsive genes[162,163]. The most abundant PPARα isoform can be found in metabolically active tissues such as the liver, heart, skeletal muscle, intestinal mucosa, and brown adipose tissue. Activation of PPARα shifts metabolic balance from lipid storage to lipid catabolism, as PPARα regulates many processes linked to mitochondrial as well as peroxisomal β-oxidation of FA, FA transport, and lipoprotein metabolism. PPARγ isoform, on the other hand, promotes lipid storage and adipogenesis, and may be found mainly in WAT. The affinity of FA towards PPAR depends on the biochemical properties of the respective FA, and it was reported to be highest in PUFA, lesser in MUFA, and lowest in SFA[164,165]. Thus, MUFA-induced activation of PPARα may increase oxidation and sequestration of potentially lipotoxic SFA, while the activation of PPARγ promotes safe storage of FA within neutral TAG molecules[166].

Unlike SFA, MUFA are also strong ligands for the enzyme diacylglycerol acyltransferase (**DGAT**), which catalyzes the final step of neutral TAG synthesis[166]. The enzyme stearoyl-CoA desaturase-1 (**SCD1**) catalyzes insertion of the double bond to the Δ7 position of the carbon chain, thus turning palmitic and stearic acid into palmitoleic and oleic acid, respectively. A presence of functional SCD1 has been shown to be obligatory for the incorporation of *de novo* synthesized FA into TAG[167]. The tendency towards rapid neutralization of MUFA

by the re-esterification into TAG is, therefore, another explanation for their benign or even beneficial effects (see **Fig. 9**); however it could also contribute to a obesogenic and steatogenic potential of MUFA, which has been reported by several studies[168]. Moreover, MUFA not only incorporate themselves into the TAG, but also promote more effective re-esterification of other FA species, which may explain the protective effects against SFA-induced lipotoxicity[152].

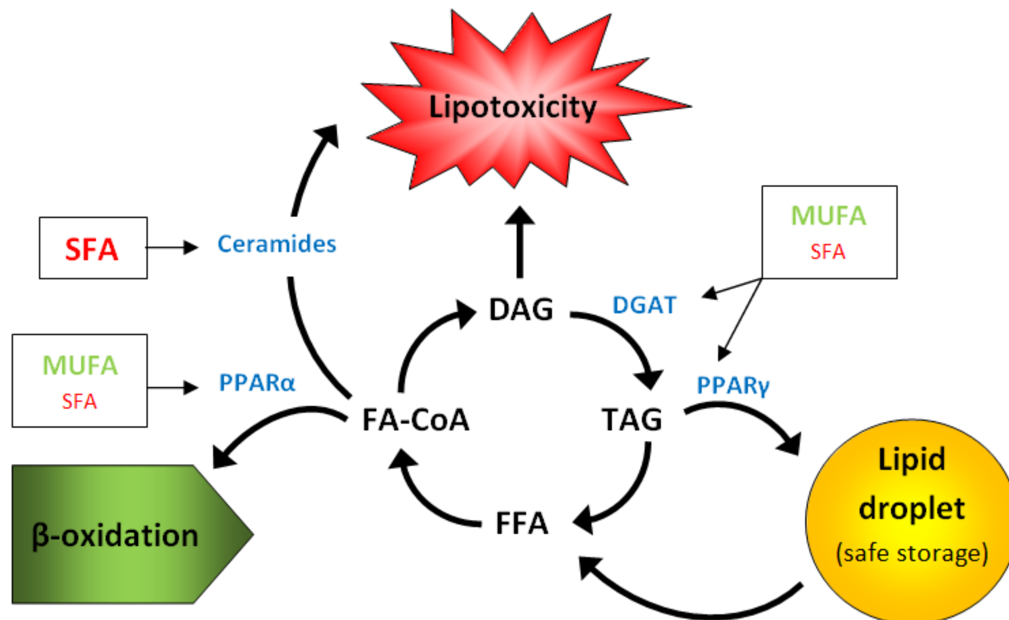


Fig. 9 Comparison of the effects of SFA and MUFA on lipid metabolism

DGAT enzymes and transcription factor PPARγ promote lipid deposition within neutral TAG molecules, while transcription factor PPARα promotes mitochondrial and peroxisomal lipid oxidation. As compared to SFA, MUFA represent stronger ligands both for PPARs and DGATs; thus, MUFA can be promptly cleared out, while SFA linger in the system and form lipotoxic metabolites, such as ceramides or DAG. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; FFA, free fatty acid; DAG, diacylglycerol; TAG, triacylglycerol; DGAT, diacylglycerol acyltrasferase; PPAR, peroxisome profeliferator-activated receptor.

Figure edited according to Nolan and Larter, J Gastroenterol Hepatol, 2009

1.5.3. *Trans* unsaturated fatty acids

Unsaturated FA occur in two different stereoisomeric configurations, i.e. *cis*, where hydrogens bound to the carbons of the double bond are on the same side, and *trans*, where the hydrogens reside on the opposite sides (**Fig. 10**). *Trans* unsaturated FA (*trans* FA) are synthesized mainly by anaerobic bacteria residing in the intestinal tract of ruminants and also by several species of aerobic bacteria in order to adapt biochemical properties of their membranes for extreme environmental conditions[169]. While *cis*-configured double bonds bend the FA chain, *trans* FA remain straight, thus their biochemical properties resemble those of SFA. Low concentrations of *trans* FA are commonly ingested in milk and meat of ruminants, while higher concentrations occur in chemically hydrogenated vegetable oils that are commonly used as the replacement of lard and butter[170].

Increased consumption of *trans* FA was linked to detrimental changes in plasma lipid profile, i.e. an increase in low-density lipoprotein (**LDL**) cholesterol and decrease in HDL cholesterol, IR, and endothelial dysfunction with increased plasma levels of inflammation markers[171]. Taken together, excessive intake of *trans* FA increases the risk of CVD, which needs to be considered with regard to the replacement of SFA by hydrogenated vegetable oils.

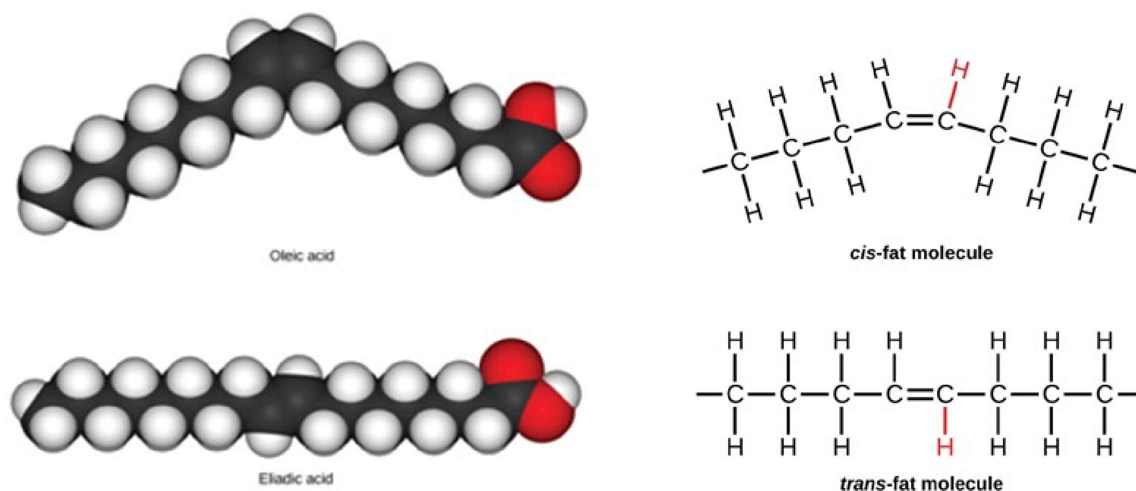


Fig. 10 *Cis* and *trans* configuration of fatty acids

Oleic acid represents a classical *cis*-configured FA with the double bond bending the molecule, while the eliadic acid represents the *trans*-configured FA resembling the biochemical structure of SFA. Figure edited according to boundless.com and prettyhealthy.co

1.5.4. Omega-6 polyunsaturated fatty acids

The carbon chain of Omega-6 contains two or more double bonds, with the first double bond being present at the 6th carbon from the methyl (-CH_3) end of the FA chain. Omega-6 belong to the group of essential nutrients, as the shortest Omega-6, linoleic acid (**LA**; C18:2 *n-6*), is synthesized by the action of Δ12 desaturase, which is present only in plant and algae cells.

Omega-6, namely arachidonic acid (**AA**; C20:4 *n-6*), are the key FA involved in cellular signaling processes. AA frequently covers the *sn-2* position of PL embedded in the inner layer of cytoplasmic membrane. Here it presents itself to the hydrolytic activity of phospholipase A2 (**PLA2**), which in a highly regulated manner cleaves AA from the phospholipid backbone and makes it available as a substrate for the synthesis of signaling molecules collectively termed as eicosanoids. Briefly, eicosanoids are synthesized via three main enzymatic pathways involving cyclooxygenases (**COX**), lipoxygenases (**LOX**), and cytochrome P450, which gives rise to prostaglandins and thromboxanes, leukotriens, and non-classical eicosanoid epoxides, respectively (**Fig. 11**). Eicosanoids generated from Omega-6 regulate many processes associated with inflammation and injury, such as vasoconstriction, pain, fever, platelet aggregation, or smooth muscle contraction[56].

Membrane PL containing AA also serve for the synthesis of endocannabinoids (**EC**), i.e. the endogenously produced lipid molecules that interact with membrane cannabinoid receptors 1 and 2 (**CB1** and **CB2**, respectively). AA bound to phosphatidyletanolamine serves for the synthesis of arachidonoyl ethanolamine, i.e. Anandamide (**AEA**), while AA bound to the *sn-1* position of phosphatidylglycerol serves as a precursor for 2-arachidonoyl glycerol (**2-AG**)[172]. Both AEA and 2-AG regulate various biological processes either as neuromodulators, i.e. on the central level, or as paracrine signaling molecules at the periphery. Together with glucocorticoids, they contribute to the restoration of local homeostasis after stress stimuli, such as neural overactivation, inflammation, or tissue damage. In terms of energy homeostasis, the endocannabinoid system (**ECS**) enables the organism to respond to fasting, as it promotes food-searching behavior and appetite on the central level, while promoting fat deposition in the periphery. Physiological activity of the ECS is strictly regulated in the time- and localization-dependent manner. In obesity, however, the ECS becomes overactive, which promotes a further weight gain and metabolic derrangements[173,174].

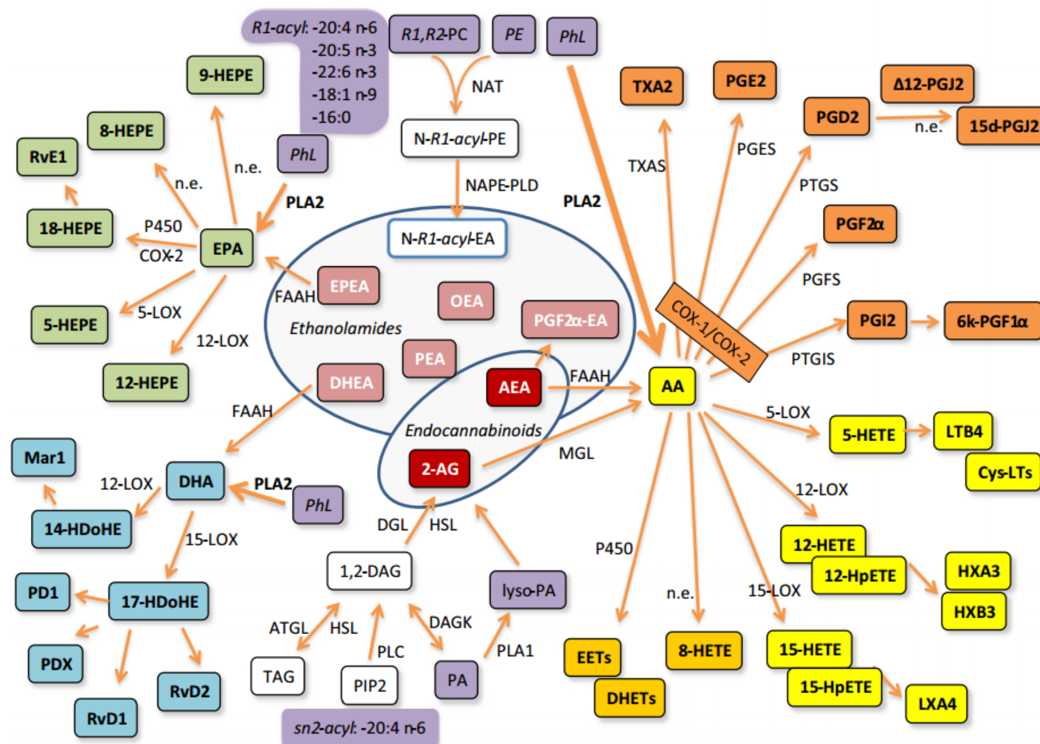


Fig. 115 Lipid signaling molecules

Membrane PL (violet) are cleaved by phospholipase A2 to yield free arachidonic (AA), eicosapentaenoic (EPA) or docosahexaenoic (DHA) acids, which are subsequently metabolized into either eicosanoids (orange, yellow, green) or docosanoids (blue) by a variety of enzymes. Endocannabinoids (red) come either from membrane PL via N-acyl-phosphatidylethanolamine intermediate or from 1,2-diacylglycerol in case of anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), respectively. 2-AG, 2-arachidonoyl glycerol; AA, arachidonic acid; AEA, arachidonoylethanolamide; ATGL, adipose triglyceride lipase; COX, cyclooxygenase; DAG, diacylglycerol; DAGK, diacylglycerol kinase; DGL, diacylglycerol lipase; DHEA, docosahexaenoylethanolamide; –EA, –ethanolamide; EPEA, eicosapentaenoylethanolamide; FAAH, fatty acid amide hydrolase; HSL, hormone-sensitive lipase; HX*, hepoxilin; LOX, lipoxygenase; LT*, leukotriene; MGL, monoacylglycerol lipase; n.e., non-enzymatic oxidation; NAPE-PLD, N-acyl phosphatidylethanolamine-specific phospholipase D; NAT, N-acyl transferase; OEA, oleoylethanolamide; P450, cytochrome P450; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEA, palmitoylethanolamide; PG*, prostaglandin; PIP2, phosphatidylinositol biphosphate; PLC, phospholipase C; TX*, thromboxane. Figure source: Massodi et al., Biochim Biophys Acta, 2015

1.5.5. Omega-3 polyunsaturated fatty acids

1.5.5.1. Biochemical and nutritional aspects of Omega-3 polyunsaturated fatty acids

Omega-3 contain three or more double bonds, with the first double bond at the 3rd carbon from the methyl end of the FA chain. The parental Omega-3, α-linolenic fatty acid (**ALA**; C18:3 *n-3*) is also an essential nutrient, as it is synthesized by the action of the above mentioned Δ12 desaturase, as well as Δ15 desaturase, which are both absent in eukaryotic cells.

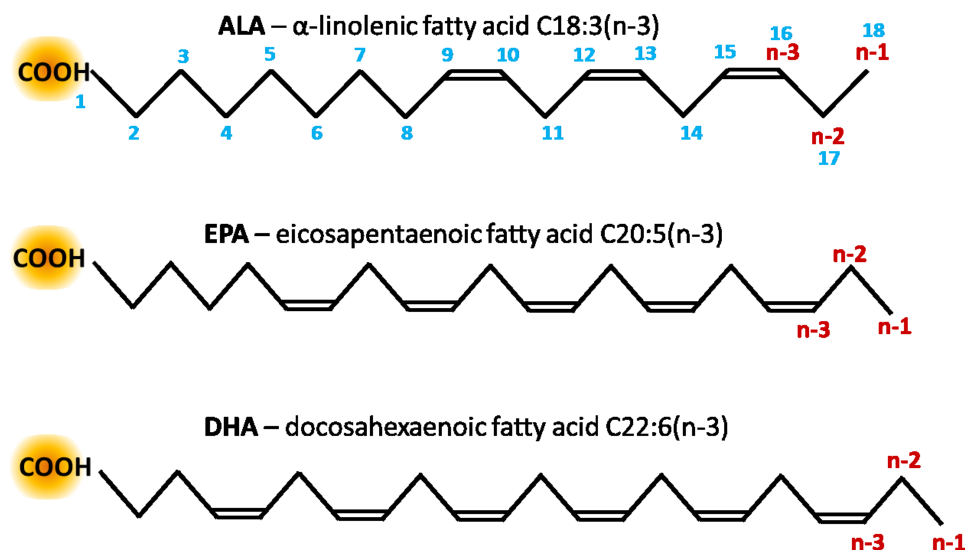


Fig. 12 Omega-3 fatty acids that play an important role in animal and human physiology.
ALA, α-linolenic acid (C18:3); EPA, eicosapentaenoic acid (C20:5); DHA, docosahexaenoic acid (C22:6). The numbering of carbons: In blue - numbering according to the α-position; in red – numbering according to the n-position (ω-position) within the carbon chain.

In mammals, ALA serves mainly as a precursor for the synthesis of FA with a longer chain, e.g. eicosapentaenoic acid (**EPA**; C20:5 *n-3*) and docosahexaenoic acid (**DHA**; C22:6 *n-3*), which are based on their biological properties the most studied Omega-3. While ALA is most abundant in higher plants (for example linseed oil contains up to 65 % of ALA), algae and even mammals possess specific elongases that can convert ALA into EPA and DHA. The dietary intake of ALA can, therefore, serve as a limited source of long-chain Omega-3[175]; however, the majority of ALA is readily oxidized and the conversion rate, which varies between species and depends strongly on total ALA intake[176], is generally low (<1 % ALA transforms into DHA in humans[177]). Therefore, the consumption of EPA- and DHA-rich foods is strongly recommended[176–179].

Significant dietary sources of ALA are linseed oil, walnuts, chia seeds, pumpkin seeds and other vegetable oils such as the soya been oil or canola oil. The main producers of long-chain Omega-3, i.e. EPA and DHA, are unicellular algae. Thus, based on the food chain hierarchy, EPA and DHA can be found mainly in marine crustaceans (Antarctic krill), herbivorous and plankton-consuming fish (e.g. herring), and marine predators such as salmon, tuna fish, and

others. Fresh-water fish meat contains smaller, but still significant amount of Omega-3; however, this is not true for the fish originating from artificial fish-farms, where the fish are fed mainly by corn and other vegetable products[180].

Recommended intake of Omega-3 according to various health organizations accounts for 400 - 500 mg EPA/DHA per day, which can be covered e.g. by two servings of sea-food per week[181]. According to the comprehensive data meta-analysis by Mozaffarian and Rimm[182], modest intake of fish meat (i.e. 1 - 2 servings per week) providing ≥250 mg Omega-3/day is sufficient to ensure primary prevention of CVD while lowering the relative risk of CVD-related death by 25 % (**Fig. 13**).

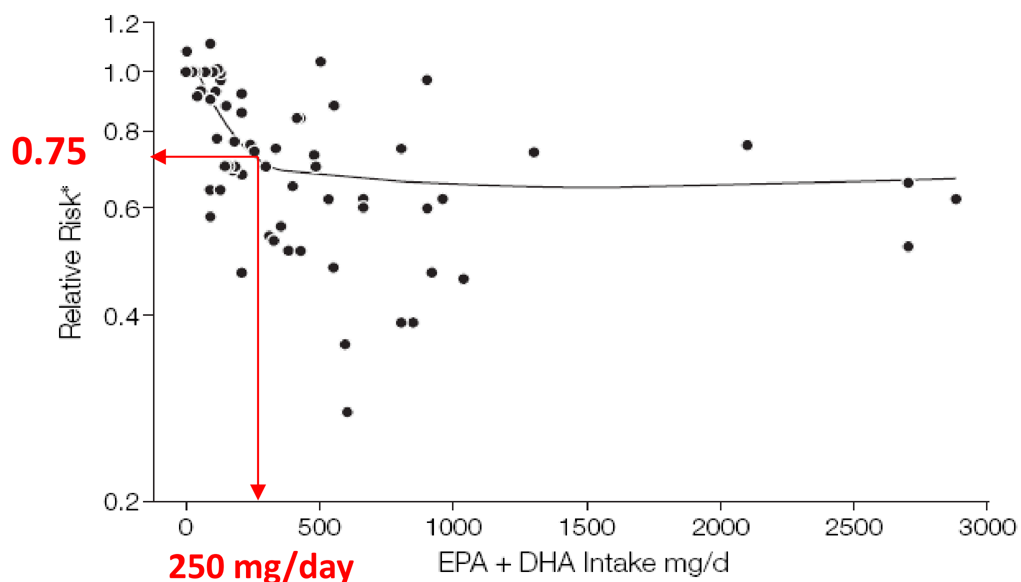


Fig. 13 Relationship between the relative risk of CVD-related morbidity and EPA/DHA intake
Figure edited according to Mozaffarian et al., JAMA, 2006

1.5.5.2. Metabolic effects of Omega-3 polyunsaturated fatty acids

Although the greatest demand for Omega-3 arises during the prenatal and early postnatal periods because of the rapid development of neural and visual structures, stable intake of EPA and DHA is necessary also in adults in order to retain the function of cellular membranes. Omega-3 deficiency, manifested as a severe decrease in membrane PL-bound DHA, can be partially compensated by a replacement of DHA by its Omega-6 counterpart (i.e. C22:5 *n-6*); however, even these small changes can result in a deterioration of cognitive and visual functions[183,184]. Increased intake of long-chained Omega-3 has been shown to exert various beneficial effects on metabolic health. This was first evident from the epidemiological studies of Greenland Inuits in 1980s, which showed that in this population, the number of deaths caused by CVD was extremely low, despite high dietary intake of cholesterol. The factor that distinguished this population from other comparable cohorts was frequent

consumption of whale meat and other seafood and, therefore, a high bioavailability of Omega-3[185,186]. As mentioned above, Omega-3 are important in the prevention of CVD; in this context, Omega-3 have been shown to reduce blood pressure[187,188], decrease the risk of thrombosis[189], lower plasma TAG concentrations[190] (reviewed in[191]), and improve cardiac function. Furthermore, very high doses of Omega-3 (i.e. ~3000 mg/day) are prescribed as a pharmacological treatment for dyslipidaemia[192]. However, the results of a number of clinical studies evaluating the impact of Omega-3 interventions on CVD-related mortality are unconvincing (reviewed in[193]), possibly due to a long-term overtreatment with drugs targeting similar mechanisms as Omega-3, inhomogeneous target group for treatment, and other reasons. On the other hand, anti-inflammatory properties of Omega-3 can be utilized in the supportive treatment of diseases such as rheumatoid arthritis, asthma, and inflammatory bowel disease.

With regard to the prevention and/or treatment of IR and glucose intolerance associated with obesity and T2DM, the role of Omega-3 remains controversial. In rodent models of obesity, Omega-3 have been frequently shown to reduce adiposity and improve various aspects of glucose homeostasis[194–198]. Hypolipidemic effects of Omega-3 are likely connected to their ability to enhance lipid catabolism, and to suppress lipid synthesis *de novo*[194,199]. Reduction of lipid levels in plasma and non-adipose tissues (i.e. amelioration of lipotoxicity), as well as the suppression of chronic inflammation is linked to the improvements in insulin sensitivity, mainly in the liver, but also in WAT, and skeletal muscle[194]. In obese and T2DM patients, Omega-3 might also reduce adiposity[187,200]; however, their effect on glycemic control and insulin sensitivity appears to be either very limited or absent[201,202].

1.5.5.3. Molecular mechanisms of Omega-3 polyunsaturated fatty acids action

Mainly DHA, and to a lesser extent also ALA and EPA, accumulate in membrane PL and sphingolipids of all tissues, with the highest abundance in neurological structures such as brain and retina[177]. Long polyunsaturated carbon chains increase membrane thickness and fluidity, thus influencing the composition of lipid rafts and the function of transmembrane proteins and ion channels[179,193,203]; an interesting example of this mechanism [204204]is the inhibition of TLR4-mediated proinflammatory responses in immune cells[205]. Omega-3 serve as ligands for the membrane-bound as well as intracellular receptors, the latter directly regulating gene expression as transcriptions factors. They also serve as substrates for the synthesis of bioactive molecules[206], and their hypolipidemic effect seems to be dependent on the presence of functional AMPK[194].

Interaction with receptors and transcription factors

PUFA, and in particular Omega-3, serve as potent ligands for the intracellular receptors/transcriptional factors such as PPARα and PPARγ, thus promoting lipid oxidation and adipogenesis, respectively[207]. While PPARα activation mediates mainly the lipid-lowering

and insulin-sensitizing properties of Omega-3[163,208], activation of PPARγ has been connected with some anti-inflammatory effects[209].

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Omega-3 also indirectly interact with other transcription factors, such as sterol regulatory element-binding protein-1 (SREBP-1), carbohydrate regulatory element binding protein (ChREBP), or Max-like factor X (MLX)[208,211]. Specifically, they can interfere with gene expression and mRNA turnover, and decrease nuclear abundance, probably by changing the phosphorylation status of these transcription factors[208,212], while some of these effects are dependent on the activation of AMPK[213]. The suppression of SREBP-1 and ChREBP/MLX activity mediates the Omega-3-induced decrease in *de novo* lipogenesis. Omega-3 also interact with the membrane-bound G-protein-coupled receptor GPR120[214,215]. This receptor superfamily is known to recognize FA of various chain length, with GPR120 binding specifically the long-chain FA such as Omega-3. Thus, Omega-3-mediated activation of GRP120 could increase the secretion of GLP-1 and stimulate endogenous insulin production, as shown under *in vitro* conditions[214]. GPR120 has also been found to be abundant in mature adipocytes and pro-inflammatory macrophages, where it mediates some of the anti-inflammatory effects of Omega-3 via the inhibition of TLR and TNFα signaling[215].

Synthesis of bioactive molecules

As described in the section 1.5.4, many paracrine signaling molecules such as eicosanoids and EC, are synthesized from AA (i.e. Omega-6 FA). Omega-6 share many biochemical properties with Omega-3, which also means that many enzymes, including some elongases, desaturases, and the enzymes for the synthesis of PL and EC, as well as PLA2, COX, and LOX, freely cross react with both classes of these PUFA. Omega-3 negate pro-inflammatory and other effects of eicosanoids and EC by three different mechanisms: displacement, competition, and counteraction.

Displacement and competition

As essential FA, Omega-3 and Omega-6 must be taken in the diet either as precursor FA (i.e. ALA and LA, respectively) or as their long-chain forms (i.e. EPA and/or DHA, and AA, respectively). Both ALA and LA can be transformed into their respective long-chain counterparts, while competing for the same enzymatic apparatus. Thus, consuming more ALA will decrease the synthesis of AA from LA. Long-chain Omega-3 and Omega-6 also compete for the active sites of enzymes involved in the acylation of phospholipid backbones, as well as for the enzymes for the synthesis of eicosanoids and EC. Thus, consumption of foods rich in Omega-6 results in an increase in membrane-bound AA and AA-derived lipid molecules, while increased consumption of Omega-3 can lead to a partial replacement of AA with EPA and/or DHA with a concomitant increase in Omega-3-derived lipid metabolites[216–219].

Counteraction

Displacement of AA in membrane PL leads to a decrease in the absolute amount of AA-derived pro-inflammatory and obesogenic eicosanoids and EC molecules. However, various Omega-3-derived signaling molecules themselves exert bioactive properties that can directly counteract the effects of Omega-6-derived molecules[220]. Increased bioavailability of EPA has been shown to induce synthesis of 5-series leukotriens[221,222] and prostaglandin PGE_3[223], in some cases at the expense of pro-inflammatory AA-derived PGE_2[224,225]. Such molecules are less biologically active than their AA-derived counterparts and thus contribute to the resolution of inflammation mainly by decreasing net pro-inflammatory activities of eicosanoids[226,227]. The 15-lipoxygenase-catalysed transformation of both EPA and DHA gives rise to molecules, which are collectively marked as resolvins and protectins, and which, on the contrary, contribute actively to the inflammation resolution and exert various biological effects (see **Fig. 11**, and reviewed in[228]). Importantly, the synthesis of active resolvins and protectins can also be triggered by Aspirin, as the catalysis by 15-LOX may be alternated by Aspirin-acetylated COX2[229].

1.6. Omega-3 polyunsaturated fatty acids in the form of marine phospholipids

Recent data have suggested that the metabolic effects of dietary lipids do not depend only on the FA composition, but also on the complex structure of the molecules, in which FA are delivered to the organism. In this regard, there is an evidence that metabolic effects of Omega-3 ingested in the form of PL (**Omega-3 PL**) are superior to Omega-3 in the form of TAG (**Omega-3 TAG**)[230–232]. Thus, slight differences in digestion and metabolism of Omega-3 PL and Omega-3 TAG, as well as the presence of other components within complex lipid molecules could explain the differential effects of these lipid forms of Omega-3 on metabolism, as will be further demonstrated.

In the organism, TAG are mainly used for storing energy; therefore, TAG are abundant in plant oils, as well as in animal fat. On the contrary, PL represent the building blocks of cellular membranes and are, therefore, evenly distributed throughout the biomass. Omega-3-rich TAG may be found mainly in fish oil, while Omega-3 PL are more concentrated in lean fish meat. Sources that are particularly rich in Omega-3 PL are quite scarce. High concentrations of Omega-3 PL can be found in marine fish roe[233] or Krill oil (**KO**), the latter being harvested from Antarctic crustacean zooplankton *Euphausia superba*, generally known as krill (**Fig.14**)[234,235]. Purified Omega-3 PL, as well as fish roe and KO, have been documented to affect favorably cognitive functions and various metabolic disturbances such as increased fat mass, dyslipidemia, and ectopic accumulation of lipids in the heart and liver, both in obese rodents and in humans; these conclusions are based on the data published until 2012 (reviewed by Burri et al.[236]), as well as on more recent articles[237,238,232,239] (and reviewed in[240]).

Fig. 14 *Euphasia superba*
Euphasia superba, alias Antarctic krill, is a 1-6 cm long marine crustacean that can form large swarms, which makes it a significant food source for whales, the largest mammals on the planet, but also it is attractive for harvesting and processing in the fishing industry. Figure source: en.wikipedia.org, krillcruise.wordpress.com

1.6.1. Triacylglycerols versus phospholipids: Differences in the molecular structure and digestion

TAG consist of three FA esterified to the glycerol backbone. Individual FA may be, therefore, attached to three different positions marked as *sn-1*, *sn-2*, and *sn-3* (**Fig. 15**), which is important, as various synthetic and degradation enzymes differ in their affinity towards respective positions. Thus, some FA are more likely to be esterified to a specific position, and, on the other hand, the position of FA on the glycerol backbone largely influences its fate during digestion and/or metabolism. [241,242241,242]Briefly, dietary TAG are degraded mainly by pre-pancreatic and pancreatic lipases. Pre-pancreatic lingual and gastric lipases cleave preferentially FA from the *sn-3* position and thus create mainly (*sn-1, sn-2)*DAG. Pancreatic lipase, which is secreted from exocrine pancreas into duodenum, then cleaves FA from both *sn-1* and *sn-3* positions, thus producing mainly FA and (*sn-2)*monoacylglycerols as the end products of TAG hydrolysis. The minor portion of TAG is degraded to FA and glycerol due to the marginal affinity of pancreatic lipase towards FA bound at the *sn-2* position of TAG molecule, occasional migration of FA from *sn-2* to *sn-1* position during the digestion process, and activity of cholesterol esterase[243–245].

The main portion of FA and monoacylglycerols is absorbed in the small intestine, namely in the upper jejunum, while a minor portion passes to the colon, where it is fermented by colonic microbiota and turned into short-chain fatty acids. Some FA enter the enterocytes by plain transmembrane diffusion; however, the facilitation of the process by transmembrane FA transporter CD36 appears to be necessary for the effective digestion[246].

Once within the enterocyte, hydrolytic products of TAG digestion travel with the assistance of FA binding proteins to the endoplasmic reticulum, where they are reassembled into nascent TAG mainly by the acylation of MAG by monoacylglycerol acyltransferase and DGAT2. Most nascent TAG are incorporated into the lumen of chylomicrons, i.e. the largest of lipoprotein particles that are characterized by the high content of TAG, very low density, and presence of apolipoproteins B48 or B100[241,242].

