Charles University

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Study program: Analytical Chemistry

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Targeted analysis and metabolism of fatty acids in mice and humans

Cílená analýza a metabolismus mastných kyselin u myší a lidí

Doctoral thesis

Supervisor: RNDr. Ondřej Kuda, Ph.D.

Prague, 2021

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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I declare that I have prepared this dissertation independently and that I have listed all used information sources and literature. Neither this work nor a substantial part of it has been submitted for another or the same academic degree.

In Prague, June 2021

Marina Oseeva

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Finally, I thank my family, my husband, and my friends for their help and for encouraging me to keep up.

Abstract

Widespread sedentary lifestyle and unhealthy eating habits in the last few decades have resulted in a dramatic increase of the number of people affected by obesity, type 2 diabetes, and cardiovascular diseases. The study of these pathological conditions revealed that impaired metabolism often causes these disorders. Lipid metabolism research has contributed significantly to determining mechanisms underlying metabolic disorders. Omega-3 fatty acids are an interesting target for lipidomics studies because they were shown to lower risk of cardiovascular diseases and are hypothesized to regulate lipid metabolism. In this work, I optimized lipid extraction and chemical modification methods for analysis of fatty acids profile of tissue samples and biofluids using comprehensive two-dimensional gas chromatography coupled to mass spectrometry (GCxGC-MS).

At first, I evaluated the relative amount of omega-3 fatty acids in red blood cells (Omega-3 index) of people living in Czech Republic in either the capital city (n=476) or the rural region (n=388). For this large-scale project, I extracted phospholipids from red blood cell (RBC) membranes, transesterified them into fatty acid methyl esters (FAMEs), and measured their profile by GCxGC-MS. The mean Omega-3 index was 3.56 mol % and I detected no significant difference in the index value between rural and urban/industrial regions. Participants who self-reported the regular consumption of fish or fish oil supplements had a higher value of the Omega-3 index compared to those who lacked fish in their diet.

Next, I tested the bioaccumulation of omega-3 fatty acids from omega-3-containing triacylglycerols, phospholipids, and wax esters in humans and mice. I found that omega-3-containing triacylglycerols positively affect the level of omega-3 fatty acids in serum, white adipose tissue, RBC, and liver. Furthermore, the consumption of omega-3 fatty acids in a form of phospholipids increased omega-3 fatty acids content in liver and RBC. The analysis of the Omega-3 index in the experiment with omega-3 fatty acids esterified into wax esters did not show any significant difference between the test and the control group of human subjects.

Beside omega-3 fatty acids studies, I also examined lipid metabolism using stable isotope labeling. I used heavy water as a source of deuterium to track the changes in lipodome in response to bioactive lipid PAHSA and cold exposure in mice. I detected higher amounts of deuterium incorporated in fatty acids associated with lipogenesis in mice that were kept at low temperature

and this effect was enhanced by PAHSA administration in comparison to other cohorts kept at thermoneutrality. In cell studies, I showed that PAHSA promoted the release of triacylglycerol breakdown products. Therefore, PAHSA affected both lipogenesis and lipolysis processes.

Abstrakt

Sedavý životní styl a nezdravé stravovací návyky v posledních několika desetiletích vedly k dramatickému nárůstu počtu lidí trpících obezitou, cukrovkou 2. typu a kardiovaskulárními chorobami. Studium těchto patologických stavů odhalilo, že tyto poruchy často způsobuje narušení metabolické rovnováhy. Výzkum lipidového metabolismu významně přispěl k určení mechanismů, které jsou základem metabolických poruch. Omega-3 mastné kyseliny jsou zajímavým tématem pro lipidomické studie, neboť se ukázalo, že snižují riziko kardiovaskulárních onemocnění a podílejí se na metabolismu lipidů. V této práci jsem optimalizovala postupy extrakce lipidů a jejich chemické modifikace za účelem analýzy profilu mastných kyselin ve vzorcích tkání a krve pomocí metody komplexní dvourozměrné plynové chromatografie spojené s hmotnostní spektrometrií (GCxGC-MS).

Nejprve jsem hodnotila relativní množství omega-3 mastných kyselin v červených krvinkách (index Omega-3) lidí žijících v České republice buď v hlavním městě (n = 476), a nebo na venkově (n = 388). Pro tento rozsáhlý projekt jsem extrahovala fosfolipidy z membrán červených krvinek (RBC), transesterifikovala je na methylestery mastných kyselin (FAME) a změřila jejich profil pomocí GCxGC-MS. Průměrný index Omega-3 byl 3,56 mol% a nezjistila jsem žádný významný rozdíl v hodnotě indexu mezi venkovskými a městskými (průmyslovými) regiony. Účastníci, kteří sami nahlásili pravidelnou konzumaci ryb nebo doplňků obsahujících rybí olej, měli vyšší hodnotu indexu Omega-3 ve srovnání s těmi, kteří takovou stravu nepreferují.

Dále jsem testovala bioakumulaci omega-3 mastných kyselin v organismu u myší a lidí po podávání triacylglycerolů, fosfolipidů a esterů vosků obsahujících omega-3 mastné kyseliny. Zjistila jsem, že triacylglyceroly obsahující omega-3 pozitivně ovlivňují hladinu omega-3 mastných kyselin v séru, bílé tukové tkáni, RBC a játrech. Dále konzumace omega-3 mastných kyselin ve formě fosfolipidů zvýšila obsah omega-3 mastných kyselin v játrech a RBC. Analýza indexu Omega-3 v experimentu s omega-3 mastnými kyselinami esterifikovanými na voskové estery neprokázala žádný významný rozdíl mezi testovanou a kontrolní skupinou lidských subjektů.

Kromě studií omega-3 mastných kyselin jsem zkoumala také metabolismus lipidů pomocí značení stabilními izotopy. Jako zdroj deuteria jsem použila těžkou vodu a sledovali jsme změny lipodomu v reakci na bioaktivní lipid PAHSA a na chladovou expozici u myší. Změřila jsem vyšší množství

deuteria zabudovaného v mastných kyselinách spojených s *de novo* lipogenezí u myší, které byly chovány při nízké teplotě, a tento účinek byl zvýšen podáváním PAHSA ve srovnání se skupinami udržovanými v termoneutralitě. V buněčných studiích jsem prokázala, že PAHSA podporuje remodelaci triacylglycerolů a zasahuje do procesů lipogeneze i lipolýzy.

Key words:

Lipidomics

Fatty acids

GCxGC-MS

Omega-3 index

Isotope labeling

Klíčová slova:

Lipidomika

Mastné kyseliny

GCxGC-MS

Index Omega-3

Značení pomocí isotopů

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ABBREVIATIONS

AA	arachidonic acid
ALA	alpha-linolenic acid
ATGL	adipose triglyceride lipase
BHT	*n*-butylated hydroxytoluene
BSA	*N*,*O*-Bis(trimethylsilyl)acetamide
BSTFA	*N*,*O*-Bis(trimethylsilyl)trifluoroacetamide
CE	cold exposure
DAG	diacylglycerol
DC	direct current
DHA	docosahexaenoic acid
DNL	*de novo* lipogenesis
EI	electron impact ionization
EPA	eicosapentaenoic acid
ESI	electrospray ionization
eWAT	epididymal white adipose tissue
FA	fatty acid
FAME	fatty acid methyl ester
FASN	fatty acid synthase
GC	gas chromatography
GC-MS	gas chromatography coupled to mass spectrometry
GCxGC	comprehensive two-dimensional gas chromatography

GCxGC-MS	comprehensive two-dimensional gas chromatography coupled to mass spectrometry
HFD	high-fat diet
HPLC	high performance liquid chromatography
HSL	hormone-sensitive lipase
LA	linoleic acid
LC	liquid chromatography
LHF	lard-based high-fat (diet)
m/z	mass-to-charge ratio
MAG	monoacylglycerol
MGL	monoacylglycerol lipase
MeOH	methanol
MRM	multiple reaction monitoring (mode)
MS	mass spectrometry
MTBE	methyl-tert-butyl ether
MUFA	monounsaturated fatty acid
NEFA	non-esterified fatty acids
PAHSA	palmitic acid ester of hydroxystearic acid
PC	phosphatidylcholine
PL	phospholipid
PUFA	polyunsaturated fatty acid
QQQ	triple quadrupole
RBC	red blood cell, erythrocytes

RF	radio frequency
RP	reversed-phase (mode)
RT	retention time
SCOT	support-coated open tubular (column)
SFA	saturated fatty acid
SIM	selected ion monitoring
SPE	solid-phase extraction
SRM	selected reaction monitoring (mode)
TAG	triacylglycerol
TCA	tricarboxylic acid
TMCS	trimethylchlorosilane
TMS	trimethylsilyl (group)
TN	thermoneutrality
TOF	Time-of-Flight (mass analyser)
UHPLC	ultra high performance liquid chromatography
UPLC	ultra performance liquid chromatography
Veh	vehicle
WCOT	wall-coated open tubular (column)
wt	weight

INTRODUCTION

1. Lipids

Lipids are a diverse class of organic compounds that are commonly insoluble in water due to hydrophobic parts. They can perform different tasks throughout the body: energy storage, formation of membranes, hormonal roles, a vehicle for fat-soluble vitamins and protection of internal body organs as a fat layer. They are the vital nutrients.

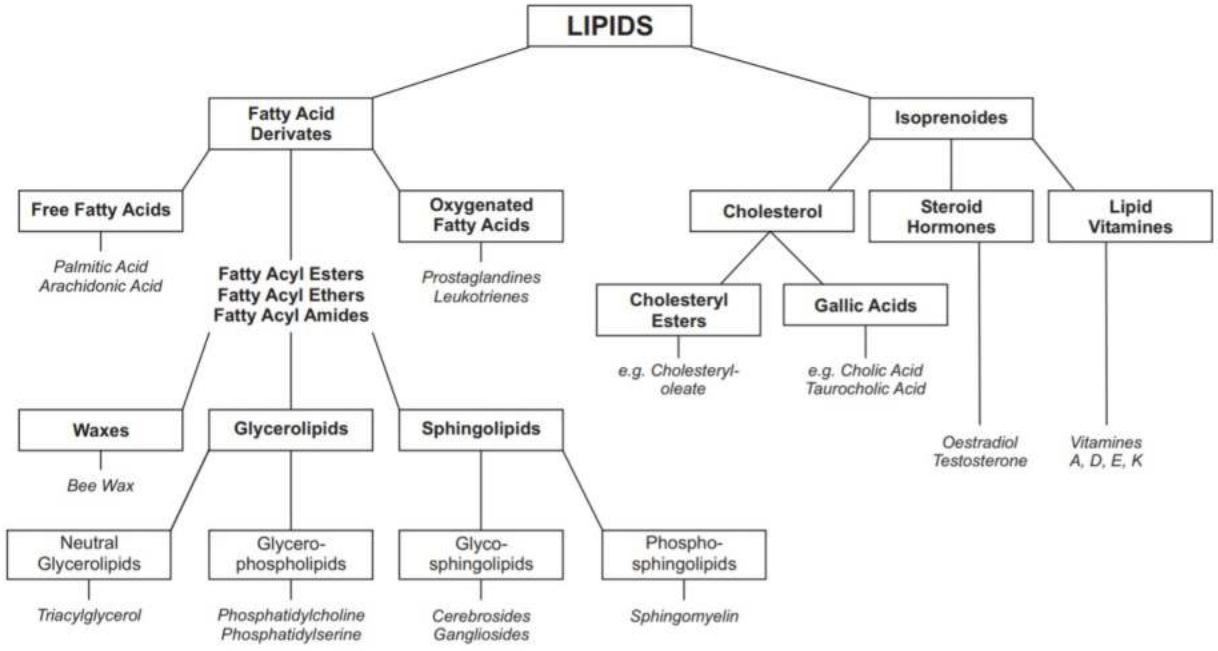


Fig. 1. Short survey of the different classes of lipids **[1]**

1.1. Fatty acids

Fatty acids are the major constituent of lipids. They mainly determine the physicochemical and physiological properties of lipids. Fatty acids consist of an aliphatic chain and a carboxyl group. Two features, the length of the chain and the degree of saturation, define the physical and biological properties of fatty acids. Concerning the length of the chain, fatty acids can be divided into short-chain fatty acids (less than six carbons), medium-chain fatty acids (from 6 to 12 carbons), long-chain fatty acids (from 14 to 20 carbons), and very long-chain fatty acids (over 22 carbons). The most common length of naturally occurring fatty acids varies from 14 to 22 carbon

atoms. The other important characteristic of fatty acids is the degree of saturation. The degree of saturation stands for the presence or absence of double bonds in fatty acid chains.

One of the most common fatty acids is hexadecanoic acid in IUPAC nomenclature, or palmitic acid $CH_3(CH_2)_{14}COOH$. It is 16-carbon saturated fatty acid (means zero double bonds) that also can be designated 'FA 16:0'.

1.1.1. Unsaturated fatty acids

The degree of saturation of fatty acids is often related to the source of lipids. Saturated fatty acids (containing no double bonds – SFAs) account for 30-50% of fatty acids of animal fat (except for the most of the fish oil **[2, 3]**) and the rest is the sum of monounsaturated and polyunsaturated **[4]**. Meanwhile monounsaturated (one double bond – MUFAs) and polyunsaturated fatty acids (two or more double bonds – PUFAs) correspond to approximately 80-90% of fatty acids in most plant oils. Exceptions are cocoa butter (*Theobroma cacao*), palm oil (*Elaeis guineensis*), and cottonseed oil (*Gossypium spp.)* **[5, 6]**.

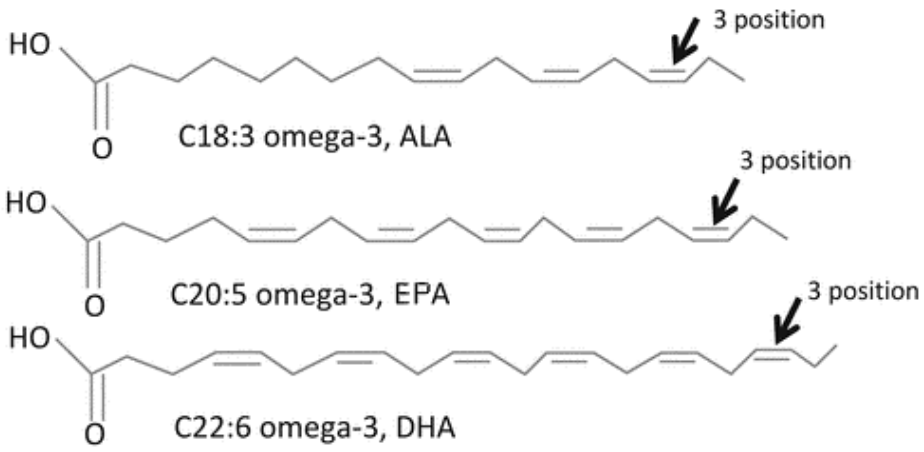
Unsaturated fatty acids have long hydrocarbon chains containing one or more double bonds, thus, they exist in *cis*- or *trans*- forms. Naturally derived unsaturated fatty acids come almost exclusively in *cis*- transfiguration **[7]**. Fatty acid in t*rans*- configuration can be a byproduct of reverse reaction that can occur during chemical reaction (e.g. oil hydrogenation). The presence of double bonds makes unsaturated fatty acids more prone to autoxidation than saturated ones.

1.1.1.1. Polyunsaturated fatty acids

Polyunsaturated fatty acids have 2 or more double bonds. The main PUFAs families are omega-3 (ω-3), omega-6 (ω-6), and omega-9 (ω-9) fatty acids. The position of the first double bond starting from the methyl group at the end (omega or n-end) of a hydrocarbon chain is one of the major characteristics of PUFAs. The numbers (e.g., '3') stand for this double bond position.

1.1.1.1.1. Omega-3 fatty acids

Omega-3 (ω-3) fatty acids are polyunsaturated fatty acids, also called n-3 fatty acids. Three of omega-3 fatty acids are of major interest in mammalian physiology research, namely alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA).



Structures of omega-3 fatty acids **[8]**

The source of ALA is plant oil, mainly linseed, rapeseed, and canola oils **[9]**. EPA and DHA can be found in marine products and game meats. The highest content of these omega-3 fatty acids has been detected in marine oily fish **[10]**.

Omega-3 fatty acids are essential and have a positive impact on health **[11, 12]**. ALA serves as a precursor for EPA and DHA synthesis **[13]**. EPA and DHA, in turn, serve as precursors of anti-inflammatory molecules, resolvins and docosatrienes **[14, 15]**. Higher levels of EPA and DHA in whole blood is related to lower risk of sudden cardiac death **[16]**, coronary heart and cardiovascular diseases **[17]**. DHA as a part of membrane phospholipids, which comprise two fatty acids in the 1 and 2 positions and a phosphorylated functional group in the 3 position on the glycerol, is the major omega-3 fatty acid in the brain. High levels of DHA in the brain can protect it from neuroinflammation **[18]**. Patients with Alzheimer's disease showed a considerable decrease of DHA content in gray matter and in the hippocampus compared to healthy controls **[19]**. However, the DHA supplementation did not slow the progression of the disease in the patients with mild-to-moderate Alzheimer's disease **[20]**.

Unfortunately, mammals lack enzymes to synthesize long-chain PUFAs *de novo* **[21]**. They also show low rates of conversion of ALA to EPA and DHA **[22]**. Therefore, dietary intake of omega-3 fatty acids is the only way to replenish the physiological needs.

1.1.1.1.2. Omega-3 index

EPA and DHA as omega-3 fatty acids showed a positive effect on the state of the cardiovascular system. In 2004 William S. Harris and Clemens von Schacky proposed a new biomarker based on the EPA and DHA content because fatty acids profile of red blood cell (RBC) membranes is similar to cardiac membrane **[23]**. They named it "Omega-3 index" and stated that the Omega-3 index reflects the relative amount of EPA and DHA in RBC. RBC have been chosen for omega-3 fatty acids content analysis due to ease of sample collection from patients and also due to ability of RBC membranes to maintain composition of fatty acids (accumulated partly from diet) up to 2-3 months **[24]**. They estimated the Omega-3 index of ≥8% as desirable for lowering the risk and of ≥4% as undesirable. The Omega-3 index represents a novel risk factor for death from coronary heart disease and sudden cardiac deaths. Harris and von Schacky additionally showed correlation between dietary intake of EPA and DHA and increase of the Omega-3 index in healthy adult volunteers.

Later Ken D. Stark and his collaborators made a comprehensive worldwide survey on the Omega-3 index **[25]** based on 298 studies published from 1980 to 2014 (Fig. 2). Countries that mostly follow the Western diet with a low amount of fish and seafood show the least Omega-3 index (≤4%). In contrast, countries and regions where people have fish and marine products in their diet on a daily basis display the highest Omega-3 index (>8%).

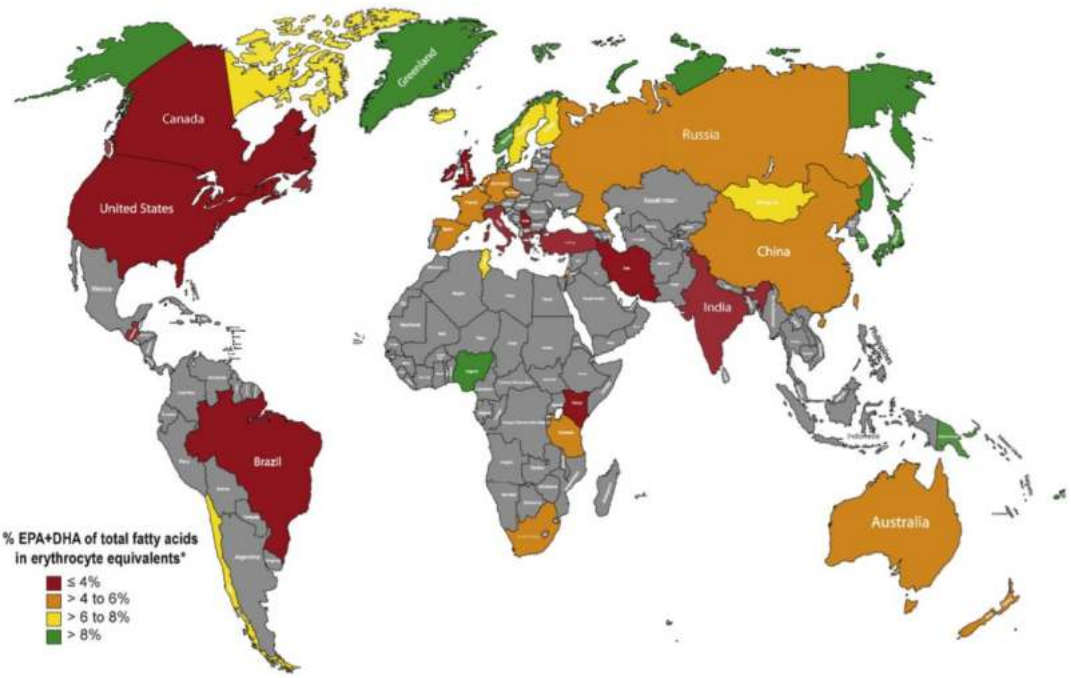
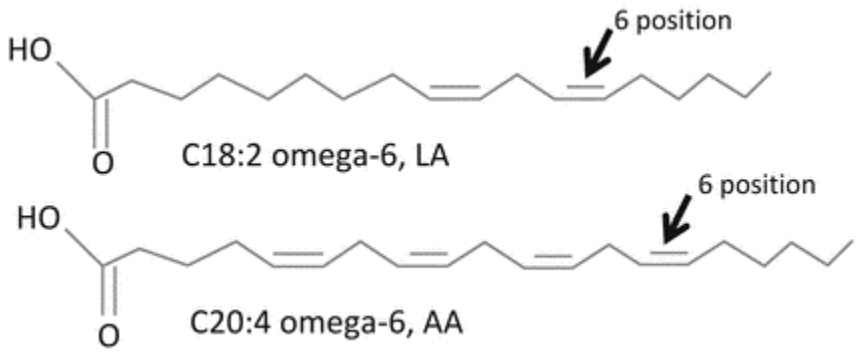


Fig. 2. Global blood levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) content. All the data were recalculated to relative weight percentages (wt. %) **[25]**.

1.1.1.1.3. Dietary omega-6/omega-3 fatty acid ratio

An early study of bioactive lipids found the relationship between a lower rate of cardiovascular diseases and an increased level of omega-3 fatty acids in plasma in Greenland Eskimos compared to Caucasian Danes. Moreover, diminution of omega-6 (ω-6) fatty acids was detected in the Eskimo patients **[26]**. This research prompted scientists to study not only the physiological function of omega-3 fatty acids but also the meaning of the omega-6/omega-3 fatty acid ratio in the body.

Like omega-3 fatty acids, omega-6 fatty acids are defined as essential **[27]**. Linoleic acid (18:2n-6; LA) is the dietary precursor of arachidonic acid (20:4n-6; AA). AA-derived eicosanoids serve as a pro-inflammatory agent important for immune response **[28]**.



Structures of omega-6 fatty acids **[8]**

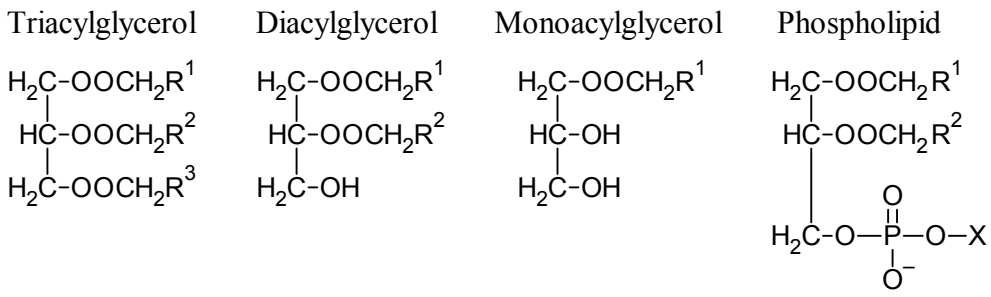
Our modern diet (Western diet) is based on cereals, vegetable oils and meat of animals fed corn and grains. This food is rich in omega-6 fatty acids but poor in omega-3 fatty acids **[29]**. This nutrition pattern results in omega-6/omega-3 ratio of 10-20:1 in Western diet **[30]**. Omega-3 fatty acids and omega-6 fatty acids compete for the same set of enzymes **[13]**. The shift to the higher amount of omega-6 fatty acids can boost the synthesis of pro-inflammatory AA derivatives and conversely, inhibit the anti-inflammatory omega-3 fatty acids derivatives production. The imbalance in the omega-6/omega-3 fatty acid ratio can lead to elevated inflammatory status due to increased level of pro-inflammatory cytokines and higher risk of the metabolic syndrome **[27]**. It has been proposed that a ratio of omega-6 to omega-3 fatty acids between 1:1 and 5:1 is supposedly favorable to health **[10]**.

1.2. Fatty acids derivatives

Typically, fatty acids remain unesterified in the body for a relatively short time and they are predominantly linked to a glycerol backbone forming acylglycerol. The most abundant type of acylglycerols is triacylglycerol (TAG), or sometimes referred to as triglyceride, the derivative made of three fatty acids esterified to glycerol. If only two fatty acids are attached to glycerol moiety, it is termed diacylglycerol (DAG). If glycerol is esterified solely to one fatty acid, they form monoacylglycerol (MAG).

If one of the fatty acids in TAG is substituted for a phosphate group head modified with nitrogenous bases of choline, ethanolamine, or serine, such compound is termed a phospholipid or a phosphoglyceride. This change in structure makes the phospholipids less hydrophobic than TAGs. The phosphate heads are polar while fatty acids are non-polar; thereby, phospholipids can

form lipid bilayers due to their amphiphilicity. These bilayers are crucial components of cell membranes.

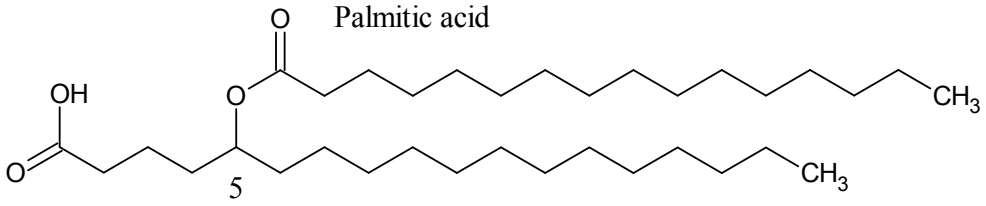


Acylglycerols and phospholipids structures, where R^1, R^2, and R^3 are fatty acids; X is nitrogenous base of choline, ethanolamine, or serine

1.2.1 Palmitic acid esters of hydroxystearic acid

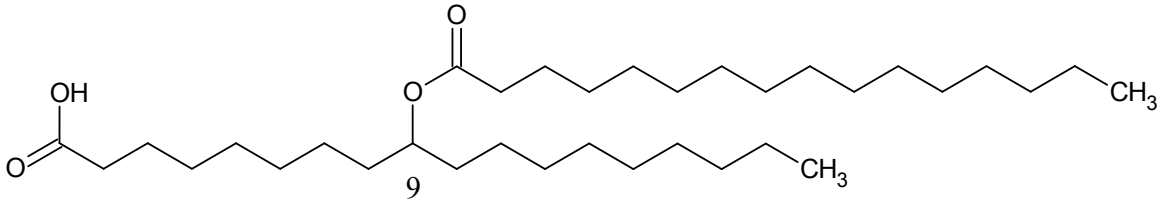
Palmitic acid esters of hydroxystearic acid (PAHSAs) are newly found endogenous lipids showing anti-inflammatory and anti-diabetic properties **[31]**. Since these lipids are recently discovered, the study of PAHSAs is still ongoing.

5-PAHSA



Hydroxystearic acid

9-PAHSA



Examples of PAHSA structures

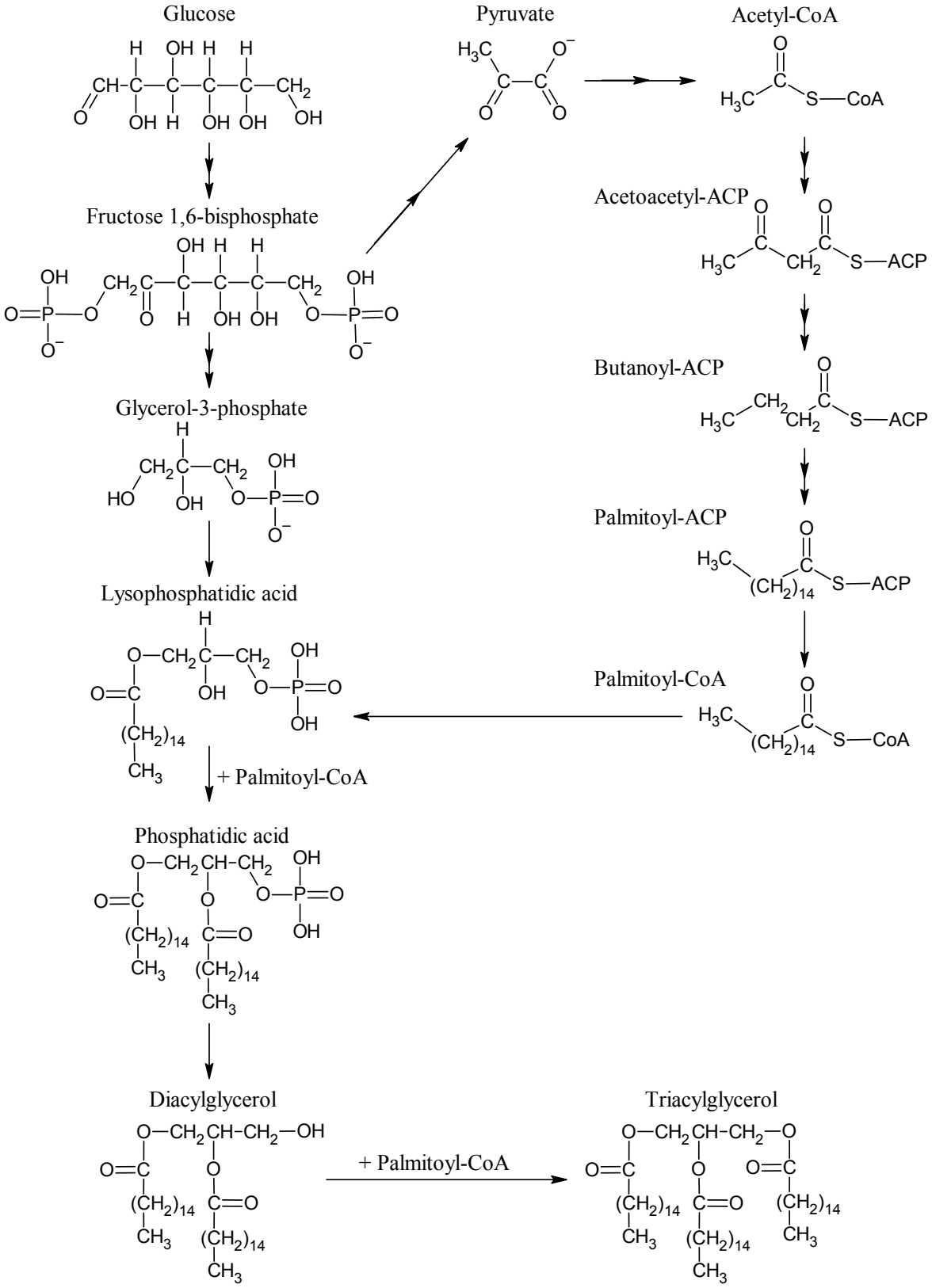
PAHSA is a lipid that consists of palmitic acid and hydroxystearic acid, which are linked together by the ester bond. 5 and 9 are numbers that represent the position of ester bond related to palmitic acid.

2. Biochemistry of lipids

Metabolism is a highly complex process with myriads of reactions occurring constantly in cells. Most of the reactions are reversible, thus, many substances are created and then degrade. Lipids are also involved in these processes, mainly in *de novo* lipogenesis and lipolysis. During *de novo* lipogenesis (DNL), fatty acids are synthesized via carbohydrates breakdown. Lipolysis is a combination of reactions in which lipids decompose releasing free fatty acids. The balance between both processes indicates unimpaired energy metabolism.

DNL starts with carbohydrate digestion. Catabolism (i.e. destructive metabolic reactions) of dietary carbohydrates provides glucose, which undergoes glycolysis and transforms into pyruvate. The latter enters the tricarboxylic acid (TCA) cycle transforming into citrate, the intermediate of TCA cycle. Citrate converts into acetyl-CoA, which is a substrate for palmitoyl-CoA synthesis. Palmitoyl-CoA, acyl-Coenzyme A thioester, is a final product of the reaction sequence because it is regulated by fatty acid synthase (FASN), the key rate-limiting multi-complex enzyme **[32]**, and serves as a precursor of a 16-carbon saturated fatty acid, palmitic acid (FA 16:0). Thus, palmitic acid is the main product of DNL. Following the synthesis, it can undergo elongation and desaturation leading to formation of other fatty acids, such as palmitoleic acid (FA 16:1n-7), cis-vaccenic acid (FA 18:1n-7), stearic acid (FA 18:0), and oleic acid (FA 18:1n-9) **[33]**.

Under normal conditions, DNL is promoted by the excess of glucose in cells. Consequently, fatty acids generated via DNL can be converted to TAGs. Glycerol-3-phosphate, which can be derived from glucose during glycolysis, reacts stepwise with 3 molecules of FA-CoA resulting in TAG molecule.



A simplified pathway of TAG 48:0 *de novo* synthesis

TAGs are mostly stored in adipocytes, which are building blocks of an adipose tissue. The main function of TAG in living organisms is energy storage. The energy obtained from the decomposition of 1 g TAGs reaches 9 kcal **[34]**. To produce this amount of energy fatty acids are needed to be liberated. TAG undergoes a series of reactions, in a process called lipolysis, breaking down into fatty acids and glycerol. The first step of lipolysis requires an enzyme, adipose triglyceride lipase (ATGL). As a result of the hydrolysis involving ATGL, one molecule of fatty acid releases and triacylglycerol turns into diacylglycerol (DAG). To convert DAG into monoacylglycerol (MAG) and one more fatty acid, another enzyme, hormone-sensitive lipase (HSL) is needed. Finally, monoacylglycerol lipase (MGL) breaks the MAG into a fatty acid molecule and a glycerol molecule.

3. Omics sciences

3.1. Lipidomics

Hundreds of various species of lipids can be found in cells throughout the whole body. Their study on a large scale, lipidomics, became possible with the progress of mass spectrometry. The concentrations of lipids and their distribution in cells may vary depending on a shift in physiological, pathological, and environmental conditions.

The first step in lipidomics studies is the sample preparation. Proper collection of biomaterial and sample storage is crucial **[35]**. Instant deep freezing in liquid nitrogen of the collected tissues stops enzymatic reactions, prevents oxidation and degradation of the majority of metabolites. After the samples are frozen, the following storage at -80°C allows to preserve the lipids and metabolites **[36]**. By contrast, storage of red blood cells at -20° promotes degradation of fatty acids, and especially PUFA; however, keeping the red blood cells sample at -80°C significantly slows all decomposition processes **[37]**.

The next step is extraction of lipids from a biological matrix. All the procedures must be done using ice-cold solvents, on ice if possible. Commonly, 10–100 µl or 1–100 mg of biomaterial is sufficient for lipidomics screening **[36]**. The recovery can be different depending on the lipid class, thus the appropriate procedure for the lipids of interest should be established. The main principle is to create a mixture of liquids insoluble in each other due to different solvent polarity. The vast majority of lipids are hydrophobic and they tend to accumulate in a non-polar phase.

In a procedure called derivatization, researchers modify lipid molecules to alter their physical properties or defragment the structure. This is followed by mass spectrometry-based analysis (alone, or coupled to chromatographic separation). The resolving power (i.e. the ability to separate two individual peaks) of modern mass spectrometry machines is noticeably high.

3.2. Metabolomics

The analysis of organic compounds in tissues and biofluids can give us information on metabolic pathways. Metabolomics is an approach that focuses on simultaneous detection of a big number of metabolites. Metabolites are the molecules formed as end products or intermediates in the body during metabolic processes. Modern types of mass-spectrometry provide an opportunity to create metabolic profiles in both control and test animals and humans allowing qualitative and quantitative analysis. The usage of stable isotopes as tracers makes it easier to track which metabolites are downregulated or upregulated. Also, new pathways can be discovered. This applies only to untargeted metabolomics, i.e. an approach allowing detection of every possible analyte in a sample simultaneously, if *m/z* values have been listed in the database.

In a case of targeted metabolomics, one chooses the metabolites of interest beforehand. Some types of mass analyzers provide the selection of daughter ions followed by the filtering of an exact product ion of interest.

The choice of experimental model is an important part of lipidomics and metabolomics studies. For example, studies of cell-based models are carried out under controlled conditions and known as *in vitro*, literally meaning “in the glass”. Researchers often add stimulating or inhibiting agents to imitate the activity taking place in real living organisms **[38]**. Although *in vitro* models can provide only limited information about the ongoing processes, they are cheaper and results can be obtained much faster compared to *in vivo* (Latin for “within the living”) models. *In vivo* studies involve whole living systems such as test animals or human subjects. Experiments *in vivo* allow to test drugs or other compounds or to explore metabolism in pathophysiological and normal conditions, providing a more comprehensive outlook of the processes occurring in the organism. However, a living organism is a complex system and it can be difficult to predict random changes. In human studies, it is almost impossible to monitor and control all the aspects of lifestyle such as diet, physical activity, taking medication, bad habits, etc. Moreover, laboratory animals are

expensive to take care of. Therefore, researchers have to consider which experimental model, e.g. cell lines or laboratory mice, fits the experimental design.

3.3. Isotope labeling

Analyzing reactions of interest in a living organism presents a challenge due to a vast number of reactions occurring simultaneously. This complication can be overcome this complication by using tracers, the molecules or atoms that are chemically indistinguishable from the naturally occurring objects of interest but can be recognizable due to physical properties. Isotopes, i.e. the atoms with the equal numbers of electrons (and protons) but different numbers of neutrons, have the same chemical properties, which allows to utilize them as tracers.

The first tracers contained radioactive isotopes for the ease of detection **[39, 40]**. Unfortunately, this advantage goes along with complications. Later the development of mass spectrometry methods made it possible to use stable, non-radioactive isotopes of carbon ^{13}C, hydrogen ^{2}H (alias deuterium D), oxygen ^{18}O, etc. They are safe and have the same chemical properties as the most abundant isotopes but different masses. This feature makes it possible to detect molecules involved in synthesis and turnover by mass spectrometry. To study metabolism, researchers use compounds that already exist in the body. ^{13}C-labeled acetate, ^{13}C-labeled glucose, or heavy water (D_2O) are the most common precursors. High doses of these compounds do not alter metabolism and are safe to introduce in a living organism.

D_2O is a stable and cheap isotope tracer and easy to handle. The distribution of deuterium in the body can be estimated by measuring D_2O level in plasma. This tracer is involved only in endogenous synthesis of fatty acids but not PUFAs (in mammalian cells) **[41]**, which makes it highly useful for the study of lipogenesis.

It should be noted that every chemical element has known isotopic forms. Some isotopes can be found in nature while others (usually radioactive) can be produced by nuclear fusion and radioactive decay. Commonly natural isotopic abundances follow the pattern, which is represented as a ratio of naturally occurring isotopes.

Table 1. Natural isotopic abundance (adopted from **[42]**)

Element	Mass number	Representative isotopic composition (mole fraction), %	Observed range of natural variations (mole fraction), %
Hydrogen, H	1 2	99.9885(70) 0.0115(70)	99.9816 – 99.9974 0.0026 – 0.0184
Carbon, C	12 13	98.93(8) 1.07(8)	98.853 – 99.037 0.963 – 1.147
Nitrogen, N	14 15	99.636(20) 0.364(20)	99.579 – 99.654 0.346 – 0.421
Oxygen, O	16 17 18	99.757(16) 0.038(1) 0.205(14)	99.738 – 99.776 0.037 – 0.040 0.188 – 0.222

Thus, molecules can be composed of not only the most abundant isotopes. If no additional treatment was applied, the profile should correspond to the natural isotopic distribution.

In the Fig. 3 the natural isotopic profile of unlabeled palmitic acid is shown. ^{13}C contributes the most to the intensity of the second highest peak M+1 because other isotopes such as deuterium or ^{17}O are even less abundant (Table 1). If the intensity of the first peak (monoisotopic mass of the most abundant isotopes) is set as 100% then the intensity of the second peak equals about 20%. This isotopic distribution profile was computed theoretically.

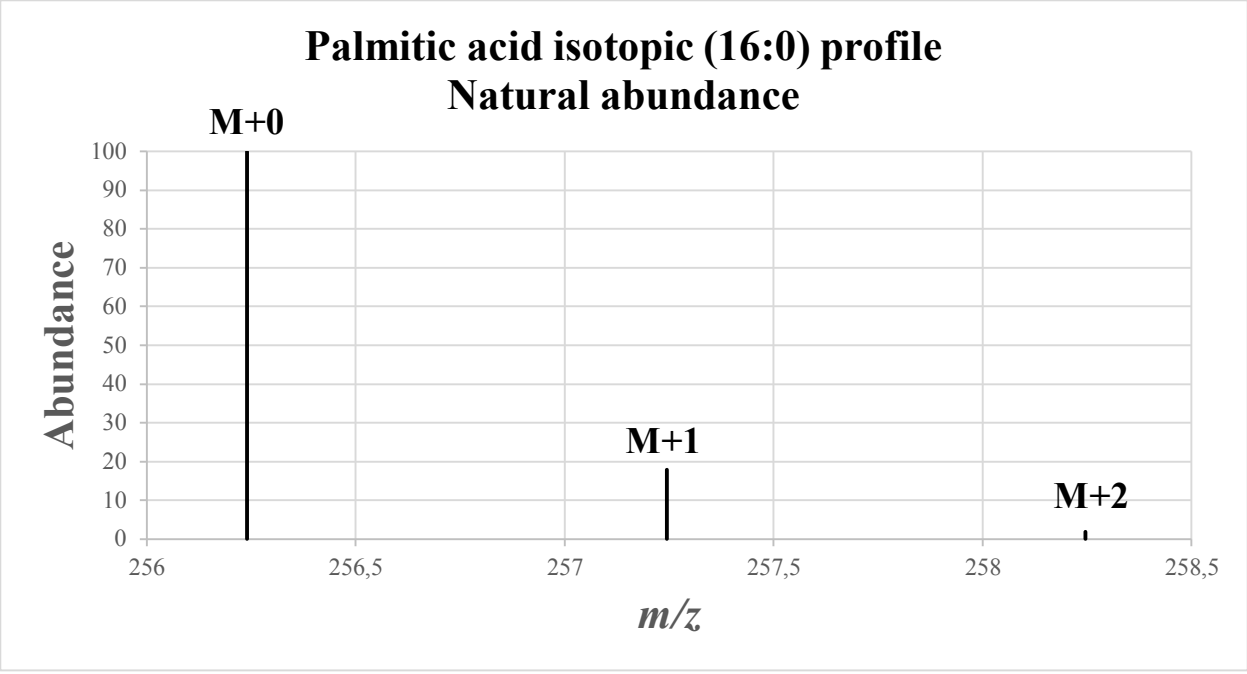


Fig. 3. Natural isotopologue profile of unlabeled palmitic acid (created with *enviPat* web interface **[43]**).

Molecules can be labeled, partially labeled, and unlabeled. The molecules of the same chemical formula that differ only in isotopic composition are called isotopologues. The examples are CH_4, CH_3D, CH_2D_2. Isotopologues should not be confused with isotopomers. Isotopomers are isomers, i.e. molecules having the same isotopic composition but different positions of isotopic atoms. For example, $CDH_2CH_2CH_2CH_3$ and $CH_3CHDCH_2CH_3$ are isotopomers of *n*-butane. Not all the mass-spectrometric methods are able to distinguish isotopomers from each other.

Calculation of the peak intensities in a natural isotopic distribution profile is relatively easy if the number of atoms in a molecule is small. For the rough estimation of the peak intensities of fatty acids, it is possible to ignore isotopes other than ^{12}C and ^{13}C. The absolute abundances for each isotopologue can be calculated as:

$$A = \frac{n!}{(a)!\times(b)!\times(c)!\cdots}(r_1)^a\times(r_2)^b\times(r_3)^c\ldots \quad (1)$$

where n is the number of atoms of the element, r_1, r_2, r_3, etc. are the abundances of each isotope, and a, b, c, etc., are the number distribution of the atoms in a given molecule **[44]**.

For example, the values of absolute abundances for palmitic acid can be obtained by following equations:

$$A(^{12}C_{\mathbf{16}}{}^{13}C_{\mathbf{0}}H_{32}O_2) = \frac{16!}{16!\times 0!}(0.9893)^{16}\times(0.0107)^{0} = 0.842$$

$$A(^{12}C_{\mathbf{15}}{}^{13}C_{\mathbf{1}}H_{32}O_2) = \frac{16!}{15!\times 1!}(0.9893)^{15}\times(0.0107)^{1} = 0.146$$

If we set the abundance of unlabeled palmitic acid $^{12}C_{\mathbf{16}}{}^{13}C_{\mathbf{0}}H_{32}O_2$ as 100% then the relative abundance of palmitic acid-1-^{13}C would be 17.3%, which corresponds to the height of the measured M+1 peak of palmitic acid presented in the Fig. 3.

More precise estimation of an isotopic profile of fatty acids or more complicated molecules can be obtained by special calculators such as "Isotope Distribution Calculator and Mass Spec Plotter" available on the Scientific Instrument Services website **[45]** or *enviPat* web interface **[43]**.

To estimate the enrichment of a selected isotope used as a tracer, a natural isotopic distribution profile of a control sample (treated with only a vehicle or not treated at all) or a theoretically calculated isotopic profile should be subtracted from a profile of a labeled sample.

IsoCor is a software for isotope correction of raw mass spectrometry data, i.e. mass fractions of the group of isotopologues, for evaluation of isotopic enrichment provided by a tracer during the experiment.

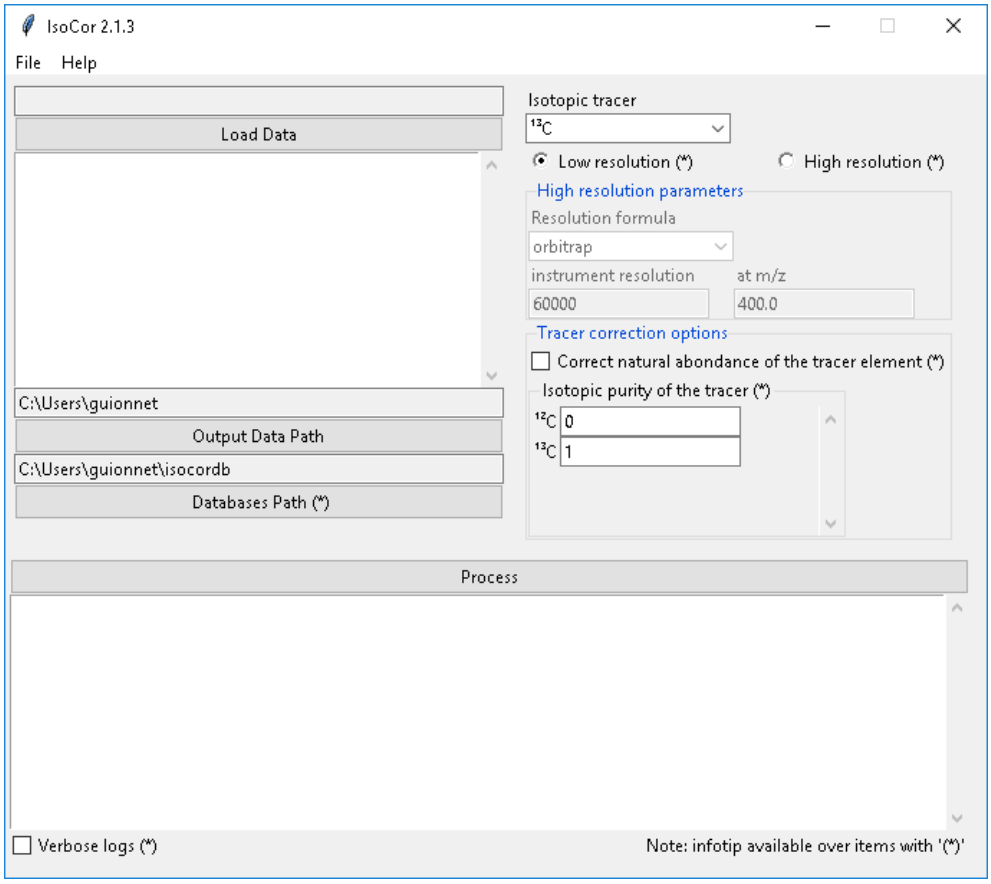


Fig. 4. The IsoCor window **[46]**

In the Fig. 4 the IsoCor window is presented. Prior to the start of data processing, it is required to choose the type of a tracer, parameters of mass spectrometry measurement, upload the raw mass spectrometry data for each molecule of interest, the database with the exact mass and natural abundance of each isotope, and the elemental formulas used for correction.

The IsoCor software calculates the mean isotopic enrichment of molecules based on parameters a user inputs. The final data represents a subtraction of theoretically calculated natural abundances of the isotopologues from the profile of isotope-labeled samples measured in the experiment.

IsoCor uses the following formula for calculation of the mean enrichment:

$$M_E = \frac{\sum_{i=0}^{n} M_i}{n} \tag{2}$$

where M_E is the mean enrichment of the molecule, M_i is the relative proportion of the isotopologue containing i atoms of the isotopic tracer, and n denotes the number of atoms of the chemical element in the molecule **[47]**.

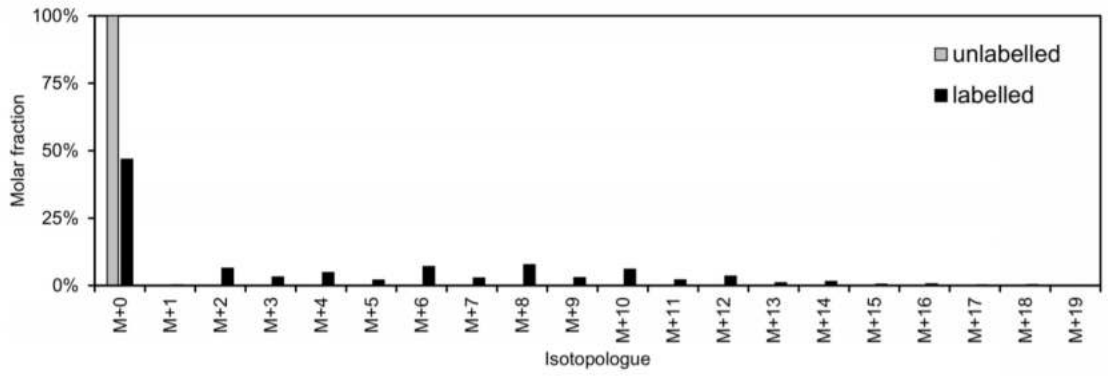


Fig. 5. Isotopologue profile of phosphatidylcholine 34:1 extracted from A549 cells grown on $^{13}C_6$-glucose (marked as labelled) and monoisotopic glucose (marked as unlabelled) created after IsoCor isotope correction **[48]**

In the Fig. 5 the data from the isotope labeling experiment in cells is shown as an example. The raw mass spectra were corrected with Isocor software. After isotope correction for natural ^{13}C abundance, the isotopologue profile of labeled phosphatidylcholine 34:1 shows that less than half of the molecules remained totally unlabeled after the isotope labeling experiment, while in the experiment with the cells grown on monoisotopic glucose the intensity of the M+0 peak (corresponds to unlabeled molecules) was 100% as expected.

4. Preparation for the analysis

4.1. Extraction

Since most of the lipids are water insoluble, the first step of lipid analysis of biological samples is the isolation of hydrophobic compounds. Various combinations of nonpolar organic solvents, polar organic solvents and water can be used for the separation of different classes of lipids. Bligh and Dyer **[49]** and Folch **[50]** procedures are considered as "gold standards" for the extraction of lipids. Both of the methods are suitable for total lipid recovery using a chloroform-methanol-water mixture. However, chloroform has higher density compared to water thus the chloroform phase containing lipids moves down at the bottom when mixing with water. Limited access to the bottom

phase leads to complications during the isolation of the chloroform phase if the aspiration of the organic fraction from the glass tube is applied. The re-extraction by adding another portion of chloroform-methanol mixture to the remaining water phase may help to increase the recovery of lipids. This step predictably increases the total time required for the sample preparation. These and other disadvantages (e.g. high toxicity of chloroform) of Bligh and Dyer and Folch methods pushed researchers to change chloroform or chloroform-methanol system for less hazardous compounds. Methyl-tert-butyl ether (MTBE) **[51]**, ethyl acetate **[52]**, butanol (BUME method) **[53]**, hexane **[54]**, dichloromethane **[55]** have been proposed as safer and faster approaches for lipid isolation. Not all of them are suitable for high-efficiency recovery of total lipids **[36]**; however, it is possible to choose a right solvent system depending on a experiment.

