Nutritional properties of protein sources on diet-induced obesity, hepatic lipid accumulation, and fatty acid composition in mice

Kristin Røen Fauske

Thesis for the Degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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Scientific environment

The present PhD work was completed at the Institute of Marine Research (IMR), Bergen, Norway, formerly known as the National Institute of Nutrition and Seafood Research (NIFES). The National Institute of Nutrition and Seafood Research and the IMR were merged into one institute on the 01.01.18 under the name of the IMR. This PhD work was undertaken in cooperation with the Department of Biomedicine, the University of Bergen, Norway.

My main supervisor was Prof. Lise Madsen, and my co-supervisors were Dr. Scient. Bjørn Liaset and Prof. Livar Frøyland.

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Contents

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Abstract

The prevalence of obesity has increased rapidly, and excess adiposity is associated with comorbidities that increase the risk of mortality. An energy-dense diet that is high in sugar and fat is one of the major explanations for the increased levels of obesity. Importantly, obesity is preventable, and diet represents one significant tool for curbing the development of excess adiposity and its related comorbidities. Epidemiological studies have indicated that the intake of various seafood, dairy, and plant-derived protein-rich food is associated with protection against long-term weight gain. In addition, cod contains the majority of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) as phospholipids (PLs), which, it has been suggested, are more bioavailable and can promote higher anti-obesogenic effects than triacylglycerol-bound EPA and DHA. In line with this, it is important to assess the role of food that could influence primary prevention of obesity. The overall aim of this thesis is to determine the impact of different sources of dietary protein for preventing diet-induced obesity and influence on metabolic changes associated with obesity development in mice.

The studies were conducted by performing dietary trials with the obesity-prone C57BL/6J mice. Various protein sources were blended in high-protein diets, while cod fillets and pork sirloins were blended in Western diets. In addition, the nutritional effects of EPA and DHA in cod were investigated by supplementing pork-containing Western diets with n-3 PUFAs to match the levels of the cod-containing diet. Body weight, energy intake, body composition, and glucose tolerance testing were measured during the experiments. The mice were sacrificed, and adipose tissue depots and liver were dissected out. Analyzes of gene and protein expression, histological investigation, and lipid and fatty acid analyses were performed in the tissues.

By comparing proteins from casein, soy, cod, beef, chicken, and pork in a high-fat/high-protein diet, we observed that casein was the only protein that completely protected against high-fat diet-induced obesity in comparison with mice fed on a low-fat diet. Casein was particularly effective in preventing the whitening of interscapular

brown adipose tissue (iBAT) and maintaining Uncoupling Protein 1 (UCP1) expression. The terrestrial proteins from pork, chicken, and meat promoted fat mass gain more than proteins from cod and soya. In addition, cod was the only protein source that significantly decreased energy intake. Furthermore, in a Western diet, we reported that exchanging pork with cod as protein source reduced energy intake by 6%, attenuated adiposity and hepatic lipid accumulation, and lowered plasma levels of 2-AG and AEA as well as the n-6:n-3 ratio in RBCs and hepatic polar lipids. Frozen storage decreased the PLs' content in the cod fillets and thus could affect the ability of cod to attenuate obesity. Compared with mice fed on a casein-based Western diet, mice fed on fresh cod, but not frozen cod with a reduced PL content, increased obesity development. In addition, exchanging pork with fresh cod did not protect against diet-induced obesity or reduce energy intake. Supplementing a pork-containing diet with PL-bound but not TAG-bound EPA and DHA to the level of a fresh cod diet led to significantly higher body weight, fat mass, and liver mass in mice compared to a diet without supplementation.

Based on the data of this research, the protein source exerted pronounced effects in attenuating obesity in the intake of high-protein diets and Western diets. Casein effectively protected against high-fat induced obesity. Despite the low content, the level of EPA and DHA in the cod-containing Western diet was sufficient to replace ARA in RBCs and hepatic polar lipids and to modulate the endocannabinoid profile. However, the frozen storage of cod seems to modulate the ability of cod intake to attenuate obesity. The combined effect of PL-bound n-3 PUFA and pork in promoting obesity needs further investigation since pork is a major component of the Western diet.

List of publications

Paper I

Liisberg*, U., Myrmel*, L. S., Fjære, E., Rønnevik, A. K., Bjelland, S., **Fauske, K. R.,** Holm, J. B., Basse, A. L., Hansen, J. B., Liaset, B., Kristiansen, K., Madsen, L. (2016): The protein source determines the potential of high protein diets to attenuate obesity development in C57BL/6J mice. *Adipocyte* 2016, 5(2), 196-211.

Paper II

Liisberg, U*., **Fauske*, K. R**., Kuda, O., Fjære, E., Myrmel, L. S., Norberg, N., Frøyland, L., Graff, I. E., Liaset, B., Kristiansen, K., Kopecky, J., Madsen, L. (2016): Intake of a Western diet containing cod instead of pork alters fatty acid composition in tissue phospholipids and attenuates obesity and hepatic lipid accumulation in mice. *Journal of Nutritional Biochemistry* 2016, 33, 119-127.

Paper III

Fauske, K. R., Bernhard, A., Fjære, E., Myrmel, L. S., Frøyland, L., Kristiansen, K., Liaset, B., Madsen, L. (2018): Effects of frozen storage on phospholipid content in Atlantic cod fillets and the influence on diet-induced obesity in mice. *Nutrients* 2018, 10(6), 695.

*Shared first authors

The Roman numerals henceforth refer to the papers.

Abbreviations

2-AG	2-Arachidonoylglycerol
AEA	*N*-arachidonoylethanolamine
ANOVA	Analysis of variance
ARA	Arachidonic acid (20: 4n-6)
BCAAs	Branched chain amino acids
CB1 (or 2)	cannabinoid receptor type 1 (or 2)
DHA	Docosahexaenoic acid (22: 6n-3)
EPA	Eicosapentaenoic acid (20: 5n-3)
FFAs	Free fatty acids
HPTLC	High-performance thin -layer chromatography
(i) BAT	(Interscapular) brown adipose tissue
IMR	Institute of Marine Research
(i)WAT	(Inguinal) white adipose tissue
LA	Linoleic acid
(LC) PUFAs	(Long-chain) Polyunsaturated fatty acids
NAFLD	Non-alcoholic fatty liver disease
(O)GTT	(Oral) Glucose tolerance test
PLs	Phospholipids
RBCs	Red blood cells
SEM	Standard error of the mean
RER	Respiratory exchange ratio
SPE	solid phase extraction
TAG	Triacylglycerol
UCP1	Uncoupling Protein 1

List of figures

Background

Obesity

Obesity is as a condition in which fat accumulates in the body to a level where it may impair health [1]. The co-morbidities associated with excess adiposity are a major public health concern and include type II diabetes, liver disease, cardiovascular disease, and certain forms of cancer [2,3]. Importantly, both overweight and obesity are associated with increased all-cause mortality [4,5]. The most common screening tool for measuring obesity entails calculating the body mass index (BMI; weight/height^2); a BMI between 25 and 30 kg/m² is classified as overweight, and a BMI greater than or equal to 30 kg/m² as obese [1]. Globally, over the past four decades obesity has increased dramatically, and the prevalence has almost tripled [1,6]. Today, more people are obese than underweight [1]. In 2016, it was estimated that 39% of adults aged 18 years and above were overweight, while 13% were obese [1]. It should be noted that if these trends continue, estimations have shown that by 2025 global obesity prevalence will reach 18% in men and surpass 21% in women [6].

The diet increases the risk of long-term weight gain

The cause of obesity is multifactorial, but suboptimal nutrition and physical inactivity have been explained as the two major factors responsible for the obesity epidemic [7]. Humans in contemporary society live in a nutritional environment that differs from that for which our genetic constitution was selected [8]. In particular, profound changes in diet and lifestyle conditions began with the introduction of agriculture over 10,000 years ago. It has been suggested that the dietary shift has occurred too recently on an evolutionary timescale for the human genome to adapt [9,10]. These observations have led to the hypothesis that some humans lack genetic protection against weight gain [11]. Over the past four decades, and parallel with the increased prevalence of obesity, the worldwide diet has become more energy-dense with a high amount of refined sugar and vegetable oils. This diet has been referred to as a Western diet [9]. Nutritional science argues that one of the major drivers of obesity is the chronic consumption engendered by this Western type diet [12,13]. As the human genome is not genetically

adapted to this particular diet pattern and lifestyle [10], it has been shown that the Western diet pattern is associated with the obese phenotype [14]. Strong positive associations between an obese-phenotype and the intake of starches, refined grains, and processed foods have been reported, whereas the intake of vegetables, nuts, and whole grain has been associated with weight loss [15]. In line with this distinction, a cross-sectional study reported that individuals consuming the Western diet, which is characterized by a high consumption of refined grains, processed meat, french fries, and regular soft-drinks, were more likely to be obese than those who followed a prudent eating pattern, characterized by a higher consumption of non-hydrogenated fats, vegetables, eggs, fish, and seafood [14].

Preventing obesity through diet

It is well known that obesity is largely preventable through diet and physical exercise and, importantly, that it is easier to prevent than reverse [16,17]. The theory that obesity is caused by an imbalance in energy intake and expenditure, has been suggested to be oversimplified and that it is rather the quality and types of food consumed that influence pathways related to weight homeostasis, such as satiety, hunger, glucose-insulin responses, and energy expenditure (Figure 1) [18]. Studies have shown that a short-term weight loss can be achieved by almost any calorie-reduced diet but that such a diet may not be biologically or behaviorally relevant for maintaining a lean phenotype from a long-term perspective [19]. In addition, the long-term prospects of maintaining weight loss in obese subjects are associated with a high risk of failure and weight regain [20]. Accordingly, the prevention of long-term weight gain, which often occurs subtly (0.45 kg/year) [15] has been suggested to be a primary, crucial strategy for reducing the prevalence of obesity and its associated co-morbidities, over and above short-term assessments of weight loss in obese individuals [19,21]. Since diet is a fundamental risk factor for developing obesity, it is crucial to understand how different sources of food may prevent long-term weight gain and, thus, the development of obesity [18].