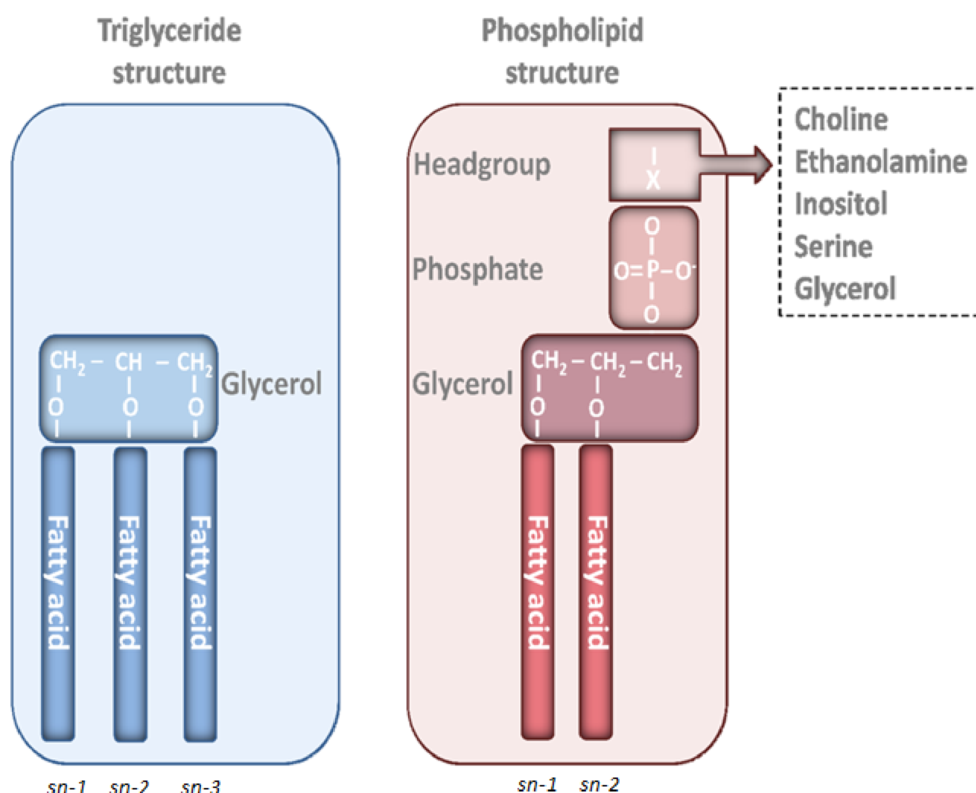


Fig. 615 The structure of the TAG and PL molecule
Figure edited according to Burri et al., Int J Mol Sci, 2012

While TAG have no polar groups and are, therefore, hydrophobic, PL molecules are amphipathic, as they consist of both the hydrophobic part (i.e. two FA esterified to the *sn-1* and *sn-2* positions of the glycerol backbone) and the polar part (i.e. the alcohol-phosphate group). Lingual and gastric lipases mostly do not interact with PL; therefore, digestion of PL is situated mainly to duodenum, where pancreatic PLA2 cleaves FA from the *sn-2* position, thus creating FA and lysophospholipids[236,242]. Digested PL are finally incorporated mainly to the surface lipid monolayer of chylomicrons; however, it has been shown that the intestine produces also HDL particles associated with ApoA-I and ApoA-IV[241], which are very small, dense, rich in cholesterol, and thus could be possible acceptors of a significant portion of dietary PL[247].

TAG in the chylomicron lumen are hydrolyzed by endothelial LPL to release FA, which can be taken up by various tissues. Thus, condensed chylomicrons gradually progress to chylomicron remnants, which are endocytosed and processed by the liver. The rate of intestinal TAG and PL absorption has been shown to be equivalent[236]; however, the lipid form of dietary FA clearly influences the localization of digested FA between lipoprotein species and their layers and, therefore, the bioavailability of FA in different tissues[248]. Indeed, many studies documented an increased bioavailability of EPA and/or DHA when supplied in the form of PL as compared to TAG[230,249,231], although the results of human studies are not conclusive[240].

1.6.2. Additional bioactive parts of Omega-3 PL molecules

While Omega-3 TAG, administered in the form of fish oil or chemically modified re-esterified TAG, deliver into the organism primarily EPA and DHA together with other FA esterified to the glycerol backbone, Omega-3 PL preparations are less homogenous and consist of other potentially bioactive constituents. For instance, it has been demonstrated that dietary supplementation with purified PL that did not contain Omega-3 exerted beneficial effects on cognitive and metabolic functions[236,250], hepatic lipid accumulation[251–253], and dyslipidemia[254]. Special attention has been paid to phosphatidylcholine (PC) that has been shown to alleviate orotic acid-induced hepatic steatosis in rats[252], while lowering intestinal cholesterol absorption in both animal and human studies[255].

Choline, representing a headgroup of the PC molecule, represents another bioactive molecule, as well as the essential nutrient, which must be ingested in the form of PC or sphingomyelin. Particularly high concentrations of choline may be found in beef or chicken liver, eggs, milk, or soybeans[256]. Choline is an intermediate in the synthesis of acetylcholine neurotransmitter; therefore, nowadays, a lot of attention is paid to choline supplementation in pregnancy, which may influence central nervous system development and cognitive functions in infants. However, some publications indicate that choline has also a beneficial impact on metabolism by influencing cholesterol homeostasis and protecting against the development of hepatic injury and hepatic lipid accumulation[257,258].

As mentioned above, the most frequently used source of Omega-3 PL is KO. It contains not only Omega-3, PC and other PL, but also a substantial amount of astaxanthin, a carotenoid produced by marine algae, which serve as a food source for Antarctic krill. Astaxanthin is an anti-oxidant that protects Omega-3 from oxidation, thus serving as a natural preservative[235]. Moreover, astaxanthin can have anti-inflammatory and neuroprotective functions in animal models of metabolic damage[259–261].

2. Aims of the thesis

The main goal, which brings together all four projects that represent the basis of this thesis, was to investigate molecular mechanisms mediating the beneficial effects of Omega-3, especially those administered in the form of Omega-3 PL, on glucose and lipid metabolism of obesity-prone C57BL/6 mice fed a high-fat diet.

The specific objectives were as follows:

1. to compare the effects of two obesogenic high-fat diets, differing mainly in the FA composition, on the development of obesity, IR, liver fat accumulation, and WAT inflammation;

2. to analyze changes in metabolism induced by long-term Omega-3 TAG supplementation, and their dependence on the type of FA contained in the background high-fat diet;

3. to verify the hypothesis that long-term dietary intervention with Omega-3 TAG increases glucose-stimulated insulin secretion by stimulating the activity of the incretin system;

4. to unveil molecular mechanisms that could explain why Omega-3 exert stronger biological effects (e.g. on glucose homeostasis and liver fat) when administered in the form of Omega-3 PL as compared to Omega-3 TAG;

5. to assess the contribution of the PC part of Omega-3 PL molecule to the metabolic efficacy of Omega-3 PL administration; and

6. to compare insulin-sensitizing properties of Omega-3 TAG and Omega-3 PL supplementation in obese mice fed a high-fat diet, using the state-of-the-art technique of hyperinsulinemic-euglycemic clamps.

3. Methods

The following methods were conducted by the author, unless stated otherwise.

3.1. Experimental setup, animals, and dietary interventions

For all subsequent experiments (exceptions are specified) male C57BL/6N mice at the age of 10 weeks were used. The animals were obtained from the Charles River Laboratories (Sulzfeld, Germany) and maintained for 2 weeks on a 12-h light/dark cycle (light from 6:00 a.m.) at 22°C with *ad libitum* access to water and a low-fat standard diet (**Chow**; extruded Ssniff R/M-H diet; Ssniff Spezialdieten GmbH, Germany) in order to adapt to local environment. At the age of 12 weeks, mice were transferred onto the experimental diets and long-term dietary interventions (7-10 weeks) including *in vivo* metabolic analyses (see section 3.2) were conducted. Throughout the experiments, body weight of single-caged mice was monitored weekly, while a fresh ration was given every 2 days. The calculation of average energy intake was based on food consumption measurements assessed in each mouse during a 24-hour period once a week. At the end of all experiments, mice were killed by cervical dislocation in diethyl ether anesthesia and blood samples were collected from cervical incision. Selected tissues were weighed and aliquots were snap-frozen in liquid nitrogen and stored at -80°C for subsequent biochemical analyses (see section 3.3). Samples for immunohistochemical analyses were fixed in 4% formaldehyde and subsequently embedded in paraffin (see section 3.4). All animal experiments were approved by the Animal Care and Use Committee of the Institute of Physiology, Czech Academy of Sciences (Approval Number: 127/2013) and followed the guidelines.

3.1.1. Experimental diets

Lean control animals were maintained on the Chow, based mainly on carbohydrates (wheat starch), protein, and fiber, and only a limited amount of lipids (~3.4 %). For the induction of obesity and various components of the MS, two different obesogenic diets (see **Tab. 1**) with a comparable content of lipids (33 – 35 % wt/wt) were used. The high-fat diet based on pork lard (**LHF**) was a commercial semi-synthetic diet (Ssniff EF acc. D12492 (I) mod., product # E15742-34; Ssniff Spezialdieten GmbH, Germany). This diet is characterized by a high content of SFA and MUFA. It also contains wheat starch and a small portion of sucrose (9 % wt/wt). The other high-fat diet based on corn oil (**cHF**) was prepared at the department of Adipose Tissue Biology in Prague. Since the majority of lipids comes from corn oil, this diet is rich in Omega-6. It also contains grinded Chow as a source of essential FA, fiber, vitamins, and minerals, infant formula as a source of protein, and corn starch as a source of carbohydrates.

Both high-fat diets were prepared with or without a supplementation with different types of Omega-3-rich products. As a source of Omega-3 TAG, we used the EPA+DHA concentrate Epax 1050 TG (DHA ~47% *wt/wt*, EPA, ~11% wt/wt; Epax, Norway). In case of Omega-3 supplementation 15 % (wt/wt) of total lipids (i.e. 52.5 g per 1 kg of diet) were

replaced by the Epax 1050 TG concentrate in order to achieve 30 g EPA+DHA per 1 kg of diet. The LHF and cHF diets supplemented with Omega-3 were marked as **LHF+F** and **cHF+F**, respectively.

In Publication C, we also tested the effects of Omega-3 administered as PL derived from herring-oil (Epax, Norway); Omega-3 PL were supplemented either alone or in combination with a low dose (i.e. 10 mg/kg diet) of the antidiabetic TZD drug rosiglitazone. Herring-derived Omega-3 PL concentrate was admixed into cHF diet, replacing ~10 % wt/wt of total lipids, resulting in the concentration of ~5 g EPA+DHA per 1 kg of diet (i.e. six times less than in the cHF+F diet). As the main PL compound in the herring-derived concentrate was PC, the Omega-3 PL supplemented diet was designated as "**PC**". The other diet supplemented with rosiglitazone (10 mg/kg of the diet) was marked as "**R**", while the diet supplemented with both Omega-3 PL and rosiglitazone was marked as "**PC+R**".

Tab. 1 Macronutrient composition of the parental diets

	Chow	LHF	cHF
Gross energy [MJ/100 g]	1.7	2.4	2.2
Metabolizable energy [MJ/100 g]	1.3	2.1	2.0
Crude protein [g/100 g]	19.3	24.1	12.1
Crude fat [g/100 g]	3.4	34.6	33.3
Soya-bean oil	3.4	3.0	1.4
Corn oil	-	-	19.8
Pork lard	-	31.6	-
Milk fat	-	-	12.1
Crude carbohydrates [g/100 g]	42.2	25.3	35.5
Starch	37.5	0.5	15.8
Sucrose	4.7	9.5	1.9
Maltodextrin	-	15.3	4.6
Lactose	-	-	13.2
Crude fiber [g/100 g]	5.0	6.0	2.6
Crude ash [g/100 g]	6.5	6.0	2.6

Chow, low-fat standard maintenance diet, Ssniff Spezialdieten; LHF, high-fat diet based on pork lard, Ssniff Spezialdieten; cHF, high-fat diet based on corn-oil, prepared at the Department of Adipose Tissue Biology in Prague. Information about the energy content and macronutrient composition are based on the data supplied by the manufacturer of the diet (Ssniff Spezialdieten) or individual components used for the preparation of the diet (cHF).

In order to decipher the contribution of different parts of the marine-derived PL concentrate, Omega-3 PL in the form of herring-derived concentrate (Epax, Norway) was compared with the EPA/DHA-free PC-rich PL concentrate derived from soya bean oil (L-α-Phosphatidylcholine, MilliporeSigma). Both concentrates, matched for the total PC

content, were admixed into cHF diet, replacing 10 % wt/wt of total lipids. The resulting diets were marked as "**PC-M**" (i.e. cHF diet supplemented with Omega-3 PL of marine origin; 3.4 g EPA+DHA per 1 kg of diet), and "**PC-S**" (cHF diet supplemented with soy-derived PC-rich PL product; see above).

In the Publication D, we continued testing the effects of Omega-3 PL; however, in this case, we used KO (Rimfrost Sublime, Rimfrost AS, Norway; ~130 g EPA + ~80 g DHA per 1 kg diet). In order to directly compare the effects of Omega-3 PL and Omega-3 TAG (Epax 1050 TG, Epax AS, Norway), differing in the total content of EPA and DHA, two doses of Omega-3 PL were used to prepare the corresponding supplemented diets: (i) ~15 % of total lipids (52.5 g per 1 kg of diet) were replaced by KO in order to match the amount of Epax 1050 TG admixed in the corresponding cHF+F diet, and (ii) the amount of replaced lipids was adjusted in order to match total content of EPA+DHA in cHF+F diet (i.e. 30 g EPA+DHA per 1 kg of diet). The resulting diets were designated as "**K-L**" (i.e. Krill-low dose; matched for the Epax 1050 TG content) and "**K-H**" (i.e. Krill-high dose; matched for the dietary EPA+DHA content).

For the macronutrient composition of the parental diets and an overview of the experimental diets see **Tab. 1** and **Tab. 2**, respectively.

Tab. 2 Overview of parental and supplemented diets used in respective experiments

Publication	Diet	Description	Supplementation	Amount [g/100 g]	EPA+DHA [g/kg]
A,B,C,D	Chow	Low-fat standard diet	-	-	-
A,B,C,D	cHF	High-fat diet based on corn oil (Omega-6)	-	-	-
A,B,C,D	cHF+F	cHF supplemented with Omega-3 TAG	Epax 1050 TG	5.2	30.0
A	LHF	High-fat diet based on lard (SFA and MUFA)	-	-	-
A	LHF+F	LHF supplemented with Omega-3 TAG	Epax 1050 TG	5.2	30.0
C	PC	cHF + Omega-3 PL	Herring PL	4.3	5.0
C	R	cHF + rosiglitazone	Rosiglitazone	0.001	-
C	PC+R	cHF + Omega-3 PL+ rosiglitazone	Herring PL Rosiglitazone	4.3 0.001	5.0
C	PC-M	cHF + Omega-3 PL	Herring PL	3.5	3.4
C	PC-S	cHF + soy-derived PL	Soy PL	3.4	-
D	K-L	cHF + KO, matched for EPAX content	Krill oil	5.2	10.8
D	K-H	cHF + KO, matched for Omega-3 content	Krill oil	14.8	30.0

3.1.2. Publication A - Experimental setup

In publication A, we investigated how metabolic effects of Omega-3 TAG depend on FA composition of lipids contained in the background high-fat diet. Two separate experiments (**Fig. 16**) were conducted in order to assess the effects of two high-fat diets, markedly differing in FA composition, primarily on IR (experiment 1.), and to analyze changes

in metabolism induced by Omega-3 TAG supplementation depending on the type of background high-fat diet (experiment 2.).

Experiment 1.

After the 2-week adaptation phase, 12-weeks-old C57BL/6N mice were randomly divided into 3 experimental groups (n = 16 - 20) and further maintained either on the Chow, cHF, and LHF diet. After 8 weeks of experimental feeding, the level of IR was assessed *in vivo* by using hyperinsulinemic-euglycemic clamp (see section 3.2.2). After the clamp (i.e. in hyperinsulinemic state), the mice were killed and dissected as described above (**Fig. 16**).

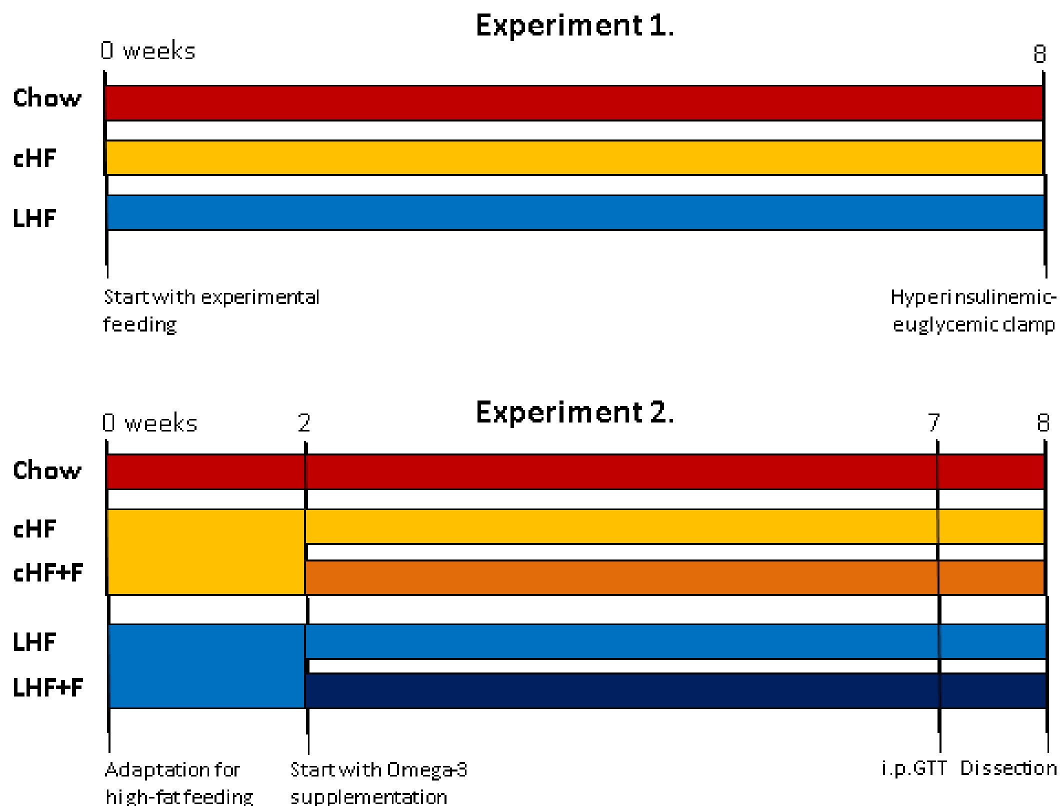


Fig. 16 Publication A - experimental setup

Chow, low-fat standard diet; cHF, corn oil-based high-fat diet; cHF+F, cHF supplemented by Omega-3 TG; LHF, lard-based high-fat diet; LHF+F, LHF supplemented by Omega-3 TG; i.p. GTT, intraperitoneal glucose tolerance test.

Experiment 2.

Twelve-weeks-old C57BL/6N mice were randomly divided into 3 experimental groups; mice serving as lean controls were further maintained on the Chow, while the rest of mice was fed either cHF or LHF diet. After 2 weeks of adaptation to high-fat feeding, each of the cHF and LHF groups was further divided into two subgroups, one continuing further on the respective parental high-fat diet (i.e. either on the cHF or LHF), while the high-fat diet of the other subgroup was supplemented with Omega-3 TAG. Thus, the study involved 5 experimental groups: Chow (n=6), cHF, cHF+F, LHF, and LHF+F (n = 9 - 10). Glucose tolerance was assessed

by intraperitoneal glucose tolerance test (i.p. GTT; see section 3.2.1) after 7 weeks of experimental feeding (including 2-weeks adaptation period). At week 8, mice were killed and dissected after 6-hour fasting period (food removed at 6:00 a.m.; see **Fig. 16**, Experiment 2.).

3.1.3. Publication B - Experimental setup

In publication B, we investigated a possible link between the effect of Omega-3 supplementation on plasma insulin levels and activation of the incretin system. We performed three different experiments in order to investigate, whether (i) gastrointestinal tract (**GIT**) is involved in the Omega-3-mediated potentiation of glucose-stimulated insulin levels (Experiment 1.), (ii) long-term Omega-3 supplementation could impact on various members of the incretin system (Experiment 2.), and (iii) the effect of Omega-3 on incretin levels could be sustained even in mice made obese prior to Omega-3 supplementation (Experiment 3.). Of note, in the experiments 1. and 3. the C57BL/6J mouse strain (bred at the Department of Adipose Tissue Biology of the Institute of Physiology) was used instead of the C57BL/6N strain.

Experiment 1.

Male C57BL/6N mice (2 - 4 mice per cage) were maintained on a 12-h light/dark cycle (light from 6:00 a.m.) at 22°C with ad libitum access to drinking water and the Chow since weaning at the age of 3 weeks. At the age of 12 weeks, mice were randomly divided into three experimental groups (n = 16) in order to continue either on the Chow, cHF, or cHF+F diet. After 8 weeks of experimental feeding, each group was randomly divided into two subgroups (n = 8), one group subjected to i.p. GTT and the other one to oral glucose tolerance test (OGTT; see section 3.2.1 and **Fig. 17**, experiment 1.).

Experiment 2.

After the 2-week adaptation phase, 12-weeks-old C57BL/6N mice were randomly divided into 3 experimental groups (i.e. Chow, cHF, cHF+F; n = 16), thus matching the setup of the Experiment 1 (see above). After 8 weeks of experimental feeding, mice were fasted for 6 hours (6:00 a.m. - 12:00 a.m.) and subjected to OGTT. After 9 weeks, mice were divided into two subgroups with homogenous body weight distribution, fasted for 6 hours, and either gavaged with 0.5 ml of saline (i.e. "fasted" group) or 0.5 ml of 30% glucose (i.e. "gavaged" group). Blood samples were collected 30 min after the gavage under the isoflurane anesthesia by the cannulation of portal vein. During the sampling, blood was immediately mixed with an inhibitor of DPP-4 (MilliporeSigma). Anesthetized mice from both subgroups were then killed by cervical dislocation and dissected as described above. For the analysis of gene expression in the gut, 2-cm long fragments of the proximal small intestine, distal small intestine, and colon were removed, rinsed with saline, snap-frozen in liquid nitrogen and stored at −80 °C (**Fig. 17**, experiment 2.).

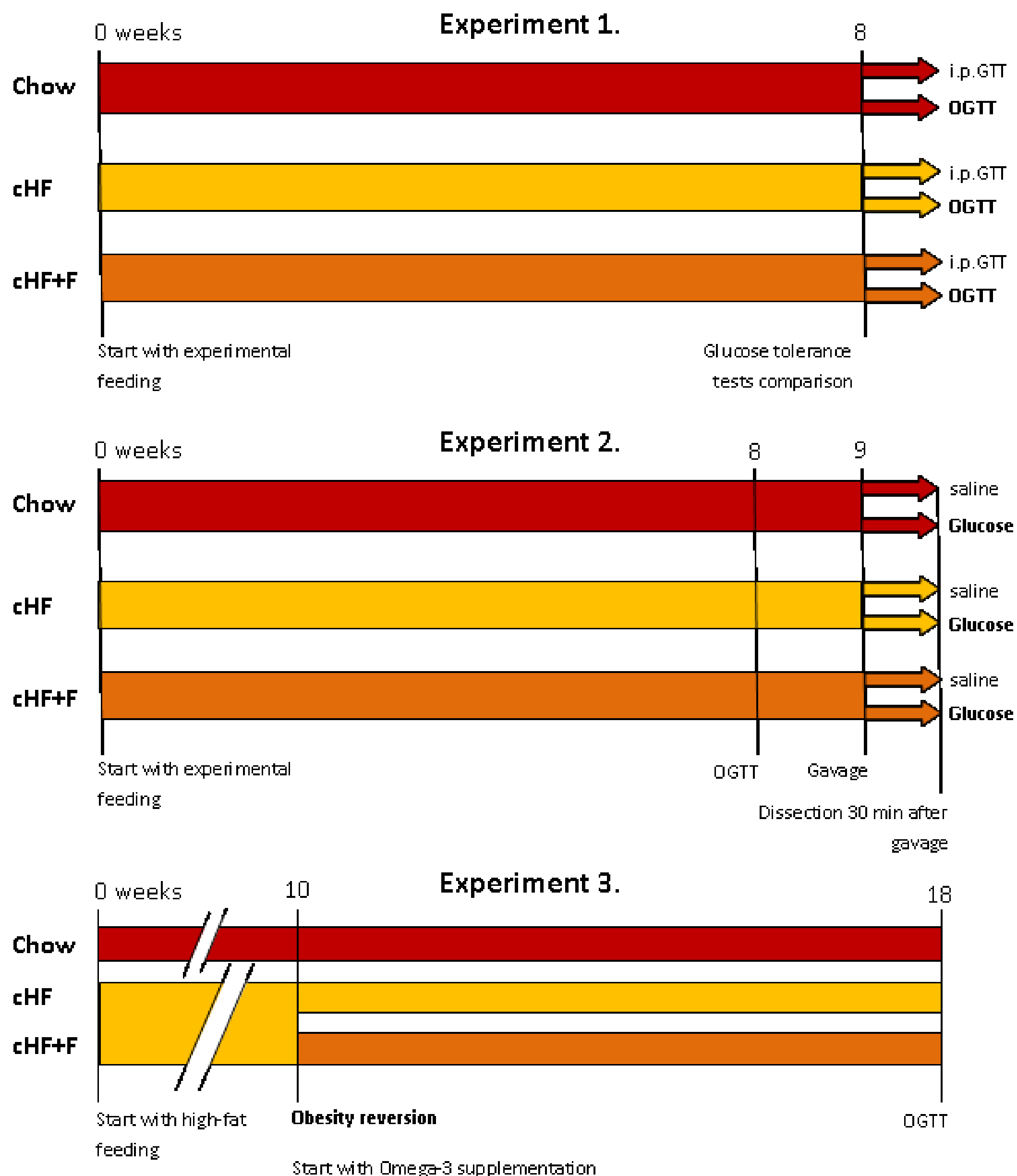


Fig. 17 Publication B - experimental setup

Chow, low-fat standard diet; cHF, corn oil-based high-fat diet; cHF+F, cHF supplemented with Omega-3; i.p. GTT, intraperitoneal glucose tolerance test; OGTT, oral glucose tolerance test.

Experiment 3.

Male C57BL/6J mice were maintained on a 12-h light/dark cycle (light from 6:00 a.m.) at 22°C with ad libitum access to drinking water and the Chow since weaning at the age of 3 weeks. At the age of 12 weeks, mice were sorted into separate cages and randomly divided into two groups, which continued either on the Chow diet (n = 6) or cHF diet (n = 24). After 10 weeks of experimental feeding, obese cHF-fed mice were further divided into two subgroups continuing either on cHF diet (n = 12) or receiving cHF diet supplemented with Omega-3 TAG (cHF+F; n = 12); the Chow-fed mice continued on their respective diet. At week 18, mice were fasted overnight (~14 hours) and subjected to OGTT (**Fig. 17**, experiment 3.).

3.1.4. Publication C - Experimental setup

In publication C, we assessed the effects of Omega-3 PL derived from herring meal on liver fat accumulation (i.e. hepatic steatosis) and tissue lipid metabolism. We conducted 3 separate experiments: (i) to evaluate the effects of Omega-3 PL administered either alone or in combination with rosiglitazone (Experiment 1.), (ii) to partially decipher the contribution of different parts of the PL concentrate to the overall metabolic effect of Omega-3 PL by directly comparing the PC-rich PL concentrates that contain or do not contain Omega-3 (Experiment 2.), and (iii) to assess the effects of Omega-3 PL on fecal lipid excretion (Experiment 3.).

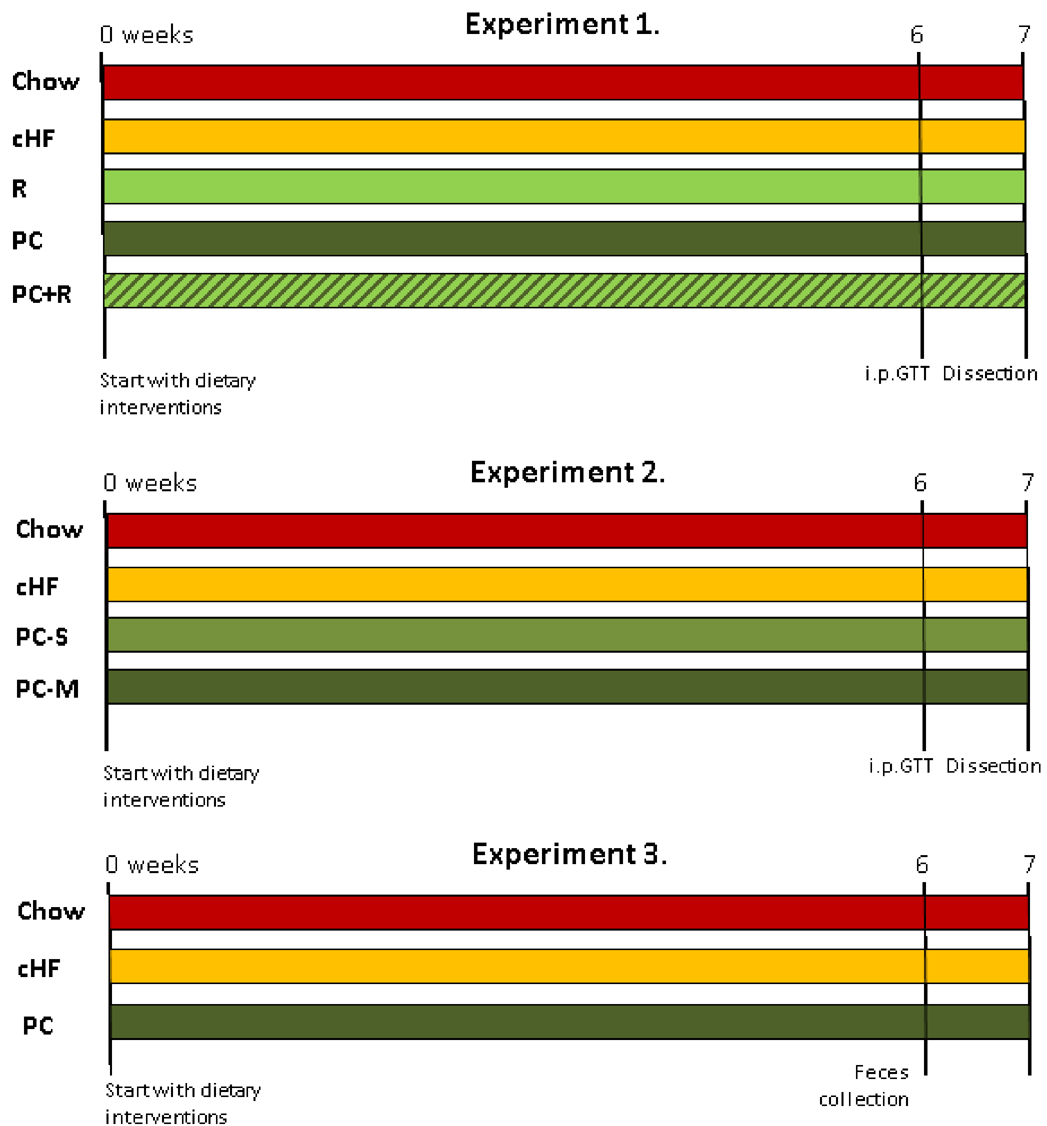


Fig. 18 Publication C - Experimental setup

Chow, low-fat standard diet; cHF, corn oil-based high-fat diet; PC, cHF diet supplemented with Omega-3 PL; R, cHF diet supplemented with rosiglitazone; PC+R, cHF diet supplemented with the combination of Omega-3 PL and rosiglitazone; i.p. GTT, intraperitoneal glucose tolerance test.

Experiment 1.

Twelve-weeks-old mice were randomly divided into 5 experimental groups (n = 8) and continued for 7 weeks on different experimental diets, i.e. Chow, cHF, and cHF-based diets supplemented either with herring-derived PC concentrate (PC), low dose of rosiglitazone (R), or both (PC+R). After 6 weeks of experimental feeding, glucose tolerance was assessed by i.p. GTT. At week 7, mice were killed and dissected as described above (**Fig. 18**, experiment 1.).

Experiment 2.

Experiment 2. was conducted similarly to the Experiment1.; however, only the following experimental diets were involved, i.e. Chow, cHF, and cHF-based diets supplemented with PC-rich concentrate either from herring (PC-M) or from soy (PC-S) (**Fig. 18**, experiment 2.).

Experiment 3.

The last experiment in this study followed the previous experimental setup, and involved the Chow, cHF, and PC group. At week 6 of experimental feeding, feces were collected over a 24-hour period in order to quantify the loss of lipids in stool (**Fig. 18**, experiment 3.).