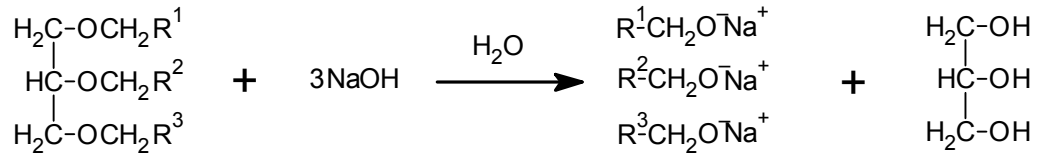
Some tasks of lipid analysis require separation of different lipid classes from each other. The most frequently used approach is the solid-phase extraction (SPE). SPE columns contain silica-based packing material, which can be modified with different groups, e.g., with chemically bonded octadecyl (C_{18}) groups. Separation is based on the affinity of a compound of interest to the stationary phase while sample matrix and undesired components are letting through the column with the mobile phase. Then the column is washed with the other solvent (e.g. different polarity) and the retained component is removed by the change of solvent nature. For the isolation of polar lipids, it is important to maintain the specific pH **[36]**. Moreover, SPE can be used for the removal of non-lipid contaminants from crude extracts of polar lipids **[56]**.

4.2. Derivatization of acylglycerols and phospholipids

For some types of analysis, it is necessary to modify compounds of interest chemically. Derivatization improves their chemical and physical properties. The most significant changes are decreasing the boiling point temperature, increasing the chemical stability, and simplification of detection.

As stated before, acylglycerols and phospholipids contain ester linkages between glycerol and molecules of fatty acids. To figure out which kinds of fatty acids are bonded to the glycerol backbone, it is necessary to break down the bonds between them. Chemical reactions such as transesterification and derivatization are commonly used prior to the analysis of fatty acids.

Reaction of fat hydrolysis is called saponification. This process was known from ancient times when people mixed and boiled oil or fat with ashes to produce soap. Later ashes were replaced by strong alkali. The reaction is based on the attack of a hydroxide ion on the molecule with ester bonds followed by liberation of glycerol and fatty acids in a form of salt. Sodium and potassium salts of fatty acids are soluble in water, they are the major components of soap; however, free fatty acids are insoluble. This makes the fatty acid analysis more complicated. Moreover, the saponification procedure is time consuming **[57]**.



Triacylglycerol — Salts of fatty acids — Glycerol

Saponification of triacylglycerol

Another approach is to perform transesterification, i.e. substitute glycerol moiety in glyceride molecule for another alkoxy group. The most common strategy is to convert triacylglycerols to methyl esters of fatty acids. As a result of this modification, the boiling point of the product is lower compared to the initial compound. Since only one fatty acid is connected to the methyl group, fatty acid methyl ester (FAME) has lower molar mass compared to acylglycerol. Thus, FAMEs can be transferred to the gaseous phase, separated, and detected.

Triacylglycerol — Alcohol — Alkyl esters — Glycerol

Transesterification of triacylglycerol

The reaction of transesterification is reversible. The excess of a reagent may help to get a higher yield of a product. Thus, an extra amount of a reactant is highly required. The reaction mixture should contain lipid extract, methanol (MeOH) and a catalyst. However, a large portion of a non-polar lipid like triacylglycerol does not fully dissolve in methanol. The addition of a sufficient amount of non-polar solvent (e.g. *n*-hexane **[58]**) may improve it and subsequently increase the

yield of the product. Moreover, heating the reaction mixture increases reaction efficiency as well. At the same time, elevating temperature of the system promotes the oxidation of lipids. Polyunsaturated fatty acids are the most susceptible to oxidation among the common lipids due to numerous unsaturated bonds **[59]**. To protect PUFAs from alteration and autoxidation, it has been advised to add *n*-butylated hydroxytoluene (BHT) as an antioxidant **[60, 61]**.

The transesterification process requires the addition of an alkali or acidic catalyst, in particular: NaOH, KOH, and sodium methoxide CH_3ONa as alkali-based catalysts, or formic acid, hydrochloric acid, acetyl chloride, sulfuric acid, and boron trifluoride in methanol as acidic-based catalysts **[62]**. The latter catalyst is the most common among the acidic-based catalysts **[24]**. When using a BF_3-based catalyst, the reaction time is brief. However, a short shelf-life of BF_3-methanol solution limits the its use.

The transesterification reaction proceeds considerably faster in the presence of alkali-based catalysts compared to acidic-based catalysts **[63]**. The acid-catalyzed transesterification reaction demands higher temperature, equal to or exceeding 100°C **[64, 65]**. Since PUFAs can undergo oxidation at high temperatures, it is more reasonable to keep the temperature as low as possible at which the reaction proceeds quantitatively. Thus, the usage of alkali-based catalysts is preferable.

Sodium methoxide (CH_3ONa) is the most frequently used basic transesterifying agent. Ten minutes at 50°C in the presence of a 100-fold excess of 0.5 to 2M sodium methoxide solution are the sufficient conditions to carry out the TAGs and phosphoglycerides transesterification **[66]**. Moreover, 0.5% (~0.07 M) CH_3ONa promotes significant FAMEs generation at 40°C even with the stoichiometric molar ratio of TAG/MeOH **[67]**. The addition of a diluted acid solution or $NaHSO_4$ (15% solution) stops the reaction by neutralizing sodium methoxide **[24]**. However, if non-esterified fatty acids present in a sample, they remain intact in basic conditions **[68]**.

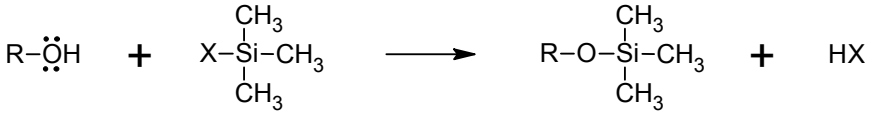
After transesterification procedure, FAMEs should be separated from the reaction mixture. FAMEs are non-polar thus they show affinity to non-polar solvents. Liquid alkanes such as *n*-pentane, *n*-hexane, or iso-octane are suitable for FAMEs extraction **[62, 69]**. Addition of water helps to create a two-phase mixture; thus, FAMEs concentrate in the organic non-polar phase. Extraction with a mixture of *n*-pentane and water in a ratio 2:1 gives 97-99% recovery of FAMEs **[65]**.

4.3. Derivatization of glycerol

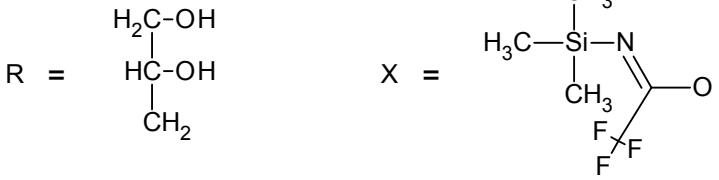
The main by-product of the transesterification reaction is glycerol. It can also be partially or fully deuterated during isotope labeling procedure. Thus, the analysis of glycerol can be an additional source of information about lipid metabolism.

Glycerol, 1,2,3-propanetriol, is a polyol compound. It is highly polar due to hydroxyl groups, therefore it has good water solubility and considerably lower solubility in organic non-polar solvents. This feature allows the separation of glycerol from FAMEs after reaction of transesterification. Apart from the glycerol, in the water phase, there is NaCl, salt, which was generated before by the neutralization reaction between acid and the excess of sodium methoxide. The high concentration of the salt does not allow the use of liquid chromatography coupled to mass spectrometry for glycerol detection. Modification of glycerol can help to overcome this problem. The hydroxyl groups of glycerol can be altered to create volatile derivatives of glycerol.

Silylation, a process of incorporation of a silyl group (R_3Si), has been proposed as one of the ways to make non-volatile molecules bearing an active hydrogen (–OH, –SH, $–NH_2$) suitable for gas chromatography due to the loss of hydrogen bonds. The most common silylating agents for GC are BSTFA [*N*,*O*-Bis(trimethylsilyl)trifluoroacetamide] and BSA [*N*,*O*-Bis(trimethylsilyl)acetamide], which introduce trimethylsilyl (TMS) groups to the molecules of interest. A catalyst such as trimethylchlorosilane (TMCS) is widely added for improving the derivatization process **[70]**. The silylation reaction driven by BSTFA proceeds faster and more completely than by BSA **[71]**. Hence, BSTFA with addition of TMCS is an optimal choice for glycerol derivatization.



where



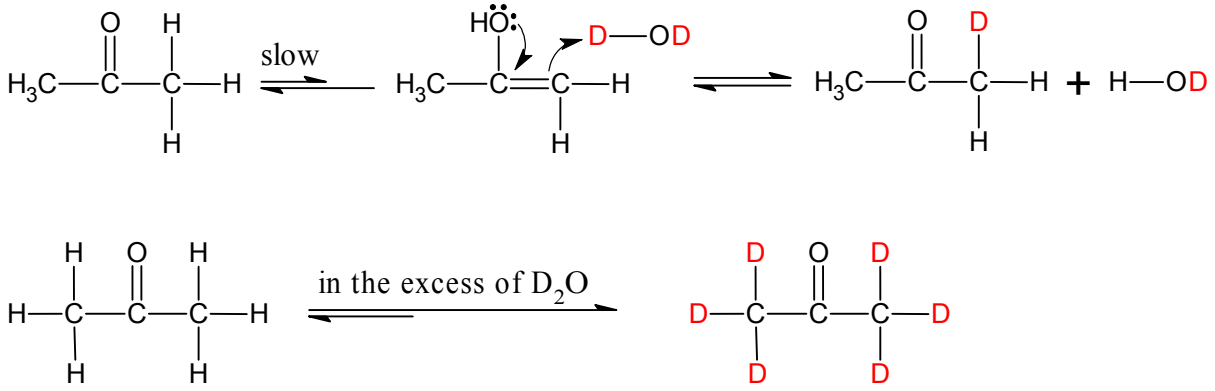
The reaction between glycerol and BSTFA

BSTFA and silyl derivatives are sensitive to moisture, i.e. unstable in presence of traces of water **[70]**. Glycerol is hygroscopic so the water should be excluded from the reaction mixture as much as possible. Thus, water from the aqueous phase collected after the transesterification must be vaporized. Glycerol is thermally stable and does not evaporate under 120°C at atmospheric pressure **[72]**. After evaporation of water, there is a dry residue containing mainly glycerol and salt crystals at the bottom of the flask. Using a portion of pyridine, glycerol is transferred to a new glassware leaving the impurities behind in the evaporation flasks. The choice of pyridine as a solvent is based on two reasons of principle. Firstly, glycerol is soluble in pyridine, whereas NaCl does not dissolve in it. Therefore, it would not be present in the reaction mixture. Secondly, pyridine is often used as a solvent in the silylation reactions **[70, 71, 73]**. BSTFA is added to glycerol dissolved in pyridine. After moderate heating of the mixture in a sealed vial, the sample is ready for measurements.

4.4. Acetone labeling for heavy water enrichment assessment

As discussed before, lipidomics and metabolomics methods usually require the usage of stable isotopes. Introduction of D_2O, heavy water, to a living system provides deuterium for tracking metabolic pathways. To incorporate deuterium in DNL, it is necessary to maintain a sufficient amount of it in body fluids. Moreover, the excess of deuterium in the organism can affect the rate of metabolism due to stronger carbon–deuterium (C-D) bonds compared to carbon–protium (C-H) **[74]**. To not disturb the experiment, the amount of deuterium should be controlled.

Determination of D_2O level in plasma corresponds to its relative amount in the body. One of the methods established for the heavy water enrichment measurement is preparation of labeled acetone via exchange reaction. Acetone exists in keto and enol forms. Under basic conditions, deuterium from heavy water can be incorporated in acetone while it is in an enol form. Acetone reacts with unlabeled water as well but the products are indistinguishable from the reagents. With an increase of D_2O to H_2O ratio the exchange reaction tends to produce more deuterium-labeled acetone. According to this, a set of solutions with the exact percent of heavy water can be prepared and mixed with acetone to create a calibration curve.



The simplified reaction of formation labeled acetone

With the addition of heavy water, not all the molecules of acetone are completely modified with deuterium, yet after a while acetone becomes proportionally deuterated. When the equilibrium of the exchange reaction is reached, i.e. the concentrations of reagents and products remain stable, acetone (both labeled and unlabeled) should be extracted with organic non-polar solvent. Hexane can be used for D-acetone extraction from plasma samples to avoid coagulation of proteins **[75]**. The extraction is an essential step prior to gas chromatography analysis to isolate acetone from the reaction mixture.

5. Separation and analysis

Chromatography is the widely used technique for separation and isolation of compounds in complex mixtures. Chromatographic columns coupled with various detectors are a powerful tool for clinical, pharmaceutical, environmental and food analysis.

Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (the stationary phase) while the other (the mobile phase) moves in a definite direction. The first chromatographic separation was performed by M. S. Tswett. He used calcium carbonate as a polar stationary phase and petrol ether as a non-polar mobile phase **[76]**. His experiment was a forerunner for the modern method of liquid chromatography (LC). Another approach is to use gas as a mobile phase instead of liquid. This method is called gas chromatography (GC).

Interaction between analyte and stationary and mobile phases underlines the principle of chromatography. Since different compounds have different affinities for the stationary and mobile phases, they tend to travel differently in a chromatographic column (or plate in a case of planar chromatography). As a result, compounds leave the column at different times (i.e. retention time, RT).

Retention time can be used as a parameter for lipid identification; however, it is incomplete for full detection and should be supported by other methods of analysis. One of the most comprehensive methods of lipid qualification and quantification is mass spectrometry. Mass spectrometry (MS) is an analytical technique that enables detection of the mass to charge (*m/z*) ratio of molecules and their fragments. A mass spectrum is the intensity plotted against *m/z*. Characteristic peaks give important information for identification of compounds.

5.1. Gas chromatography

Lipid molecules are non-volatile and boiling them results in their oxidation and degradation. Nevertheless, there are options to adopt gas chromatography (GC) for lipid separation as well; even the mobile phase here is gas. The chemical modification of lipids makes lipid molecules volatile so they can be transferred to the gas phase. The gas itself should be pure and inert (helium, nitrogen, less often hydrogen) to not react with the molecules of interest and not compromise the result.

Stationary phase in GC generally is a liquid siloxane-based polymer with different functional groups such as methyl, methyl phenyl, methyl trifluoropropyl, methyl cyanoethyl, etc. **[77]**. Another polymer used as a stationary phase is polyethylene glycol **[78]**. These polymers are stable at high temperatures maintained inside the column oven. High temperature (up to 250°C **[79, 80]**)

in GC is required to transfer FAMEs and other compounds to a gas phase and to keep them volatile during the separation process.

The materials of stationary phases in GC can be of different polarity, similarly as in LC. Since a mobile phase is an inert gas, the nature of a stationary phase significantly influences the way analytes are retained in the column. Polar stationary phases allow separating fatty acids having the same number of carbons but different saturation degrees with saturated fatty acids first to emerge and then unsaturated fatty acids in ascending order of number of double bonds **[68]**.

There are two ways of stationary phase organization, namely packed columns and capillary columns. They are made of glass, fused-silica, or stainless steel. Glass is more inert than metal so it can be used in highly precise analyses. Columns in GC are substantially longer than in LC because of the higher speed of a mobile phase. Packed columns contain fine inert solid particles (commonly diatomaceous earth) itself or covered with non-volatile polymeric, chemically inert, and thermally stable liquid. The common length of packed columns is 1.5–10m in length, and an internal diameter is 2–4mm **[81]**. Although they have low resolution, their capacity is higher compared to capillary columns, which makes packed columns suitable for preparative chromatography. Capillary columns are hollow and named open-tubular. The carrier gas is going through them easily without the resistance. The length of capillary columns ranges from 5 to 100 m; columns of 25–30 m are the most common in practice. The internal diameter of capillary columns is a few tenths of a millimeter **[78]**. The inner walls of open-tubular columns are coated with the stationary phase, which can be viscous liquid applied directly on walls or sorbent without chemical binding. Furthermore, it can be chemically bonded onto the inner walls of the tube and cross-linked throughout the polymer matrix.

There is another way to distinguish open-tubular columns: there are wall-coated open tubular (WCOT) and support-coated open tubular (SCOT) columns. Walls of WCOT are coated with a thin layer of a liquid stationary phase. In the SCOT columns, a stationary phase is absorbed on support material such as diatomaceous earth, for instance. The separation efficiency of WCOT columns is higher than SCOT columns **[82]**.

5.1.1 Comprehensive two-dimensional gas chromatography

The longer the column, the higher the resolution can be achieved. However, long columns still can be inefficient if analytes are of a similar nature. In planar chromatography, it is possible to increase resolution by rotating a plate 90° after the first development and redeveloping with the second mobile phase. In GC the change of the mobile phase during the separation process is impossible; however, the first column and the additional one mounted sequentially may improve the resolution. Coupling two or more columns is a complicated task; however, it gives greater separation in a case of compounds of similar nature. It also requires a specific way of connection between the columns. The heart cutting technique has been the first approach of the connection. A portion of the gas stream with analyte is taken from the first column and focused into the second one. The polarity of the second column can differ from the first one; moreover, the second column can be set at higher or lower temperature compared to the first column. This technique makes the separation of compounds more efficient. However, it allows to separate only a few compounds in a sample. Thus, it is suitable only if the analysis of an entire sample is not required. Additionally, the valve between the columns should be at the same operating temperature as columns but also should be manually operated from the outside **[83]**. Moreover, it is necessary to trap the analyte in the first column before entering the second column to produce the final peaks sharper.

In 1991, Phillips and Liu published an article on comprehensive two-dimensional gas chromatography (GCxGC) **[84]**. In this method, all the substances are passing through both open-tubular columns with different stationary phases. To create this technique, they placed a special modulator at the interface between the columns. The modulator keeps the portion of the gas stream with analytes emerging from the first column for a few seconds and then releases it into the second column. While the next portion is kept, the previous one passes through the columns and goes to the detector. In the Fig. 6 the principal parts of the GCxGC instrumentation are shown. Typically, the primary column phase is less polar than the secondary column phase **[69]**. The second column is shorter than the first one making the chromatographic development is rapid enough for analytes to leave the second column.

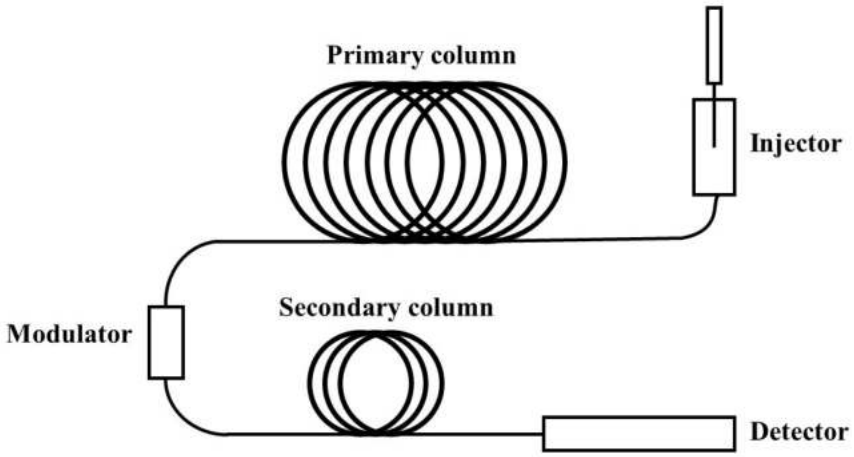


Fig. 6. GCxGC instrumentation

After detection and data processing, a two-dimensional contour plot chromatogram is created. Axes represent retention times on columns, i.e. one axis is retention time on the primary column and the other is retention time on the secondary column. In the Fig. 7 GCxGC 2D chromatogram of the standard mixture of 45 FAMEs is shown as an example. The separation power of this method is strong enough to develop isolated peaks of different c*is* and *trans* isomers of unsaturated fatty acids. The height of a peak corresponds to the intensity of the signal.

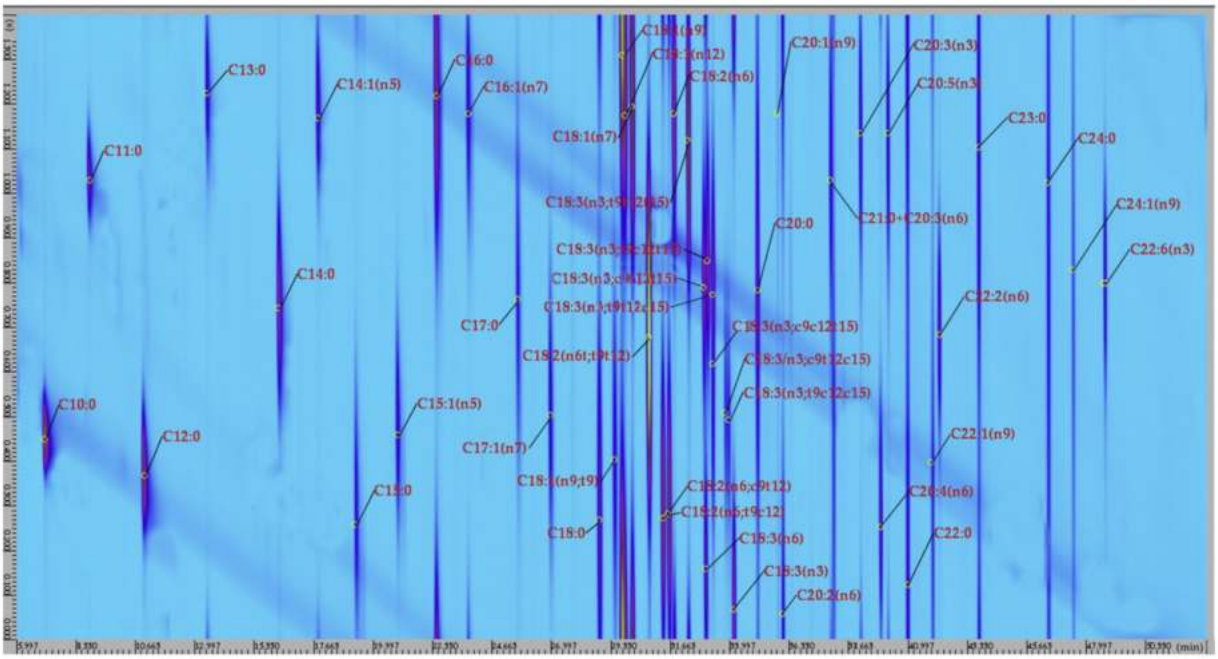


Fig. 7. GCxGC 2D chromatogram of the standard mixture of 45 FAMEs (adapted from **[69]**)

5.2. Liquid chromatography

The most common type of chromatography used for lipid analysis is liquid (i.e. liquid chromatography, LC), since lipids are non-volatile and the size of molecules is relatively large. LC is an effective method for lipid separation and identification. For this task, the reversed-phase (RP) mode is used. In this approach, a stationary phase is non-polar and a mobile phase is polar. There is a great variety of stationary phases: chemically modified silica-based or organic polymer-based materials. The most common ligand is n-octadecyl (C_{18}); it is satisfactory for the most routine tasks. Due to the intermolecular forces, lipid molecules have an affinity to this non-polar material so they can be retained in the column.

Chromatographic columns in LC are much shorter compared to GC because of the difference in viscosity of liquid and gas. The higher viscosity of liquid results in a lower flow rate of the liquid mobile phase in comparison to the gas mobile phase. Thus, the chromatographic separation might be time consuming which is not optimal for a big set of samples.

As stated before, the mobile phase should be much more polar. In case of lipid separation, mobile phases based on acetonitrile, methanol, water, and isopropanol are generally used **[35]**. The solvents should be completely miscible; otherwise, the droplets of the one phase can cause instability of the separation. The solution in which extracted lipids are dissolved also should be compatible with the mobile phase. Some lipid identification methods require using gradient elution when one solution of the mobile phase is gradually replaced by the other mixture of solvents.

The efficiency of chromatographic separation inversely correlates with a particle size of the stationary phase **[85]**. The change of particle size entails the shift of the other characteristics of the system. To compensate for this, the flow rate must be adjusted to the desired efficiency. The flow rate is inversely proportional to the particle size: as the particle size decreases, the flow rate increases, the analysis time decreases, the width of the peaks becomes narrower, and more resolution is achieved. However, according to the Van Deemter equation, the flow rate of the mobile phase itself is inversely proportional to the efficiency **[86]**. With the increase the flow rate, not all the molecules have enough time to move into and out of the pores of the stationary phase. The increase in the flow rate also broadens the peaks in a chromatogram. Therefore, the optimal flow rate should not be too low or too high. All these considerations resulted in the two types of

modern column LC: High Performance Liquid Chromatography (HPLC) and Ultra Performance Liquid Chromatography (UPLC), or Ultra High Performance Liquid Chromatography (UHPLC).

In HPLC the size of the adsorbent particles is smaller compared to the traditional LC and ranges from 2 to 20 μm. The HPLC setup requires using high operational pressure (up to 400 bar or 40 MPa) **[87]**. In UPLC the size of the particles is sub-2 μm, which provide the better resolution, higher efficiency and reduced time of analysis. However, to achieve the optimal result it is necessary to build up higher back pressure in the system because smaller particles have stronger resistance to flow. The pressure in UPLC reaches up to 100 MPa **[88]**. Despite the advantages of UPLC, the UPLC machine is more expensive than HPLC; thus, HPLC is still widely used.

5.3. Mass spectrometry

Chromatography coupled to mass spectrometry is a comprehensive method for analysis of organic molecules. It is fast and accurate and even complex samples can be examined thoroughly. The molecules that are possible to transfer to the gas phase and ionize afterwards move to a mass analyzer. In a mass analyzer, ionized molecules and their fragments migrate at different velocities under the influence of electric and magnetic fields. The difference of mass and charge between various ions leads to their segregation. Eventually, ions are detected by an electron multiplier.

As a result of the ionization process, ions are generated. Most methods provide formation of positive ions; however, negatively charged molecules can be produced and detected by other means as well. The ion M^{n+} (or $M^{n\bullet+}$) is called the molecular ion, or the parent ion. The formed ions are not entirely stable and they show the tendency to decay into smaller particles. Some of them are not charged and consequently do not participate in the separation and detection. The other particles that carry some charge travel through the instrument and can be observed in the mass spectrum. These ions are named daughter ions.

There are numerous ion sources providing ionization of molecules in mass spectrometry. One of the major distinctions between all of them is how hard the ionization proceeds. Hard ionization methods produce daughter ions due to a high degree of fragmentation. Generally, break of bonds follows some rules and can be characterized. Thus, the pattern of the fragmentation is mainly predictable and serves as a good approach for structural identification. However, some spectra are

too complicated to interpret. Soft ionization methods provide less energy to the ionization process so it generates molecular ions with only a few daughter ions.

The most widely used ionization techniques for lipid analysis are Electron Impact Ionization (EI) and Electrospray Ionization (ESI). EI is the hard ionization technique causing analytes fragmentation. EI is mostly used in gas chromatography-mass spectrometry (GC-MS) since analytes need to be volatile. The process of ionization is as follows: a molecule (mass range of it should not exceed 1000 Da) comes into an ionization chamber, the beam of high-energy electrons (70 eV) knocks electrons out of the molecule, generating the positive ions. In contrast, ESI is a soft ionization technique suitable for non-volatile and thermally labile molecules. This method is suitable for molecules of high molecular weight as long as the sample is completely dissolved. The flow of liquid from a chromatographic column enters inside of a stainless steel or quartz silica capillary. The fine capillary (nebulizer) maintained at a high voltage (e.g. 2.5–6.0 kV) **[89]** releases the charged droplets. Due to elevated temperature and low pressure in a chamber, solvent evaporates leaving ionized molecules in the gas phase. Additionally, the coulombic forces in drops are too high for them to remain stable and they eventually explode to create gas state analytes. Nebulizer can be charged positively or negatively, thus the emitted ionized particles can have a positive or negative charge (i.e. $[M+H]^+/[M-H]^-$), respectively. The positive ESI ionization is more commonly used for routine studies of the majority of lipids **[90]**. The modifiers such as ammonium acetate, ammonium formate, and formic acid or acetic acid added in the mobile phase in prior chromatographic separation of lipids provide stable adduct ions using ESI in positive or negative modes. TAGs mostly form $[M+NH4]^+$ or $[M+Na]^+$ adduct ions **[35]**. Other positive adduct ions are possible as well depending on modifiers presence in the mobile phase.

Moving from the ionization chamber, ions are accelerated and focused into the mass analyzer by the electric field. There are several types of mass analysers to be used for the analysis of biological samples. They all have the thing in common: they sort the ions according to their mass-to-charge ratio. The most common analyzer is a quadrupole made of four cylindrical metal rods placed in parallel. The opposing rods are connected to each other electrically; the constant DC (direct current) voltage and alternating RF (radio frequency) voltage are applied to each pair of rods of different polarities. Passing from the ion source, ions enter the space between the rods. Ions start to oscillate by the electric field within an abruptly varying phase.

The ions that are in resonance move in a stable trajectory and then they will be spotted and counted. Meanwhile, the others leave the quadrupole without detecting. The segregation happens because only the ions with the selected *m/z* can travel through the space between rods and not be neglected. If you keep the constant voltage applied to the rods, the only one *m/z* value will be measured. The name of this mode is Selected Ion Monitoring (SIM). Another mode is the Scan mode where the voltage is increasing so all the *m/z* range is scanned.

The other type of mass analyzer is a triple quadrupole (QQQ or QqQ). QqQ MS is a tandem mass spectrometer consisting of 3 parts connected in series: 2 quadrupoles working on the same principle as described before and a cylinder between them. The first quadrupole Q1 usually selects a precursor ion with the single *m/z*. Q2 is a collision cell filled with inert gas (Ar, N_2, He) where precursor ions are breaking down to generate daughter ions. The third quadrupole Q3 can select and separate the specific ion [Selected Reaction Monitoring mode (SRM) or Multiple Reaction Monitoring (MRM) mode] or scan all the ions generated during fragmentation of the precursor ion (Product Ion Scan). Beside these modes, it is possible to use Precursor Ion Scan (precursors ions are not selected but daughter ions of the exact *m/z* are focused to the detector after the fragmentation) and Neutral Loss Scan (no ion selecting occurs, detecting of all ions).

Orbitrap is another type of mass analyzer. It consists of an outer barrel-shaped electrode and an inner electrode. The ions entering inside the Orbitrap start to oscillate around the axis. The ions with different *m/z* oscillate at different frequencies, resulting in their separation. When the voltage on the central electrode changes, ions with a specific *m/z* value are ejected from the ion trap and detected by an electron multiplier. Moreover, the Orbitrap can be both a mass filter and a detector. By measuring the oscillation frequencies induced by the ions on the outer electrodes, the mass spectra of the ions are obtained using image current detection. The acquired data is processed by the Fourier transform method.

Time-of-Flight (TOF) mass analyzer does not select the exact *m/z*; the full scan can be done. All the ions are pushed and accelerated from the ion source into the vacuum tube. Escaping from the ion source, the ions have the same kinetic energy but distinct masses. The velocity of flight is related to the mass-to-charge ratio. Therefore, close to the end of the tube, the ions are moving at different speeds.

$$\text{Time-of-Flight} = \frac{L}{\upsilon} = L\left(\frac{m}{2zeV}\right)^{½} \quad (3)$$

where L is a distance to travel (i.e. the length of the vacuum tube), υ is the velocity of the ion, m is mass of the ion, z is the charge on the ion, e is the charge of an electron, V is the magnitude of the high voltage.

The equation (2) shows that the time of flight of an ion depends on the length of the tube and mass and charge of the ion. A group of ions with the same *m/z* comes at the same time point, while it takes a longer time for the heavier ions to arrive. The resolution increases with the increase of the path that the ions should pass. Mass spectrometer equipped with a TOF mass analyzer lacks resolution compared to the mass analyzers discussed before but it is the simplest setup among them. TOF measures all the range of *m/z* practically simultaneously, no ion is discarded as it occurs in quadrupole mass analyzers.

AIMS OF THE THESIS

Obesity, type 2 diabetes, and cardiovascular diseases, which are largely associated with impaired lipid metabolism, have become major public health problems worldwide. Dietary habits and lifestyle can affect the lipidome – the total composition of lipids in the body. Modern analytical methods allow to monitor the lipidome as well as selected lipids to control the impact of exogenous factors on normal or pathological state of lipid metabolism. This thesis focuses on optimization of biological sample preparation for lipidomics analysis by comprehensive two-dimensional gas chromatography coupled to mass spectrometry.

Here I aimed to (1) assess the Omega-3 index in Czech population because it represents the relative amount of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), bioactive fatty acids, linked to lower risk of type 2 diabetes and cardiovascular diseases, in red blood cells. Since EPA and DHA, omega-3 fatty acids, are found predominantly in an esterified form, I also explored (2) how bioavailable omega-3 fatty acids esterified in triacylglycerols, phospholipids, and wax esters. Furthermore, using a stable isotope of hydrogen, I examined (3) the impact of PAHSA, a novel bioactive lipid, on lipid metabolism.

LIST OF PUBLISHED ARTICLES RELATED TO THE THESIS

1. **Oseeva, M.**, Paluchova, V., Zacek, P., Janovska, P., Mracek, T., Rossmeisl, M., Hamplova, D., Cadova, N., Stohanzlova, I., Flachs, P., Kopecky, J., Kuda, O. Omega-3 index in the Czech Republic: No difference between urban and rural populations. *Chemistry and physics of lipids*, 2019, *220*, pp.23-27.

2. Rossmeisl, M., Pavlisova, J., Janovska, P., Kuda, O., Bardova, K., Hansikova, J., Svobodova, M., **Oseeva, M.**, Veleba, J., Kopecky Jr, J., 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. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 2018, *1863*(7), pp.712-725.

3. Kroupova, P., van Schothorst, E.M., Keijer, J., Bunschoten, A., Vodicka, M., Irodenko, I., **Oseeva, M.,** Zacek, P., Kopecky, J., Rossmeisl, M., Horakova, O. Omega-3 phospholipids from krill oil enhance intestinal fatty acid oxidation more effectively than omega-3 triacylglycerols in high-fat diet-fed obese mice. *Nutrients*, 2020, *12*(7), p.2037.

4. Sistilli, G., Kalendova, V., Cajka, T., Irodenko, I., Bardova, K., **Oseeva, M.**, Zacek, P., Kroupova, P., Horakova, O., Lackner, K., Gastaldelli, A., Kuda, O., Kopecky, J., Rossmeisl, M. Krill oil supplementation reduces exacerbated hepatic steatosis induced by thermoneutral housing in mice with diet-induced obesity. *Nutrients*, 2021, *13*(2), p.437.

5. Brezinova, M., Cajka, T., **Oseeva, M.**, Stepan, M., Dadova, K., Rossmeislova, L., Matous, M., Siklova, M., Rossmeisl, M., Kuda, O. Exercise training induces insulin-sensitizing PAHSAs in adipose tissue of elderly women. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 2020, *1865*(2), p.158576.

6. Paluchova, V., **Oseeva, M.**, Brezinova, M., Cajka, T., Bardova, K., Adamcova, K., Zacek, P., Brejchova, K., Balas, L., Chodounska, H., Kudova, E., Schreiber, R., Zechner, R., Durand, T., Rossmeisl, M., Abumrad, N. A., Kopecky, J., Kuda, O. Lipokine 5-PAHSA is regulated by adipose triglyceride lipase and primes adipocytes for de novo lipogenesis in mice. *Diabetes*, 2020, *69*(3), pp.300-312.

RESULTS AND DISCUSSION

Omega-3 fatty acids are vital substances for human well-being. I used the Omega-3 index as an indicator of the omega-3 fatty acid content in the human body. The Omega-3 index represents the relative amount of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in red blood cells. Previously, it was reported that EPA and DHA positively affect lipid metabolism. To estimate EPA and DHA content, I optimized the method of extraction and following transesterification of lipids from biological samples and measured fatty acid methyl esters (FAMEs) profile by comprehensive two-dimensional gas chromatography coupled to mass spectrometry (GCxGC-MS). In a large-scale experiment, I tested a group of volunteers from Czech Republic to determine the Omega-3 index in Czech population. In the second study I controlled the Omega-3 index in overweight/obese type 2 diabetic patients before and after omega-3 fatty acids treatment. For this research, I used EPA and DHA esterified into triacylglycerols obtained from marine fish oil. EPA and DHA can also be found in a form of phospholipids and wax esters. To test its bioavailability, we fed mice with a diet containing omega-3 phospholipids from krill oil. After the diet administration, I measured the level of EPA and DHA in RBC and liver as a marker of omega-3 fatty acids accumulation from krill oil. In the other experiment, we provided calanus oil as a source of wax esters of omega-3 fatty acids to a group of human females who performed physical exercises. To examine the synergic effect of calanus oil and physical activity, I estimate the Omega-3 index before and after the study. I used a similar approach of lipidomics analysis to examine the effect of PAHSA (a novel lipid class with a potential beneficial effect on mammalian health) administration on lipid metabolism in mice and cell culture (3T3 L1 adipocytes).

Article I

Omega-3 index in the Czech Republic: No difference between urban and rural populations

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are essential fatty acids. The Omega-3 index reflects the relative amount of EPA and DHA in red blood cell membranes. In this study, I estimated the Omega-3 index in Czech Republic. We hypothesized that people may have different values of the Omega-3 index depending on whether they live in a city or a rural area.

476 volunteers from the capital city (Prague) and 388 volunteers from the rural region (České Budějovice and surrounding countryside), 864 human subjects in total donated blood samples. Additionally, the participants responded to the survey concerning their lifestyle and eating habits.

The blood sample collection was processed in a slightly different manner in these areas due to the lack of equipment in small medical facilities in the rural region compared to the capital city. In Prague central laboratory the samples were immediately frozen and stored at -80°C, whereas the medical workers in České Budějovice countryside had to refrigerate samples in -20°C freezers and then within 1-2 days transfer them on dry ice to the central laboratory for storage.

From the donated blood, red blood cells were isolated by centrifugation. Phospholipids, building blocks of red blood cell membranes, were extracted with methyl-tert-butyl ether. Then separated lipids were transesterified with sodium methoxide in methanol. Fatty acid methyl esters, the principal products of the reaction, were isolated with hexane. The FAMEs profile was measured by the means of comprehensive two-dimensional gas chromatography coupled to mass spectrometry. The Omega-3 index was calculated as a ratio of EPA and DHA to the total amount of detected fatty acids (expressed as mol %).

The mean Omega-3 index of the study population was 3.56%. The value ranged from 1.12% to 8.10%. The Omega-3 index in Czech Republic is relatively low compared to global statistics.

Considering the number of participants and the large studied area, the chosen approach of blood collection was optimal but not ideal. The different methods of freezing and storage of the samples immediately followed by the blood collection could lead to fluctuations in results. Moreover, the limited number of laboratory workers can reduce the probability of random errors. Unfortunately, based on the design of the experiment, we had to seek assistance from numerous medical workers. They were fully instructed on the experiment; however, if errors occurred during this part of the project, they remained unrecorded.

To reduce the risk of other inaccuracies during the extraction, derivatization, and chromatographic measurement, I added phosphatidylcholine 19:0 (PC 19:0) as an internal standard for data normalization prior to the extraction procedure. PC 19:0 comprises nonadecanoic acid ($C_{19}H_{38}O_2$), a saturated fatty acid, that is nearly undetectable in the human body **[91]**. Thus, methyl ester of nonadecanoic acid derived from PC 19:0 does not affect the overall fatty acids profile.

For quality control, I used blood samples aliquoted prior to the entire extraction process donated by the only person. I processed an aliquot for each set of samples as if it was a real sample.

Some impurities, e.g. plastic residues, long hydrocarbon chains, can interfere and affect the result. To eliminate this outcome, I used a blank sample, i.e. an empty plastic Eppendorf tube was filled with solvents needed for the extraction but an aliquot of RBC was excluded. Then I performed the whole procedure as if the real sample was present there.

We hypothesized that the region of living would affect the Omega-3 index. Moreover, České Budějovice and its surroundings are famous for local ponds and fish farms. However, I could not detect a significant difference between urban and rural cohorts.

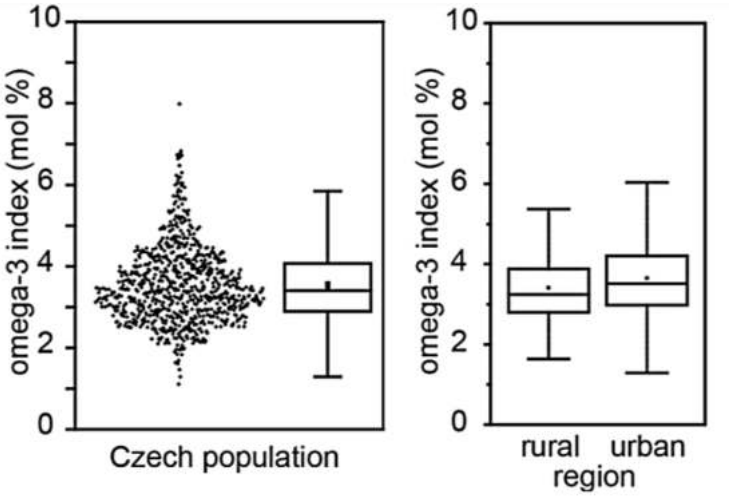


Fig. 8. Omega-3 index in Czech population (left) and Omega-3 index in rural and urban regions of Czech Republic (right) expressed as mol %

As a part of the project, participants filled the questionnaires that included questions about their fish consumption and omega-3 supplements intake. People were divided into six groups related to the frequency of fish dishes in their diet. Subjects who reported eating fish at least two times per week had the average Omega-3 index 4.10%. Moreover, The Omega-3 index within the groups showed a tendency towards decrease when less fish was consumed. People who avoid fish in their diet had the lowest Omega-3 index (2.58%) within the cohorts.

In addition, participants were divided into groups according to their self-reported intake of omega-3 supplements. I found that people who consumed pills with omega-3 fatty acids had a

higher Omega-3 index (4.22%) comparing to people who did not take any fish-oil supplements (3.46%).

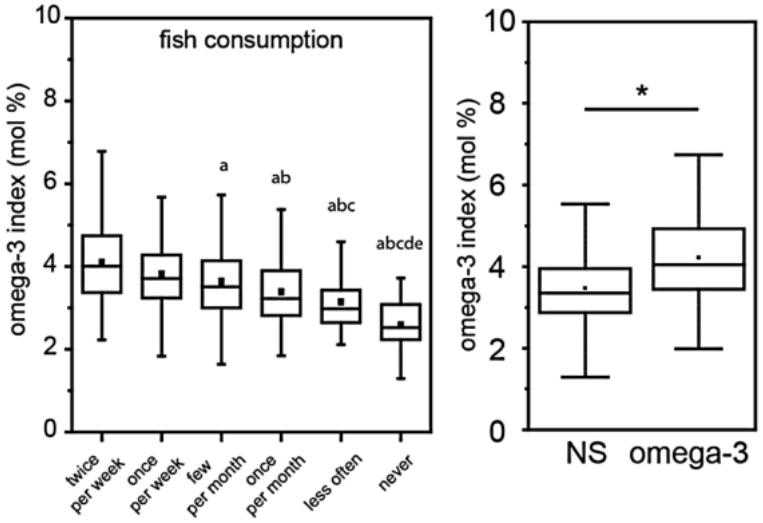


Fig. 9. Omega-3 index expressed based on the frequency of fish meal consumption. ANOVA on ranks: a, statistically significant from the 1st group; b, statistically significant from the 2nd group, etc. up to e, statistically significant from the 5th group (left). Omega-3 index of participants who reported omega-3 supplementation (omega-3) or no supplements (NS) (right).

Article II

Differential modulation of white adipose tissue endocannabinoid levels by n-3 fatty acids in obese mice and type 2 diabetic patients

Type 2 diabetes is a metabolic disorder accompanied by chronic inflammation. So far, there is no cure for this disease. Even so, a diet, physical activity and weight management may beneficially affect the condition. EPA and DHA as marine omega-3 fatty acids are known for their anti-inflammatory properties. In this study 69 overweight/obese type 2 diabetic patients were treated for 24 weeks with a dose of omega-3 fatty acids concentrate or corn oil as a placebo. Serum and white adipose tissue were collected before and after the treatment to control the Omega-3 index. To measure the relative amount of EPA and DHA by comprehensive two-dimensional gas chromatography coupled to mass spectrometry, I modified fatty acids incorporated in triacylglycerols into FAMEs. In both types of samples, I detected increase of the Omega-3 index in relation to the EPA and DHA administration. In serum it was increased ~2.5-fold and

in WAT – 2.2-fold. On the contrary, the placebo group did not show any significant change in the Omega-3 index.

In this study the number of participants (n=29) was considerably smaller than in the project concerning the Omega-3 index in Czech Republic, which allowed the experiment to be more controlled. The sampling of human samples was under strict monitoring in the hospital where biopsies and blood collection were made.

Article III

Omega-3 Phospholipids from Krill Oil Enhance Intestinal Fatty Acid Oxidation More Effectively than Omega-3 Triacylglycerols in High-Fat Diet-Fed Obese Mice

Fish oil, the most available omega-3 supplement, mainly comprises triacylglycerols. However, there are other lipid classes that can be administered as a source of EPA and DHA. Krill oil contains omega-3 fatty acids in the form of phospholipids. In this study of intestinal metabolism, we fed C57BL/6N mice with 4 different high-fat-based (HFD) diets [lipids ~35% (wt/wt)]: 1) HFD; 2) HFD-based diet, in which 15% (wt/wt) of lipid content were substituted by TAG-based EPA and DHA (ω3TG diet) compound to reach ~30 mg/g EPA and DHA concentration; 3) HFD-based diet, in which 45% (wt/wt) of lipid content were substituted by Atlantic krill oil containing PL-based EPA and DHA (ω3PL-H diet) compound to reach ~30 mg/g EPA and DHA concentration; and 4) HFD-based diet, in which 15% (wt/wt) of lipid content were substituted by Atlantic krill oil containing PL-based EPA and DHA (ω3PL-L diet) compound to reach ~10 mg/g EPA and DHA concentration.

As a test of their bioavailability, after 8 weeks of the diet administration, I measured the Omega-3 index in the cohorts. For this purpose, mice RBC were collected to analyze omega-3 fatty acids content. After GCxGC-MS analysis of FAMEs followed by phospholipids extraction and derivatization, I calculated the Omega-3 index. The group of mice fed with HFD was considered as a control since they were not treated with omega-3 supplements. ω3TG and ω3PL-L mice showed a similar Omega-3 index (7.5% and 6.5%, respectively), although the ω3PL-L diet contained 3 times less EPA and DHA. The ω3PL-H mice had the highest Omega-3 index (12.5%) despite consuming the same amount of dietary omega-3 fatty acids as ω3TG mice. Thus, EPA and

DHA in a phospholipid form are more prone to be incorporated into the body than omega-3-containing triacylglycerols.

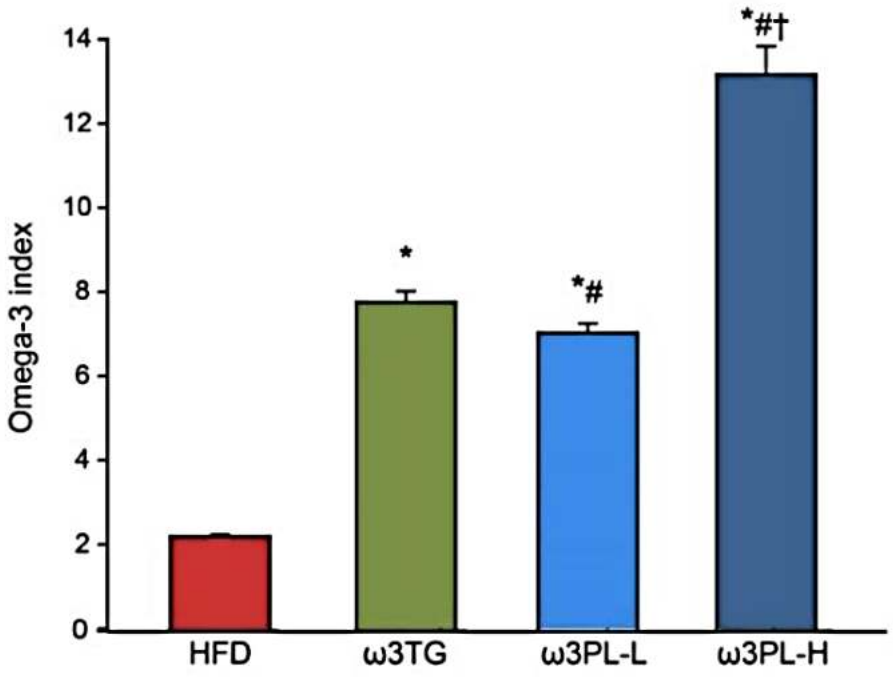


Fig. 10. Omega-3 index within the cohorts based on their diets. Data are mean percentage ± SEM (n = 8). *, significantly different vs. HFD; #, significantly different vs. ω3TG; †, significantly different vs. ω3PL-L ($p < 0.05$, one-way ANOVA).

Article IV

Krill Oil Supplementation Reduces Exacerbated Hepatic Steatosis Induced by Thermoneutral Housing in Mice with Diet-Induced Obesity

Liver synthesizes and metabolizes a lot of lipids. Moreover, impaired hepatocytes tend to store increased amounts of triacylglycerols. Thus, liver can be a target tissue in a study of omega-3 fatty acids accumulation.

In this study, C57BL/6N mice were fed with 3 different diets: 1) lard-based high-fat diet LHF, 2) LHF-based diet supplemented with krill oil as a source of omega-3 fatty acids-containing phospholipids (ω3PL diet), 3) LHF-based diet supplemented with omega-3 fatty acids in the form of triacylglycerols (ω3TG diet). Total content of EPA and DHA in both supplemented diets was

adjusted to ~30 mg/g. The duration of the diet administration was 24 weeks; one cohort of mice received a LHF diet, the other one received an ω3PL diet. Besides these groups, two other cohorts were fed with LHF for 8 weeks and then the diet was replaced with omega-3-enriched diets (ω3PL or ω3TG) until the end of the experiment. These groups were labeled as ω3PL-R and ω3TG-R, respectively. After 24 weeks of administration of mentioned diets, the mice were dissected. Collected liver samples were flash frozen and stored for analysis.

Using MTBE-based extraction, I obtained total lipid extract, which was divided into the neutral lipid fraction (mostly TAGs) and the polar lipid fraction (mostly phospholipids) by solid-phase extraction (SPE). Extracted lipids were transesterified for the following GCxGC-MS analysis.

Table 2. Omega-3 fatty acids content in the polar and neutral fraction of liver lipids

	LHF group (control)	ω3PL group	ω3PL-R group	ω3TG-R group
	Molar percentage of omega-3 fatty acids in the polar lipid fraction, %			
EPA	0.14±0.01	9.48±0.20[a]	8.86±0.48[a]	6.75±0.14[abc]
DHA	7.20±0.23	12.1±0.3[a]	12.2±0.4[a]	12.1±0.5[a]
	Molar percentage of omega-3 fatty acids in the neutral lipid fraction, %			
EPA	0.060±0.004	2.09±0.37[a]	2.70±0.26[a]	2.00±0.11[a]
DHA	0.58±0.07	3.33±0.57[a]	3.92±0.44[a]	3.28±0.17[a]

where a,b,c different from LHF, ω3PL, ω3PL-R, respectively (one-way ANOVA or Kruskal-Wallis test).

The content of EPA and DHA was higher in the all liver samples (both the polar and neutral fraction) taken from mice fed omega-3 supplemented diets compared to mice fed the control lard-based high-fat diet (in the Table 2). Thus, I showed that liver accumulates omega-3 fatty acids from dietary sources. However, the form of omega-3 fatty acids (TAGs or PLs) does not significantly affect the level of EPA and DHA.

For the confirmation of omega-3 fatty acids presence in diets, I isolated total lipids by two-step extraction with hexane and mixture of methanol and dichloromethane. I used this approach to maximize the number of lipid classes and increase lipid recovery. Extracted lipids were converted into FAMEs for GCxGC-MS analysis. FAMEs profile showed the absence of a detectable amount

of EPA and DHA in LHF diet samples. In case of omega-3 supplemented diets, EPA (3.74±0.02 for ω3PL diet and 3.93±0.20 ω3TG diet) and DHA (1.32±0.04 for ω3PL diet and 1.76±0.13 ω3TG diet) concentrations were comparable.

Article V

Exercise training induces insulin-sensitizing PAHSAs in adipose tissue of elderly women

Aging is a complex process accompanied by changes in metabolism. Often they give rise to low-grade inflammation and metabolic disorders. Healthy lifestyle, i.e. balanced diet and physical activity, may diminish the effects of negative changes in elderly people.

To test the combinatorial effect of physical training and omega-3 supplementation, woman aged 65-80 (n=55) exercised with professional trainers for 4 months. During this period, they also obtained capsules with Calanus oil as a source of omega-3 fatty acids, or capsules with sunflower oil as a placebo. Calanus oil provided omega-3 fatty acids in a form of wax esters. To control bioavailability of Calanus oil, I analyzed the fatty acid profile of RBC samples taken before and after the treatment and physical activity program. For this purpose, I extracted phospholipids from RBC with methyl-tert-butyl ether and then transesterified them with sodium methoxide in methanol. FAMEs formed in the reaction of transesterification were analysed by GCxGC-MS. The Omega-3 index was determined as a ratio of EPA and DHA to the total amount of detected fatty acids (expressed as mol %).

The Omega-3 index did not show a significant difference between the Calanus group and the placebo group. The results are presented in the Table 3.

Table 3. Omega-3 index within the Calanus oil group and the control group

Experimental group	Calanus		Placebo	
Experimental status	before	after	before	after
Omega-3 index, mol %	5.1±1.4	6.1±1.7	5.0±1.0	6.4±1.2

Comparing this research to the study of the Omega-3 index in Czech Republic, I found out that the Omega-3 index of aged women was higher than overall in the country. The possible explanation

is the different approaches of sample collection and storing. In this project, the blood sampling was done in a more controlled environment, immediately frozen at -80°C, stored for a short time period, and then quickly processed. Thus, degradation of omega-3 fatty acids could occur in the big comprehensive study due to limitations discussed before.