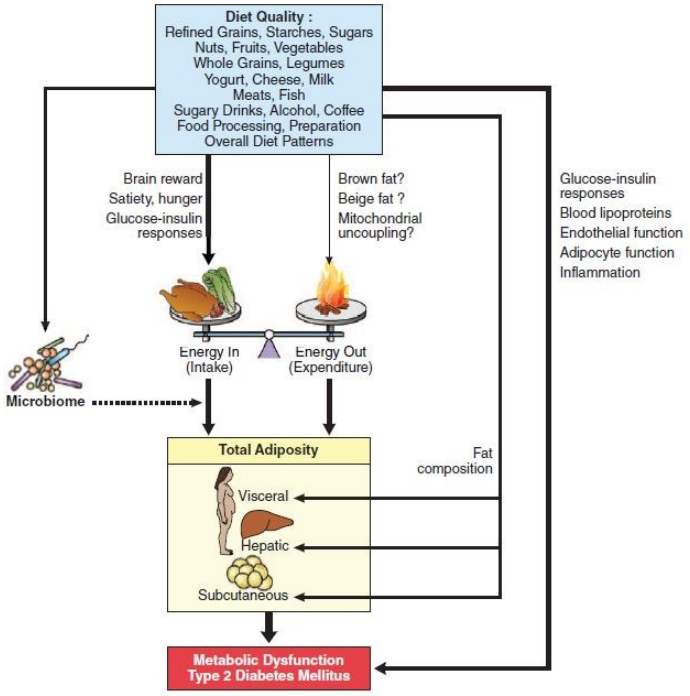

Figure 1 (as in [18]). Diet quality influences the risk of adiposity and metabolic dysfunctions through multiple pathways. This includes altering energy intake, energy expenditure, microbiome-host interactions, and glucose-insulin responses.

Adipose tissue

As obesity development has increased worldwide, adipose tissue has become a subject of increased research in the last decades. The discovery that leptin and adiponectin are secreted by adipocytes has shifted the scholarly focus on the function of adipose tissue—from being a simple storage depot of energy to being, in addition, an important endocrine organ, with multiple metabolic roles in regulating whole-body physiology [22,23]. Adipose tissues are organized as a large organ with a discrete anatomy, specific vascular and nerve supplies, complex cytology, and a high physiological plasticity [24]. The adipose organ contains several subcutaneous and visceral depots and can be broadly divided into white adipose tissue (WAT), brown adipose tissue (BAT) [25], and, additionally, white areas that contain a variable amount of brown-like adipocytes (also called beige or brite adipocytes) [26].

White adipose tissue

The WAT depot stores energy from nutrient overload in a single large lipid droplet in the cytoplasm within the adipocytes [25]. These unilocular adipocytes are surrounded by various different cell types, the WAT depot is connected to adrenergic nerve fibers and blood vessels [22]. When nutrients are required, triacylglycerols (TAGs) in adipocytes undergo hydrolyzation, and non-esterified fatty acids are then secreted into the bloodstream and supplied to other tissues [27]. In addition, adipocytes secrete multiple different proteins with endocrine functions that are designated as adipokines [28]. Among others, leptin, the most studied adipokine, regulate energy intake by suppressing hunger, adiponectin modulate glucose regulations and the adipokines TNF-α, IL-6, and MCP-1 exert inflammatory responses and cell migration. Accordingly, an imbalanced secretion of some of these adipokines is associated with obesity and metabolic dysfunctions [29].

In obese individuals, WAT undergoes profound changes: it expands, becomes dysfunctional, and develops a low-grade inflammatory state. White adipocytes expand rapidly to alterations in a surplus nutrient status [22], and the expansion of white adipocytes occurs both through an increase in size of existing adipocytes (hypertrophy) and the formation of new white adipocytes (hyperplasia) from preadipocytes [30]. Hyperplasia and/or hypertrophy of adipocytes is strongly associated with low-grade inflammation, which is, in turn, associated with insulin resistance through a variety of molecular mechanisms [31]. In human obesity as well as in genetically-altered and high-fat diet-induced mouse models, obesity-associated inflammatory changes in white adipose tissue are predominantly due to macrophage infiltration [32,33]. M1-stage macrophage-infiltration is regarded as a key mechanism for insulin resistance in obesity [34]. The majority of macrophage infiltration arranges around dead adipocytes and forms characteristic crown-like structures. Furthermore, in addition to the absolute fat quantity, cellular composition, the rate of dysfunction (Figure 2) and distribution of the adipose tissue are important predictors of the metabolic consequence of obesity [31]. Visceral fat is infiltrated more by low-grade inflammation than subcutaneous fat is [35]; the result is that visceral adipose tissue has been shown to have a more

important role in the development of insulin resistance [36]. Obesity may lead to lipid accumulation within non-adipose tissues, which then form ectopic fat depots, especially in the liver, skeletal muscles, heart, and pancreas, as well as within blood vessel walls. Consequently, this may alter the function of organs and lead to pathological conditions [22]; in addition, ectopic fat accumulation is strongly related to insulin resistance [37].

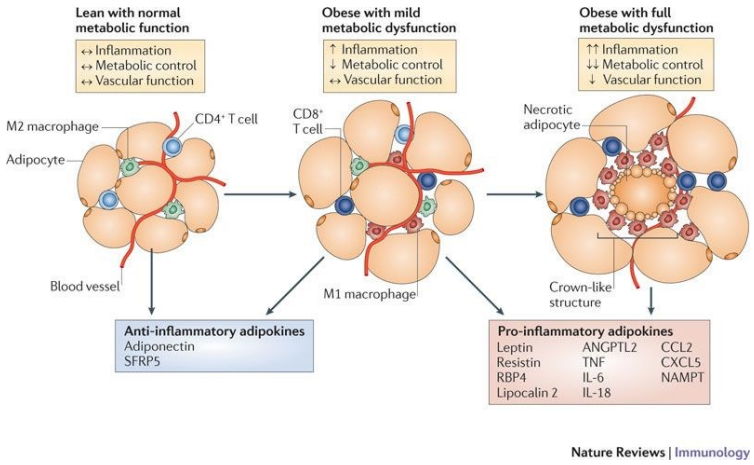

Figure 2 (as in [31]). Adipose tissue from lean individuals with normal metabolic function, and adipose tissue from obese individuals with mild or full metabolic dysfunction. In states of obesity, adipose tissue generates large amounts of pro-inflammatory factors. Obese individuals possessing adipose tissue with mild metabolic dysfunction have improved metabolic parameters, diminished inflammatory marker expressions, and better vascular functioning compared to individuals who have metabolically dysfunctional adipose tissue. Metabolically dysfunctional adipose tissue can be associated with higher levels of adipocyte necrosis, (M1 macrophages are arranged around these dead cells in crown-like structures).

Brown adipose tissue

BAT is composed of multilocular brown adipocytes that contain numerous large mitochondria and store TAG in small vacuoles. Brown adipocytes use lipids and carbohydrates to produce heat through non-shivering thermogenesis. Brown adipocytes convert energy to heat by the tissue-specific uncoupling protein 1 (UCP1);

this protein uncouples oxidative phosphorylation from ATP synthesis, which results in thermogenesis [35]. The molecular pathway for thermogenesis in brown adipocytes is promoted by norepinephrine, which acts on β3-adrenoceptors in brown adipocytes [38]. It has been demonstrated that cold-exposed or β-adrenoceptor agonist treated rodents express UCP1 in WAT depots [39,40]. This brown-like adipocytes located within the WAT is often termed beige adipocytes and have a low expression of UCP1 in a basal unstimulated state; however, they respond to cAMP so as to activate a thermogenic program to reach levels similar to those seen in the brown cells [41]. In obese individuals, BAT content and activity decline, mainly as a result of the conversion of brown adipocytes to white-like unilocular cells [42]. Both classical brown and beige adipocytes have been shown to have therapeutic potential for protecting against obesity and type II diabetes. This is also illustrated in genetically manipulated mice with higher brown and beige adipocytes that are protected against obesity and diabetes [43].

Obesity-related co-morbidities

Non-alcoholic fatty liver disease

The liver regulates the energy metabolism in response to insulin and other metabolic hormones. Obesity is strongly related to dysfunctions in insulin signaling and in other metabolic functions that cause or predispose an individual to non-alcoholic fatty liver disease (NAFLD) [44]. Non-alcoholic fatty liver disease is the broad term for those liver abnormalities that are characterized by an increase in intrahepatic TAG content (hepatic steatosis) [45,46]. Steatosis is chemically defined as occurring when intrahepatic TAG content is greater than 5% of liver weight [47]. Non-alcoholic steatohepatitis (NASH) is the form of liver disease that occurs when excess fat is present in the liver and is accompanied by inflammation and possibly fibrosis; consequently, NAFLD may ultimately lead to irreversible liver failure [48].

Obesity is closely linked to development of NAFLD [45]; therefore, the prevalence of NAFLD is growing in parallel with the global obesity epidemic [49]. Non-alcoholic fatty liver disease is now regarded as the most prevalent chronic liver disease in the

developing world. Current epidemiological data shows that the prevalence of NAFLD in the populations of the US and Europe has reaches 30% and 24%, respectively [49]. Currently, lifestyle interventions including weight loss and increased physical activity are used as therapeutic agents in the treatment of NAFLD [50].

The liver does not serve as a storage depot for lipids, and the concentration of hepatic TAG is low under physiological conditions [51]. The small concentrations of fatty acids are incorporated into lipid droplets and membrane structures of hepatocytes [44]. Levels of TAG in hepatocytes are decreased by mitochondrial fatty acid oxidation and through packaging and secretion of VLDL particles that are secreted into the circulation [48]. Obesity may lead to an imbalance in the hepatic fatty acid regulation and may progress ectopic accumulation—primarily through TAG in the liver [52]. An increase in intrahepatic TAG content occurs when the rate of hepatic fatty acid uptake from plasma and *de novo* lipogenesis in the liver is greater than the rate of fatty acid oxidation and export within VLDLs [45,53].