3.1.5. Publication D - Experimental setup

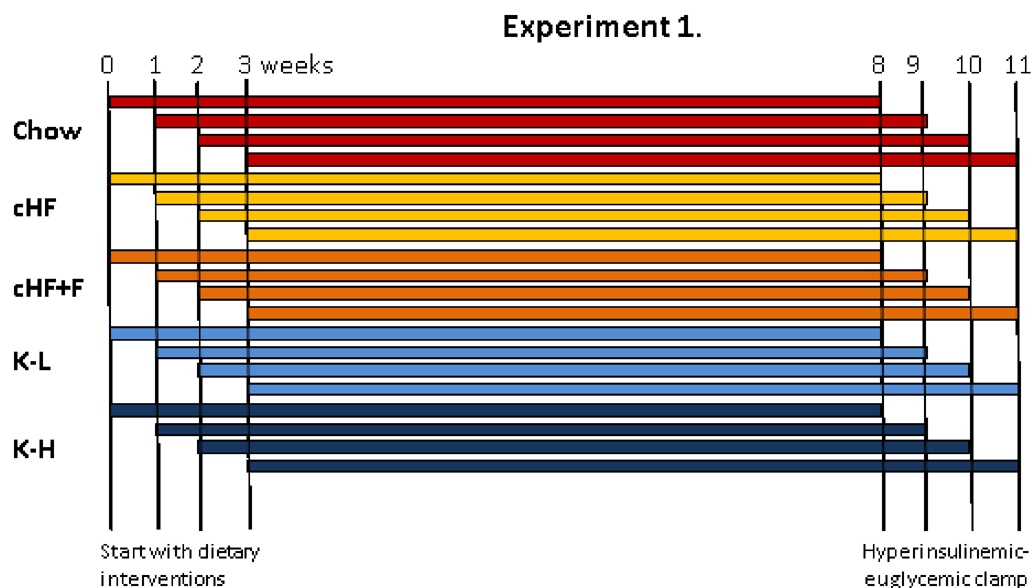


Fig. 19 Publication D - experimental setup
Chow, low-fat standard diet; cHF, corn oil-based high-fat diet; cHF+F, cHF supplemented with Omega-3 TAG; K-L, Krill oil-low dose; K-H, Krill oil-high dose.

Twelve-weeks-old C57BL/6N mice were randomly divided into 5 experimental groups, i.e. the Chow, cHF, cHF+F, K-L, and K-H (n = 16). Because the clamp procedure allows analysis of up to 20 mice per week, the animals were randomized and the experimental feeding was initiated gradually. After 8 weeks of experimental feeding, the level of IR was assessed *in vivo* by hyperinsulinemic-euglycemic clamp technique (see section 3.2.2). After the clamp, the mice were killed and dissected as described above (**Fig. 19**).

3.2. *In vivo* testing

3.2.1. Glucose tolerance tests

Fasted mice (the length of fasting is specified within each experiment) were given a single dose of glucose either by oral gavage (OGTT; 500 µl of 30% D-glucose, 150 mg/animal) or intraperitoneal injection (i.p. GTT; 10 µl of 10% D-glucose, i.e. 1 mg/g body weight). For the analysis of hormone levels in plasma, blood samples were collected through the incision at the tail tip before and 30 min after glucose administration. For the analysis of glucose tolerance, blood glucose levels were measured using a handheld glucometer (Contour™PLUS; Bayer, Germany) before glucose administration to evaluate fasting blood glucose (**FBG**) and 15, 30, 60, 120, and 180 minutes after glucose administration. Glucose tolerance was quantified as the area under the glycemic curve (**AUC**), expressed as either incremental or total (i.e. including the value of FBG) AUC.

3.2.2. Hyperinsulinemic-euglycemic clamp

After 7 weeks of experimental feeding, mice were anesthetized by isoflurane (Forane, AbbVie, Czech Republic) and surgically equipped with a permanent catheter in the jugular vein. After the recovery period (>2 days), during which the animals had an *ad libitum* access to drinking water and their respective experimental diets, mice were fasted for 6 hours and then connected to an infusion device. During the infusion, mice were conscious and freely moving. Blood samples (5µl) were collected from the tail tip incision and mixed with 125 µl 2N $ZnSO_4$ (MilliporeSigma). After that, mixed infusion of insulin (HumulinR, Eli Lilly, USA) and radioactive D-[3-^{3}H]glucose (Perkin Elmer, USA) was started, with a constant rate of 4.8 mU/min per kg body weight and 0.26 µCi/min in case of insulin and radioactive glucose, respectively. Throughout the infusion, glucose concentration was monitored in blood samples collected from the tail, and euglycemia (~5.5 mmol/l) was maintained by periodically adjusting a variable infusion of unlabeled D-glucose solution at a concentration of 30% for lean animals and 15% for their obese counterparts. After reaching a stable blood glucose level (i.e. after ~80-140 min of infusion), blood samples (5 µl) were collected from the tail at 10 min intervals for one hour and mixed with 125 µl 2N $ZnSO_4$ (MilliporeSigma). At the end of the clamp, mice were killed and dissected as described above, while specific measures were taken due to tissue radioactivity.

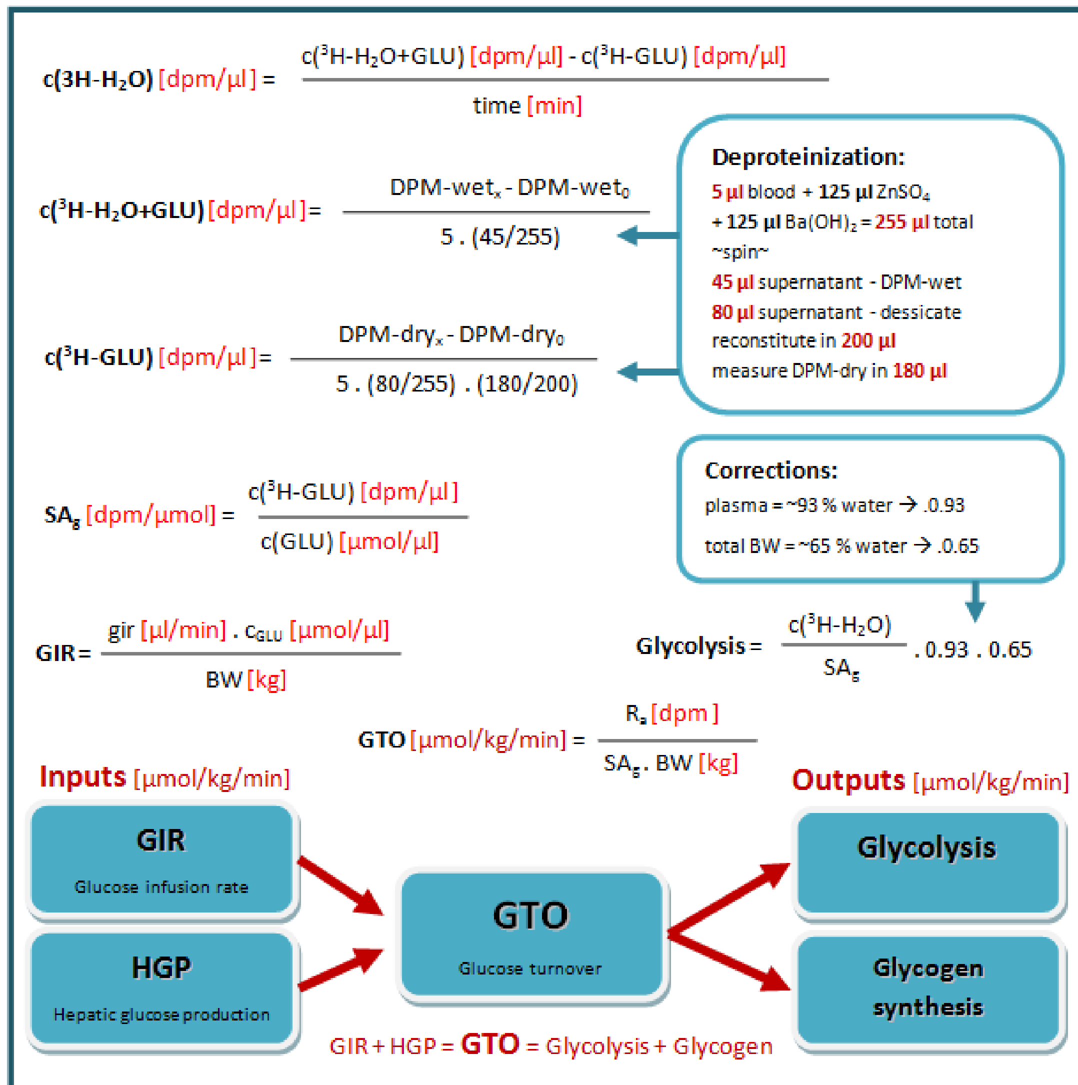


Fig. 20 The procedure used to calculate various parameters linked to insulin sensitivity and glucose metabolism, which is based on the data obtained by hyperinsulinemic-euglycemic clamps

Glucose turnover (GTO) presents a sum of glucose inputs, i.e. exogenous glucose (GIR) and glucose produced from endogenous sources, mainly from the liver (HGP), as well as a sum of glucose metabolized via different pathways, e.g. glycolysis and glycogen synthesis. GIR, glucose infusion rate; HGP, hepatic glucose production; GTO, glucose turnover; R_a, total radioactivity in the infusion; SA_g, specific activity of glucose; BW, body weight; c_{glu}, glucose concentration in the infusion; c(^{3}H-GLU), plasma concentration of ^{3}H-labeled glucose; c(^{3}H-H_2O+GLU), plasma concentration of ^{3}H-labeled glucose and water; c(^{3}H-H_2O), plasma concentration of ^{3}H-labeled water; dpm, dose per minute; DPM-wet$_x$, total radioactivity in plasma at the time point x; DPM-wet$_0$, total radioactivity in plasma at the time point 0´; DPM-dry$_x$, activity of ^{3}H-labeled glucose in plasma at the time point x; DPM-dry$_0$, activity of ^{3}H-labeled glucose in plasma at the time point 0´.

The blood-$ZnSO_4$ mixtures (see above) were mixed with 125 µl of 2N $Ba(OH)_2$ (MilliporeSigma), centrifuged at 5000 x g, 4°C, for 10 min, and the resulting supernatant that was free of proteins and blood cells and contained only freely soluble glucose was stored at -80 °C. In deproteinized plasma samples, the concentration of total glucose, as well as the activities associated either with radioactive glucose or radioactive water were then determined as follows: the total glucose concentration was determined spectrophotometrically using the enzymatic kit GLU 500 (Erba Lachema, Czech Republic);

Beta counter (Hewlet-Packard, USA) was used to assess the total activity of the sample as well as the activity originating only from D-[3-^{3}H]glucose, the latter being assessed in 80 µl-aliquot of the original supernatant that was dried and reconstituted in distilled water (i.e. "dry activity"). Subsequently, the activity of ^{3}H-water, i.e. the glycolytic product of D-[3-^{3}H]glucose metabolism, was calculated as the difference between total activity and "dry activity". Total glucose turnover as well as the other parameters linked to insulin sensitivity and glucose metabolism, i.e. glucose infusion rate (**GIR**), hepatic glucose production (**HGP**; mainly linked to insulin sensitivity of the liver and associated with the process of gluconeogenesis), and the rate of glycolysis and whole-body glycogen synthesis were calculated according to the formulas shown in **Fig. 20**.

3.3. Biochemical analyses and *ex vivo* measurements

3.3.1. Plasma metabolites and hormones

Plasma levels of various lipid metabolites and hormones were quantified using mainly commercially available kits (summarized in **Tab. 3**). In Publication A, total adiponectin was analyzed by ELISA, while a more detailed distribution of adiponectin multimeric complexes shown in Publication C was analyzed by Western blotting (for details on the procedure see ref. Kuda et al., Diabetologia 2009[198]).

Tab. 3 Overview of commercial kits used for the quantification of hormones and metabolites in mouse plasma

Analyte	Kit	Manufacturer	Publication
Lipid metabolites			
Total glycerolipids (TAG)	Bio-La-Test TG L 250S	Erba-Lachema, Czech Republic	A, C, D
Total cholesterol (CHOL)	Bio-La-Test CHOL L 250S	Erba-Lachema, Czech Republic	A, C, D
HDL-cholesterol	BIO-La-Test HDL Cholesterol Direct Liquid	Erba-Lachema, Czech Republic	C
Non-esterified fatty acids	Wako NEFA-HR	Wako Chemicals USA, USA	A, C, D
Total bile acids	Mouse Total Bile Acids Kit	Crystal Chem, USA	C
Hormones			
Total adiponectin	Mouse Adiponectin ELISA kit	MilliporeSigma, USA	A, C
Insulin	Sensitive rat insulin RIA kit	MilliporeSigma, USA	A, C, D
Insulin	MILLIPLEX MAP Mouse Gut Hormone Panel	MilliporeSigma, USA	B
Total GIP	MILLIPLEX MAP Mouse Gut Hormone Panel	MilliporeSigma, USA	B
Active GLP-1	MILLIPLEX MAP Mouse Gut Hormone Panel	MilliporeSigma, USA	B
Total GLP-1	Total GLP-1 ELISA	Alpco, USA	B
Other metabolites			
Glucose	Bio-La-Test GLU 500	Erba-Lachema, Czech Republic	A

In publication B, plasma levels of insulin, GLP-1 (active), and GIP (total) were assessed by multiplex magnetic-beads assay MILLIPLEX MAP Mouse Gut Hormone Panel (MilliporeSigma, St. Louis, MO, USA). The analysis was performed by Annelies Bunschoten at the Department of Human and Animal Physiology (Wageningen University, Netherlands).

The homeostasis model assessment was applied to quantify the level of IR (i.e. the **HOMA-IR** index) using the following formula: fasting plasma insulin (mU/l) x fasting plasma glucose (mmol/l)/22.5.

3.3.2. Tissue lipid content and the analysis of fatty acids composition

Concentration of glycerolipids in the liver was determined spectrophotometrically following a hydrolysis of tissue lipids by KOH. Pre-weighed liver samples (~50 mg) were digested in 150 µl of 3M KOH (dissolved in 65% ethanol) at 70 °C for 2 hours. The resulting lysates were cleared of debris by a brief centrifugation at low g and the amount of released glycerol was analyzed by the enzymatic kit Bio-La-test TG L 250S (see above). In publication A, Experiment 2, the total lipids were isolated from the liver using the Folch´s method[262], and subsequently quantified by gravimetry.

To estimate the bile acids (**BA**) content in the liver, pre-weighed liver samples (~100 mg) were homogenized in 1 ml of 75% ethanol. The resulting homogenates were then incubated at 50 °C for 2 hours, cleared of debris by centrifugation at 6000 x g, 4°C for 10 min, and analyzed for the bile acid content by the Mouse Total Bile Acids Kit (see above).

The cholesterol content in the liver and feces was analyzed after the lipid extraction using the modified Folch's method; pre-weighed samples (~100 mg) were homogenized in 400 µl of distilled water. The lipid fraction was obtained by mixing the homogenate with 6 ml of the chloroform/methanol/3% KH_2PO_4 (2:1:1.2) mixture, followed by overnight incubation at 4°C, while stirring gently on a rotating mixer. One ml of the bottom chloroform phase was dried under the nitrogen stream and re-dissolved in 100 µl of absolute isopropanol. Total cholesterol content in the samples was then analyzed by the Bio-La-Test CHOL L 250S enzymatic kit (see above). Total lipids in feces were isolated by the Folch´s method and quantified by gravimetry.

The analysis of FA composition in the experimental diets and plasma/tissues was conducted by E. Tvrzicka and her colleagues at the 4th Department of Internal Medicine, 1st Faculty of Medicine, Charles University in Prague, using gas chromatography technique. Before the analysis, total lipids were extracted by the Folch´s method; in case of the liver, TAG and PL lipid fractions were further separated by thin-layer chromatography.

3.3.3. Gene expression analysis

Aliquots of the liver were stored in RNAlater solution (Qiagen, Germany), while WAT samples were snap-frozen in liquid nitrogen; the samples were then stored in -80 °C. Total RNA was isolated using a TRIzol reagent (MilliporeSigma) according to the manufacturer´s instructions. Briefly, the samples were homogenized in TRIzol and cleared of debris by centrifugation at 12 000 x g, 4°C, for 10 min. Following the addition of chloroform into TRIzol and subsequent centrifugation at 14 000 x g, 4°C, for 15 min, the organic phase containing mainly proteins and the aqueous phase containing RNA were separated. The upper aqueous phase was collected and mixed with absolute isopropanol in order to precipitate RNA. After further centrifugation at 12 000 x g, 4°C, for 10 min, the pellets containing RNA were rinsed twice with 75 % ethanol, dried, and reconstituted in sterile deionized water. The concentration of isolated RNA was determined by NanoDrop (Thermo Scientific, USA), and its purity was assessed as the ratio of the absorbances measured at 260 nm and 280 nm.

Reverse transcription of total RNA into cDNA was done by using Oligo thymidine (OligoT) primers (Generi Biotech, Czech Republic) and the M-MLV reverse transcriptase kit (Invitrogen, USA) according to the manufacturer´s instructions. Briefly, 1 µg of RNA was mixed with OligoT. After a short denaturation phase (i.e. 65 °C, 5 min), reverse transcriptase was added into the reaction and transcription proceeded at 37°C for 50 min. Reaction was terminated by denaturing the enzyme at 70 °C for 15 min and final cooling of the reaction to 4 °C.

Relative amounts of the individual transcripts were quantified by quantitative RT-PCR (**qPCR**) using the LightCycler 480 II instrument (Roche Diagnostics, Germany) and the LightCycler 480 SYBR Green I Master kit (Roche Diagnostics, Germany). The conditions of the PCR reaction were set as follows: initial denaturation (95 °C, 6 min); 45 cycles of denaturation (95 °C, 10 sec), primer annealing (55 – 60 °C depending on the primer used, 10 sec), and elongation (72 °C, 20 sec). The specificity of the amplified PCR products was determined by a melting curve analysis at the end of each PCR. Results were calculated as a relative change in gene expression as compared to the internal standard with unknown concentration, and also normalized using a geometric mean of the expression of several housekeeping genes.

Oligonucleotide primers were designed using the Lasergene Software (DNASTAR, USA). Primer sequences are listed in **Tab. 4**.

Tab. 4 Primer sequences

Abbreviation	Gene ID	Forward sequence (5´ → 3´)	Reverse sequence (3´ → 5´)
Abcg5	27409	GACCGCGTGGGGCTGCTCTA	CTGAAAATGACCGTGGCGATGAC
Acaca	107476	CAGATCCAGGCCATGTTGAGACG	TCGCTGGGTGGGTGAGATGTG
Acc2	100705	TGCGCTCCACCATCCGTGAAAACAT	TTCCCAAAATAAGCCCGTGTCC
Acly	104112	TGTGCTCGGGCTGGGAAGGAC	GTGGCGGGGAAGTGCTGTTTGA
Acot1	26897	AGCGCTGGCATGCACCTCCTG	TTCCCCAACCTCCAAACCATCATA
Acox1	11430	GCTGGGCTGAAGGCTTTTACTACC	CACCTGCTGCGGCTGGATAC
Adgre1	13733	CTCGCCTGCTTCTTCTGGATGC	TAAACCCCGTCTCTGTATTCAACC
Ccl2	20296	GTTAACGCCCCACTCAC	GGTTCCGATCCAGGTTT
Cyp7a1	13122	GTAGAGGCTGGAGGTGATGTTGAGT	GGGTAAATGCCAGGAGGATGTG
Eef2	13629	GAAACGCGCAGATGTCCAAAAGTC	GCCGGGCTGCAAGTCTAAGG
Ehhadh	74177	CCCCAATTGCTGATATGCTCTGTG	ATGCGGAATGCCTCGTTGATAAG
Ef1a	13627	TGA CAG CAA AAA CGA CCC ACC AAT	GGG CCA TCT TCC AGC TTC TTA CCA
Elovl5	68801	CCTCTCGGGTGGCTGTTCTTCC	AGGCTTCGGCTCGGCTTGTC
Fasn	14104	GGC TGC CTC CGT GGA CCT TAT C	GTC TAG CCC TCC CGT ACA CTC ACT CGT
Fdps	110196	ATGCCATCAACGACGCTCTGCT	TGGCCCTGGGGTGCTGTCA
Gipr	381853	GCCCCTGCGGTTGCTGCTTCTG	GGTGTTGGCGGCCGTGTAGTTCC
Hmgcs1	208715	GAGGCCTTCAGGGGTCTAAA	GGGAGGCCTTGGTCTTCTG
Lcad	11363	TGGCATCAACATCGCAGAGAAACA	ACCGATACACTTGCCCGCCGTCAT
Mcad	11364	TCGCCCCGGAATATGACAAAA	GCCAAGGCCACCGCAACT
Nos2	18126	CTTTGCCACGGACGAGACGGATAGG	CGGGCACATGCAAGGAAGGGAACT
Nr0b2	23957	AGCGCTGCCTGGAGTCTTTCTGG	AGGCCTGGCACATCTGGGTTGA
Nr1h4	20186	CTTCGTTCGGCGGAGATTT	ACAGAGGAGCGGGGTGAACT
Ppib	19035	ACTACGGGCCTGGCTGGGTGAG	TGCCGGAGTCGACAATGATGA
Progip	14607	TCAGGGAAAGGAGGACAAAGAGG	GACCAGGGCGGAAGCAGGAG
Scarb1	20778	ATGGGCCAGCGTGCTTTTATGAAC	ACGCCCGTGAAGACAGTGAAGACC
Scd1	20249	ACTGGGGCTGCTAATCTCTGGGTGTA	TAACAAACCCACCCAGAGATAAAGCC
Sqle	20775	GCTTTCTGTATTTTAAACTTGGTGGAGAG	AGTGGAAATAGGATAGAACACGCTTTG
Srebf1	20787	TACCCGTCCGTGTCCCCCTTTTC	TGCGCTTCTCACCACGGCTCTG
Srebf2	20788	GCTGTGCGCTCTCGTTTTACTGAA	GTGCCGCTGACGTTGAGACTGCT

3.3.4. Determination of the DPP-4 enzyme activity

The activity of DPP-4 activity was assessed *ex vivo* in samples of EDTA-plasma as well as in tissue homogenates. Tissue samples (~100 mg) were homogenized in 300 µl of the PBS/Triton-X100 (100:1) mixture containing Aprotinin (100 KIU/ml; MilliporeSigma). The homogenate was cleared of debris by centrifugation at 100 x g, 4°C, for 10 min, and the resulting supernatant was further purified by double centrifugation at 20 000 x g, 4°C, for 10 min. Clear supernatant or EDTA-plasma samples (15 µl) were mixed with 35 µl of Tris-buffer and 50 µl of DPP-4 substrate solution. The DPP-4 substrate solution was prepared by mixing 10 mM DPP-4 substrate (i.e. H-Gly-Pro-pNA • p-tosylate; Bachem AG, Switzerland) dissolved in DMSO with a Tris-buffer in a ratio 1:50. All reagents were heated

to 37°C prior mixing. The kinetics of DPP-4 substrate degradation was then measured by spectrophotometry (n = 405 nm; 37 °C; 30 min) and quantified as the slope of the linear segment of the kinetics curve normalized to tissue sample weight.

3.4. Light microscopy and immunohistochemical analysis

All immunohistochemical analyses were performed by K. Bardova (Department of Adipose Tissue Biology, Institute of Physiology CAS, Prague, Czech Republic). Briefly, samples of epididymal WAT (**eWAT**), liver, and pancreas were fixed in 4 % formaldehyde and subsequently embedded in paraffin. Five µm-thin sections were stained by hematoxylin and eosin in order to visualize cell nuclei and cytoplasm, respectively. In eWAT, a marker of activated macrophages MAC-2/galectin-3 was visualized by using specific antibodies (Cedarlane Laboratories, USA) in order to quantify a relative density of CLS, i.e. the aggregates of macrophages surrounding dead adipocytes in inflamed WAT. In the pancreas, GIPR in the islets of Langerhans was quantified with a fluorescent dye-labeled antibody against GIPR (Novus Biologicals, USA), while co-staining with a fluorescent antibody against insulin (MilliporeSigma). Digital images were captured using Olympus AX70 light microscope and a DP 70 camera (Olympus, Japan). Adipocyte morphometry was performed using a NIS Elements v3.0 (Laboratory Imaging, Czech Republic).

3.5. Statistics

Data are presented as means ± SEM. The comparisons were judged to be significant at $p \leq 0.05$. All statistical evaluations were performed using the SigmaStat 3.5 Software. For the particular statistical tests used in different studies, see below.

Publication A

The effects of obesogenic high-fat diets in mice fed the cHF and LHF diet were compared to those observed in mice fed the Chow, while the effects of EPA+DHA supplemented diets (i.e. cHF+F and LHF+F) were compared to those induced by their parental diets (i.e. cHF and LHF, respectively). Statistical significance was determined using the Student´s t-test or Mann-Whitney test for the data with and without normal distribution, respectively. To compare all the parental high-fat diets together with their EPA/DHA-supplemented versions, two-way ANOVA followed by the Holm-Sidak method for pair-wise multiple comparisons was used.

Publication B

The effects of obesogenic high-fat diet (i.e. cHF) were compared to those of the Chow, while the effects of Omega-3-supplemented diet (i.e. cHF+F) were compared to those induced by its parental cHF diet or the Chow; statistical significance was determined using the Student´s t-test or Mann-Whitney test for the data with and without normal distribution, respectively. Differences in the gene expression of *Gip* between different segments of the gut were evaluated by the Duncan´s modification of two-way ANOVA with the data being expressed as the fold-change relative to mice fed the Chow.

Publication C + D

The effects of the obesogenic cHF diet relative to those observed in mice fed the Chow were evaluated by the Student´s t-test or Mann-Whitney test for the data with and without normal distribution, respectively. The effects of Omega-3 supplementation were determined by one-way ANOVA followed by the Holm-Sidak post-hoc test, similarly to Publication A. In Publication D, Dunn´s modification of one-way ANOVA was applied due to the variation in the size of the groups.

4. Results

4.1. Publication A (*published*). Corn oil versus lard: metabolic effects of omega-3 fatty acids in mice fed obesogenic diets with different fatty acid composition.

4.1.1. Obesogenic diets based on either SFA or Omega-6 are distinguished by their impact on hepatic steatosis, but not by the induction of obesity and IR.

In order to evaluate the ability of two different obesogenic diets to induce obesity and IR, a long-term (8 weeks) dietary intervention was conducted, and changes in insulin sensitivity were analyzed at the end of experiment using the hyperinsulinemic-euglycemic clamp (see section 3.2.2.).

As shown in Fig. 21a, both the cHF and LHF diet induced a significant but comparable weight gain, even though the energy intake in LHF mice was decreased by 8 % as compared to cHF mice (Fig. 21b). Both obesogenic diets also affected various aspects of glucose homeostasis and induced IR, as documented by the results of hyperinsulinemic-euglycemic clamps (Fig. 21c); thus, GIR needed to maintain euglycemia was reduced by ~60 % in mice fed either high-fat diet. Whole-body GTO, representing the sum of glucose producing as well as glucose consuming processes, was reduced by ~40 % in response to high-fat feeding, while HGP increased ~3-fold in both the cHF and LHF group. Whole-body glycogen synthesis was decreased up to 25 % in both groups of obese mice as compared to lean controls fed the Chow, while the rate of whole-body glycolysis was unaffected by high-fat feeding (data not shown). Taken together, these data show a significant impairment of insulin sensitivity and glucose metabolism in both groups of obese mice fed high-fat diets, in which reduced glucose clearance from the blood stream, impaired regulation of gluconeogenesis, and decreased insulin-stimulated glycogen synthesis were observed; however, the deleterious effects of cHF and LHF diets on glucose metabolism were fully comparable. In contrast, the total lipid content in the liver increased significantly only in the LHF group as compared to mice fed the Chow (see Fig. 21d and the section 4.1.3. below).

4.1.2. The beneficial effects of Omega-3 supplementation on adiposity and glucose homeostasis are influenced by the type of FA in the background diet.

While consumption of the two obesogenic diets differing in macronutrient and in particular lipid composition led to comparable deleterious effects on glucose homeostasis of obese mice (see above), the lipid background could still influence the effects of dietary Omega-3 supplementation on the organism. Thus, another long-term dietary intervention was conducted in order to assess metabolic effects of Omega-3 administered for 6 weeks following a 2-weeks-adaptation period, during which the mice were fed either the cHF or LHF diet.

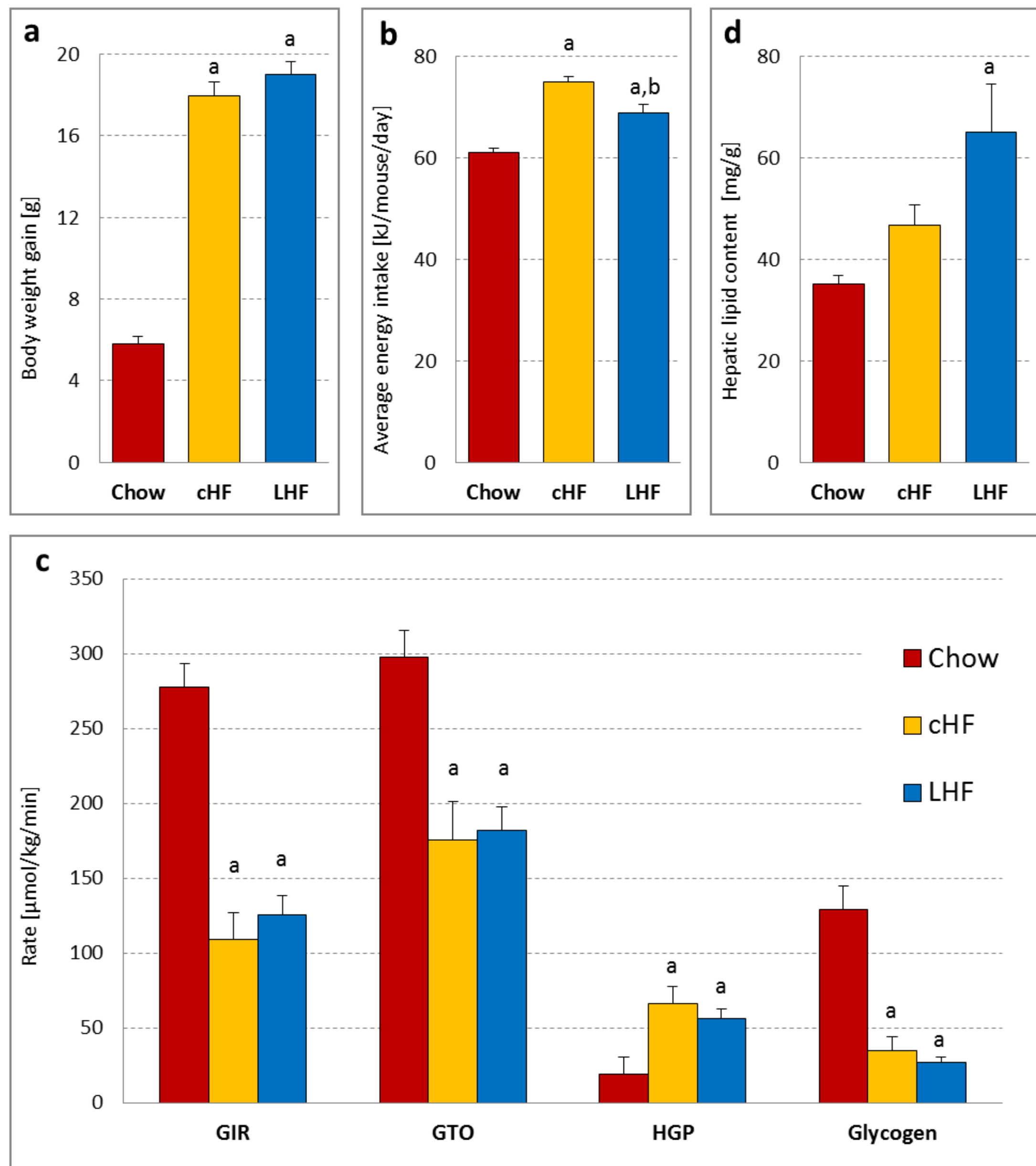


Fig. 21 Pub. A, Exp. 1. Body weight gain, energy intake, hepatic lipids, and the parameters of insulin sensitivity and glucose metabolism in mice fed different obesogenic high-fat diets.

The effects of 8-weeks-long dietary interventions with either the cHF and LHF diet on body weight gain (A), energy intake (B), various parameters of insulin sensitivity and glucose metabolism assessed by hyperinsulinemic-euglycemic clamps (C), and lipid accumulation in the livers of clamped mice (D). Data are means ± SEM, n = 11 – 12, except Chow, where n = 8. [a]$p \leq 0.05$ cHF or LHF vs. Chow; [b]$p \leq 0.05$ LHF vs. cHF (t-test). GIR, glucose infusion rate; GTO, glucose turnover; HGP, hepatic glucose production; Glycogen, whole-body glycogen synthesis.