Article VI

Lipokine 5-PAHSA Is Regulated by Adipose Triglyceride Lipase and Primes Adipocytes for De Novo Lipogenesis in Mice

Lipid metabolism is based mainly on lipolysis and *de novo* lipogenesis. Under normal conditions, these processes are in balance if calorie intake is equal to calorie expenditure. Thus, the change in conditions (e.g. shift in temperature conditions, change in diet) can affect fat accumulation and its loss. Supplementation of bioactive compounds is another way to modify lipid metabolism.

In this study I wanted to test the impact of cold exposure, bioactive lipid 5-PAHSA, and a combination of both on lipid metabolism in C57BL/6J mice and in 3T3-L1 adipocytes. For this purpose, mice were divided into 2 groups and each group was kept either at 30°C (thermoneutrality) or at 6°C (cold exposure) for 7 days before dissection. These groups were divided into 2 subgroups based on planned treatment of either 5-PAHSA oral gavage or saline gavage as a placebo. Gavage treatment was performed for 3 days before dissection. To track the changes in lipid metabolism after cold exposure and/or 5-PAHSA administration, I used heavy water as a source of deuterium. Two days before dissection mice were injected with D_2O and the drinking water was substituted for 10% (v/v) D_2O solution to reach the constant D_2O level in the organism.

After mice were dissected, collected epididymal white adipose tissue (eWAT) samples were processed for GCxGC-MS analysis of labeled FAMEs and labeled glycerol. Prior to gas chromatography analysis, glycerol was silanized by BSTFA in pyridine to make it volatile. To evaluate the change in lipids due to lipogenesis, I calculated the deuterium enrichment in the collected samples. For mass isotopomer analysis, the raw MS spectra were corrected for background natural abundances with Isocor software to obtain isotopologue profiles. As a control, I used eWAT of mice that were not involved in D_2O treatment. They did not receive any deuterium-

containing injection and heavy water solution for drinking. Therefore, their lipidomic profile is of the natural isotopic distribution. The amount of deuterium involved in metabolism can be measured indirectly by estimation of D_2O content in plasma. For this task, a portion of plasma was mixed with acetone in acetonitrile and concentrated alkaline solution. The reaction is based on keto-enol tautomerism of acetone. This conversion is a rate-limiting reaction, and thus, for the reaction to reach the equilibrium, it is recommended to incubate the mixture overnight. When the concentration of D-acetone remains stable, the reaction is considered being completed. Acetone extracted by hexane should be analyzed by GCxGC-MS as soon as possible due to volatilization of acetone. The value of deuterium enrichment in plasma is required for calculation of fractional synthesis of fatty acids and glycerol to estimate DNL. Fractional synthesis equals the ratio of deuterium mean enrichment of the molecule to deuterium enrichment in plasma since we consider the level of heavy water as pseudo steady-state **[92]**. Mass isotopomer analysis revealed that cold exposure (CE) promotes the higher rate of palmitic and stearic acids synthesis. Palmitic acid is the major product of *de novo* lipogenesis and can be elongated to stearic acid. Moreover, PAHSA enhances this effect except for stearic acid synthesis at thermoneutrality. Measurement of deuterium enrichment in glycerol showed the effect of cold exposure on glycerol synthesis but PAHSA administration did not have an impact on it.

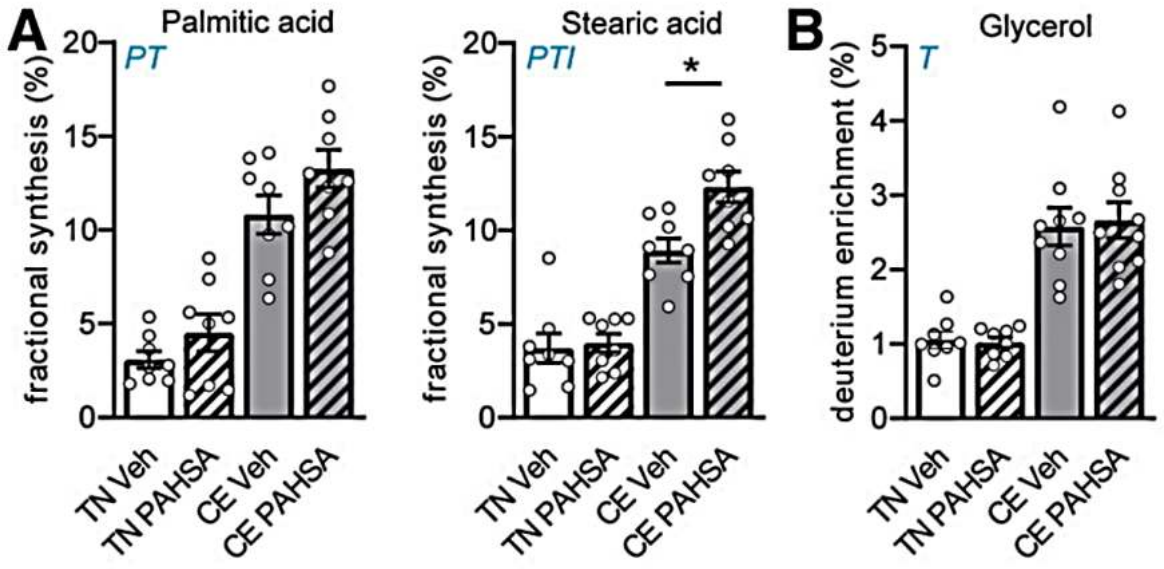


Fig. 11. 5-PAHSA stimulated DNL in eWAT during cold exposure. (A) Fractional synthesis of palmitic and stearic acid measured in hydrolysates of TAG fraction of eWAT. Two-way ANOVA with multiple comparison test (Sidak). Letters within the graphs denote a statistically significant effect of 5-PAHSA (P), temperature (T), or interaction of factors (I); *, planned multiple comparison of the effect of 5-PAHSA at the given temperature statistically different at $p < 0.05$. (B) Deuterium enrichment of glycerol measured in hydrolysates as above. Data are mean±SEM. *$P < 0.05$ by Student t test (n = 8-9). TN, thermoneutrality; Veh, vehicle; CE, cold exposure.

As a part of the experiment, I examined the effect of 5-PAHSA on 3T3-L1 adipocytes metabolism. To mimic the processes ongoing *in vivo*, we used various agents as insulin, isoproterenol, and forskolin. Insulin promotes DNL and inhibits lipolysis. By contrast, isoproterenol induces lipolysis releasing free fatty acids and glycerol from TAGs. Like isoproterenol, forskolin stimulates lipolysis.

In this experiment, we first treated adipocytes with insulin, 5-PAHSA, or their combination and then a half of plates with cells were incubated with isoproterenol. After the incubation, I took the medium above the cells. Commercial colorimetric kits provide reagents for enzymatic-based methods of determination of non-esterified fatty acids (NEFA) and glycerol content in the medium samples. The enzymatic reactions are highly specific, and thus each metabolite should be measured by a specific kit. Reaction products in both cases are of purple color; hence, the absorbance can be

measured at the wavelength of 550 nm for NEFA and 520 nm for glycerol. All assays were run twice in duplicates.

As a result of the measurements, I could detect that 5-PAHSA enhanced the glycerol release in all the setups. The NEFA concentration did not alter in the presence of 5-PAHSA in adipocytes treated by forskolin. However, it was elevated in the presence of 5-PAHSA when isoproterenol was used as a lipolysis-inducing agent. Therefore, 5-PAHSA affects the lipolysis mechanisms resulting in increased concentration of TAGs breakdown products.

CONCLUSIONS

Here I studied fatty acids involved in human and mice metabolism by extraction of fatty-acids-containing lipids and their derivatization to generate volatile fatty acid methyl esters suitable for GCxGC-MS analysis.

For the large-scale study of the Omega-3 index in Czech population, I processed 864 red blood cells samples from people living in urban areas or countryside. I found out that no significant difference in the Omega-3 index was detected between urban and rural regions: the mean Omega-3 index in Czech Republic was 3.56 mol%. Values below 4 mol% are associated with the highest risk for death from coronary heart disease **[23]**. The self-reported data about dietary habits of participants showed that EPA and DHA content in red blood cells correlated with omega-3 fatty acids consumed as fish and/or omega-3 supplements.

I tested bioavailability of EPA and DHA acids esterified in triacylglycerols, phospholipids, and wax esters in human and mice studies. Administration of omega-3 fatty acids in a form of triacylglycerols increased the content of EPA and DHA in serum, WAT, RBC, and liver. The consumption of EPA and DHA acids esterified in phospholipids positively affected the level of omega-3 fatty acids in RBC and liver. EPA and DHA in a phospholipid form are more prone to be incorporated into RBC than omega-3-containing triacylglycerols; however, consumption of EPA and DHA in a form of triacylglycerols resulted in a similar level of omega-3 fatty acids in liver compared to consumption of omega-3-containing phospholipids. In the experiment with administration of omega-3 fatty acids in a form of wax esters, I detected nearly the same levels of EPA and DHA in both control and test groups, thus, no observable incorporation of omega-3 fatty acids in RBC occurred.

In the lipid metabolism study, I used a deuterium-labeling method to track changes in mice lipidome in response to cold exposure and/or PAHSA treatment. The rate of fractional synthesis of palmitic acid, stearic acid, and glycerol, the major products of lipogenesis, showed that PAHSA stimulates DNL, and this effect is more pronounced at low temperatures. In murine adipocytes, PAHSA promoted the release of glycerol and fatty acids, the products of triacylglycerol breakdown. Therefore, PAHSA affects both lipogenesis and lipolysis processes.

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RESEARCH ARTICLES

Article I

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Omega-3 index in the Czech Republic: No difference between urban and rural populations

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ABSTRACT

Naturally occurring long-chain omega-3 PUFA such as eicosapentaenoic acid (EPA; 20:5 ω-3) and docosahexaenoic acid (DHA; 22:6 ω-3) exert multiple effects on health, which are related to the intake of these lipids in the diet and correlate with the levels of omega-3 PUFA in the body. These levels are reflected by the omega-3 PUFA index, i.e. the EPA and DHA content as % of all fatty acids in red blood cells. The aim of this study was to evaluate omega-3 index in the Czech Republic, using blood samples collected from the capital city (n = 476) and the rural region (n = 388). The mean omega-3 index was 3.56 mol % with a maximal value of 8.10% and a minimal value of 1.12%. There was no difference in the index value between rural and urban / industrial regions, but this value was higher in subjects who reported eating fish or omega-3 PUFA supplements. In conclusion, the results indicated suboptimal values of the omega-3 index in the Czech population independent of the sampling region.

1. Introduction

Naturally occurring long-chain omega-3 PUFA, namely eicosapentaenoic acid (EPA; 20:5 ω-3) and docosahexaenoic acid (DHA; 22:6 ω-3), exert multiple biological effects that are mediated either by these PUFA themselves or their bioactive metabolites including specialized pro-resolving mediators, oxidized derivatives, ethanolamines, acylglycerols, and branched-esters of fatty acids, exerting mostly anti-inflammatory effects (reviewed in (Calder, 2013; Kuda, 2017; Kuda et al., 2018)). In humans, omega-3 PUFA attenuate systemic inflammation (Calder, 2013; Hung et al., 2015), ameliorate non-alcoholic fatty liver disease (Scorletti et al., 2014), and protect against cardiovascular disease (reviewed in (Mozaffarian et al., 2013; Tribulova et al., 2017; von Schacky, 2014)). The role of omega-3 PUFA in the primary prevention of cardiovascular diseases was supported by the recent randomized, placebo-controlled VITAL trial (see Discussion and refs. (Bassuk et al., 2016; Manson et al., 2018)). Therefore, several (inter)national health authorities have recommended dietary omega-3 PUFA intake to be between 0.2 g and 2.0 g per day (Mozaffarian et al., 2012; Perk et al., 2012; Smith et al., 2011) and omega-3 PUFA supplementation is advised as part of the secondary prevention of coronary heart disease (Siscovick et al., 2017).

Omega-3 PUFA index, i.e. the EPA and DHA content adjusted to the total content of other fatty acids in red blood cells (RBC), was defined as a risk factor for death from coronary heart disease (Harris and Von Schacky, 2004; von Schacky, 2014). This value serves also as a biomarker of omega-3 PUFA intake within the timeframe of erythrocyte half-life (~60 days) and can be used by clinicians to trace dietary habits of their patients. Recently, a systematic world-wide analysis of omega-3 fatty acids in the blood stream of healthy adults revealed differences between populations adapted to Westernized eating habits and indigenous coastal diet (Stark et al., 2016). Very low blood levels of EPA + DHA (< 4%) were observed in Europe, including the Czech Republic (Stark et al., 2016).

Although there were published several papers describing omega-3 index in small clinical trials in the Czech Republic (Crispim et al., 2011; Hlavaty et al., 2008; Rossmeisl et al., 2018; Stankova et al., 2018; Veleba et al., 2015), a population-based survey was missing. We were

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able to collect and analyze RBC samples from a large socio-physiological survey QUALITAS focused on wellbeing, health and disease, and therefore to explore levels of omega-3 index in the Czech population. The general aim of the survey was to compare two distinct populations – people living in the capital city and people living in rural areas and small villages. Our hypothesis was that differences in living style, socio-economic factors and nutritional habits (e.g. availability of omega-3 PUFA-rich marine products in central European villages) might result in a difference in omega-3 PUFA availability, and thus in a difference in omega-3 index.

2. Material and methods

2.1. Study population

The study population was defined within a combined socio-physiological survey project "QUALITAS - Wellbeing in health and disease" using quota sampling (sex, age, education, place of residence size). Detailed analysis of the survey is out of scope of this study and will be published separately. Briefly, sex, age, and education quota followed data from the Czech population and housing census 2011 and the place of residence was defined either as highly urbanized area (Prague, ~1.4 million inhabitants) or rural region (Ceske Budejovice and neighboring villages, up to 100,000 inhabitants). Participants (aged 18 years and older) were asked to fill a questionnaire related to their health and socio-economic status and to provide blood samples. In total, 864 RBC samples were collected and included in the study.

2.2. Study design

The period of data and sample collection was from September 2016 to June 2017. Fasted blood samples were routinely processed at participating medical facilities. One ml of RBC was frozen at −80 °C at the central laboratory in Prague or frozen at -20 °C upon collection at remote facilities and in 1–2 days delivered on dry ice to the central laboratory to be stored at −80 °C to prevent PUFA degradation (Pottala et al., 2012). This was the optimal logistics of sample collection for this large study as small medical facilities in rural areas do not have −80 °C freezers. Among other data, participants responded to a question "How often do you eat fish?" allowing 6 possible answers: more than twice a week / once a week / few per month / once a month / less often / never; and "Do you take any omega-3 PUFA supplements?" Y/N, optionally followed by detailed description of the form and quantity.

2.3. RBC fatty acid composition

An aliquot of 100 mg of RBC was extracted according to Matyash (Matyash et al., 2008). The amount was a frozen sample equivalent of 80–250 µl of fresh RBC (Pottala et al., 2012). The sample was milled with 250 µl of water, 100 µl of methanol with 0.001% butylated hydroxytoluene and 200 µl of 0.1 mg/ml 19:0 phosphatidyl choline internal standard (MM400, Retsch, Germany) for 1 min at 30 Hz. One ml of cold methyl-tert-butyl ether was added and samples extracted for 15 min using an orbital shaker at 1 Hz. Tubes were centrifuged at 2500 g for 10 min at 4 °C, the organic phase collected and dried in SpeedVac (refrigerated CentriVap, Labconco). Extracted lipids were trans-esterified with 0.5 N sodium methoxide in methanol (Sigma-Aldrich) for 10 min at room temperature. Reaction was quenched with 3 M HCl and fatty acid methyl esters (FAME) extracted to 1 ml of hexane after 10 min in the orbital shaker.

FAME were analyzed using comprehensive two-dimensional gas chromatograph with mass detection (Pegasus 4D, LECO, USA) (Rossmeisl et al., 2018). FAME sample was injected onto Tr-FAME column, 60 m, 250 µm ID, 0.25 µm PT (Thermo, USA) coupled to Rxi-5MS column, 1.7 m, ID- 250 µm ID, 0.25 µm PT (Restek, USA). Temperature program was as follows: 50 °C (1 min) – 20 °C/min – 180 °C (0 min) – 10 °C/min – 250 °C (6 min), He flow 1 ml/min. Injection temperature was 250 °C, transfer line temperature was 280 °C, modulation period 4 s, offset between primary and secondary column 15 °C, hot pulse time 1.5 s. Data files were automatically processed in ChromaTOF software using S/N ratio 10 and omega-3 index was calculated as a sum of EPA and DHA levels divided by total levels of all fatty acids (see Table 1) according to a methodology used in a recent review to present directly comparable values (Stark et al., 2016). FAME standard mixture GLC 744 (Nu Check Prep, USA) was used to optimize the assay (Rossmeisl et al., 2018). Figure S1 shows representative 2D chromatograms and mass spectra of EPA and DHA.

Table 1
Fatty acid composition of RBC.

Fatty acid	Mol %
14:00	0.47 ± 0.23
16:00	24.38 ± 1.30
18:00	17.35 ± 1.30
20:00	0.08 ± 0.03
22:00	0.01 ± 0.01
16:1 n-7	0.81 ± 0.41
18:1 n-9	18.25 ± 1.60
20:1 n-9	0.24 ± 0.06
18:2 n-6	13.56 ± 1.84
18:3 n-6	0.20 ± 0.10
20:2 n-6	0.21 ± 0.06
20:3 n-6	1.60 ± 0.34
20:4 n-6	15.02 ± 1.58
22:4 n-6	2.22 ± 0.49
18:3 n-3	0.08 ± 0.06
20:5 n-3	0.56 ± 0.27
22:5 n-3	1.62 ± 0.33
22:6 n-3	3.00 ± 0.77

2.4. Statistical analyses

The data are expressed as boxplots (median, 25th and 75th percentile) with the whisker range defining the outer-most data point that falls within 1.5-times the inter-quartile range or as mean ± standard deviation for tabular data. Statistical analysis was performed with OriginPro 2018. The fish meal consumption data were evaluated using ANOVA on ranks with Dunn's all-pairwise test. Omega-3 PUFA supplements data were evaluated using unpaired two-tailed Student's *t*-test and $p < 0.05$ was considered significant. Data on fish production and consumption were extracted from FishStatJ, a tool for fishery statistics analysis, Release: 3.04.7 (FAO UN, 2018).

3. Results

The characteristics of our study population providing blood samples showed that the age was equally distributed (Fig. 1A) and that according to body mass index (BMI), 1.4% of the studied subjects were underweight (BMI ≤ 18.5), 47.1% normal weight (BMI between 18.5–25), 34.5% overweight (BMI between 25–30) and 17.0% obese (BMI ≥ 30; Fig. 1B), corresponding to the results of European health interview survey 2014 for the Czech Republic. Participants (366 males and 498 females) lived either in mostly rural South Bohemian region with administrative center Ceske Budejovice (~94,000 inhabitants) or in the capital city of Prague (~1,4 million inhabitants) and the selection followed the quota (Fig. 1C).

Analysis of RBC fatty acid composition revealed that the mean omega-3 index of the study population was 3.56% with maximal value 8.10% and minimal value 1.12% (Fig. 1D and Table 1). There was no difference detected in omega-3 index between the rural (n = 388) and the urban / industrial (n = 476) region (Fig. 1E) and no difference between males and females (males 3.50 ± 0.98%; females 3.60 ± 0.89%).

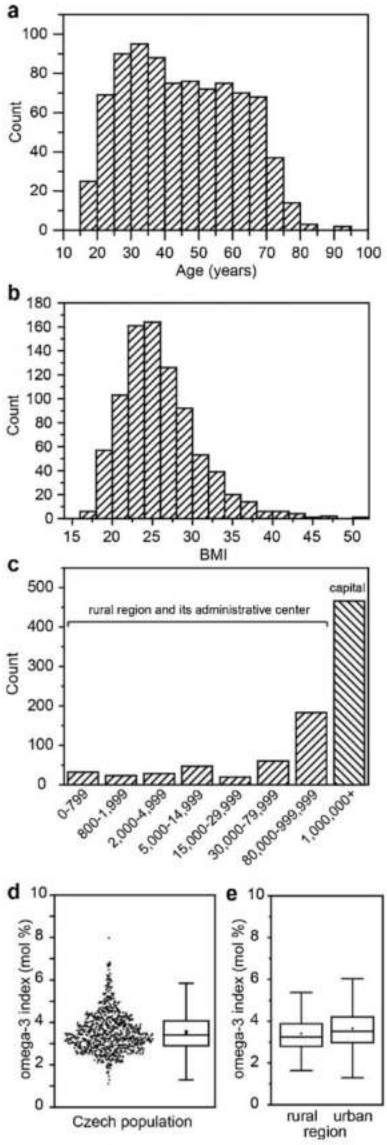

Fig. 1. Characteristics of the study population. (a) Distribution of participants according to age; (b) self-reported body mass index expressed as kg/m^2; (c) place of residence size / number of inhabitants (i.e. small villages up to capital city). Rural region was defined as a sum of all residences smaller than 1 million inhabitants. (d) Omega-3 index in RBC expressed as mol %. (e) Omega-3 index in rural and urban regions of the Czech Republic.

The study population was divided into 6 groups based on the self-reported fish consumption frequency. Participants, who consumed fish meal at least two times per week, had mean omega-3 index 4.10% and the value linearly decreased to 2.58% in participants, who don't eat fish. This trend was progressively statistically significant (Fig. 2A).

Participants (10%), who reported consumption of omega-3 PUFA

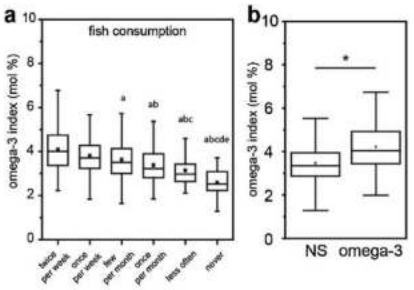

Fig. 2. Self-reported fish and omega-3 PUFA consumption. (a): Omega-3 index expressed based on the frequency of fish meal consumption. ANOVA on ranks: a, statistically significant from the 1 st group; b, statistically significant from the 2nd group, etc. up to e, statistically significant from the 5th group. (b): Omega-3 index of participants who reported omega-3 PUFA supplementation (omega-3) or no supplements (NS).

supplements, had significantly higher omega-3 index than those not taking any supplementation, 4.22% versus 3.46%, respectively (Fig. 2B). Brand, form and dose of omega-3 supplements were not statistically evaluable, but reflected the omega-3 PUFA dietary supplements available on the market in general.

4. Discussion

We explored the omega-3 index in the Czech population and compared the urban and the rural regions. The median omega-3 index is lower than 4% and should be considered as very low when compared to world-wide statistics (Harris and Von Schacky, 2004; von Schacky, 2014). Previously, the omega-3 index within the Czech population was quantified in plasma phospholipids of healthy individuals (4.2%; n = 118) (Crispim et al., 2011) and also in clinical trials: plasma phospholipids of obese women (4.0%; n = 40) (Hlavaty et al., 2008), plasma phospholipids of diabetic patients (5.1%; n = 60) (Veleba et al., 2015), and RBC of typical urban population (5.1%; n = 95) (Stankova et al., 2018). Results of the present study using 864 respondents are in agreement with the previous data and highlight very low levels of omega-3 PUFA in the Czech population. However, the main objective of the socio-physiological survey was to compare rural and urban regions in the Czech Republic. Our data showed that there was no difference in omega-3 index between these two regions. Sociological evaluation of the data (the effect of sex, age, self-reported diseases and medications, etc.) was beyond the scope of this paper and will be published later.

The low levels of omega-3 PUFA are striking and could be partially explained by dietary habits of Czech citizens. According to Fishery and Aquaculture Statistics 2013 (FAO UN, 2018), food supply quantities of freshwater fish and marine fish in the Czech Republic were 2.96 kg/capita/year and 0.56 kg/capita/year, respectively. Majority of the Czech freshwater aquaculture production in ponds was focused on common carp (~95%), followed by rainbow trout or pike in 2016 (FAO UN, 2018), while imported marine species were unknown. Carp and its farmed variants is a very poor source of omega-3 PUFA, especially of DHA, according to US Department of Agriculture (USDA, 2015) and the related characterization of fish products on the market (Strobel et al., 2012). Moreover, the South Bohemian region is famous for carp production in local ponds. Therefore, respondents who reported fish meal consumption are most probably eating farmed carps.

One of the limitations of our study is that the number of participants, who reported use of omega-3 PUFA dietary supplements (10%), and the variability of their responses regarding the brand, dose and frequency of use of these supplements, did not allow a clear assessment of the effects of this type of dietary supplementation. Previously, we

have reported omega-3 index ˜8% in diabetic patients supplemented with omega-3 PUFA capsules (Rossmeisl et al., 2018). Therefore, the omega-3 index ˜4% in subjects who self-reported omega-3 supplementation is probably not sufficiently informative. Also, we were not able to evaluate any associations of omega-3 index with diseases, because we do not have access to the medical records.

Results of the present study substantiate the importance of direct evaluation of omega-3 PUFA in the organism, namely the use of the omega-3 index as an omega-3 PUFA intake indicator and a direct correlate of biological effects of omega-3 PUFA. Furthermore, the recent randomized, placebo-controlled VITAL trial in 25,871 men and women across the U.S. has examined whether the daily intake of dietary supplements containing vitamin D3 (2000 IU) and/or omega-3 PUFA (Omacor® fish oil, 1 g) reduces the risk of developing cancer, heart disease, and stroke in people who do not have a prior history of these illnesses (Bassuk et al., 2016; Manson et al., 2018). Although the primary outcome was negative, the analysis of a subgroup of subjects (non-Hispanic whites) revealed that people with a very low omega-3 index (total fish intake < 1.5 servings per week) benefit from the omega-3 PUFA supplementation as the risk of major cardiovascular events decreased (Manson et al., 2018). The observation that omega-3 PUFA could help at a specific combination of factors is further supported by the REDUCE-IT clinical trial where patients with elevated triacylglycerol levels had lower incidence of ischemic events when treated with EPA ester (Bhatt et al., 2018).

5. Conclusion

In conclusion, this was the first large-scale study of omega-3 index in the Czech Republic and we found suboptimal values of omega-3 index in the Czech population independent on the sampling region. Our idea that the urban and rural populations will have different omega-3 index due to differences in lifestyle and nutrition was not confirmed. Although fish consumption and usage of omega-3 PUFA nutritional supplements resulted in elevated omega-3 index, even higher intake of omega-3 PUFA in general is needed to achieve optimal levels of omega-3 PUFA in the organism (Harris and Von Schacky, 2004; Stark et al., 2016; von Schacky, 2014) and to fully explore their beneficial effects on health (Calder, 2013; Harris and Von Schacky, 2004; Kuda et al., 2018).

Author contributions

Conceptualization, DH, PF, JK, OK; Data curation, PJ, NC, IS, OK; Funding acquisition, MR, DH, JK, OK; Methodology, MO, VP, PZ, PJ, DH, OK; Project administration, PJ, TM, OK; Resources, TM, DH, JK; Writing – review & editing, all authors.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chemphyslip.2019.02.006.

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Article II

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Differential modulation of white adipose tissue endocannabinoid levels by n-3 fatty acids in obese mice and type 2 diabetic patients

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ABSTRACT

n-3 polyunsaturated fatty acids (**n-3 PUFA**) might regulate metabolism by lowering endocannabinoid levels. We examined time-dependent changes in adipose tissue levels of endocannabinoids as well as in parameters of glucose homeostasis induced by n-3 PUFA in dietary-obese mice, and compared these results with the effect of n-3 PUFA intervention in type 2 diabetic (T2DM) subjects. Male C57BL/6J mice were fed for 8, 16 or 24 weeks a high-fat diet alone (cHF) or supplemented with n-3 PUFA (cHF + F). Overweight/obese, T2DM patients on metformin therapy were given for 24 weeks corn oil (Placebo; 5 g/day) or n-3 PUFA concentrate as above (Omega-3; 5 g/day). Endocannabinoids were measured by liquid chromatography-tandem mass-spectrometry. Compared to cHF-fed controls, the cHF + F mice consistently reduced 2-arachidonoylglycerol (up to −2-fold at week 24) and anandamide (−2-fold) in adipose tissue, while the levels of endocannabinoid-related anti-inflammatory molecules *N*-eicosapentaenoyl ethanolamine (EPEA) and *N*-docosahexaenoyl ethanolamine (DHEA) increased more than ~10-fold and ~8-fold, respectively. At week 24, the cHF + F mice improved glucose tolerance and fasting blood glucose, the latter being positively correlated with adipose 2-arachidonoylglycerol levels only in obese cHF-fed controls, like fasting insulin and HOMA-IR. In the patients, n-3 PUFA failed to reduce 2-arachidonoylglycerol and anandamide levels in adipose tissue and serum, but they increased both adipose tissue and serum levels of EPEA and DHEA. In conclusion, the inability of n-3 PUFA to reduce adipose tissue and serum levels of classical endocannabinoids might contribute to a lack of beneficial effects of these lipids on glucose homeostasis in T2DM patients.

1. Introduction

Treatment of diseases associated with obesity, mainly type 2 diabetes mellitus (**T2DM**) and cardiovascular disease, represents a major challenge for medicine today. Endocannabinoid (**EC**) system is a promising therapeutic target, since it regulates food intake, energy balance, as well as lipid and glucose metabolism and inflammation; both central and peripheral effects are involved (reviewed in [1,2]). The whole EC

Abbreviations: 2-AG, arachidonoyl glycerol; AEA, *N*-arachidonoyl ethanolamine (anandamide); AMPK, AMP-activated protein kinase; cHF, high-fat diet based on corn oil; cHF + F, high-fat diet supplemented with EPA + DHA in the form of triacylglycerol concentrate; Chow, standard low-fat diet; DHA, docosahexaenoic acid; DHEA, *N*-docosahexaenoyl ethanolamine; DNL, de novo lipogenesis; EC(s), endocannabinoid(s); EPA, eicosapentaenoic acid; EPEA, *N*-eicosapentaenoyl ethanolamine; eWAT, epididymal white adipose tissue; FBG, fasting blood glucose; FPG, fasting plasma glucose; GTT, glucose tolerance test; HbA_1c, glycated heamoglobin; HOMA-IR, homeostatic model assessment of insulin resistance; iAUC, incremental area under the glucose curve; LC-MS/MS, liquid chromatography-tandem mass spectrometry; mWAT, mesenteric white adipose tissue; n-3 PUFA, n-3 polyunsaturated fatty acids of marine origin; NEFA, non-esterified fatty acids; OEA, *N*-oleoyl ethanolamine; Omega-3, n-3 PUFA-containing capsules; Omega-3 index, sum of EPA and DHA relative to total fatty acids measured in the total lipid fraction from serum or adipose tissue; PCA, principal component analysis; PEA, *N*-palmitoyl ethanolamine; Placebo, corn oil-containing capsules; PPAR, peroxisome proliferator-activated receptor; qPCR, real-time quantitative PCR; scWAT, subcutaneous white adipose tissue; T2DM, type 2 diabetes mellitus; tAUC, total area under the glucose curve; WAT, white adipose tissue

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system is comprised of endocannabinoids (**ECs**) such as *N*-arachidonoyl ethanolamine (anandamide; **AEA**) and 2-arachidonoyl glycerol (**2-AG**), the enzymes that regulate the production and degradation of ECs, and the corresponding cannabinoid receptors such as CB1 and CB2. In obesity and T2DM, the EC system becomes dysregulated, including its major alteration in white adipose tissue (**WAT;** refs. [3–7]); this was observed in both mice [3–5] and humans [6,7].

The EC-dependent changes in WAT could have systemic consequence, because aberrant glucose [8] and lipid metabolism [9] in WAT, as well as obesity-associated low-grade inflammation of WAT linked to alterations in the pattern of adipokines and various lipid mediators secreted from WAT [10], could all disrupt metabolic homeostasis and systemic insulin sensitivity. Indeed, CB1 receptor antagonist improved the insulin responsiveness of adipocytes due to modification of cytokine production in WAT macrophages [11], and pharmacological down-regulation of the CB1 activity using rimonabant has been successfully used to promote weight loss and normalisation of glucose and lipid homeostasis in obese, T2DM patients (reviewed [12,13]); however rimonabant had to be withdrawn due to neuropsychiatric side effects. In contrast, activation of CB2 receptor improves glucose homeostasis [14].

An essential component in the prevention of metabolic impairments in obesity remains lifestyle modifications. For instance, increased physical activity and dietary manipulations lowered the incidence of T2DM development in pre-diabetic subjects by as much as 60% [15]. With respect to the diet, lipids that are abundant in sea fish exert numerous beneficial effects on health, depending mainly on the content of long-chain polyunsaturated fatty acids of *n*-3 series (**n-3 PUFA**) such as docosahexaenoic acid (**DHA**; 22:6n-3) and eicosapentaenoic acid (**EPA**; 20:5n-3; reviewed in [16]). n-3 PUFA reduce inflammation, one of the key factors involved in the development of obesity-associated diseases (reviewed in [10,16,17]), while higher circulating n-3 PUFA levels have been associated with lower total mortality, especially deaths from coronary heart disease [18,19]. Part of these complex effects of n-3 PUFA is probably mediated by the modulation of the EC system activity in peripheral tissues (reviewed in [20]), which could be a consequence of a suppression in tissue ECs levels, observed also in WAT of laboratory rodents [21–25]. Nevertheless, whether this effect of n-3 PUFA could be also observed in human WAT, and how it is related to changes in glucose homeostasis is not known.

Beneficial effects of n-3 PUFA might be explained in part by reduced plasma triacylglycerol levels, as observed in both rodents [21,26,27] and humans [28], as well as by suppression of liver fat accumulation in rodents (e.g. [21,26,27,29,30]) and humans [31]. In obese rodents fed a high-fat diet, n-3 PUFA administration elicited beneficial effects on insulin sensitivity and/or glucose metabolism [21,26,29,32–34], and even reverted glucose intolerance [34]. In contrast, in humans, n-3 PUFA could only prevent T2DM in obese children and young obese individuals, but were not able to revert insulin resistance in T2DM patients (reviewed in [35]). Accordingly, our recent study in overweight/obese T2DM patients already treated with metformin showed no effect of n-3 PUFA on insulin sensitivity [36], although the postprandial lipid metabolism was improved when the combination of n-3 PUFA and antidiabetic drugs (i.e. pioglitazone) was used [36]. An explanation for the absence of beneficial effects of n-3 PUFA on glucose metabolism in the T2DM subjects is missing.

We hypothesized that the differential effects of n-3 PUFA on insulin sensitivity in obese rodents and in human subjects with T2DM could be related, at least in part, to the modulation of the activity of EC system in WAT. Therefore, the potential link between the EC system activity in WAT and glucose homeostasis was characterized in dietary-obese mice and overweight/obese, T2DM patients, both subjected to a prolonged n-3 PUFA supplementation of the same duration and using the same type of n-3 PUFA concentrate.

2. Materials and methods

2.1. Dietary interventions in mice and tissue sampling

Male C57BL/6J mice (from the colony maintained at the Institute of Physiology, Czech Academy of Sciences, Prague) were fed ad libitum a standard low-fat diet (**Chow**; $n = 8$; 3.4% wt/wt as lipids; extruded Ssniff R/M-H diet; Ssniff Spezialdieten GmbH, Soest, Germany; for fatty acid composition, see Kuda et al. [34]) and maintained on a 12-h light-dark cycle (light from 6:00 a.m.) at 22 °C. At 3 months of age, animals were randomly divided into three different cohorts that were subjected to dietary interventions lasting 8, 16, and 24 weeks, respectively. Each cohort comprised three groups of mice fed (i) the Chow ($n = 8$), (ii) a corn oil-based high-fat diet (**cHF**; ~32% wt/wt as lipids; $n = 16$), or (iii) cHF-based diet, in which 15% (wt/wt) of dietary lipids (corn oil) was replaced by the EPA + DHA concentrate Epax 1050 TG (**cHF + F**; $n = 8$; Epax 1050 TG contained ~12% EPA and ~47% DHA, wt/wt, in the form of triglycerides; Epax, Aalesund, Norway) to achieve the total EPA + DHA concentration of ~30 g/kg diet. For the macronutrient composition of the diets, see Supplementary Table 1, for fatty acid composition, see ref. [34].

Chow-fed mice served as lean controls for the obesogenic effect of the cHF diet. Body weight of single-caged mice and their food intake during a 24-h period were monitored weekly, and fresh rations (stored under N_2 atmosphere in sealed bags at −20 °C) were given every second day. At the end of experiments, ad libitum fed mice in each cohort were killed by cervical dislocation under diethylether anesthesia (9:00 a.m.–11:00 a.m.), and WAT from the epididymal (**eWAT**), mesenteric (**mWAT**) and subcutaneous dorsolumbar (**scWAT**) fat depots, as well as interscapular brown fat, heart and liver were dissected and snap-frozen in liquid nitrogen. Tissues and EDTA-plasma were stored at −80 °C for further analyses. The experiments followed the guidelines for the use and care of laboratory animals of the Institute of Physiology and were approved under the protocol no. 127/2013.

2.2. Human samples

Adipose tissue and serum samples were acquired in a clinical trial (EudraCT number 2009-011106-42), which was aimed to evaluate the effect of a combined intervention using antidiabetic drug pioglitazone and n-3 PUFA on lipid and glucose homeostasis in overweight/obese T2DM patients (40–70 years of age) who were treated with stable metformin doses before and during the study [36]. The patients ($n = 69$; 66% men) were randomly assigned to 24-week-intervention using (i) 5 g/day corn oil (**Placebo**; $n = 13$), (ii) 5 g/day n-3 PUFA concentrate Epax 1050 TG (**Omega-3**; contained ~15% EPA and ~40% DHA, wt/wt; i.e. 2.8 g of EPA + DHA; $n = 16$), (iii) pioglitazone, or (iv) pioglitazone + Omega-3 [36]. Within this study, only the samples and some of the previously obtained data [36] from the Placebo and Omega-3 subgroups were used. The serum samples and biopsies of abdominal subcutaneous WAT were collected before and at the end of intervention after an overnight fast and stored at −80 °C. All patients provided written informed consent prior to their participation.

2.3. Blood and plasma parameters

Plasma levels of (i) triglycerides and total cholesterol were determined using the enzymatic colorimetric assays from Erba Lachema (Brno, Czech Republic), (ii) non-esterified fatty acids (**NEFA**) were assessed with a kit NEFA-HR(2) from Waco Chemicals GmbH (Neuss, Germany), and (iii) insulin were determined using the Sensitive rat insulin RIA kit from Linco Research (St. Charles, MO, USA). Fasting blood glucose (**FBG**; see also Section 2.4.) as well as blood glucose levels in mice fed ad libitum (at dissection) were measured by glucometers OneTouch Ultra (LifeScan, Milpitas, CA, USA). The homeostasis model assessment was applied to quantify insulin resistance

(**HOMA-IR**) using the following formula: fasting plasma insulin (mU/l) × fasting glucose (mmol/l)/22.5.

2.4. Glucose tolerance tests

One week before the end of each dietary intervention (i.e. at week 7, 15, and 23), glucose tolerance test (**GTT**) was performed on overnight fasted mice as described before [37]. Results were expressed as area under the curve for glucose (**AUC**), either as total (**tAUC**) or incremental (**iAUC**) area.

2.5. The composition of fatty acids in human adipose tissue and serum

The fatty acid composition was analysed in total lipids in serum (~100 μl) as well as bioptic samples of abdominal subcutaneous WAT (~30–50 mg) of type 2 diabetic subjects using gas chromatography as before [21]. The complete sets of data (in %) are presented in Supplementary Table 2 (serum) and Supplementary Table 3 (WAT). Omega-3 index in serum and WAT was calculated based on the content of EPA + DHA relative to all fatty acids in the fraction of total lipids extracted from the respective biological material.

2.6. The analysis of ECs and related lipid mediators in adipose tissue and serum

Adipose tissue (mice and patients; aliquots ~50–100 mg) and serum (only patients; aliquots ~100 μl) were extracted into acidified methanol with appropriate internal standards, and the extracts were further purified using solid phase extraction columns as before [17,38]. Samples were analysed on UPLC-MS/MS platform (Ultimate 3000 RSLC, Dionex/Thermo and QTRAP 5500, AB SCIEX, Framingham, MA, USA) similarly as before [17]. The levels of classical ECs (i.e. 2-AG and anandamide), as well as of ECs-related molecules, *N*-eicosapentaenoyl ethanolamine (**EPEA**) and *N*-docosahexaenoyl ethanolamine (**DHEA**), that are derived from EPA and DHA, respectively, were quantified. The levels of *N*-palmitoyl ethanolamine (**PEA**) and *N*-oleoyl ethanolamine (**OEA**) were also measured.

2.7. Lipid content in the liver

Tissue pieces (~50 mg) were dissolved in ethanolic KOH and the released glycerol was quantified in tissue extracts by the use of a colorimetric assay as before [37]. Thus, all glycerol-containing lipids were measured (referred to as "hepatic lipids" in the following text).

2.8. RNA isolation and gene expression

Total RNA was isolated from eWAT and liver samples (~100 mg) by the use of TRI Reagent (Sigma-Aldrich, Prague, Czech Republic). Real-time quantitative PCR (**qPCR**) was performed using the LightCycler Instrument (Roche Diagnostics, Mannheim, Germany). The data were normalized to the mean signal of 2 reference genes, i.e. eukaryotic translation elongation factor 1 alpha 1 (*Eef1a1*) and eukaryotic translation elongation factor 2 (*Eef2*) in case of eWAT, and *Eef1a1* and peptidylprolyl isomerase B (*Ppib*) in case of the liver. The geometric means of the respective reference genes did not significantly differ between the dietary groups and different time-points (not shown). For the gene names and corresponding sequences of oligonucleotide primers, see Supplementary Table 4.

2.9. Statistical analysis

All values are means ± SEM. Logarithmic transformation was used to stabilise variance when necessary. Data from mice were first analysed by the two-way ANOVA (with Holm-Sidak post hoc tests) to reveal the interactions between the type of diet (i.e. Chow, cHF or cHF + F) and the time-point (i.e. 8, 16 or 24 weeks), which provided also information regarding potential time-dependent changes in a given variable within each dietary group. Subsequently, for each time-point separately, one-way ANOVA on all three dietary groups or *t*-test on the cHF vs. cHF + F group was also performed using SigmaStat 3.5 software. Data obtained from T2DM patients (i.e. the Placebo and Omega-3 groups) before and after the intervention were analysed by two-way ANOVA. Spearman's rank correlation coefficients were calculated to assess correlations between various parameters in both the mouse and human study. Comparisons were judged to be significant at $p \leq 0.05$.

In the murine experiments, the principal component analysis (**PCA**) was performed using R statistical software to assess distance and relatedness between mouse populations with different type of diet, to reveal global relationships between studied variables, and to evaluate the complex effect of the type of diet.

In the human study, the number of subjects necessary for the evaluation of the effect of n-3 PUFA supplementation on the ECs levels was calculated based on the published differences in total plasma AEA concentrations [39], observed in obese men before and after 24 weeks of the n-3 PUFA treatment. Thus, the sample size of 14 per group was calculated using G*Power software (power 0.95, $\alpha = 0.05$; see ref. [40]).

3. Results

3.1. The absence of effects on weight gain and adiposity, but reductions in plasma and hepatic lipids in mice fed a high-fat diet supplemented with n-3 PUFA

Subgroups of three different cohorts of adult C57BL/6J male mice were fed the Chow, cHF, or cHF + F diet for a period of 8, 16, and 24 weeks. As compared to lean Chow-fed controls at week 8, 16, and 24, the weight gain in cHF-fed mice was increased ~5.1-fold, ~5.3-fold, and ~4.1-fold, respectively, and did not significantly differ from the cHF + F mice at any time point (Table 1; see also Fig. 1A). Cumulative energy intake was slightly reduced in the cHF + F mice at week 8 and 16 vs. the cHF mice (Table 1), although feeding efficiency did not significantly differ between these two groups at any time point (Table 1).

The changes in the adiposity index, i.e. the sum of weights of eWAT, mWAT and scWAT (Table 1) expressed relative to body weight, correlated with those observed for weight gain (Fig. 1B). Compared to the Chow-fed lean mice, the weight gain in the cHF mice at week 8 was primarily reflected in the enlargement of eWAT (~4-fold), while the longer periods of feeding did not cause further increases of eWAT, but it did so in case of scWAT and mWAT (Table 1). A detailed characterization of eWAT revealed an expected increase in mean size of adipocytes as well as the content of tissue macrophages in response to cHF diet, independent of n-3 PUFA supplementation (Supplementary Fig. 1).

Accumulation of hepatic lipids gradually increased in cHF mice during the course of study (Fig. 1C), being ~2.1-fold, ~3.9-fold, and ~5.0-fold higher than in the Chow mice at week 8, 16, and 24, respectively. Hepatic steatosis was reduced by ~35% in the cHF + F vs. cHF mice (Fig. 1C).

Regarding the effect of n-3 PUFA on plasma markers of lipid metabolism measured in ad libitum fed mice, only NEFA levels were persistently reduced by 21–33% in the cHF + F vs. cHF mice. The observed reductions in triglycerides and total cholesterol levels did not reach statistical significance (except at week 16; Table 1).

3.2. Improved FBG and glucose tolerance after a prolonged period of dietary n-3 PUFA supplementation in obese mice

Although no differences in blood glucose levels in the fed state were observed among the subgroups at any time-point (Table 1), FBG levels in overnight fasted mice (see the time 0 in the glycemic curves derived

Table 1
Parameters of energy balance, adiposity and metabolite levels in mice fed high-fats diets with or without n-3 PUFA.

	8 weeks			16 weeks			24 weeks		
	Chow	cHF	cHF + F	Chow	cHF	cHF + F	Chow	cHF	cHF + F
Energy balance									
BW – initial (g)	27.4 ± 0.6	27.4 ± 0.4	27.8 ± 0.7	27.7 ± 0.6	27.8 ± 0.4	27.9 ± 0.6	28.0 ± 0.6	28.1 ± 0.4	28.3 ± 0.7
BW – final (g)	28.8 ± 0.6[a]	35.1 ± 1.2	32.7 ± 1.9	30.4 ± 0.8[a]	42.2 ± 1.8[c]	41.2 ± 2.3[b,c]	32.4 ± 1.3[a]	46.3 ± 2.0[c]	44.6 ± 2.5[b,c]
BW – gain (g)	1.5 ± 0.3[a]	7.7 ± 1.2	4.9 ± 1.8	2.7 ± 0.5[a]	14.4 ± 1.7[c]	13.3 ± 2.4[b,c]	4.4 ± 0.8[a]	18.2 ± 2.1[c]	16.3 ± 2.5[b,c]
cEI (MJ)	3.7 ± 0.1[a]	3.9 ± 0.1	3.6 ± 0.1[a]	7.4 ± 0.2[a,c]	8.6 ± 0.2[c]	7.8 ± 0.2[a,c]	11.2 ± 0.2[a,c,d]	12.7 ± 0.2[c,d]	12.5 ± 0.5[b,c,d]
Feeding efficiency	0.39 ± 0.09[a]	1.87 ± 0.27	1.28 ± 0.49	0.36 ± 0.06[a]	1.58 ± 0.17	1.62 ± 0.30[b]	0.39 ± 0.07[a]	1.37 ± 0.15	1.24 ± 0.19[b]
Adipose tissues									
eWAT (g)	0.37 ± 0.03[a]	1.46 ± 0.21	1.15 ± 0.28[b]	0.45 ± 0.04[a]	1.88 ± 0.16	2.07 ± 0.26[b,c]	0.57 ± 0.07[a]	1.96 ± 0.14	2.14 ± 0.20[b,c]
mWAT (g)	0.26 ± 0.02	0.49 ± 0.07	0.39 ± 0.10	0.26 ± 0.02[a]	0.92 ± 0.14[c]	0.80 ± 0.15[b,c]	0.35 ± 0.03[a]	1.04 ± 0.13[c]	0.97 ± 0.16[b,c]
scWAT (g)	0.20 ± 0.01[a]	0.46 ± 0.05	0.39 ± 0.08[b]	0.23 ± 0.02[a]	0.85 ± 0.11[c]	0.80 ± 0.14[b,c]	0.27 ± 0.04[a]	1.18 ± 0.13[c,d]	1.24 ± 0.23[b,c]
BAT (mg)	82 ± 8	112 ± 7	107 ± 12	105 ± 5[a]	159 ± 11[c]	147 ± 17	104 ± 12[a]	183 ± 19[c]	192 ± 21[b,c]
Blood and plasma parameters									
TAG (mmol/l)	1.06 ± 0.13	1.10 ± 0.11	0.76 ± 0.13	1.06 ± 0.07	1.33 ± 0.08	0.85 ± 0.09[a]	1.11 ± 0.20	1.45 ± 0.27	0.94 ± 0.11
NEFA (mmol/l)	0.57 ± 0.05[a]	0.74 ± 0.04	0.55 ± 0.04[a]	0.60 ± 0.05[a]	0.87 ± 0.04[c]	0.59 ± 0.04[a]	0.56 ± 0.07[a]	0.87 ± 0.04[c]	0.69 ± 0.04[a]
TC (mmol/l)	2.13 ± 0.14[a]	3.79 ± 0.25	3.16 ± 0.26[b]	1.91 ± 0.16[a]	4.73 ± 0.23[c]	3.66 ± 0.32[a,b]	1.88 ± 0.16[a]	4.66 ± 0.25[c]	3.96 ± 0.30[b]
Glucose (mmol/l)	10.4 ± 0.4	11.1 ± 0.4	10.3 ± 0.5	9.2 ± 0.8	10.0 ± 0.3	9.6 ± 0.5	9.5 ± 0.9	9.4 ± 0.3	9.0 ± 0.2
Insulin (mmol/l)	0.55 ± 0.02[a]	0.81 ± 0.05	0.76 ± 0.03	0.54 ± 0.01[a]	1.29 ± 0.20[c]	0.90 ± 0.11	0.57 ± 0.03[a]	1.39 ± 0.14[c]	1.11 ± 0.14[b]

Ad libitum fed mice were killed between 9 a.m. and 11 a.m. Each cohort of mice (8, 16 and 24 weeks) comprised 3 groups of mice fed either Chow, cHF, or cHF + F. BW gain was calculated as a difference in body weight determined at the start and at the penultimate week of dietary interventions within each mouse cohort, when glucose tolerance was assessed. Cumulative energy intake (cEI; per mouse) was calculated using the 24-hr food consumption data collected once weekly from the start till the penultimate week of dietary interventions. Abbreviations: BAT, interscapular brown fat; BW, body weight; cHF, high-fat diet; cHF + F, cHF-based diet supplemented with n-3 PUFA concentrate; Chow, standard low-fat diet; eWAT, mWAT, and scWAT, epididymal, mesenteric and subcutaneous white adipose tissue, respectively; TAG, triglycerides; TC, total cholesterol.
[a,b] Significant difference (two-way or one-way ANOVA) compared with cHF or Chow within the same time-point, respectively.
[c,d] Significant difference (two-way ANOVA) compared with mice fed the same diet at week 8 or week 16, respectively.
Data are means ± SEM (Chow and cHF + F, $n = 8$; cHF, $n = 16$).

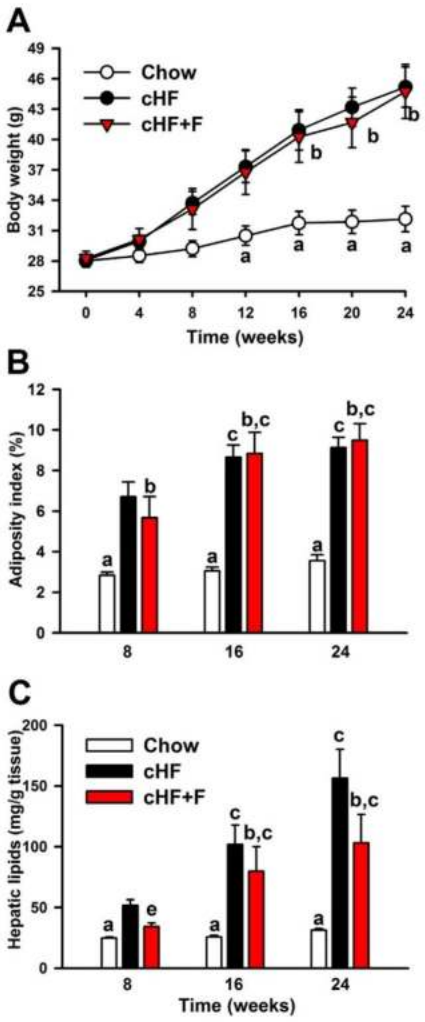

Fig. 1. Body weight, adiposity and liver lipid content in mice. Data from three experimental cohorts (i.e. 8, 16 and 24 weeks), each of which comprised mice fed either the Chow, cHF, or cHF + F diet (see the Section 2.1. for details). (A) Body weight development (only the cohort fed for 24 weeks is shown). (B) Time-dependent changes in the adiposity index (see Section 3.1. for details). (C) Accumulation of lipids in the liver. Data are means ± SEM (Chow and cHF + F, $n = 8$; cHF, $n = 16$). [a,b]Significant difference (two-way or one-way ANOVA) compared with cHF or Chow within the same time-point, respectively. [c,d]Significant difference (two-way ANOVA) compared with mice fed the same diet at week 8 or week 16, respectively. [e]Significant difference (*t*-test) when cHF and cHF + F are compared within the same time-point. Data are means ± SEM (Chow and cHF + F, $n = 8$; cHF, $n = 16$).

from GTT; Fig. 2A–C) were significantly (~1.6-fold) increased in the cHF vs. Chow mice at both week 16 and 24 (Fig. 2D); in the cHF + F mice, FBG levels were reduced at week 24 when compared to the cHF mice. As expected, cHF mice were characterized by impaired glucose tolerance at all time-points, as evidenced by higher tAUC (calculated from the GTT glycemic curves; Fig. 2A–C) when compared to Chow mice (Fig. 2E). In contrast, at both week 16 and 24, tAUC was significantly lower in the cHF + F than in cHF mice (Fig. 2E). Plasma insulin levels in the fasting state gradually increased by ~47%, ~139%, and ~144% in the cHF-fed vs. Chow mice at week 8, 16, and 24, respectively (Table 1). In the cHF mice, the level of insulin resistance, assessed as HOMA-IR (Fig. 2F; see Section 2.3. for details), corresponded to the development of glucose intolerance, while only a non-significant decrease in HOMA-IR was observed in the cHF + F mice at all time-points (Fig. 2F).