The pathology of NAFLD is illustrated in Figure 3. In obese individuals, the increased release of non-esterified fatty acids from adipose tissue to the liver results in an increase in hepatic fatty acid uptake. The non-esterified fatty acids is mainly taken up in the liver by fatty acid transport proteins (FATPs) and fatty acid translocase/cluster of differentiation 36 (FAT/CD36) [54]. In addition, NAFLD is associated with an increased hepatic expression of several genes involved in *de novo* lipogenesis [55]. Hepatic *de novo* lipogenesis is regulated by insulin and glucose through the activation of SREBP-1c and ChREBP [45]. Hepatocytes are thought to contain between 500 and 4,000 mitochondria per cell, and as mitochondria play a crucial role in hepatic metabolism, a decrease in their capacity to oxidize fatty acids in the liver might also contribute to the development of hepatic steatosis [48].

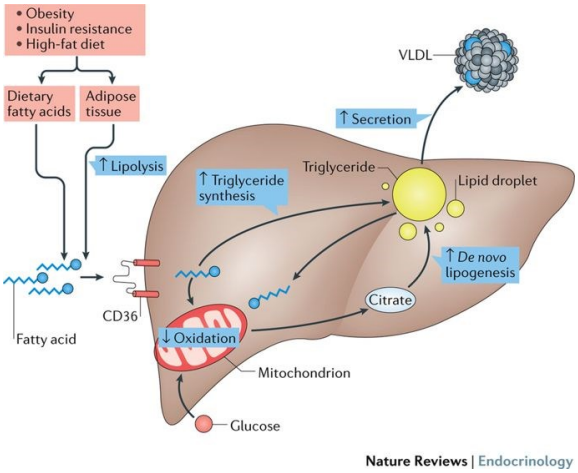

Figure 3 (as in [48]): The lipid storage and disposal imbalance in non-alcoholic fatty acid disease (NAFLD). The hepatic lipid content is regulated by a complex interplay between the delivery of lipids to the liver and the hepatic uptake, synthesis, oxidation, and secretion of lipids. In NAFLD, oxidation in the mitochondria is decreased, while lipolysis, triglyceride synthesis, *de novo* lipogenesis, and VLDL secretion are all increased.

Insulin resistance and diabetes type II

In addition to NAFLD, obesity is also strongly associated with insulin resistance [56] and type II diabetes [57]. Hepatic lipid content has been shown to be a strong predictor of the development of insulin resistance and often precedes the onset of other known mediators of insulin resistance in skeletal muscle, liver and adipose tissue [48]. Insulin is an essential anabolic hormone in the regulation of glucose homeostasis. The pancreatic β-cells secret insulin in response to elevated blood glucose levels. The increase in blood glucose is caused mainly by dietary carbohydrates but also by other nutrients, hormones, and neurotransmitters [58]. Skeletal muscle, liver, and adipose tissue are insulin-sensitive tissues. Some of the most important pathways of insulin for stimulating the storage of energy from nutrients in these tissues are shown in Figure 3.

In healthy individuals, insulin stimulates the storage of nutrients by permitting the entry of glucose in the cells and increasing glycogen synthesis in skeletal muscle [59];

indeed, it is estimated that 75% of whole-body insulin-stimulated glucose uptake is taken up by skeletal muscle [60]. In addition, insulin inhibits gluconeogenesis, while it promotes glycogen synthesis and *de novo* lipogenesis in the liver. Finally, insulin suppresses lipolysis and promotes lipogenesis in adipose tissue [59].

However, in obese individuals, this nutrient pathway may be impaired because the excess adiposity may lead to adipocyte dysfunction and increases the risk of ectopic lipid accumulation in liver and skeletal muscle. Furthermore, ectopic lipid accumulation impairs the nutrient storage pathways through inhibiting insulin action in liver, adipose, and muscle tissue [37]. Figure 3 illustrates how impaired insulin-sensitivity hinders the nutrient storage capacity of insulin in individuals with diabetes type II. Increased insulin secretion from the β-cells initially functions to compensate for the dysregulated processes in the impaired nutrient pathway. However, the hyperinsulemia may contribute to pancreatic β-cell failure, that may finally lead to progress of developing diabetes type II [61].

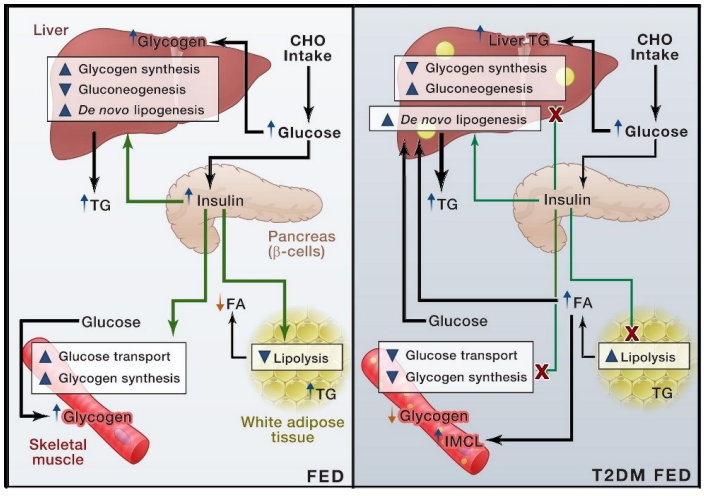

Figure 4 (modified from [59]): Pathways that insulin stimulate nutrient storage in tissue. Illustrated in the tissue from non-diabetic individuals (FED); the figure also illustrates how impaired insulin sensitivity hinders the nutrient storage capacity in type II diabetes (T2DM).

Dietary proteins

Dietary proteins contribute more to higher thermic effect [62,63] and satiety [64,65] than fat and carbohydrates. Thus, increasing the ratio of proteins rather than carbohydrates has been of great interest to weight-management strategies [66]. It has been shown that the satiety effect of proteins is mediated by a synergistic effect of the satiety gut hormones glucagon-like peptide 1 (GLP-1) and peptide tyrosine-tyrosine (PYY) [65,67,68]. Accordingly, increasing the intake of dietary proteins has been shown to be beneficial to weight management and weight loss [69-71]. However, the literature on the effects of different protein sources is scarce [72]. Therefore, there has been a growing interest to investigate how various types of protein sources affect body composition [73]. In addition, results from studies in rodents have shown that different sources of proteins have different obesogenic potential [74-77]. In line with this, long-term monitoring of weight gain from three large prospective cohort studies has shown that various sources of protein-rich food have different associations with long-term weight gain [78]. For example, dairy- and plant-derived proteins and various seafood sources were associated with protection against weight gain, whereas a high intake of meat protein predicted higher weight gain [78].

Protein-rich food such as legumes, dairy, seafood, and meat differ in amino acid composition. Amino acids have been shown to have complex roles in regulation of body composition, bone health, gastrointestinal function, bacterial flora, glucose homeostasis, cell signalling, and satiety [79]. Compared to terrestrial protein sources, seafood is characterized by high levels of taurine [80]. In rodents, supplementation of taurine has been shown to prevent diet-induced weight gain and steatosis and to improve insulin sensitivity [81-83]. In addition, for obesogenic diets that contain protein sources varying in taurine concentrations (chicken, cod, crab, and scallop), the intake of taurine and glycine correlated negatively with body mass [75]. Furthermore, rats given diets with fish protein hydrolysate, rich in taurine, and glycine exhibited reduced adiposity—possibly through an increased bile acid concentration in plasma [77,84]. Compared with proteins from meat and seafood, casein and whey have a high content of the branched chain amino acids (BCAAs), valine, leucine, and isoleucine.

Leucine is recognized as a nutrient signal that has been suggested to mediate, at least in part, the beneficial effects of high-protein diets on metabolism [85,86]. Increasing leucine content in a high-fat diet with regular-protein content to a level matching a diet with a high-whey content attenuated obesity development in mice [87]. However, the effect of BCAA on metabolism is complex and not fully understood [72].

Lean seafood has been investigated in relation to weight management and the prevention of diabetes. It has been demonstrated that the inclusion of 150 g cod for 3 days per week in an energy-restricted diet was as efficient as salmon and supplementation of marine n-3 PUFAs in increasing weight loss by approximately 1 kg more in overweight young males after 4 weeks, compared to a similar diet without seafood or supplements of marine origin [88]. In addition, increasing the cod intake to 150 g for 5 days per week in an energy-restricted diet resulted in a 1.7 kg significantly more weight loss after 8 weeks than in the intake of an isocaloric diet with no seafood [89]. Furthermore, another study has shown that a short-term supplementation of a low dose of cod proteins in overweight adults had beneficial effects on glucose and lipid metabolism; moreover, body muscle percent increased, and body fat percent was reduced [90]. Proteins from lean seafood have also been suggested to have positive influences on glucose tolerance and insulin sensitivity in rodents [91,92].

A population-based cohort study of Norwegian women has shown that the consumption of 75–100 g of lean fish per day reduced the risk of type II diabetes mellitus by approximately 30%. Intake of fatty fish or cod liver oil supplement had no effect on type II diabetes mellitus [93]. In a crossover study with two 4-week diet periods in which the participants were given daily lunch and dinner meals with either lean seafood or non-seafood (mainly lean meat), there were no observable differences in body composition between diets in healthy adults; however, 4 weeks of high-lean seafood intake when compared to no seafood intake altered lipid and glucose metabolism—as was evident from changes in fasting and postprandial serum metabolites [94,95]. Thus, cod intake might affect metabolism and glucose regulation.

Cod fillets have a lipid content less than 1% and, hence, are classified as a lean fish. In contrast to fatty fish, cod uses the liver as a lipid storage organ, and the flesh in cod does not represent an energy storage [96]. Consumption of fatty fish for 1–2 servings per week gives a dose of EPA and DHA of 250 mg per day that has been documented to have beneficial health effects in humans [97]. However, it has been shown that a short-term daily intake of 150 g cod for 15 days provided a daily dose of 134 mg EPA/DHA that significantly increased levels of EPA and DHA in plasma PLs [98].

Dietary lipids

Lipids consists of a large number of organic compounds [99] and are defined as "fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds" [100].