Tab. 5 Overview of the effects of two different high-fat diets with and without the supplementation with Omega-3 on body mass, adiposity, glucose homeostasis, and plasma levels of selected metabolites and hormones

	cHF	cHF+F	LHF	LHF+F	Chow
Energy balance					
Body weight – *initial* (g)	23.3 ± 0.2	23.4 ± 0.3	23.2 ± 0.2	23.4 ± 0.2	23.4 ± 0.3
Body weight – *final* (g)	43.2 ± 1.2 [a]	42.0 ± 1.0	44.4 ± 1.5 [a]	45.5 ± 0.7 [d]	30.7 ± 0.5
Weight gain (g)	18.2 ± 1.2 [a]	16.8 ± 0.9	19.5 ± 1.4 [a]	20.4 ± 0.7 [d]	5.9 ± 0.4
Energy intake (kJ/mouse)	77.2 ± 1.9 [a]	73.2 ± 1.6	76.7 ± 2.7	85.9 ± 1.6 [b,d]	68.1 ± 0.7
WAT					
eWAT (g)	2.56 ± 0.11 [a]	2.02 ± 0.11 [b]	2.53 ± 0.13 [a]	2.32 ± 0.07	0.54 ± 0.04
Subcutaneous WAT (g)	0.83 ± 0.04 [a]	0.77 ± 0.05	0.94 ± 0.06 [a]	1.00 ± 0.05 [d]	0.24 ± 0.01
Mesenteric WAT (g)	1.18 ± 0.15 [a]	1.11 ± 0.12	1.20 ± 0.18 [a]	1.28 ± 0.07	0.28 ± 0.01
Adiposity index (%)	10.5 ± 0.4 [a]	9.3 ± 0.5 [b]	10.4 ± 0.4 [a]	10.1 ± 0.3	3.4 ± 0.1
Glucose homeostasis					
FBG (mmol/l)	8.7 ± 0.4 [a]	8.5 ± 0.4	9.0 ± 0.6 [a]	10.3 ± 0.4 [b,d]	5.8 ± 0.7
Incremental AUC (mol x 180 min)	1.90 ± 0.21 [a]	1.73 ± 0.19	1.79 ± 0.25 [a]	1.69 ± 0.22	0.91 ± 0.06
Total AUC (mol x 180 min)	3.49 ± 0.24 [a]	3.28 ± 0.22	3.42 ± 0.32 [a]	3.55 ± 0.26	1.94 ± 0.15
HOMA-IR	15.7 ± 1.6 [a]	11.6 ± 1.6	15.4 ± 2.5 [a]	17.9 ± 1.5 [d]	2.8 ± 1.0
Metabolites and hormones					
TAG (mmol/l)	0.75 ± 0.05 [a]	0.61 ± 0.03 [b]	0.60 ± 0.03 [c]	0.46 ± 0.02 [b,d]	0.58 ± 0.05
NEFA (mmol/l)	0.72 ± 0.03	0.63 ± 0.04 [b]	0.67 ± 0.04 [a]	0.59 ± 0.01	0.83 ± 0.05
Cholesterol (mmol/l)	4.04 ± 0.13 [a]	3.16 ± 0.18 [b]	4.48 ± 0.22 [a]	3.75 ± 0.14 [b,d]	2.11 ± 0.05
Insulin (pmol/l)	318 ± 24 [a]	241 ± 29 [b]	297 ± 35 [a]	313 ± 19	79 ± 15
Adiponectin (total; µg/ml)	8.87 ± 0.54	11.88 ± 0.77 [b]	11.03 ± 0.77 [a]	14.7 ± 1.49 [b,d]	8.80 ± 0.25

Publication A, Experiment 2. Data are means ± SEM, *n* = 11 – 12, except Chow, where *n* = 8. [a]$p \leq 0.05$ for difference from Chow; [b,c,d]$p \leq 0.05$ for difference from parental diets (either cHF or LHF), cHF diet, and cHF+F diet, respectively (two-way ANOVA). Adiposity index (%) was calculated as the sum of weights of the subcutaneous, mesenteric, and eWAT fat depots, divided by body weight.

As shown in **Tab. 5**, metabolic effects of the parental high-fat diets, i.e. the cHF and LHF diet, were quite similar. As compared with lean Chow-fed controls, both the cHF and LHF diet induced significant but comparable increases in body weight, energy intake, and adiposity, as well as an impairment of glucose homeostasis, demonstrated as increased FBG, HOMA-IR, and plasma insulin levels in the fasting state. On the contrary, metabolic effects of Omega-3 depended on the type of high-fat diet, in which they were supplemented to the organism. Thus, in the cHF+F group, energy intake and body weight tended to decrease, while the amount of body fat expressed as the adiposity index decreased significantly as compared to cHF, mostly because of the preferential impact of Omega-3 administration on the weight of eWAT. No such effects were seen in case of the LHF diet, where Omega-3 supplementation was associated with an increase in energy intake by ~12 % and a tendency for increased body weight in the LHF+F mice (**Tab. 5**).

A similar pattern of changes induced by the Omega-3-supplemented cHF+F and LHF+F diets could be observed with regard to various parameters of glucose homeostasis. For instance, on the cHF diet, Omega-3 supplementation decreased plasma insulin levels, while these remained unchanged and FBG even increased in LHF+F mice, which also resulted in a marked increase in HOMA-IR index (**Tab. 5**).

Taken together, while the Omega-3 supplementation on the cHF background (i.e. the cHF+F diet) improved a number of parameters such as adiposity and plasma insulin levels, and, in general, tended to improve glucose homeostasis, Omega-3 supplementation on the SFA-rich dietary background (i.e. the LHF+F diet) worsened the degree of IR as compared to its parental LHF diet and did not significantly reduce adiposity. Of note, the specific effects of the cHF+F and LHF+F diet on glucose homeostasis did not correspond to changes in plasma levels of insulin-sensitizing hormone adiponectin, since Omega-3 supplementation led to an increase in plasma adiponectin levels irrespective of the background diet (**Tab. 5**).

In contrast to the effect of Omega-3 on parameters of glucose homeostasis, plasma concentrations of lipid metabolites such as TAG, NEFA and total cholesterol were reduced by Omega-3 supplementation on both types of high-fat diets, i.e. in mice fed the cHF+F and LHF+F diet (**Tab. 5**).

4.1.3. The SFA-rich LHF diet potentiates the development of hepatic steatosis, which is accompanied by the elevated enzyme activity of SCD1.

Regarding the differences in hepatic lipid accumulation previously observed in clamped mice fed either the cHF or LHF diet (see section 4.1.1.), we further evaluated the effects of these two high-fat diets on hepatic lipid metabolism in 6-hour-fasted mice. Compared to Chow-fed mice, both high-fat diets increased the accumulation of lipids in the liver; however, similarly to Experiment 1, this increase was much greater in the LHF than in the cHF group (**Fig. 22a and Fig. 22b**) this was also clearly visible on the histological sections of liver tissue stained with hematoxylin-eosin (**Fig. 23**). On the other hand, Omega-3 supplementation ameliorated hepatic steatosis regardless of the type of lipids in the background diet (**Fig. 22a and Fig. 22b**).

To elucidate possible mechanisms behind the differential effects of the cHF and LHD diet on hepatic lipid accumulation, we analyzed the expression of genes linked to lipid metabolism, namely *de novo* lipogenesis (i.e. *Srebf1, Fasn, Acaca, Acly, Scd1*, and *Elovl5*), β-oxidation of FA (i.e. *Hadhb, Ehhadh, Acot1*, and *Acox1*), VLDL formation (i.e. *Mttp, Apob*), and FA import (i.e. *Cd36*). As shown in **Fig. 22e**, the level of expression of genes associated with the lipogenic pathway was mostly comparable between the cHF and LHF mice (except *Scd1* and *Elovl5*, both significantly higher in LHF mice), as was the expression of genes for β-oxidation of FA (except *Ehhadh* - higher in LHF mice). The expression of genes involved in VLDL formation and FA import was similar between the groups (not shown). However, in case of Omega-3 supplementation either in the form of the cHF+F or LHF+F diet, the expression of lipogenic enzymes tended to decrease, while the expression of genes involved in β-oxidation of FA was significantly increased (**Fig. 22e**).

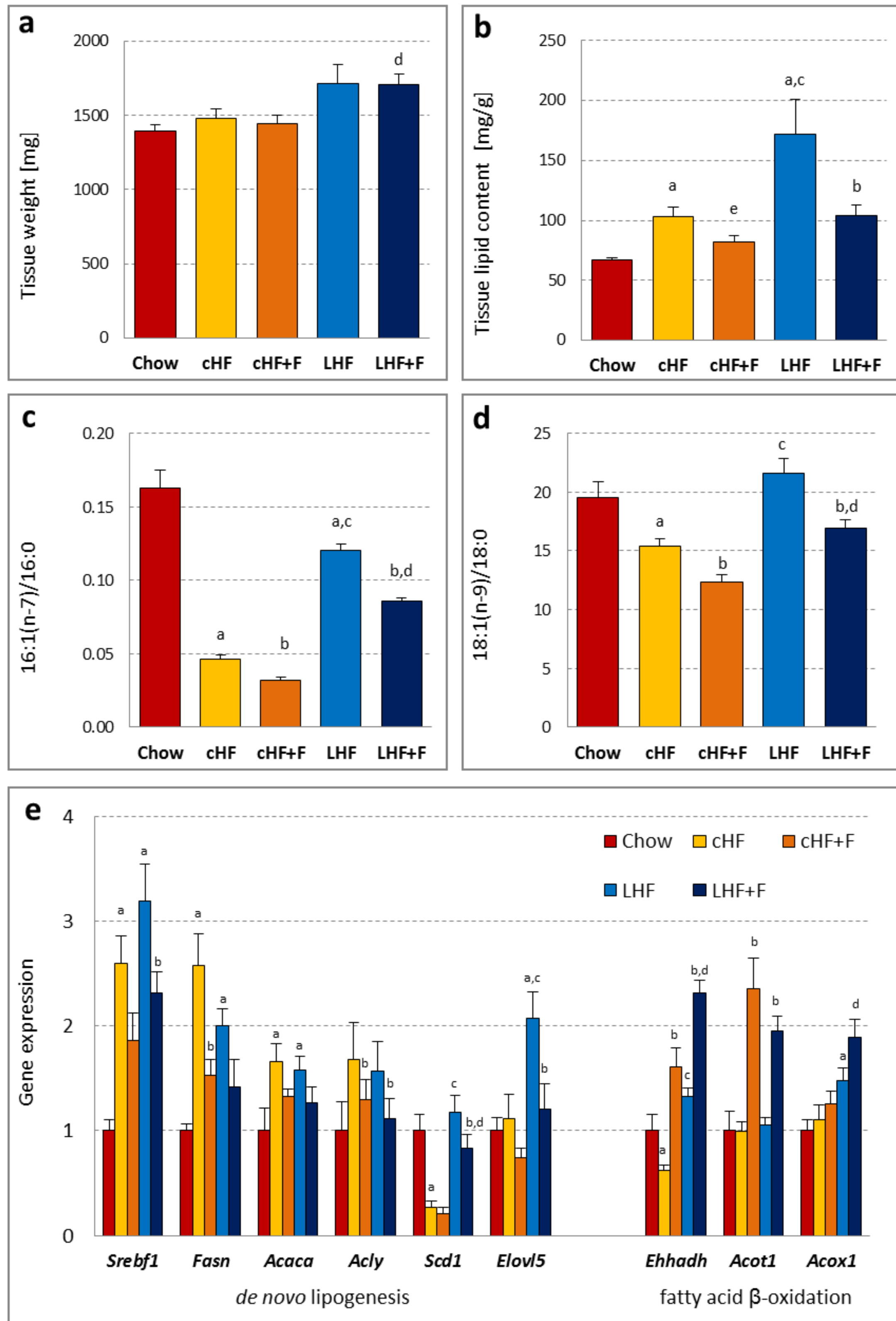


Fig. 22 Pub. A, Exp. 2. Wet weight of the liver, tissue lipid content, SCD1 activity indexes, and the expression of genes involved in lipid metabolism

Wet weight (A), total lipid content (i.e. hepatic steatosis) (B), and the activity of SCD1 based on the measurement of FA composition in the TAG fraction and the calculation of the desaturation indexes, i.e. 16:1(n-7)/16:0 (C) and 18:1(n-9)/18:0 (D). Hepatic gene expression of the key enzymes involved in *de novo* lipogenesis and FA oxidation, expressed as a fold-change relative to Chow-fed mice (E). Data are means ± SEM (*n* = 9-10 except Chow, *n* = 6). $^{a}p\leq0.05$ cHF or LHF vs. Chow; $^{b}p\leq0.05$ cHF or LHF vs. cHF+F or LHF+F, respectively; $^{c}p\leq0.05$ cHF vs. LHF; $^{d}p\leq0.05$ cHF+F vs. LHF+F (two-way ANOVA); $^{e}p\leq0.05$ cHF vs. cHF+F (t-test).

As mentioned above, the only genes, the expression of which corresponded with the observed differences in lipid content in the liver between the cHF and LHF mice, were *Scd1* and *Elovl5*. Both of these enzymes are involved in the transformation of SFA into MUFA and were strongly upregulated in the LHF group; in contrast, Omega-3 supplementation as the LHF+F diet decreased significantly the expression of these genes, while the effect of cHF+F diet was less pronounced. Importantly, the changes in *Scd1* expression corresponded well with changes in the activity of SCD1, based on desaturation indexes C16:1/C16:0 and C18:1/C18:0, derived from the measurement of FA composition in the fraction of hepatic TAG (Fig. 22c and Fig.22d), as well as in hepatic PL (data not shown).

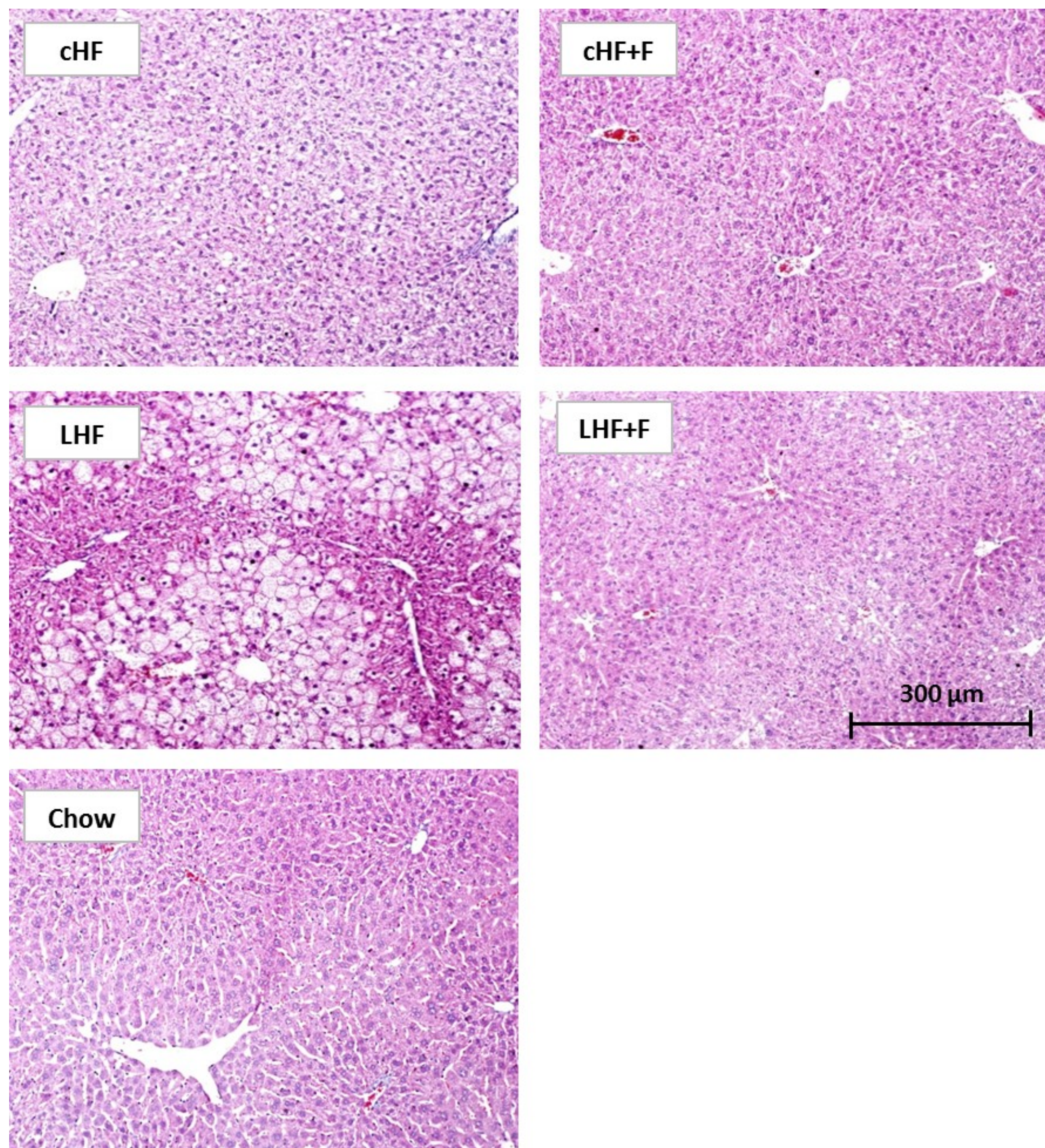


Fig. 23 Pub. A, Exp. 2. Histological sections of the liver stained with hematoxylin-eosin
Representative histological sections of the liver stained with hematoxylin-eosin. Scale bar = 300 µm.

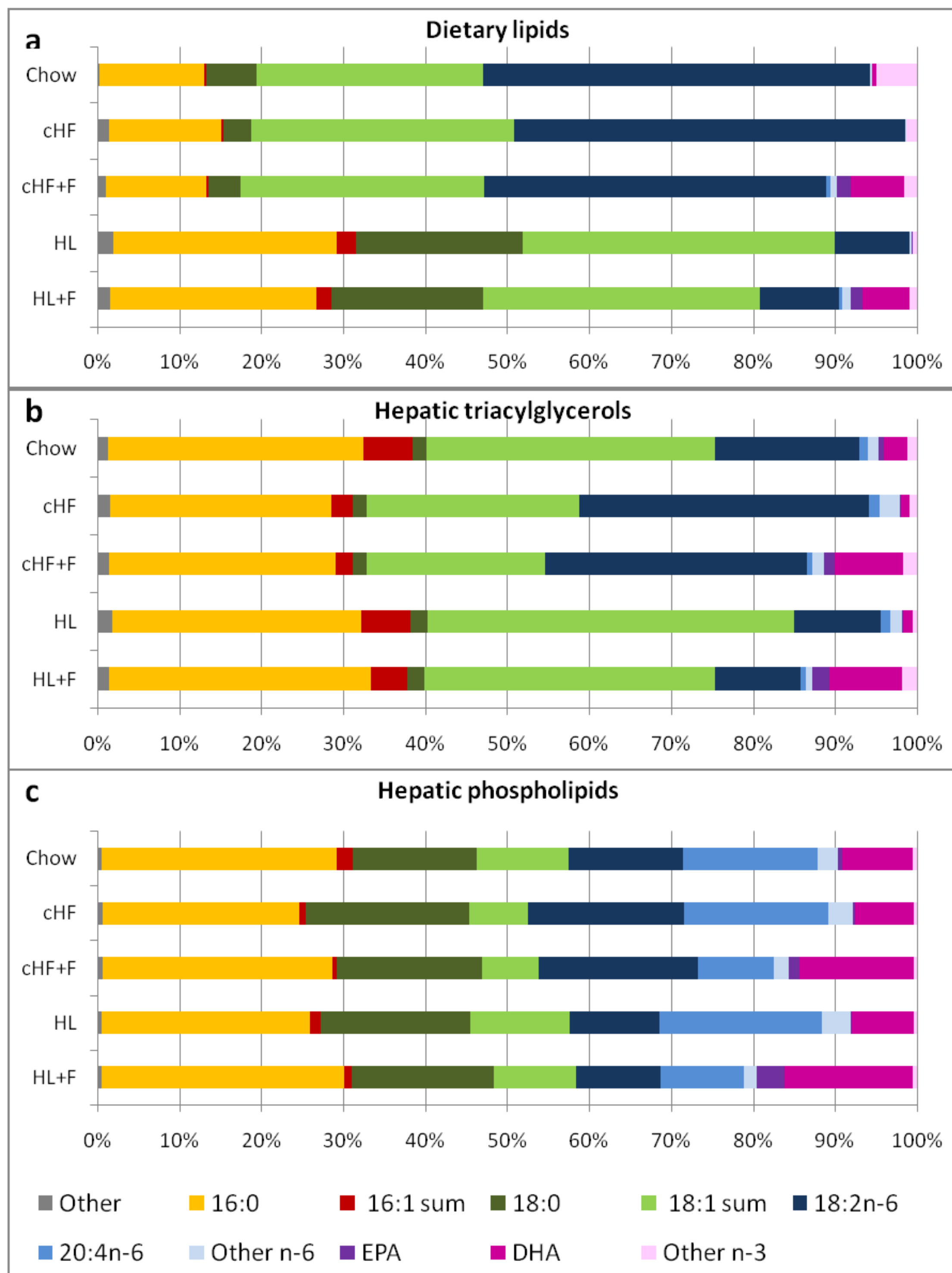


Fig. 24 Pub. A, Exp. 2. Graphical representation of FA composition in the diets and major lipid fractions in the liver
The relative content of individual FA was analyzed by gas chromatography in the standard low-fat Chow as well as in the parental high-fat diets cHF and LHF, and in their Omega-3-supplemented variants cHF+F and LHF+F (a); and in the liver lipid fractions including the TAG (b) and the PL (c) fraction. Hepatic profiles of SFA and MUFA, especially in the TAG fraction, correspond to changes in hepatic *Scd1* expression (see Fig. 22). The values are expressed as mol%. "Other", the sum of FA containing less than 16 carbons; "16:1 sum" and "18:1 sum", sum of n-7 and n-9 isomers, respectively.

4.1.4. Dietary background determines the effect of Omega-3 on the size of adipocytes and inflammatory state of WAT.

In agreement with the changes in the weight of eWAT (see the section 4.1.2. and **Tab. 5**), the size of adipocytes increased significantly but comparably in the cHF and LHF group, while Omega-3 decreased the size of adipocytes only when they were supplemented in the cHF diet (**Fig. 26a**). The analysis of gene expression in eWAT showed that Omega-3 decreased the expression of lipogenic genes including *Acaca, Acly*, and *Fasn*, but only when supplemented on the cHF background (**Fig. 26d**); there were no consistent changes in the expression of genes involved in β-oxidation of FA (data not shown).

High-fat diets induced inflammation of eWAT, as suggested by an increased number of CLS (**Fig. 26b**; for details on methodology, see section 3.4.); representative images of immunochemical analysis of eWAT sections is shown in **Fig. 25** (red arrows denote the presence of CLS). While Omega-3 supplementation on the cHF background had no effect on the number of CLS in eWAT, it even increased the number of CLS when supplemented on the LHF background (**Fig. 26b**). Similar results could be observed at the level of gene expression of selected inflammatory cytokines and macrophage markers (**Fig. 26c**). On the cHF background, Omega-3 decreased the expression of chemoattractant *Ccl2* as well as *Nos2*, a marker of pro-inflammatory M1 macrophages. On the LHF background, Omega-3 did not change the expression of the above genes, while the expression of *Adgre1*, encoding a M1-specific membrane molecule F4/80 was even increased. The expression of a marker of anti-inflammatory M2 macrophages *Arg1* was increased by Omega-3 independently of a dietary background. Taken together, similarly to the effects on glucose homeostasis, Omega-3 reduced the size of adipocytes and eWAT inflammation only when supplemented on the cHF background. When supplemented within the SFA-rich LHF diet, Omega-3 had either no effect or even worsened the inflammatory state of eWAT.

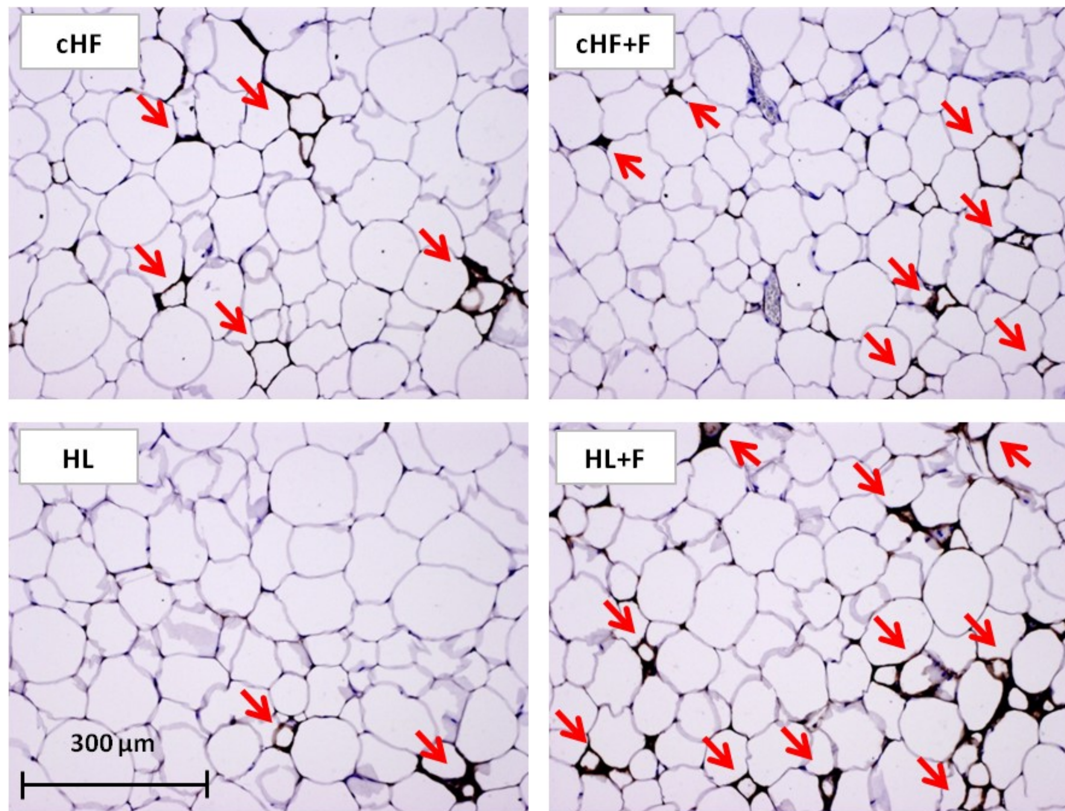


Fig. 25 Pub. A, Exp. 2. The morphology of adipocytes and macrophages accumulation in adipose tissue
Morphology of eWAT and the accumulation of macrophages in the tissue. Representative histological sections of eWAT, stained with a specific antibody against a macrophage marker MAC-2/galectin-3 in order to visualize macrophages and calculate the number of CLS (marked with red arrows).

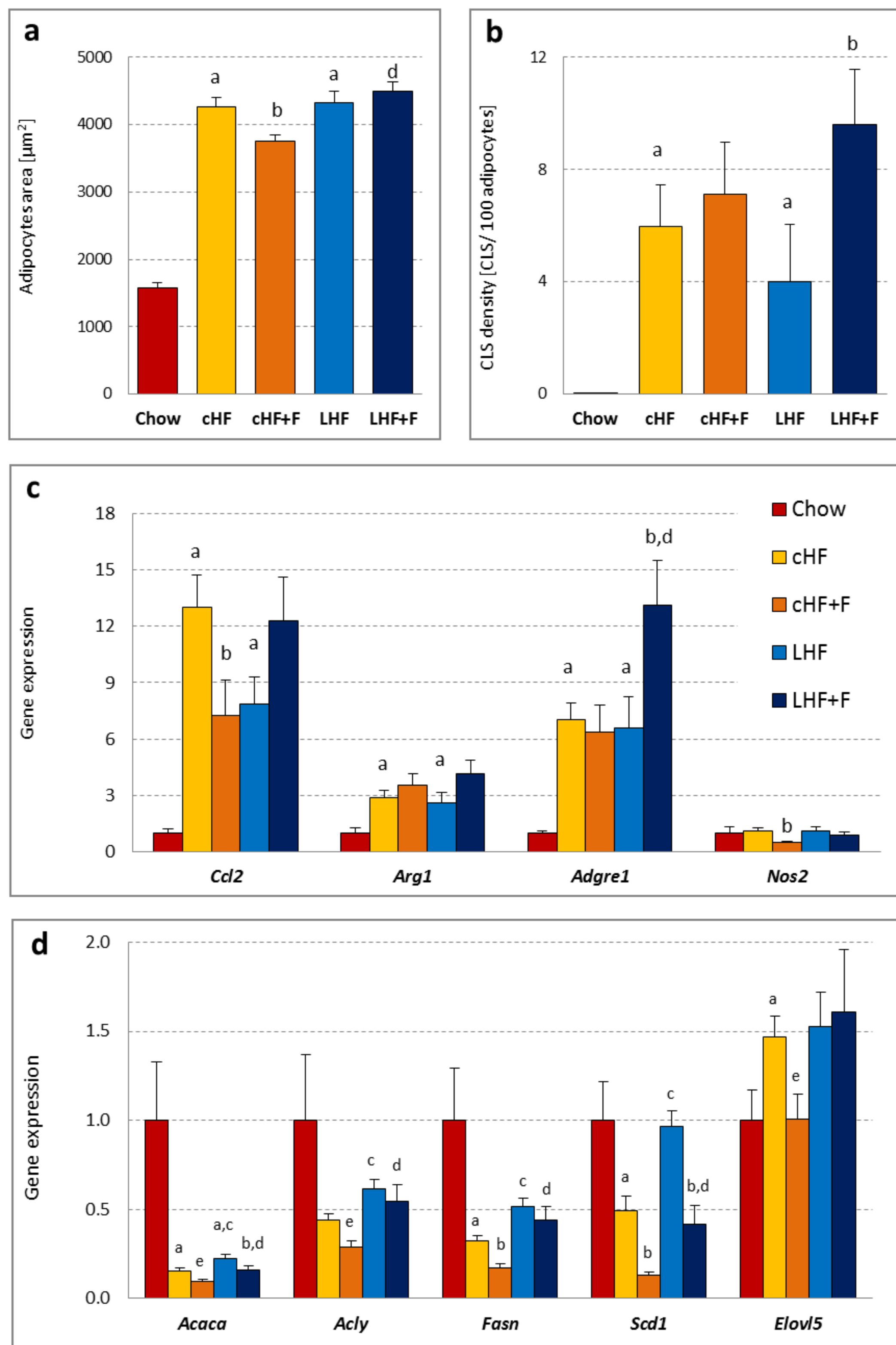


Fig. 26 Pub. A, Exp. 2. Major characteristics of eWAT including the size of adipocytes, quantification of tissue macrophages based on the immunohistochemical detection, and gene expression analysis

Morphological analysis of histological sections of eWAT was used to evaluate the average size of adipocytes in the tissue (A), while the accumulation of macrophages was assessed by calculating the relative density of CLS in the tissue (B). Gene expression analysis of the markers of pro-inflammatory M1 macrophages (*Ccl2, Adgre1, Nos2*) and anti-inflammatory M2 macrophages (*Arg1*; C), and of the key enzymes of *de novo* lipogenesis (D); results are expressed as a fold change relative to the Chow-fed mice. Data are means ± SEM (n = 9-10 except Chow, where n = 6). [a]p≤0.05 cHF or LHF vs. Chow; [b]p≤0.05 cHF or LHF vs. cHF+F or LHF+F, respectively; [c]p≤0.05 cHF vs. LHF; [d]p≤0.05 cHF+F vs. LHF+F (two-way ANOVA); [e]p≤0.05 cHF vs. cHF+F (t-test).

4.2. Publication B (In preparation): The impact of long-term Omega-3 supplementation on the incretin system of dietary obese mice.

4.2.1. Long-term Omega-3 supplementation increases plasma insulin levels after oral but not intraperitoneal administration of glucose.

In the preliminary experiment, the effect of long-term Omega-3 supplementation on the incretin system was examined by applying either i.p. GTT or oGTT (see section 3.2.1. for details) and measuring plasma levels of insulin, GIP and GLP-1 in the basal (fasting) state and 30 min after glucose administration. As expected, feeding mice with the cHF diet for 8 weeks lead to the development of obesity; thus, the cHF mice gained ~13 g, which was 3 times more than in the lean controls fed the Chow, while Omega-3 supplementation decreased the weight gain by 30 % as compared to cHF-fed counterparts (**Tab. 6**). The results of both i.p. GTT and oGTT demonstrated an impairment of glucose tolerance in the cHF mice, which was partially normalized by Omega-3 supplementation (**Fig. 27a, Fig. 27e**); however, the beneficial effect of Omega-3 was more pronounced and significant only in the cHF+F mice subjected to oGTT (**Tab. 6**, iAUC and tAUC).