3.3. Persistent reduction of classical ECs and elevations of EPA/DHA-derived ECs-related lipid mediators in adipose tissue of obese mice in response to n-3 PUFA

Levels of selected ECs and related lipid mediators were evaluated in eWAT, the typical WAT depot in mice. Levels of 2-AG in eWAT of the cHF mice increased from week 8 to week 16 and then remained stable till week 24, while in the Chow mice, these levels showed an opposite profile decreasing from week 8 to week 24 (Fig. 3A); thus, at week 24, 2-AG levels in the cHF mice were ~2-fold higher than in the Chow mice. Supplementation of the cHF diet by n-3 PUFA decreased 2-AG levels at all time-points, reaching the maximum ~2-fold decrease in the cHF + F vs. cHF mice at week 24. Of note, the course of 2-EPA-glycerol in eWAT (DHA-containing glycerol species were undetectable; not shown) had a completely different profile as compared to 2-AG, with a marked increase in the cHF + F mice at week 8 and a subsequent lowering to the levels observed either in Chow or cHF mice at week 16 and 24 (Supplementary Fig. 2). The levels of AEA were relatively stable in eWAT during week 8 to week 24 in all groups of mice, and they did not differ between the cHF and Chow mice; however, the AEA levels in the cHF + F mice were ~2-fold lower as compared with the other two subgroups throughout the study (Fig. 3B). Tissue levels of the ECs-related anti-inflammatory molecules EPEA and DHEA were steadily reduced by ~1.5-fold–3.4-fold, respectively, in eWAT of the cHF vs. Chow mice (Fig. 3C and D), while n-3 PUFA supplementation in the cHF + F mice increased (vs. cHF mice) the eWAT levels of EPEA and DHEA up to 10- and 8-fold, respectively (Fig. 3C and D). Of note, eWAT levels of either PEA or OEA were not affected by n-3 PUFA supplementation (not shown).

Quantification of the selected EC system-related gene transcripts in eWAT was also performed (Fig. 3E). There were no differences between the groups in mRNA levels of *Cnr1* (i.e. endocannabinoid CB1 receptor) at any time-point (not shown). While the gene expression of CB2 receptor (*Cnr2*) was stable in the Chow mice throughout the study, it increased ~2.0-fold between the week 8 and 16 in the cHF mice, and it was independent of the n-3 PUFA supplementation. The expression of *Mgll*, i.e. the gene for 2-AG degrading enzyme monoacylglycerol lipase, was decreased at week 24 in both cHF and cHF + F mice (in the latter mice also at week 16), as compared to their counterparts analysed at week 8, while no such changes were observed in Chow mice. Similarly in case of *Faah*, i.e. the gene for AEA degrading enzyme fatty acid amid hydrolase, only at week 16 and 24 was its expression down-regulated in cHF + F mice as compared to week 8, however no significant differences were observed between the cHF and cHF + F mice (Fig. 3E).

3.4. Restricted effects of n-3 PUFA on the EC system in T2DM patients

Levels of ECs and related lipids were evaluated in serum and abdominal subcutaneous WAT of metformin-treated overnight fasted T2DM patients supplemented for 24 weeks with either Placebo or Omega-3 capsules (Fig. 4 and Supplementary Table 5). At the end of the intervention, no differences in either serum (Fig. 4A) or WAT (Fig. 4B) levels of classical ECs, i.e. 2-AG and AEA, were observed between the Placebo and the Omega-3 group. In contrast, the levels of EPEA in serum (Fig. 4A) and the DHEA levels in both serum (Fig. 4A) and WAT (Fig. 4B) were higher in the Omega-3 group. The stimulatory effect of n-3 PUFA supplementation on the EPEA and DHEA levels was also

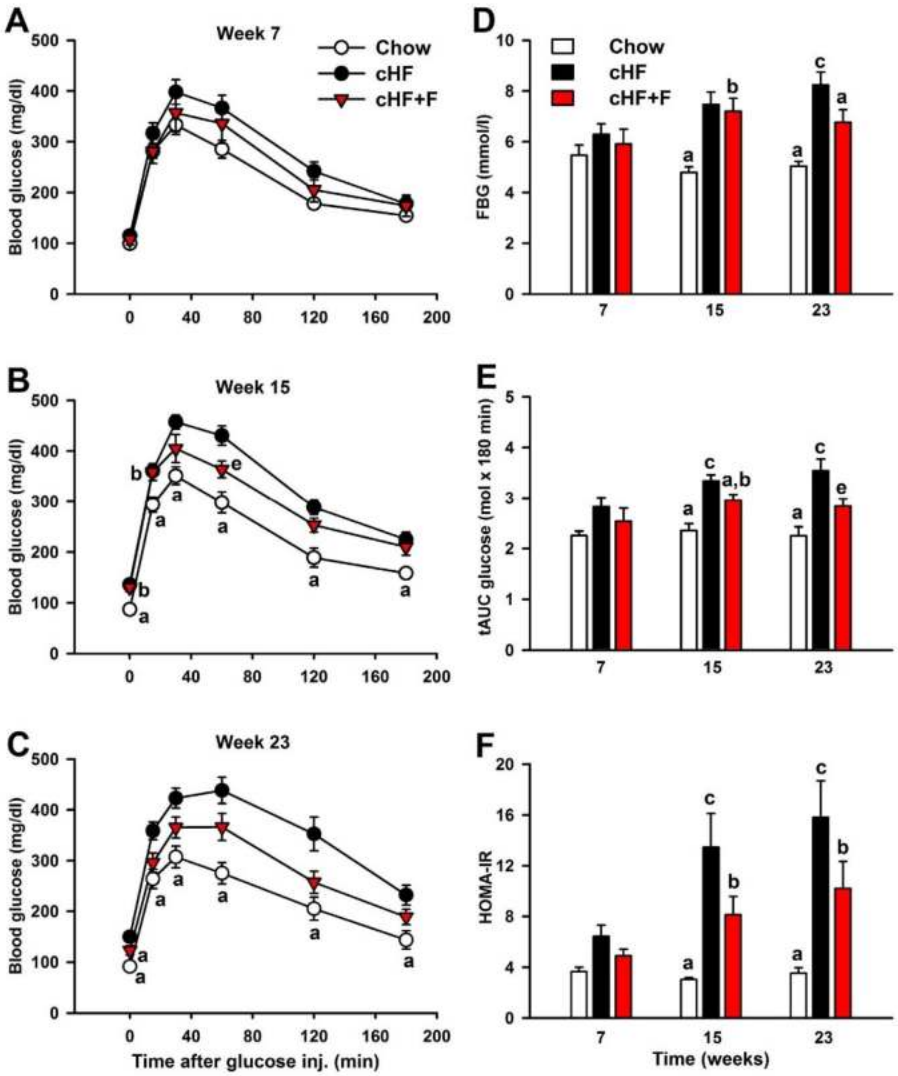

Fig. 2. Glucose homeostasis in mice. GTT was performed in mice from different cohorts (see Fig. 1 and Sections 2.3 and 2.4) and glucose tolerance was analysed at week 7 (A), 15 (B) and 23 (C). Changes in FBG (D), tAUC (E), and HOMA-IR (F). Data are means ± SEM. See the legend to Fig. 1 for the number of animals and description of symbols of significance.

observed when the data were expressed as the delta values of the levels measured before and after the intervention, both in serum (Fig. 4C) and WAT (Fig. 4D) of the individual patients. As in case of the mouse study, n-3 PUFA supplementation did not affect PEA and OEA levels in WAT (as well as in serum) of T2DM patients (not shown).

In order to correlate the effects of n-3 PUFA supplementation with the changes of EPA and DHA in serum and WAT, fatty acid composition in the total lipid fraction extracted from the patient's samples were measured (see Supplementary Tables 2 and 3) and the Omega-3 index in serum (Fig. 5A) and WAT (Fig. 5B) was calculated. In response to n-3 PUFA supplementation in the Omega-3 group, the Omega-3 index in serum and WAT increased ~2.5-fold and 2.2-fold, respectively, while no significant changes were observed in the Placebo group. A moderate positive correlation was noted between the change in the Omega-3 index in WAT and the tissue EPEA levels (Fig. 5C), and a strong positive correlation in case of DHEA levels (Fig. 5D).

Moreover, possible correlations between the serum and WAT levels of the individual lipid mediators were analysed before and after the n-3 PUFA supplementation. Only the serum levels of AEA and DHEA after the intervention correlated positively with their corresponding levels in WAT (Supplementary Table 6).

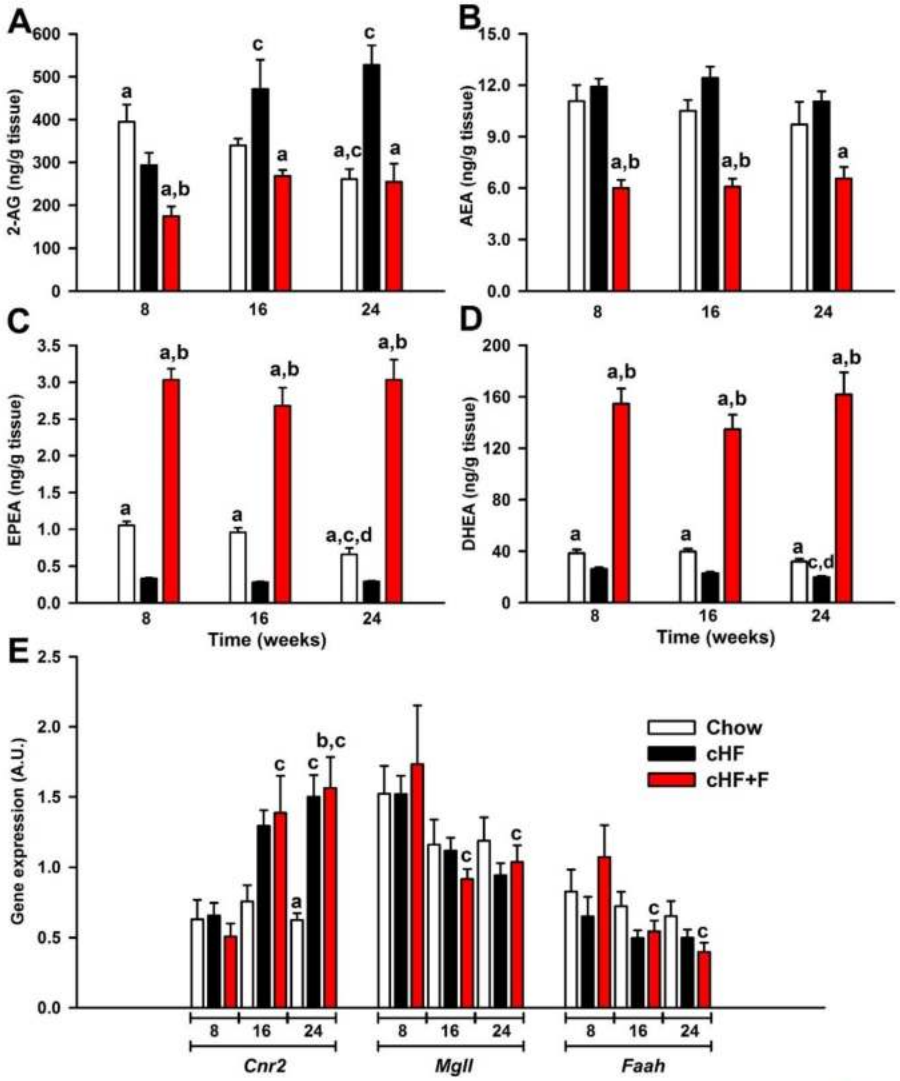

Fig. 3. The EC system activity in adipose tissue of mice. The eWAT depots of mice fed different diets for 8, 16 or 24 weeks was analysed (see Fig. 1). Concentrations of 2-AG (A) and AEA (anandamide; B), as well as ECs-related molecules EPEA (C) and DHEA (D), in tissue extracts. (E) mRNA levels of selected genes encoding the components of the EC system. Data are means ± SEM. See the legend to Fig. 1 for the number of animals and description of symbols of significance, and Supplementary Table 4 for the gene names and primer sequences.

3.5. The analysis of a potential link between WAT levels of lipid mediators and glucose homeostasis in mice and T2DM patients

Next, the general relationship between the levels of ECs and related molecules in WAT, and the state of glucose homeostasis was investigated in both mice and humans. In the mouse study, we first performed PCA of tissue endocannabinoid levels, parameters of glucose homeostasis as well as other variables measured in all three dietary groups of mice (Fig. 6), and constructed the biplots for each time-point, i.e. for week 7–8 (Fig. 6A), week 15–16 (Fig. 6B), and week 23–24 (Fig. 6C), in order to reveal the response patterns of individual observations (points) and relationships between variables (arrows). This global analysis of the data revealed that separation (according to the first principal component) between the Chow and the cHF mice developed until after 8 weeks of dietary intervention, and it was affected by most of the measured variables, the influence of which has changed over time. On the other hand, a clear separation of the cHF + F mice from the other groups (primarily according to the second principal component) was apparent at all time-points, thus indicating an early onset of n-3 PUFA's effects; this separation could be explained in large by eWAT levels of EPEA, DHEA, 2-AG and AEA, with varying contribution of the other variables over time.

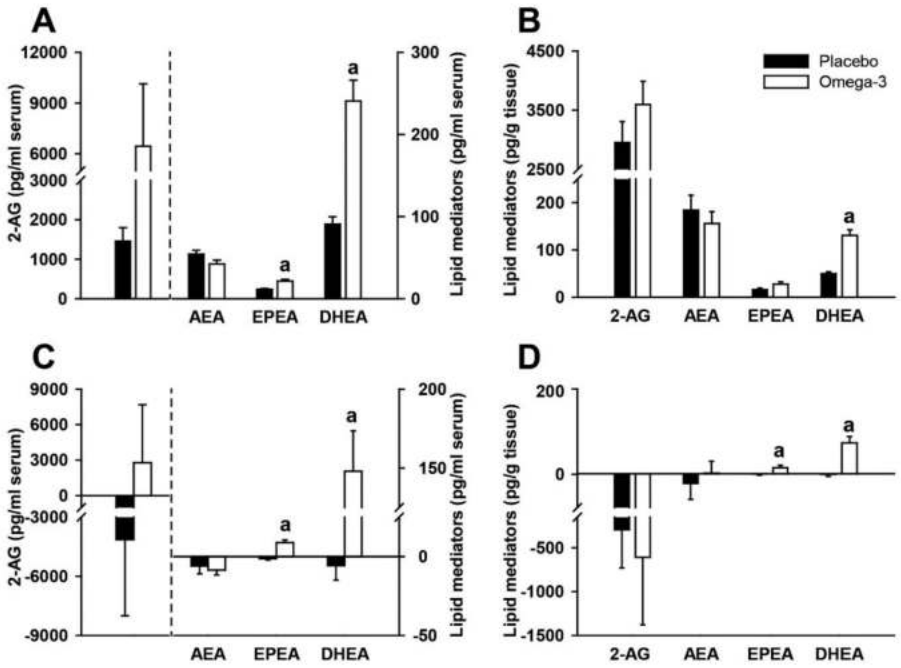

Fig. 4. Serum and adipose tissue levels of ECs and ECs-related lipids in patients with T2DM. The levels of 2-AG, AEA, EPEA and DHEA were evaluated in serum (A) and in biopsies of abdominal subcutaneous WAT (B) collected from fasted patients after 24 weeks of the intervention with n-3 PUFA concentrate (Omega-3) or corn oil (Placebo). Data in serum (C) and WAT (D) expressed as the delta values of the levels assessed after and before the intervention; see also Supplementary Table 5. Data are means ± SEM (Placebo, $n = 13$; Omega-3, $n = 16$). [a]Significant difference (two-way ANOVA) compared with Placebo.

Separate correlation analyses were then performed for all three dietary groups and all time-points analysed (i.e. at weeks 7–8, 15–16, and 23–24; Fig. 7). In addition to various parameters of glucose homeostasis, including FBG, fasting plasma insulin, HOMA-IR, tAUC and iAUC, data on hepatic expression of selected gene markers of metabolism (Supplementary Fig. 3), namely phosphoenolpyruvate carboxykinase (*Pck1*; gluconeogenesis), glucoso-6-phosphatase (*G6pc*; glycogenolysis) and fatty acid synthase (*Fasn*; *de novo* lipogenesis) were also included in the analysis. Only few significant correlations were found in the Chow mice. Consistent results emerged in the case of the cHF mice, showing strong positive correlations between 2-AG levels and either FBG, plasma insulin or HOMA-IR both at week 15–16 and week 23–24, i.e. the association of increased 2-AG levels with the induction of impairment in glucose homeostasis during the development of cHF-induced obesity; moreover, positive correlations between the levels of 2-AG and those of either *G6pc* or *Fasn* transcript at week 24 suggested the role of 2-AG in WAT in metabolic disturbances induced in the liver in response to a prolonged cHF-feeding. Conversely, both AEA and DHEA levels at week 23–24 correlated negatively with FBG, plasma insulin and HOMA-IR, suggesting a protective effect of these lipid mediators against the deterioration of glucose homeostasis induced by cHF diet. Also in the cHF + F mice positive correlations between 2-AG levels and most of the markers of impaired glucose homeostasis were detected at week 7–8, but not later on during the cHF + F diet feeding, when such correlations prevailed in the cHF mice (see above). These results are consistent with the n-3 PUFA-induced protection against the rise of 2-AG levels otherwise seen in animals fed the cHF diet (see Fig. 3A), as well as with the prevention of glucose intolerance during the development of diet-induced obesity by n-3 PUFA (see Fig. 2).

In general, the mouse data above were consistent with a tight link between the activity of EC system in WAT and glucose homeostasis, namely in the context of overt obesity induced by prolonged cHF feeding. The data supported the involvement of 2-AG in the deterioration of glucose homeostasis as opposed to EPEA and DHEA. Linear regression plots in Fig. 8 further illustrate the strong correlations between the levels of selected lipid mediators in eWAT and FBG in cHF-fed mice, i.e. a positive correlation between 2-AG and FBG (Fig. 8A), and a negative correlation between DHEA and FBG (Fig. 8B).

The associations between the levels of ECs and related molecules in abdominal subcutaneous WAT and parameters of glucose homeostasis, which were recorded in the original clinical study in the overweight/obese patients with T2DM [36], was also analysed. Data from both the Placebo and Omega-3 group at the end of the 24-week-intervention were used (Fig. 9). In sharp contrast with the mouse data (see above), only a few significant correlations have been identified, and in the opposite direction than expected. Thus, negative correlations between 2-AG levels and markers of impaired glucose homeostasis were found, including FPG in the Placebo group, and both glucose tolerance assessed using a meal test and fasting plasma levels of glycated hemoglobin ($\mathbf{HbA_{1c}}$) in the Omega-3 group; in addition, DHEA levels correlated positively with both insulin and C-peptide responses during the meal test. These results suggest that T2DM and/or metformin treatment could modulate the link between the EC system in WAT and glucose homeostasis in patients (see Discussion section).

4. Discussion

The present study demonstrated that a 24-week-long supplementation with the DHA-enriched, triacylglycerol-based n-3 PUFA concentrate administered either to C57BL/6J mice during the induction of

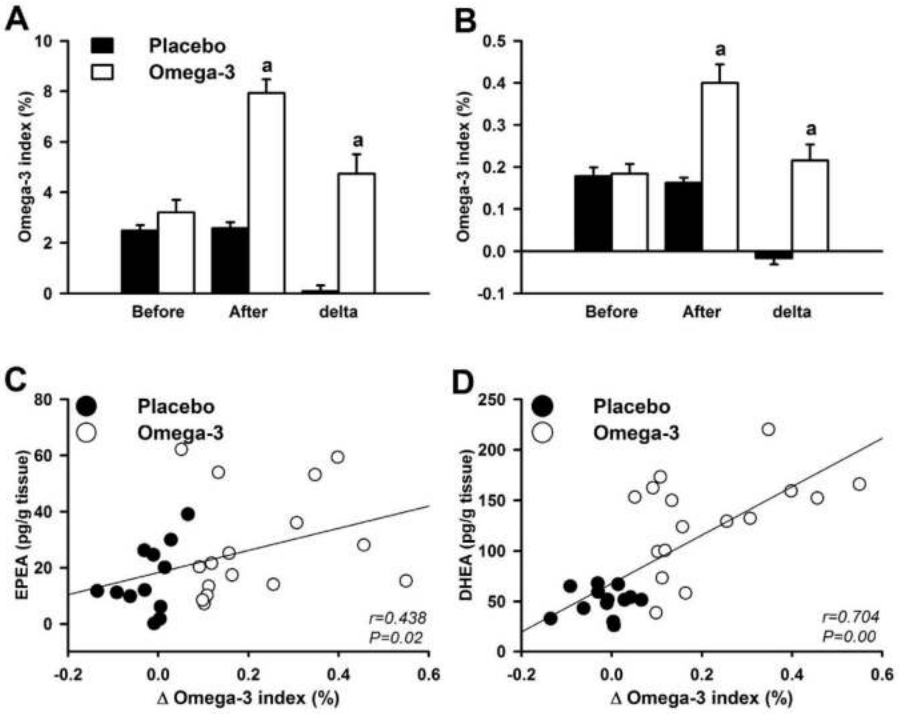

Fig. 5. Omega-3 index in serum and WAT of T2DM patients. The relative levels of EPA + DHA in serum (A) and in biopsies of abdominal subcutaneous WAT (B) collected from fasted patients before and after 24 weeks of intervention with n-3 PUFA (Omega-3) or corn oil (Placebo). Data are means ± SEM (Placebo, $n = 13$; Omega-3, $n = 16$). (C) Linear regression plot showing the relationship between the change in Omega-3 index (delta value "After-Before") in subcutaneous WAT and tissue levels of EPEA (C) and DHEA (D) measured 24 weeks after the intervention in the Placebo (filled circles) and Omega-3 (open circles) group. [a]Significant difference (two-way ANOVA) compared with Placebo.

dietary obesity or to overweight/obese patients with T2DM had the same stimulatory effect on WAT levels of EC-related anti-inflammatory molecules such as EPEA and DHEA, but markedly different effects on the levels of classical ECs such as 2-AG and AEA. The differential effect of n-3 PUFA on classical ECs in obese mice and diabetic patients correlated with the beneficial effect of n-3 PUFA supplementation on glucose homeostasis in mice and the absence of such effects in patients.

The EC system regulates energy homeostasis through the control of appetite and food intake, mainly via CB1 receptors in the brain (reviewed in [1]). In obesity and T2DM, the overactivity of this system favours lipid storage and insulin resistance while inhibiting energy expenditure, which could be reversed by the use of CB1 receptor antagonists (see Introduction section; reviewed in [12,13]). Using this strategy, involvement of the EC system in the regulation of metabolism directly in peripheral tissues was demonstrated, namely in the liver [4,41–43], skeletal muscle [4,44,45], pancreas [3], as well as WAT [11,46,47]. Moreover, inhibition of CB1 receptor activity could reduce adipocyte proliferation and differentiation [48]. Therefore, the use of peripherally-restricted antagonists of CB1 receptors might represent an effective therapy for obesity-associated metabolic disorders, including insulin resistance and β-cell failure ([5,49]; reviewed in [12,13]). In this respect, n-3 PUFA are known to modulate tissue ECs levels, possibly by limiting availability of ECs biosynthetic precursors, i.e. arachidonic acid (i.e. 20:4n-6), in membrane phospholipids (reviewed in [2,20]). This holds true also for adipose cells, as demonstrated in 3T3-L1 adipocyte cell line [50,51] and in WAT of animal models of obesity [21–25]. However, to our knowledge, our current study is the first to characterize the effect of n-3 PUFA on the levels of ECs and ECs-like lipid mediators in WAT of human subjects. Moreover, it is unique in that it compares the effects of the same type of the DHA-enriched n-3 PUFA concentrate in mice and humans while in both cases it uses interventions of the same duration.

Although n-3 PUFA had no effect on the levels of the classical ECs in abdominal subcutaneous WAT of T2DM patients, in obese cHF-fed mice n-3 PUFA induced a decrease in eWAT levels of 2-AG and AEA. In case of 2-AG, this decrease was most pronounced at week 24, since it coincided with the highest levels of 2-AG as well as with the maximal level of glucose intolerance observed in the obese cHF-fed controls. In fact, the 2-AG levels in eWAT of these animals showed a clear upward trend during the study (i.e. opposite to the lean Chow-fed mice), which was blunted in mice fed n-3 PUFA. Time-dependent modulation of 2-AG levels in eWAT of Chow and cHF mice, as well as their modulation by n-3 PUFA supplementation in cHF + F mice, was different from modulation of 2-EPA-glycerol levels, and it did not correspond to the time-dependent profile in the expression of *Mgll*, their degrading enzyme. The reason for the above differential modulation of 2-AG levels is to be determined. Although tissue AEA levels decreased in response to dietary n-3 PUFA, they were indistinguishable between the lean Chow and obese cHF mice and did not change throughout the study. These results are consistent with those published by Matias et al., showing an increase of 2-AG levels in visceral WAT of both obese mice and humans, but no change in AEA levels, when compared to lean subjects [52]. In addition, our current study demonstrated positive correlations between 2-AG levels in eWAT and impairment of glucose homeostasis in the

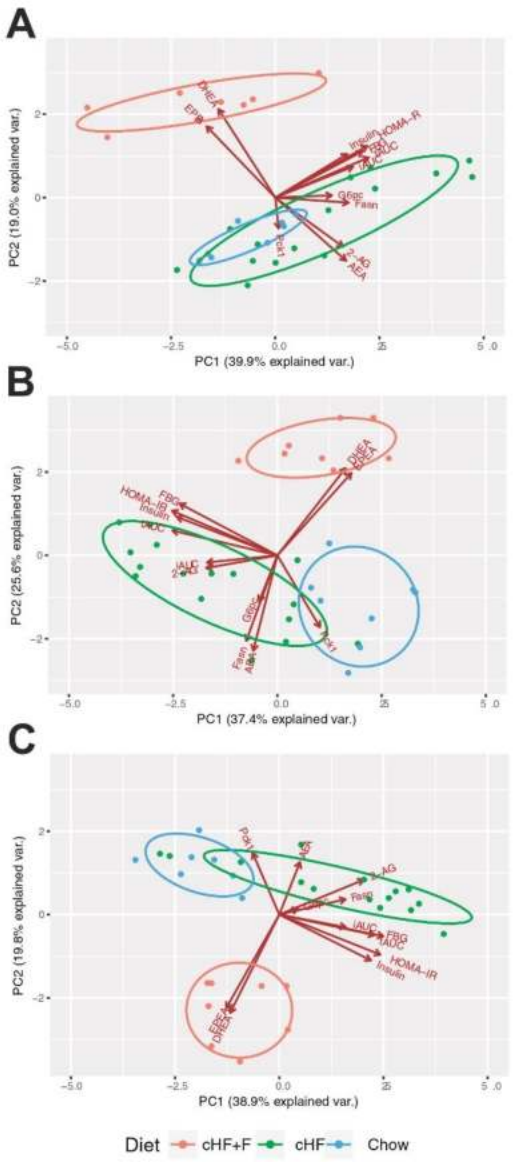

Fig. 6. The PCA analysis of eWAT levels of ECs and ECs-related lipids as well as parameters of glucose homeostasis in the mouse study. The results are plotted as biplots, each of which including all three dietary groups (i.e. Chow, cHF, cHF + F) for a given time-point, i.e. for week 7–8 (A), week 15–16 (B), and week 23–24 (C). Points represent the projection of observations on the first two principal components (PCs), with different colors corresponding to the type of experimental diet. Ellipses indicate Normal contour lines with probability 0.68. The relationships between different variables are depicted by arrows; the angle between the arrows is inversely proportional to the magnitude of the correlation, e.g. 90° = no correlation; 180° = correlation −1.

obese cHF-fed mice. The results support the view that 2-AG in abdominal WAT is among the major causative factors contributing to impaired glucose tolerance in obesity, at least in mice. On the other hand, our results do not indicate any role of AEA in WAT in the obesity-associated impairments of glucose metabolism.

The differential effect of n-3 PUFA supplementation on the ECs levels in WAT of the obese mice and diabetic patients could not be probably explained by the fat depot-specific properties of WAT, because the supplementation by n-3 PUFA as Krill oil (i.e. EPA + DHA in the form of phospholipids) resulted in a uniform decrease of 2-AG levels in both eWAT and abdominal subcutaneous WAT of obese mice [23]. Moreover, that n-3 PUFA failed to decrease 2-AG and AEA levels in serum of T2DM subjects is in contrast with our results in obese mice, as well as with other human studies showing (i) reduced plasma AEA levels in mildly obese men [39] and plasma 2-AG levels in obese subjects [53] in response to n-3 PUFA administration either in the form of Krill powder or Krill oil, and (ii) a negative correlation between plasma 2-AG concentrations and insulin sensitivity in human non-diabetic subjects [7].

Part of the explanation for the discrepancies above could reflect the dose-response relationship. The dose of EPA + DHA adjusted to body weight was much higher in the mouse as compared with the human studies, as in our case: ~2000 mg/kg vs. ~30 mg/kg in the obese mice and T2DM patients, respectively. This corresponded to an approximately 6- and 50-fold increase in the relative concentration of EPA + DHA (i.e. Omega-3 index) in the total lipid fraction in plasma and eWAT of cHF + F mice as compared with the cHF-fed controls (measured at week 9; see the data for cHF and cHF + ω3TG mice in Tables S4 and S7 of ref. [21]). In contrast, the measurements of fatty acid composition in the total lipid fraction in serum and WAT of the patients in this study revealed that the increase of the Omega-3 index in response to n-3 PUFA supplementation was lower than in mice (using the data from mice after 9 weeks of n-3 PUFA supplementation; see ref. [21]; see above), i.e. 2.5-fold vs. 6-fold for serum/plasma, and 2.2-fold vs. 50-fold for WAT. The incorporation of EPA and DHA into WAT lipids of mice fed n-3 PUFA-containing diet is characterized by an extended period of time before reaching its maximum [54]; thus, it is likely that EPA and DHA concentrations in eWAT of cHF + F mice would be further increased at week 16 and 24 in our current study, when the difference in tissue levels of ECs between the cHF and cHF + F mice was the biggest. Nevertheless, the dose of n-3 PUFA supplementation has obviously a great impact concerning the elevation of Omega-3 index both in circulation and especially in WAT; however, although n-3 PUFA failed to significantly affect the WAT levels of ECs in T2DM subjects, who showed a relatively low induction of Omega-3 index in the tissue, it was still sufficient to induce anti-inflammatory molecules such as DHEA (see below).

However, the above quantitative differences regarding the dose of n-3 PUFA are not likely to explain in full the different responses of the EC system to n-3 PUFA supplementation observed in WAT of obese mice and T2DM patients. The link between the EC system activity in T2DM patients and glucose homeostasis could be affected by metformin used as the anti-diabetic treatment in all subjects included in the Placebo and the Omega-3 group, and/or by the presence of overt diabetes in these patients. While in case of obese mice the eWAT levels of 2-AG detected in the present study are very similar to those observed in the previous studies on dietary-obese mice (e.g. [21,23,24].), i.e. they are in the range of 100–600 ng 2-AG/g tissue, the levels of 2-AG in WAT of T2DM patients measured in this study (i.e. 3.0–4.2 ng 2-AG/g tissue) are much lower as compared with the corresponding 2-AG levels previously found in the subcutaneous WAT of obese non-diabetic patients (~40 ng 2-AG/g tissue; ref. [52]). Regarding the potential effect of metformin, it has been shown in women with the polycystic ovary syndrome that metformin treatment reversed the observed increase in plasma AEA levels [55], while breast cancer cells with acquired resistance to metformin were characterized by transcriptome reprogramming that

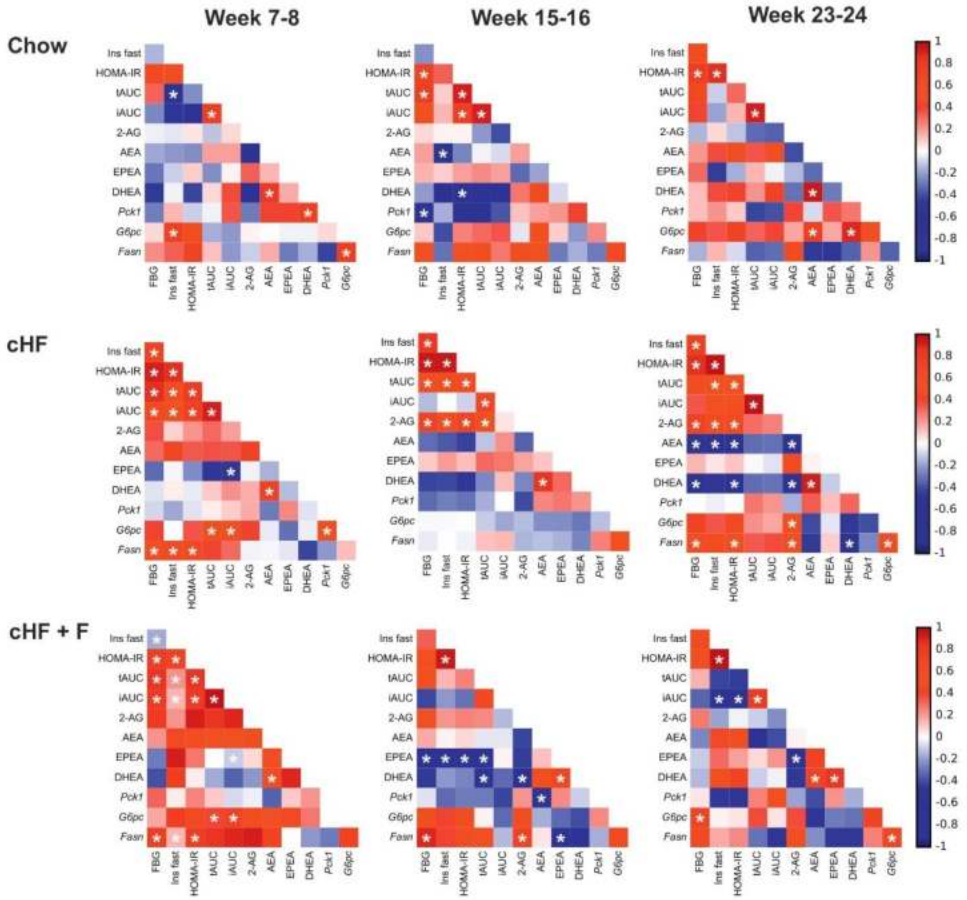

Fig. 7. The general relationship between the levels of ECs and ECs-related lipids in adipose tissue and different parameters of glucose homeostasis in mice. Data from mice fed the Chow (n = 8), cHF (n = 16) or cHF + F (n = 8) diet for a period of 8, 16 and 24 weeks were used. Data from Fig. 2 (i.e. glucose homeostasis at week 7, 15, and 23) and Fig. 3 (i.e. ECs levels in eWAT at week 8, 16, and 24)) were replotted as heatmaps, where different colors correspond to the values of Spearman's rank correlation coefficients calculated for the respective correlations of parameters grouped at week 7–8, 15–16, and 23–24. In the correlation matrices, the relationships between the levels of ECs and related lipids and hepatic expression of selected genes related to glucose/lipid homeostasis is also shown. Asterisks denote significant correlations; see the legend to Fig. 1 for the number of animals in each group, Supplementary Table 4 for the gene names and primer sequences, and Supplementary Fig. 3 for the data on hepatic gene expression.

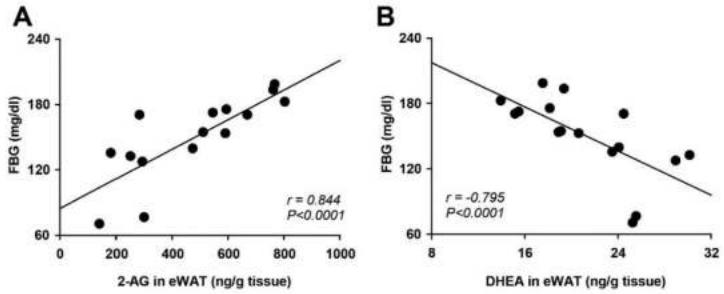

Fig. 8. Linear regression plots documenting the relationship between FBG and the levels of 2-AG (A) or DHEA (B) in eWAT of mice fed the cHF diet for 24 weeks (n = 16; see also Figs. 6 and 7 for these results in the context of the complex analysis of the mouse data).

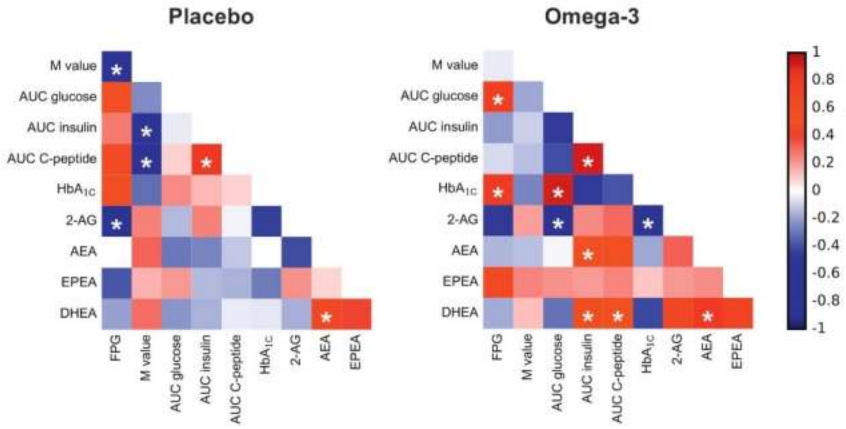

Fig. 9. The general relationship between adipose tissue levels of ECs and ECs-related lipids and glucose homeostasis in humans. The data from metformin-treated T2DM patients who were given capsules containing either corn oil (Placebo; $n = 13$) or n-3 PUFA (Omega-3; $n = 16$) for 24 weeks were used. The correlations are based on the data derived from Fig. 4B (i.e. WAT levels of ECs and related molecules; see also Supplementary Table 5) and from the previous clinical trial ([36]; parameters of glucose homeostasis). The data were replotted as heatmaps, where different colors correspond to the values of Spearman's rank correlation coefficients calculated for the respective correlations. Abbreviations: FPG, fasting plasma glucose; M value, glucose disposal rate assessed by hyperinsulinemic-euglycemic clamp (it is the amount of infused glucose necessary to maintain plasma concentrations of glucose during the last 20 min of the clamp; in mg/kg body weight × min^{-1}); AUC glucose, insulin, C-peptide, area under the plasma glucose, serum insulin and serum C-peptide curve, respectively, based on the respective measurements during 120 min of the meal test; HbA_{1c}, glycated hemoglobin. Asterisks denote significant correlations.

included the induction of pro-metastatic lipases such as 2-AG degrading enzyme monoacylglycerol lipase [56]. On the other hand, it was shown in high-fat diet-fed mice with obesity induced prior to initiation of treatment, that the 2-AG and AEA levels were reduced in WAT and plasma by a co-treatment with metformin and n-3 PUFA, as compared to mice treated with metformin alone [21]. Thus, the presence of diabetes rather than metformin could abolish or even reverse the expected link between the 2-AG levels in WAT and the parameters of glucose homeostasis, as was the case in both the Placebo and Omega-3 group (Fig. 9).

We have found a sustained decrease in the levels of both EPEA and DHEA, which are known to be formed by WAT and to exert anti-inflammatory properties [21,51,57], in eWAT of the obese cHF mice vs. the lean Chow mice, while n-3 PUFA supplementation elevated their tissue levels (up to ~5-fold) above those found in lean mice. The levels of both EPEA and DHEA in eWAT were negatively correlated with glucose homeostasis in the cHF and cHF + F mice, suggesting a beneficial effect of these lipid mediators on systemic glucose metabolism, especially when acting together with the reduction of both adiposity and plasma levels [25] of the classical ECs. Previous studies showed that (i) 3T3-L1 adipocytes are capable of converting DHA and EPA to their respective *N*-acyl ethanolamines DHEA and EPEA, and both DHEA and EPEA reduced the secretion of pro-inflammatory cytokines from lipopolysaccharide-stimulated adipocytes [51], (ii) supplementation of a high-fat diet with n-3 PUFA in mice increased the levels of DHEA in eWAT adipocytes [17], (iii) DHEA can exert immunomodulatory effects in cultured macrophages [57], and (iv) DHEA-derived products activate CB2 preferentially over AEA [58]. In accord with the above results in mice, we show for the first time that n-3 PUFA intervention is capable of increasing the levels of DHEA and EPEA in both serum and WAT of T2DM patients. As mentioned above [2,20], dietary n-3 PUFA could reduce the levels of ECs by limiting availability of their biosynthetic precursors, i.e. replacing arachidonic acid by EPA and/or DHA in membrane phospholipids; the same mechanism should be involved in the generation of ethanolamines such as EPEA and DHEA. Therefore, the induction of DHEA and EPEA by n-3 PUFA intervention in the absence of any effects on the levels of classical ECs suggests that a relatively low saturation level of plasma membrane phospholipids by n-3 PUFA is sufficient for the production of these ECs-like mediators. Interestingly, WAT levels of either PEA or OEA were not affected by n-3 PUFA supplementation, neither in mice nor in the patients, which is in agreement with our previous mouse study [21], showing no significant changes in plasma and eWAT levels of either PEA or OEA in mice fed for 9 weeks the cHF diet supplemented with n-3 PUFA.

Our finding that the differential effect of n-3 PUFA on the levels of 2-AG in WAT of the obese mice and the T2DM patients was mirrored by the modulation of glucose homeostasis supports the key role of WAT metabolism and its secretory features in whole-body insulin sensitivity (see Introduction section). Emerging evidence suggests that stimulation of glucose uptake in WAT adipocytes by insulin, which results in augmented de novo lipogenesis (**DNL**), positively influences whole-body insulin sensitivity [8,9,59]. Importantly, compromised insulin response of adipocytes from WAT of obese rats could be rescued by CB1 receptor antagonists, reflecting a modification of cytokine production in WAT macrophages (see Introduction section and ref. [11]). A direct involvement of the EC system in the regulation of DNL in WAT has not been proven yet. However, we have shown previously that n-3 PUFA supplementation counteracted the cHF diet-induced decrease of insulin-dependent DNL in eWAT of mice [60], thus suggesting that this effect could involve the interaction of n-3 PUFA with the EC system. It was postulated [59,61] that DNL in adipocytes is essential for the production of novel lipid mediators including branched fatty acid esters of hydroxy fatty acids, which have multiple favourable effects on systemic glucose homeostasis and insulin sensitivity [62]; our recent study [38], utilizing the same serum samples from T2DM patients as in the current work, has demonstrated increased levels of novel DHA-containing lipid mediators from this family [38]. All these findings support the adipocentric view of the control of glucose homeostasis as well as the novel concept that the immuno-metabolic cross-talk within WAT is able to affect whole-body metabolism and insulin sensitivity by secreting various adipokines and lipokines (reviewed in [10,63]). New experiments to better characterize the role of EC system in WAT and this complex homeostatic mechanism are required.

In conclusion, our results demonstrate that a long-term

supplementation of n-3 PUFA in dietary-obese mice as well as in overweight/obese T2DM patients is capable of elevating WAT levels of anti-inflammatory molecules EPEA and DHEA, but it differentially affects the levels of classical ECs such as 2-AG and AEA. While n-3 PUFA decreased WAT levels of 2-AG and AEA in obese insulin-resistant mice, which was associated with the improvements in glucose homeostasis, they failed to do so in WAT as well as in serum of T2DM patients. This novel finding might help to explain, why n-3 PUFA have limited efficacy with regard to the effects on insulin sensitivity and/or the regulation of glucose homeostasis in these subjects. However, various anti-inflammatory lipid mediators were induced by n-3 PUFA even in T2DM patients, thus supporting the notion that n-3 PUFA supplementation can be used to treat not only their impaired postprandial lipid metabolism, but possibly also various T2DM-associated inflammatory conditions.

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Article III

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Omega-3 Phospholipids from Krill Oil Enhance Intestinal Fatty Acid Oxidation More Effectively than Omega-3 Triacylglycerols in High-Fat Diet-Fed Obese Mice

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Abstract: Antisteatotic effects of omega-3 fatty acids (Omega-3) in obese rodents seem to vary depending on the lipid form of their administration. Whether these effects could reflect changes in intestinal metabolism is unknown. Here, we compare Omega-3-containing phospholipids (krill oil; ω3PL-H) and triacylglycerols (ω3TG) in terms of their effects on morphology, gene expression and fatty acid (FA) oxidation in the small intestine. Male C57BL/6N mice were fed for 8 weeks with a high-fat diet (HFD) alone or supplemented with 30 mg/g diet of ω3TG or ω3PL-H. Omega-3 index, reflecting the bioavailability of Omega-3, reached 12.5% and 7.5% in the ω3PL-H and ω3TG groups, respectively. Compared to HFD mice, ω3PL-H but not ω3TG animals had lower body weight gain (−40%), mesenteric adipose tissue (−43%), and hepatic lipid content (−64%). The highest number and expression level of regulated intestinal genes was observed in ω3PL-H mice. The expression of FA ω-oxidation genes was enhanced in both Omega-3-supplemented groups, but gene expression within the FA β-oxidation pathway and functional palmitate oxidation in the proximal ileum was significantly increased only in ω3PL-H mice. In conclusion, enhanced intestinal FA oxidation could contribute to the strong antisteatotic effects of Omega-3 when administered as phospholipids to dietary obese mice.

Keywords: krill oil; Omega-3 phospholipids; high-fat diet; Omega-3 index; small intestine

1. Introduction

Obesity, i.e., excessive accumulation of white adipose tissue (WAT) in the body, is associated with insulin resistance and metabolic disorders (i.e., "metabolic syndrome"), which in turn increase the risk of type 2 diabetes, cardiovascular disease and premature death. While effective pharmacological interventions for the treatment of metabolic consequences of obesity require the use of multiple agents and are often associated with adverse side effects, lifestyle changes remain an essential component of

any prevention or treatment strategy. For instance, increased physical activity and dietary changes could lower the incidence of type 2 diabetes in subjects with elevated fasting glucose concentrations and impaired glucose tolerance by as much as 60% [1].

In terms of the effect of dietary fatty acids (FA) on metabolism, supplementation with polyunsaturated FA of n-3 series (Omega-3) such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), which are found in marine fish and fish oils, could reduce the risk of cardiovascular disease [2]. Several studies in obese humans also demonstrated a reduction of adiposity after Omega-3 supplementation [3,4], while Omega-3 prevented the development of obesity, insulin resistance, and dyslipidemia in rodents fed a high-fat diet [5–9]. Moreover, Omega-3 may reduce liver steatosis ([5,10], and reviewed in [11]). Beneficial effects of Omega-3 on metabolism mainly involve lipid-lowering and anti-inflammatory mechanisms [12]. While the molecular targets of Omega-3 in liver [8], WAT [5,6,9] or muscle [7,13] have been intensively studied, the contribution of other organs to the overall metabolic effect of Omega-3 is still little characterized. Previous studies suggest that dietary interventions with Omega-3 supplemented in the form of triacylglycerols (TAG) may also affect intestinal metabolism [14,15]. For instance, fish oil supplementation (i.e., Omega-3 as TAG) induced dose-dependent expression of genes involved in lipid metabolism and FA oxidation in the small intestine but not in the colon of C57BL/6 mice fed a high-fat diet [14]. Enhancement of FA oxidation in the intestine has been shown to protect mice from visceral fat accumulation and impaired glucose homeostasis induced by high-fat feeding [16]. These data suggest that the small intestine may contribute significantly to the metabolic, antisteatotic and possibly also the anti-obesity effects of Omega-3 administration.

Further evidence suggests that the efficacy of Omega-3 in modulating metabolism may depend on the lipid class in which these FA are delivered into the organism. In statin-treated dyslipidemic subjects, EPA and DHA administered as TAG showed a stronger hypolipidemic effect as compared to their ethyl ester form [17]. The superior effect of Omega-3 given as TAG might be attributable to a better bioavailability of EPA and DHA, determined as a relative percentage of EPA and DHA in plasma phospholipids (PL) or red blood cell (RBC) membranes (i.e., Omega-3 index [18]). Moreover, administration of a single dose of EPA and DHA to healthy young men either as re-esterified TAG, ethyl esters or marine PLs resulted in an increase in the Omega-3 index with the following efficacy: PL > TAG > ethyl esters [19]. Importantly, improved bioavailability of DHA and especially EPA in plasma following Omega-3 administration as PL was demonstrated in both human subjects [19,20] and laboratory rodents [21]. In line with improved EPA and DHA bioavailability, Omega-3 PL were able to alleviate many aspects of the metabolic syndrome including dyslipidemia, impaired glucose homeostasis, adipocyte hypertrophy as well as hepatic steatosis in rodents fed a high-fat diet [10,21–23]. Importantly, administration of Omega-3 PL contained in krill oil or herring meal extract resulted in a more effective reduction of ectopic fat accumulation in the livers of obese animals, compared to Omega-3 TAG [10,21,24]. Reduction of lipid content in the liver due to administration of Omega-3 PL was associated with strong suppression of gene expression in the de novo FA synthesis and cholesterol biosynthesis pathways [10,22]. However, despite strong evidence for the beneficial effects of Omega-3 PL on metabolism and fat accumulation, the potential role of the small intestine and its metabolism in these effects has not yet been addressed.

Given the available evidence for stimulation of FA catabolism in the small intestine in response to Omega-3 supplementation [14], we hypothesized that the potent antisteatotic effects of Omega-3 PL supplementation, often observed in preclinical models of obesity, may also be related to more effective regulation of intestinal lipid metabolism. Therefore, in this study, we determined the global changes in gene expression as well as FA oxidation in the small intestine of dietary obese mice supplemented with EPA and DHA administered as Omega-3 PL (in the form of krill oil), and compared them with those elicited by the same dose of EPA and DHA but given as TAG.

2. Materials and Methods

2.1. Animals and Diets

Male C57BL/6N mice (Charles River Laboratories, Sulzfeld, Germany) at the age of 12 weeks were fed a standard low-fat diet (Chow; 3.4% wt/wt as lipids; Rat/Mouse—Maintenance extrudate; Ssniff Spezialdieten GmbH, Soest, Germany). After one week of adaptation to a corn oil-based high-fat diet [HFD; lipids ~35% (wt/wt)], mice were randomly assigned to one of the four experimental diets: (i) HFD; (ii) HFD-based diet, in which 15% (wt/wt) of dietary lipids was replaced (at the expense of the corn oil component) by the TAG-based EPA and DHA concentrate Epax 1050 TG (ω3TG diet; Epax 1050 TG contained ~11% EPA and ~47% DHA (wt/wt); Epax, Ålesund, Norway) to achieve a total EPA and DHA concentration of ~30 mg/g diet (tocopherol content ~0.02% (wt/wt]; (iii) HFD-based diet, in which 45% (wt/wt) of dietary lipids was replaced by PL-based EPA and DHA in the form of Antarctic krill oil (ω3PL-H diet; krill oil contained ~13% EPA and ~7% DHA (wt/wt); Rimfrost USA, Merry Hill, NC, USA) to achieve a total EPA and DHA concentration of ~30 mg/g diet (astaxanthin content ~0.005% (wt/wt)); and (iv) HFD-based diet, in which 15% (wt/wt) of dietary lipids was replaced by Antarctic krill oil (ω3PL-L diet), this time at a dose corresponding to ~10 mg EPA and DHA per g diet (astaxanthin content ~0.002% (wt/wt); see Supplementary Materials Table S1 for macronutrient composition of the diets). Chow-fed mice served as lean controls. The mice were kept in controlled conditions (i.e., 22 °C; 50% humidity; 12 h/12 h light/dark cycle) with free access to food and water. Body weight and 24-h food consumption were recorded every week. Mice were fed the respective diets for 8 weeks and then sacrificed by cervical dislocation under diethyl ether anesthesia (n = 8 per group). Blood was collected into EDTA-coated tubes and plasma obtained by centrifugation. The entire small intestine from the pylorus to the ileocecal valve, as well as liver and skeletal muscle (*m. quadriceps femoris*) were removed and snap frozen in liquid nitrogen. The dissected small intestine, excluding the sample for microarray analysis, was subdivided into several segments, namely the duodenum, proximal and distal jejunum, as well as the proximal and distal ileum. Segments were snap frozen in liquid nitrogen and stored at −80 °C for qPCR. All samples were stored at −80 °C for further analysis. In a second experiment of the same experimental design (n = 8 per group), the small intestine was dissected and subdivided into several segments, while the proximal ileum was used for explants to measure FA β-oxidation ex vivo (see Section 2.8) and the proximal jejunum was fixed in 4% paraformaldehyde and stored at −80 °C for histological analysis (see Section 2.5). The experiments followed the guidelines for the use and care of laboratory animals of the Institute of Physiology of the Czech Academy of Sciences and were approved under the protocol no. 81/2016.