Triacylglycerols and phospholipids

Nutritionally, neutral and polar lipids are the two major sub-classes of lipids in a diet. Neutral lipids are molecules with long hydrophobic hydrocarbon chains; the most well-known are the fatty acid esters of glycerol, which includes tri-, di-, and monoacylglycerols, where the main class in the diet is TAG. In addition, we consume the majority of dietary fat as TAG [99]. Polar lipids consist of a hydrophobic hydrocarbon chain but importantly also include a polar-hydrophilic group (Figure 5) [101]. The main class of polar lipids is glycerophospholipids, known in nutrition science as phospholipids (PLs). Phospholipids are amphiphilic lipids, and they arrange the lipids bilayers found in all plant and animal cells [102]. A normal diet consists of approximately 2–8 g PLs per day, where the main sources of PLs include seafood, meat, and egg and dairy products [103]. The most prevalent PL found in seafood and meat is phosphatidylcholine [102]. Phospholipids consist of two fatty acid molecules esterified in the *sn*-1 and the *sn*-2 position of a glycerol back bound (Figure 5). The *sn*-3 position consists of a phosphate group with a hydrophilic head-group [104]. Phospholipids (either from plant or animal origin) predominantly contain an unsaturated fatty acid in the sn-2 position, such as oleic, linoleic acid, linolenic acid,

the pro-inflammatory ARA, or the anti-inflammatory EPA, while the sn-1 position predominantly carries a saturated FA, such as stearic acid or palmitic acid [104].

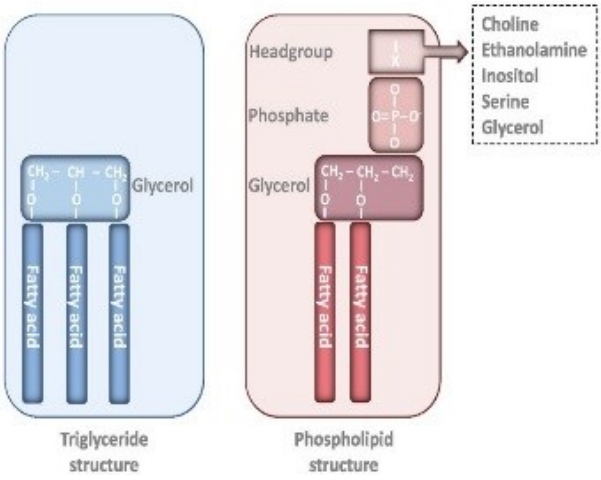

Figure 5 (modified from [105]): Triglyceride and phospholipid structure.

Long-chain polyunsaturated fatty acids

There are two classes of fatty acids that must be provided by the diet because we cannot synthetize them from other substrates, n-6 and n-3 PUFAs, for which linoleic acid (LA) and α-linolenic acid (ALA) are, respectively, the parent fatty acids [99]. Linoleic acid and ALA are obtained from the diet and converted to n-6 and n-3 PUFA families by a series of alternating desaturations and elongation reactions, whereby both pathways of desaturations and elongation of n-6 and n-3 PUFAs compete for the same enzymes. It is believed that the imbalance in the ratio of n-6 to n-3 PUFA in the Western diet pattern during the 20th century is closely connected to obesity and major diseases occurring today in the Western world [106]. It has been suggested that the optimal ratio of n-6 to n-3 PUFAs in the diet should be 1–4:1 [9]; today, the n-6:n-3 PUFA ratio reaches 20:1 or even higher [107]. Linoleic acid occurs in almost every dietary fat, in especially high proportions in soybean, corn, and sunflower oils. Thus, the high ratio of n-6:n-3 PUFA has been suggested to be caused by increased consumption of primarily soybean oil [108]. The intake of dietary fatty acids from PUFAs changes the membrane composition of PLs [109,110]. Therefore, an increase in LA and ARA in the diet is

accompanied by an increase in the ARA:EPA+DHA ratio in the PL membrane of tissue; this leads to increased production of pro-inflammatory ARA eicosanoids including prostaglandins (PGs), thromboxanes (TXs), prostacyclins, and lipoxins [111] and also to the endocannabinoids 2-AG and AEA, which are involved in energy homeostasis [107]. The pathways of endocannabinoids and pro-inflammatory eicosanoids from ARA are illustrated in figure 6.

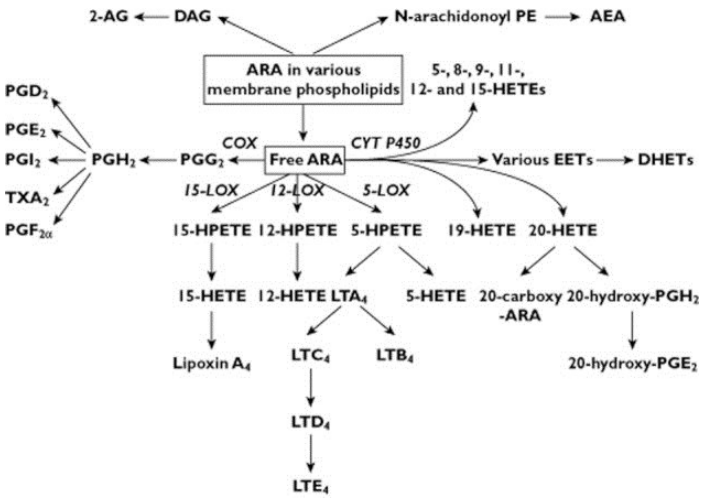

Figure 6 (as in [112]). The pathways of eicosanoid and endocannabinoid synthesis from arachidonic acid.

There are two versions of n-3 PUFAs: long-chain (≥C_{20}; LC) and short-chain (≤C_{18}; SC). Long chain n-3 PUFAs include EPA (20: 5n-3), DPA (22: 5n-3), and DHA (22: 6n-3) [113]. The efficacy for converting ALA to EPA and, in particular, DHA appears to be low. Thus, an adequate dietary intake of EPA and DHA is suggested to be important for maintaining optimal tissue function [114]. The LC n-3 PUFAs EPA and DHA exert multiple, self-mediated biological effects by acting as regulator ligands for the peroxisome proliferator-activated receptors (PPAR). Furthermore, EPA and DHA are ligands for liver X receptors (LXRs) and for sterol regulatory element-binding proteins (SREBPs) [115]. Further, EPA and DHA exert effects through their multiple bioactive metabolites which, in turn, exert mostly anti-inflammatory effects. These bioactive compounds could be divided into several sub-families including specialized

pro-resolving mediators, epoxides, electrophilic oxo-derivatives, ethanolamines and acylglycerols, among others [116]. It is well established from studies in both humans and rodents that EPA and DHA have positive influences on various inflammatory diseases and in the prevention of cardiovascular disease because of their anti-inflammatory properties [117]. The mechanisms underlying the anti-inflammatory actions of EPA and DHA have been suggested to mediate via activation of the GPR120 receptor/FFAR4 [118]. However, studies have also suggested that GPR120/FFAR4 may not be the only effector [119,120] and that additional mechanisms likely play a role.

In addition, EPA and DHA have been investigated as therapeutic agents for attenuating and reversing obesity. Clinical trials using n-3 PUFAs as weight-reducing agents have shown conflicting findings, with some studies showing a positive effect on weight loss [121-123] and others showing no effect [124]. However, a number of studies in rodents have indicated that incorporating EPA and DHA into high-fat obesogenic diets can attenuate diet-induced obesity [110,125-132]. Studies in mice have shown that the anti-obesity effects of EPA and DHA may involve a switch in adipocytes that includes an increase in β-oxidation and the upregulation of mitochondrial biogenesis [133]. In addition, n-3 PUFAs have been shown to suppress appetite [134]. Furthermore, studies in rodents have reported greater insulin sensitivity with fish oil supplementation in a high-fat diet [132,135,136]. In humans, a high n-3 PUFA index in RBC is associated with increased insulin sensitivity [137]. Moreover, n-3 PUFAs have been shown to effect and, potentially, to ameliorate NAFLD . The mechanisms behind these potential effects include the activation of PPARs that increase hepatic fatty acid β-oxidation. Eicosapentaenoic acid and DHA may also reduce hepatic *de novo* lipogenesis by inhibit SREBP-1c and ChREBP activity. In addition, LC n-3 PUFAs may reduce inflammation in NAFLD through inhibiting the production of ARA–derived eicosanoids [138] .

Dietary EPA and DHA are provided mostly by fatty fish and fish oils, where they are mainly esterified in TAG. In other sources, including krill and herring roe, the major proportion of these LC n-3 PUFA is esterified in PLs, but PLs are also present in fish to some degree [139]. There are also other chemicals forms of LC n-3 PUFAs,

including ethyl esters and free fatty acids. It has been reported that the bioavailability of EPA and DHA, their ability to modulate endocannabinoid signaling, and their anti-obesogenic effect are higher when they are PLs than when they are TAG-bound [140-142]. In addition, studies have shown that the FFA form is more bioavailable than the TAG form, which, in turn, is more bioavailable than the ethyl ester form [143]. However, Ghasemifard et al. [143] have concluded that PL-bound n-3 PUFAs seem to be more bioavailable than TAG-bound n-3 PUFAs in animal studies, while human studies have not given any conclusive results regarding the bioavailability of n-3 PUFAs provided as PLs versus TAG-bound. The authors of this review have concluded that human studies are significantly different in their design and that this makes it difficult to draw substantiated conclusions [143]. Moreover, it is not known what the mechanism is behind an increased tissue uptake of LC n-3 PUFAs that are bound to PLs [105].

Eicosapentaenoic acid and DHA are not physiologically active in the bloodstream itself but are incorporated in the membrane tissue. Consequently, one cannot determine the levels of LC n-3 PUFAs that are not incorporated in membranes (in other words, in serum and plasma) to assess the extent to which EPA and DHA are absorbed into the target tissue. To be clear, EPA and DHA levels in serum and plasma rather represent EPA and DHA status after a single-dose treatment of EPA and DHA [144]. In contrast, EPA and DHA levels in membrane RBCs are better indicators of long-term bioavailability. The omega-3 index, which is defined as the sum of EPA and DHA expressed as a percentage of the total FA in membrane RBCs, is regarded as an adequate measure of the long-term incorporation of EPA and DHA in tissue [145], and this EPA and DHA level correlates well with other tissues [146,147].