Tab. 6 Publication B, Experiment 1. The parameters of energy balance and glucose homeostasis

	Chow	cHF	cHF+F
Energy balance			
Body weight - initial (g)	25.37 ± 0.63	25.12 ± 0.55	24.74 ± 0.38
Body weight - final (g)	29.87 ± 0.59	38.06 ± 1.61[a]	33.97 ± 1.02[a,b]
Weight gain (g)	4.50 ± 0.24	12.94 ± 1.39[a]	9.23 ± 0.87[a,b]
Energy intake (kJ/day/mouse)	50.03 ± 1.19	56.01 ± 1.09	57.30 ± 1.22[a]
i.p. GTT			
FBG (mg/dl)	92.11 ± 5.80	127.38 ± 12.98[a]	103.67 ± 5.07
Incr. AUC (mol x 180 min)	1421.40 ± 103.20	2008.61 ± 126.31[a]	1818.37 ± 138.02[a]
Total AUC (mol x 180 min)	2341.59 ± 114.78	3281.09 ± 207.49[a]	2854.00 ± 135.62[a,]
oGTT			
FBG (mg/dl)	102.89 ± 6.50	119.00 ± 13.55	93.00 ± 3.11
Incremental AUC (mol x 180 min)	2333.64 ± 95.64	3076.09 ± 155.29[a]	2459.58 ± 115.49[b]
Total AUC (mol x 180 min)	3356.13 ± 120.18	4257.05 ± 169.24[a]	3372.37 ± 101.26[b]

Data are means ± SEM (energy balance, *n* = 16; glucose homeostasis, *n* = 8). Average energy intake was based on 24-hour-measurements of food consumption performed weekly during the study. Both i.p. GTT and oGTT were performed in overnight fasted animals after 8 weeks of dietary intervention. i.p. GTT, intraperitoneal glucose tolerance test; oGTT, oral glucose tolerance test; FBG, fasting blood glucose; AUC, area under the glycemic curve. [a]$p \leq 0.05$ for the difference from Chow, [b]$p \leq 0.05$ for the difference between cHF and cHF+F (t-test).

Plasma fasting insulin levels showed a similar profile in both groups of mice subjected either to i.p. GTT or oGTT; thus, in the cHF mice insulin levels were increased ~4-fold when compared to the Chow-fed group, while in the cHF+F mice they were reduced to ~50 %

of those found in the cHF mice (**Fig. 27b, 27f**). While glucose administration via intraperitoneal injections resulted in a ~2-fold increase in plasma insulin concentrations in the cHF+F mice, no measurable increase was seen in the Chow and cHF group (**Fig. 27f, 27g, 27h**); thus, plasma insulin levels 30 min after glucose administration were comparable between the cHF and cHF+F mice (**Fig. 27g**). On the contrary, oral administration of glucose increased plasma insulin levels more than 6-fold in the cHF+F mice, while only a ~2.5-fold increase was detected in the cHF group (**Fig. 27b, 27c, 27d**); thus, oral administration of glucose resulted in plasma insulin levels being ~70 % higher in the cHF+F group than in their cHF counterparts (**Fig. 27c**).

4.2.2. The impact of high-fat feeding and Omega-3 supplementation on basal and glucose-stimulated plasma incretin concentrations.

As long-term Omega-3 supplementation strongly potentiated the stimulatory effect of orally administered glucose on plasma insulin levels, the next experiment aimed to examine whether Omega-3 supplementation could affect the incretin system. The experimental setup was similar to the preliminary experiment. However, mice of the C57BL/6N strain were used instead of C57BL/6J mice, since mice of the former strain are known to achieve higher and more homogenous body weight gains in response to high-fat feeding.

As shown in **Tab. 7**, both the cHF and cHF+F mice markedly increased their body weight when compared to lean Chow-fed controls; however, the weight gain was significantly lower in the cHF+F than in cHF mice. Changes in body weight were then reflected in the weight of all major fat depots (**Tab. 7**), namely mesenteric WAT (**mWAT**) and eWAT (i.e. abdominal fat depots) and subcutaneous WAT dissected from the dorsolumbal region (**sWAT**). Despite the beneficial effect on body weight gain and adiposity, Omega-3 supplementation did not significantly affect glucose tolerance as compared to cHF mice (**Tab. 7**).

As incretin hormones undergo rapid degradation by the action of ubiquitously present DPP-4, the concentration of insulin, active GLP-1, total GLP-1, and total GIP was analyzed in plasma isolated from DPP-4 inhibitor-treated blood samples collected by the cannulation of the portal vein (this vein collects blood from the intestines and thus it is very close to the site of incretin secretion). Because the collection of blood from the portal vein represents a terminal procedure, the usual protocol for oGTT was adapted so that mice were fasted for 6 hours, then administered either 0.5 ml saline or 0.5 ml of 30 % glucose by gavage and dissected 30 min later in order to evaluate plasma concentrations of hormones in the basal as well as glucose-stimulated conditions (for the experimental setup, see section 3.1.3. In the cHF and cHF+F mice the basal and glucose-stimulated plasma insulin concentrations were increased as compared to lean Chow-fed controls (**Fig. 28A**); however, unlike the previous experiment, only the trend related to the decrease in plasma insulin in the basal state and their increase in glucose-stimulated conditions was observed in the cHF+F mice as compared to obese cHF controls (**Fig. 28A**).

There was no difference between the cHF and cHF+F group in fasting concentrations of total GLP-1 (i.e. the sum of the active and inactive forms of the hormone), however they were significantly higher as compared to those found in lean Chow-fed mice (**Fig. 28B**). Also under stimulated conditions, both cHF and cHF+F mice had increased levels of total GLP-1 when compared to Chow-fed mice, however, no difference in GLP-1 levels was observed in the cHF and cHF+F mice (**Fig. 28B**). We also analyzed plasma levels of the active form of GLP-1; however, the measured concentrations were often under the detection limit (data not shown). Finally, plasma levels of total GIP were similar among the groups in the basal conditions but significantly elevated in both the cHF and cHF+F mice in glucose-stimulated conditions as compared to Chow-fed lean controls (**Fig. 28C**).

Tab. 7 Publication B, Experiment 2. Parameters of energy balance, glucose homeostasis and adiposity

	Chow	cHF	cHF+F
Energy balance			
Body weight - initial (g)	26.46 ± 0.47	26.23 ± 0.50	26.73 ± 0.43
Body weight - final (g)	30.37 ± 0.64	45.49 ± 0.67[a]	42.03 ± 0.80[a,b]
Weight gain (g)	3.90 ± 0.42	19.26 ± 0.45[a]	15.30 ± 0.61[a,b]
Energy intake (kJ/day/mouse)	63.10 ± 0.66	71.54 ± 0.93[a]	70.40 ± 1.00[a]
Glucose homeostasis			
FBG (mg/dl)	183.86 ± 4.84	243.94 ± 9.12[a]	228.18 ± 6.36[a]
Incremental AUC (mol x 180 min)	1031.82 ± 73.81	1969.85 ± 176.89[a]	1666.27 ± 187.44[a]
Total AUC (mol x 180 min)	2868.56 ± 63.49	4406.77 ± 212.33[a]	3945.76 ± 203.96[a]
Fat depots			
eWAT (g)	543.21 ± 44.40	2059.29 ± 51.73[a]	1764.47 ± 59.06[a,b]
sWAT (g)	225.64 ± 14.83	884.59 ± 26.56[a]	757.00 ± 34.21[a,b]
mWAT (g)	270.78 ± 18.77	1427.88 ± 58.70[a]	1093.06 ± 60.32[a,b]
Adiposity index (%)	3.39 ± 0.19	9.62 ± 0.11[a]	8.61 ± 0.15[a,b]

Data are means ± SEM (energy balance and fat depots, *n*=16; glucose homeostasis, *n*=8). The average energy intake was based on 24-hour measurements of food intake performed weekly during the study. Glucose tolerance tests and dissection of mice were performed after 8 and 9 weeks of dietary interventions, respectively. In both cases, the animals were deprived of food for 6 hours prior to testing. Adiposity index (%) was calculated as the sum of weights of the sWAT, mWAT, and eWAT fat depot, divided by body weight. FBG, fasting blood glucose; AUC, area under the glycemic curve; WAT, white adipose tissue; eWAT, epididymal WAT; sWAT, subcutaneous WAT; mWAT, mesenteric WAT. [a]$p \leq 0.05$ for the difference from Chow, [b]$p \leq 0.05$ for the difference between cHF and cHF+F (t-test).

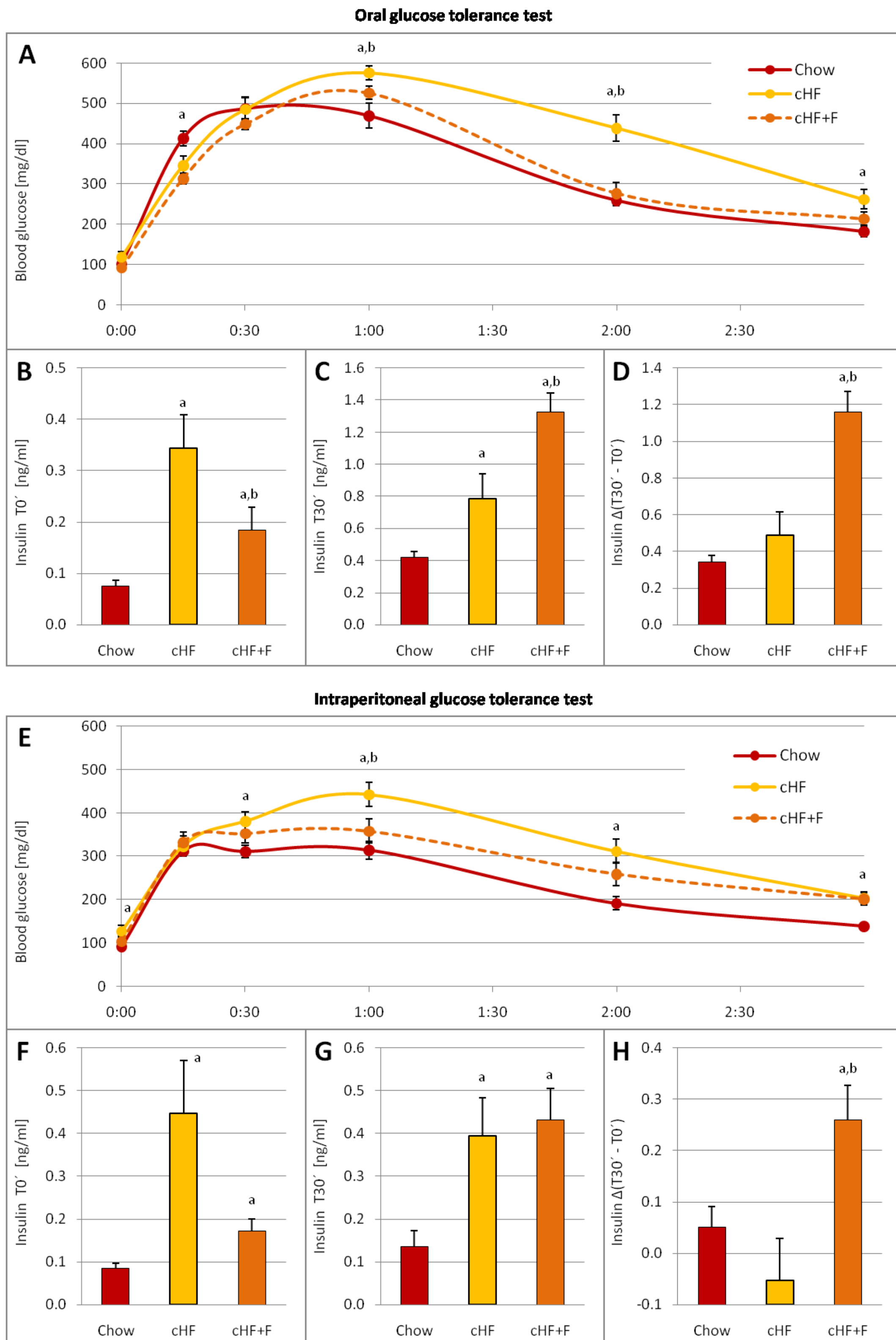


Fig. 27 Pub. B, Exp. 1. Glucose tolerance and plasma insulin levels in mice subjected to either i.p. GTT or oGTT

After 8 weeks of dietary interventions with the Chow, cHF, and cHF+F diet, mice were divided into two subgroups with a homogenous body weight distribution, then fasted overnight (~14 hours) and subjected to either i.p. GTT or oGTT. The level of glucose tolerance was evaluated as the area under the glucose curve (i.e. AUC; see **Tab. 6** above). Glycemic curves during oGTT (A) and corresponding plasma insulin levels in the basal (fasted) state (B), 30 min after glucose load (C), and expressed as the difference between these two states (i.e. Δ(T30' – T0'); D). Glycemic curves during i.p. GTT (E), and corresponding plasma insulin levels in the basal (fasted) state (F), 30 min after glucose load (G), and expressed as the difference between these two states (i.e. Δ(T30´-T0´); H). [a]*p*≤0.05 for the difference between Chow and cHF (t-test); [b]*p*≤0.05 for the difference between cHF and cHF+F (t-test).

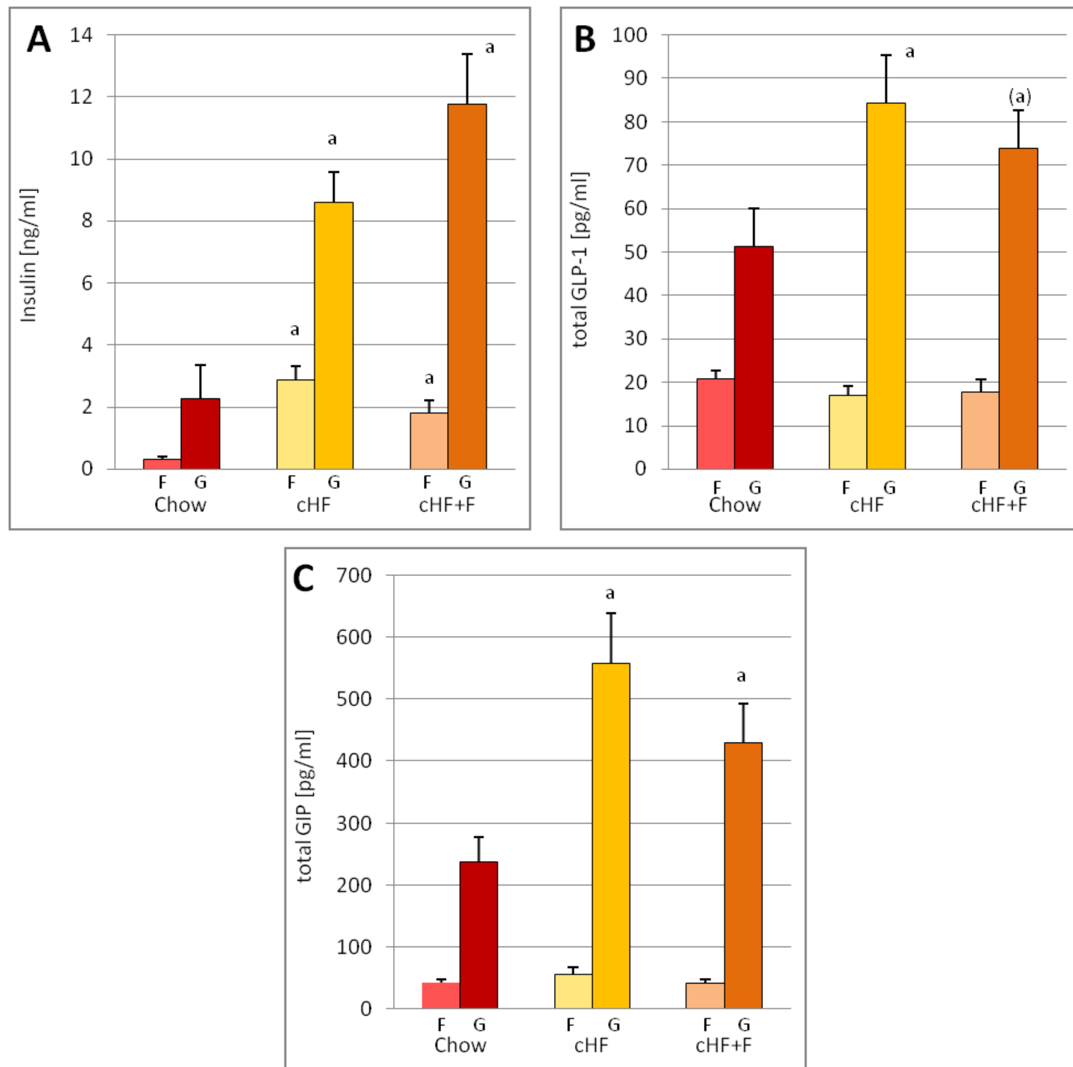


Fig. 28 Pub. B, Exp. 2. The basal and glucose-stimulated plasma levels of insulin and incretin hormones in mice after intragastric administration of either saline or glucose

After 9 weeks of dietary interventions, mice were divided into two subgroups, fasted for 6 hours, and then given either 0.5 ml of saline (F) or 0.5 ml of 30 % glucose (G) by gavage. Blood was collected via cannulation of the portal vein 30 min after the gavage in isoflurane anesthesia, and the concentration of insulin (A), total GLP-1 (B), and total GIP (C). $^{a}p \leq 0.05$ for the difference between Chow and cHF (t-test), $^{b}p \leq 0.05$ for the difference between cHF and cHF+F (t-test)

4.2.3. Tissue-specific activity of DPP-4 is elevated in obesity, but it is not influenced by Omega-3 supplementation.

Tissue-specific activity of DPP-4, i.e. the enzyme involved in the inactivation of incretin hormones, was examined in plasma, the gut (i.e. in the ileum), and eWAT. In the gut, the activity of DPP-4, quantified as the slope of the linear segment of the kinetic curve (see **Fig. 29A**), was significantly increased in the cHF and cHF+F groups as compared to Chow, but no difference was observed between the cHF and cHF+F mice (**Fig. 29B**). Similar data were obtained, when DPP-4 activity was analyzed in plasma (**Fig. 29C and Fig. 29D**). In eWAT, only the cHF and cHF+F groups were analyzed, as the properties of adipocytes from lean Chow-fed mice were incomparable to those of hypertrophied adipocytes from obese mice. The cHF and

cHF+F groups showed a comparable activity of DPP-4 in eWAT (**Fig. 29E**). However, since Omega-3 supplementation decreased the total weight of eWAT (**Tab. 7**), the kinetic curve normalized to the total eWAT weight revealed a higher DPP-4 activity in the cHF+F mice as compared to obese cHF controls (**Fig. 29F**)

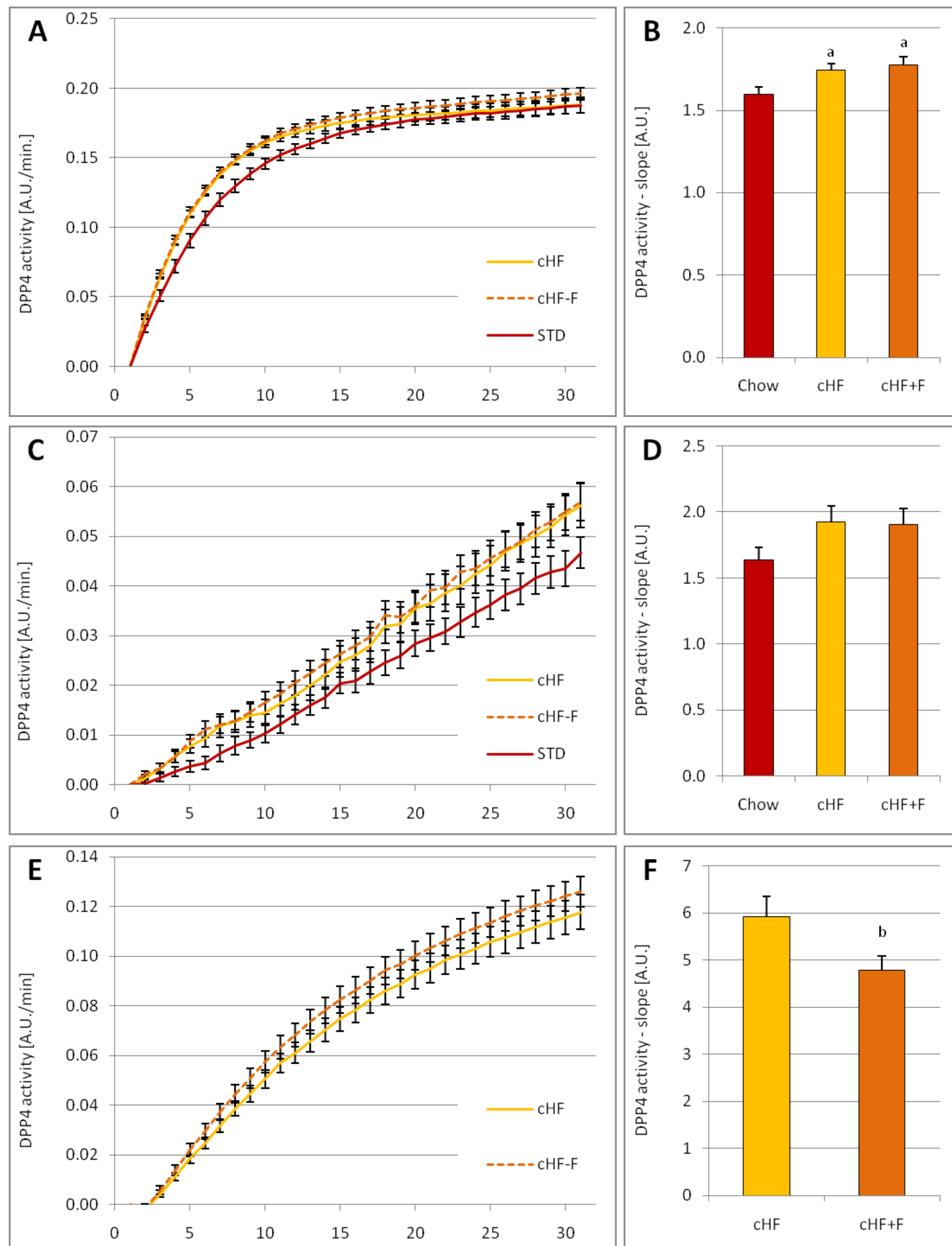


Fig. 29 Pub. B, Exp. 2. The activity of DPP-4 in tissues and plasma

The activity of DPP-4 was assessed in samples of intestine (A and B), plasma (C and D), and eWAT (E and F), which were collected during the dissection after 9 weeks of dietary interventions. The results are presented either as the kinetic curves (A, C, and E) or the quantification of the slope of the linear segment of the kinetic curve normalized to tissue weight (B). The activity of DPP-4 in eWAT was normalized to the total weight of eWAT depot (F). [a]$p \leq 0.05$ for the difference from Chow (t-test), [b]$p \leq 0.05$ for the difference between cHF and cHF+F (t-test)

4.2.4. Omega-3 supplementation decreased gene expression of *Progip* in the gut as well as GIPR in white adipose tissue.

As Omega-3 showed a strong tendency to prevent cHF-induced increase in plasma GIP concentrations in the fasted as well as glucose-stimulated state, gene expression of *Progip*, i.e. the propeptide precursor of GIP synthesis, was examined in various segments of the small and large intestine. In the proximal segment of the small intestine (i.e. duodenum/jejunum), which is the main site of GIP secretion, a significant increase in *Progip* expression was observed in the cHF mice when compared to Chow-fed animals (**Fig. 30A**). However, this increase was not observed when a more distant segment of the small intestine (i.e. ileum) was analyzed (**Fig. 30B**). The greatest differences in *Progip* expression among the groups were observed in the proximal part of the colon (**Fig. 30C**), in which the expression of *Progip* was relatively low but still detectable; thus, cHF mice had increased expression of *Progip* as compared to the Chow or cHF+F mice, while Omega-3 supplementation in the cHF+F mice normalized the expression of *Progip* in this intestinal segment (**Fig. 30C**).

In connection with the effect of Omega-3 on adiposity, gene expression of *Gipr* in eWAT was also analyzed; it was significantly increased in the cHF mice when compared to the Chow-fed mice, while Omega-3 supplementation (i.e. in the cHF+F mice) tended to decrease it (**Fig. 30D**).

4.2.5. Long-term Omega-3 supplementation reversed elevated plasma GIP levels in mice with already established obesity due to high-fat feeding.

The results of the experiment described above strongly suggested that Omega-3 supplementation could modulate the function of the incretin system, namely in terms of the regulation of GIP secretion. While the analysis of *Progip* expression in the gut revealed significant differences between the cHF and cHF+F mice, the results regarding the measurements of plasma GIP concentrations were not conclusive. The aim of the next study was to reveal whether dietary intervention with Omega-3, performed in mice that were already obese due to a previous consumption of a high-fat (cHF) diet, would unmask the effects of Omega-3 supplementation on the incretin system.

As shown in **Tab. 8**, the initial body weight of obese mice (i.e. in mice assigned to the cHF and cHF+F groups) was ~40 g, which was further increased by 6.4 g in the cHF mice but only by 2.8 g in the cHF+F mice with Omega-3 supplementation; this resulted in a significantly lower body weight of cHF+F mice at the end of the study as compared to the cHF group. On the other hand, lower body weight gain in the cHF+F mice was accompanied by a ~6 % decrease in energy consumption in these animals.

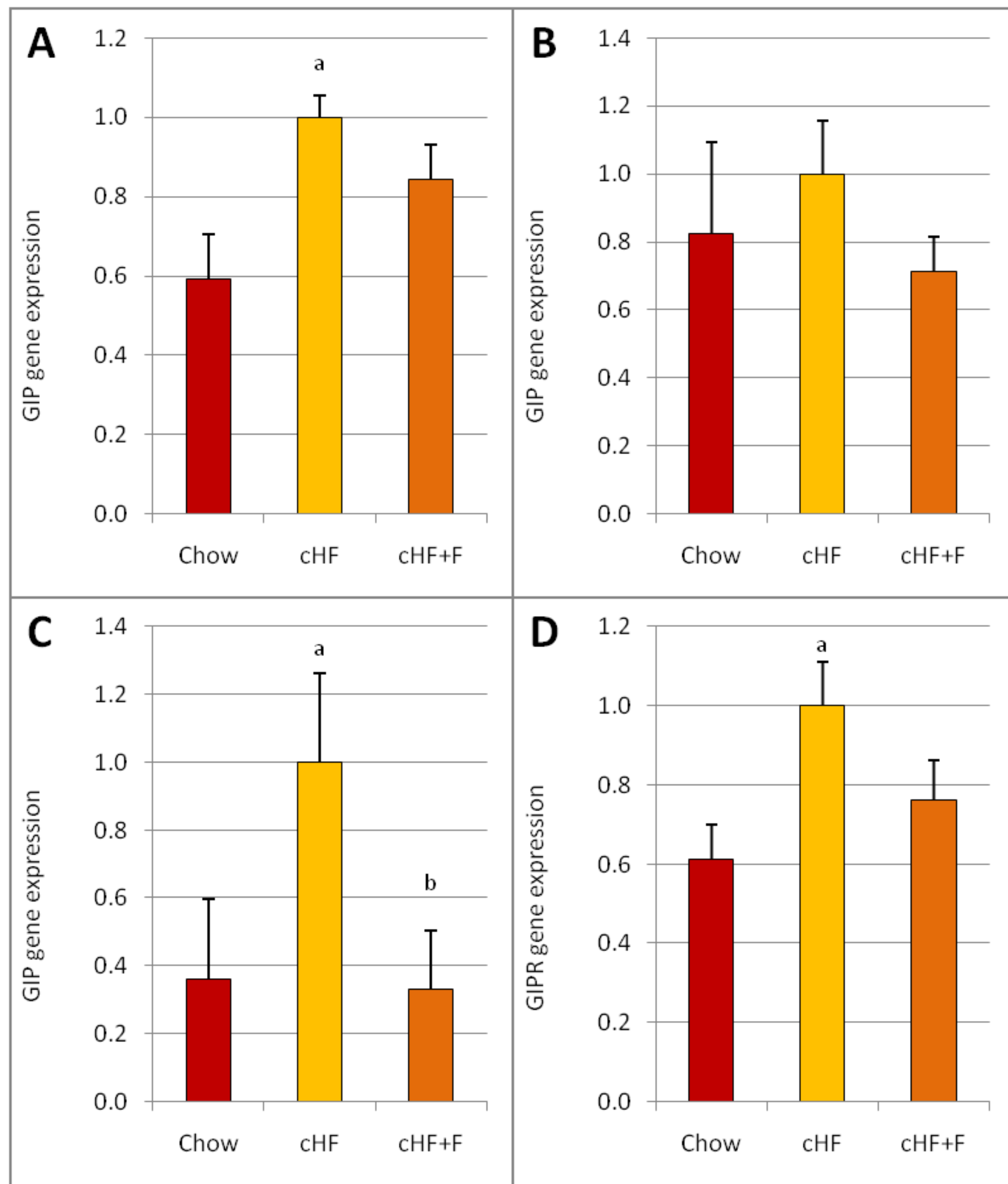


Fig. 30 Pub. B, Exp. 2. Gene expression analysis of *Progip* in the intestine and *Gipr* in eWAT

Gene expression analysis of *Progip*, i.e. the precursor for the GIP synthesis, was analyzed in the proximal segment of the small intestine (A), distal segment of the small intestine (B), and proximal segment of the colon (C). Gene expression of *Gipr* was measured in eWAT (D). Tissue samples were obtained during the dissection that followed 9-weeks-long dietary interventions. [a]$p \leq 0.05$ for the difference from Chow (t-test), [b]$p \leq 0.05$ for the difference between cHF and cHF+F (t-test)

Tab. 8 Publication B, Experiment 3. Parameters of energy balance

	Chow	cHF	cHF+F
Energy balance			
Body weight - initial (g)	28.72 ± 0.82	40.50 ± 0.64[a]	40.18 ± 0.68[a]
Body weight - final (g)	30.66 ± 0.68	46.90 ± 0.85[a]	43.00 ± 0.87[a,b]
Weight gain (g)	1.97 ± 0.35	6.40 ± 0.61[a]	2.82 ± 0.88[b]
Energy intake (kJ/day/mouse)	65.44 ± 1.00	79.05 ± 0.83[a]	74.30 ± 1.32[a,b]

The profile of insulin secretion was consistent with the results of both previous experiments. In the fasting state, plasma insulin concentrations in the cHF mice were significantly increased when compared either to the Chow or cHF+F group (**Fig. 31A**). When calculated as the increment in plasma insulin concentrations following the oral glucose load (**Fig. 31B**), it was ~55 % higher in the cHF+F than in cHF mice; thus, 30 min after glucose administration, plasma insulin levels in the cHF+F group exceeded those in the cHF group (p=0.05; **Fig. 31A and Fig. 31B**).

Fasting concentrations of GIP were significantly increased in both cHF and cHF+F groups when compared to Chow with no difference between cHF and cHF+F (**Fig. 31C**). High-fat feeding induced ~90 % higher increment in GIP secretion after the oral glucose load when compared to Chow, while in this experimental setup, Omega-3 supplementation lead to almost complete normalization of stimulated GIP secretion (**Fig. 31D**).