2.2. Biochemical Analysis of Plasma and Tissue Samples

Plasma levels of (i) TAG and total cholesterol were determined using the colorimetric enzymatic assays from Erba Lachema (Brno, Czech Republic), and (ii) non-esterified FA (NEFA) were assessed with a NEFA-HR(2) kit from Waco Chemicals GmbH (Neuss, Germany). Blood glucose levels in both fasting and *ad libitum* fed mice were measured by OneTouch Ultra glucometers (LifeScan, Milpitas, CA, USA).

Liver and muscle TAG content was estimated in ethanolic KOH tissue solubilisates as before [25]. Briefly, tissue samples (~50 mg) were digested with 150 mL of 3 M KOH in 65% ethanol at 70°C for 2 h. Resulting homogenates were cleared from debris by a brief centrifugation and the concentration of total glycerolipids was assessed by the Bio-La-Test TG L250S (Erba Lachema, Brno, Czech Republic).

2.3. Oral Glucose Tolerance Test

One week before the end of dietary intervention (i.e., at week 7), oral glucose tolerance test (GTT) was performed after an overnight fast (~15–16 h), as described earlier [26]. A bolus of 200 mg of glucose was administered orally by gavage and blood glucose levels were measured using Contour

Plus glucometers (Bayer, Leverkusen, Germany) at time 0 (i.e., before injection) and 15, 30, 60, 120 and 180 min after the gavage. Results were expressed as incremental area under the glucose curve (AUC).

2.4. FA Composition in RBC

Extraction of lipids from RBC, subsequent extraction of FA methyl esters and their analysis using comprehensive two-dimensional gas chromatography with mass detection Pegasus 4D (LECO, St. Joseph, MI, USA) was performed as described earlier [27]. The Omega-3 index was calculated as the sum of EPA and DHA levels divided by the total levels of all FA.

2.5. Histology

Villi length, crypt height and the thickness of muscular layer were measured in the proximal jejunum. The frozen jejunum embedded in Tissue-Tek block was cut in 20 µm-slices attached to Superfrost Plus (Thermo Scientific, Waltham, MA, USA) slides and stained using hematoxylin. The above intestinal parameters were measured using a Leica LMD 6000 optical microscope and Leica Microdissection software v 6.5 (Leica Microsystems CMS GmbH, Wetzlar, Germany). At least 10 individual measurements for each parameter were performed for each sample. Two intestinal samples (200 µm apart) were taken and measured from each mouse. The villi length was defined as the length from lamina propria to the top of the villus. The crypts were measured from the bottom to the top of the crypt. All histological analyses were performed by a pathologist blinded to dietary groups.

2.6. Transcriptome Analysis of the Small Intestine

Total RNA was isolated from small intestine samples using TRI Reagent (Sigma-Aldrich, Prague, Czech Republic) and purified using RNeasy columns (Qiagen, Venlo, The Netherlands). 200 ng RNA of each sample ($n = 8$ per group) was assayed using the Agilent's Whole Mouse Genome 8 × 60 K Oligo microarrays (Agilent Technologies Inc., Santa Clara, CA, USA), as earlier [10]. All microarray data are available at Gene Expression Omnibus (GEO; GSE93151). Pathway analysis using all differentially expressed genes (DEG) with $p < 0.05$ was performed using the DAVID database [28]. Statistical analysis was performed by a standard procedure using log 2 normalized data.

2.7. Real-time Quantitative PCR (RT-qPCR)

To confirm the results of microarray analysis, RT-qPCR was performed as described [9]. Data were normalized to the level of villin expression. An overview of the primer sequences is given in Table S2.

2.8. Fatty Acid Oxidation

The level of mitochondrial FA β-oxidation in the intestine (i.e., proximal ileum) was measured as before [14].

2.9. Statistical Analysis

Data are presented as means ± SEM. Statistical analysis was performed using SigmaStat software v. 4.0 (Systat Software Inc., San Jose, CA, USA). Data were analysed by two-tailed Student's *t*-test or one-way ANOVA followed by the Holm-Sidak test. Specific p values are stated in the Results section and the legends to figures. A $p < 0.05$ was considered significant.

3. Results

3.1. Parameters of Energy Balance and Adiposity

First, we characterized the ability of Omega-3 administered as either krill oil (ω3PL-H and ω3PL-L diet) or TAG concentrate (ω3TG diet) to prevent the development of HFD-induced obesity

and metabolic disorders. As expected, the rate of body weight gain (Figure 1A), as well as the final weight gain (Figure 1B), were markedly reduced in the Chow-fed mice as compared to HFD group.

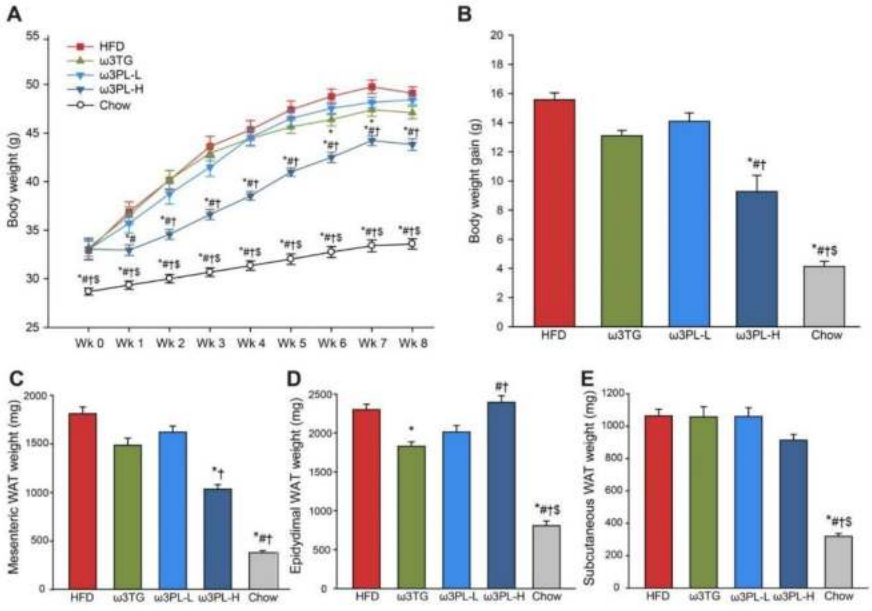

Figure 1. The effect of high-fat diet (HFD) feeding and its supplementation by Omega-3 on body weight (**A**), body weight gain (**B**), mesenteric WAT (**C**), epididymal WAT (**D**) and subcutaneous WAT (**E**) in mice. Tissues were weighed after 8 weeks of dietary intervention, while body weight gain was calculated as difference between Week 7 and 0. Data are means ± SEM (n = 8). *, significantly different vs. HFD; #, significantly different vs. ω3TG; †, significantly different vs. ω3PL-L, $, significantly different vs. ω3PL-H. ($p < 0.05$, one-way ANOVA).

Among the groups fed HFD-based diets, ω3PL-H mice had significantly lower body weight compared to other groups of mice, starting from week 1 (week 2 in the ω3PL-L group) until the end of the study (Figure 1A). In addition, both ω3PL-H and ω3TG mice exhibited a reduced cumulative food intake of ~10% during the first 7 weeks of the study (Table 1). To determine to what extent HFD-induced weight gain reflected an increase in adiposity, the weights of various fat depots were analyzed. As expected, at the end of the 8-week dietary intervention period all analyzed fat depots were significantly larger in animals fed HFD-based diets than in lean chow-fed mice (Figure 1C–E and Table 1), with the largest difference observed in the case of mesenteric WAT, showing a ~5-fold increase. Only ω3TG and ω3PL-H diets with higher Omega-3 content were able to reduce the weight of some of the visceral WAT depot, as compared to obese HFD-fed controls (Figure 1C, D). However, there was a depot-specific effect of different lipid classes used to administer Omega-3; while the ω3TG group exhibited reduced epididymal and retroperitoneal WAT by 20 and 23%, respectively (Figure 1D and Table 1), ω3PL-H mice showed a preferential reduction in mesenteric WAT by 43% (Figure 1C). Besides the reduced weight of mesenteric WAT, ω3PL-H mice also had lower liver weight (by 17%; Table 1).

Table 1. Body mass, tissue weights, tissue TAG content and plasma parameters in mice fed HFD alone or supplemented with Omega-3.

	HFD	ω3TG	ω3PL-L	ω3PL-H	Chow
Energy balance					
Body weight initial (g)	32.94 ± 0.93	33.18 ± 0.86	33.02 ± 1.05	33.06 ± 1.13	28.68 ± 0.36 *
Body weight final (g)	49.09 ± 0.66	47.09 ± 0.64	48.41 ± 0.50	43.81 ± 0.60 *#†	33.58 ± 0.52 *#†$
Cumulative food intake (MJ/animal)	4.32 ± 0.06	3.93 ± 0.05 *	4.27 ± 0.07 #	3.90 ± 0.07 *†	3.60 ± 0.06 *#†$
Tissue weight (mg)					
Liver	2110 ± 151	2293 ± 172	2523 ± 160	1747 ± 84 #†	1504 ± 87 *#†
Brown adipose tissue	182 ± 11	150.3 ± 12	192 ± 5 #	174 ± 12	82 ± 6 *#†$
Perirenal WAT	1218 ± 83	940 ± 51 *	1052 ± 67	1002 ± 40	206 ± 24 *#†$
Plasma-fasted state					
NEFA (mmol/L)	0.61 ± 0.03	0.56 ± 0.02	0.65 ± 0.03	0.81 ± 0.03 *#†	0.94 ± 0.03 *#†$
Cholesterol (mmol/L)	3.72 ± 0.09	3.05 ± 0.07 *	3.93 ± 0.10 #	3.05 ± 0.07 *†	1.87 ± 0.06 *#†$
TAG (mmol/L)	0.75 ± 0.04	0.86 ± 0.05	0.92 ± 0.05 *	1.08 ± 0.05 *	0.72 ± 0.05 †$
Glucose (mmol/L)	9.16 ± 0.43	8.59 ± 0.53	9.33 ± 0.47	9.25 ± 0.38	5.81 ± 0.34 *#†$
Insulin (pmol/L)	2.18 ± 0.32	1.53 ± 0.28	1.49 ± 0.36	0.95 ± 0.20 *	0.15 ± 0.46 *#†
Glucose homeostasis					
Glucose AUC (mol/L × 180 min)	3232 ± 249	2561 ± 323 *	2324 ± 228 *	949 ± 97 *#†	1805 ± 231 *#$

Data are means ± SEM (n = 8). Cumulative energy intake was assessed during the initial 7-week period of dietary interventions. Plasma parameters were measured after an overnight fast. *, significantly different vs. HFD; #, significantly different vs. ω3TG; †, significantly different vs. ω3PL-L, $, significantly different vs. ω3PL-H ($p < 0.05$, one-way ANOVA).

To examine the effects of HFD feeding and Omega-3 supplementation in terms of induction or prevention of ectopic lipid storage, we measured the TAG content in tissues such as the liver and skeletal muscle (Figure 2). In the liver (Figure 2A), the TAG content was ~3-fold higher in HFD mice than in the chow-fed animals. While the level of hepatic lipid accumulation in ω3TG and ω3PL-L mice was comparable to HFD-fed controls, ω3PL-H mice were completely protected against hepatic steatosis (Figure 2A). Similar effects of krill oil supplementation (i.e., ω3PL-H) were observed also in the skeletal muscle (Figure 2B).

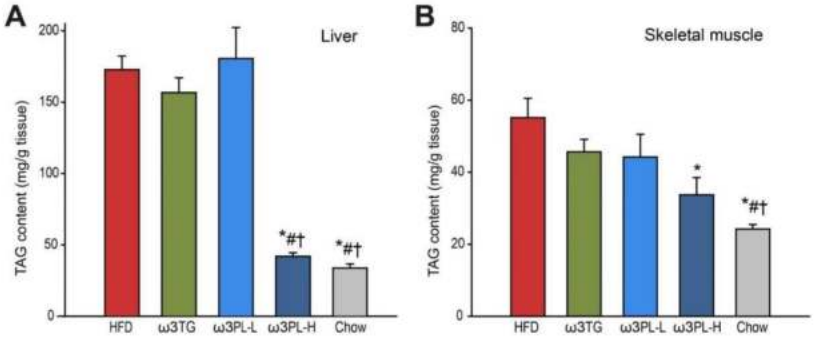

Figure 2. The effect of long-term Omega-3 supplementation on ectopic fat accumulation in dietary obese mice. After 8 weeks of dietary interventions, the level of TAG accumulation was assessed in the liver (**A**) and skeletal muscle (**B**). Data are means ± SEM (n = 8). *, significantly different vs. HFD; #, significantly different vs. ω3TG; †, significantly different vs. ω3PL-L, ($p < 0.05$, one-way ANOVA).

3.2. Effect of Omega-3 Supplementation on Lipid and Glucose Homeostasis

To evaluate potential changes in systemic lipid metabolism due to Omega-3 supplementation, we measured plasma levels of TAG, NEFA and total cholesterol in the fasted state (Table 1). HFD feeding significantly impaired the fasting-induced increase in NEFA plasma levels, while Omega-3 supplementation in ω3PL-H mice normalized this defect (Table 1). Plasma cholesterol levels were increased ~2-fold in HFD mice as compared to lean Chow-fed animals; Omega-3 supplementation in either ω3PL-H or ω3TG mice partially normalized plasma cholesterol levels (Table 1). Despite the fact that plasma levels of TAG did not generally differ between the chow and HFD groups, they were partially increased in both krill oil-supplemented groups and unchanged in ω3TG mice (Table 1).

While HFD feeding substantially increased both fasting blood glucose and plasma insulin levels, the latter parameter was reduced by ~50% in ω3PL-H mice compared to HFD-fed controls (Table 1). Omega-3 supplementation did not change fasting plasma glucose level (Table 1). Furthermore, in ω3PL-H animals, glucose clearance during oral GTT was significantly faster than in other groups of mice fed HFD-based diets, and thus the level of glucose intolerance expressed as AUC was markedly reduced (Table 1).

3.3. Lipidomic Analysis of RBC and the Omega-3 Index

Next, lipidomic analysis of FAs contained in RBC PLs was performed in order to determine the Omega-3 index. The analysis revealed distinct changes in FA profiles as a result of various dietary interventions (Figure 3A and Table S3). Thus, HFD feeding led to a reduction in oleic acid (18:1) and palmitoleic acid (POA; 16:1), as well as to specific changes in the content of saturated FAs (SFA), including palmitic acid (16:0) reduction and increase in stearic acid (18:0) content, which resulted in an unchanged percentage of total SFA as compared to Chow (Table S3). The total content of Omega-6 and Omega-3 FAs remained unchanged in HFD mice. Omega-3, regardless of the form of supplementation, decreased the percentage of total Omega-6, while increasing the percentage of total SFA only in the ω3PL-H group and the percentage of POA in both krill oil-supplemented groups (Table S3).

To characterize in detail the impact of different lipid classes used to administer Omega-3, we analyzed the data on FAs composition in RBC by partial-least-squares discriminant analysis (PLS-DA). When only mice fed HFD-based diets were considered, PLS-DA separated the mice into distinct groups (Figure 3B), with DHA (C22:6), arachidonic acid (AA; C20:4), dihomo-γ-linolenic acid (C20:3), margaric acid (C17:0) and docosapentaenoic acid (DPA; C22:5) identified as the most discriminant metabolites (data not shown).

We then calculated the Omega-3 index, defined as the sum of EPA and DHA in RBC expressed as a percentage of total FAs. As expected, it was increased due to Omega-3 supplementation in the lipid-form-dependent manner, from 2% in HFD mice to 7.5% and 12.5% in the ω3TG and ω3PL-H group, respectively (Figure 3C). Moreover, in ω3PL-L mice the Omega-3 index increased to 6.5%, similar to ω3TG mice whose diet contained three times more EPA and DHA (Figure 3C). Upon close inspection, EPA content in RBC PLs increased from 0.03% in HFD mice to 1.6% and 2.6% in ω3TG and ω3PL-L, respectively, while in the ω3PL-H group EPA content increased to 7.2% (Figure 3D and Table S3). Furthermore, DHA incorporation in RBC PLs increased from 2% in HFD mice to 4.0%, 5.3% and 5.8% in ω3PL-L, ω3PL-H and ω3TG mice, respectively (Figure 3D and Table S3). The content of α-linolenic acid (ALA; C18:3), an essential FA that may serve as a precursor for longer-chain Omega-3, was significantly increased only in ω3PL-H mice (Figure 3D and Table S3), while DPA content increased from 0.11% in HFD fed animals to 0.22%, 0.37% and 0.54% in ω3TG, ω3PL-L and ω3PL-H mice, respectively (Figure 3D and Table S3).

As expected, correlation analysis using pooled data from all Omega-3-supplemented mice suggested an inverse relationship between the level of Omega-3 index and the hepatic TAG content ($r = -0.80$, $p < 0.0001$; Figure S1A).

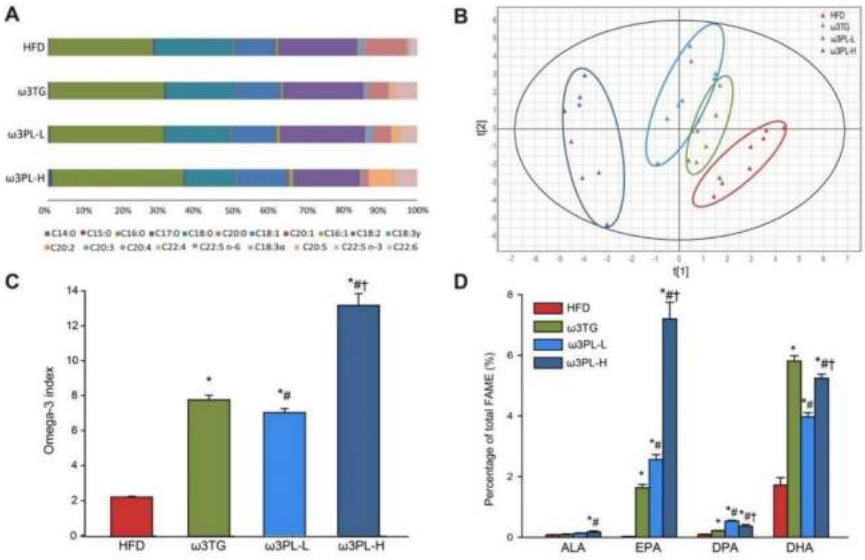

Figure 3. The long-term Omega-3 dietary supplementation affected FA composition of cell membranes. Distribution of FA in the phospholipid fraction of RBCs (**A**) and score plot as assessed by partial-least-squares discriminant analysis (PLS-DA) based on the FA composition in total phospholipids of RBC (**B**) from mice after 8 weeks of dietary intervention. Incorporation of Omega-3 in the RBC membranes expressed as the Omega-3 index (**C**) or relative content of individual Omega-3, ALA, EPA, DPA and DHA (**D**). Data are mean percentage of total FA in phospholipid fraction (**A**) or mean percentage ± SEM (**C**,**D**) (n = 8). *, significantly different vs. HFD; #, significantly different vs. ω3TG; †, significantly different vs. ω3PL-L ($p < 0.05$, one-way ANOVA). For detailed FA composition of RBCs phospholipids including Chow-fed mice, see Table S3.

3.4. The Effect of Omega-3 Supplementation on Intestinal Morphology

As indicated above (see Section 3.1), the abdominal WAT depots are differentially affected by dietary interventions using Omega-3 as TAG or PL. Thus, unlike ω3TG, ω3PL-H feeding resulted in a preferential reduction of mesenteric WAT, which is directly linked to the small intestine. Therefore, we first investigated possible negative changes in intestinal morphology associated with HFD feeding, as well as possible changes associated with Omega-3 supplementation (Figure 4A–D). However, the muscular-serous layer and intestinal crypts in the proximal ileum showed no significant alterations in size or shape between the groups. On the other hand, intestinal villi of the mucosa in both ω3TG and ω3PL-H mice were significantly shorter than those in the control HFD group (Figure 4E); moreover, the small intestine was also significantly longer in the case of ω3PL-H and ω3TG mice (Figure 4F).

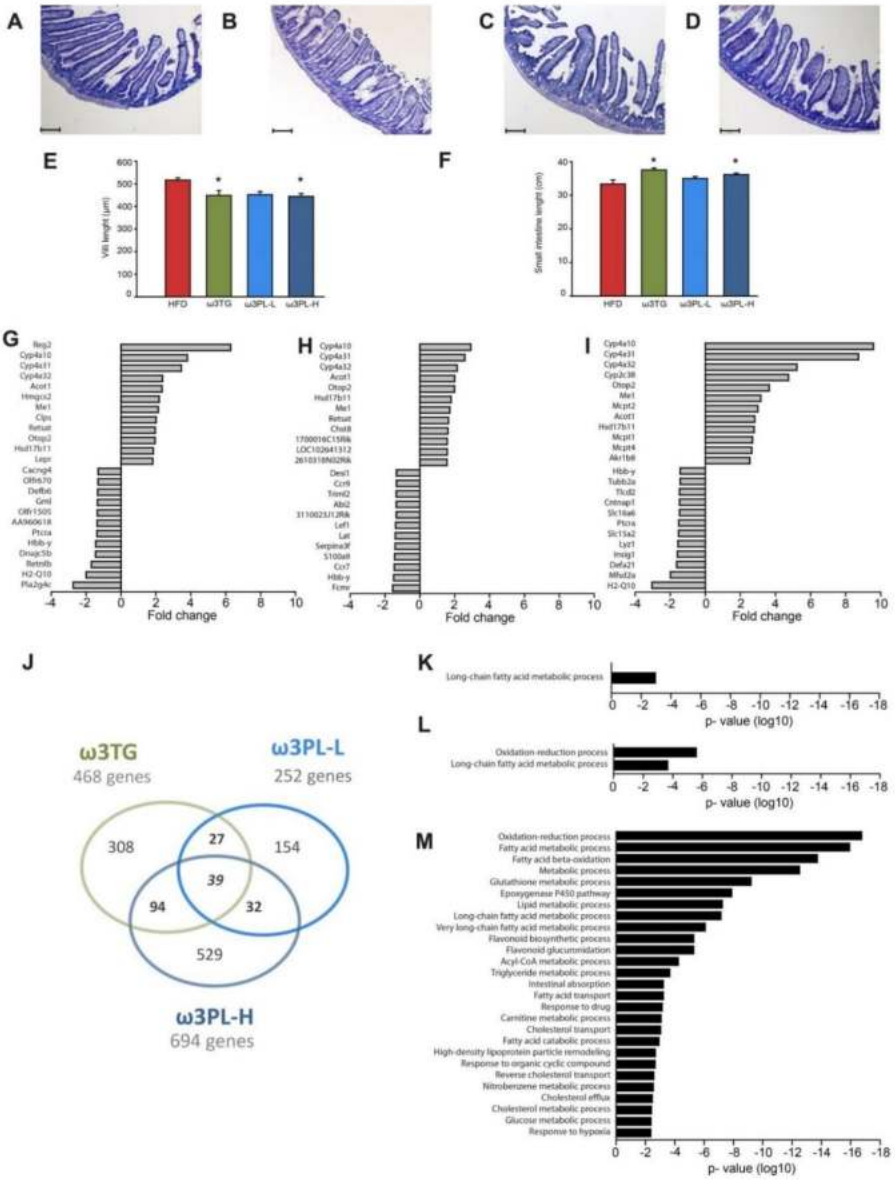

Figure 4. Small intestine as target of Omega-3 dietary intervention in dietary obese mice. (**A**–**D**) Representative hematoxylin-eosin sections of proximal ileum from HFD (**A**), ω3TG (**B**), ω3PL-L (**C**) and ω3 PL-H (**D**) mice; original magnification, 100x; scale bar, 200 µm (**E**) Length of villi in proximal ileum and (**F**) length of small intestine of mice fed HFD with and without supplementation for 8 weeks. Data are means ± SEM (n = 8). *, significantly different vs. HFD; #, significantly different vs. ω3TG; †, significantly different vs. ω3PL-L ($p < 0.05$, one-way ANOVA). (**G**–**I**) The 12 most up- and down-regulated genes compared to HFD as assessed by microarray analysis in whole length of small intestine

from mice fed ω3TG (**G**), ω3PL-L (**H**) or ω3PL-H (**I**) diet for 8 weeks ($p < 0.05$). (**J**) Venn diagram illustrating the overlap in differentially expressed genes compared to HFD mice as determined by microarray-based RNA analysis in the intestinal samples from ω3TG, ω3PL-L and ω3PL-H mice ($p < 0.05$). (**K**–**M**) Enrichment for Gene Ontology Process terms of genes differentially expressed comparing to HFD in (**K**) ω3TG, (**L**) ω3PL-L and (**M**) ω3PL-H small intestine samples identified by DAVID analysis. Gene Ontology (GO) terms were sorted based on *p*-values ($p < 0.005$). For detailed effects of HFD compared to chow-fed mice, see Figure S2.

3.5. Effects of Omega-3 Supplementation on Intestinal Gene Expression

To analyze potential differences in the effect of various lipid forms of Omega-3 on the small intestine, we performed whole genome gene expression profiling using the whole small intestine of ω3TG, ω3PL-L and ω3PL-H mice in comparison to the control HFD mice. The highest number of DEG was found in the ω3PL-H group with 694 DEGs, while the expression of 468 and 252 genes was significantly changed in ω3TG and ω3PL-L mice, respectively (Figure 4J). Expression of only a small number of these genes was altered at least two-fold in one or more dietary groups (i.e., 10, 4 and 35 genes in the ω3TG, ω3PL-L and ω3PL-H group, respectively). Moreover, 7 of the 12 most upregulated genes were common to all groups, and included *Cyp4a10*, *Cyp4a31*, *Cyp4a32*, *Otop2*, *Me1*, *Acot1*, and *Hsd17b11*. However, these genes were upregulated to varying degrees, mostly in line with the level of Omega-3 incorporation in each group (Figure 4G–I and Figure 5A–C).

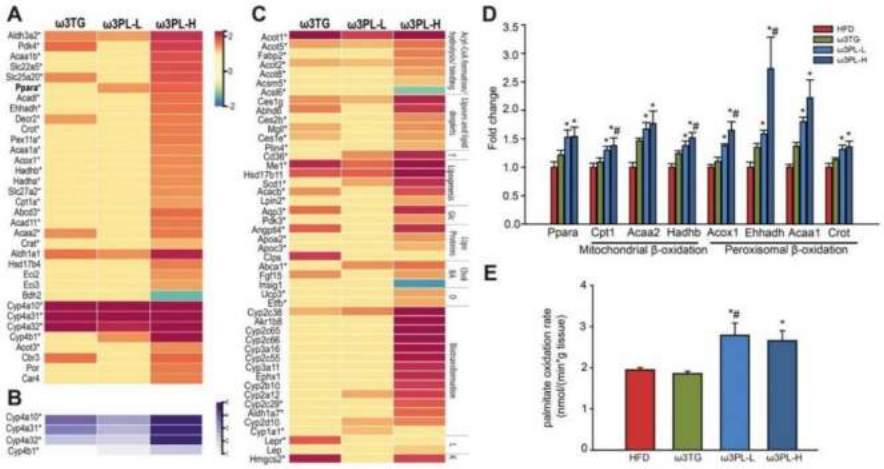

Figure 5. The effect of Omega-3 supplementation on lipid metabolism in the small intestine of dietary obese mice. (**A**) Heatmap showing the gene expression of (**A**) FA oxidation genes, (**B**) ω-oxidation genes (includes also in **A** - for better resolution in different color scale compared to **A**) and (**C**) genes involved in different processes related to lipid metabolism (as indicated on right side) relative to HFD ($p < 0.05$). T, lipid transport; Glc, glucose/glycerol transport and metabolism; Chol BA; metabolism and transport of cholesterol and bile acids; O, oxidative phosphorylation; L, leptin signaling; K, ketogenesis. (**D**) The expression of selected genes of mitochondrial and peroxisomal FA oxidation in proximal ileum. Data were expressed as relative to HFD; HFD = 1. (**E**) Quantification of FA oxidation rate in proximal ileum. Data are means ± SEM ($n = 8$). *, significantly different vs. HFD; #, significantly different vs. ω3TG ($p < 0.05$, one-way ANOVA). For detailed effects of HFD compared to Chow-fed mice, see Figure S2.

Regenerating islet-derived 2 (*Reg2*), colipase (*Clps*), leptin receptor (*Lepr*) and phospholipase A2 gamma (*Pla2g4c*) were among the most regulated genes in the ω3TG group (Figures 4G and

5A–C), while the ω3PL-L group showed specific up-regulation of a gene related to glycosylation in ER (*Chst8*) and down-regulation of genes related to immune response (e.g., *Fcmr*, *Ccr7*, *Ccr9*, *Lat*, *Lef1*, and *Abl2*; Figure 4H). In contrast, overexpression of mast cells-related genes *Mcpt2*, *1* and *4*, and reduced expression of *H2-Q10*, *Mfsd2a*, *Defa21*, *Insig*, *Lyz1*, *Slc15a2*, *Pctra*, and *Slc16a6* genes were characteristic of ω3PL-H mice (Figure 4I).

3.6. Functional Enrichment Analysis of DEGs

Next, we performed functional analysis of gene expression data using DAVID in order to determine which biological processes were affected in the small intestine by exposure to Omega-3 supplementation versus control HFD. All genes showing at least 1.2-fold differential expression were included in the analysis. Functional annotation clustering analysis revealed 1, 2 and 27 significantly affected GO biological processes in the ω3TG, ω3PL-L and ω3PL-H group, respectively (Figure 4K–M). Long-chain FAs metabolism was the only enriched process in ω3TG mice (Figure 4K). Despite the lower number of differentially expressed genes in ω3PL-L compared to ω3TG mice, oxidation-reduction was identified as a highly enriched process, in addition to long-chain FAs metabolism, in these mice (Figure 4L). In ω3PL-H mice the highest number of regulated genes along with the highest level of expression corresponded primarily to 27 affected processes, which included metabolism of FAs, lipids, cholesterol and glucose, as well as intestinal transport and absorption, epoxygenase P450 and glutathione pathways (Figure 4M).

With respect to the effect of Omega-3 supplementation on genes related to FAs and lipid metabolism, the greatest changes were observed in ω3PL-H mice (Figure 5A–C). Specifically, genes belonging to several cytochrome P450 families were among the most upregulated genes in this group of mice. While genes in the CYP4 family, which is involved in FA ω-oxidation, were up-regulated in all Omega-3-supplemented groups (Figure 5A,B), the genes in the CYP2 and CYP3 family, which are capable of catalyzing the oxidative biotransformation of most drugs and other xenobiotics, were upregulated only in ω3PL-H mice (Figure 5C). Furthermore, a high number of genes involved in mitochondrial as well as peroxisomal FA β-oxidation was primarily affected in ω3PL-H mice (Figure 5A), indicating a high level of FA oxidation in the small intestine of these animals. Accordingly, the expression of peroxisome proliferator-activated receptor (PPAR) α, the major transcriptional regulator of FA catabolism, was highest (~1.8-fold increase) in the ω3PL-H group (Figure 5D).

RT-qPCR analysis was used to verify the results of DNA microarrays and to identify possible segment-specific changes in gene expression in the intestine. Eight genes were selected based on microarray analysis, which included the most up-regulated lipid metabolism genes (i.e., *Cyp4a32*, *Me1*, *Acot1*, *Hmgcs2*), genes associated with FA oxidation (*Cpt1*), FAs transport (*Cd36*), lipogenesis (*Scd1*), and finally Pparα transcription factor itself. While the results of RT-PCR analysis were generally in accordance with the data obtained by DNA microarrays (see Table S4), consensus was particularly pronounced in the case of the proximal ileum (Figure 5D). Both ω3PL-L and ω3PL-H mice had increased expression of *Pparα* and its target gene *Cpt1* (i.e., the rate-limiting enzyme for mitochondrial FA β-oxidation) in the proximal ileum (Figure 5D). Therefore, we measured the expression of other FA β-oxidation-related genes in the proximal ileum, namely *Hadhb*, *Acox1*, *Ehhadh*, *Acca1*, *Crot* and *Fabp2*. Consistent with *Cpt1* expression, all these genes were up-regulated in both ω3PL-L and ω3PL-H mice, while their expression was much less affected in ω3TG mice (Figure 5D).

We further correlated the expression of selected genes in the proximal ileum with the Omega-3 index in all Omega-3-supplemented mice. Thus, the expression of genes related to FA ω-oxidation (*Me*, *Cyp4a*) strongly and positively correlated with the Omega-3 index (r = 0.84 and 0.85, respectively; $p < 0.00001$; Figure S1B) and EPA (r = 0.86 and 0.87, respectively; $p < 0.00001$; Figure S1B). We further observed positive correlations, especially with EPA but also with the Omega-3 index, in the case of genes involved in peroxisomal FA β-oxidation (for details see Figure S1B). In contrast, the expression of genes related to mitochondrial FA β-oxidation as well as *Ppara* itself showed only a weak association with EPA or Omega-3 index (for details see Figure S1B).

3.7. Effects of Omega-3 Supplementation on Intestinal Fatty Acid Oxidation

To link up-regulation of FA oxidation genes with an increased pathway flux at the functional level, we measured the rate of FA oxidation ex vivo using proximal ileum explants and ^{14}C-palmitate as substrate. Explants from ω3PL-L and ω3PL-H mice had a higher ability to oxidize palmitate (1114 ± 120 and 1062 ± 97 nmol × min^{-1} × g^{-1}, respectively) as compared to HFD mice (778 ± 23 nmol × min^{-1} × g^{-1}). However, administration of Omega-3 in the form of ω3TG diet did not increase palmitate oxidation above that observed in HFD mice (Figure 5E). The above data suggest that activation of the krill oil-induced intestinal FA β-oxidation transcription program is associated with increased in situ oxidation of FAs, which may help prevent weight gain and glucose intolerance in mice fed a HFD supplemented with this type of Omega-3 PL.

4. Discussion

In this study using mice fed a HFD, we showed that chronic Omega-3 PLs supplementation in the form of krill oil induced specific changes in the small intestine, which were characterized by marked up-regulation of the expression of lipid metabolism-related genes, especially those encoding enzymes involved in ω- and β- oxidation of FAs. The above changes were most pronounced in the proximal ileum, and were accompanied by increased in situ FA oxidation, regardless of the krill oil dose. Omega-3 supplementation in the form of TAG, providing the same total amount of EPA+DHA as krill oil (ω3PL-H), failed to induce consistent changes in the expression of FA catabolism genes, except for less pronounced modulation of gene expression of enzymes involved in ω-oxidation of FAs. Thus, some of the effects of krill oil in the intestine were unique, and could also contribute to a preferential reduction of mesenteric WAT and hepatic lipid content in krill oil-supplemented mice.

We showed here that Omega-3 PLs supplementation in the form of krill oil induced a higher degree of Omega-3 incorporation in plasma membranes of RBC (i.e., Omega-3 index) compared to Omega-3 administered as TAG, despite the same EPA+DHA content in the diets. In a previous study, feeding mice a high-fat diet supplemented with another type of marine PLs (i.e., from herring) resulted in higher plasma levels of DHA and especially EPA compared to those achieved by Omega-3 supplementation as TAG [21]. Other studies in rodents and obese subjects suggest that the bioavailability of EPA and DHA from krill oil seems to be higher compared to fish oil [29,30]. However, the fact that various Omega-3 concentrates also differ in the EPA:DHA ratio makes the interpretation difficult. A previous study indicated that Omega-3 concentrate containing EPA and DHA at a 1:1 ratio reached the highest level of Omega-3 absorption [31]. Accordingly, similar levels of EPA and DHA in plasma and RBC were achieved when comparing fish and krill oil with similar ratios of EPA and DHA [32,33], indicating that the type of lipid carrier is not the only factor affecting the bioavailability of Omega-3. In the present study, the Omega-3 index in the ω3PL-H group reached 12.5%, which was associated with an improved metabolic profile, particularly in terms of decreased weight gain, reduced lipid accumulation in ectopic, non-adipose, tissues, and improved glucose tolerance. In humans, it has been shown that an Omega-3 index of ≥8% was associated with the greatest cardio-protection, whereas an index of <4% was associated with the least [34]. This is in contrast to our data in the ω3TG or ω3PL-L group, indicating that the Omega-3 index of 7.5% was not associated with a significant improvement in metabolic profile. Obviously, there are differences between rodents and humans regarding optimal levels of the Omega-3 index. In addition, the Omega-3 index increased to 8% due to the use of highly-enriched Omega-3 concentrate, which was associated with a significant reduction in liver fat content in subjects with relatively low levels of hepatic steatosis [35]. In agreement, we observed a strong positive correlation between the Omega-3 index and the lipid content of the liver. At the same time, mice with Omega-3 index higher than 8% showed a reduction of the liver TAG content to the level similar to that observed in Chow-fed mice, so further reduction cannot be expected.

In terms of the effect of Omega-3 supplementation on body weight, our study showed that ω3PL-H significantly reduced body weight (gain), which was accompanied by decreased weights of liver and mesenteric WAT. In contrast, ω3TG supplementation, providing the same amount of

EPA and DHA, significantly reduced the weight of epididymal and perirenal WAT. Such findings are consistent with a previous study in rats showing that fish oil administration reduced epididymal and perirenal WAT without affecting body weight [36]. Krill oil supplementation reduced body weight in various animal models of obesity [37,38] while administering krill powder to mildly obese men tended to decrease the amount of visceral fat [39]. Specific reduction of mesenteric WAT in ω3PL-H mice might be associated with improved glucose tolerance in these animals since previously a stronger involvement of mesenteric WAT in insulin resistance compared to other abdominal WAT depots was shown [40]. Moreover, the improved whole-body glucose tolerance in ω3PL-H mice could also be, at least in part, attributed to increased plasma levels of POA, a lipokine known to enhance insulin signaling [41]. Reduction of hepatic steatosis in ω3PL-H mice is likely to be associated with reduced mesenteric WAT only indirectly, through improved insulin sensitivity and modulation of intestinal lipid transport and metabolism. Although mesenteric WAT is anatomically adjacent to the portal vein, it contributes a relatively small percentage of the total amount of FA taken up by the liver [42].

The main focus of our study was on the effects of various lipid forms of Omega-3 supplementation on intestinal morphology and function. We observed the general effect of Omega-3 in preventing the negative influence of HFD feeding on intestinal length and intestinal villi. Feeding a high-fat diet has been shown to shorten the small intestine, increase villi length, and stimulate the absorption and transport of FA and lipoprotein synthesis [43–45]. Such an intestinal adaptation to macronutrient availability/digestibility is more common than only for lipids; for instance, a low versus high digestible starch-containing diet showed an increased small intestinal length, suggestive of enhanced digestion and absorption [46]. Moreover, high-fat feeding is known to impair the integrity of the gut in mice, while Omega-3 supplementation maintains intestinal barrier integrity [47]. In this context, we observed that ω3PL-H upregulated genes related to intestinal barrier function (e.g., *Cyp2c* family members) while ω3TG induced leptin-mediated antimicrobial defense that involves Reg peptide and LepR [48]. The improvement in the intestinal barrier observed in Omega-3 supplemented groups of mice may also play a role in reducing hepatic steatosis, while disruption in the gut-liver barrier (aka "leaky gut") is common in non-alcoholic steatohepatitis [49].

The metabolic effects of Omega-3 are partially mediated by direct modulation of gene expression. Our transcriptomic analysis used to determine the mechanistic basis of the action of Omega-3 in the small intestine revealed that dietary supplementation with krill oil altered the expression of more genes than fish oil supplying similar amounts of Omega-3. These findings are supported by previous studies suggesting similar superior effects of krill oil on the hepatic transcriptome in mice [50] and on gene expression in peripheral blood mononuclear cells in humans [51]. In the present study, ω3PL-induced DEGs in the small intestine were significantly enriched in 27 GO biological processes, including FAs metabolic processes, intestinal absorption, FAs transport, cholesterol transport, HDL remodeling and glucose metabolism, indicating effective adaptation of the small intestine to lipid overload. However, among the most induced DEGs in all Omega-3-supplemented groups were genes involved in FA ω-oxidation. This finding is supported by previous studies [14,15], where ingestion of fish oil increased expression of members of the cytochrome P450 family 4A (CYP4A) in the small intestine after only two weeks. Although significantly increased expression of the malic enzyme suggests induction of lipogenesis, other lipogenic genes were not affected by dietary interventions. Thus, these data support the idea that malic enzyme is the producer of nicotinamide adenine dinucleotide phosphate (NADPH) needed for CYP4A function [52]. Activation of FA ω-oxidation is important in preventing lipotoxicity during periods of substrate overload, e.g., in the case of high-fat feeding or insufficient mitochondrial FA β-oxidation [53,54]. Moreover, members of the CYP4A family can alleviate obesity-related inflammation, mainly through the anabolism and catabolism of critical anti-inflammatory and pro-resolving lipid mediators [55,56]. Interestingly, although CYP4A members are PPARα targets [57], CYP4A expression corresponded rather to differences in Omega-3 index between the groups, but not with the expression of PPARα itself. In fact, except for several genes primarily involved in lipid synthesis and transport (*Cd36*, *Scd1*, and *Abca1*) and mitochondrial FA β-oxidation in

proximal ileum (*Cpt1*, *Acaa2* and *Hadhb*), the expression level of most PPARα-regulated DEGs was more dependent on the Omega-3 index achieved than on the expression of PPARα itself. In previous studies, no relationship between the Omega-3 index and the intestine was characterized besides the effect on intestinal microbial diversity [58] and intestinal barrier function [59]. Omega-3-induced changes in microbiome composition observed in humans did not correlate with the Omega-3 content in RBCs [60]. An association between changes in intestinal metabolism induced by Omega-3 supplementation and bioavailability of EPA and DHA in the gastrointestinal tract can be expected. It is known that the relative content of EPA and DHA in RBC (i.e., the Omega-3 index) correlates strongly with their content in the plasma membranes of the small intestine [61]. Thus, in our study also the relative amounts of EPA and DHA in the gastrointestinal tract of mice should reflect the intergroup differences in the Omega-3 index. Here, we observed for the first time a strong positive association between the Omega-3 index and the expression of genes involved in intestinal ω- and peroxisomal β-oxidation of FAs.

Although the changes in gene expression do not indicate significant changes in the expression of entire metabolic pathways, ω3PL-H regulated several key genes that themselves regulate processes such as the absorption, transport, and metabolism of FA and cholesterol in the small intestine. For example, *Cd36*, well known as FA transporter, plays a role in the absorption and chylomicron formation, secretion of intestinal peptides and is important for maintenance of intestinal homeostasis and epithelial barrier integrity [62]. Furthermore, increased expression of the *Hmgcs2* gene can help maintain a mitochondrial acetyl-CoA pool, strongly affected by enhanced FA oxidation, that is regulated by the spillover pathway of ketogenesis [63]. In addition, β-hydroxybutyrate, the main product of ketogenesis, not only contributes to intestinal cell differentiation [64] but may also improve gut lining integrity impaired by HFD feeding [65,66].

While the results of our transcriptomic analysis reflect global changes at the level of the whole intestine (see above), Omega-3 supplementation can be expected to induce specific changes in different intestinal segments. Therefore, we analyzed the expression of PPARα and its regulated genes in four different segments of the small intestine. Surprisingly, in the proximal ileum, PPARα expression was increased to the same extent in both krill oil-supplemented groups, but not in the ω3TG group. Oxidation of FAs in the intestine has been shown to be regulated by PPARα through control of *Cpt1* and *Acox* expression [67], and mRNA and protein levels of these enzymes have been shown to be strongly correlated in a number of cell types [68,69]. Here we show that krill oil supplementation stimulated mitochondrial FA β-oxidation in conjunction with induction of *Cpt1* expression in a segment- and not dose-specific manner. This is in contrast to the genes involved in peroxisomal FA β-oxidation, which were up-regulated by krill oil in a dose-dependent manner. In addition, it has also been shown in the liver that krill oil stimulates the expression of mitochondrial and peroxisomal FA β-oxidation genes more effectively than fish oil [70]. Although previous studies have indicated that intestinal *Cpt1* expression and oxidation of FAs can also be stimulated by fish oil [14,15], the Omega-3 dose used in both studies was much higher than in the current study. Interestingly, EPA, but not DHA, was able to induce *Cpt1* expression in the intestinal Caco-2/TC7 cell line [71] and rat liver parenchymal cells [72]. Since the incorporation of EPA from krill oil, even when administered at the lower dose (ω3PL-L), exceeds the incorporation of EPA from Omega-3 TAG (ω3TG), this may explain the superior effects of krill oil supplementation in stimulating intestinal mitochondrial FA β-oxidation. However, the fact that the correlation between EPA and CPT1 is only modest suggests that in addition to the EPA content there is another unknown regulatory mechanism for mitochondrial FA β-oxidation induced by krill oil supplementation. Krill oil also contains astaxanthin, a powerful antioxidant with great anti-inflammatory activity, previously associated with lowering TAG levels in the liver, which might be the result of increased hepatic FA β-oxidation [73,74]. However, since the long-term treatment of diet-induced obese mice with astaxanthin administered at a dose four times higher than that used in our experiment did not affect the expression of genes related to FA oxidation in the liver [74], it is unlikely that astaxanthin was responsible for stimulating intestinal mitochondrial FA β-oxidation in our present study. It has been shown that high-fat feeding predominantly up-regulated the activity of FA

β-oxidation enzymes in obesity-resistant A/J as compared to obesity-prone C57BL/6J mice, suggesting that the inability to catabolize lipids in the small intestine is associated with the development of diet-induced obesity [75]. In our study, krill oil administration stimulated palmitate oxidation in only one of the five small intestine segments analyzed (i.e., proximal ileum). Despite previous reports showing that constitutive up-regulation of FA β-oxidation throughout the intestine but not selectively in the proximal ileum improved whole-body glucose homeostasis [16], we observed improved glucose tolerance only in mice fed the ω3PL-H diet. Thus, it is more likely that segment-specific stimulation of FA oxidation is closely related to the regulation of intestinal TAG transport, as observed after activation of PPARα in the small intestine by fenofibrate [76]. For this reason, we consider the stimulation of FA oxidation in the small intestine together with changes in the absorption, transport and metabolism of FA to be crucial for the antisteatotic effect of krill oil. Based on available evidence from various transgenic mouse models (reviewed in [77]), the PPAR family of nuclear receptors, as well as histone deacetylases, are key regulators of genes for lipid oxidation in enterocytes, and their genetic manipulation has led to reduced weight gain, hepatic steatosis and serum TAG levels, and improved glucose tolerance. Although it is not clear whether similar changes can be achieved in humans, targeting intestinal metabolism by dietary interventions based on the administration of Omega-3, especially those involving krill oil, remains a promising approach to the treatment of metabolic disorders associated with obesity and metabolic syndrome.

5. Conclusions

By using gene expression profiling in the small intestine, we provide evidence that Omega-3 supplemented as PLs from krill oil are more effective than Omega-3 administered at the same dose as re-esterified TAG in terms of effects on intestinal gene expression, while also enhancing mitochondrial β-oxidation of FAs. Despite the fact that both forms of Omega-3 supplementation affected pathways involved in ω-oxidation, krill oil supplementation had more pronounced effects on gene expression and its effect was much broader in terms of the number of pathways affected. This effect of krill oil was linked to improved bioavailability of Omega-3, primarily EPA, and may be responsible for superior metabolic effects of marine PLs, especially when the reduction of hepatic steatosis and improved glucose tolerance are concerned. Thus, our results point to the small intestine and gut-mesenteric WAT-liver interaction as one of the major targets of krill oil administration.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/12/7/2037/s1, Table S1: Macronutrient composition and energy content in the experimental diets, Table S2: Primers used for RT–qPCR, Table S3: Distribution of fatty acids in phospholipid fraction of red blood cells, Table S4: Comparison of quantified mRNA expressions obtained using microarray analysis in the whole length of small intestine and qPCR in specific segments of small intestine, Figure S1: Potential involvement of Omega-3 bioavailability in metabolic effects of Omega-3. Figure S2. The effect of HFD feeding on the small intestine.

Author Contributions: O.H., P.K., J.K. (Jan Kopecky) and M.R. made substantial contributions to conception and design. P.K. and O.H. performed the experiments. E.M.v.S., J.K. (Jaap Keijer), A.B. and P.K. performed microarray analysis. P.Z. and M.O. performed lipidomic analysis. M.V. and I.I. performed histological analysis. P.K. and O.H. made substantial contributions to analysis and interpretation of data. P.K., E.M.v.S., J.K. (Jaap Keijer), M.R. and O.H. wrote the manuscript. M.R. provided conceptual advice and supervised the manuscript. All authors have approved the final version of the article. All authors have read and agreed to the published version of the manuscript.

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Article IV

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Krill Oil Supplementation Reduces Exacerbated Hepatic Steatosis Induced by Thermoneutral Housing in Mice with Diet-Induced Obesity

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Abstract: Preclinical evidence suggests that n-3 fatty acids EPA and DHA (Omega-3) supplemented as phospholipids (PLs) may be more effective than triacylglycerols (TAGs) in reducing hepatic steatosis. To further test the ability of Omega-3 PLs to alleviate liver steatosis, we used a model of exacerbated non-alcoholic fatty liver disease based on high-fat feeding at thermoneutral temperature. Male C57BL/6N mice were fed for 24 weeks a lard-based diet given either alone (LHF) or supplemented with Omega-3 (30 mg/g diet) as PLs (krill oil; ω3PL) or TAGs (Epax 3000TG concentrate; ω3TG), which had a similar total content of EPA and DHA and their ratio. Substantial levels of TAG accumulation (~250 mg/g) but relatively low inflammation/fibrosis levels were achieved in the livers of control LHF mice. Liver steatosis was reduced by >40% in the ω3PL but not ω3TG group, and plasma ALT levels were markedly reduced (by 68%) in ω3PL mice as well. Krill oil administration also improved hepatic insulin sensitivity, and its effects were associated with high plasma adiponectin levels (150% of LHF mice) along with superior bioavailability of EPA, increased content of alkaloids stachydrine and trigonelline, suppression of lipogenic gene expression, and decreased diacylglycerol levels in the liver. This study reveals that in addition to Omega-3 PLs, other constituents of krill oil, such as alkaloids, may contribute to its strong antisteatotic effects in the liver.

Keywords: NAFLD; obesity; omega-3; krill oil; phospholipids; high-fat diet; C57BL/6N mice; thermoneutral temperature

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1. Introduction

Obesity is frequently associated with non-alcoholic fatty liver disease (NAFLD), a spectrum of conditions ranging from increased intrahepatic accumulation of triacylglycerols (TAGs; i.e., fatty liver or hepatic steatosis) to steatohepatitis (NASH) and end-stage liver disease [1]. Prevalence of hepatic steatosis and NASH in extremely obese subjects may reach up to 85% and 40%, respectively [2,3], while the presence of metabolic syndrome is associated with a potentially progressive, severe liver disease [4,5]. NAFLD is a serious public health problem [6] for which currently no approved drug therapy exists [7].

Dietary fatty acids (FAs) can differentially affect the body's ability to store lipids in certain fat depots as well as in extra-adipose tissues [8]. In humans, overeating saturated FAs (SFAs) promoted hepatic and visceral fat storage [9–11]. Differential effects of various types of FAs are also observed in the case of regulation of inflammatory responses; thus SFAs and polyunsaturated FAs (PUFAs) of n-6 series are more pro-inflammatory, while PUFAs of n-3 series (omega-3 PUFAs) such as docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3) exert anti-inflammatory and hypolipidemic effects ([12–14], and reviewed in [15–17]). At the same time, omega-3 PUFA supplementation may reduce de novo lipogenesis (DNL) and increase FA oxidation in the liver [18], with the transcription factor peroxisome proliferator-activated receptor (PPAR)α playing a crucial role in the latter effect [19]. For these reasons, omega-3 PUFA supplements could be effective in preventing and treating NAFLD [20]. Indeed, in NAFLD patients treated with EPA and DHA as ethyl esters, a decrease in the percentage of liver fat was linearly correlated with the amount of omega-3 PUFAs taken [21]; however, no improvement in markers of liver function/injury or the fibrosis scores was detected. Similar results were obtained in subjects with non-cirrhotic NASH treated with omega-3 PUFAs as TAGs [22], in which a decrease in liver fat but no improvements in histological activity were observed. In general, omega-3 PUFAs administered in the form of TAGs or ethyl esters have been shown to partially limit hepatic steatosis in some studies [23].

Omega-3 PUFAs also alleviated hepatic steatosis in various rodent models of obesity (e.g., [14,24–26], and reviewed in [27]). Interestingly, the efficacy of omega-3 PUFAs may depend on the lipid form of their supplementation. For instance, compared to their TAG form, omega-3 PUFAs administered via phosphatidylcholine-rich phospholipids (PLs), either in the form of krill oil extracted from the Antarctic krill *Euphausia superba* [28] or as an extract of herring meal [26], had stronger effects in reducing the TAG content in the liver of rodents with genetically- or diet-induced obesity [25,26,29]. Moreover, in mice fed a corn oil-based high-fat diet, a significant reduction in hepatic TAGs was achieved only by administration of marine PLs containing omega-3 PUFAs and not soybean-derived phosphatidylcholine that did not contain EPA or DHA [30]. The higher efficacy of omega-3 PUFA-containing PLs in reducing hepatic steatosis could be related to the improved bioavailability of omega-3 PUFAs, in particular EPA and docosapentaenoic acid (22:5n-3), both in plasma and in target organs ([26,29,31] and reviewed in [32]). At the same time, supplementation of omega-3 PUFAs as PLs led to a stronger downregulation of liver gene expression in the DNL pathway [29,33,34] and significantly reduced activities of the corresponding lipogenic enzymes as well as of the mitochondrial citrate carrier [35]. However, despite its strong effects on TAG accumulation in the liver, it is not clear whether administration of omega-3 PUFAs in the form of PLs is able to affect advanced stages of NAFLD such as NASH and fibrosis, which remain unaffected in response to more traditional forms of omega-3 PUFAs such as TAGs or ethyl esters (see above).