Encocannabinoids

The endocannabinoid system refers to the endocannabinoids and the proteins that regulate the endocannabinoid production and degradations and the receptor as the endocannabinoids mediate signaling [148]. The main endocannabinoids 2-arachidonoylglycerol (2-AG) and *N*-arachidonoylethanolamine (AEA) are endogenous

lipid mediators formed enzymatically on demand from ARA in membrane phospholipids [142]. The endocannabinoids bind to the G protein-coupled cannabinoid receptors CB1 and CB2. The CB1 receptor is widely distributed in different brain areas and peripheral tissues [148]. CB1 receptor activation by increased endocannabinoid levels or exogenous agonists induces hyperphagia, increased lipogenesis, and peripheral adiposity [110,149,150]. In the liver, CB1 activation increases *de novo* lipogenesis through stimulating cytosolic fatty acid synthase activity, which then leads to fatty liver and obesity [149]. Thus, a pharmacological blockade of the CB1 receptor is effective in treating obesity and its related metabolic derangements; however, serious psychiatric side effects have been reported, and thus this blockade is not used in clinics [151]. A dietary intake of EPA and DHA decreases the membrane-based content of ARA and increases the content of EPA and DHA, and this results in changed patterns of production of endocannabinoids [110,152]. Endocannabinoids control lipid and glucose metabolism in several peripheral organs, particularly the liver and adipose tissue [153]. Thus, different studies have supported the hypothesis that the composition of dietary fatty acids can affect energy homeostasis through changes in the endocannabinoid system (ECS) and that this might then be a strategy for weight management [139].

Aims of the project

Long-term weight gain contributes to the growing obesity epidemic. Obesity is preventable through diet, and studies have shown that maintaining weight loss is challenging for obese individuals. Accordingly, it is important to investigate food that may contribute to protection against long-term weight gain. Protein-rich food has been shown to have different potentials for maintaining long-term weight homeostasis. In line with this, the overall aim of this thesis is to determine the impact of different sources of dietary protein for preventing diet-induced obesity and influence on metabolic changes associated with obesity development in mice. This overall aim was divided into secondary aims which each paper in this dissertation investigates in turn.

- The aim of Paper I is to determine the efficiency of high-fat/high-protein diets by using casein, soy, cod, beef, chicken, or pork as protein sources in the prevention of diet-induced obesity in mice. Furthermore, by decreasing the protein:carbohydrate ratio in a casein- and pork-based diet, we also aim to analyze the influence of protein intake and protein sources on energy expenditure and brown adipose tissue.

- The aim of Paper II is to investigate whether exchanging the lean protein source of pork with the lean fish protein source of cod sufficiently attenuates obesity, influences hepatic lipid accumulation, and modulates fatty-acid composition and endocannabinoid profiles in mice that have been fed on Western diets.

- The aim of Paper III is to investigate whether the frozen storage of fresh cod influences the bioavailability of EPA and DHA that is present in the cod fillets and the ability of cod to modulate energy intake, the development of diet-induced obesity, and hepatic lipid accumulation in mice that have been fed on Western diets. We also aim to investigate whether PL-bound EPA and DHA, which had been supplemented to a level comparable to that in fresh cod, can affect the development of obesity and hepatic lipid accumulation in mice.

Methodology

Ethical considerations

All animal experiments were approved by the Norwegian Animal Research Authority (FOTS id.nr; Paper I: 3750; Paper II: 5358; and Paper III: 7882). Animal care and handling were performed in accordance with national and international guidelines, as described in Paper I–III.

Animal experiments

All feeding experiments took place at the animal facilities at IMR under closely monitored conditions, except for the metabolic chamber study in Paper I that was performed in the animal facilities at the University of Copenhagen. Paper I was based on four separate animal studies, Paper II one animal study, and Paper III two separate animal studies.

In all of the experiments, the obesity prone C57BL/6 mice were used as experimental models. This inbred mouse strain rapidly develops obesity, glucose intolerance, and insulin resistance on a high-fat diet and on a diet high in fat and sucrose [154,155]. In addition, the C57BL/6 mice retain their lean phenotype on a low-fat diet [155]. Male mice were chosen because many key determinants of energy balance in female mice were affected by hormonal variations associated with the estrous cycle [156]. Mice have an enormous capacity for cold-induced thermogenesis [38], and housing mice at thermoneutrality has been claimed to be an advantageous step towards aligning mouse energy metabolism to human energy metabolism [157]. To avoid any cold stress, the animal experiments were performed in a thermoneutral environment, 29°C (± 1°C).

Free water, lean mass, and fat mass were measured in live mice by using a Bruker minispec LF50 Body Composition Analyzer mq 7.5 (Bruker Optik GmBh, Germany), as described by Halldorsdottir et al. [158]. The time points for the measurements of body composition are described in Paper I–III.

The mice were 8 weeks old at arrival and were obtained for all experiments from Taconic (Ejby, Denmark). The mice were housed in individual cages with a 12 h light/dark cycle with 50% relative humidity. After 5–7 days acclimatization on a low-fat diet, which is based on the Sniff ER R/M control diet ([E15000-04], Germany), the mice were divided into feeding groups that were based on the measurements of body weight in Paper I and body weight, fat, and lean mass in Paper II and III.

In all animal experiments, fresh water was provided twice per week, and fresh feed three times per week. Body weight was recorded once per week, and feed intake recorded three times per week. Apparent digestibility of nitrogen and fat was measured after 6-7 weeks of feeding, as described in Paper III, based on [75]. The mice were sacrificed by cardiac puncture under isoflurane anesthesia (Isoba vet, Schering-Plough, Denmark) after 11 weeks in Paper I Experiment I and 2, and after 12 weeks of feeding in Paper II and III. Blood was collected from the hearts into tubes containing EDTA. The RBC and plasma fractions were prepared by centrifugation (1500 G, 15 min, 4°C) and were stored at -80°C until further analyses. Liver and adipose tissues were dissected, weighed, snap frozen in liquid nitrogen, and stored at -80°C until further analyses.

In addition, in Paper I, diet-induced metabolic changes were measured before the onset of obesity in open-circuit, indirect calorimetry cages, as described by Lillefosse et al. [159]. The respiratory exchange ratio (RER) was calculated from VO_2 and VCO_2, and spontaneous locomotor activity was defined as total counts of light-beam breaks. Energy expenditure was calculated as described in Paper I, based on [160].

Experimental diets

Various protein sources were used to increase our knowledge of how different protein sources influence diet-induced obesity and its associated metabolic changes in mice fed from different background diets. Hence, in Paper I, We used casein powder (Sigma, C-8654), soy powder (Ssniff Spezialdiäaten, Soest, Germany), cod fillet powder (Seagarden AS), beef tenderloin (H. Bragstad A/S, Bergen), chicken breast fillet (Prior, Norway), or pork sirloin (H. Bragstad A/S, Bergen) as protein sources in a high-

fat/high-protein diet (Paper I, Table 2). In addition, casein and pork sirloins were blended in a high-fat/high-sucrose diet, (Paper I, Table 2) and in a low-fat diet (Paper I, table 2). In Paper II, pork sirloins (H.Bragstad A/S, Bergen) and frozen cod fillets (Lerøy, Bergen) were blended in Western diets (Paper II, Table 1). In Paper III, pork sirloins (H.Bragstad A/S, Bergen), frozen cod fillets (Lerøy, Bergen), fresh cod fillets (Lerøy, Bergen) and, additionally, casein powder (Sigma, C-8654) were blended in Western diets (Paper III, Table S4 and S9).

Paper I–III provides detailed descriptions of the preparation, the various analyses of the different protein sources, the experimental diets, and the composition of the experimental diets. In brief, beef tenderloin, chicken breast fillets, pork sirloins, and frozen and fresh cod fillets were heated in a steamer to a core temperature of 70°C, freeze dried, homogenized to powder, and mixed into the different background diets. Given the varying content of nitrogen in the protein sources, different amounts of powder were required to prepare isonitrogenious diets. Hence, crude protein/kg was calculated from measurements of nitrogen from the powders; this was determined by means of the Dumas method, using Leco FP 628 nitrogen analyzer (Leco Corporation Svenska AB, Sweden). Based on measurements of endogenous fat in the protein powders (described in Paper I-III), diets were balanced with corn oil in Paper I (Paper I, table 2), and equal parts of margarine, lard and milk fat in paper II and III (Paper II, Table 1, and Paper III, Table S4 and S9) in order to balance the diets with respect to fat content.

C57BL/6 mice maintain their lean phenotype on a low-fat diet [161], and thus a casein-based low-fat diet served as a reference group for the obesogenic effects of the high-fat/high-protein, the high-fat/high-sucrose (Paper I), and Western diets (Paper II and III). The mice fed on a low-fat diet were not included in analyses of fatty acid composition in RBC and liver lipids in Paper II and III.

Histological investigation of tissue

A morphology investigation of inguinal white adipose tissue (iWAT), interscapular brown adipose tissue (iBAT) (Paper I), and liver samples (Paper II) was performed, as

described by [162]. In brief, tissues were fixed in 4% formaldehyde in a 0.1 mol/L phosphate buffer overnight, washed in the phosphate buffer, gradually dehydrated by increasing the concentrations of alcohol, cleared in xylene, and embedded in paraffin blocks. Subsequently, 3–5 µm thick sections of the embedded tissue were stained with Hematoxylin-Eosin for morphology investigations. In addition, in Paper I, an immunohistological detection of UCP1-positive cells in iBAT was performed by an avidin-biotin peroxidase method, as described by Fjære et al. [163].

Gene and protein expression in tissue

RNA was extracted from the tissues, cDNA was synthesized, and gene expression was analyzed with RT-qPCR, as was described by Lillefosse et al. [159]. The primer sequences used in Paper I and II are not described, but available on request. The expression of mRNA was normalized to that of the housekeeping gene TATA box-binding protein (*Tbp*) in Paper I and to the housekeeping gene *Calnexin* in Paper II. In addition, Western blotting was performed in the iBAT samples by using antibodies against glyceraldehyde 3-phosphate dehydrogenase (Ab824545, Abcam) and UCP1 (Ab10983, Abcam), as described by [164].