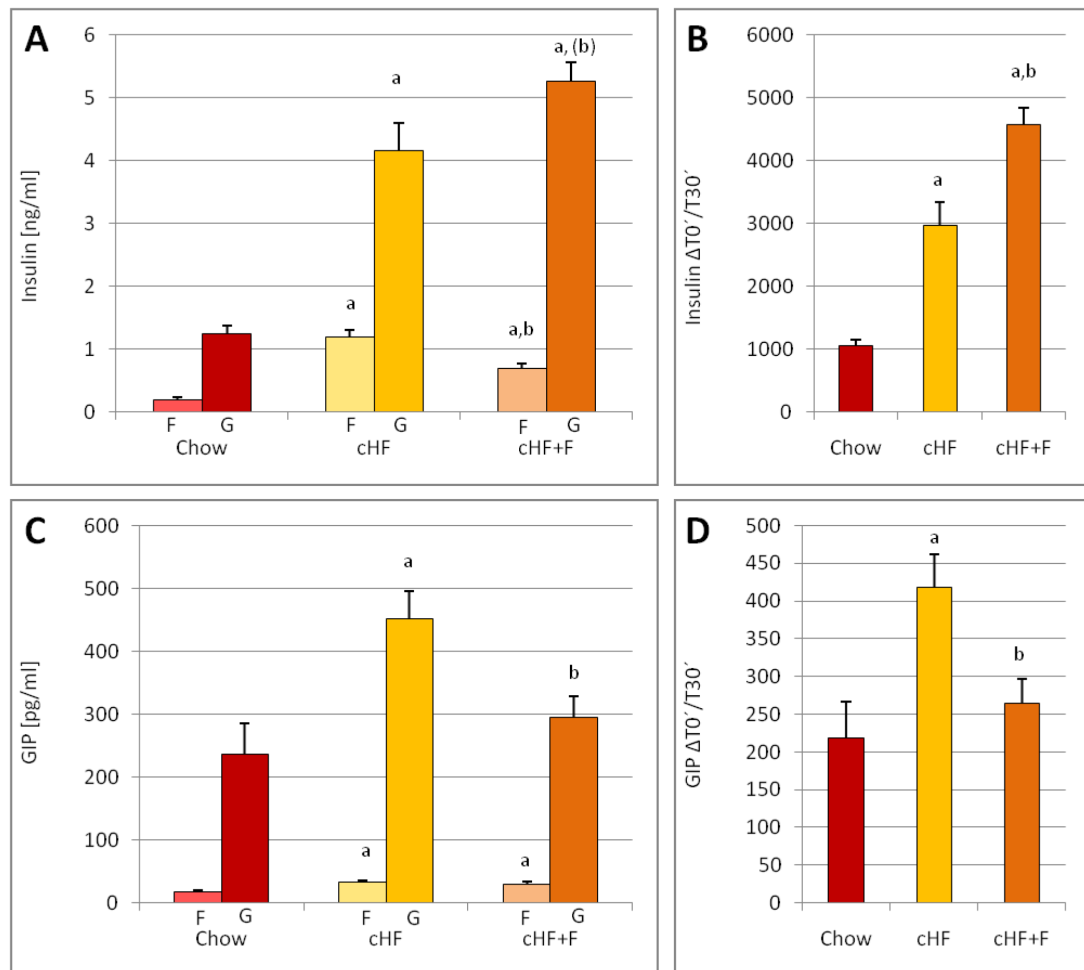


Fig. 31 Pub. B, Exp. 3. Basal and glucose-stimulated plasma concentrations of insulin and GIP in mice with dietary obesity induced prior to Omega-3 supplementation

To induce obesity, mice were first fed the cHF diet for 8 weeks, and then differential dietary interventions were initiated for a further 8 weeks. At the end of study, mice were fasted overnight and blood samples were collected from the tail tip before (i.e. in the fasting state; F) and 30 min after administration of glucose by gavage (G). Plasma concentrations of insulin (A and B) as well as of total GIP (C and D) were measured, and the change in plasma levels of insulin (B) and GIP (D) measured before and 30 min after glucose administration was expressed as ΔT30´ - T0´. [a]$p \leq 0.05$ for the difference between Chow and cHF (t-test), [b]$p \leq 0.05$ for the difference between cHF and cHF+F (t-test).

4.3. Publication C (*published*): Omega-3 phospholipids from fish suppress hepatic steatosis by integrated inhibition of biosynthetic pathways in dietary obese mice.

Although part of the results included in this already published article, e.g. gene expression screening by microarrays and the analyses linked to the Experiment 2. (i.e. the comparison of metabolic effects of the PC-based concentrates derived from marine sources or soy), were not obtained by the author of this thesis, they were still included in the Results as well as the Discussion sections of this thesis, since they are important for the overall understanding of this study.

4.3.1. Omega-3 PL prevent obesity, dyslipidemia, glucose intolerance, and inflammation in white adipose tissue.

Several 7-weeks-long intervention studies were performed in order to analyze metabolic effects of Omega-3 PL derived from herring meal. In the Experiment 1., we tested the effects of a high-fat diet supplemented with Omega-3 PL (i.e. the PC diet) alone, anti-diabetic drug rosiglitazone (R), and their combination (PC+R). As shown in **Tab. 9**, both cHF and R induced a significant increase in body weight as compared to Chow, which was completely prevented either by Omega-3 PL administered alone (PC) or in combination with rosiglitazone (PC+R). The changes in body weight correlated well with the weights of both major WAT depots, i.e. eWAT and sWAT, as well as with the size of adipocytes in eWAT adipocytes (**Tab. 9**). This was despite the fact that the energy intake in the PC and PC+R mice was significantly increased when compared to either the obese cHF controls or mice in the R group.

Potential energy losses in stool were analyzed in the Experiment 3. using the quantification of total lipids, TAG, and cholesterol in feces. Interestingly, total fecal lipids were decreased in the PC group as compared to cHF mice, which could be explained to a great extent by the changes in the content of fecal cholesterol (**Tab. 10**).

Only in the PC and PC+R groups cholesterol concentrations in plasma were reduced as compared to cHF mice, in which the cholesterol levels were much higher than in the lean Chow-fed animals (**Tab. 9**). All interventions, i.e. the PC, R, and PC+R groups, normalized the cHF-induced increases in plasma TAG levels; however, these interventions, especially in the PC and PC+R group, also decreased plasma NEFA levels as compared to either the cHF or Chow group (**Tab. 9**).

Glucose tolerance and insulin sensitivity were strongly impaired by cHF feeding when compared to Chow, while all interventions, i.e. the PC, R, and PC+R groups, were able to normalize this defect, as documented by the results of i.p. GTT (**Tab. 9; Fig. 32A and Fig. 32B**), and the lower fasting plasma insulin levels (**Fig. 32C**) as well as HOMA-IR index (**Fig. 32D**). All interventions had also a beneficial impact on WAT inflammation, which was manifested as a decrease in the number of CLS in eWAT and an increase in plasma concentrations of the anti-inflammatory hormone adiponectin, which was highest in the PC+R combination group (**Tab. 9**).

Tab. 9 Publication C, Experiment 1. Parameters of energy balance, adiposity and markers of glucose and lipid metabolism in plasma

	cHF	PC	R	PC+R	Chow
Energy balance					
Body weight – *initial* (g)	28.5 ± 0.5	28.7 ± 0.5	28.6 ± 0.5	28.9 ± 0.6	30.3 ± 1.0
Body weight – *final* (g)	44.4 ± 1.3	33.6 ± 0.9[ab]	40.7 ± 1.8	30.9 ± 0.8[ab]	34.3 ± 1.3[d]
Weight gain (g)	15.9 ± 1.2	4.9 ± 1.1[ab]	12.1 ± 1.9	2.0 ± 0.9[ab]	4.0 ± 0.5[d]
Food intake (MJ/mouse)	4.1 ± 0.1	5.1 ± 0.2[ab]	4.1 ± 0.3	5.0 ± 0.2[ab]	3.4 ± 0.1[d]
Adipose tissues					
eWAT (g)	2.70 ± 0.14	1.2 ± 0.17[ab]	1.86 ± 0.25[a]	0.71 ± 0.12[ab]	0.79 ± 0.08[d]
Adipocyte size (μm^2)	4066 ± 146	2772 ± 281[a]	3120 ± 285[a]	1851 ± 168[abc]	1984 ± 207[d]
Inflammation (CLS no.)	2.55 ± 0.61	0.29 ± 0.12[a]	0.73 ± 0.30	0.07 ± 0.03[a]	0.04 ± 0.02[d]
sWAT (g)	0.91 ± 0.09	0.45 ± 0.05[a]	0.71 ± 0.13	0.35 ± 0.04[ab]	0.30 ± 0.03[d]
Metabolites and hormones in plasma					
TAG (mmol/l)	2.16 ± 0.14	1.05 ± 0.22[a]	1.25 ± 0.12[a]	1.02 ± 0.22[a]	1.33 ± 0.05[d]
NEFA (mmol/l)	1.00 ± 0.06	0.44 ± 0.04[ab]	0.67 ± 0.04[a]	0.38 ± 0.04[ab]	0.92 ± 0.06
Cholesterol (mmol/l)	4.31 ± 0.07	3.35 ± 0.27[a]	4.02 ± 0.26	3.27 ± 0.23[a]	2.60 ± 0.14[d]
Glucose (mmol/l)	6.66 ± 0.36	5.32 ± 0.38[d]	5.22 ± 0.23[d]	5.54 ± 0.50	4.97 ± 0.21[d]
Insulin (ng/ml)	4.18 ± 0.48	1.73 ± 0.38[a]	1.58 ± 0.23[a]	1.68 ± 0.16[a]	0.94 ± 0.19[d]
Adiponectin - HMW (A.U.)	0.34 ± 0.03	0.56 ± 0.09[a]	0.82 ± 0.11[a]	1.13 ± 0.18[ac]	0.33 ± 0.03
Adiponectin - HMW: total	0.39 ± 0.02	0.47 ± 0.03	0.50 ± 0.03[a]	0.59 ± 0.03[ac]	0.42 ± 0.02

Data are means ± SEM (*n* = 8 except Chow, *n* = 5). Cumulative energy intake was assessed during a 7-week period of dietary interventions. Inflammation was assessed as the number of crown-like structures (CLS) per 100 adipocytes. Blood glucose was measured after an overnight fast right before glucose tolerance testing. A.U., arbitrary units; HMW, high-molecular weight; WAT, white adipose tissue. [a]$p \leq 0.05$ for difference from cHF (ANOVA); [b]$p \leq 0.05$ for difference from R (ANOVA); [c]$p \leq 0.05$ for difference from PC (ANOVA); [d]$p \leq 0.05$ for difference from cHF (t-test).

Tab. 10 Publication C, Experiment 3. Parameters of energy balance and fecal lipid excretion.

	Chow	cHF	PC
Energy balance			
Body weight - initial (g)	25.71 ± 0.33	25.61 ± 0.23	25.87 ± 0.29
Body weight - final (g)	29.69 ± 0.41	41.85 ± 1.92[a]	36.73 ± 0.27[b]
Weight gain (g)	2.56 ± 0.68	15.54 ± 1.68[a]	10.66 ± 0.44[b]
Energy intake (kJ/day/mouse)	62.38 ± 0.74	72.03 ± 1.71	75.00 ± 2.23
Fecal lipids			
TAG (mg/24 h)	3.90 ± 0.55	1.87 ± 0.17[a]	4.09 ± 0.18[b]
CHOL (mg/24 h)	8.95 ± 0.38	19.16 ± 1.33[a]	8.44 ± 0.76[b]
Total lipids (mg/24 h)	75.41 ± 8.77	71.05 ± 10.2	51.01 ± 1.85

Data are means ± SEM (n = 9 except Chow, n = 6). Average energy intake was assessed during a 7-week period of dietary interventions. TAG, triacylglycerols; CHOL, cholesterol ap≤0.05 for the difference between Chow and cHF (t-test), bp≤0.05 for the difference between cHF and cHF+F (t-test).

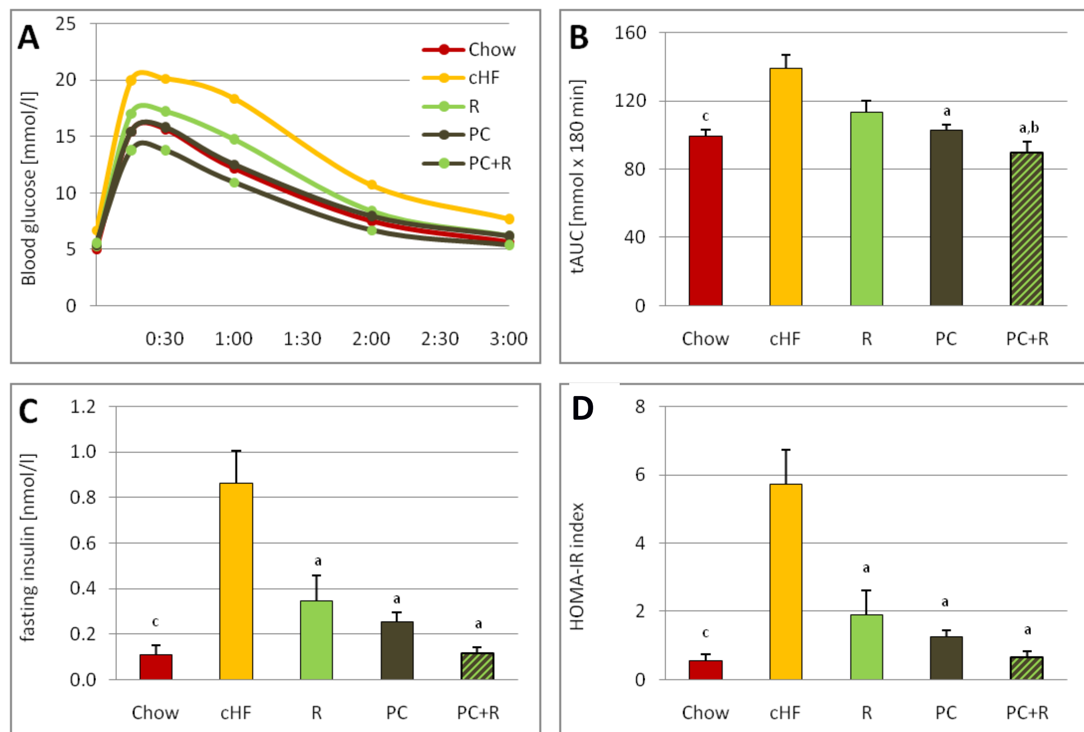


Fig. 32 Pub. C, Exp. 1. Prevention of glucose intolerance and insulin resistance by Omega-3 PL supplementation
Glucose tolerance was analyzed by i.p. GTT performed in overnight fasted mice, and the results are shown as glycemic curves (A) or they are expressed as total AUC (B). Plasma levels of insulin in the fasted state (C) as well as HOMA-IR index of insulin resistance (D) are also shown. Data are means ± SEM (n = 8, except Chow, where n = 5). $^{a}p≤0.05$ vs. cHF; $^{b}p≤0.05$ vs. R (ANOVA). $^{c}p≤0.05$ vs. cHF (t-test).

4.3.2. Distinct effects of Omega-3 PL and rosiglitazone on hepatic steatosis and a complex down-regulation of biosynthetic pathways by Omega-3 PL supplementation.

Despite the fact that no differences were observed in the weight of liver between the cHF and Chow groups (**Fig. 33A**), the liver weight was significantly reduced in both groups fed Omega-3 PL (i.e. in the PC and PC+R group). However, the cHF mice were characterized by a marked accumulation of TAG (i.e. hepatic steatosis; **Fig. 33B**) and cholesterol (Fig. **33C**) in the liver tissue. While in the PC and PC+R groups the content of both of these lipid classes was normalized, a tendency for an aggravation of hepatic steatosis was observed in the R group. The total content of BA in the liver was decreased in the cHF group as compared to Chow-fed mice, while in the PC and PC+R groups it was almost normalized (**Fig. 33D**).

The global screening of gene expression by DNA microarrays (performed by a colleague D. Medrikova, Ph.D. in the frame of our collaboration with J. Keijer, Wageningen University, The Netherlands), revealed a complex regulation of lipid metabolism pathways including *de novo* lipogenesis, β-oxidation of FA, as well as cholesterol biosynthesis (**Fig. 34**). The microarray data were then verified by qPCR, showing a good consistency between these two methods.

Thus, based on the data obtained by qPCR, rosiglitazone alone increased the expression of a number of lipogenic genes including *Acacb*, *Fasn,* and *Scd1,* while in the PC group the expression of all analyzed lipogenic enzymes (i.e. *Acly, Acacb, Fasn, Scd1, and Elovl5*) was decreased when compared to cHF mice.

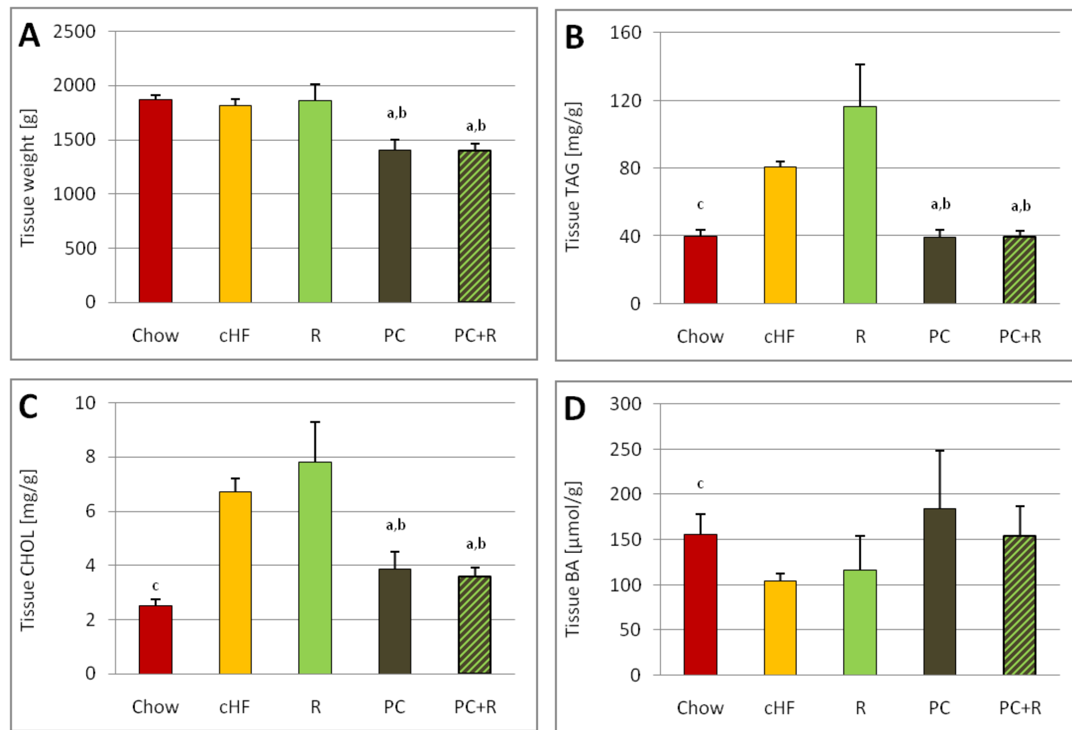
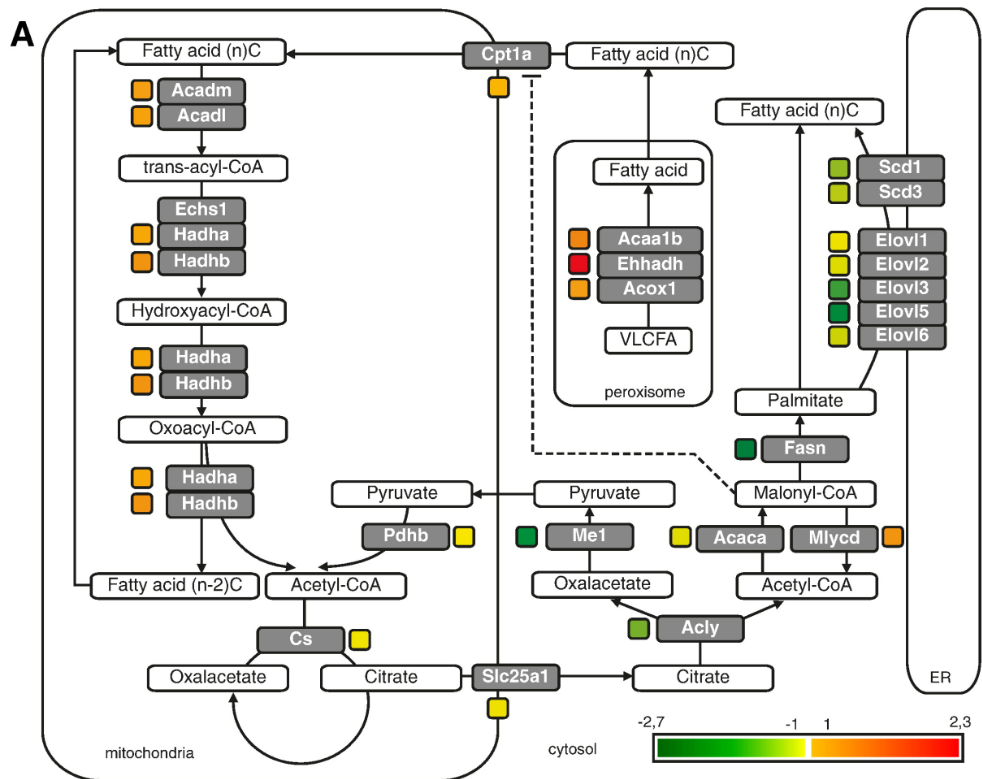


Fig. 33 Pub. C, Exp. 1. Liver parameters in mice fed a high-fat diet supplemented or not with Omega-3 PL, rosiglitazone or both Reduced hepatic lipid content in response to Omega-3 PL. The weight of liver (A) and tissue content of TAG (B), cholesterol (C), and BA (D) was assessed in mice killed in *ad libitum* fed state. Data are means ± SEM (n = 8, except for Chow, where n = 5). $^{a}p \leq 0.05$ vs. cHF; $^{b}p \leq 0.05$ vs. R (One-way ANOVA). $^{c}p \leq 0.05$ vs. cHF (t-test). CHOL, cholesterol.

In the PC+R group, the effect of Omega-3 PL overrode the prolipogenic effect of rosiglitazone, and thus the expression of some genes was either reduced to the level observed in the PC group (i.e. *Acly*, *Acacb*, *Fasn*, and *Elovl5*) or at least reduced as compared to mice treated with rosiglitazone alone (*Scd1*; **Fig. 35A**).

Both Omega-3 PL and rosiglitazone exerted stimulatory effects regarding the gene expression of selected enzymes involved in β-oxidation of FA (e.g. *Acadm, Acot1,* and *Ehhadh*)*,* while the effect of rosiglitazone slightly exceeded that of Omega-3 PL alone in the PC group (**Fig. 35A**). The combination of Omega-3 PL and rosiglitazone in the PC+R group retained the stimulatory effect of individual treatments, especially when the expression of *Acadl* and *Acox1* is concerned (**Fig. 35A**).



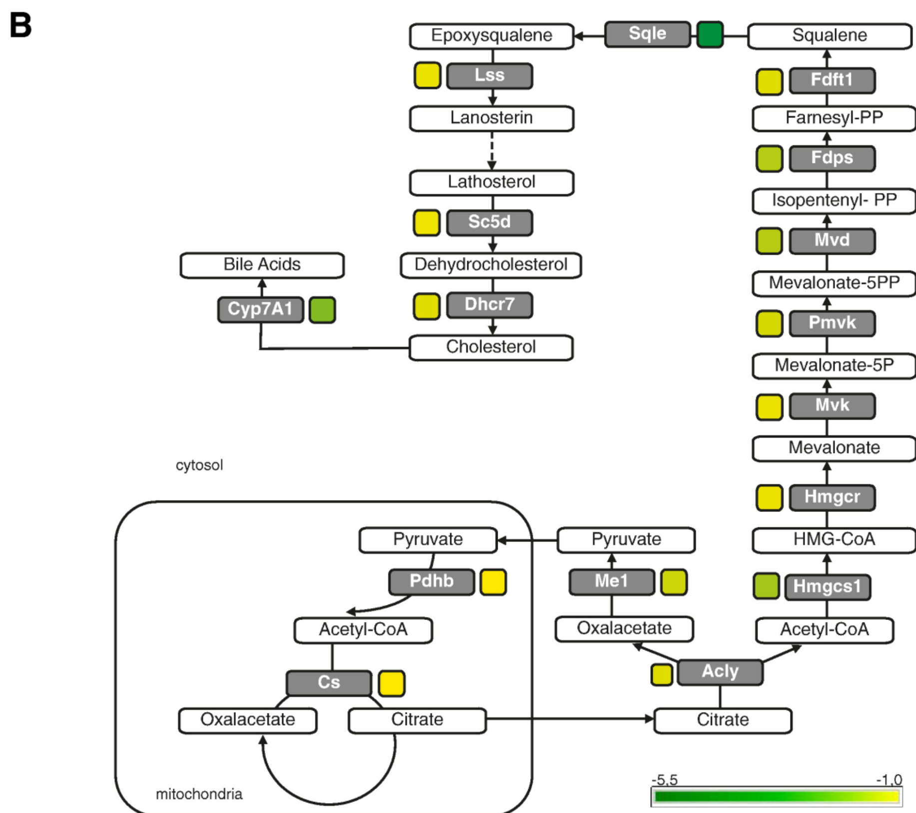


Fig. 34 Pub. C, Exp. 1. Modulation of hepatic FA metabolism and the cholesterol biosynthesis pathway by Omega-3 PL

DNA microarray analysis of gene expression within the major metabolic pathways affected by dietary supplementation with Omega-3 PL, i.e. the pathways of FA synthesis and oxidation (A) and cholesterol biosynthesis (B). Genes up- and down-regulated in response to dietary Omega-3 PL (i.e. in the PC group) are depicted in red and green, respectively (color bars indicate the fold-change). Only the genes showing a significant regulation (i.e. False discovery rate, $p < 0.05$) were used.

The expression of genes encoding enzymes involved in the biosynthesis of cholesterol (i.e. *Hmgcs1, Fdps, Sqle*) and BA (i.e. *Cyp7a1*) was significantly lower in the PC and PC+R groups as compared to cHF mice, while in the R group rosiglitazone alone had no effect (**Fig. 35B**). On the contrary, the expression of *Scarb1* and *Abcg5,* which are responsible for cholesterol uptake and excretion, respectively, as well as of *NrOb2*, which encodes the small heterodimer partner protein, i.e. an inhibitor of *Cyp7a1* expression, was significantly increased only in the PC and PC+R groups but not in the R group. Gene expression of the transcription factors *Srebf2* and *Nr1h4*, which encode the sterol regulatory element-binding protein 2 (involved in the regulation of cholesterol biosynthesis) and farnesoid X receptor (BA receptor involved in the negative feedback loop regulating BA synthesis), respectively, remained unchanged in all experimental groups (**Fig. 35B**).

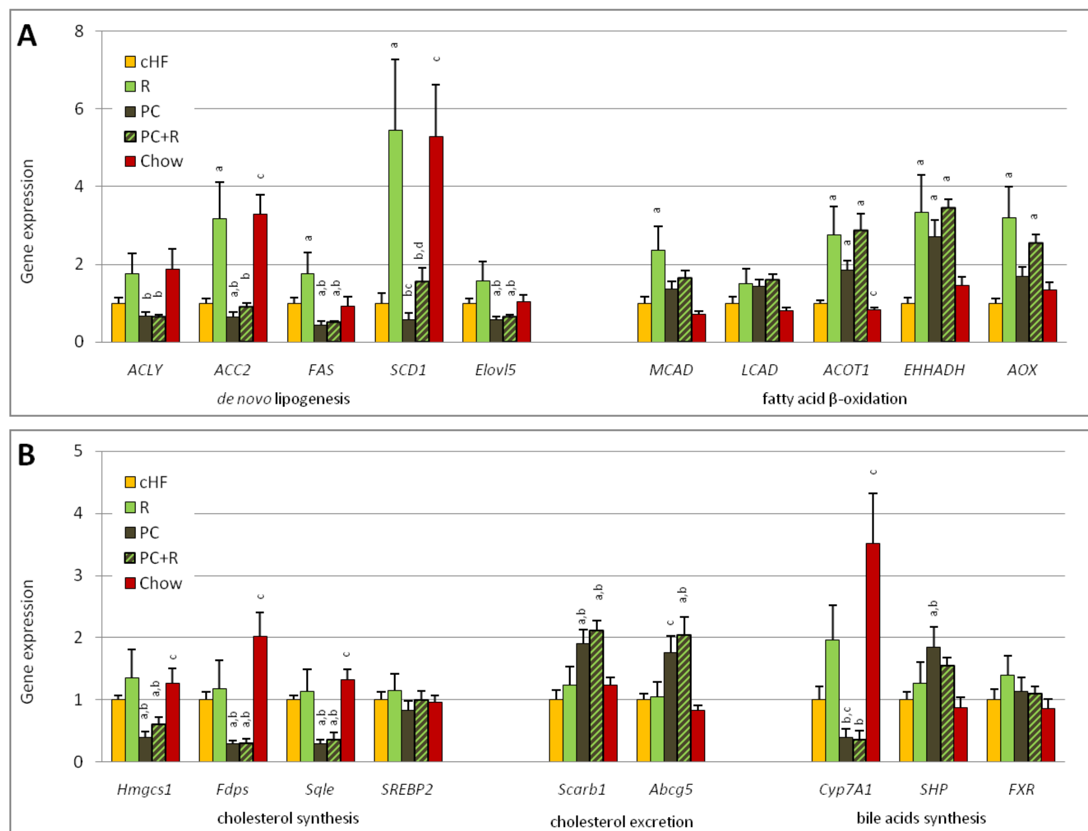


Fig. 35 Pub. C, Exp. 1. Dietary interventions regulate hepatic expression of genes involved in FA and cholesterol metabolism
Hepatic expression of selected genes involved in FA (A) and cholesterol (B) metabolism was assessed using qPCR. Data are means ± SEM (*n* = 8, except for Chow, where *n* = 5). $^{a}p\leq0.05$ vs. cHF; $^{b}p\leq0.05$ vs. R (ANOVA). $^{c}p\leq0.05$ vs. cHF (t-test).

4.3.3. Omega-3 are indispensable for the beneficial effect of marine Omega-3 PL on metabolism of obese mice.

In previous experiments, various beneficial metabolic effects of the PC-rich Omega-3 PL concentrate derived from herring meal were observed in dietary obese mice. To evaluate the contribution of the specific parts of the PC component contained in the Omega-3 PL preparation, we performed an additional experiment (i.e. the Experiment 2.), in which we

compared the effects of cHF-based diets supplemented by PC-rich products derived either from herring fish (i.e. PC-M) and soy (i.e. PC-S). As shown in **Fig. 36A**, the major FA contained in the herring-derived PC concentrate were PA and, naturally, EPA and DHA, while the soy-derived concentrate contained mainly PA, LA, and ALA (C18:3 *n-3*).

As compared to Chow-fed mice, the cHF group was characterized by a marked increase in the body weight gain (**Fig. 36B**), adiposity (**Fig. 36C**), TAG content in the liver (**Fig. 36D**), as well as by a deterioration of insulin sensitivity as indicated by increased HOMA-IR (**Fig. 36E**). All these adverse effects of high-fat feeding were significantly lowered in the PC-M group, while no effect was observed in mice from the PC-S group, where even a further deterioration of some parameters was noticed, as in the case of insulin sensitivity (**Fig. 36E**).

Furthermore, PC-M but not PC-S lowered plasma TAG and total cholesterol content as compared to cHF (data not shown), and decreased gene expression of selected enzymes involved in *de novo* lipogenesis (*Acly, Fasn*, and *Elovl5*) and cholesterol synthesis (*Hmgcs1, Fdps*, and *Sqle*), as is shown in the **Fig. 36F**.

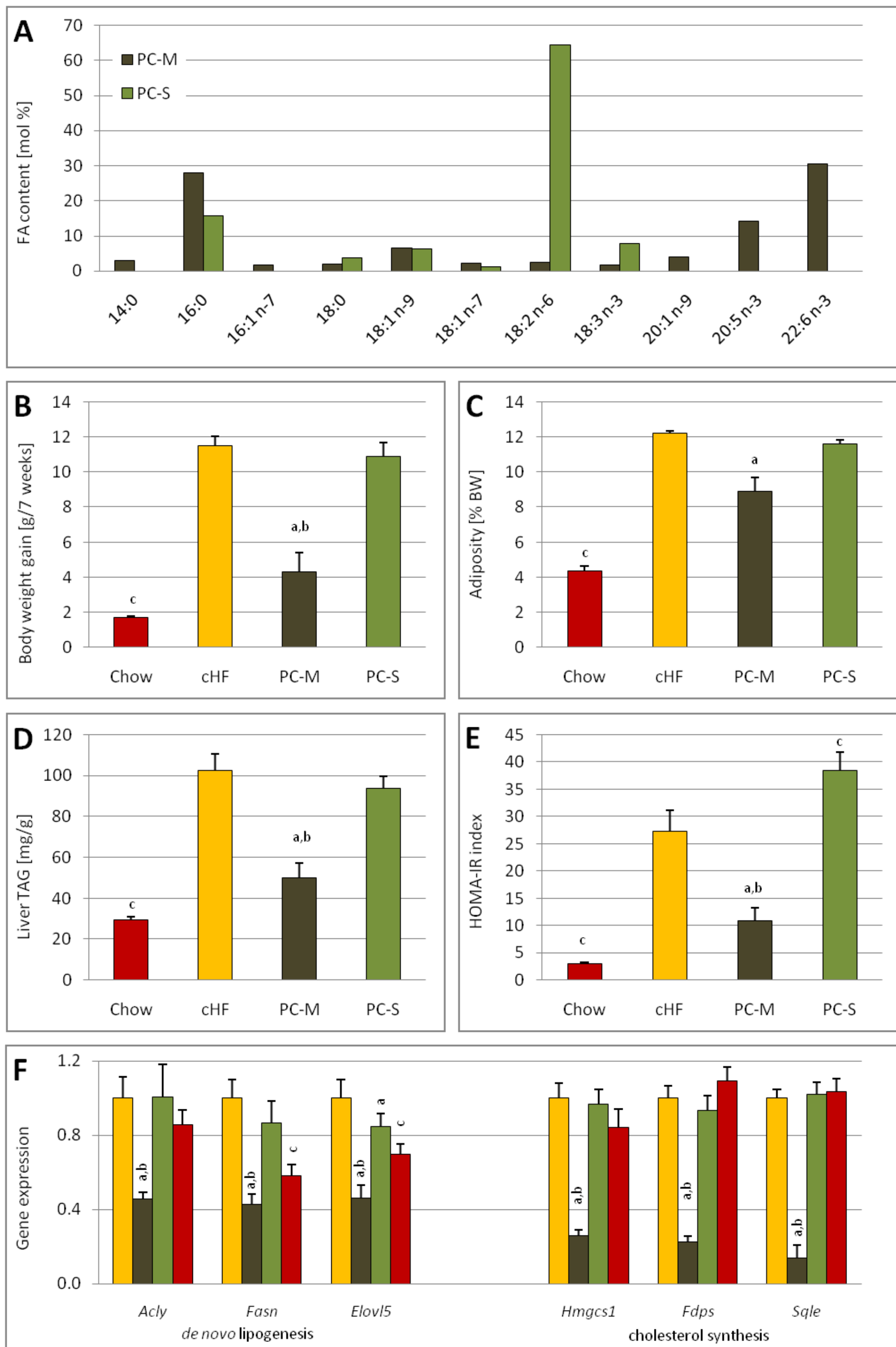


Fig. 36 Pub. C, Exp. 2. The effects of various PC-rich concentrates differing in their FA profiles on obesity-associated metabolic disorders and gene expression in the liver.