Recently, a mouse model of obesity-associated exacerbated NAFLD based on the administration of a lard-based high-fat diet in a thermoneutral environment was introduced [36]. This experimental model is characterized by lower stress-driven production of corticosterone, augmented mouse pro-inflammatory immune responses and markedly exacerbated high-fat diet-induced NAFLD pathogenesis, which should recapitulate the severe end of the disease spectrum in humans. Thus, in the present study, the above experimental conditions were used to examine whether omega-3 PUFAs supplemented as PLs via krill oil could beneficially affect NAFLD-related phenotypes and hepatic insulin sensitivity, and what is the potential mechanism of action. At the same time, for comparison, other mice were administered omega-3 PUFAs in the form of a TAG-based concentrate, which was similar to krill oil in terms of the amount and ratio of EPA and DHA, thus representing the group receiving omega-3 PUFAs in one of the traditional lipid forms used for this purpose.

2. Materials and Methods

2.1. Animals and Diets

Male C57BL/6N mice (Charles River Laboratories, Sulzfeld, Germany) were obtained at the age of ~10 weeks. After arrival, mice were individually housed in cages and maintained at ~22 °C on a 12-h light/dark cycle (light from 6:00 a.m.), with ad libitum access to water and a standard diet (Chow; ~14 kJ/g; fat content ~3.6% (*w/w*); Rat/Mouse-Maintenance extrudate; ssniff Spezialdiäten GmbH, Soest, Germany). After one week of adaptation, the animals were transferred to a thermoneutral environment (~30 °C) and fed the following experimental diets: (i) a lard-based high-fat diet (LHF diet; ~21 kJ/g; fat content ~35% (*w/w*); product "DIO-60 kJ% fat (Lard)", Cat. No. E15742-34; ssniff Spezialdiäten GmbH, Soest, Germany), (ii) a LHF-based diet supplemented with omega-3 PUFA-containing PLs (ω3PL diet), using krill oil (Rimfrost Sublime; EPA ~13%, DHA ~8%; Rimfrost AS, Ålesund, Norway), and (iii) a LHF-based diet supplemented with omega-3 PUFAs in the form of re-esterified TAGs (ω3TG diet), using the product Epax 3000 TG (EPA ~18%, DHA ~11%; Epax Norway AS, Ålesund, Norway). Experimental diets were prepared at the ssniff facility in Germany. The total content of EPA and DHA in both supplemented diets was ~30 mg/g diet. For details on macronutrient and FA composition of the experimental diets, see Supplementary Materials Tables S1 and S2, respectively.

2.2. Experimental Setup

The experimental setup is shown in Figure 1. After moving to a thermoneutral environment, the mice were divided into four groups with the same average weight ($n = 8$; see Figure 1A). One group was fed the LHF diet for 24 weeks, as was the group fed the ω3PL diet from the beginning of the experiment (i.e., "preventive" approach). However, the other two groups first received the LHF diet for 8 weeks, and only then was this diet replaced with omega-3 PUFA-supplemented diets (ω3TG or ω3PL) administered for the remaining 16 weeks (i.e., "reverse" approach; marked with the letter "R" at the end of the group name). The total duration of all dietary interventions was therefore 24 weeks. Chow-fed mice served as lean controls. Body weight was recorded weekly and a fresh ration of diet was administered every other day. The calculation of cumulative energy intake was based on weekly measurements of food consumption over 24 h. Fasting plasma insulin and blood glucose levels were measured at week 21. Mice were killed by cervical dislocation under diethyl ether anesthesia between 9:00 a.m. and 11:00 a.m. Truncal blood was collected into tubes containing EDTA for plasma isolation, liver and white adipose tissue (WAT) samples from the epididymal, mesenteric and subcutaneous (dorso-lumbar) fat depots were dissected, weighed, and adiposity index was calculated as the sum of the weights of all analyzed WAT depots divided by body weight. Liver and epididymal WAT samples were snap-frozen in liquid nitrogen and stored at −80 °C for subsequent analyses, while one aliquot of tissue was used for histological evaluation. In a separate study using mice from the Chow, LHF and ω3PL groups (see Figure 1B), pyruvate tolerance, hepatic production of TAGs contained in very low-density lipoproteins (VLDL) and insulin sensitivity were determined at weeks 21, 23, and 24, respectively. Animal experiments were approved by the Institutional Animal Care and Use Committee and the Committee for Animal Protection of the Ministry of Agriculture of the Czech Republic (Approval Number: 81/2016).

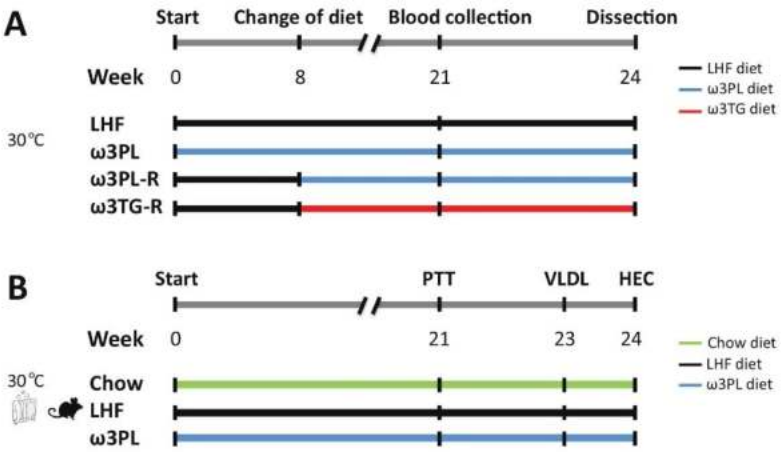

Figure 1. Overview of the experimental setup. (**A**) Four groups of mice (n = 8) housed in a thermoneutral environment (~30 °C) were used: (i) the control LHF group, which was fed a lard-based high-fat diet (i.e., LHF diet) for 24 weeks; (ii) ω3PL group fed a LHF-based diet supplemented with omega-3 PUFAs as PLs in the form of krill oil (i.e., ω3PL diet) for the duration of the experiment (i.e., "preventive" approach); (iii) ω3PL-R group fed the LHF diet for the first eight weeks and then from the ninth week on the ω3PL diet until the end of the experiment (i.e., "reverse" approach; marked with the letter "R" at the end of the group name); and (iv) ω3TG-R group fed the LHF diet for the first 8 weeks and then from the ninth week on the LHF-based diet supplemented with omega-3 PUFAs in the form of a concentrate of re-esterified TAGs (i.e., ω3TG diet) until the end of the experiment. (**B**) Three groups of mice (n = 8) housed in a thermoneutral environment (~30 °C) were used: (i) Chow group, in which mice were fed a standard low-fat diet and served as lean controls; (ii) the control LHF group, which was fed a lard-based high-fat diet (i.e., LHF diet) for 24 weeks; and (iii) ω3PL group fed the ω3PL diet for the duration of the experiment. Further details in Section 2.2. PTT, pyruvate tolerance test; VLDL, liver VLDL-TAGs secretion test; HEC, hyperinsulinemic-euglycemic clamp.

2.3. Pyruvate Tolerance Test

The level of gluconeogenesis was estimated using pyruvate tolerance test. Mice fasted overnight (~14 h) were injected i.p. with pyruvate (1.5 mg/g body weight) and blood glucose levels were measured using glucometers Contour Plus (Bayer, Leverkusen, Germany) at time 0 (i.e., before injection), and then 15, 30, 60, 120, and 180 min after injection. The response to pyruvate administration was quantified as area under the glucose curve (AUC).

2.4. Light Microscopy and Immunohistochemical Analysis

Liver and epididymal WAT samples were fixed in 4% formaldehyde, embedded in paraffin, and sections of 5 µm thickness were stained using hematoxylin-eosin. The NAFLD histological scoring system [37] was used to assess the effect of omega-3 PUFAs administration on NAFLD progression. In epididymal WAT, macrophage marker MAC-2/galectin-3 was detected using specific antibodies (Cedarlane Laboratories; Burlington, NC, USA; 1:4000 dilution) and the number of crown-like structures (CLS) counted as before [38]. Morphometric analysis of WAT was performed using the imaging software NIS-Elements AR3.0 (Laboratory Imaging, Prague, Czech Republic).

2.5. Hepatic Production of VLDL-TAGs

The procedure was the same as before [39]. After an overnight fast (~16 h), mice were injected i.p. with a solution of 15% Tyloxapol (Triton WR-1339; Sigma-Aldrich; Prague, Czech Republic; dissolved in 0.9% saline) at a dose of 500 mg per kg body weight and blood

was collected from the tail vein under basal conditions and 2, 4, and 6 h after Tyloxapol injection. Plasma TAG concentrations were measured at 500 nm using the Triglycerides kit from Erba Lachema (Brno, Czech Republic) and the Sunrise microplate reader (Tecan Group, Männedorf, Switzerland).

2.6. Insulin Sensitivity Measured by Hyperinsulinemic-Euglycemic Clamp

Hyperinsulinemic-euglycemic clamp was performed in awake mice as before [14,29]. Briefly, a week before the end of the study, a permanent catheter was inserted into the *v. jugularis*. After a postoperative period of 4–7 days, mice were fasted for 6 h (6:00 a.m.–12:00 p.m.) and then infused with insulin Actrapid (Novo Nordisk) and D-[3-^{3}H]glucose (Perkin Elmer, Boston, MA, USA) at a constant rate of 4.8 mU/min per kg body weight and 0.26 µCi/min, respectively. Euglycemia (~5.5 mmol/L) was maintained by periodical adjusting the variable infusion of glucose solution (30% for lean animals, 15% for obese animals), while blood glucose levels were regularly monitored using glucometers (see Section 2.3). Blood samples taken every 10 min during the last hour of the 3-h infusion period were used to analyze specific D-[3-^{3}H]-glucose activity.

2.7. Metabolites and Hormones

Plasma levels of lipid metabolites (i.e., TAGs, total cholesterol, non-esterified fatty acids), as well as aspartate transaminase (AST) and alanine aminotransferase (ALT), were measured using the appropriate assays from Roche or Wako (for the measurement of non-esterified fatty acids) and a Clinical Chemistry analyzer Roche/Hitachi 902 (Roche Diagnostics; Basel, Switzerland). Plasma levels of insulin were quantified using xMAP technology and MILLIPLEX MAP Mouse Metabolic Hormone Magnetic Bead Panel (MMHMAG-44K; Merck-Millipore; Burlington, MA, USA). Plasma levels of total adiponectin were measured by Mouse Adiponectin ELISA kit (EZMADP-60K; Sigma-Aldrich). Fasting plasma insulin and blood glucose levels were used to quantify Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), using the following formula: fasting plasma insulin (mU/L) × fasting plasma glucose (mmol/L)/22.5.

2.8. TAG Content in the Liver

Approximately 50 mg of tissue was dissolved in 150 µL 3M KOH (dissolved in 65% ethanol) at 70 °C for 2 h. The resulting homogenate was diluted 10× in redistilled water and the TAG content was measured (see Section 2.5) and the results were related to tissue weight.

2.9. Gene Expression Analysis

Gene expression was analyzed in the liver (stored in RNA later; Ambion, Austin, TX, USA) by real-time quantitative PCR as before [29,40]. Transcript levels were normalized to the expression level of a housekeeping gene for 18S ribosomal RNA (*Rn18s*). Gene names and sequences of the oligonucleotide primers are listed in Table S3.

2.10. Composition of FAs in Experimental Diets and Liver

Total lipids were extracted from aliquots of experimental diets (100 mg) by two-step extraction using hexane and a mixture of methanol and dichloromethane (see Supplementary Materials for details). The methyl tert-butyl ether (MTBE)-based extraction of total lipids from the liver (50 mg) was performed as before [31], and neutral and polar lipid fractions were obtained by using SPE Columns (Discovery). Trans-esterification of extracted lipids, FAs methyl esters (FAME) extraction and their analysis using comprehensive two-dimensional gas chromatography with mass detection (Pegasus 4D, LECO, USA) was performed as before [41].

2.11. LC-MS Analysis of Liver Samples

Metabolomic and lipidomic profiling of liver samples was conducted using a combined targeted and untargeted workflow for the lipidome, metabolome, and exposome analysis (LIMeX) [42,43]. Extraction was carried out using a biphasic solvent system of cold methanol, MTBE, and 10% methanol. Four different LC-MS platforms were used for metabolomic and lipidomic profiling: (i) lipidomics of complex lipids in positive ion mode, (ii) lipidomics of complex lipids in negative ion mode, (iii) metabolomics of polar metabolites in positive ion mode, and (iv) metabolomics of polar metabolites in negative ion mode. Details of sample preparation, LC-MS conditions, raw data processing and curation, and list of annotated complex lipids and polar metabolites are in Supplementary Materials.

2.12. Data Processing and Statistics

Results are means ± SEM. To compare the groups fed experimental LHF-based diets, One Way ANOVA (for normally distributed data sets) or Kruskal–Wallis test (non-normally distributed data sets) followed by Student–Newman–Keuls post-hoc test was used (SigmaStat 3.5 software; Systat Software Inc., San Jose, CA, USA). Differences were considered significant when $p \leq 0.05$. Pearson's correlation coefficient (r) was calculated to measure the strength of the association between the two variables. Multivariate analysis was performed using partial least-squares discriminant analysis (PLS-DA) using MetaboAnalyst 4.0 [44]. Statistical models were created for metabolomic and lipidomic sets separately after logarithmic transformation (base 10) and Pareto scaling. Exported variable importance in projection (VIP) scores were used for evaluation.

The number of animals required to evaluate the effect of krill oil supplementation on liver steatosis was based on a previous publication [29], namely the difference in liver TAGs between control mice fed a corn oil-based high-fat diet (i.e., cHF diet) and mice fed a cHF-based diet containing krill oil (i.e., the ω3PL-H diet), supplemented at the same dose as in the current study. Thus, the minimal sample size of 6 animals per group was calculated using G*Power software (power 0.95, $\alpha = 0.05$; see [45]).

3. Results

3.1. Basic Parameters of Energy Balance, Adiposity, as well as Lipid and Glucose Homeostasis

Table 1 and Figure 2 show the general characteristics of obese mice from the control group (LHF) and the three intervention groups (ω3PL, ω3PL-R and ω3TG-R; description of groups—see Section 2.2 and Figure 1) that differed in the lipid form and/or timing of omega-3 PUFA administration. In the control LHF group, high-fat feeding for 24 weeks caused a weight gain of ~30 g, which was reduced by 15% in the ω3PL group supplemented with krill oil since the start of the study (Table 1); this reduction in weight gain was mainly due to weight loss during the second half of the study (Figure 2A). Although no changes in average daily food intake (Figure 2B) and cumulative energy intake (Table 1) were observed between the groups, the average feeding efficiency, calculated by dividing the weight gain by the amount of energy consumed each week, was significantly reduced in ω3PL mice (Figure 2C). The adiposity index did not change in response to omega-3 PUFA administration (Table 1), but specifically in the ω3PL group, mesenteric WAT weight was reduced by ~27% (a similar trend was also observed in ω3PL-R mice), while the weight of epididymal WAT was increased in the ω3PL and ω3PL-R groups (Table 1). Despite the increased weight of epididymal WAT in both krill oil-supplemented groups, the average size of adipocytes in this fat depot remained unchanged (Figure 2D) while tissue accumulation of inflammatory macrophages, analyzed by MAC-2/galectin-3 immunodetection (see Supplementary Figure S1) and assessed by CLS counting, was reduced (Figure 2E); no such changes were observed in the ω3TG-R group. With regard to lipid metabolism markers in the circulation, omega-3 PUFAs reduced total cholesterol levels, regardless of the lipid form of their supplementation (Table 1). Given the role of obesity and WAT inflammation in impaired glucose metabolism, we next evaluated the effect of omega-3 PUFAs on glucose homeostasis; it was improved specifically in krill oil-supplemented

mice (i.e., ω3PL and ω3PL-R), as evidenced by lower FBG and non-fasting plasma insulin (Table 1). Furthermore, these mice also showed stronger induction of plasma adiponectin levels (Figure 2F) in association with significantly reduced HOMA-IR (Figure 2G).

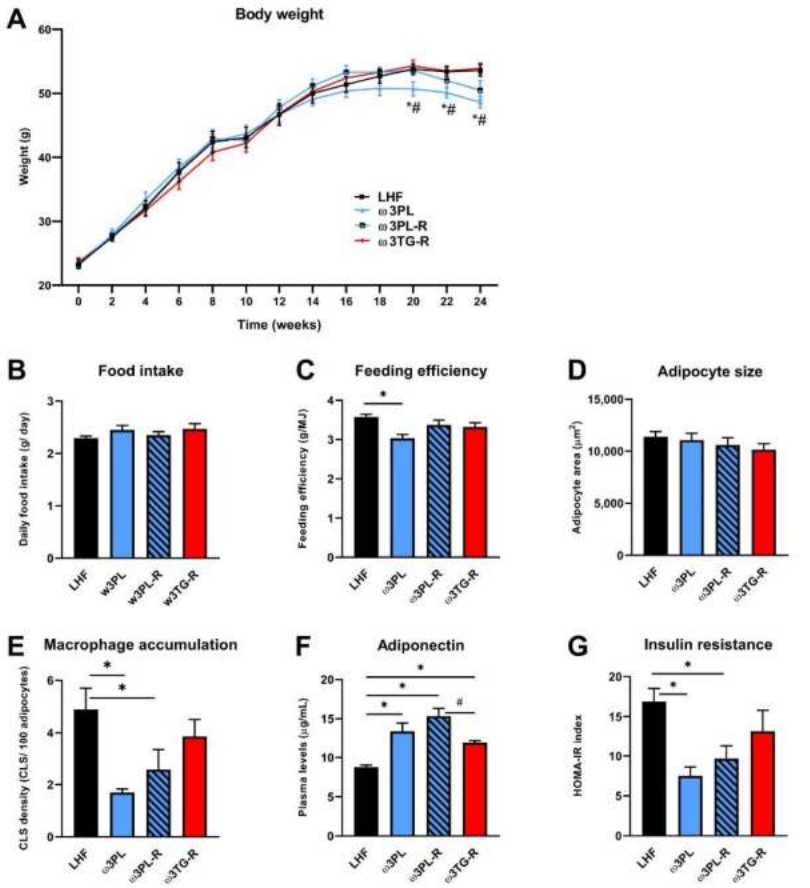

Figure 2. The effect of omega-3 PUFA supplementation on parameters related to energy balance, adipose tissue health and insulin sensitivity: changes in body weight during the study (**A**), average daily food intake (**B**), feeding efficiency (**C**), average size of adipocytes (**D**) and macrophage accumulation in epididymal WAT (**E**), plasma adiponectin levels (**F**), and insulin resistance based on the HOMA-IR index (**G**). Data are means ± SEM (n = 7–8). *, significant effect of omega-3 PUFAs (vs. LHF); #, significant difference from ω3TG-R (One Way ANOVA or Kruskal–Wallis).

Table 1. Energy balance, adiposity, and biochemical parameters in mice fed high-fat diets supplemented or not with omega-3 PUFA concentrates.

	LHF	ω3PL	ω3PL-R	ω3TG-R
Body weight (g)				
Week 0	23.3 ± 0.7	23.3 ± 0.6	23.1 ± 0.5	23.6 ± 0.7
Week 24	53.6 ± 0.9	48.6 ± 0.9 [a]	50.5 ± 1.5	53.9 ± 0.8 [b]
Gain	30.3 ± 0.8	25.3 ± 1.3 [a]	27.4 ± 1.6 [b]	30.2 ± 0.4 [b]
En. intake (MJ/mouse/study)	8.1 ± 0.1	8.1 ± 0.3	7.9 ± 0.3	7.7 ± 0.3
WAT depots (g)				
Epididymal WAT	2.11 ± 0.06	2.54 ± 0.15 [a]	2.52 ± 0.12 [a]	2.07 ± 0.07 [b,c]
Subcutaneous WAT	1.59 ± 0.06	1.45 ± 0.06	1.55 ± 0.06	1.75 ± 0.10
Mesenteric WAT	1.50 ± 0.07	1.09 ± 0.06 [a]	1.36 ± 0.11 [b]	1.48 ± 0.07 [b]
Adiposity index (%)	9.7 ± 0.2	10.5 ± 0.4	10.7 ± 0.4	9.8 ± 0.3
Clinical biochemistry				
TAGs (mmol/L)	1.14 ± 0.12	0.91 ± 0.05	1.02 ± 0.11	0.97 ± 0.04
NEFA (mmol/L)	0.61 ± 0.05	0.57 ± 0.05	0.60 ± 0.07	0.64 ± 0.06
Cholesterol (mmol/L)	6.22 ± 0.18	5.10 ± 0.30 [a]	5.49 ± 0.14 [a]	5.87 ± 0.14 [b]
FBG (mmol/L)	5.19 ± 0.12	4.33 ± 0.09 [a]	4.46 ± 0.21 [a]	5.00 ± 0.20 [b,c]
Insulin (ng/mL)	4.80 ± 0.60	2.65 ± 0.29 [a]	3.92 ± 0.64	5.74 ± 0.66 [b]

Data are means ± SEM (n = 7–8). Except FBG, measured in overnight fasted mice, biochemical parameters were determined in plasma of mice fed ad libitum. [a,b,c] different from LHF, ω3PL, ω3PL-R, respectively (one-way ANOVA or Kruskal–Wallis). FBG, fasting blood glucose; NEFA, non-esterified fatty acids.

Thus, in obese mice kept in a thermoneutral environment, administration of krill oil, unlike omega-3 PUFAs supplemented in the TAG form, led to redistribution of WAT and improvement of its function, which corresponded to positive effects on glucose homeostasis.

3.2. Histological Analysis of NAFLD-Related Phenotypes

We further examined the effect of krill oil and omega-3 PUFAs supplemented as TAGs on the development of NAFLD using biochemical and histological analyses of the liver (Figures 3 and 4).

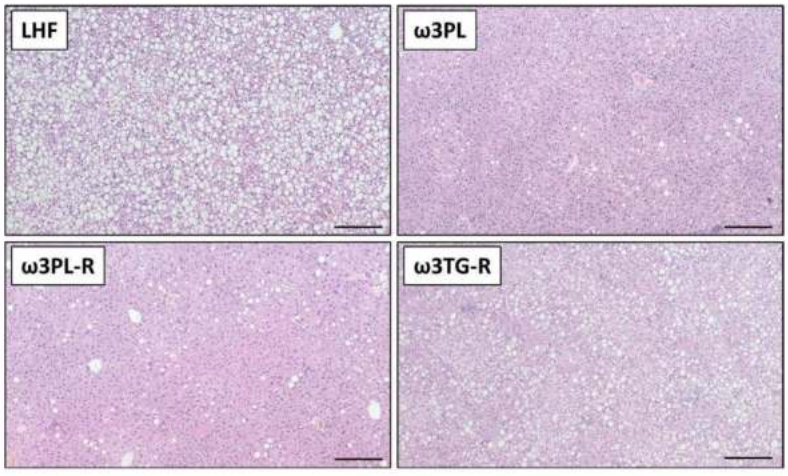

Figure 3. Representative histological sections of liver stained with hematoxylin and eosin. Bars = 200 μm.

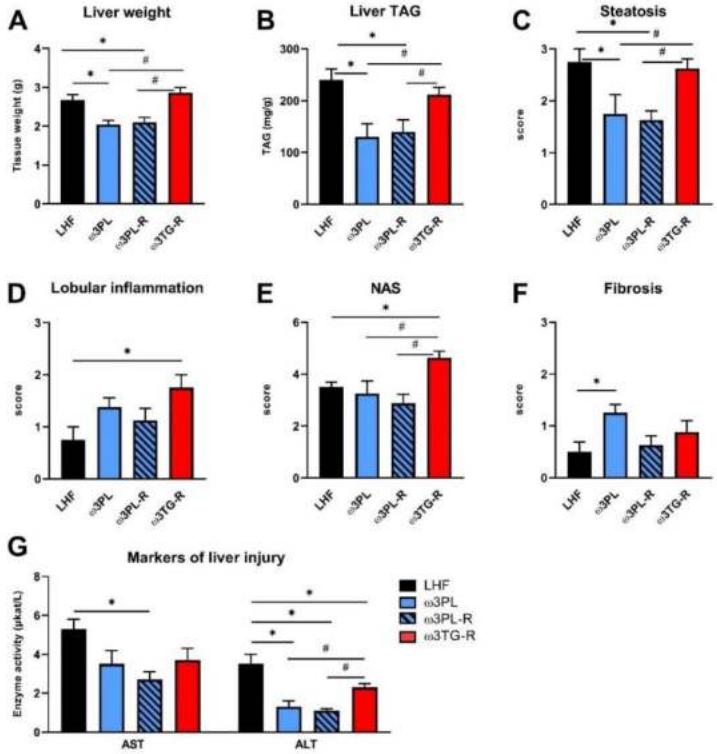

Figure 4. The effect of omega-3 PUFA supplementation on NAFLD-related parameters: liver weight (**A**), liver TAG content (**B**), steatosis (**C**), lobular inflammation (**D**), NAFLD activity score—NAS (**E**), fibrosis (**F**), and plasma AST and ALT levels (**G**). The results presented in panels C–F are based on histological analysis of liver sections. Data are means ± SEM (n = 7–8). *, significant effect of omega-3 PUFAs (vs. LHF); #, significant difference from ω3TG-R (one-way ANOVA or Kruskal–Wallis).

Despite having similar plasma TAG levels (Table 1), liver weight (Figure 4A) and the TAG content in the liver (Figure 4B; quantified biochemically) were reduced in both experimental groups that received krill oil compared either to LHF controls or ω3TG-R mice supplemented with omega-3 PUFAs as TAGs. Histological analysis of hematoxylin-eosin-stained liver sections (Figure 3) confirmed reduced levels of steatosis in the livers of ω3PL and ω3PL-R mice compared to the LHF and ω3TG-R groups, where the steatosis score reached almost the maximum value of 3 (Figure 4C). In addition to the degree of steatosis, histological analysis was also used to assess other components of the NAFLD activity score (NAS), which includes lobular inflammation and hepatocyte ballooning. Lobular inflammation was increased in ω3TG-R mice and unchanged in krill oil-supplemented mice compared to LHF-fed controls (Figure 4D), while hepatocyte ballooning was relatively less frequent (score < 0.5) and was similar among the groups (not shown). As a result, the NAS score was higher in ω3TG-R mice and unchanged in ω3PL and ω3PL-R animals compared to LHF-fed controls (Figure 4E). We also evaluated the degree of fibrotic changes in the liver, which was generally relatively low in all groups (Figure 4F); however, ω3PL mice supplemented with krill oil during the development of obesity showed significantly higher fibrosis score compared to control animals fed LHF (Figure 4F). On the other hand, plasma levels of transaminases, especially ALT, were reduced due to krill oil supplementation, with a weaker effect observed in mice receiving omega-3 PUFAs as TAGs (Figure 4G).

These data primarily document the excellent efficacy of krill oil in reducing severe hepatic steatosis induced by administration of a high-fat diet in a thermoneutral environment.

3.3. Analysis of Parameters Related to the Efficacy of Omega-3 PUFAs in the Liver

Next, we analyzed some parameters that may determine the metabolic effects of krill oil in the liver (Figure 5). First, the bioavailability of FAs such as arachidonic acid (ARA), EPA, and DHA, which are substrates for the formation of biologically active lipid mediators, was measured in the neutral (i.e., mainly TAGs) and polar (i.e., mainly PLs) lipid fractions of the liver (Figure 5A,B; for complete data on FAs composition in hepatic TAGs and PLs, see Tables S4 and S5, respectively).

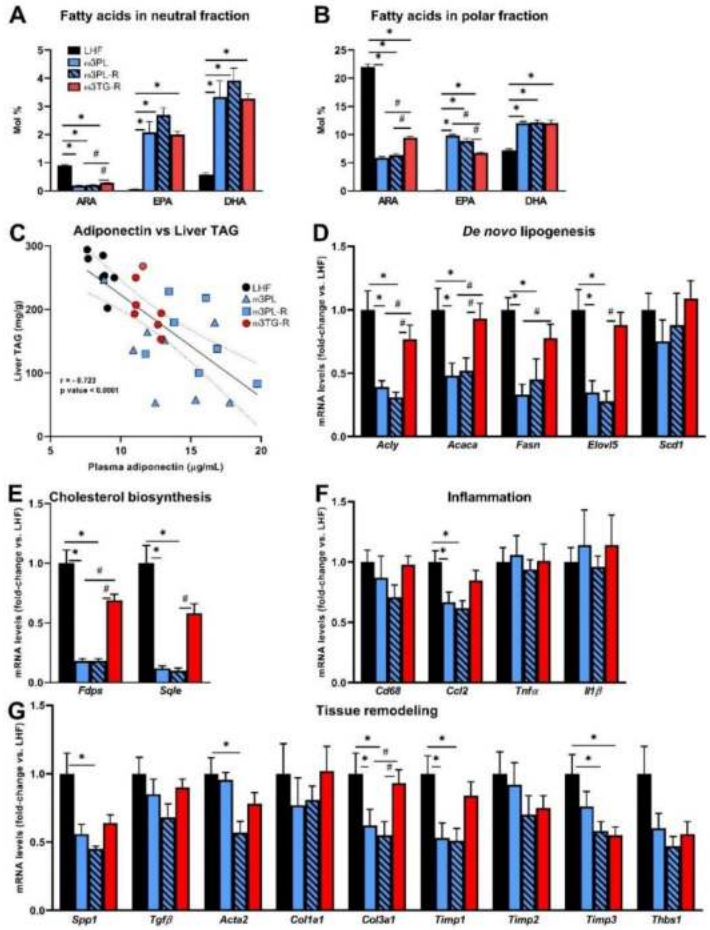

Figure 5. Selected parameters determining the effects of omega-3 PUFAs in the liver: bioavailability of FAs such as arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) measured in the neutral (**A**) and polar (**B**) fraction of liver lipids; correlation between plasma adiponectin levels and the degree of TAG accumulation in the liver (**C**); hepatic expression of genes related to DNL (**D**), cholesterol biosynthesis (**E**), inflammation (**F**), and tissue remodeling (**G**). Data are means ± SEM (n = 7–8). *, significant effect of omega-3 PUFAs (vs. LHF); #, significant difference from ω3TG-R (one-way ANOVA or Kruskal–Wallis).

In general, the relative content of ARA was reduced while the content of EPA and DHA was increased in response to administration of both krill oil and omega-3 PUFAs as TAGs; however, the ARA reduction efficacy in both the neutral and polar fractions was significantly higher in the krill oil-supplemented groups (Figure 5A,B), and the same situation was observed in the case of an increase in EPA primarily in the polar fraction (Figure 5B). We also examined the relationship between plasma adiponectin levels and the degree of TAG accumulation in the liver (Figure 5C), as adiponectin can activate 5′-AMP-activated protein kinase (AMPK) and PPARα and thus stimulate lipid catabolism. Indeed, in the livers of mice fed various LHF-based diets, plasma adiponectin levels and liver TAG levels showed a strong negative correlation ($r = -0.723$; $p < 0.0001$; Figure 5C).

Furthermore, the above findings regarding NAFLD-related phenotypes were then related to changes in gene expression of key enzymes of lipid and cholesterol metabolism, as well as markers of inflammation and tissue remodeling with a known relationship to the development of NAFLD/NASH (Figure 5D–G). Expression of the genes related to DNL (Figure 5D) and cholesterol biosynthesis (Figure 5E) was reduced up to 10-fold in krill oil-supplemented mice (ω3PL and ω3PL-R mice) compared to LHF-fed controls. In contrast, no such changes were observed in ω3TG-R mice receiving omega-3 PUFAs as TAGs (Figure 5D,E). Although histological analysis did not reveal a significant degree of NASH/fibrosis induced in C57BL/6N mice fed the LHF diet in a thermoneutral environment (Figure 4D,F), the expression of both inflammatory genes and tissue remodeling genes in the liver was analyzed in order to see whether supplementation with krill oil or omega-3 PUFAs as TAGs could affect these processes at this level. There were no significant differences between groups in the expression of inflammation-related genes (Figure 5F), with the exception of chemokine (C-C motif) ligand 2 (CCL2; also known as monocyte chemoattractant protein 1), whose expression was reduced in groups ω3PL and ω3PL-R. No consistent effects on the expression of genes related to tissue remodeling were observed (Figure 5G); in general, the administration of krill oil reduced the mRNA levels of some of the measured genes (e.g., Spp1, Col3a1, Timp1, Timp3), with occasional effects (e.g., Timp3) also observed in ω3TG-R mice given omega-3 PUFAs as TAGs. Decreased expression of genes involved in DNL may therefore help explain the beneficial effects of krill oil supplementation on liver fat accumulation, while its effect on the expression of inflammatory genes, and in particular tissue remodeling genes, was not conclusive.

3.4. In Vivo Analyses Related to Liver Function and Insulin Sensitivity

Given the significant reduction in TAG levels in the livers of mice supplemented with krill oil (Figure 4B,C) and a likely reduction in insulin resistance (i.e., HOMA-IR; Figure 2G) in these animals, we initiated an additional experiment (see also Figure 1B), in which we performed a series of in vivo functional assays in mice fed either the Chow, LHF or ω3PL diet for 24 weeks at 30 °C (Figure 6). At the end of the study, mice in the Chow, LHF and ω3PL groups weighed 41.3 ± 1.1, 57.6 ± 0.5, and 54.5 ± 0.8 g ($p < 0.01$ vs. LHF for both other groups), which corresponded to a weight gain of 14.5 ± 1.0, 30.7 ± 0.4, and 27.5 ± 0.6 g ($p < 0.0001$ vs. LHF), respectively. Thus, the weight gain in LHF and ω3PL mice was similar to that observed in the previous experiment (see Table 1). We first evaluated whether lower levels of hepatic steatosis in ω3PL mice, supplemented with krill oil during the development of obesity, can be explained by changes in hepatic production of TAGs contained in VLDL (Figure 6A,B). However, the VLDL-TAG secretion test in overnight fasted mice did not reveal any significant differences in lipemic curves between the groups, especially LHF and ω3PL mice (Figure 6A), although basal plasma TAG levels were decreased by 15% in ω3PL compared to LHF mice (Figure 6B). We further examined whether the potent effect of krill oil supplementation on hepatic steatosis is associated with changes in gluconeogenesis and/or improved insulin sensitivity. Thus, we evaluated the glycemic response to pyruvate injection to determine gluconeogenic activity; glycemic curves (Figure 6C), as well as quantification of the glycemic response based on AUC values (Figure 6D), indicated a reduced level of pyruvate-stimulated gluconeogenesis in both

Chow and ω3PL mice compared to LHF-fed controls. Finally, we used the state-of-the-art hyperinsulinemic-euglycemic clamp technique in combination with a radioactive glucose tracer to analyze whole-body and hepatic insulin sensitivity (Figure 6E,F). Whole-body insulin sensitivity was markedly impaired in obese LHF-fed control animals compared to Chow-fed mice, as documented by changes in glucose infusion rate (GIR; Figure 6F), reflecting the amount of exogenous glucose required to maintain euglycemia during the clamp (i.e., under insulin-stimulated conditions) and which showed a ~2.7-fold reduction in LHF mice.

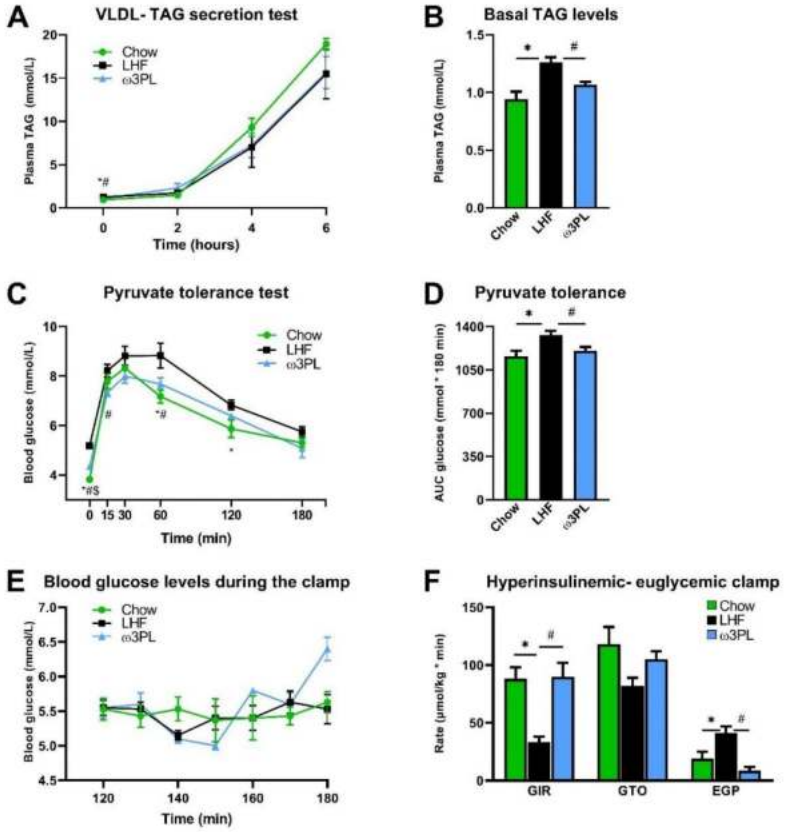

Figure 6. Effect of krill oil supplementation on hepatic VLDL-TAG production (**A**), fasting plasma TAG levels (**B**), glycemia during the pyruvate tolerance test (**C**), the level of pyruvate-driven gluconeogenesis (**D**), as well as glycemia during the last hour of hyperinsulinemic-euglycemic clamp (**E**) and clamp-related parameters including glucose infusion rate (GIR), glucose turnover (GTO) and endogenous glucose production (EGP; **F**). Data are means ± SEM (n = 6–7). *, significant difference between LHF and Chow; #, significant difference between LHF and ω3PL; $, significant difference between ω3PL and Chow (one-way ANOVA or Kruskal–Wallis).

However, the total glucose turnover (GTO) in the organism was not significantly changed in LHF mice compared to the Chow group, primarily because of an ~2.2-fold increase in endogenous glucose production (EGP; Figure 6F). In contrast, krill oil supplementation in ω3PL mice led to normalization of insulin sensitivity at the whole-body level and in the liver, as shown by increased GIR and decreased EGP levels under hy-

perinsulinemic conditions (Figure 6F). Therefore, the above data suggest that the potent antisteatotic effects of krill oil supplementation in the livers of mice with diet-induced obesity and exacerbated hepatic steatosis are associated with improved whole-body and tissue sensitivity to insulin, but cannot be explained by changes in VLDL-TAG secretion.

3.5. Hepatic Metabolome in Relation to Tissue TAG Accumulation and Insulin Sensitivity

To better understand the underlying mechanisms of the potent antisteatotic and insulin-sensitizing effects of krill oil supplementation in mice with exacerbated hepatic steatosis, and to determine how these mechanisms differ in mice given omega-3 PUFAs as TAGs (see also Figure 1A), we analyzed the metabolipidomic profiles of the liver using four different LC-MS platforms (see Section 2.11 for details). First, the annotated data were analyzed using PLS-DA, supervised classification technique, to gain a general view on the impact of omega-3 PUFAs supplementation on both complex lipids and polar metabolites (Figure 7; see Table S7 for a complete list of annotated metabolites).

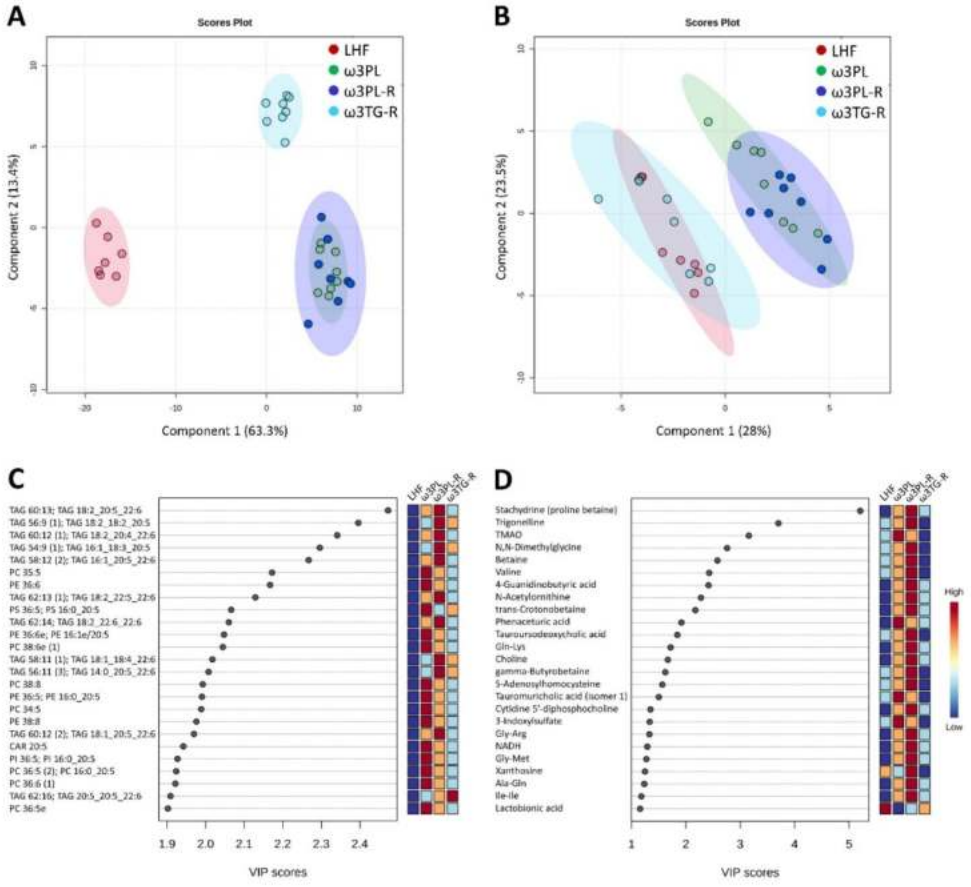

Figure 7. Four-class PLS-DA score plots of complex lipids (**A**; n = 507) and polar metabolites (**B**; n = 157) in the liver in response to dietary challenges, and the most discriminating complex lipids (**C**) and polar metabolites (**D**) based on VIP scores from PLS-DA.

In the case of complex lipids (Figure 7A), the analysis revealed a distinct separation of both krill oil-supplemented mice and mice given omega-3 PUFAs as TAGs from LHF-fed controls within the first Component (C1), which describes 63% of the total variation between these groups. Moreover, both groups fed with krill oil (i.e., ω3PL, ω3PL-R) separated from the ω3TG-R group (omega-3 PUFAs as TAGs) within C2 (Figure 7A). A separate analysis of polar metabolites (Figure 7B) showed a weaker separation of mice fed with krill oil from both LHF controls and mice fed a diet containing omega-3 PUFAs as TAGs within C1, which accounted for 28% of the total variation between the LHF and ω3TG-R groups on the one hand and the ω3PL and ω3PL-R groups on the other. Thus, krill oil supplementation had major effects on both complex lipids and polar metabolites, with omega-3 TAGs affecting mainly the lipidome. Subsequent VIP analysis indicated that several TAG species containing DHA and/or EPA (e.g., TAG 60:13; TAG 18:2_20:5_22:6, TAG 56:9; TAG 18:2_18:2_20:5 or TAG 60:12; TAG 18:2_20:4_22:6) were the most discriminating factors in terms of complex lipids (Figure 7C), while alkaloids stachydrine and trigonelline, as well as trimethylamine N-oxide (TMAO), represented the most discriminating factors among polar metabolites (Figure 7D). In the case of alkaloids, their concentration was significantly increased in the liver of ω3PL and ω3PL-R mice compared to the LHF and ω3TG mice (see left panels in Figure 8A,B), which is due to the increased concentration of these substances in the krill oil-containing diet (right panels in Figure 8A,B).

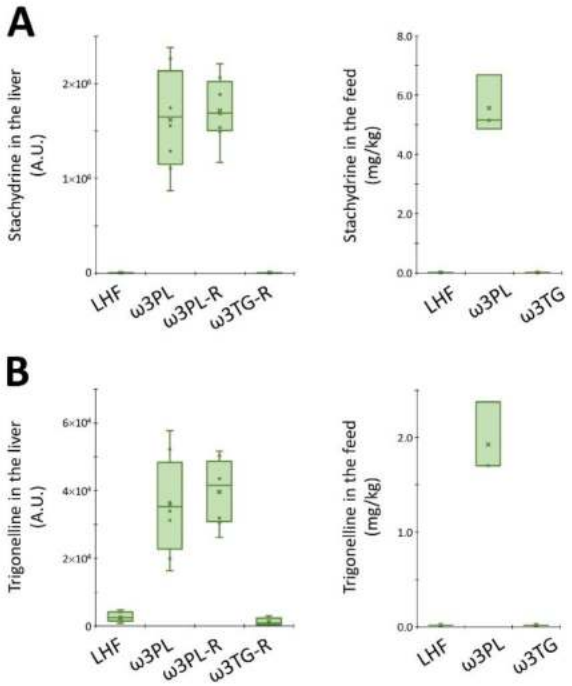

Figure 8. Box plots for stachydrine (**A**) and trigonelline (**B**) levels in the liver (arbitrary units; left panel) and in experimental diets (mg/kg; right panel).

Furthermore, we also performed a subanalysis of certain lipid classes with a known relationship to insulin resistance (e.g., diacylglycerols; DAGs) or DNL (e.g., short/medium-chain TAGs containing 38 to 48 carbons and 0 to 3 double bonds; see Section 4 for details) in the liver (Figure 9). We found that krill oil administration reduced the total DAG levels in liver tissue of ω3PL and ω3PL-R mice (Figure 9A), and in particular the content of

DAGs containing SFA (Figure 9B,C), while supplementation of omega-3 PUFAs as TAGs in the ω3TG-R group was ineffective. In contrast, ω3TG-R mice showed increased levels of DAG species containing primarily DHA (e.g., DAG 44:12; DAG 22:6_22:6), with lower increases observed in the krill oil-supplemented groups (Figure 9D). However, DAG species containing at least 1 SFA represented the majority of all DAGs (20 out of 37), while the levels of SFA-containing DAGs showed a very strong correlation with the total levels of all DAGs (Figure 9E). In addition, total levels of short-chain TAGs were analyzed and found to be reduced in both krill oil-supplemented groups (i.e., ω3PL, ω3PL-R) compared to LHF-fed controls (Figure 9F), while the strongest effect was observed at the level of only SFA-containing TAGs (Figure 9G,H). No such effects were observed in ω3TG-R mice supplemented with omega-3 PUFAs as TAGs.

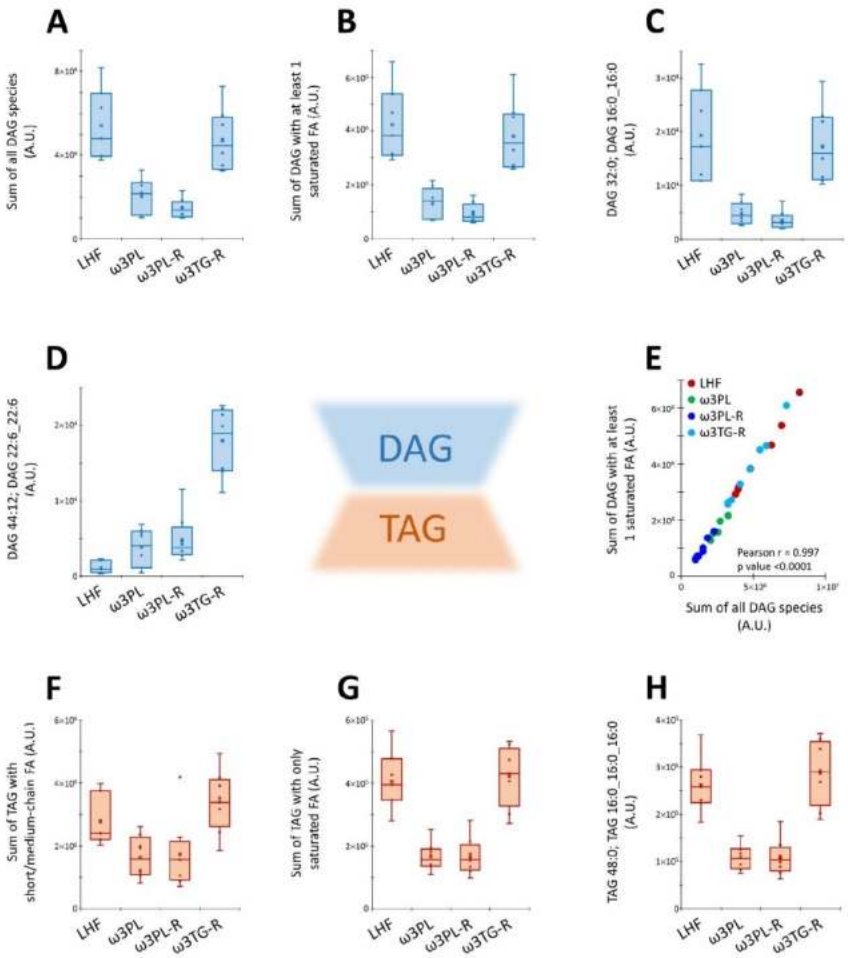

Figure 9. Box plots and correlation analysis for DAGs and TAGs lipid classes in the liver in response to dietary challenges: the sum of all DAG species (**A**; $n = 37$), the sum of DAGs with at least 1 SFA (**B**; $n = 20$), and representative species of DAGs containing either SFA (i.e., DAG 32:0; DAG 16:0_16:0; **C**) or omega-3 PUFAs (i.e., DAG 44:12; DAG 22:6_22:6; **D**). Correlation between the sum of all DAG species ($n = 37$) and the sum of DAGs with at least 1 SFA ($n = 20$; **E**). The sum of TAG species with short/medium-chain FAs (**F**; $n = 9$), the sum of TAG species with only SFAs (**G**; $n = 3$), and a representative SFA-containing TAG species TAG 48:0; TAG 16:0_16:0_16:0 (**H**). Lipid intensities are in arbitrary units (A.U.).

Overall, the above data suggest that krill oil administration has led to profound changes in the levels of complex lipids as well as polar metabolites in the liver, while affecting lipid species that are either involved in the induction of insulin resistance or are established markers of DNL.

4. Discussion

This study aimed to assess the ability of krill oil supplementation to affect NAFLD-related phenotypes in mice with diet-induced obesity and exacerbated NAFLD. At the same time, we wanted to find out whether the effects of krill oil administration on NAFLD are associated with changes in insulin sensitivity, and to look for possible determinants of these effects. Our results clearly demonstrate the ability of krill oil supplementation to alleviate hepatic steatosis, even in a situation when the accumulation of fat in the liver is maximally stimulated due to the combination of high-fat feeding and thermoneutral housing. In contrast, similar doses of omega-3 PUFAs administered via a TAG-based concentrate did not significantly reduce hepatic fat accumulation under the severe obesogenic and steatosis-promoting conditions. In addition, the potent antisteatotic effects of krill oil were observed in a situation when insulin sensitivity in the liver and at the whole-body level was maintained, and which was associated with a hepatic lipidomic signature characterized by reduced concentrations of both short/medium-chain TAGs and total DAGs.

To evaluate the efficacy of krill oil administration in influencing NAFLD-associated phenotypes, we adopted a recently established model of exacerbated NAFLD in C57BL/6J mice [36], which combines the administration of a lard-based high-fat diet with thermoneutral animal housing (i.e., ambient temperature ~30 °C). However, instead of using the "J" substrain of C57BL/6 mice as in Giles et al. [36], we used the "N" substrain (i.e., C57BL/6N mice) due to its apparent ability to accumulate a larger amount of TAGs in the liver when fed a high-fat diet. In fact, when comparing high-fat diet-fed mice of the C57BL/6J [36] and C57BL/6N [46] substrains, kept at 22 °C, C57BL/6N mice accumulated more fat in the liver despite being fed a corn oil-based diet rich in n-6 PUFA, which has a lower potential to induce hepatic steatosis compared to an SFA-rich lard-based diet [9,10,14]. Indeed, in terms of liver fat accumulation, the use of C57BL/6N mice in the current study led to the induction of very severe hepatic steatosis with TAG levels reaching ~250 mg/g, which was much more than in the corresponding group of C57BL/6J mice in the reference study by Giles et al. (i.e., ~130 mg/g in the HFD group at 30 °C; see Figure 2e in [36]). Thus, the use of the C57BL/6N mouse substrain in combination with thermoneutral housing and lard-based high-fat feeding allowed remarkably high accumulation of TAGs in the liver.

Notwithstanding the above differences in liver fat accumulation between the two substrains of C57BL/6 mice, our current study demonstrated the ability of krill oil administered to C57BL/6N mice to induce strong antisteatotic effects even in the presence of pronounced hepatic steatosis. Although some previous studies have already shown the beneficial effects of dietary omega-3 PUFAs as PLs on liver fat accumulation in various rodent models of obesity [24–26,29,30], none of those models reached the level of liver fat accumulation observed in our current study (see above). Even in genetically obese Zucker *fa/fa* rats, the fat content in the liver did not exceed 200 mg/g [25], while in mice fed different high-fat diets [24,26,29,30], it ranged from ~50 to ~160 mg/g, depending on the type and percentage of fat in the diet and the duration of its administration. This work, together with previous studies that involved supplementation of omega-3 PUFAs as PLs using either krill oil [24,25,29] or herring meal extract [26,30], thus demonstrates the excellent ability of krill oil to positively affect liver steatosis, at a wide range of tissue concentrations of TAGs. Importantly, in our present study, the antisteatotic effects of krill oil supplementation were observed regardless of whether krill oil was added to the LHF diet from the very beginning of the dietary interventions or after previous administration of the LHF diet when the animals were already obese.