Glucose tolerance test

A glucose tolerance test (GTT) was performed in Paper I–III, as described by [165]. Paper I–III list the details regarding the dose of glucose, the time points of blood sampling, and the kits for the quantification of plasma insulin collected during the GTT. An intraperitoneal GTT was performed in Paper I, and an oral GTT (OGTT) was performed in the experiments of Paper II and III. In brief, after 6 hours of fasting, the mice were given glucose by intraperitoneal administration or gavage. Blood was collected from the tail vein of conscious mice, and blood glucose was measured using a glucometer. Blood glucose was measured in a fasting state, and again at 15, 30, 60, and 120 min after the glucose injection. Plasma was sampled at various timeouts during the GTT, as is described in Paper I–III, to quantify plasma insulin with mouse insulin ELISA kits.

Lipid class composition in diets and livers

In Paper II and III, lipid class composition of the diets, the mouse livers, and additionally in Paper III dietary protein sources, was measured through high-performance thin-layer chromatography (HPTLC), as described by Jordal et al. [166]. Lipids were extracted from the samples by adding 20 x the amount of a sample (v/w) of chloroform:methanol (2:1) with 0.01% BHT. After extraction of the lipids, the samples were filtered, taken to dryness, and diluted in chloroform with 0.01% BHT in order to obtain a final concentration of 5 mg/ml. One µl of the solution was applied to a 20 x 10 cm HPTLC Silica 60 plate, which had been pre-run in chloroform and activated at 110°C for 30 min. The plates were developed to 48 mm in a polar solution of chloroform, isopropanol, methyl acetate, methanol, and 0.25% (w/v) aqueous KCl (25:25:25:10:9, by volume) in order to separate polar from neutral lipid classes. After drying, the plates were fully developed in isohexane, diethyl ether, and acetic acid (80:20:1.5, by volume) to separate neutral lipids and cholesterol. The plates were developed in 3% copper acetate (w/v), then in 8% (v/v) phosphoric acid. Lipid classes were visualized by charring for 15 min at 160°C, and the classes were identified by comparison with commercially available standards. Lipid classes were quantified by using a densitometer (CAMAG TLC Scanner) and were calculated by using an integrator (winCATS Planar Chromatography Manager). Furthermore, a quantitative determination of lipid classes (mg lipid class/g tissue) was performed by establishing standard equations for each lipid class within a linear range of area. The quantification was limited to 0.01 mg lipids/g sample.

Fatty-acid composition in the neutral and polar lipid fractions

Lipids were extracted from protein sources, the diets, RBCs, and mouse livers by adding 20 × the amount from a sample (v:w) of chloroform:methanol (2:1). After the filtration of the extracts of protein sources (Paper III), diets (Paper II and III), and mouse livers (Paper II and III), solvents were evaporated, and the residue was dissolved in chloroform (2% methanol) and separated into polar and neutral fractions through using solid phase extraction (SPE). Lipids recovered in chloroform to yield 50 mg lipid/ml. The SPE cartridge was conditioned with 5 mL of hexane. The sample was

then loaded and eluted with 10 mL 2% methanol in chloroform, and the neutral fraction was collected. Subsequently, 15 mL of methanol was added, and the fraction was collected as the polar lipids.

Methyl ester from C19:0 (nonadecanoic acid) was added to each fraction/sample as an internal standard before the lipid samples were saponified with NaOH and the fatty acids were methylated by using a 12% BF_3 in methanol. The quantity of fatty acids in each fraction/sample was determined by gas chromatography coupled with a flame ionization detector, identified by retention time by using standard mixtures of methyl esters, and quantified toward the internal standard, under the conditions previously described by Torstensen et al. [167], which are themselves based on the work of Lie et al. [168]. The limit of quantification was 0.01 mg FA/g per sample.

Oxylipins and endocannabinoids

We measured oxylipins and endocannabinoids from mouse plasma and liver in Paper II. Plasma was prepared as is described in Paper II. In brief, lipids were extracted by using strata-X SPE columns and were analyzed with a ultra-performance liquid chromatography system, according to a published method [169].

Statistical analyses

Data was analyzed in Graphpad and Statistica. All data was presented as a mean ± SEM, if not presented otherwise. Data was compared between groups through using a one-way ANOVA; this was followed by Tukey's multiple comparison post-hoc test in Paper I; and Fisher's LSD multiple comparison post-hoc test in Paper II and III. Body weight development and the GTT, were analyzed by repeated measurements through using a one-way ANOVA; this was followed by Tukey's post-hoc test in Paper I, while cumulative energy intake in Paper II and III was analyzed by repeated measurements through using one-way ANOVA and then Fisher's LSD post-hoc test. Differences between groups means were considered significant when $p < 0.05$. Paper I–III provide details on the control group, the n per group, and the statistics in the animal experiments.

Summary of results

Paper I

By comparing proteins from casein, soy, cod, beef, chicken, and pork in a high-fat/high-protein diet, we observed that in comparison with mice that had been fed a low-fat diet, casein was the only protein that completely protected against high-fat diet-induced obesity. The terrestrial proteins from pork, chicken, and meat promoted fat mass gain to a greater extent than proteins from cod and soya. Cod was the only protein source that significantly reduced energy intake. Decreasing the protein:carbohydrate ratio increased high-fat-induced obesity, independent of whether casein or pork was uses as a protein source. In addition, the intake of a low-fat diet with casein but not pork reversed diet-induced obesity. The high-protein diet based on casein was particularly effective in preventing the whitening of iBAT and in maintaining UCP1 expression. Thus, the effects of high-fat/high-protein diets to protect against obesity in mice is greatly determined by the protein source. Compared to pork, proteins from casein excel by maintaining a brown phenotype iBAT with a high UCP1 expression in mice.

Paper II

Exchanging pork with cod as protein source in a Western diet, which was fed *ad libitum* to mice, led to a reduced gain of body weight, fat mass, and a 6% lower energy intake. In addition, mice fed cod compared to mice fed pork *ad libitum* revealed a reduced hepatic lipid accumulation, higher levels of EPA and DHA, lower levels of ARA, and a reduction in the n-6:n-3 PUFA ratio in the polar lipid fraction in the liver and in RBCs. In cod-fed mice, we recorded lower circulating levels in plasma of the two major endocannabinoids, 2-AG and AEA, and a higher level of DHEA produced from DHA. In pork-fed mice that were subjected to a 6% reduction in energy intake, matching the energy intake in the cod-fed mice, we observed that body weight, fat mass, and feeding efficiency in the mildly energy-restricted pork-fed group were not significantly different from *ad libitum* pork-fed mice or cod-fed mice. Additionally, the accumulation of hepatic lipids was in between the *ad libitum* fed mice, but the levels

of EPA, DHA, sum n-3 PUFAs, ARA, and the n-6:n-3 ratio in the polar lipid fraction in the liver and RBC were similar to *ad libitum* pork-fed mice. Taken together, the results suggest that the amount of EPA and DHA in cod fillets was sufficient to replace ARA with EPA and DHA in hepatic polar lipids and RBCs in mice and was also sufficient to modulate the endocannabinoid profile. Pork-fed mice subjected to an energy intake similar to the cod-fed mice were either significantly different in obesity development from *ad libitum* pork fed mice or cod-fed mice. This suggests that decreased energy intake contributed but did not fully explain the anti-obesogenic effect of cod.

Paper III

Frozen storage of cod fillets led to decreased levels of polar lipids and higher levels of FFAs in the fillets. Western diets containing fresh cod contained more PL-bound EPA and DHA (3.12 mg/g) than diets containing frozen cod (1.9 mg/g). In a Western diet, exchanging casein with fresh cod or pork (but not frozen cod) as a protein source led to significantly more body and fat mass. The levels of EPA and DHA were similar in RBCs and liver lipids of mice that had been fed frozen cod and fresh cod; this suggests that the bioavailability of EPA and DHA present in the cod fillets was not influenced by the storage conditions. Compared to casein, energy intake was reduced in both fresh and frozen cod-fed mice but not pork-fed mice. Supplementing a pork-based diet with a PL-bound, but not TAG-bound, EPA and DHA to the level of a fresh cod diet led to significantly higher body weight, fat mass, and liver mass in mice compared to no supplementation. There were no differences in the levels of sum EPA and DHA in RBCs that were collected from mice fed on fresh cod or pork diets that were supplemented with PL- or TAG-bound n-3 PUFAs. Mice fed on fresh cod were not different in fat, liver mass, or energy intake to pork-fed mice without supplements. Thus, compared with mice fed on a casein-based Western diet, mice fed on fresh cod, but not frozen cod with reduced PL, increased the development of obesity. Furthermore, supplementation with phospholipid-bound n-3 PUFAs did not protect against but rather promoted obesity development in mice fed on a pork-based diet.

Discussion

Methodological considerations

Design of the animal experiments

It is difficult to establish a control diet in nutritional studies. However, a casein-based chow or low-fat diet is regarded as the closest physiological diet or, in any case, the most used for laboratory rodents for the sake of maintaining normal growth and healthy development [161]. Hence, in Paper I–III, I included mice fed on a low-fat diet as a reference for normal weight and healthy development. Diets with a high protein:carbohydrate ratio have been shown to be beneficial in preventing high-fat diet-induced obesity development in rodents [170-173]. In Paper I, Experiment I, we aimed to investigate the potential for various protein sources in a high-protein diet to attenuate obesity development; therefore, the low-fat (regular) diet was included in the statistics as a control for a development of a lean phenotype. We concluded that the casein-based high-fat/high-protein diet prevented obesity just as effectively as a low-fat diet.

However, in the three Western-diet experiments in Paper II and III, the low-fat fed mice were used as a reference diet, and this data was, accordingly, not included in the statistics. A diet high in sugar and fat, a Western diet, is known to be obesogenic in C57BL/6J mice when compared to a low-fat diet [155]. In Paper II, comparing the Western diet to a low-fat diet could reveal whether it was the cod diet that decreased adiposity and hepatic lipids or the pork diet that induced changes. However, feeding obesity-prone mice a Western diet in a thermoneutral environment for 12 weeks increased the adiposity to a relatively high level. Thus comparing low-fat fed mice to Western diet fed mice may be physiologically irrelevant due to the large difference in the obese phenotype between the mice. If to statistically compare the Western diet fed mice to low-fat diet, an alternative would be to perform the experiments over a shorter duration.