Mice were fed for 7 weeks the cHF diet (i.e. obese controls) or cHF-based diets supplemented with PL concentrates isolated either from herring (PC-M) or soy (PC-S). (A) The profiles of major FA contained in the PL concentrates used in this study. (B) Body weight gain during a 7-week-long period of differential dietary interventions. (C) Adiposity index was calculated as a sum of weights of all three major fat depots in the body (eWAT, sWAT, and mWAT), and expressed as a percentage of body weight. (D) The content of TAG in the liver. (E) The degree of insulin resistance expressed as the HOMA-IR index. (F) The mRNA levels of selected genes within the lipogenic (i.e. FA synthesis) and cholesterol biosynthesis pathways. All data are means ± SEM (n = 6–8). [a]$p \leq 0.05$ vs. cHF; [b]$p \leq 0.05$ vs. R (ANOVA). [c]$p \leq 0.05$ vs. cHF (t-test).

4.4. Publication D (*unpublished*): Omega-3 PL but not Omega-3 TAG, improve insulin sensitivity in obese C57BL/6 mice fed a high-fat diet.

In this project, we performed an 8-weeks-long dietary study, in which we explored the effects of Omega-3, administered in different lipid forms, on insulin sensitivity using the hyperinsulinemic-euglycemic clamp. At the beginning of the study, 16 mice were assigned to each group; however, only those mice, in which the catheterization of the jugular vein was associated with a weight reduction of no more than 10 % and which completed a successful clamping procedure and subsequent biochemical measurements, were included in the data analysis. Thus, in the end, the Chow, cHF, and cHF+F groups consisted of 8 animals, while the K-L and K-H groups consisted of 9 and 11 mice, respectively.

4.4.1. Omega-3 PL reduce obesity and prevent dyslipidemia more efficiently than Omega-3 TAG.

As shown in **Tab. 11** and **Fig. 37a**, high-fat feeding for 8 weeks led to the development of marked obesity in cHF mice, with their body weight gain averaging ~20 g. In general, Omega-3 supplementation partially decreased the weight gain, with Omega-3 TAG, administered to mice in the cHF+F group at the dose of ~30 mg/g diet, showing comparable effects with the low-dose of KO (i.e. in the K-L group) despite the fact that the dose of EPA+DHA was ~3-fold lower in the latter group. On the other hand, the lowering of weight gain in response to the high-dose (i.e. 30 mg/g diet) of KO in the K-H group was much stronger than in the cHF+F mice receiving the same dose of EPA+DHA. The induction of obesity in the cHF group was accompanied by a significant rise in the amount of consumed calories as compared to Chow; however, neither of Omega-3-based dietary interventions induced significant changes in energy intake when compared to the cHF-fed obese controls (**Tab. 11** and **Fig. 37b**). The changes in the weight of eWAT and sWAT reflected the changes in body weight induced by the respective dietary interventions.

The concentration of lipid metabolites (**Tab. 11**) was measured in plasma that was collected at the end of hyperinsulinemic-euglycemic clamps, i.e. in the hyperinsulinemic state, when exogenously applied glucose represents the only available energy substrate. Administration of a high-fat diet lead to a significant increase in plasma concentration of total TAG, cholesterol, and NEFA in the cHF mice as compared to lean Chow-fed controls. KO-based supplementation of a high-fat diet reduced plasma TAG levels in a dose-dependent manner, i.e. TAG levels were lower in the K-H than in the K-L group, and its effects were superior to those of Omega-3 TAG (i.e. in the cHF+F mice). On the other hand, all Omega-3-based interventions proved their efficacy regarding the reduction of plasma NEFA levels; in this case the effect of Omega-3 TAG in the cHF+F mice was superior to that of KO in the K-L group and comparable to that in the K-H group. Neither Omega-3 TAG nor Omega-PL supplementation lead to a decrease in plasma cholesterol levels as compared to obese cHF-fed mice (**Tab. 11**).

Tab. 11 Publication D, Experiment 1. Parameters of energy balance, adiposity, and lipid metabolites in plasma

	Chow	cHF	cHF+F	K-L	K-H
Energy balance					
Body weight - initial (g)	28.50 ± 0.24	27.99 ± 0.40	28.53 ± 0.58	28.31 ± 0.58	28.15 ± 0.37
Body weight - final (g)	34.32 ± 0.35	48.17 ± 0.75[a]	45.63 ± 0.69[c]	45.19 ± 0.98[c]	41.42 ± 1.61[b]
Weight gain (g)	5.81 ± 0.36	20.18 ± 0.57[a]	17.11 ± 0.55[b]	16.88 ± 0.84[b]	13.27 ± 1.26[b]
Energy intake (kJ/day/mouse)	61.14 ± 0.85	72.38 ± 2.06[a]	72.02 ± 1.03	76.7 ± 2.37	71.35 ± 1.47
Adipose tissues					
eWAT (mg)	863 ± 57	2688 ± 125[a]	2202 ± 52[c]	2229 ± 92[c]	2086 ± 167[b]
sWAT (mg)	299 ± 19	1084 ± 69[a]	915 ± 25[c]	802 ± 48[b]	720 ± 85[b]
Lipid metabolites in plasma					
TAG (mg/dl)	53.9 ± 4.05	73.72 ± 4.44[a]	66.65 ± 5.23	54.50 ± 4.32[c]	39.70 ± 3.41[b]
Total cholesterol (mmol/l)	1.48 ± 0.11	2.83 ± 0.15[a]	2.45 ± 0.13	2.42 ± 0.17[c]	2.45 ± 0.18
NEFA (mmol/l)	0.38 ± 0.03	0.62 ± 0.09[a]	0.38 ± 0.04[b]	0.44 ± 0.05[c]	0.39 ± 0.04[b]

Data are means ± SEM. Tissues and blood samples were collected at the end of the clamp, i.e. in the hyperinsulinemic state. sWAT was dissected from the dorso-lumbal region; [a]$p \le 0.05$ for difference from Chow (t-test); [b]$p \le 0.05$ for difference from cHF (One-way ANOVA); [c]$p \le 0.05$ for difference from cHF (t-test).

4.4.2. Omega-3 PL but not Omega-3 TAG improve insulin sensitivity and various aspects of glucose metabolism as assessed by hyperinsulinemic-euglycemic clamp.

High-fat feeding induced mild hyperglycemia, because plasma glucose levels assessed right before the clamping procedure, i.e. after 6 hours of fasting, were ~20 % higher as compared to lean Chow-fed controls (**Fig. 37c**). Both Omega-3 PL-supplemented diets, but not Omega-3 TAG-supplemented diet, significantly reduced glycemia in a dose-dependent manner, with the high-dose of KO in the K-H mice normalizing glucose concentrations to the level observed in lean Chow-fed controls (**Fig. 37c**). Furthermore, insulin sensitivity was significantly impaired in obese cHF-fed mice as compared to lean Chow-fed controls, as GIR, GTO, as well as whole-body glycolysis and glycogen synthesis were all decreased by ~75 %, ~60 %, ~40 %, and ~90 %, respectively (**Fig. 37d**). Only a tendency for increased HGP (i.e. a surrogate marker of IR in the liver) was observed in obese cHF mice, which was, however, largely due to the large variability of data within the Chow group. While cHF+F mice showed no significant changes in any of the parameters of glucose homeostasis (see above), in both the K-L and K-H group GIR, GTO, glycolysis and glycogen synthesis were partially restored by KO supplementation in a dose-dependent manner. However, HGP was normalized only in the K-H group (**Fig. 37d**).

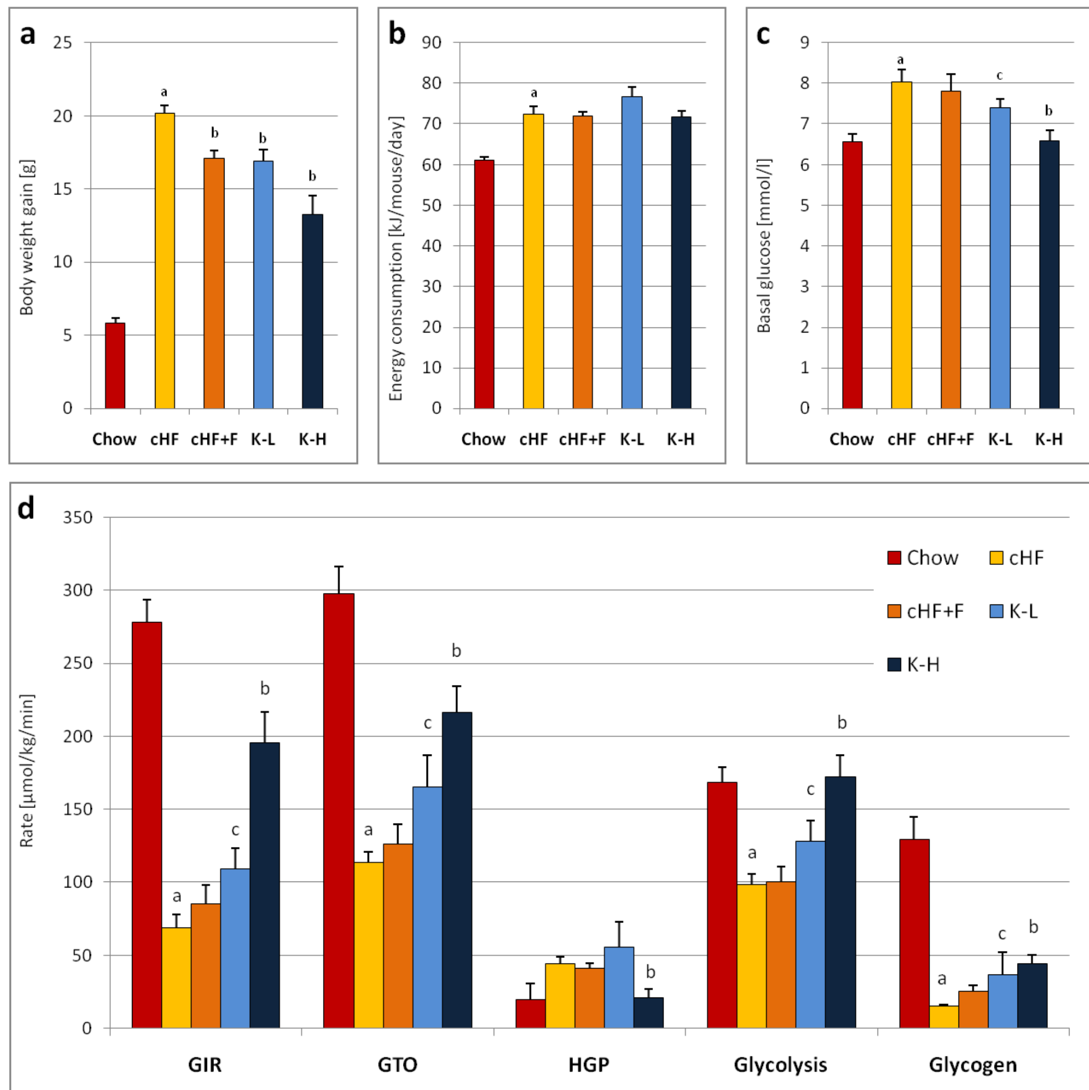


Fig. 37 Pub. D, Exp. 1. Insulin sensitivity and parameters of glucose metabolism assessed by the hyperinsulinemic-euglycemic clamp

Body weight gain (a) and average energy intake (b) were calculated after 8 weeks of dietary interventions. In the 8th week, insulin sensitivity of mice was analyzed by the hyperinsulinemic-euglycemic clamp technique, while various parameters of glucose metabolism were also assessed (d). Before the clamping procedure, mice were fasted for 6 hours and FBG was measured after tail incision (c). GIR, glucose infusion rate; GTO, glucose turnover; HGP, hepatic glucose production. [a]*p*≤0.05 for difference from Chow (t-test); [b]p≤0.05 for difference from cHF (One-way ANOVA); [c]p≤0.05 for difference from cHF (t-test).

5. Discussion

5.1. Publication A

In the Publication A, we investigated the metabolic effects of two high-fat diets with a similar energy content (~22 kJ/g), which however differed by their macronutrient composition, and in particular by the composition of lipids, i.e. they were based on either corn-oil rich in Omega-6 (the cHF diet) or pork lard rich in SFA and MUFA (the LHF diet). Furthermore, we also investigated whether and how the different types of FA contained in the background high-fat diet influence the metabolic effects of Omega-3 supplementation.

Direct experimental comparison of the cHF and LHF diet without Omega-3 supplementation demonstrated that despite the differences in the macronutrient and FA composition, the consumption of these two high-fat diets lead to comparable increases in the body weight gain and adiposity. Moreover, the effects of these diets were indistinguishable with regard to parameters of glucose homeostasis and insulin sensitivity assessed by the state-of-the-art technique hyperinsulinemic-euglycemic clamp. Such results were unexpected, since numerous publications have demonstrated deleterious effects of SFA on abdominal adiposity, inflammation, and insulin sensitivity in animal models of dietary obesity[143,144] as well as in humans consuming this type of FA[140,141]. Several studies, however, support our results, by showing that SFA contained in a mixed meal do not cause a higher body weight, adiposity, or inflammation when compared to PUFA[263,264]. It can also be speculated, that the total amount of lipids, which accounted for ~60 % of energy in both the cHF and LHF diet, masked the potential difference between the two diets, as was the case in the so called "KANWU study", where a differential impact of SFA and MUFA on insulin sensitivity of human subjects could be observed only when the total lipid content did not exceed 37 % of energy[139].

In our study, the cHF and LHF diets differed substantially only in the degree of ectopic lipid accumulation in the liver (i.e. hepatic steatosis). The potent induction of hepatic steatosis by SFA feeding is, indeed, firmly established in the literature[139,140,144,141,263]. The ectopic accumulation of lipids may be caused by alterations in a number of processes including the import, export, synthesis, as well as oxidation of FA; however, when assessed at the level of gene expression, none of the processes including FA transport, *de novo* synthesis of FA, mitochondrial and peroxisomal β-oxidation of FA, and VLDL synthesis were differentially affected by the cHF and LHF diet in our study. On the other hand, LHF diet increased gene expression of *Scd1* and *Elovl5* in the liver as well as the enzyme activity of SCD1 (evaluated as an increase in desaturation indices such as C18:1/C18:0 and C16:1/C16:0). Thus, up-regulation of SCD1 enzyme activity could explain an increased accumulation of liver lipids in the LHF mice, as the SCD1 deficiency has been linked to serious disruptions of *de novo* TAG synthesis[115,204], and MUFA synthesized endogenously by SCD1 are considered to be the main substrate for hepatic TAG and cholesterylesther synthesis[265]. We also observed that increased accumulation of TAG in the liver is associated with decreased plasma concentrations of TAG in the LHF mice, which could suggest a buffering role of the liver regarding the storage

of excessive lipids. This finding is, however, not supported by the literature, which shows that the over-expression of SCD1 is connected to hypertriglyceridemia in humans[266], while the SCD1 deficiency protects against the accumulation of TAG in liver, as well as in plasma[265].

It is also known that TAG accumulation associated with an increased SCD1 activity protects against lipotoxic effects of SFA[152,267]. Thus, the up-regulation of SCD1 activity in mice consuming a high-fat diet rich in SFA could represent a protective mechanism by which lipotoxic SFA are transformed into MUFA. This is also documented by the changes in FA composition; while the SFA content in both the LHF and LHF+F diets exceeds that in the cHF and cHF+F diets (**Fig. 24**), there are no differences in the SFA content measured in the fraction of hepatic PL and TAG between the LHF (and LHF+F) mice vs. cHF (and cHF+F) mice, but the content of MUFA rises in the LHF (and LHF+F) mice as compared to cHF (and cHF+F) mice (**Fig. 24)**. Such a protective mechanism could also explain why the LHF diet rich in SFA did not disrupt glucose homeostasis and insulin sensitivity more than the diet rich in Omega-6 (i.e. the cHF diet). These considerations are of utmost importance from the clinical point of view, since the inhibition of SCD1 has been shown to protect against obesity, hepatic steatosis, and dyslipidemia in obese rodents, and, therefore, pharmacological inhibition of SCD1 has been proposed as a treatment for obesity-associated metabolic disorders[268–270]. However, the beneficial effects of such treatment may cause further damage when combined with an inappropriate diet, as evidenced in the study of Brown et al., who showed that SCD1 inhibition in a mouse model of hyperlipidemia and atherosclerosis attenuated obesity, hepatic steatosis, and IR, but at the same time aggravated the atherosclerosis by promoting SFA accumulation and inflammation[271].

In our study, we also evaluated the metabolic effects of Omega-3 TAG supplementation, which proved to be partially dependent on the background diet. Irrespective of the background diet, Omega-3 supplementation proved to be efficient in reducing hepatic steatosis and plasma TAG concentrations. On the contrary, only on the Omega-6-rich dietary background (i.e. the cHF+F diet) Omega-3 managed to reduce adiposity, namely the weight of eWAT, decrease the size of adipocytes in eWAT, and improve glucose homeostasis, as evidenced by a decrease in fasting plasma insulin levels. Such effects have been also observed in our previous studies using the same model of dietary obesity in mice[194,198]. On the other hand, Omega-3 supplemented on the SFA-rich dietary background (i.e. the LHF+F diet) had no effect on adiposity and eWAT weight, and even tended to worsen IR (assessed as HOMA-IR index), as they did not influence plasma insulin concentrations and even increased FBG. It is important to note, however, that the energy intake in the LHF+F group was ~12 % higher when compared to either the LHF or cHF+F group.

Diet-independent effects of Omega-3 supplementation on the accumulation of hepatic lipids was not surprising, as it agreed with previously published articles[272–275], as well as with the consistent changes in the gene expression of selected enzymes of FA metabolism in the liver, namely with the reduction of lipogenic enzymes and the up-regulation of enzymes for mitochondrial as well as peroxisomal β-oxidation of FA. These effects were

likely linked to the ability of Omega-3 to regulate hepatic gene expression via direct suppression of pro-lipogenic SREBP-1c activity[273] and promotion of PPARα[208].

Omega-3-mediated regulation of gene expression was much less pronounced in eWAT, where Omega-3 supplementation induced no consistent changes in the expression of genes coding for enzymes involved in β-oxidation of FA. Suppression of pro-lipogenic genes by Omega-3 was more pronounced on the cHF than the LHF dietary background, which could partially explain the differences regarding the impact of Omega-3 supplementation on the eWAT weight. Interestingly, the suppression of *Scd1* expression was relatively strong in eWAT and it was independent of the background diet. It is also likely that the reduction of SCD1 activity by Omega-3 supplementation plays an important role in the Omega-3-mediated amelioration of hepatic steatosis; however, given the potentially protective role of SCD1 when mice are fed the SFA-rich diet, the reduced SCD1 activity in response to Omega-3 supplementation could also contribute to deteriorations of glucose metabolism observed in the LHF+F mice.

Obesity and the size of adipocytes have been shown to correlate with WAT inflammation and infiltration of the tissue with proinflammatory macrophages[276]. In agreement with this finding, we found that eWAT of LHF+F mice contained significantly more macrophages surrounding dead adipocytes (assessed as the number of CLS) when compared to the control LHF group. However, the absolute number of CLS measured in eWAT of mice fed different high-fat diets was quite low, which could be explained by a relatively short period of high-fat feeding[277]. The results of the immunohistochemical evaluation of eWAT were supported by changes in gene expression of various inflammatory markers, as the mRNA levels for MCP-1 (i.e. *Ccl2*) and inducible nitric oxide synthase (i.e. *Nos2*) decreased in the cHF+F as compared to cHF mice, but they tended to rise or remain unchanged in the LHF+F as compared to LHF mice. While the antigen F4/80 (i.e. *Adgre1*), another marker of proinflammatory M1 macrophages, was unchanged in the cHF+F mice, it increased significantly in the LHF+F as compared to either cHF or LHF mice. Since plasma concentrations of the anti-inflammatory and insulin-sensitizing hormone adiponectin were elevated in both Omega-3-supplemented groups (i.e. in the cHF+F and LHF+F mice), the beneficial metabolic effects of this hormone were probably suppressed by other factors. Taken together, Omega-3 supplemented onto the cHF background were more likely to alleviate WAT inflammation, while this effect was absent when Omega-3 were supplemented onto the LHF dietary background.

Given the known proinflammatory properties of SFA which can be neutralized by exogenously administered or endogenously produced MUFA[140,152,166], the negative regulation of SCD1 by Omega-3 could explain the background diet-dependent differences in the Omega-3-mediated suppression of WAT inflammation (i.e. the "SCD1 hypothesis"). Inflammation in WAT is believed to play a central role in the development of whole-body IR[52,277]; therefore, changes in WAT inflammation induced by Omega-3 supplementation on different dietary backgrounds may account for the corresponding differences regarding

the effects on glucose homeostasis. Without using the technique of hyperinsulinemic-euglycemic clamps, we were not able to localize the epicenter of the effects of Omega-3 supplementation on insulin sensitivity in this study (i.e. Publication A, Experiment 2); however, plasma NEFA concentrations in 6-hours-fasted mice were lowered by Omega-3 supplementation on both dietary backgrounds, although this decrease was not significant in case of the LHF+F mice (as compared to LFH controls); this possibly implies the absence of significant changes in insulin-mediated suppression of lipolysis in WAT and thus insulin sensitivity of the tissue. On the other hand, Omega-3 supplementation has been previously shown to ameliorate systemic IR by targeting primarily the liver[163,194,198], which seems to be more relevant to our study, since we observed significant increases in FBG, i.e. a surrogate marker of (mainly hepatic) gluconeogenesis, in the LHF+F mice as compared to either cHF+F mice or LHF controls. The accumulation of lipotoxic DAG in the liver is considered to be one of the most important pathophysiological factors in the development of hepatic IR. Therefore, in this context, the "SCD1 hypothesis" could be relevant also for the liver, since endogenously synthesized MUFA not only neutralize the inflammatory properties of SFA but also protect against the accumulation of DAG by promoting re-esterification of FA and TAG synthesis[166]. This concept is also in agreement with the general opinion, that the capacity to neutralize lipids by storing them as TAG protects the organism against lipotoxicity, even in case of ectopic lipid accumulation, which could represent in some instances a protective mechanism rather than the primary cause of metabolic damage.

Finally, the differences in other macronutrients contained in the cHF and LHF diet must be taken into consideration. Thus, the cHF diet was based exclusively on vegetable oils and was, therefore, free of cholesterol, while the LHF diet contained 290 mg cholesterol per kg diet. Interestingly, plasma cholesterol concentrations increased in both groups fed the parental high-fat diets (cHF and LHF), independently of the cholesterol content in the diet. However, a plausible explanation for elevated cholesterol levels in mice consuming the cHF diet is lacking. In terms of the protein and carbohydrate content, the LHF diet contained more proteins (i.e. 21 vs. 12 weight %), and also contained sucrose (9 weight %), while carbohydrates in the cHF diet consisted mainly of starch and lactose. Sucrose is considered a highly obesogenic and lipogenic nutrient[278–280], and high-sucrose diets are often used to induce hepatic steatosis in animal models[278]. The study of Ma et al. showed that a high dietary content of sucrose (~45 weight %) counteracted the anti-inflammatory effects of Omega-3 supplementation, but did not affect the suppression of hepatic steatosis[280]. These results closely resemble the data from our study; however, it is important to note that the high-sucrose diets used in the above-mentioned publications had the sucrose content ranging from 45 to 60 weight %, and thus only 9 % of sucrose in our LHF diet was unlikely to exert such deleterious effects on metabolism.

5.2. Publication B

In the Publication B, we investigated the impact of long-term Omega-3 administration on various components of the incretin system. The pilot study (i.e. the experiment 1) showed, that in agreement with our previously published results[194,196,231], high-fat feeding for 8 weeks induced obesity, glucose intolerance, and IR in male C57BL/6N mice, which also manifested as an increase in both fasting and glucose-stimulated concentrations of insulin. Omega-3 TAG supplementation on the background of a high-fat diet (i.e. cHF) led to a partial normalization of the metabolic state, including a decrease in the body weight gain, improvements in glucose tolerance, and a significant decrease in fasting plasma insulin concentrations, as compared to obese cHF controls.

In the Experiment 1, obese mice with and without Omega-3 supplementation (i.e. cHF+F and cHF mice, respectively), as well as lean Chow-fed controls, were subjected to two different variants of glucose tolerance test in order to compare insulin secretion in response to either intraperitoneal or oral glucose challenge, and the insulin response was quantified as a difference between the fasting and glucose-stimulated state (i.e. Δ(T30´ - T0´)). When compared to obese cHF-fed controls, Omega-3 supplementation increased glucose-stimulated insulin levels irrespective of the route of glucose administration; however, the effect was much stronger when glucose was applied orally, thus leading to ~70 % higher plasma insulin concentrations in the cHF+F than in cHF mice, corresponding to a significant improvement in glucose tolerance (quantified as the AUC). In contrast, following the intraperitoneal glucose administration, glucose tolerance was not significantly improved in the cHF+F mice, while the glucose-stimulated plasma insulin concentrations were equal between the cHF and cHF+F group 30 min after glucose administration. In this context, the published data describing the direct effect of Omega-3 on glucose-stimulated insulin levels (or GSIS) are scarce and controversial; however, the interaction of glucose and FA regarding the stimulation of insulin secretion is generally well described. Presence of FA in physiological concentrations is essential for the functional GSIS[281]. However, prolonged elevation of NEFA in the circulation impairs GSIS by the lipotoxic damage of pancreatic β-cells[282]; in contrast, the acute elevation of NEFA, such as in the fasting state, has the opposite effect[282]. The potency of FA to augment GSIS depends on the degree of saturation and the chain length of FA, with long-chain SFA being the most effective[283,284]. Thus, regarding their immediate effects, Omega-3 are not likely to mediate stronger GSIS than other types of FA.

However, the long-term administration of Omega-3 has been shown to improve GSIS in several models, including transgenic *Fat-1* mice that express *Caenorhabditis elegans Fat-1* gene encoding *ω-3* desaturase, an enzyme involved in the conversion of Omega-6 into Omega-3. Thus, the *ex vivo* study using pancreatic islets isolated from the *Fat-1* mice showed increased GSIS when compared to wild-type mice[285], while *in vivo* the *Fat-1* mice were protected against streptozotocin-induced β-cell damage and hypoinsulinemia[286]. EPA has been shown to protect insulin-producing pancreatic β-cells against palmitate-induced

lipotoxic cellular damage and disruption of insulin signaling pathway, which is otherwise connected to the long-lasting reduction of GSIS through suppression of the activity of the transcription factor SREBP-1c[287]. Apart from suppressing SREBP-1c activity, there is a [288288]whole range of plausible molecular mechanisms, which could lead to Omega-3-mediated increase in GSIS, as suggested by the comprehensive review from Wang and Chan from 2015[288]; however, more mechanistic studies are needed to confirm these hypotheses.

Omega-3-mediated protection against β-cells damage could partially explain the increase in GSIS after both intraperitoneal and oral glucose administration; however, the magnitude of the response to orally administered glucose suggests an involvement of yet another factor, which is triggered by the passage of glucose through GIT. Therefore, we hypothesized that long-term Omega-3 administration stimulates the activity of the incretin system, which in turn increases secretion of insulin after ingestion of nutrients.

Therefore, in the Experiment 2., we focused directly on the incretin system, i.e. on the two most studied incretin hormones GLP-1 and GIP, as well as on their degrading enzyme DPP-4. Several studies showed GLP-1 levels to be decreased in obesity and T2DM, suggesting that decreased GLP-1 secretion could lead to the disruption of incretin effect in obese and T2DM individuals[100,289,290]; however, this was not the case in our study. We measured the concentrations of both active and total (i.e. active + truncated) forms of GLP-1. When compared to lean Chow-fed controls, the glucose-stimulated levels of total GLP-1 were significantly increased in obese mice and not significantly in cHF+F. The analysis of active GLP-1 was hampered by intense degradation, which was not prevented even though the blood was collected directly from the portal vein and the DPP-4 inhibitor was added immediately after the blood collection; nevertheless, the obtained data suggested an increase of active GLP-1 in the cHF and cHF+F groups, as compared to Chow.

Incretins are able to execute most of their biological functions only in the active state before they are degraded by the ubiquitous protease DPP-4[65]. The activity of DPP-4 has been shown to be increased in obese mice as well as humans[111,291,292], while the inhibition of DPP-4 has already been introduced to the clinical practice as an efficient way to increase the activity of the incretin system[132]. Correspondingly, we observed an increased activity of DPP-4 in both plasma and the gut of obese cHF control mice, but also in the cHF+F mice, as compared to lean Chow-fed controls. We also analyzed the activity of DPP-4 in WAT; when normalized to tissue weight, the DPP-4 activity in eWAT was, like in the gut and plasma, comparable between the cHF and cHF+F mice. However, since Omega-3 administration led to a decrease in eWAT weight and total adiposity, reducing the overall activity of DPP-4 in WAT could be a mechanism by which Omega-3 beneficially influence the activity of the incretin system. It is unlikely, however, that a decreased activity of DPP-4 in WAT would influence the plasma concentrations of active GLP-1, since the majority of GLP-1 gets degraded immediately after entering the blood stream, thus causing GLP-1 to act in a highly localized manner[65]. Comparable concentrations of total GLP-1 together with the comparable

activity of DPP-4 in plasma and the gut in both the cHF and cHF+F mice suggest that Omega-3 could hardly execute their beneficial metabolic effects by increasing the concentration of active GLP-1.

Absence of Omega-3 effect in terms of increased glucose-stimulated GLP-1 levels may be a surprising finding, as Omega-3 have been reported to stimulate GLP-1 release from the enterocrine L-cells by the activation of GPR120 receptor, which was observed both *in vitro* and *in vivo*[214,293]. However, only ALA, the most potent of different Omega-3 functioning as ligands of GPR120, has been also shown to increase GLP-1 secretion following its oral administration in C57BL/6 mice[214], while the EPA- and DHA-induced effects have been observed only after the intracolonic application[89]. Such results suggest that the physiological significance of the direct interaction of Omega-3 with GPR120 on the surface of L-cells is doubtful. Most FA are ingested in the proximal segment of the small intestine, while the highest concentration of L-cells is found in the colon; however, L-cells are scattered throughout the entire length of the intestine, and thus the involvement of a minority of these cells, located in the proximal intestinal segments, could still play a significant role in the effects of Omega-3. Nevertheless, direct stimulation of GLP-1 release by activation of GRP120 would be relevant in the experimental setup, where glucose would be co-administered with Omega-3. This was not the case in our study, where Omega-3 were supplemented in the diet, while the glucose challenge was performed in mice that were fasted for several hours. GLP-1 could, however, still be involved in the stimulatory effects of chronic Omega-3 supplementation on glucose-induced insulin levels observed in our study, as palmitate-induced lipotoxicity in β-cells has been associated with down-regulation of GLP-1R expression, while EPA restored insulin secretion in response to glucose as well as GLP-1 administration[294]. Thus, in the cHF+F mice with Omega-3 supplementation, GLP-1-mediated insulin secretion could be enhanced, even though the concentration of GLP-1 itself was not increased.