Krill oil, containing significant amounts of PLs, especially phosphatidylcholine [31], was used in the current study to administer primarily EPA and DHA. The type of krill

oil used in the current study is characterized by the presence of two main fractions, i.e., phosphatidylcholines and TAGs, which represent 49 and 28%; furthermore, free FAs, DAGs, ether-linked phosphatidylcholines, cholesterol, phosphatidylethanolamines, phosphatidylinositols and lysophosphatidylcholines represent minor fractions of 4.9%, 3.5%, 3.5%, 3.4%, 3.2%, 2.3%, and 2.0%, respectively [31]. Because TAGs, along with ethyl esters, represent lipid classes traditionally used for supplying omega-3 PUFAs into the organism and to treat NAFLD in humans [21,23], we also included in our current study a group of obese mice given EPA and DHA as a TAG-based concentrate. Specifically, we used Epax 3000 TG concentrate with an EPA and DHA content of 29%, which corresponds to the relative content of these FAs in krill oil [47]. Thus, similar amounts of lard in the LHF diet had to be replaced by one or the other concentrate in order to prepare respective supplemented diets. Furthermore, the EPA:DHA ratio was approximately 1.6:1 in both krill oil and Epax 3000 TG, which facilitates the interpretation of our results compared to previous reports (e.g., [26,29]), where EPA and DHA content, as well as their ratio, differed significantly between the omega-3 PUFA concentrates based on TAGs or PLs. However, despite the similarity in the concentration and ratio of EPA and DHA, krill oil and the TAG-based omega-3 PUFA concentrate differed dramatically in terms of their effects on hepatic steatosis. As determined by biochemical analysis of TAG content in the tissue and histological evaluation (i.e., steatosis score), dietary supplementation with krill oil resulted in a 42% reduction in TAG (glycerolipids) accumulation in the liver of obese mice (i.e., ω3PL-R mice), while in mice with omega-3 PUFAs supplemented as TAGs (i.e., ω3TG-R mice) only an insignificant decrease of 5–12% was found. While this difference cannot be explained by the effects on energy intake, body weight or overall adiposity, adipose tissue functionality was improved specifically in krill oil-supplemented mice. A more pronounced reduction in macrophage accumulation in epididymal WAT of ω3PL-R mice was accompanied by higher plasma adiponectin levels (up to ~15 μg/mL) compared to their counterparts treated with the same dose of omega-3 PUFAs given as TAGs. These data are consistent with previous reports documenting the excellent efficacy of krill oil (vs. omega-3 PUFAs as TAGs; [29]) and its dose-dependent effects [24,29] in stimulating plasma adiponectin levels in high-fat diet-fed mice housed under standard thermal conditions. In this context, krill oil administered at approximately 3-fold lower dose to mice fed a 21% fat diet caused an increase in adiponectin levels to only 7.5 μg/mL [24], which is about half of the values achieved in our current study (see above). The role of elevated adiponectin levels in the antisteatotic effects of krill oil supplementation in our study is supported by the presence of a strong negative correlation between plasma adiponectin levels and the degree of liver TAG accumulation. A similar relationship has been shown, for example, in type 2 diabetic patients before and after treatment with insulin sensitizers thiazolidinediones, which resulted in a reduction in liver fat and improved insulin sensitivity while plasma adiponectin increased [48]. Thus, improved WAT function combined with a substantial increase in plasma adiponectin levels may play a role in the potent effects of krill oil supplementation on both liver steatosis and insulin sensitivity in our mice with exacerbated NAFLD.

Adiponectin levels are negatively associated with hepatic and peripheral insulin resistance and hepatic fat content [49,50]. It is known that adiponectin activates AMPK in both skeletal muscle and liver [51]. Activation of AMPK in turn leads to inhibition of acetyl-CoA carboxylase, a key enzyme in the DNL pathway, as well as to induction of FA oxidation and suppression of lipogenic enzymes [51–53]. This is in line with our current data and results from previous rodent studies using various forms of omega-3 PUFAs as PLs [29,30,33,34], which show decreased gene expression primarily within the DNL and cholesterol biosynthesis pathways. In addition to effects on FA metabolism pathways, AMPK is also important for the suppressive effect of adiponectin on hepatic glucose production and for maintaining normal fasting glucose levels [54], as well as for the beneficial effect of omega-3 PUFAs on hepatic insulin sensitivity [53]. Our results showing increased plasma adiponectin associated with decreased TAG accumulation and lipogenic gene expression in the liver are therefore consistent with the involvement of the adiponectin-

AMPK axis in the antisteatotic effects of krill oil supplementation. Furthermore, activation of this axis could also explain the observed reduction in tissue DAG levels (especially those containing SFAs) in krill oil-supplemented mice. This effect may be directly related to the improvement of hepatic insulin sensitivity, as DAGs are known to be strongly involved in the development of hepatic insulin resistance [53,55]. Because SFA-containing DAGs represented the majority of DAG species in the liver, a marked reduction in total DAG levels in mice supplemented with krill oil may be a direct consequence of its inhibitory effects on DNL (see below).

In addition to elevated adiponectin levels, there are likely to be other mechanisms by which krill oil effectively alleviates exacerbated liver steatosis. Although krill oil supplementation does not seem to change the level of VLDL-TAG secretion (our current data and [34]), it can reduce the mitochondrial citrate carrier activity, as previously observed in rats fed a lard-based high-fat diet [35], and which also showed reduced activities of DNL enzymes such as acetyl-CoA carboxylase and fatty acid synthase. This carrier acts upstream of cytosolic lipogenic processes [56], and its inhibition could thus explain the strong antilipogenic properties of krill oil. In fact, our lipidomics data from the livers of mice fed with krill oil document significantly reduced levels of short/medium-chain TAGs, a subset of TAGs previously proposed as a DNL marker [57]. Therefore, inhibition of this pathway may be one of the main mechanisms of the antisteatotic effects of krill oil. Furthermore, krill oil administration can lead to effective stimulation of FA oxidation in the liver [35], which in turn may be related to the ability of EPA, but not DHA, to increase FA oxidation while inhibiting 1,2-diacylglycerol esterification and thus TAG synthesis in hepatocytes [58]. This is in line with our current and previous [29] results documenting the improved bioavailability of EPA at the level of liver PLs. Furthermore, a recent study on mice fed a high-fat diet based on corn oil suggests that intestinal FA oxidation, which was more effectively stimulated by krill oil compared to omega-3 PUFAs supplemented as TAGs, could also be involved in the antisteatotic effects of this marine oil [59]. It is worth mentioning that other bioactive constituents are present in krill oil, which may possess antisteatotic and insulin-sensitizing properties. Our metabolomics analysis revealed that stachydrine and trigonelline are among the top two polar metabolites that most distinguish the krill oil-supplemented groups from LHF-fed controls, as well as mice supplemented with omega-3 PUFAs as TAGs. Here we show that both of these alkaloids are enriched in the diet supplemented with krill oil, and both have previously been shown to have positive effects on NAFLD, probably by restoring hepatic autophagy [60,61]. The increase in hepatic concentrations of TMAO in mice fed krill oil was probably due to its increased biosynthesis from choline by intestinal bacteria [62]. Moreover, palmitoleic acid contained in krill oil may contribute not only to the positive effects of this marine oil on glucose homeostasis and insulin sensitivity [29], but also on liver steatosis due to its stimulatory effects on PPARα and AMPK activation [63]. The complex composition of krill oil and the role of its constituents in influencing liver fat accumulation and insulin sensitivity is shown in Figure 10 below. Recently, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) has been reported as a plasma metabolite whose levels increased with omega-3 PUFAs intake and which was able to alleviate hepatic steatosis when administered to mice [64,65]. Using our metabolomics method we detected CMPF at very low signal intensities in the liver samples. Higher fold changes of 1.7 and 1.9 were observed in the ω3TG-R group compared to the ω3PL and ω3PL-R groups, respectively. However, in the control LHF group, high biological variability of CMPF was noticed, and thus, this metabolite was not ranked among the most discriminating ones.

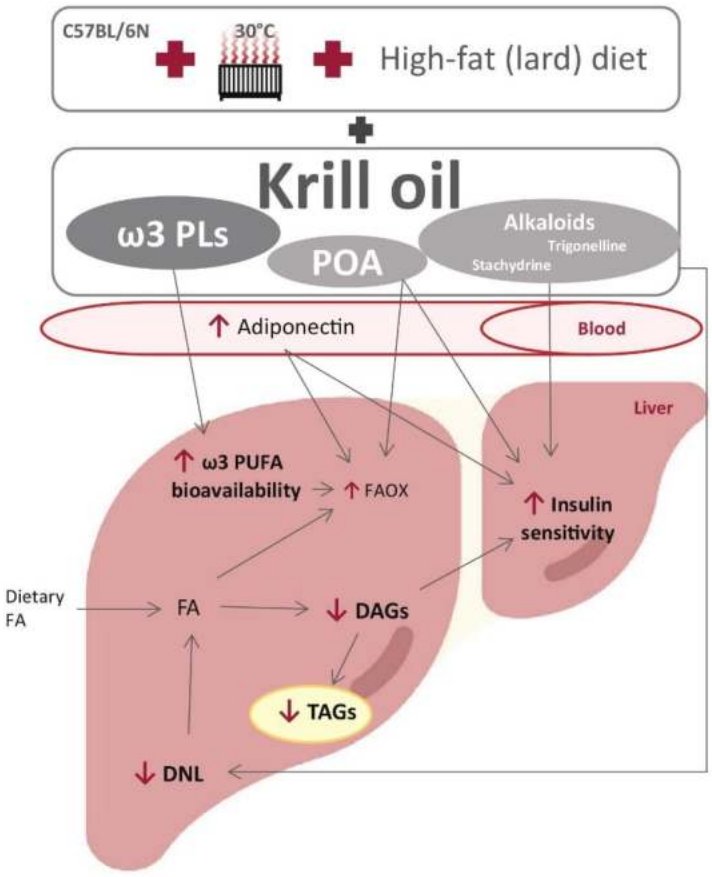

Figure 10. The potential mechanisms involved in the effects of krill oil supplementation on liver fat accumulation and insulin sensitivity in a mouse model of exacerbated hepatic steatosis induced in C57BL/6N mice fed a high-fat (lard) diet in a thermoneutral environment. The effects of krill oil are determined not only by omega-3 PUFA-containing PLs (ω3 PLs) in this marine oil, but also by its other bioactive constituents, including palmitoleic acid (POA) and the alkaloids stachydrine and trigonelline, and may involve direct or indirect mechanisms. The livers of mice fed a high-fat diet supplemented with krill oil have markedly reduced TAG accumulation and improved insulin sensitivity, which is associated with increased bioavailability of omega-3 PUFAs, suppressed DNL, decreased tissue DAG levels, and stimulated FA oxidation (FAOX). While many of these changes may be due to indirect mechanisms based on the beneficial effect of krill oil on WAT functionality associated with markedly elevated plasma adiponectin levels, direct mechanisms may include the effect of stachydrine and/or trigonelline, i.e., alkaloids contained in krill oil, which have previously been shown to have positive effects on NAFLD, presumably by restoring hepatic autophagy.

It is not clear why supplementation with omega-3 PUFAs in the form of a TAG-based concentrate did not reduce liver fat in this model of exacerbated hepatic steatosis, despite the fact that the content of both EPA and DHA in the liver was significantly increased. In this regard, we can speculate that the stronger effects of krill oil on liver steatosis are based on a combination of a number of factors, including higher adiponectin levels along with better bioavailability of EPA in liver tissue, as well as the specific effect of alkaloids

contained in krill oil. In addition, choline contained as phosphatidylcholine in krill oil can also contribute to the strong antisteatotic effects of this oil in the liver, as compared to omega-3 PUFAs supplemented as TAGs. Poor availability of hepatic choline/phosphatidylcholine is known to promote steatosis by various mechanisms, including increased DNL and impaired synthesis and secretion of hepatic VLDL (reviewed in [66]). In this context, our previous study in mice fed a corn oil-based high-fat diet showed that the antisteatotic effects of dietary phosphatidylcholine in the liver were unique to PLs containing DHA and EPA, whereas these effects were not present in animals fed soy-derived phosphatidylcholine, which contained mainly PUFAs of n-6 series such as linoleic acid [30].

Among the main weaknesses of our study is the fact that, although we used an established model of exacerbated NAFLD, whose characteristics should include NASH and liver fibrosis [36], we were not able to sufficiently induce these characteristics in our experimental mice. While the exact cause is not obvious, it may be related to the fact that a different substrain of C57BL/6 mice was used in our current study. Thus, despite a maximum steatosis score of about 3, LHF-fed control mice of the C57BL/6N substrain showed only minimal lobular inflammation (score < 1), and the overall NAS score of less than 4. This is in sharp contrast to the reference study performed on C57BL/6J mice, where the mean NAS score was ~7 [36]. Therefore, it was not possible to assess the effect of various forms of omega-3 PUFA supplementation on NASH/fibrosis in our current study. Interestingly, however, in LHF-fed control mice, plasma levels of ALT, a marker of liver damage, were almost comparable in our and the reference study. Therefore, the marked decrease in plasma ALT levels in the krill oil-supplemented groups may be due to both the potential protective effect of this oil on liver tissue and its inhibitory effect on hepatic gluconeogenesis [67]. Furthermore, the strengths of our study include: (i) mouse model with marked hepatic steatosis; (ii) evaluation of the relative efficacy of krill oil versus omega-3 TAGs in terms of effects on liver fat; (iii) comprehensive methodological approach, including various in vivo techniques such as hyperinsulinemic-euglycemic clamps, which revealed a number of potential mechanisms of action of krill oil on liver steatosis; and (iv) metabolomic analysis that identified, in addition to omega-3 PLs, other constituents of krill oil that may contribute to the potent antisteatotic effects of this oil.

5. Conclusions

By using C57BL/6N mice in combination with thermoneutral housing and lard-based high-fat feeding, we achieved remarkably high levels of TAG accumulation in the liver. Despite these extreme conditions, severe hepatic steatosis was markedly reduced in response to krill oil administration, but not in response to omega-3 PUFAs using a TAG-based concentrate. The potent antisteatotic effects of krill oil, which have been observed in both the prevention and reversal of hepatic steatosis, were associated with improved insulin sensitivity in the liver and at the systemic level. Mechanistically, high plasma adiponectin levels, as well as improved EPA bioavailability, strong repression of DNL, and decreased levels of DAGs in the liver may explain the above beneficial effects of krill oil on liver fat and insulin sensitivity. Furthermore, the role of polar metabolites contained in krill oil, including alkaloids trigonelline and stachydrine, cannot be excluded. Thus, our results suggest that in addition to omega-3 PUFAs contained in PLs, other constituents of krill oil may contribute to its strong antisteatotic effects in the liver.

Supplementary Materials: The following are available online at https://www.mdpi.com/2072-6643/13/2/437/s1, Table S1: Macronutrient composition of the experimental diets; Table S2: Composition of fatty acids in dietary lipids; Table S3: Gene names and sequences of the oligonucleotide primers; Table S4: Fatty acid composition of the neutral lipid fraction in the liver; Table S5: Fatty acid composition of the polar lipid fraction in the liver; Table S6: Concentration of trigonelline and stachydrine in experimental diets; Table S7: List of annotated complex lipids and polar metabolites in liver samples; Figure S1: Representative histological sections of epididymal white adipose tissue from mice housed in a thermoneutral environment and fed various experimental diets for 24 weeks.

Author Contributions: Conceptualization, J.K. and M.R.; Methodology, T.C., K.B., P.Z., K.L., O.K. and M.R.; Software, T.C.; Validation, T.C., P.Z. and M.R.; Formal analysis, G.S., V.K., T.C., I.I., P.Z., O.H., K.L., O.K., and M.R.; Investigation, G.S., V.K., T.C., I.I., K.B., M.O., P.Z., P.K., O.H., K.L., and M.R.; Resources, M.R.; Data curation, G.S. and M.R.; Writing—original draft preparation, G.S. and M.R.; Writing—review and editing, G.S., V.K., K.B., O.H., K.L., A.G., O.K., J.K, and M.R.; Visualization, G.S., T.C., and M.R.; Supervision, M.R.; Project administration, M.R.; Funding acquisition, J.K. and M.R. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The experiments were performed according to the European Guidelines for the care and use of laboratory animals (Directive 2010/63/EU), and approved by the Animal Care and Use Committee of the Institute of Physiology CAS (Approval no. 81/2016).

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Conflicts of Interest: The authors declare no conflict of interest.

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Article V

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Exercise training induces insulin-sensitizing PAHSAs in adipose tissue of elderly women

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ABSTRACT

Adverse effects of aging can be delayed with life-style interventions. We examined how exercise training (ET) alone or combined with omega-3 polyunsaturated fatty acid (PUFA) affects serum and adipose tissue (AT) lipidome in older women. Fifty-five sedentary older women were included in the physical activity program and given either sunflower (Placebo) or wax esters-rich (Calanus) oil capsules for 4 months. Serum and subcutaneous abdominal AT samples were acquired while maximum rates of oxygen consumption (VO_2 max), insulin sensitivity (hyperinsulinemic-euglycemic clamps) and comprehensive lipidome profiles were determined before and after the study.

ET increased VO_2 max in both groups. Lipidomics profiling revealed unusual serum triacylglycerols and phospholipids with ether-bound alkyls in the Calanus group, while ET generally induced shorter-chain triacylglycerols in AT, suggesting increased de novo lipogenesis. The latter was positively associated with whole-body insulin sensitivity. Unexpectedly, insulin-sensitizing lipokines from the family of branched palmitic acid esters of hydroxy stearic acid (PAHSAs) were elevated in both serum and AT after ET, while PAHSAs-containing triacylglycerols were detected in AT.

ET stimulated beneficial changes in AT, including PAHSAs synthesis. Although the added value of omega-3 PUFA supplementation was not proven, our discovery can help understand the nature of the metabolic benefits of exercise.

1. Introduction

Aging is associated with redistribution of adipose tissue (AT), characterized by increased visceral and ectopic fat deposition, which may be independent of changes in body weight due to concomitant decreases in muscle mass (sarcopenia) [1]. These changes are then related to an increased risk of metabolic diseases such as type 2 diabetes and cardiovascular disease [1]. Thus, AT dysfunction appears to be one of the important contributors to impaired metabolic status in the elderly; it is characterized by altered lipid storage, impaired de novo lipogenesis (DNL) and lipolysis, and increased pro-inflammatory state due to changes in innate immunity [2,3]. Circulating pro-inflammatory cytokines secreted by AT have been suggested to promote sarcopenia in the elderly, and obesity was the main factor explaining poorer physical performance in older adults with metabolic syndrome [4].

In the elderly, lifestyle interventions based on the increased physical activity are primarily aimed to improve muscle function and/or cardiovascular fitness, but recent data suggest that AT may also contribute to the beneficial effects of exercise on systemic inflammation and overall health [5]. Accordingly, it has been shown that exercise-induced lipokines increasing muscle fatty acid (FA) uptake are produced in brown AT [6], while transplantation of AT from mice subjected to exercise training (ET) into their sedentary counterparts improved glucose homeostasis of the recipients [5,7]. Moreover, AT was identified as a source of branched FA esters of hydroxy FA (FAHFA), i.e. a growing family of endogenous lipids with documented anti-inflammatory and insulin-sensitizing effects at the systemic level [8–10], whose regulation and relevance to the beneficial effects of exercise are currently unknown.

The pro-inflammatory phenotype may also be affected by natural

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substances such as omega-3 polyunsaturated fatty acids (PUFA) and specialized pro-resolving mediators [11–13]. Omega-3 PUFA supplementation reduced AT and systemic inflammation in obese non-diabetic subjects [13], and it could represent a potential strategy for the treatment/prevention of sarcopenia through increased muscle protein synthesis [14]. Calanus oil represents a novel source of omega-3 PUFA, which is unique in its combination of PUFA and alcohols [15]. Moreover, in dietary obese mice, supplementation of omega-3 PUFA-containing wax esters from Calanus oil ameliorated AT dysfunction more effectively than the same dose of omega-3 PUFA administered as ethyl esters [16,17].

Thus, the objective of this study was to evaluate in older sedentary individuals the effect of ET alone, or in combination with Calanus oil, on serum and AT lipidome and its relationship to insulin sensitivity as well as other clinical parameters.

2. Materials and methods

2.1. Study design

This work is based on the clinical study EXODYA (Effect of Exercise training and Omega-3 fatty acids on metabolic health and Dysfunction of Adipose tissue in elderly; NCT number: NCT03386461), and focuses primarily on the presentation of lipidomics data and their association with clinical parameters. Briefly, fifty-five healthy sedentary women aged 65–80 were enrolled in the physical activity program (i.e. ET) that consisted of supervised combined aerobic (mainly nordic walking, moderate intensity 60–85% VO_2 peak) and resistance training (mainly functional muscle training adapted for elderly and stretching) for 1 h, 3 times a week for 4 months. Details of the study including changes in other anthropometric parameters and the function of cardiovascular system and muscle will be published elsewhere. During the study duration, subjects were taking either 5 capsules of Calanus oil (Calanus) or sunflower oil (Placebo). The dose of omega-3 PUFA in the Calanus group was approximately 230 mg EPA and DHA per day. All measurements, procedures and sample collection were performed at week 0 (before) and week 16 (after), on an outpatient basis, after an overnight (10-12 h) fasting with water ad libitum. At both visits, serum, red blood cells (RBC), subcutaneous abdominal AT samples, and anthropometric parameters were acquired. Measurement of maximum oxygen consumption (VO_2 max) and hyperinsulinemic-euglycemic clamps (HEC) were performed as before [18,19]. Fifty paired serum samples and 46 paired AT samples were successfully processed through the lipidomics and metabolomics pipelines (see below), while the remaining samples were either not collected in pairs or their quantity was not sufficient for the analysis. The study was conducted according to Helsinki declaration and approved by the Ethical Committee of Kralovske Vinohrady University Hospital in Prague. All subjects signed informed consent before the start of the study.

2.2. Sample extraction

Extraction of serum and AT samples was carried out using a biphasic solvent system of cold methanol, methyl *tert*-butyl ether (MTBE), and water [20], with some modifications. See Supplemental methods for details.

2.3. LC–MS analysis

The LC-MS systems consisted of a Vanquish UHPLC System (Thermo Fisher Scientific, Bremen, Germany) coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) and UltiMate 3000 RSLC UHPLC system coupled to a QTRAP 5500/ SelexION mass spectrometer (SCIEX, Darmstadt, Germany). See Supplemental methods for technical details [9,18,20–24].

2.4. Data processing and statistics

LC-MS data from global metabolomics and lipidomics profiling were processed through MS-DIAL v. 2.52 and 2.80 software [25]. Metabolites were annotated using in-house retention time–*m/z* library and using MS/MS libraries available from public sources (MassBank, MoNA). Raw data were filtered using blank samples, serial dilution samples, and quality control (QC) pool samples with relative standard deviation (RSD) < 30%, and then normalized using LOESS approach by means of QC pool samples for each matrix injected regularly between 10 actual samples. LC-MS/MS data from targeted analysis were processed using MultiQuant software (SCIEX). Data were further processed via MetaboAnalyst [26], Box-Cox transformation [27] and GraphPad PRISM 8 using Two Way Repeated Measures ANOVA or Mixed effects models pipelines. False discovery rate (FDR) was set to FDR < 0.1. Multiple comparisons (Sidak's test) were used to compare the groups and to test the effect of ET. For simplicity, the factors were referred to as "Exercise" (E), "Diet" (D), and their interaction (I).

3. Results

3.1. Exercise improved physical fitness and insulin sensitivity

We aimed to reveal whether ET, alone or combined with omega-3 PUFA supplementation, could affect adiposity and whole-body parameters of physical fitness and glucose metabolism. Although we did not observe significant changes in body weight, BMI, fat mass and fat free mass in response to either ET or ET with omega-3 PUFA, physical fitness was improved by ET regardless of omega-3 PUFA supplementation, as indicated by changes in VO_2 max (Table 1). Moreover, whole body insulin sensitivity, evaluated as glucose disposal rate during the HEC (i.e. M-value), was improved by ET, with statistically significant effect in the Calanus group (Table 1).

Table 1
Clinical characteristics of the participants.

	Placebo						Calanus					
	Before			After			Before			After		
Age (years)	70.0	±	3.7	70.4	±	3.7	71.0	±	4.2	71.7	±	3.8
BMI (kg m^{-2})	27.1	±	4.1	26.7	±	3.9	27.1	±	3.8	27.3	±	4.4
Weight (kg)	71.9	±	13.1	71.0	±	12.8	71.5	±	10.6	71.5	±	11.7
Fat mass (kg)	26.5	±	8.7	25.4	±	8.0	26.9	±	8.0	26.7	±	7.9
Fat free mass (kg)	45.5	±	5.2	45.6	±	5.6	44.6	±	3.2	44.8	±	4.7
VO_2 max (l min^{-1})	19.5	±	3.0	23.0	±	3.2[a]	20.0	±	4.5	22.1	±	4.2[a]
Fasting glucose (mM)	5.73	±	0.62	5.54	±	0.49	5.46	±	0.48	5.52	±	0.56
M (mg kg^{-1} min^{-1})	5.38	±	1.91	5.78	±	2.29	5.46	±	2.39	6.37	±	2.36[a]

[a] Statistically significant effect of ET (before vs. after); two way repeated measures ANOVA; $n = 23$; means ± SD.

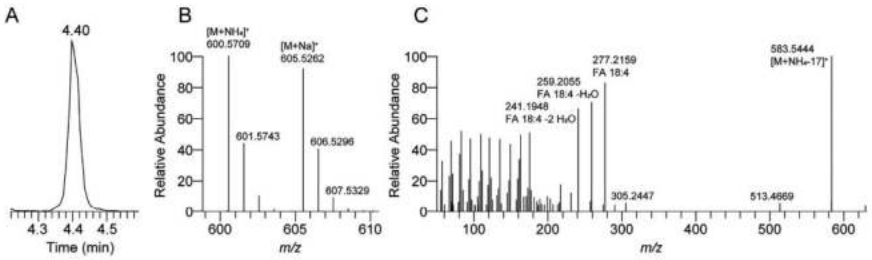

Fig. 1. An example of long-chain wax ester extracted from Calanus oil. A) Extracted ion chromatogram of FA 18:4-WE 40:5 (m/z 600.571), B) MS1 spectrum showing presence of $[M + NH_4]^+$ and $[M + Na]^+$ adducts, C) MS/MS spectrum of precursor ion m/z 600.571 acquired at a normalized collision energy (NCE) of 20%.

3.2. Calanus oil is rich in free FA and polyunsaturated wax esters

Since no public LC-MS/MS data were available for the Calanus oil, and to competently assess intervention-induced changes in serum and AT lipidome, we first explored the lipid composition of capsules using a methodology focused on intact lipids. Placebo capsules contained sunflower oil, mainly linoleic and oleic acids bound in triacylglycerols (TAG), while the Calanus capsules consisted of long-chain FA and alcohols, including wax esters. We have identified six abundant long-chain wax esters (Table S1); characterization of one representative is included in Fig. 1.

3.3. Calanus oil did not affect ET–induced changes in the production of anti-inflammatory lipid mediators

We aimed to explore, whether ET alone or in combination with Calanus supplementation could be linked to changes in the production of various lipid mediators in AT. Calanus oil contains omega-3 PUFA, and thus we explored a hypothesis that supplemented omega-3 PUFA could serve as substrates for the synthesis of omega-3 PUFA-derived anti-inflammatory specialized pro-resolving mediators (SPMs) [12]. Although we detected SPMs in several samples, we were unable to obtain a complete profile for the entire cohort. Therefore, we focused on the SPM precursors, monohydroxylated PUFA (Fig. 2). Using targeted lipidomics, we found that eicosanoids, represented by 5-, 12- and 15-hydroxyeicosatetraenoic acids, were affected by ET in both serum and AT (Fig. 2A,C). Docosanoids, represented by 7-, 14-, and 17-hydroxydocosahexaenoic acids, were relatively scattered before the intervention and consolidated to lower levels after the intervention in both serum and AT (Fig. 2B,D), suggesting the selective effects of the intervention in individuals with higher baseline docosanoid levels.

3.4. Serum and AT metabolome were affected by diet and exercise, respectively

Next, we have measured serum and AT metabolomic profiles using LIMeX, a targeted and untargeted workflow combining the lipidome, metabolome and exposome. Annotated data were processed via MetaboAnalyst Time Series module [26]. Venn diagrams revealed that serum metabolome was primarily affected by the type of dietary supplementation (Placebo vs. Calanus) while AT metabolome was affected by ET (Fig. 3A and B). A complete overview of statistically significant metabolites is presented as Tables S2 and S3.

Serum levels of lipids containing ether-bound FA, e.g. unusual TAGs 58:2e and 60:3e, and phosphatidylcholine (PC) 36:6e (Fig. 3C) were elevated in the Calanus group, and we have also identified 15 unique TAGs with ether-bound alkyls (Table S4). This suggests that fatty alcohols from the Calanus oil were efficiently incorporated into circulating lipids, especially TAGs. Although there were many glycerophospholipid species containing omega-3 PUFA (especially DHA) significantly elevated by Calanus oil supplementation (Table S2), the omega-3 index (sum of EPA and DHA in erythrocyte membranes expressed as a percentage of total erythrocyte FA [28]) showed no difference between the Calanus and Placebo group (from 5.1 ± 1.4 to 6.1 ± 1.7 and from 5.0 ± 1.0 to 6.4 ± 1.2%, respectively). This suggests that Calanus oil supplementation had no additional benefits in terms of increasing bioavailability of omega-3 PUFA.

Metabolite profiles in AT were affected by ET only. Although this effect was less pronounced when compared to serum (Fig. 3A), several metabolites could be linked to the intrinsic metabolic pathways of AT. Thus, tissue levels of arginine, previously linked to the activation of lipolysis and fat mass reduction [29](Table S3), were significantly elevated. Interestingly, a sequence of three TAGs 46:0, 48:0, and 50:0 was also elevated (Fig. 3D), suggesting activation of de novo lipogenesis in AT by exercise [30,31].

3.5. Insulin sensitivity correlated with short-chain TAGs in AT after ET

Next, we took advantage of the clinical characteristics comprising metabolic parameters of the participants and calculated correlations between the clinical and metabolomics data. We focused our attention on the metabolic status induced by ET at the end of the study, regardless of dietary supplementation. The strongest correlations were observed between the M value (a measure of whole-body insulin sensitivity; see above) and AT metabolite profiles (Fig. 4). Thus, M values positively correlated with short/medium-chain TAGs containing 38 to 48 carbons and 0 to 3 double bonds, which could serve as markers of DNL in AT [30,31], and also with diacylglycerols (DAGs) and ether-containing phosphatidylethanolamines (PEe). On the contrary, the M value correlated negatively with ether-containing phosphatidylcholines (PCe), TAGs containing 18:1 (oleic acid) and long-chain sphingomyelins.

3.6. Serum and AT levels of insulin-sensitizing PAHSAs were elevated by exercise

The presence of strong positive correlations between short-chain TAGs and whole-body insulin sensitivity has led us to explore potential changes in the levels of novel antidiabetic FAHFA mediators, in particular those from the palmitic acid esters of hydroxystearic acid (PAHSA) family [8], which could be a part of the mechanistic link between ET and increased insulin sensitivity. Except for 12/13-PAHSA, the other four PAHSA regioisomers as well as their total levels were markedly elevated by ET, both in serum and AT (Fig. 5).

3.7. TAG estolides, which represent the FAHFA metabolic reservoir, were elevated by ET

Based on the recent discovery of FAHFA-containing triacylglycerol estolides (FAHFA-TG or TAG EST) in AT [[32] and our unpublished

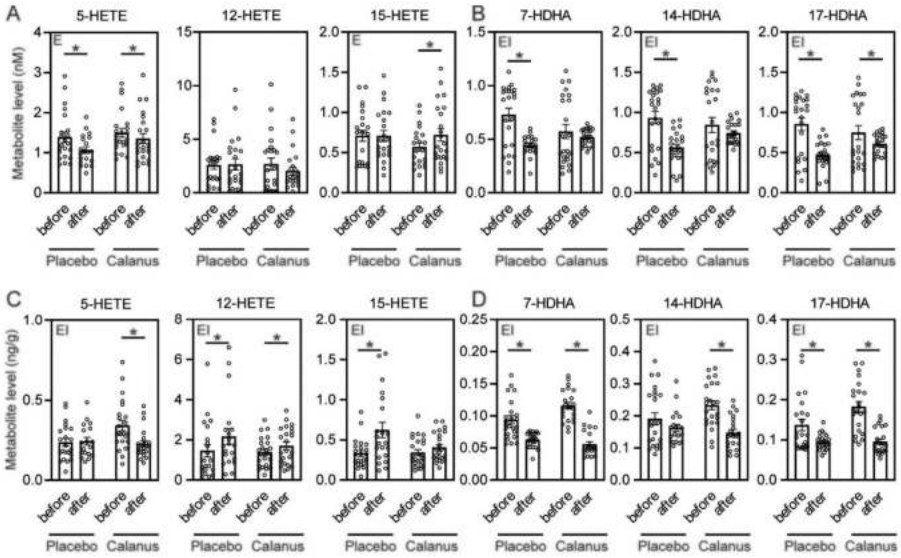

Fig. 2. The levels of eicosanoids and docosanoids in serum and AT. A) Serum levels of hydroxyeicosatetraenoic acid (HETE) isomers. B) Serum levels of hydroxydocosahexaenoic acid (HDHA) isomers. C) HETE levels in AT. D) HDHA levels in AT. Mixed effects models: E, significant effect of the main factor exercise; EI, significant effect of exercise & factor interaction; *, significant effect of exercise (before vs after, multiple comparisons); n = 20–23; bars are means ± SEM.

results], we further explored the lipidomic data and successfully identified 22 metabolites from the TAG EST family (Table S6). Total levels of TAG EST tended to be higher after ET, as documented by two TAG EST (Fig. 6A). Furthermore, we explored the relationship between the level of whole-body insulin sensitivity (i.e. M value) at the end of study, regardless of supplementation, and different TAG EST by calculating the respective correlations. It was revealed that TAG EST 68:1 had the strongest positive correlation with the M value (Fig. 6B), further confirming the potential role of elevated PAHSAs levels in ET-induced changes in insulin sensitivity.

4. Discussion

The aim of this part of the EXODYA project was to analyze the effects of ET on serum and AT metabolome and lipidome in elderly women, and to explore potential synergy between ET and omega-3 PUFA supplementation in these effects. The analysis of anthropometric and biochemical parameters showed a beneficial effect of ET on whole-body fitness, but failed to prove an additive effect of a low dose of omega-3 PUFA. There could be several reasons for this finding. The form of omega-3 PUFA used in this study was Calanus oil, a natural lipid extract from the marine copepod *Calanus finmarchicus* [15], with a

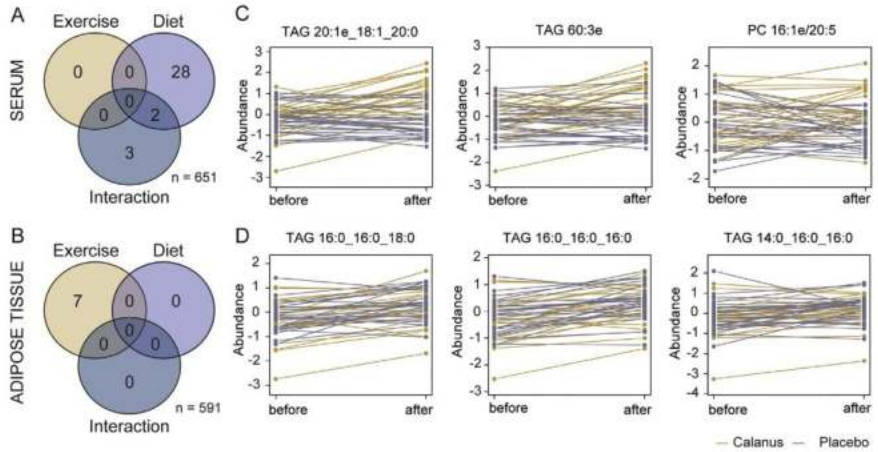

Fig. 3. An overview of serum and AT metabolomes. A) Venn diagram of 651 annotated serum metabolites; B) Venn diagram of 591 annotated AT metabolites; C) Representatives of the most discriminating lipids with ether-bound fatty acids; D) Representative TAGs affected the most by ET. Blue: Placebo; Yellow: Calanus. Two Way Repeated Measures ANOVA; n = 23. See Tables S2 and S3 for details.

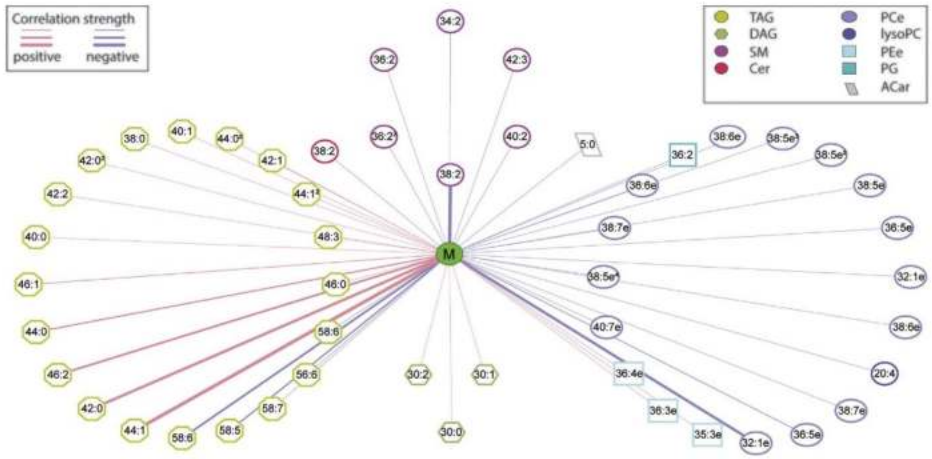

Fig. 4. An overview of moderate to strong correlations between the M value and AT metabolites. Metabolites from different lipid classes are visualized in a network using nodes of different shape and color, while the strength of their positive (pink lines) or negative (purple lines) correlations with the M value (selected on the basis of Pearson's correlation coefficient $r \geq 0.5$, $p \leq 0.05$, and false discovery rate FDR < 0.1) is indicated by lines of different thickness. A number X:Y in each frame denotes the number of carbons and the number of double bonds in the corresponding metabolite; "e" suffix marks ether species. Tabular overview in Table S5.

unique combination of FA, fatty alcohols and wax esters. Although we detected elevated serum levels of lipids with ether-bound alkyls, thus proving bioavailability of Calanus oil, there was no increase in the omega-3 index and PUFA-derived lipid mediators in the Calanus group. Therefore, it is possible that higher doses of Calanus oil are needed to significantly increase the omega-3 index and to serve as an adequate source of omega-3 PUFA when compared to e.g. re-esterified TAGs [18]. The dose of 230 mg EPA and DHA per day provided by Calanus oil capsules was on the lower border of recommended dietary intake [33]; apparently, despite its unique composition, Calanus oil as vehicle could not boost the bioavailability of omega-3 PUFA at this low dose of supplementation. Furthermore, we were able to detect markers of Calanus oil in serum but not in AT, which further suggests that the dose was not high enough for the omega-3 PUFA to enter the slow-turning lipid pool in AT.

This interpretation is further supported by the measurement of eicosanoids and docosanoids in serum and AT samples, where we observed the effect of ET but not the expected increase in SPMs in Calanus oil-supplemented participants. The levels of anti-inflammatory docosanoids were either unchanged or even tended to decrease, primarily in subjects with high baseline docosanoid levels, which was possibly due to suppression of low-grade inflammation in response to ET. This

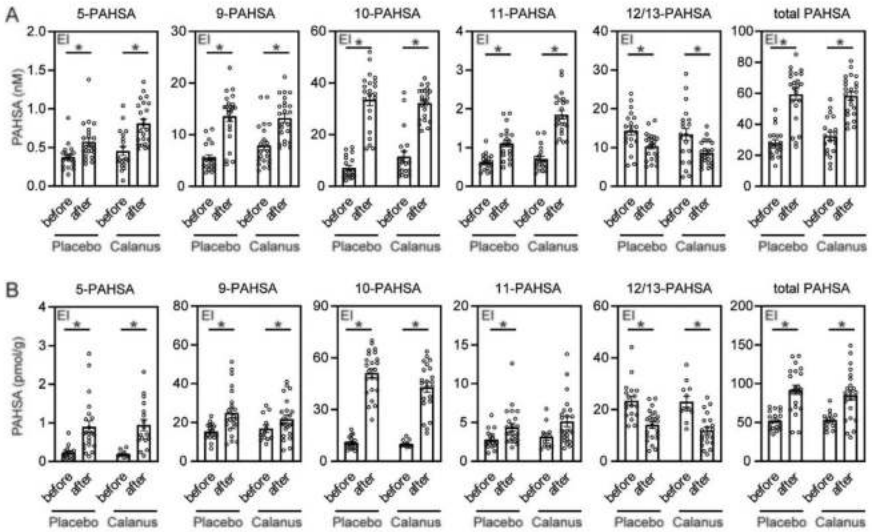

Fig. 5. PAHSA levels in serum (nM) and AT samples (pmol/g). A) Serum levels of PAHSA regioisomers. B) PAHSA levels in AT. Mixed effects models: EI, statistically significant effect of exercise & factor interaction (exercise/diet); *, significant effect of exercise (before vs after, multiple comparisons); n = 12–23; bars are means ± SEM.

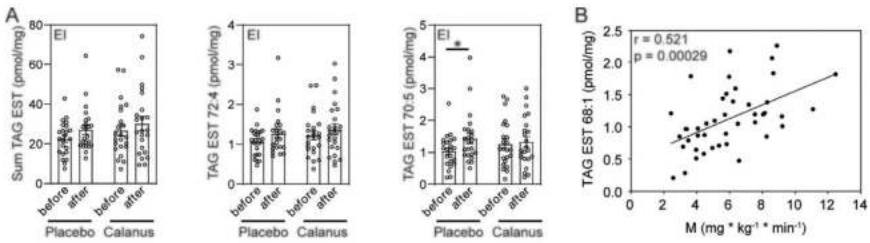

Fig. 6. TAG EST levels in AT samples (pmol/mg). A) Total TAG EST levels and two representative compounds. B) Correlation between TAG EST 68:1 in AT and M value from HEC. Two Way Repeated Measures ANOVA: EI, statistically significant effect of exercise & factor interaction (exercise/diet); *, statistically significant effect of exercise (before vs after, multiple comparisons); $n = 23$; bars are means ± SEM.

hypothesis is supported by a recent study that failed to demonstrate additive effects of omega-3 PUFA supplementation combined with resistance training in older men [34].

Metabolomics and lipidomics analysis of serum samples from overnight fasting patients revealed that the serum profiles were affected mainly by Calanus oil supplementation, reflecting probably the short-term changes in serum lipid composition after consuming oil capsules. The involvement of the liver and intestine in the wax ester metabolism and systemic availability of EPA and DHA is unknown. However, the difference between incorporation of EPA and DHA into very low-density lipoprotein TAGs and chylomicrons in postprandial period has been documented in humans [35], suggesting a possible partitioning of FA into different lipid pools within the liver before hepatic TAG synthesis and systemic availability. Regardless of these aspects, we were able to describe several novel and unusual lipids including ether TAGs and phospholipids, which were enriched in serum of Calanus oil-supplemented participants. However, these specific markers of Calanus oil intake (e.g. ether lipids, stearidonic acid 18:4 n-3) were not observed in AT of these subjects. On the other hand, there was a clear pattern of TAGs with short acyl chains, which was associated with the effect of exercise in AT. Interestingly, TAGs enriched in palmitic acid and myristic acid were previously linked with increased DNL from carbohydrates both in the liver and AT [30,31,36,37]. Thus, the presence of these particular TAGs in AT of subjects undergoing the ET regimen in our current study suggests a previously unrecognized relationship between the effects of exercise and the induction of this particular metabolic pathway in AT. The mechanism of this induction is not clear, but could be secondary to ET-induced improvements in insulin sensitivity of AT.

Importantly, the ET-induced changes in whole-body insulin sensitivity provided the highest correlations with AT metabolites. Specifically, we found a long list of short-chain TAGs, potentially related to DNL, which positively correlated with the M value at the end of the study, regardless of supplementation. On the other hand, the M value correlated negatively with long-chain polyunsaturated TAGs and ether-containing PCs. While the DNL pathway could be linked to beneficial changes in AT during exercise [38], the role and negative associations of various PCe species with the M value is puzzling due to the lack of information on the biological effects of PCe.

It is now well documented that AT produces a family of FAHFA lipid mediators such as PAHSAs that exert potent anti-inflammatory and insulin-sensitizing effects while improving glucose metabolism in AT [8,9,39,40]. Here we show that all PAHSA regioisomers except one were elevated in response to ET. This effect could be linked to improved glucose metabolism in AT due to exercise and thus contribute to changes in whole-body insulin sensitivity in the elderly. To this point, it has been shown that a single dose of PAHSA improved insulin sensitivity in aged, glucose-intolerant chow-fed mice [8]. The PAHSAs levels are regulated by multiple factors, depending also on the nutritional status and anatomical location of the fat depot [8]. Recently, a FAHFA-containing metabolic reservoir of TAG EST was discovered [32]. When we explored the levels of TAG EST in AT samples from the participants in our study, the total levels of these lipids tended to increase in response to ET, and the levels of TAG EST 68:1 positively correlated with the M values after the ET. In contrast to previous work by Yore et al. [8], positive correlations between the M value and various PAHSAs before and after ET were weak ($r < 0.4$). This was most probably because the range of values of various anthropometric parameters and the M values within our cohort was relatively narrow and more compact than the cohort of insulin-sensitive and diabetic patients reported before [8,10].

In conclusion, our data suggest that ET stimulates beneficial metabolic changes in AT, including the synthesis of PAHSAs and TAG EST. Although the added value of omega-3 PUFA supplementation in terms of these effects has not been demonstrated, our discovery of ET-induced positive metabolic changes in AT lipidome, linked to increased production of potent anti-inflammatory and insulin-sensitizing lipid mediators, could improve our understanding of the mechanisms underlying the metabolic benefits of exercise. While the above changes were uncovered in the elderly subjects participating in the specific ET regimen, it is possible that they represent a general phenomenon associated with the effects of exercise regardless of its type or age of the target population.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

Conceptualization: K.D., M.Si., M.R. and O.K.; Data curation: M.B., T.C., M.O., M.S., K.D., L.R., M.M., M.Si. and O.K.; Formal analysis: M.B., T.C., M.O., M.S., K.D., L.R., M.M., M.Si. and O.K.; Funding acquisition: K.D., M.Si., M.R. and O.K.; Investigation: M.B., T.C., M.O., M.S., K.D.,

L.R., M.M., M.Si. and O.K.; Methodology: T.C., K.D., M.M., M.Si. and O.K.; Project administration: M.Si. and M.R.; Resources: M.Si. and O.K.; Software: T.C.; Supervision: M.Si. and O.K.; Validation: M.R.; Visualization: O.K.; Writing – original draft: O.K.; Writing - review & editing: M.B., T.C., M.O., M.S., K.D., L.R., M.M., M.Si., M.R. and O.K.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbalip.2019.158576.

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Article VI

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Lipokine 5-PAHSA Is Regulated by Adipose Triglyceride Lipase and Primes Adipocytes for De Novo Lipogenesis in Mice

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Branched esters of palmitic acid and hydroxystearic acid (PAHSA) are anti-inflammatory and antidiabetic lipokines that connect glucose and lipid metabolism. We aimed to characterize involvement of the 5-PAHSA regioisomer in the adaptive metabolic response of white adipose tissue (WAT) to cold exposure (CE) in mice, exploring the cross talk between glucose utilization and lipid metabolism. CE promoted local production of 5- and 9-PAHSAs in WAT. Metabolic labeling of de novo lipogenesis (DNL) using $^{2}H_{2}O$ revealed that 5-PAHSA potentiated the effects of CE and stimulated triacylglycerol (TAG)/fatty acid (FA) cycling in WAT through impacting lipogenesis and lipolysis. Adipocyte lipolytic products were altered by 5-PAHSA through selective FA re-esterification. The impaired lipolysis in global adipose triglyceride lipase (ATGL) knockout mice reduced free PAHSA levels and uncovered a metabolite reservoir of TAG-bound PAHSAs (TAG estolides) in WAT. Utilization of ^{13}C isotope tracers and dynamic metabolomics documented that 5-PAHSA primes adipocytes for glucose metabolism in a different way from insulin, promoting DNL and impeding TAG synthesis. In summary, our data reveal new cellular and physiological mechanisms underlying the beneficial effects of 5-PAHSA and its relation to insulin action in adipocytes and independently confirm a PAHSA metabolite reservoir linked to ATGL-mediated lipolysis.

The dysregulation of both glucose and lipid metabolism in white adipose tissue (WAT) contributes to the development of obesity-associated type 2 diabetes, which represents one of the most serious health threats. However, the mechanistic links between altered glucose and lipid metabolism in the WAT of obese patients and the development of systemic insulin resistance are not fully explored. De novo lipogenesis (DNL) converts carbohydrates to energy-dense neutral lipids both in the liver and in WAT (1–4). Although hepatic DNL is usually associated with systemic insulin resistance, DNL in WAT correlates with insulin sensitivity and obesity resistance (5–8). We recently demonstrated in WAT that DNL, when combined with triacylglycerol (TAG)/fatty acid (FA) cycling activity, contributes to a lean phenotype in mice (6). This supports the notion that adipose tissue–specific regulation of DNL is critical for metabolic homeostasis (reviewed in Yilmaz et al. [4]).

DNL in WAT might also serve as a source of signaling molecules, i.e., bioactive lipids (lipokines), including palmitoleate (5), alkyl ether lipids (9), and FA esters of hydroxy FAs (FAHFAs), namely, palmitic acid hydroxystearic acids (PAHSAs) (8), molecules that promote insulin sensitivity and ameliorate insulin resistance. Structurally, FAHFAs consist of an FA (e.g., palmitic acid) esterified to a hydroxy FA (e.g., hydroxystearic acid), and the position of the branching carbon defines the regioisomer (e.g.,

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5-PAHSA, positional isomer). Many families of regioisomers have been identified in humans, rodents, and plants (8,10,11). The 5- and 9-PAHSA regioisomers have been the most studied for their anti-inflammatory and insulin-sensitizing effects (12,13).

During cold exposure (CE), adipose triglyceride lipase (ATGL) catalyzes TAG hydrolysis in WAT and the released FAs fuel thermogenesis in brown adipose tissue (14) while TAGs in WAT are replenished by DNL from glucose (6). Here, we explored the potential involvement of 5-PAHSA in the adaptive metabolic response of WAT to CE in mice. We tested the hypothesis that CE could associate with increased generation of PAHSA that affects both DNL and TAG/FA recycling and influences the metabolic profile of WAT.

RESEARCH DESIGN AND METHODS

Reagents

All chemical reagents were from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. FAHFA standards were from Cayman Europe (Tallinn, Estonia), and 5-PAHSA (Supplementary Fig. 1) was synthesized as previously described (15). Heavy water (^2H_2O) was from CortecNET (Voisins-le-Bretonneux, France), and ^{13}C-labeled glucose and glutamine and ^2H-labeled glucose were from Cambridge Isotope Laboratories (Tewksbury, MA). TAG estolide tag 16:0/16:0/9-PAHSA [*sn*-3-((9-(palmitoyloxy)octadecanoyl)oxy)propane-1,2-diyldipalmitate] was synthesized from 1,2-dipalmitoyl glycerol and 9-PAHSA (Sigma) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and 4-dimethylaminopyridine in CH_2Cl_2.

Animal Studies

Two-month-old male B6 (C57BL/6JBomTac; Taconic Biosciences, Ejby, Denmark) mice fed standard chow were maintained close to thermoneutrality (TN) at 30°C for 1 week. Thereafter, subgroups of mice were either maintained at 30°C (control animals) or exposed to cold (CE animals) at 6°C for 7 days before killing (6). After 3 days, mice were further divided into subgroups and received an oral gavage of 5-PAHSA or saline (in a polyethylene glycol 400/TWEEN 80 formulation [8]) (Fig. 1). The 5-PAHSA dose was 45 mg/kg. EDTA plasma and various tissues were collected, including liver and epididymal WAT (eWAT). Samples were flash frozen and stored in liquid nitrogen. Global ATGL knockout (AKO) mice were generated as previously described (14). Female AKO mice and wild-type (WT) littermates fed standard chow were fasted for 12 h before dissection (fed vs. fasted state) or sacrificed at ad libitum fed state (acute and chronic CE) (14).

In Vivo Lipid Synthesis in Murine eWAT

Two days prior to dissection, mice were injected intraperitoneally with a bolus of ^2H_2O in saline (3.5 mL of 0.9% NaCl w/v in 99.9%-enriched ^2H_2O/100 g body weight) and 10% of their drinking water was replaced with ^2H_2O for the rest of the experiment to stabilize ^2H_2O content in body water as previously described (6). Deuterium enrichment of mouse plasma was assayed by exchange with acetone using GC×GC-TOFMS (two-dimensional gas chromatography time-of-flight mass spectrometry) (16). Total lipids from eWAT were extracted using the methanol/methyl *tert*-butyl ether/water protocol and the TAGs purified by solid-phase extraction (17,18). An aliquot of the TAG extract was hydrolyzed in KOH, the free FA converted to methyl esters, and free glycerol derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide before analysis using GC×GC-TOFMS (19). The fractional synthesis of FA from ^2H_2O was calculated according to mass isotopomer analysis (20). A separate aliquot of the lipid extract was processed for lipidomics and metabolomics.