Furthermore, the Western diets in Paper II and III were balanced and prepared to a specific macronutrient composition: 40% of energy as fat, 44% of energy as carbohydrates, and 16% of energy as protein—which is based on the 5TJN Western

diet for rodents (Test Diet, USA). The pork sirloins were obtained from H. Bragstad, A/S (Bergen, Norway), at different time points for all the Western diet experiments. Although the experiments were performed separately at different time points, the use of pork sirloins as a protein source induced remarkably similar energy intake and obesity development in all three Western-diet experiments (Figure 7). Pork was somewhat higher in the n-6:n-3 ratio in RBCs in Paper II than the experiments in Paper III (Figure 7). This could mean, however, that the cod-fed mice in Paper II and III varied in obesity development.

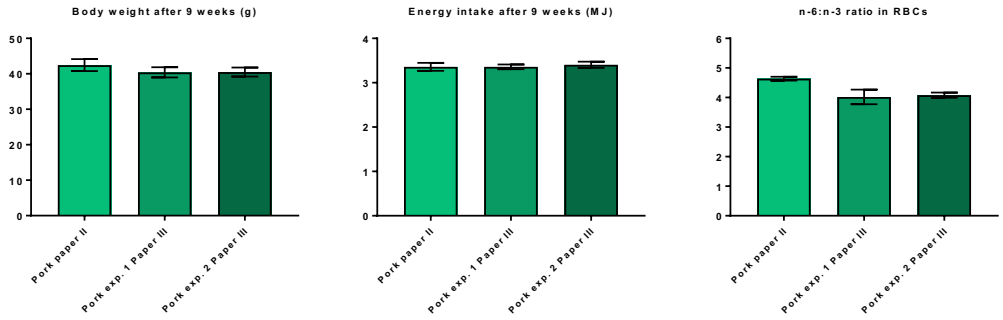

Figure 7. Pork as a protein source in a Western diet, with respect to impact on body weight, energy intake, and n-6:n-3 ratio in red blood cells after 9 weeks of experimental feeding in Paper II and III.

All animal experiments were performed in a thermoneutral environment (28 ± 1 C°). Mice have an enormous capacity for cold-induced thermogenesis [38]. Thus, housing mice in thermoneutrality affords one a unique opportunity to study the obese phenotype without any major influence of the beneficial metabolic effects from BAT; in addition, housing mice at thermoneutrality has been claimed to be an advantageous step towards aligning mouse energy metabolism to human energy metabolism [157]. However, possible effects of LC n-3 PUFAs on energy expenditure in brown or brown-like adipocytes [174,175] in paper III may be masked by the thermoneutral environment. Most studies investigating the anti-obesity effects of PL-bound n-3 PUFAs have been conducted in mice maintained at 20°C [141,176]—that is to say, under conditions that activate cold-induced thermogenesis in mice [65]. Nevertheless, the anti-obesity effects

of n-3 PUFAs have been shown to be independent of housing temperatures [177]. In the latter study, however, casein was used as the dietary protein source, and the n-3 PUFAs were TAG-bound [177]. Thus, combining PL-bound n-3 PUFAs with protein sources other than casein, in combination with housing in a thermoneutral environment, might influence the anti-obesity effects previously observed in mice fed on n-3 PUFAs. To investigate this would require a parallel feeding experiment involving temperature variables (both 20°C and 30°C), protein source variables (pork and casein), and with/without the supplementing of PL-bound n-3 PUFAs.

One strength in the feeding experiment of this research is that the mice were housed alone. This is of significant value since energy intake per mouse was an important endpoint in the experiments. However, C57BL/6J mice that have been housed alone have displayed increased locomotor activity and a decreased habituation response [178]. Thus housing mice separately might affect the results in some respect.

Studies have shown that cod as a protein source may improve insulin sensitivity in rodents [91,92] and humans [179]. In Zucker (fa/fa) rats, replacing 25% of the total dietary protein intake with cod resulted in a lower 2-h postprandial glucose level than for rats that had been fed on casein [180]. Interestingly, in this study, compared to casein-fed rats, cod-fed rats had a higher body weight without this leading to higher adipose tissue depots [180]. Compared with low-fat fed mice impaired glucose tolerance and mild insulin resistance were observed in mice fed on Western diets with lean meat, but not with mice fed on lean seafood [74]. In Paper I–III, a GTT was performed to detect differences in maintaining homeostasis of blood glucose. However, the glucose tolerance mirrored fat mass in Experiment I (not shown), while no differences were detected in Paper II (not shown) and III (see, Paper III, Supplementary Figure 1). The measuring of insulin sensitivity could have been performed with a Matsuda index calculated from plasma insulin and glucose collected from the GTT. The calculation of the Matsuda index from an OGTT has been shown to be a reasonable approximation of whole-body insulin sensitivity [181]**.** However, a Matsuda index calculation was not measured in Paper I-III.

It has been suggested that a high-fat diet induces hyperinsulemia before the onset of obesity and not as a consequence of obesity [182]. Therefore, the diets capacity to influence insulin secretion could be detected before onset of obesity. The primary outcomes of Paper II and III concerned energy intake. Thus, performing a GTT earlier in the experiment could interfere with weight development and the calculation of cumulative energy intake. Therefore, the GTT is performed after the energy intake and the body scan are calculated and measured. Lastly, glucose tolerance was not measured in Experiment 2, Paper III. Accordingly, important information regarding glucose tolerance and insulin sensitivity might have been missed.

Experimental diets

Importantly, in the studies of this research, lean fish and lean meat have been investigated as whole food sources. Nutritional studies investigating single components from food sources may give important information about effects from the particular studied components. When studying such single components, the synergistic functioning between components in the given food and their resulting effects on metabolism may be missed since humans consume whole food and not single components. However, there are disadvantages in the approach of this research: for example, when protein-rich food is investigated, it is impossible to control for all components that are present in the respective whole food that is being studied. Studies have reported that cod protein might improve insulin sensitivity in rodents; however, in these experiments, diethyl ether was used to remove the small amount of endogenous fat that was present in the cod fillet [91,92]. Thus, the diethyl ether treatment might influence the nutrient composition of the cod fillets.

To minimize nutrient losses, protein sources from meat and lean fish were gently heated in a steamer to 70°C, processed into a freeze-dried powder, and stored at -20°C before being blended in the experimental diets. Both protein and fat from the protein sources were present in the experimental diets and occurred as two simultaneous variables in the experimental diets. As a result of using protein-rich food sources, nutritional properties from the freeze-dried protein sources may be caused either by the protein or the fat or a synergic effect between these variables. One study, for example,

has shown that the combination of proteins and lipids from herring roe significantly reduced the fat pad weight of the mice compared with control-fed mice; however, this reduction did not occur when protein and lipids from herring roe were added separately to the diets [183]. The latter study has proposed that protein and lipids may have synergic effects on metabolism, and this might also be the case of the diets employed in Paper I–III. In addition, other quantitative and qualitative factors must be taken into account, and both desirable and undesirable components can also be found in the freeze-dried protein sources.

In addition to marine n-3 PUFAs and protein, cod fillets contain desirable components such as various vitamins and minerals, but they also contain contaminants such as methylmercury. Increased blood mercury concentrations have been shown to be associated with visceral adiposity; however, the research in this field of enquiry remains conflicting [184]. Furthermore, cod does contain iodine, and while little is known about the relationship between BMI and iodine status, obesity was recently associated with a higher risk of iodine deficiency, which might, in turn, lead to hypothyroidism [185]. Thus the obesogenic potential of different protein sources can be caused by a combination of amino acid composition, fatty acid composition, and other factors. Moreover, the different protein sources were used as sole proteins in the experimental diets to avoid contamination with other protein sources. However, lowering the level of the dietary protein being investigated would better reflect a normal human consumption of that dietary protein. The background diet is also significant when one investigates the obesity-related effects of n-3 PUFA supplements [186] since the beneficial effects of n-3 PUFAs have been shown to be abrogated by sucrose [187] and high-glycemic carbohydrates [172]. Thus, potentially, the sucrose content in the pork-based Western diet could have affected the n-3 PUFAs in terms of the development of obesity.

In Paper II and III, the amounts of casein powder as well as pulverized freeze-dried cod fillets and pork sirloins that were added to achieve a 200 g crude protein/kg diet were calculated from measurements of nitrogen from the powders, which was determined by the Dumas method. The nitrogen to protein conversion factors used for the

calculation of crude protein in the diets were Nx6.15 for casein and Nx5.6 for cod and pork [188]. However, in Paper I, nitrogen to protein conversion factors were Nx6.25 for all protein sources. In addition, results from Paper III revealed that the nitrogen content in the frozen cod powder contained somewhat lower amounts of nitrogen compared to the fresh cod powder; this resulted in minor differences in added cod powder in the Western diet (Paper III, Supplementary Table S4). Moreover, lipid levels were reduced upon heating in the frozen cod fillets, and this resulted in minor differences when balancing milk fat, margarine, and lard between the frozen and fresh cod diet (Paper III, Supplementary Table 4).

All experimental diets used in Paper I–III were blended at IMR and, consequently, we were not able to provide the feed as pellets, which would have made it easier to collect residual feed from the cages. The experimental diets were fed to the mice as a "mass" in a small glass that the mice subsequently spread throughout the cage. The glass containing old feed was replaced with fresh feed three times per week. The cod-based Western diet contained approximately 3 g n-3 PUFAs/kg diet, and we also supplemented pork diets with n-3 PUFAs at the same level as the cod-containing diet. Marine n-3 PUFAs are highly prone to oxidation due to their large number of double bonds and their position within the fatty acid chain [189]. Accordingly, it is possible that the fatty acids in the feed were somewhat oxidized before the old feed was replaced with new feed. Furthermore, it is possible that the mice might have eaten some of the old feed that remained in the cage.