While we observed no changes in plasma GLP-1 levels, which could potentially explain the increase in glucose-stimulated insulin levels in mice fed Omega-3-supplemented cHF+F diet, the changes in plasma GIP concentrations showed an interesting pattern. GIP is more resistant towards the cleavage by DPP-4, and a significant amount of active GIP remains in the blood stream to act on the systemic level. GIP levels are consistently reported to be elevated in obesity[97,295,296]; however, the prediabetic and diabetic states are associated with GIP resistance, when even supraphysiological doses of GIPR agonists are unable to increase the early-phase of insulin secretion[103]. In the Experiment 2., glucose-stimulated levels of total GIP were increased in both groups of animals fed a high-fat diet, i.e. in the cHF and cHF+F mice, as compared to lean Chow-fed controls; however, Omega-3 supplementation tended to normalize plasma GIP concentrations; in this regard, the absence of a significant difference between the cHF and cHF+F mice was mainly due to large in-group variations. Quite recently, a similar finding was made in obese men treated by the combination of Omega-3 and calorie restriction[297]. These results were supported in our study by the corresponding changes in *progip* gene expression in the gut, namely in the proximal

segments of the small intestine and colon. In accordance with the literature[65], the *progip* expression in the proximal segment of colon was relatively weak when compared to the proximal segment of the small intestine; however, its contribution to the overall GIP secretion could still be significant.

GIP is not just a mediator of the incretin effect, but it also possesses adipogenic properties; its secretion is strongly stimulated by FA[90,298], and, in turn, GIP stimulates lipid storage in adipocytes[93–95]. Hypersecretion of GIP in obesity as well as administration of GIPR agonists is linked to increased adiposity and body weight[96–98]. High tonus of adipogenic GIP signaling in obesity is partially caused by increased expression of GIPR in WAT, which is mediated by PPARγ and correlates with the amount of WAT mass[92]. In our study, *Gipr* expression in eWAT was significantly increased in the cHF but not in the cHF+F group. Since long-term dietary intake of Omega-3 also decreased the eWAT weight without lowering energy intake, it is plausible that part of this anti-lipogenic effect was mediated by a suppression of GIP signaling in WAT, possibly as a response to lower levels of GIP in plasma and a reduced number of GIPR in WAT.

Assuming that increased levels of GIP in plasma compensate for the development of GIP resistance, Omega-3 supplementation could stimulate the activity of the incretin system by alleviating the resistance towards GIP. As the GIP resistance manifests as a decrease in GIPR expression in pancreatic β-cells[105,106], we performed immunohistochemical staining of insulin and GIPR using histological sections of pancreatic tissue; however, we found no difference in GIPR number between the Chow-fed lean and cHF-fed obese controls (data not shown). Therefore, diet-induced obesity in C57BL/6 mice does not seem to be a suitable model for an assessment of the effect of Omega-3 on GIP resistance. This conclusion is not surprising given the recent publications showing that GIP resistance is likely caused by long-term hyperglycemia typical for pre-diabetic and diabetic conditions rather than obesity as such[105]. In agreement with the work of Irwin et al.[109], we observed that in obesity GIP hypersecretion and GIP resistance are not connected. Nevertheless, the possibility to alleviate GIP resistance by Omega-3 supplementation is highly relevant, since Omega-3 are potent activators of the transcription factor PPARα that is involved in sensitization to GIP after the normalization of hyperglycemia[107]. This hypothesis should be pursued using more appropriate model.

The results of the Experiment 2 suggested that long-term Omega-3 administration could influence GIP secretion from the intestine. Therefore, in the next Experiment 3, C57BL/6 mice were fed the cHF diet to induce obesity before the initiation of Omega-3 supplementation in order to verify the effects of Omega-3 on GIP using a model of "reversal of obesity". The data confirmed the results of both previous experiments, i.e. it showed a marked increase in fasting plasma insulin in the cHF group, which was partially normalized by Omega-3; furthermore, the change in plasma insulin levels in response to glucose administration during oGTT, quantified as the delta value of insulin levels measured in the baseline (fasting) conditions and after glucose administration, was significantly increased in the cHF+F group,

again confirming the previous results. Importantly, using this experimental setup, a clear effect of Omega-3 supplementation on glucose-stimulated GIP levels was detected, as GIP hypersecretion observed in obese cHF-fed controls was markedly reduced in the cHF+F mice, almost reaching the levels of lean Chow-fed controls.

Taken together, we were not able to link increased glucose-stimulated insulin levels (potentially reflecting the increased level of GSIS) with corresponding changes in plasma levels of incretin hormones in Omega-3-treated mice. We did not detect either increased GLP-1 secretion, decreased activity of the degrading enzyme DPP-4, or changes in sensitivity towards GIP. However, we have found that long-term Omega-3 supplementation reduces GIP signaling by partially normalizing obesity-induced GIP hypersecretion and decreasing the expression of GIPR in WAT, which could contribute to lipid-lowering effects of dietary Omega-3 intake.

5.3. Publication C

In the Publication C, we investigated metabolic effects of Omega-3 PL (i.e. the PC group), a herring-derived Omega-3 concentrate that is particularly rich in PC and compared these effects with those of the anti-diabetic TZD drug rosiglitazone (i.e. the R group) as well as those elicited by the combined administration of Omega-3 PL and rosiglitazone (i.e. the PC+R group). The TZD drug class has a great potential in the restoration of insulin sensitivity, as it mediates storage of potentially lipotoxic compounds in the form of neutral lipids via activation of PPARγ transcription factor[116]; however, many TZD drugs, including rosiglitazone, have been banned from the market due to their adverse effects[118]. By lowering their dosage and combining them with natural products, TZD could still provide insulin-sensitizing properties while potentially exerting synergistic metabolic effects of the combination treatment and avoiding adverse effects of the therapy at the same time.

In the Experiment 1., we have shown that dietary supplementation with Omega-3 PL improved various metabolic disorders that are otherwise induced by high-fat feeding in C57BL/6 mice; namely, Omega-3 PL prevented the weight gain, dyslipidemia, glucose intolerance, hyperinsulinemia and WAT inflammation, and lowered the deposition of lipids both in WAT and in the liver. In accordance with our previous study, in which we compared the effects of Omega-3 PL and Omega-3 TAG[231], the effect of herring-derived Omega-3 PL on weight gain and adiposity could not be explained by changes in energy intake or thermogenic activity of brown adipose tissue, as neither the weight of this tissue nor gene expression of thermogenic uncoupling protein 1 was increased in the PC group (data not shown). Losses of lipids in stool, which were actually lower in the PC mice, are also unlikely to play a role. Reduction in adiposity and body weight could be due to increased β-oxidation of FA in the liver, as suggested by the work of Fiamoncini et al.[299]. Accordingly, the expression of genes encoding enzymes of mitochondrial and especially peroxisomal FA oxidation was markedly up-regulated in the livers of the PC mice in our study.

In our previous study[231], we investigated the effects of two different doses of Omega-3 PL. Although the content of EPA/DHA was 2-fold and 6-fold higher, and the content of total PL was 1.4-fold and 4.3-fold higher, in the groups with low- and high-dose Omega-3 PL supplementation, respectively, the effects on body weight and lipid accumulation in WAT and the liver were weaker when compared to our current study. This difference could be due to a different composition of the PL concentrate, as the PC diet contained higher amounts of lysophosphatidylcholine that is known to be well absorbed in the intestine, thus enhancing the bioavailability of lysophosphatidylcholine-bound FA[300].

Furthermore, in our previous publications, we showed that Omega-3 TAG could improve hepatic insulin sensitivity[194,198]. When combined with the TZD drug rosiglitazone, Omega-3 TAG were able additively reduce the weight gain, adiposity, WAT inflammation, and dyslipidemia, and to increase plasma concentrations of the insulin-sensitizing hormone adiponectin while improving muscle insulin sensitivity[198]. In our current study, we used the same low dose of rosiglitazone, which by itself could not ameliorate IR, but it increased the expression of lipogenic genes in the liver and induced mild hepatic steatosis in the R mice, as observed before[301]. In fact, all of the previously seen additive effects of the combination treatment with rosiglitazone and Omega-3 TAG have been recapitulated in this study using Omega-3 PL, while the lipogenic properties of rosiglitazone were completely overridden by the effect of Omega-3 PL in the combination PC+R group. The DNA microarray analysis of gene expression in the liver revealed a complex regulation of several metabolic pathways that were markedly affected by Omega-3 PL; thus, in regard to their effect on the deposition of lipids in the liver, the pathway of β-oxidation of FA was enhanced, while *de novo* lipogenesis was strongly suppressed in both the PC and PC+R group. Both these metabolic pathways are likely to contribute to the reduction of hepatic lipids in response to Omega-3 PL supplementation, as the involvement of *de novo* lipogenesis in the development of hepatic steatosis is well established[302]. The lowering of FA delivery to the liver could be another contributing factor, since the concentration of plasma NEFA was significantly reduced in the PC and PC+R group, possibly due to improvement of insulin sensitivity in WAT. We also examined the rate of VLDL production, which could also influence the deposition of hepatic lipids; however, dietary Omega-3 PL had no impact on VLDL secretion (data not shown). It should be noted that Omega-3 TAG were not capable of reducing cHF-induced hepatic steatosis in our previous studies[198,231], despite the dietary EPA/DHA content being several-fold higher than in the PC diet used in our current experiment. Also the lowering of plasma TAG in the PC and PC+R mice was probably a consequence of a shift from anabolic to catabolic metabolism of FA in the liver and possibly in other tissues, such as the small intestine and WAT[196,198], since it could not be explained by the loss of lipids in stool.

As revealed by DNA microarray analysis and then confirmed by qPCR, Omega-3 PL caused the biggest changes in the pathway of cholesterol metabolism. The expression of cholesterol biosynthetic genes was consistently reduced, while the expression of *Scarb1* and *Abcg5,* which take part in cholesterol excretion to the intestines, was increased. Correspondingly,

both plasma and liver cholesterol levels were significantly reduced, as was, surprisingly, cholesterol excretion in the feces. However, this phenomenon could be a result of cholesterol-saving compensatory mechanisms at the level of enterocytes in the small intestine, which have been observed in mice fed a low-cholesterol or cholesterol-free high-fat diet[303,304]. These results also correspond to the data in a published study, in which dietary supplementation with KO, i.e. an alternative source of Omega-3 PL, down-regulated the expression of cholesterol biosynthetic genes in lean Chow-fed mice, while Omega-3 TAG-containing fish oil had an opposite effect[305]. The only difference was that in the published study the expression of *Srebf2*, which is the major transcriptional regulator of cholesterol biosynthesis genes, was down-regulated[305], while in our study the expression of *Srebf2* remained unchanged.

Omega-3 PL supplementation also regulated gene expression of enzymes associated with the metabolism of BA in the liver. Namely the expression of *Cyp7a1*, i.e. the key enzyme in bile acid synthesis, was strongly down-regulated, while that of *Nr0b2*, which encodes small heterodimer partner SHP that is involved in the repression of *Cyp7a1* and BA synthesis[306], was increased. Despite of these changes in gene expression, the BA content in the liver of PC mice was increased; thus, the precise regulation of BA pool in response to dietary Omega-3 PL supplementation remains to be clarified.

Apart from EPA and DHA, Omega-3 PL molecules contain other bioactive compounds represented by the PL backbone itself. In particular, PC alone has been shown to alleviate hepatic steatosis[251–253,307], decrease hepatic FA synthesis[308], and affect intestinal cholesterol absorbtion[255]. Thus, to evaluate the potential contribution of PC itself to the overall metabolic effects of the Omega-3 PL concentrate, we compared two different PC-rich concentrates including Omega-3 PL of marine origin (i.e. the PC-M group) and the PL concentrate derived from soy (i.e. the PC-S group). In both of these products, PC represented the major PL fraction, however, the composition of PL-bound FA was completely different. Thus, the PC-M contained mainly EPA and DHA, and a minor portion of PA, while PC-S was particularly rich in LA with minor content of PA, stearic acid (C18:1), and ALA. Surprisingly, in contrast to PC-M, PC-S completely failed to prevent the weight gain, body fat accumulation, IR, dyslipidemia, and hepatic steatosis, and it failed to down-regulate hepatic expression of genes encoding FA synthesis and cholesterol biosynthesis enzymes. This would suggest that EPA and DHA are the only components necessary for the induction of the above-mentioned metabolic effects; however, as shown in our previous study[231], even the higher dose of EPA and DHA (~30 g/kg of the diet) in the form of Omega-3 TAG failed to significantly down-regulate hepatic genes involved in FA synthesis and cholesterol biosynthesis in association with a much weaker effect on hepatic and plasma lipids when compared to Omega-3 PL (unpublished data). Thus, it seems that it is primarily the combination of marine FA, i.e. EPA and DHA, and the proper lipid carrier, i.e. the PC-rich PL as in this case, which provides the maximum metabolic effect of Omega-3-based dietary supplements.

In summary, Omega-3 PL improved various aspects of obesity-related metabolic comorbidities in the manner that is significantly more efficacious than that of either Omega-3 TAG or PL themselves. In an agreement with the comprehensive review of Burri et al.[236], the integrated repression of genes encoding the enzymes for *de novo* lipogenesis and cholesterol biosynthesis seems to be the main mechanism by which Omega-3 PL regulated lipid content in the liver. Both Omega-3 and PC have been shown to act as ligands for the transcription factor PPARα. Accordingly, in our study, both mitochondrial and peroxisomal β-oxidation of FA was up-regulated in the liver of Omega-3 PL-treated mice. Thus, the improvement of hepatic steatosis as well as dyslipidemia at the systemic level can be associated with the shift from anabolic to catabolic metabolism of lipids. Several molecular mechanisms that might explain the difference between Omega-3 TAG and Omega3 PL have been described and they will be discussed in detail in the discussion section of the Publication D. In the presented study (i.e. Publication C), the co-administration of the low dose of rosiglitazone and Omega-3 PL was primarily used as an instrument to study the molecular mechanisms of Omega-3 PL actions, since rosiglitazone itself has been already banned from the market; however, several synergistic effects of the combination treatment have been observed and all of the less wanted effects of rosiglitazone, e.g. the induction of *in situ* lipogenesis and mild hepatic steatosis, were suppressed by the co-administration of Omega-3 PL. Thus, the combination of some alternative types of TZD and Omega-3 PL may still be of interest for potential clinical use, but it requires further studies. Our current study also showed that Omega-3 PL either alone or in combination with TZD drugs may be efficient in the treatment of patients with non-alcoholic steatohepatitis.

5.4. Publication D

It has been already shown in articles published by our group[231] as well as others [230,232,309–311], that various metabolic effects of Omega-3 PL are superior to those of Omega-3 TAG. In this project, we focused on the effect of KO-derived Omega-3 PL on insulin sensitivity assessed by hyperinsulinemic-euglycemic clamps, i.e. the gold standard in assessing IR and glucose homeostasis in general, using our well-established model of diet-induced obesity in C57BL/6 mice[194,196,231]. The dose of EPA+DHA contained in the Omega-3 TAG concentrate, i.e. the type of Omega-3 supplementation used in the cHF+F group, was consistent with our previous publications[231] and was equal to 30 mg/g diet; in case of KO supplementation, it was set to match either the total amount of the Omega-3 TG concentrate used to replace part of corn oil in the cHF+F diet or the total amount of EPA+DHA in the cHF+F diet, thus resulting in a dose of 10 mg or 30 mg EPA+DHA/g diet in the K-L and K-H groups, respectively.

In agreement with our earlier studies[231,312], both Omega-3 TAG and Omega-3 PL were able to reduce the body weight gain and adiposity without causing a reduction in energy intake. As we showed in the previous study with Omega-3 PL[312], neither lipid losses in stool nor changes in thermogenic activity of brown adipose tissue could explain the decrease in the body weight gain, while the Omega-3-induced suppression of *de novo* lipogenesis and,

on the other hand, activation of β-oxidation of FA represent a plausible explanation. Moreover, we have shown that dietary Omega-3 decrease obesity-induced elevations of plasma GIP levels and increased GIPR concentrations in eWAT, which could also contribute to the lowering of WAT weight, as GIP represents a lipogenic and adipogenic hormone[93–95], the activation of which is linked to the development of obesity[96–98].

In healthy individuals, the hyperinsulinemic state should be marked by maximal stimulation of glucose uptake, glycolysis, glycogen synthesis, and, on the other hand, a lowering of hepatic gluconeogenesis and lipolysis. As expected, in our study high-fat feeding induced a variety of disturbances associated with IR in the skeletal muscle and the liver. Due to a significantly lower whole-body GTO, less exogenous glucose was needed to maintain euglycemia in hyperinsulinemic conditions, while the glucose-consuming processes, i.e. the whole-body glycolysis and glycogen synthesis, were also decreased in the cHF group. The level of HGP (i.e. endogenously produced glucose), which could be used as an indirect marker of hepatic IR[313], tended to be increased in obese cHF-fed mice, which was, however, largely due to a high variability of data within the Chow group. Finally, the concentration of plasma NEFA in hyperinsulinemic conditions was also increased in the cHF group, thus suggesting the development of IR also in WAT[17,18].

Despite marginal body weight- and adiposity-lowering effect, Omega-3 TAG were much less efficient in ameliorating IR, as shown by the results obtained by hyperinsulinemic-euglycemic clamps. This does not correspond with our previous results using the same method, in which the comparable dose of Omega-3 TAG significantly lowered HGP in hyperinsulinemic conditions in obese cHF-fed mice[194]. Such discrepancy can be explained by slight differences in the inbred strain of mice used in the respective studies; thus, in the current study, we used C57BL/6N mice from the Charles River Laboratories (Sulzfeld, Germany), which are particularly prone to the development of diet-induced obesity and exert substantial and homogenous body weight gains when fed a high-fat diet, while in the previous study, we used C57BL/6J mice bred in our animal facility, which respond to high-fat feeding less markedly and with a higher heterogeneity. In the current study, cHF+F mice gained ~17 g as compared to 1.2 g in the former study[194]; thus, higher metabolic burden could hide the potential metabolic benefits of Omega-3 TAG supplementation, which are relatively mild. However, the ability of fish oil, i.e. the most common source of Omega-3 TAG, to improve insulin sensitivity is still a subject of controversy, as fish oil supplementation has been shown to improve insulin sensitivity and glucose tolerance in animal models[314–316] and in some human studies[317–320], while other human studies fail to show the benefits of fish oil supplementation in terms of improvement of insulin sensitivity[321–323].

On the contrary, Omega-3 PL supplementation in the form of KO improved in a dose-dependent manner various aspects of glucose homeostasis and insulin sensitivity, while the lower dose of KO (i.e. in the K-L group) proved to be more efficient than Omega-3 TG supplementation in the cHF+F mice, despite the fact that dietary content of EPA+DHA in the K-L group was ~3-fold lower as compared to the cHF+F group. Current

research focuses mainly on the impact of dietary supplementation with KO on dyslipidemia and hepatic lipid metabolism, while studies examining the impact of KO on insulin sensitivity are quite scarce. Despite this, KO supplementation was shown to improve glucose tolerance in dietary obese mice[231], obese castrated rabbits[239], as well as in healthy young people[324], while reducing HOMA-IR in T2DM subjects[325]. Unlike our current study, Ivanova et al. showed that in the rabbit model of castration-induced obesity, KO and fish oil induced comparable metabolic effects even when administered at comparable EPA+DHA doses[239]. This result is, however, quite rare, as other studies showed that Omega-3 PL achieved comparable metabolic changes as Omega-3 TAG, but using lower doses of EPA+DHA[309,326].

Increased plasma NEFA are believed to result in the development of IR in the early stages of metabolic disease, while the normalization of NEFA levels leads in some studies to the restoration of insulin sensitivity[21,22]; however, more factors must be involved in case of our study, as both Omega-3 TAG and Omega-3 PL as KO comparably lowered plasma NEFA levels in the hyperinsulinemic state. The development of IR is also triggered by cytokines and metabolites produced by various cell types in obese WAT with low-grade chronic inflammation[52]. As shown in several of our previous publications, diet-induced obesity in C57BL/6 mice is associated with the accumulation of pro-inflammatory M1 macrophages in WAT as well as the elevation in inflammatory markers[327]. While Vigerust et al. observed no significant effect of either fish oil or KO on inflammatory markers in hTNFα transgenic mice that represent an animal model of low-grade chronic inflammation[311], other studies showed KO to be effective in suppressing chronic inflammation in the animal model of ulcerative colitis[328] as well as in patients suffering from arthritis[329]. It was suggested that KO could ameliorate systemic inflammation by strengthening intestinal epithelial integrity[330]. Dietary Omega-3 were also shown to lower the content of AA in plasma membrane PL, thus decreasing the substrate pool for the synthesis of pro-inflammatory eicosanoids and EC[231,327]. Finally, Liu et al. examined metabolic effects of marine PL rich in EPA, which significantly improved glucose tolerance in dietary obese mice, while decreasing markers of inflammation and increasing the levels of insulin-sensitizing hormone adiponectin[331].Since the suppression of low-grade chronic inflammation might play an important role in the restoration of insulin sensitivity by Omega-3 PL, a detailed analysis of inflammatory markers and adiponectin concentrations will be performed in this study.

We have shown in our previous publication[312] that Omega-3 are indispensable for the effect of Omega-3 PL in terms of restoring lipid homeostasis in the liver, since soy-derived PC by itself had no beneficial effect on metabolism in obese C57BL/6 mice. Here we can show that the effects of Omega-3 PL supplementation were superior to the effects of Omega-3 TAG, even though the dose of EPA/DHA was equal (i.e. in the K-H group) or even lower (i.e. K-L group) in the former type of Omega-3 supplementation. The molecular mechanisms behind the superior effects of Omega-3 PL, especially on glucose homeostasis, are not yet fully understood; however, the literature offers several explanations. As have been shown by our [231], as well as other laboratories[240,310,326,332], dietary supplementation with Omega-3 esterified to PL is characterized by an increased bioavailability of EPA and DHA at

the level of cellular membranes, which is likely linked to different partitioning of digested lipids between nascent apolipoprotein species and their layers (see also section 1.6.1. Triacylglycerols versus phospholipids: Differences in the molecular structure and digestion). Moreover, KO is rich in carotenoid astaxanthin, an antioxidant that can increase the bioavailability of functional Omega-3 by protecting them from oxidative degradation[235] while exerting neuroprotective and anti-inflammatory actions on its own[259–261]. Although we observed no beneficial metabolic effects of soy-derived PC[312], other studies show that PL *per se* may exert various biological effects, e.g. by serving as ligands of PPARα[333] and other nuclear receptors[334]. Apart from containing the PL moieties, the EPA/DHA ratio is higher in KO as compared to Epax 1050 TG (i.e. the Omega-3 TAG concentrate used in our studies; see also section 3.1.1. and ref.[235]), which could also play a role in the differential metabolic effects of these Omega-3 concentrates. Finally, dietary supplementation with KO is associated with a relatively strong suppression of the EC system activity in animals[231,336] as well as in men[237,337], which is probably linked to the replacement of AA in cellular membranes (see above and ref.[336]).

In summary, Omega-3 PL-containing KO but not Omega-3 TAG markedly and dose-dependently prevented IR in obese C57BL/6 mice, as revealed by the results of hyperinsulinemic-euglycemic clamps. This project will continue with a detailed analysis of inflammatory markers and the EC system activity in order to explain at the molecular level the differences in the effects of Omega-3 TAG vs. Omega-3 PL on insulin sensitivity in mice with diet-induced obesity.

6. Conclusions

According to the specific aims of this thesis, the following conclusions can be made:

Two obesogenic diets that were similar in energy content and total amount of lipids, but differed mainly in the composition of fatty acids, i.e. the cHF diet was based on Omega-6 while the LHF diet contained primarily SFA and MUFA, induced a comparable weight gain, the level of IR and glucose intolerance, and WAT inflammation. They, however, differed in the impact on accumulation of lipids in the liver. Severe hepatic steatosis was, in fact, the main distinguishing factor of LHF-fed animals. It could be linked to up-regulation of SCD-1 activity, which could enable transformation of potentially harmful SFA into MUFA, thus protecting the organism against inflammation and lipotoxic damage.

Metabolic effects of long-term Omega-3 TAG supplementation proved to be partially dependent on the composition of lipids in the background diet. Irrespective of the background diet, Omega-3 supplementation proved to be efficient in reducing hepatic steatosis and plasma TAG concentrations. On the contrary, only when the animals were fed the Omega-6-rich diet (i.e. the cHF+F diet), Omega-3 supplementation reduced adiposity and improved glucose homeostasis. On the other hand, Omega-3 supplemented within the SFA-rich diet (i.e. the LHF+F diet) had no effect on adiposity and even tended to worsen WAT inflammation and IR assessed as HOMA-IR index. It was hypothesized that the suppression of SCD-1 activity by Omega-3 TAG could be harmful when substantial amounts of SFA are present in the diet.

Long-term supplementation of obese C57BL/6 mice with Omega-3 TAG leads to increased plasma concentrations of insulin after oral load of glucose; however, the involvement of the incretin system in this effect is still unclear, since we were not able to detect a substantial increase in total GLP-1 secretion or decrease in the DPP-4 degrading enzyme activity. It is still possible that plasma concentrations of active GLP-1 will be different between mice fed the Omega-3-supplemented diet as compared to high-fat diet-fed controls. Also alleviating the resistance towards GIP by Omega-3 TAG could not be confirmed, as the GIP resistance was not present in our model of "*prevention of obesity*". On the other hand, Omega-3 TAG normalized GIP over-secretion and GIPR overexpression in WAT of mice with obesity established prior to the initiation of Omega-3 feeding. These effects could represent novel molecular mechanisms by which Omega-3 TAG influence whole-body adiposity.

Marine omega-3 PL improved various aspects of obesity-related metabolic comorbidities in the manner that is significantly more efficacious than that of Omega-3 TAG. Using the DNA microarray analysis and subsequent re-evaluation by qPCR we have revealed integrated repression of genes encoding the enzymes of *de novo* lipogenesis and cholesterol biosynthesis in the liver while the genes for both mitochondrial and peroxisomal β-oxidation enzymes were up-regulated. Thus, the marked improvement of hepatic steatosis as well as systemic

dyslipidemia by marine PL can be associated with the shift from anabolic to catabolic metabolism of lipids.

To assess the contribution of the PC part of marine Omega-3 PL molecule to the metabolic efficacy of Omega-3 PL administration we compared metabolic effects of two different lipid supplements, where PC represents the main phospholipid fraction, but the composition of FA is completely different; i.e. the PC-M concentrate was rich in EPA/DHA while the PC-S was rich in Omega-6 and other FA. In contrast to PC-M, PC-S completely failed to prevent the weight gain and obesity-associated comorbidities, and it also failed to down-regulate hepatic expression of genes encoding the enzymes of FA synthesis and cholesterol biosynthesis. Together with our previous study where even the higher dose of EPA and DHA (~30 g/kg diet) in the form of Omega-3 TAG failed to significantly down-regulate hepatic genes involved in FA synthesis and cholesterol biosynthesis, these results show that it is primarily the combination of EPA and DHA and the proper lipid carrier, i.e. the PC-rich PL as in this case, which provides the maximum metabolic efficacy of Omega-3 supplements.

We used the state-of-the-art technique of hyperinsulinemic-euglycemic clamps to assess insulin-sensitizing properties of Omega-3 TAG and Omega-3 PL in obese C57BL/6 mice. Unlike Omega-3 TAG, Omega-3 PL contained in KO induced consistent and dose-dependent amelioration of IR, characterized as the increase in whole-body GTO and glycogen synthesis and decrease in HGP. The PL lipid carrier showed to be indispensable for Omega-3 PL effect, as Omega-3 PL showed to be effective even when the total amount of EPA/DHA in the diet was 3-fold lower than in the diet containing Omega-3 TAG.

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List of scientific results

Bellow follows the list of scientific projects, in which I personally participated, and the list of scientific results, i.e. poster and oral presentations, which I personally performed, or publications and manuscripts in writing, to which I significantly contributed during my PhD studies. The list also includes projects that are not a part of this thesis, as they are thematically unrelated to the main topic of my Ph.D. studies.

Project 1. Corn oil vs. pork lard

Publication: Pavlisova J, Bardova K, Stankova B, Tvrzicka E, Kopecky J, Rossmeisl M.: Corn oil versus lard: Metabolic effects of omega-3 fatty acids in mice fed obesogenic diets with different fatty acid composition. Biochimie. 2016; **IF = 3,188**

My personal contribution to this project includes the supervision of both long-term animal experiments including animal handling, body-weight and food consumption monitoring. I supervised and participated in *in vivo* analyses (i.e. i.p.GTTs and hyperinsulinemic-euglycemic clamps), and dissections, and performed all *ex vivo* biochemical analyses except light microscopy, immunohistochemistry, and FA composition analyses. I also performed statistical analysis and participated in the creation of the manuscript.

Oral presentation: Hepatic steatosis and dietary lipids
presented on local mini-symposium "Bioenergetika 2013"

Poster presentation: Corn oil vs. lard: metabolic effects of obesogenic diets with different fatty acid composition
presented on international conferences: " The Crossroads of Lipid Metabolism and Diabetes, Keystone series" Denmark; "European congress on obesity 2015", Czech Republic

Project 2. The impact of Omega-3 TAG supplementation on incretin system

Manuscript in writing: The impact of long-term Omega-3 polyunsaturated fatty acids supplementation on incretin system in dietary obese mice

Oral presentation: Incretins - more than insulin assistants
presented on local mini-symposium "Bioenergetika 2012"

Poster presentation: The impact of Omega-3 polyunsaturated fatty acids on incretin system
presented on local conference "50.Diabetologické dny Luhačovice 2014"

Project 3. Research of Omega-3 phospholipids (main Ph.D. project)

Publication: Rossmeisl M, Medrikova D, van Schothorst EM, Pavlisova J, Kuda O, Hensler M, Bardova K, Flachs P, Stankova B, Vecka M, Tvrzicka E, Zak A, Keijer J, Kopecky J.: Omega-3 phospholipids from fish suppress hepatic steatosis by integrated inhibition of biosynthetic pathways in dietary obese mice. Biochim Biophys Acta. 2014; **IF = 4,966**

My personal contribution to this project includes the supervision of Experiment 3. including body-weight and food consumption monitoring and feces collection. I participated in *in vivo* analyses and dissections, performed the analyses of hepatic and fecal lipid content, and verified the results of microarray gene expression scanning by qPCR. I also performed part of statistical analysis and participated in the creation of the manuscript.

Manuscript in writing: Omega-3 phospholipids, but not Omega-3 triacylglycerols, improve insulin sensitivity in C57Bl/6 mice with high-fat diet-induced obesity.

Oral presentation: n-3 PUFA phospholipids in the combination with a low-dose of Rosiglitazone: The impact on biosynthetic pathways in the liver.
presented on local conference "Fyziologické dny 2013" and the II Doctoral Workshop on Molecular Nutrition BIOCLAIMS, Spain

Poster presentation: Metabolic effects of dietary n-3 fatty acids supplied as phospholipids reflect down-regulation of biosynthetic pathways in the liver of mice fed a high-fat diet.
presented on international conferences: "Metabolism 2012", Germany; EASD 2012, Germany; "European congress on obesity 2015", UK; Metabolism, "Mitochondria and Fatty Acids 2013", UK

Project 4. The impact of Omega-3 on the endocannabinoid system

Publication: Rossmeisl M, Pavlisova J, Janovska P, Kuda O, Bardova K, Hansikova J, Svobodova M, Oseeva M, Veleba J, Kopecky J Jr, Zacek P, Fiserova E, Pelikanova T, Kopecky J: Differential modulation of white adipose tissue endocannabinoid levels by n-3 fatty acids in obese mice and type 2 diabetic patients. Biochim Biophys Acta. 2018; **IF = 4,966**

Oral presentation: Endocannabinoid system
presented on local mini-symposium "Bioenergetika 2011"

Project 5. Impact of food supplementation with purified lignin on metabolism and obesity

Oral presentation: Lignin, (not only) a by-product of paper production.
presented on local mini-symposium "Bioenergetika 2015"

Patent: Use of Preparation for Oral Administration Containing Lignin
Patent no. 18153496.7
Priority: CZ/26.01.17/CZA 20170014

Appendix

Publication enclosed in full text:

Pavlisova J, Bardova K, Stankova B, Tvrzicka E, Kopecky J, Rossmeisl M.: Corn oil versus lard: Metabolic effects of omega-3 fatty acids in mice fed obesogenic diets with different fatty acid composition. Biochimie. 2016

Rossmeisl M, Medrikova D, van Schothorst EM, Pavlisova J, Kuda O, Hensler M, Bardova K, Flachs P, Stankova B, Vecka M, Tvrzicka E, Zak A, Keijer J, Kopecky J.: Omega-3 phospholipids from fish suppress hepatic steatosis by integrated inhibition of biosynthetic pathways in dietary obese mice. Biochim Biophys Acta. 2014