Cell Culture

3T3-L1 murine adipocytes were differentiated according to a standard protocol and mature adipocytes kept in DMEM complete medium (25 mmol/L glucose, 10% calf serum, 850 nmol/L insulin, penicillin/streptomycin) (10).

In Vitro Lipid Synthesis

3T3-L1 adipocytes were maintained in DMEM complete medium prepared from powder (D5648; Sigma-Aldrich), ^2H_2O, and water for cell cultures (50/50, v/v) (21) for 3 days. Total lipids were extracted as above and processed for lipidomics, and the raw data were analyzed with MS-DIAL version 2.52 software (22). Data from labeling experiments (^{13}C and ^2H) were adjusted for C, H, and O natural abundance and tracer purity using IsoCor 2.0.5 (23), and the fractional synthesis of intact lipids was estimated according to the mass isotopomer analysis (20,24). Parallel reaction monitoring and tandem mass spectrometry (MS/MS) scanning modes of the Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) were used to confirm M+3 isotopologue identity and glycerol backbone labeling.

Glucose Uptake and PAHSA Treatment

Mature 3T3-L1 adipocytes were grown in full DMEM for 3 days in the absence or presence of 5-PAHSA, similar to the duration of 5-PAHSA gavage in mice. Adipocytes were serum starved in DMEM with 0.1% (w/v) BSA for 15 h, washed, and kept in DMEM without glucose and glutamine (A1443001; Gibco) for 30 min. Cells were then labeled with $^{13}C_6$-glucose (5.5 mmol/L in A144301 medium) in the presence or absence of 40 μmol/L 5-PAHSA for 0, 5, 10, and 15 min in a reverse time course fashion, similar to that described by Krycer et al. (25). Metabolism was quenched in a water bath (0°C), and cells were washed twice with ice-cold PBS, lysed in the methanol/water fraction of the methyl *tert*-butyl ether extraction mixture chilled at −20°C, and frozen in liquid nitrogen. Alternatively, cells were preincubated with 10 nmol/L insulin in the glucose-free media and labeled with $^{13}C_6$-glucose (5.5 mmol/L final) with or without 10 nmol/L insulin, and the metabolism was quenched after 10 min.

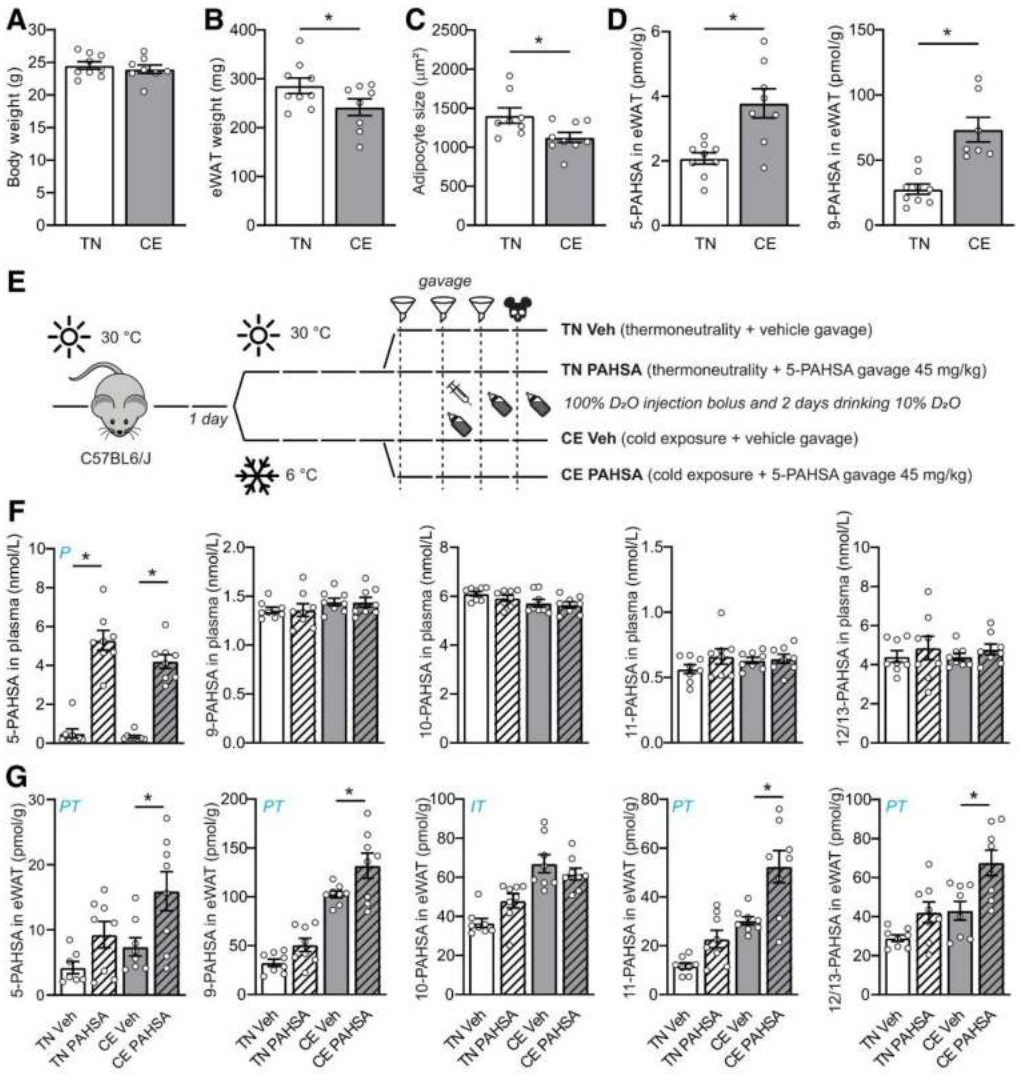

Figure 1—PAHSA levels were increased in cold. *A*: Body weight of male C57BL/6J mice maintained at TN (30°C) and then either kept at TN or exposed to cold (6°C) for 7 days. *B*: Weight of eWAT. *C*: Size of adipocytes in eWAT. *D*: Concentration of 5- and 9-PAHSA in eWAT. Data are means ± SEM (n = 8–9). *$P < 0.05$ by Student t test. *E*: Experiment combining TN, CE, gavage of 5-PAHSA, and deuterium metabolic labeling. *F*: Levels of PAHSA regioisomers in plasma. *G*: Levels of PAHSA regioisomers in eWAT. Two-way ANOVA with multiple comparison test (Sidak) was used. Letters within the graphs denote a statistically significant effect of 5-PAHSA (*P*), temperature (*T*), or interaction of factors (*I*). Data are means ± SEM (n = 8–9). *Planned multiple comparison of the effect of 5-PAHSA at the given temperature statistically different at $P < 0.05$. D_2O, heavy water; Veh, vehicle.

To explore the contribution of glutamine carbons, the labeling media were supplemented with 5.5 mmol/L glucose and 4 mmol/L $^{13}C_5$-glutamine, and the metabolism was quenched after 10 min of glucose uptake.

FAHFA Analysis

Plasma, eWAT, cells, and media were processed according to published methods (10,18) with special attention paid to known methodological issues (26,27).

Metabolomics, Lipidomics, Bioinformatics, and Statistical Analyses

The liquid chromatography (LC)-MS system consisted of a Vanquish UHPLC System (Thermo Fisher Scientific) coupled to a Q Exactive Plus mass spectrometer. See Supplementary Data for details.

LC-MS and LC-MS/MS data were processed through the software MS-DIAL, version 2.52 (22), using its isotope tracking features. Metabolites were annotated using an in-house retention time charge/mass ratio (*m/z*) library and using MS/MS libraries available from public sources (MassBank of North America [MoNA]). Normalized (locally estimated scatterplot smoothing procedure, Python script) peak heights/data from labeling experiments (^{13}C and ^{2}H) were adjusted for C, H, and O natural abundance and tracer purity using IsoCor 2.0.5 (23) when needed. An in silico library of theoretical TAG estolides was calculated using EnviPat (28) and in-house Python scripts. GraphPad Prism 8.0.2 software was used to compare groups (Student *t* test, ANOVA, etc.).

Data and Resource Availability

The data sets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request. No applicable resources were generated or analyzed during the current study.

RESULTS

PAHSA Levels Are Increased by CE

We previously showed that induction of DNL in WAT during CE was associated with a lean phenotype in mice (6). To explore the involvement of PAHSAs in this metabolic adaptation, we examined mice kept at TN or exposed to cold for 7 days. In response to CE, body weight was not affected, while the weight of eWAT and adipocyte size decreased (Fig. 1*A–C*), similar to previous studies (6,29). Levels of both 5- and 9-PAHSA in eWAT were elevated by CE (Fig. 1*D*). Therefore, we explored the effect of 5-PAHSA, administered by gavage for 3 days, on DNL in eWAT of mice kept either at TN or exposed to cold for 7 days. In these studies heavy water ($^{2}H_2O$) was used as a tracer for lipogenesis (Fig. 1*E*). The 5-PAHSA gavage led to an approximately fivefold increase in plasma 5-PAHSA levels with no changes in other PAHSA regioisomers at either TN or cold temperature (Fig. 1*F*). Intriguingly, we noticed that the levels of nearly all PAHSA regioisomers were increased in eWAT after 5-PAHSA gavage, especially in CE animals (Fig. 1*G*), suggesting a modulation of DNL. Of note, the

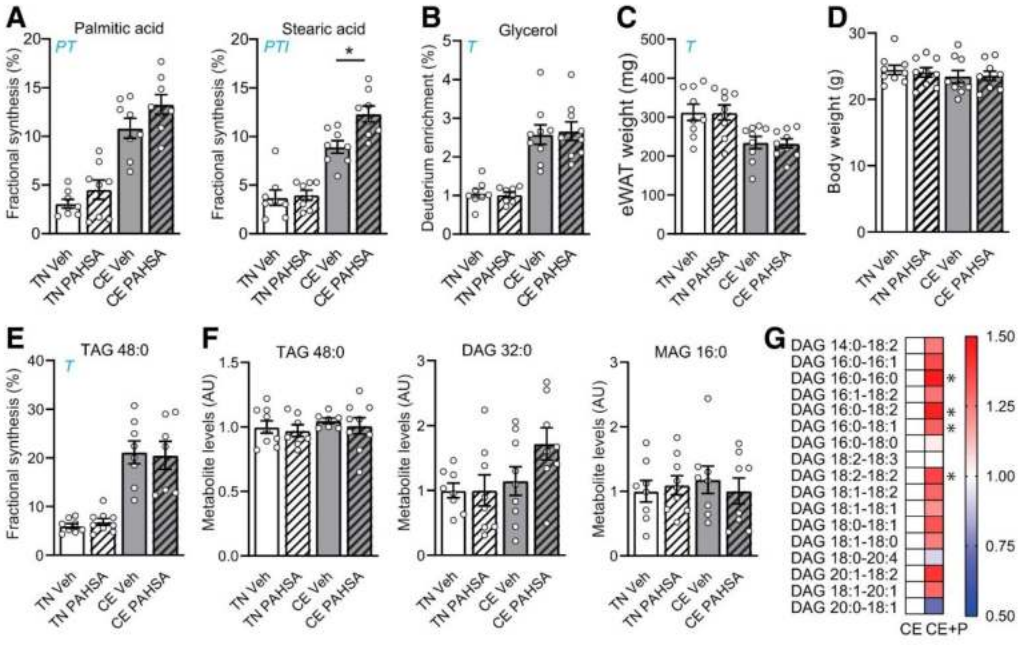

Figure 2—5-PAHSA stimulated DNL in eWAT during CE. *A*: Fractional synthesis of palmitic and stearic acid measured in hydrolysates of the TAG fraction of eWAT (see experiment in Fig. 1*E*). Two-way ANOVA with multiple comparison test (Sidak) was used. Letters within the graphs denote a statistically significant effect of 5-PAHSA (*P*), temperature (*T*), or interaction of factors (*I*). *Planned multiple comparison of the effect of 5-PAHSA at the given temperature statistically different at $P < 0.05$. *B*: Deuterium enrichment of glycerol measured in hydrolysates as above. *C*: Weight of eWAT. *D*: Body weight of animals. *E*: Fractional synthesis of TAG 48:0 (TAG 16:0_16:0_16:0). *F*: Relative levels of TAG 48:0, DAG 32:0 (16:0_16:0), and MAG 16:0. *G*: Profile of DAGs comparing CE and CE 5-PAHSA (CE + P) groups. Data are means ± SEM. *$P < 0.05$ by Student *t* test (n = 8–9). AU, arbitrary units; Veh, vehicle.

concentration and ionization efficiency of 5-PAHSA is lower compared with other PAHSAs. Therefore, the variations between experiments are larger than for other PAHSA regioisomers.

5-PAHSA Stimulates DNL and Lipid Remodeling in eWAT During CE

We took advantage of the in vivo deuterium labeling of cellular lipids in both TN and CE mice (see above) and analyzed (gas chromatography–mass spectrometry) the deuterium enrichment of *1*) FAs liberated by hydrolysis of eWAT lipid extract and *2*) plasma water to calculate the fractional rate of palmitate and stearate synthesis (Fig. 2*A*). The palmitate synthesis data showed a clear uptrend in both TN and CE mice, while the stearate data showed that 5-PAHSA stimulated DNL in the CE mice but not in the TN mice. Glycerol deuterium enrichment (Fig. 2*B*) and eWAT weight (Fig. 2*C*) were only affected by CE and not by 5-PAHSA treatment, indicating an effect independent of glyceroneogenesis. In addition, body weight was unaffected (Fig. 2*D*), leading us to conclude that the 5-PAHSA stimulation of DNL promoted not lipid storage but, rather, a form of energy-consuming DNL and TAG/FA remodeling.

We then performed a lipidomic analysis of deuterium-labeled intact lipids extracted from eWAT using LC-MS. Many lipid species were found to be enriched with deuterium, but the TAGs with shorter-chain FAs, especially TAG 16:0_16:0_16:0 (TAG 48:0) and 16:0_16:0_16:1 (TAG 48:1), were the most labeled species. In the CE mice, the fractional synthesis of TAG 48:0 was significantly higher than with the TN mice (Fig. 2*E*). The absolute levels of TAG 48:0 were similar in all the groups, but the levels of diacylglycerol (DAG) 16:0_16:0 (DAG 32:0), the lipolytic product of TAG 48:0, tended to be higher in the CE PAHSA group, while there was no difference in monoacylglycerol (MAG) 16:0 levels (Fig. 2*F*). The same pattern applied to TAG 48:1 (Supplementary Fig. 2*A*). Detailed analysis of DAG levels in the CE animals showed that 5-PAHSA induced a global increase in acylglycerol remodeling (Fig. 2*G* and Supplementary Fig. 2*B*).

FAHFAs Are Liberated From TAG Estolides During Lipolysis via ATGL

Yore et al. (8) reported the puzzling findings that levels of PAHSA increased during fasting while being associated with DNL during refeeding. Here, we observed that PAHSA levels were also elevated during CE when eWAT metabolism balances lipolysis and lipogenesis. Moreover, when we tried to measure PAHSA synthesis using the ^2H_2O approach during fasting in mice, we were unable to detect any deuterium enrichment of PAHSAs. This observation suggests that PAHSAs were released from a specific cellular pool during lipolysis. We hypothesized that the best chemical form for FAHFA storage would be a TAG estolide, a TAG-like molecule containing esterified FAHFA (30). To test this, we analyzed mouse eWAT lipid extracts and focused our LC-MS/MS measurement on the higher m/z range. Indeed, we found several analytes matching our in silico library and were able to identify them by the number of carbons and double bonds (Table 1). The structure of TAG estolide 16:0_18:2_18:0-(O-16:0) and the minor components containing 16:0/18:0/18:1/18:2 and -(O-18:1) acyls was assigned based on the structure and fragmentation patterns of a synthetic standard (Fig. 3*A* and *B* and Supplementary Fig. 3). It was identical to a recently reported PAHSA-containing TAG species (31). Several coeluting analytes were identified as FAHFA-containing TAG estolides, but further method development will be needed for detailed structural analysis including the position of acyl chains and hydroxy FA branching carbon of the isomers. We hypothesized that the FAHFAs might be released via lipolysis. When 3T3-L1 adipocytes differentiated in vitro were stimulated with forskolin, free FAHFA levels increased, while the ATGL inhibitor atglistatin (32) prevented the release of glycerol, FAs, and FAHFAs (Fig. 3*C*). Therefore, we explored this further using eWAT of ATGL-deficient (AKO) mice in the fed and fasted state (Fig. 3*D*). Interestingly, TAG estolides were higher in the fasted than in the fed state in WT animals, while this regulation was absent in the AKO mice and also the TAG EST levels were minimal in the AKO mice. Furthermore, the levels of free 5- and 9-PAHSA followed the same pattern (Fig. 3*E*). We also analyzed

Table 1—List of TAG estolides containing an FAHFA regioisomer detected in mouse eWAT

RT (min)	*m/z*	ID
5.67	1,126.9990	TAG EST 68:4
5.83	1,129.0135	TAG EST 68:3
5.96	1,131.0292	TAG EST 68:2
5.68	1,153.0143	TAG EST 70:5
5.84	1,155.0234	TAG EST 70:4
5.95	1,157.0442	TAG EST 70:3
5.83	1,157.0435	TAG EST 70:3
5.97	1,159.0581	TAG EST 70:2
5.68	1,179.0291	TAG EST 72:6
5.81	1,181.0447	TAG EST 72:5
5.94	1,183.0615	TAG EST 72:4
5.69	1,181.0448	TAG EST 72:5
5.83	1,183.0594	TAG EST 72:4
5.96	1,185.0747	TAG EST 72:3
6.01	1,187.0918	TAG EST 72:2
5.66	1,205.0460	TAG EST 74:7
5.69	1,207.0609	TAG EST 74:6
5.82	1,209.0753	TAG EST 74:5
5.95	1,211.0929	TAG EST 74:4

Identification is based on the number of carbons and double bonds, *m/z* as ammonium adducts $[M + NH_4]^+$, retention time (RT), MS/MS spectra, and fragmentation patterns of the synthetic standard TAG EST 16:0/16:0/9-PAHSA. EST, estolide; ID, identifier.

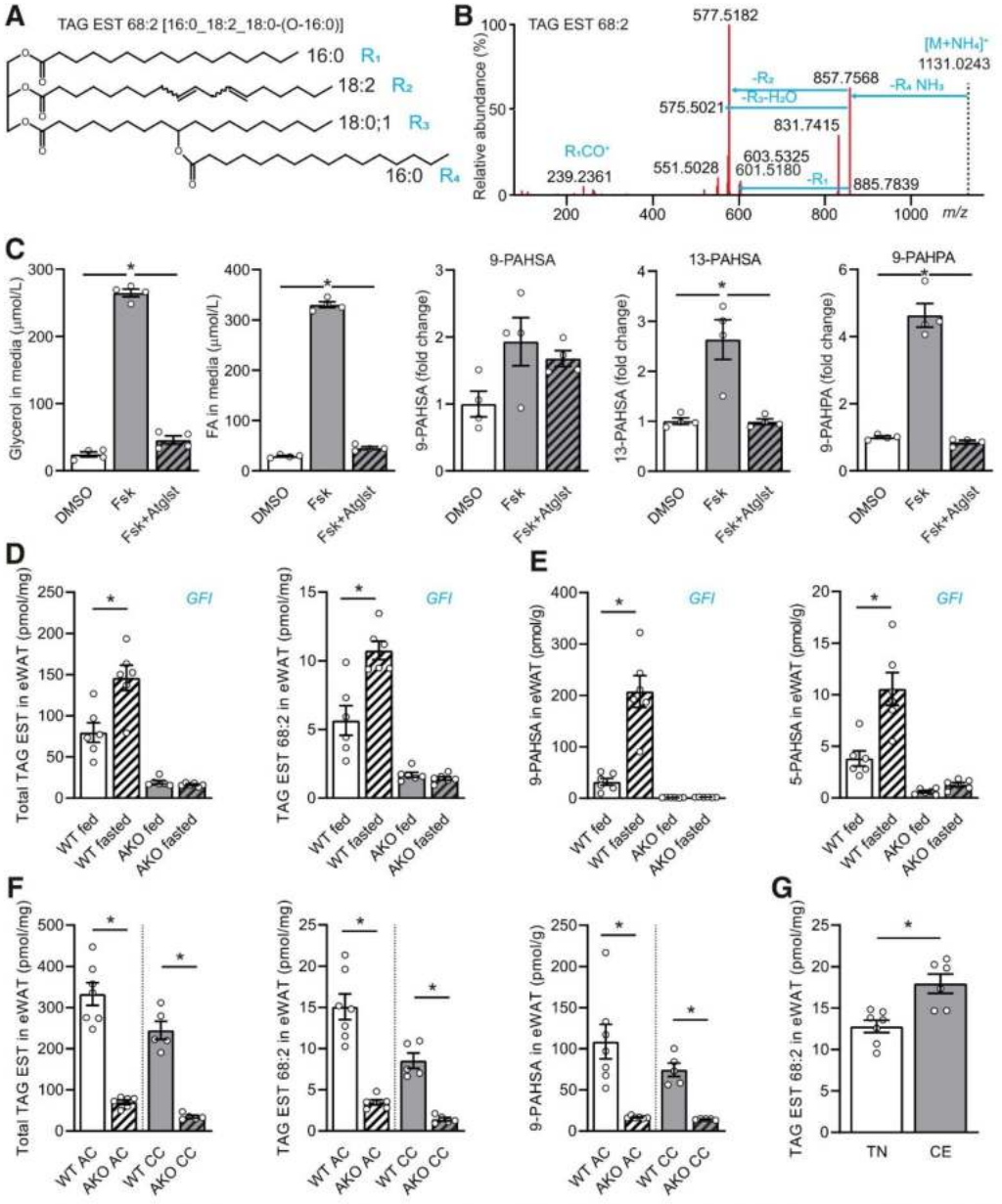

Figure 3—FAHFAs are liberated from TAG estolides during lipolysis. *A*: Tentative structure of a TAG estolide containing 9-PAHSA. This structure was deduced from MS/MS spectra observed in eWAT mouse extracts. The technique does not allow us to assign acyl position, double-bond position, and geometry. *B*: MS/MS spectrum of the measured analyte (see Supplementary Fig. 3 for annotations). *C*: 3T3-L1 adipocytes were preincubated with or without atglistatin (Atglst) and stimulated with forskolin (Fsk) for 2 h, and levels of glycerol and free FAs were determined in the media. Cells and media were extracted, and levels of free FAHFAs were measured using LC-MS/MS. DMSO indicates control cells incubated with DMSO. Data are means ± SEM ($n = 4$). One-way ANOVA with multiple comparison test (Dunnett) was used to compare means with the mean of the Fsk group. *Statistically different at $P < 0.05$. *D*: eWAT samples from WT and AKO mice were harvested in the fed and fasted state and analyzed using LC-MS/MS. Levels of TAG estolides containing PAHSAs (TAG EST 68:2 as a representative) and total levels of TAG EST are shown. *E*: Levels of free 9-PAHSA and 5-PAHSA in eWAT of WT and AKO mice analyzed in the same extract

TAG estolides and 9-PAHSA in WT and AKO animals exposed to acute cold (6 h) and chronic cold adaptation (3 weeks) (14) (Fig. 3*F*) as well as in TN and CE mice (Fig. 3*G*). Acute cold increased total levels of TAG estolides above those in the fasting state, and levels remained high in cold-adapted WT animals. In AKO mice, total TAG estolide levels slightly increased in response to cold, but values were much lower compared with WT mice (Fig. 3*F*). A difference in the changes in total TAG estolide levels, TAG estolide 68:2, and 9-PAHSA (as the representatives of all isomers) suggested that free FAHFA levels were increased through ATGL-mediated release from TAG estolides and that multiple levels of substrate specificity are involved.

5-PAHSA Modulates Both Lipogenesis and Lipolysis

Not all DAGs were affected the same way by 5-PAHSA (Fig. 2*G*), so we investigated whether there was an FA-specific metabolic pattern. Differentiated 3T3-L1 adipocytes were grown in a culture medium containing 50% ^2H_2O for 3 days with or without 5-PAHSA. The deuterium enrichment of TAGs showed that 5-PAHSA–treated cells incorporated more deuteria into shorter and saturated/monounsaturated "nascent" TAGs (Fig. 4*A*), but there were also differences in the position of incorporated deuteria within the molecule. Both the TAG acyl chains and the glycerol backbone can be labeled with deuterium through different pathways (33). We observed that TAG 48:0 was labeled mainly on the acyl chains (produced through DNL) and not as the M+3 isotopologue, which represents the TAG glycerol backbone labeled during glycolysis (Fig. 4*B* and Supplementary Fig. 4 for MS/MS spectra explanation).

Both lipolysis and FA esterification contribute to eWAT lipid metabolism during CE, balancing effects of catecholamines and insulin. Catecholamines stimulate release of FAs from WAT to supply brown adipose tissue heat production. The mice had access to food and therefore, in parallel, insulin stimulated glucose uptake into WAT to promote DNL and the TAG/FA cycle to buffer lipolysis. We examined the effect of 5-PAHSA on both pathways. 3T3-L1 adipocytes were grown with or without 5-PAHSA for 3 days, serum starved, preincubated with 10 nmol/L insulin or 5-PAHSA for 30 min, and subsequently exposed to forskolin for 2 h. Glycerol release into the medium showed that insulin partially countered lipolysis but 5-PAHSA slightly enhanced the effect of forskolin. Release of FAs was completely inhibited by insulin, while the effect of 5-PAHSA was not significant (Fig. 4*C*). Similar effects were observed with acute 5-PAHSA stimulation and isoproterenol on cultured and freshly isolated adipocytes (Supplementary Fig. 4*E* and *F*). Both markers of lipolysis suggested that 5-PAHSA affects FA re-esterification. Detailed analysis of media FA composition revealed a strong effect of 5-PAHSA on re-esterification of monounsaturated FA, especially 16:1 (i.e., palmitoleic acid), while the predominant FAs in TAGs (16:0, 18:1) accounted for net lipolysis (Fig. 4*D*). This effect was not observed using an acute preincubation with 5-PAHSA (Supplementary Fig. 4*G*). Furthermore, we tested the effect of combination of 5-PAHSA and insulin on isoproterenol-stimulated 3T3-L1 adipocytes (34) and found that 5-PAHSA stimulated glycerol release and counteracted insulin action (Fig. 4*E*). We conclude that 5-PAHSA significantly increased glycerol release and influenced FA release and re-esterification.

We added a $^{13}C_6$-glucose tracer in the forskolin experiment to explore metabolite labeling in the cells. We found that both insulin and 5-PAHSA prevented the decline during lipolysis of TAG 48:1, which proved to be a sensitive marker of DNL and lipid remodeling with the FA 16:1 (Fig. 4*F*). The M+3 isotopologue of TAG 48:1, corresponding to ^{13}C labeling of the glycerol backbone provided by glycolysis (Fig. 4*F* and Supplementary Fig. 4), proved that insulin drove glucose to the FA esterification pathway. In contrast, the levels of DAG 32:1, the lipolytic product of TAG 48:1, were higher in 5-PAHSA–treated cells, again pointing to TAG remodeling. Interestingly, the ^{13}C-labeling profile of citrate and its levels suggested that in contrast to insulin, 5-PAHSA directs glucose carbons to DNL instead of the glycerol backbone for esterification to TAGs (Fig. 4*F*).

5-PAHSA Primes Adipocytes for Glucose Metabolism and DNL

We next performed a glucose uptake experiment using $^{13}C_6$-glucose as a tracer and a major carbon source. Serum-starved 3T3-L1 adipocytes were preincubated with or without 5-PAHSA for 30 min, and then the metabolism of $^{13}C_6$-glucose was monitored for 0, 5, 10, and 15 min. The metabolites that were positively or negatively affected by 5-PAHSA are highlighted in green and red, respectively, in Fig. 5*A*. The levels of glucose and hexose 6-phosphates, which estimate glucose uptake, were higher in 5-PAHSA–treated cells, in agreement with previous data (8). 5-PAHSA stimulated ^{13}C enrichment and exchange of three carbon units within the lower part of the pentose phosphate pathway and glycolysis (Fig. 5*B*). In contrast, levels and labeling of

as above. Data are means ± SEM (n = 6). Two-way ANOVA with multiple comparison test (Tukey) was used. Letters in the graphs denote statistically significant effect of genotype (G), feeding status (F), or interaction of factors (I). *Multiple comparison of the effect of the condition with the given genotype statistically different at $P < 0.05$. Additional statistics are presented in Supplementary Table 1 for clarity. *F*: eWAT samples from WT and AKO mice were harvested in the fed state after acute cold (AC) (3 h) and chronic cold (CC) (3 weeks) and analyzed using LC-MS/MS. Levels of TAG estolides containing PAHSAs (TAG EST 68:2 as a representative), total levels of TAG EST, and levels of free 9-PAHSA are shown. Data are means ± SEM (n = 5–7). *$P < 0.05$ by Student t test. Vertical line separates AC and CC experiments. *G*: Levels of TAG EST 68:2 in eWAT samples from WT mice kept at TN or exposed to cold for 7 days as in Fig. 1. Data are means ± SEM (n = 6–7). *$P <$ 0.05 by Student t test. EST, estolide.

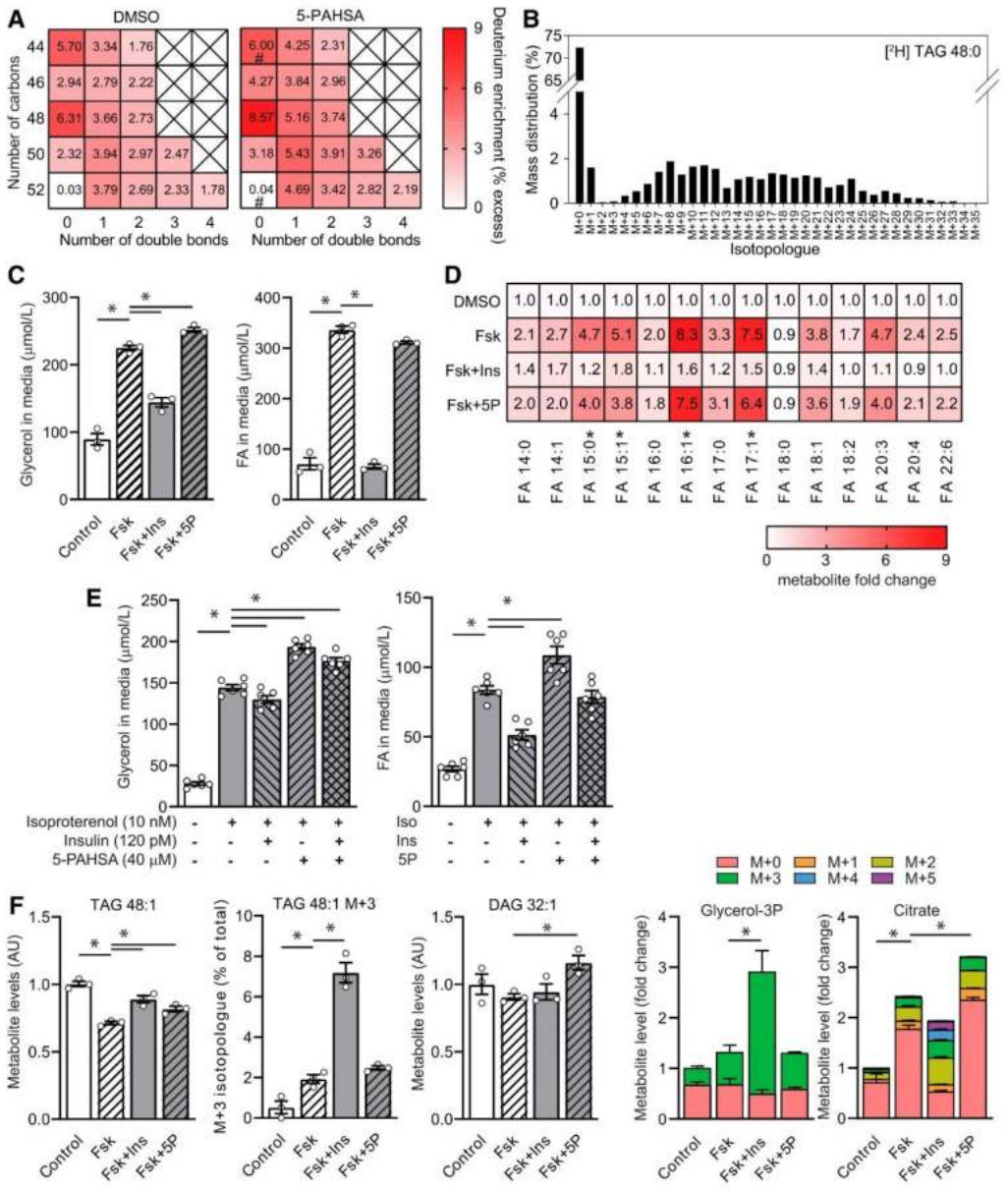

Figure 4—5-PAHSA–stimulated lipogenesis and selectively modulated lipolysis. *A*: 3T3-L1 adipocytes were grown in the presence or absence of 40 μmol/L 5-PAHSA for 3 days and labeled with 50% ^2H_2O. The deuterium enrichment of intact TAG molecules, marking the rate of lipogenesis, was measured using LC-MS/MS and is expressed as a heat map of TAGs sorted according to the number of carbons and double bonds. All fields were significantly different at $P < 0.05$ (Student *t* test, DMSO vs. 5-PAHSA) except for those marked with #, where the numbers in the fields are means ($n = 6$). *B*: Illustrative profile of deuterium-labeled TAG 48:0. *C*: Lipolysis: 3T3-L1 adipocytes were preincubated with insulin (Ins) or 40 μmol/L 5-PAHSA (5P) and stimulated with 1 μmol/L forskolin (Fsk). Glycerol and free FAs in media were measured using colorimetric kits. One-way ANOVA with multiple comparison test (Dunnett) comparing means with the mean of the Fsk group was used. Data are means ± SEM ($n = 3$). *Statistically different at $P < 0.05$. *D*: Lipolysis: levels of individual FAs in media were measured using LC-MS/MS; numbers in the fields are means ($n = 8$–9). *Statistically different at $P < 0.05$ by one-way ANOVA as above. *E*: Lipolysis: 3T3-L1 adipocytes were preincubated with 120 pmol/L Ins, 40 μmol/L 5P, or their combination and stimulated with 10 nmol/L isoproterenol

glycerol-3-phosphate were reduced by 5-PAHSA, consistent with the lipid-labeling data of Fig. 4. 5-PAHSA also channeled glucose carbons to the Krebs cycle through both pyruvate dehydrogenase and pyruvate carboxylase rather than to lactate. This was similar to the effect of insulin pretreatment on adipocytes, which primed metabolism for lipid synthesis and NADPH production through malate-pyruvate recycling (25). [4-^{2}H]glucose as a malic enzyme tracer confirmed that 5-PAHSA treatment significantly increased the ^{2}H-labeled fraction of cytosolic NADPH compared with control cells (Supplementary Fig. 5). The intermediates of the Krebs cycle decreased over time, probably due to of absence of glutamine in the medium and to lack of related anaplerotic reactions.

We then repeated the uptake experiment to test the effect of 10 nmol/L insulin (Fig. 5*C*) and quenched metabolism after 10 min, when the differences were maximal. Although insulin was a much stronger stimulant of glucose metabolism than 5-PAHSA, the specific 5-PAHSA pattern of three-carbon-unit metabolism was preserved. 5-PAHSA diverted carbon flux from glycerol-3-phosphate and rapid FA esterification downstream to the Krebs cycle.

Adipocytes use several carbon sources to supply the demands of DNL, and glutamine is an important player. Therefore, we repeated the glucose uptake experiment in the presence of 5.5 mmol/L glucose and 4 mmol/L $^{13}C_5$-glutamine and quenched metabolism after 10 min. When supply of major carbon sources was not limited, 5-PAHSA–treated adipocytes used significantly more carbon from glutamine (Fig. 6*A*). Interestingly, $^{13}C_5$-glutamine was metabolized both into succinate (M+4 labeling) and into M+5 citrate by reductive carboxylation, in good agreement with previously reported results (35) (Fig. 6*B* and *C*).

The above-described effects of 5-PAHSA on adipocyte metabolism are summarized in the scheme of Fig. 6*D*. While insulin (in red) stimulates glucose uptake and the utilization of glycerol-3-phosphate for rapid TAG synthesis, 5-PAHSA (in green) supports a longer, more energy-consuming path that involves DNL and TAG remodeling.

DISCUSSION

Here we demonstrate that 5-PAHSA, a lipokine with beneficial metabolic effects, primes adipocytes for glucose utilization and DNL while enhancing metabolically specific TAG/FA cycling. We used a combination of mouse experiments with metabolic labeling of lipogenesis and lipolysis together with dynamic metabolomics in cultured adipocytes to explore the pathways connecting glucose and lipid metabolism.

The energy-consuming combinations of TAG/FA cycling and DNL in adipocytes represent key components of a metabolically "healthy adipocyte" and are induced by CE (6,36). Our initial observation that CE increased PAHSA levels in eWAT suggested that these lipokines and DNL could be the mechanistic link underlying the metabolic changes. The experiment with 5-PAHSA oral gavage in mice at two housing temperatures with deuterium labeling of lipid synthesis proved that the effect of 5-PAHSA at TN is minimal, while CE is associated with higher PAHSA levels in eWAT. In addition, 5-PAHSA gavage increased levels of other PAHSA regioisomers in eWAT.

5-PAHSA stimulated DNL beyond the effect of CE, but it did not potentiate FA re-esterification with glucose-derived glycerol (direct TAG synthesis, fat accumulation). There was limited deuterium incorporation (only up to four deuteria) into palmitic and stearic acids in CE mice. Therefore, the effect observed at the level of FA hydrolyzed from TAGs was not clear at the level of intact lipids, where the deuterium tracer was diluted among all TAG FA combinations and insufficient tracer sensitivity was a limitation. Total metabolite level analysis suggested that 5-PAHSA affected lipid remodeling between TAGs and DAGs. The simplest interpretation is that 5-PAHSA stimulated de novo lipid synthesis from acetyl-CoA and acylglycerol remodeling, inducing a futile TAG/FA cycle that did not support lipid storage. In line with this, no effect on body weight was observed in mice chronically treated with PAHSAs (37). Our data suggest that two concurrent overlapping mechanisms were stimulated: *1*) futile (energy wasting) TAG/FA cycling (6), which builds and breaks TAG completely, and *2*) lipid remodeling of the TAG acyls, including FAHFAs and TAG estolides. Therefore, with respect to the metabolic rearrangements that are associated with obesity, 5-PAHSA positively affects adipocyte metabolism.

FAHFA levels rise during CE and with fasting in mice and rats (8,18). Because we were unable to detect any deuterium-labeled PAHSAs in either CE animals or fasted/refed mice, we looked for a metabolic storage pool from which PAHSA would be liberated during stimulated lipolysis. Using lipidomic profiling, we were able to identify several members of the TAG estolide lipid class in which PAHSAs are esterified to the glycerol backbone alongside two other acyl chains. The structural patterns were confirmed using a synthetic standard. We estimate that there are dozens of TAG estolides that contain other FAHFAs, but a targeted analytical approach using multistage fragmentation (MS^4) will be needed to explore their complexity. It is difficult to separate all FAHFA regioisomers, and

(Iso). Glycerol and free FA in media were measured using colorimetric kits. One-way ANOVA with multiple comparison test (Dunnett) comparing means with the mean of the Iso-alone group was used. Data are means ± SEM (n = 6). *Statistically different at $P < 0.05$. *F*: Lipolysis: levels of metabolites related to TAG 48:1 (16:0_16:0_16:1) were measured in the harvested cells using LC-MS/MS. $^{13}C_6$-glucose was used as a tracer and citrate as a precursor of DNL. Data are means ± SEM (n = 3). *Statistically different at $P < 0.05$ by one-way ANOVA as above. AC, acute CE; AU, arbitrary unit; CC, chronic CE; Glycerol-3P, glycerol 3-phosphate.

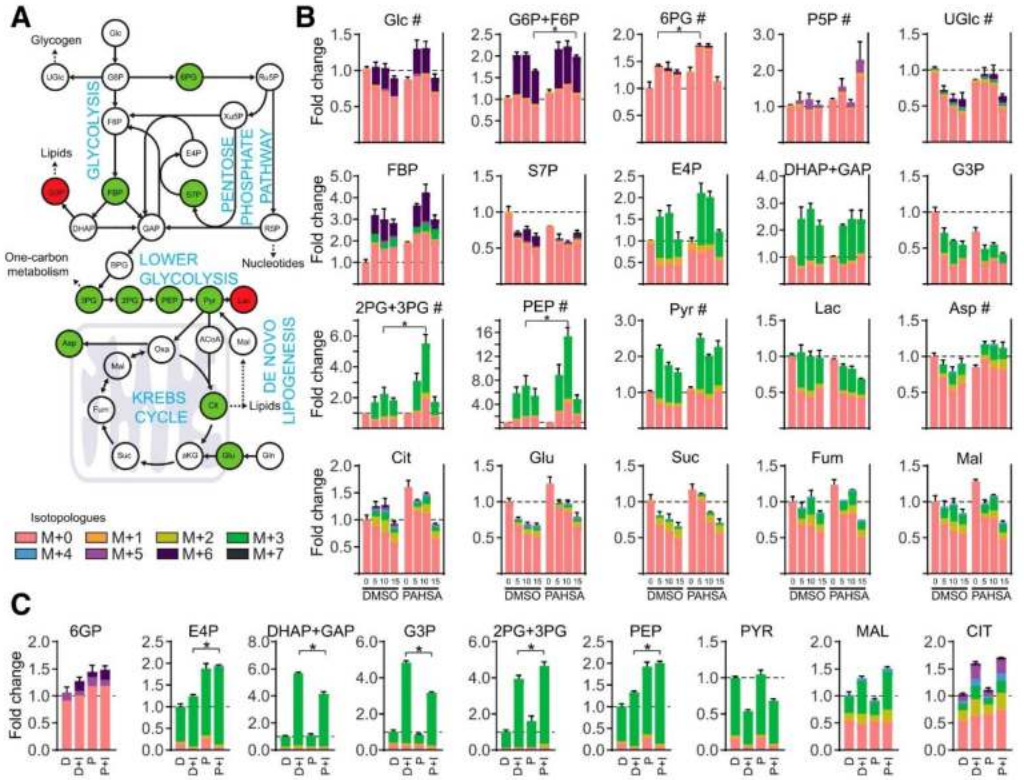

Figure 5—5-PAHSA primes adipocytes for glucose (Glc) utilization toward DNL and away from FA esterification. *A*: Glc uptake. Schematic representation of the metabolic pathways affected by 5-PAHSA treatment. Statistically significant differences between DMSO and 5-PAHSA treatments are highlighted in green (upregulation) and red (downregulation) (see below). Major pathways are labeled in blue. See Supplementary Table 2 for abbreviations of the metabolites. Once Glc enters the cell and is phosphorylated, the majority of the G6P is metabolized through glycolysis to three carbon units (e.g., GAP), but some Glc molecules are processed through ancillary pathways (e.g., the pentose phosphate pathway, glycogen synthetic pathway, lipid synthesis). GAP can be further metabolized to Pyr through the lower glycolysis pathway and converted to Lac or oxidized in the Krebs cycle. Mitochondrial Cit can be exported to the cytosol and serve as a substrate for DNL, while Pyr-Mal recycling generates NADPH to fuel the DNL. *B*: Glc uptake (UGlc): time profiles of isotopologues of selected metabolites. $^{13}C_6$-Glc uptake was measured in serum-starved 3T3-L1 adipocytes preincubated without DMSO or 5-PAHSA (40 μmol/L), and the metabolism was quenched at specific time points (0, 5, 10, and 15 min). Whole bars indicate means ± SEM of the total metabolite levels, and individual isotopologues are colored according to the key (n = 3). Glc was the only major source of carbons. Two-way repeated-measures ANOVA with Sidak multiple comparison test was performed on total metabolite levels: *statistically significant difference between DMSO and 5-PAHSA at the specific time point, #statistically significant interaction between time and treatment; additional statistics are presented in Supplementary Table 2 for clarity. *C*: Glc uptake: the same experiment was repeated with or without insulin (10 nmol/L), and cell metabolism was quenched at 10 min (n = 6). Whole bars indicate means ± SEM of the total metabolite levels, and individual isotopologues are colored according to the key (n = 6). Two-way ANOVA with Tukey multiple comparison test was performed on total metabolite levels: *Statistically significant difference between DMSO and 5-PAHSA in combination with insulin. For clarity, additional statistics are presented in Supplementary Table 3. D, DMSO; D+I, DMSO and insulin; P, 5-PAHSA; P+I, 5-PAHSA and insulin.

separation and structural analysis of low-abundance TAG estolides is at the edge of current technological limits. Our data are in agreement with and confirm those recently reported on FAHFA TAGs (identical to TAG estolides) (31).

Liberation of FAHFAs from TAG estolides during lipolysis and fasting/CE was prevented by inhibition or absence of ATGL. The FAHFA increase during fasting/CE is linked to the lipolytic activity of ATGL or hormone-sensitive lipase (31). Low levels of TAG estolides in AKO mice suggest that ATGL might also be involved in their metabolism via acyltransferase activity (38,39), independent of acyl CoA:DAG acyltransferases (31). The effect of atglistatin on forskolin-treated adipocytes was more pronounced on free FAHFA levels, and TAG estolides were not changed during this short incubation (data not shown), further

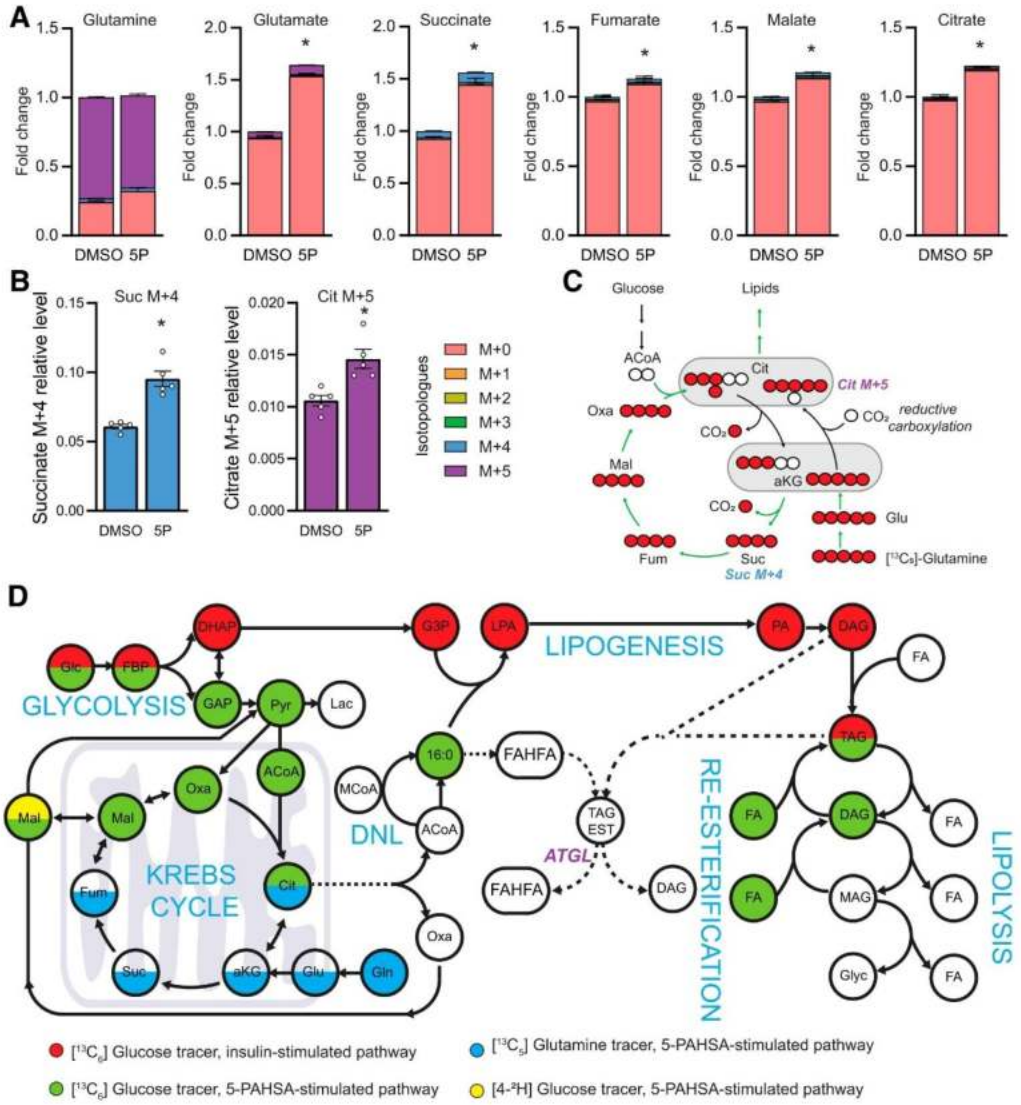

Figure 6—5-PAHSA promotes glutamine (Gln) utilization for DNL. *A*: Glucose (Glc) uptake II was measured in serum-starved 3T3-L1 adipocytes preincubated with or without 5-PAHSA (40 μmol/L), and metabolism was quenched at 10 min. $^{13}C_5$-Gln (4 mmol/L) was used as a tracer. Levels of traced metabolites and their isotopologue profiles are colored according to the key. Bars indicate means ± SEM of the total metabolite levels. *B*: Glc uptake II: ^{13}C-labeled intermediates of Krebs cycle, succinate (Suc), and citrate (Cit), illustrating the fate of Gln carbons. Data are means ± SEM ($n = 5$). *$P < 0.05$ by Student *t* test. *C*: Atom transition map depicting a model of carbon labeling within the first turn of the Krebs cycle. $^{13}C_5$-Gln carbons enter the Krebs cycle as α-ketoglutarate (aKG), produce $^{13}C_4$-Suc (Suc M+4 [as in *B*]), and label lipids (green path). Alternatively, Cit M+5 (as in *B*) is formed through a reductive carboxylation process. *D*: Metabolic scheme summarizing metabolite labeling data and the effects of 5-PAHSA. Dotted lines represent a sequence of reactions. ACoA, acetyl-CoA; LPA, lysophosphatidic acid; MCoA, malonyl-CoA; Oxa, oxaloacetate. See Supplementary Table 2 for abbreviations of the other metabolites.

supporting a dual role of ATGL in FAHFA metabolism. The new role of ATGL in controlling the release of FAHFAs from TAG estolide stores in adipocytes, together with the stimulation of TAG/FA remodeling by FAHFAs that we describe, is compatible with the previously unexplained observation that ATGL is required for

stimulation of DNL, TAG/FA cycling, and mitochondrial electron transport by the β3-adrenergic pathway in adipose tissue (29). Innovative lipidomic analyses capable of quantifying individual TAG estolides and FAHFA isomers will be needed to explore substrate specificity of ATGL and its contribution to FAHFA metabolism.

A study of lipogenesis and lipolysis in 3T3-L1 adipocytes showed that the prolipogenic effect of 5-PAHSA is significant but does not promote excess lipid storage, in agreement with previous data (40). Newly synthesized short FAs are primarily incorporated into short-chain TAGs, probably on the surface of small lipid droplets, allowing high turnover. Intriguingly, although the effect of 5-PAHSA on net lipolysis was mild, the selective re-esterification of monounsaturated FAs highlighted fine-tuning of the TAG/FA remodeling cycle and the ability of 5-PAHSA to spare or rebuild specific TAGs, possibly also TAG estolides.

A comparison with the effect of insulin using the ^{13}C-tracer data, specifically involving the labeling pattern of glycerol-3-phosphate and citrate, prompted us to thoroughly explore the glucose utilization pathway. Dynamic metabolomic profiling of 3T3-L1 adipocytes preincubated with insulin showed how cells select specific metabolic pathways to optimize metabolic flux and secure NADPH-producing reactions for DNL (25). We performed an analogous experiment with 5-PAHSA preincubation and found that the lipokine activated similar pathways, with an important difference in the fate of glucose carbons. In contrast to insulin, 5-PAHSA specifically reduced carbon flux to glycerol-3-phosphate, away from TAG accumulation, while enhancing carbon flux to the Krebs cycle and DNL precursors. Although the effect of 5-PAHSA was small compared with the net effect of insulin, the critical metabolic pattern was observed even when both 5-PAHSA and insulin were present. The combination of insulin and PAHSA could potentially be used therapeutically in metabolic situations where glycolysis is dysregulated.

In contrast to Krycer et al. (25), we performed the labeling experiment in media with limited carbon sources to unmask 5-PAHSA effects. The addition of glutamine, which is an important lipogenic source in adipocytes, revealed that the flux toward DNL might be even higher when the Krebs cycle can use anaplerotic substrates. This alternative fate of glucose carbons might lower fat accumulation in WAT and contribute to other antidiabetic effects of PAHSAs (8).

In summary, we propose that FAHFAs are synthesized via DNL through hydroxylated intermediates (18) and stored as TAG estolides in lipid droplets. ATGL-mediated lipolysis releases free FAHFAs, which limit FA esterification into TAG while promoting TAG acylglycerol remodeling and the fine-tuning of lipolysis. These effects prime adipocytes for glucose metabolism, in a different way from insulin, once it becomes available, and promote metabolically "healthy adipose tissue."

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