Casein and whey are the most commonly used protein sources in rodent-based nutritional studies. In paper I-III, we used casein salt from bovine milk (C8654 SIGMA). However, this powder was not for human consumption. Thus, we cannot draw any conclusions with regard to human consumption since the casein source was not suited for human consumption. If one were to study dietary protein so as to attain greater relevance for human consumption, freeze-dried cottage cheese could be an alternative.

Importantly, the high-fat/high-protein diet contained a high amount of sucrose. With regard to investigating a high-fat diet, the background diet could have been more relevant to humans in this experiment. In addition, the high-fat/high-protein diet contained a high amount of n-6 PUFAs—these predispose individuals to the development of obesity. In line with the finding that a high-fat diet containing a high amount of saturated fatty acids is as effective as a low-fat diet in reversing obesity and its associated metabolic disorders [190], it would be of great interest for researchers to combine a high-fat/high-protein diet with a more relevant background diet for human consumption. Replacing the majority of n-6 PUFAs with mono-unsaturated fats and saturated fats and replacing the sucrose with a low-glycemic carbohydrate source would also be of great interest.

Frozen storage of cod fillets and powder

Cod as protein source originated from different places: freeze-dried cod powder was provided from Seagarden AS in Paper I, while in Paper II and III cod fillets were provided from Lerøy AS and were processed into cod powder at IMR.

It was important to expose the mice to the same batch of cod fillets in Paper III, Experiment 1. Half of the batch of raw fresh cod was stored at -20°C for 12 weeks to initiate hydrolysis of the PL fraction in the cod fillets. Hence, the freeze-dried fresh cod powder was immediately prepared from the other half of the batch (as described in Paper III). The fresh cod powder was stored at -20°C for 12 weeks prior to being blended into a Western diet. Freeze-drying preserves most of the initial properties of the raw material such as appearance, shape, taste, and flavor [191]. However, although the fresh cod powder was freeze-dried to a dryness greater than 97% before storage, it is possible that the 12 weeks of storage may have affected the powder in the interim. The lipid class composition of the fresh freeze-dried powder was not measured before the 12-week storage. In contrast, the fresh cod powder in Experiment 2 in Paper III was blended directly into the diet after preparation. Thus, the fresh cod powder had minimum storage temperature of -20°C, prior blended in the diet. Hence, there were differences in storage conditions for the fresh cod powder before it was blended into

the diets that might have affected the energy intake and diet-induced obesity in the mice.

To avoid any further possible enzymatic hydrolysis of the powder and the cod-containing diets, the diets and powder should have been stored in vacuum bags during storage at -20°C. The FFA contents of the diets were measured after the end of the feeding experiment, and the FFA percent of total lipids in the frozen cod diet had increased from 1.05% to 1.9%, while in the fresh cod diet the FFA content had increased from < 0.01% to 0.2% of total fatty acids.

Analyses of fatty acid and lipid class composition

In Paper II and III, lipids were separated from protein sources, feed, dietary oils, and liver in the neutral and polar lipid fraction by using solid phase extraction. Subsequently the fatty acid compositions were measured in the fractions. The methyl ester C19:0 (nonadecanoic acid) was used as an internal standard and was added to each fraction after the separation of the polar and neutral lipid fractions. Then the fatty acids were in each fraction was determined by gas chromatography.

In analytic methods, the internal standards should undergo the same sample preparation procedure as analyses being assayed; hence, the internal standard should be added to the sample as soon as possible during the analytic method. In this case, the internal standards should be added before the SPE extraction; however, the internal standard used in this method is non-polar, and, therefore, the internal standard would be extracted in the neutral fraction during the solid phase extraction, so leaving the polar fraction with no internal standard. Therefore, the internal standard is added after the solid phase extraction. Off note, we did not find it essential to separate the RBC fraction in the neutral and polar lipid fraction as the majority of lipids are present as polar lipids in RBCs [192].

In the HPTLC method, the different lipid classes are detected and quantified by using the visualization of double bond oxidation. Hence, the concentration of unsaturated fatty acids in a sample will affect the detection of the lipid classes. A comparison of the number of double bonds in the different samples could be performed by using the

results from the fatty acid analysis of samples [193]. However, this was not performed in this research.

Discussion of findings

The protein source influence on energy intake

A diet high in fat and sucrose increase energy intake through numerous signaling pathways [194]. Increasing the protein:carbohydrate ratio in a high-fat diet has been shown to decrease *ad libitum* energy intake and thus to contribute to lower adiposity in rodents [170,173]. However, in Paper I, we observed no significant difference in energy intake when I increased the protein:carbohydrate ratio in a high-fat diet that was based on pork or casein as protein sources. Yet, increasing the protein:carbohydrate ratio decreased feed efficiency and attenuated obesity development—irrespective of the protein source.

We detected no differences in energy expenditure during indirect calorimetry measurements that could explain this difference in feed efficiency or adiposity (Paper I, Figure 3). However, it is possible that minor differences in energy expenditure were undetectable during the 48 h indirect calorimetry measurements and that over time they could have accumulated and contributed to the total fat gain in the present study. Furthermore, compared with casein-fed mice, mice fed on diets containing pork had a tendency toward a lower locomotor activity level, which over time could have an impact on feed efficiency and obesity development. Furthermore, the diets based on casein were particularly effective in preventing the whitening of iBAT and maintaining UCP1 expression. In addition, this was accompanied by an increased expression of genes involved in futile cycling of fatty acids. Brown adipose tissue oxidizes lipids and glucose and dissipates the energy as heat [38], and high UCP1 expression in BAT promotes thermogenesis and overall energy expenditure while maintaining leanness [43]. Investigating the nutrients that maintain UCP1 expression in BAT might represent one strategy for preventing the development of obesity. At the obese state and when mice are kept under thermoneutral conditions, “whitening” of the brown adipose organ can be observed [195]. It has been shown that both the amount and activity of BAT

decline in obese subjects—mainly as a result of the conversion of brown adipocytes to white-like unilocular cells. The whitening of BAT is induced by multiple factors, including high ambient temperature, leptin receptor deficiency, beta-adrenergic signaling impairment, and lipase deficiency, each of which is capable of inducing macrophage infiltration, brown adipocyte death, and crown-like structure formation [42]. Therefore, it is uncertain whether the whitening of BAT in pork-fed mice causes the obesity or the obese state leads to greater lipid accumulation in all adipose depots.

Although increasing the protein:carbohydrate ratio did not affect energy intake in this experimental study (Paper I), different protein sources might be of importance in the regulation of energy intake. We observed that the replacement of casein with cod, but not any other protein sources, led to a reduced energy intake in the high-fat/high-protein diet. In this experiment, pork-fed mice were significantly different from either casein- or cod-fed mice (Paper I, Figure 1d). Isoenergetic servings of protein-rich food have been shown to differ greatly in their satiating capacities [196]. Moreover, increased satiety [196] and reduced energy intake [197] have been reported through the consumption of fish protein when compared to terrestrial protein. Compared to mice fed on lean meat, energy intake is shown to be reduced in scallop-fed mice [75] and also in mice fed on a mixture of ling, rosefish, cod, wolf fish, and scallop as protein sources in a Western diet [74]; this suggests that lean seafood intake might reduce energy intake. Human intervention studies have been performed with lean seafood as part of a lean white meat diet in comparison to lean red meat diet. These studies have indicated that the inclusion of lean white meat at the expense of lean red meat reduced energy intake [198-200].

In line with this, results from Paper II showed that, concomitant with the attenuated obesity development, cumulative energy intake was 6% lower in mice fed on Western diets containing cod compared with *ad libitum* pork-fed mice (Paper II, Figure 1a). In this experiment, the EPA and DHA in the cod-diet was sufficient to replace ARA in RBCs (Paper II, Table 4) and hepatic polar lipids (Paper II, Figure 3). In addition, we observed that cod-fed mice had lower levels of the endocannabinoids 2-AG and AEA in the plasma compared to *ad libitum* pork-fed mice.

In keeping with this, 2-AG and AEA are ligands for the cannabinoid receptor CB1, which is well known to regulate satiety and energy intake [201-203]. It has been shown that C57BL/6J mice fed on a Western diet, with a macronutrient composition similar to that used in our studies, displayed an increase in energy intake compared to mice fed on a diet with low levels of fats and sugar [194]. In this study, mice fed the Western diet had elevated levels of 2-AG and AEA in plasma, and the study, accordingly, suggests that the hyperphagia associated with Western diet-induced obesity is driven by enhanced endocannabinoid signaling at the peripheral CB1 receptor [194]. On account of the importance of the endocannabinoid profile in satiety and energy intake. We speculated if the PL-bound EPA and DHA content in cod diet might attenuate diet-induced obesity by reducing energy intake that is mediated by a reduced endocannabinoid tone.

A large fraction of EPA and DHA in cod fillets is present in the form of PLs [168]. PL-bound EPA and DHA have been suggested to have a higher bioavailability and ability to modulate the endocannabinoid tone than TAG-bound [140-142]. Frozen storage initiates the hydrolysis of the small amount of EPA and DHA present in the cod fillets [204,205]. Therefore, in Paper III, we aimed to investigate if frozen cod influenced the bioavailability of EPA and DHA to modulate energy intake and adiposity. Western diets with either frozen cod, fresh cod, or pork were prepared. Compared to mice fed on casein, lower energy intake was observed in both frozen and fresh-cod-fed mice, but not pork-fed mice (Paper III, Figure 1f). Furthermore, in the second experiment, mice fed on fresh cod and pork were compared, and no differences in energy intake or diet-induced obesity were observed between the mice (Paper III, Figure 3). In line with this, when C57BL/6J mice were fed high-fat diets with chicken breast fillets, cod, crab, or scallop, both obesity development and energy intake in chicken- and cod-fed mice were similar [75]. When one considers the variable outcomes in effect of cod in reducing energy intake and attenuating diet-induced obesity in comparison to casein and lean meat, the frozen storage of cod fillets might influence the energy intake and thereby modulate obesity development.

Frozen storage of cod fillets resulted in an increase in the level of FFAs and protein denaturation in the frozen fillets over time [206-208]. Lipid oxidation and ice crystal formation have been shown to play important roles in protein denaturation, and, consequently, they can change the texture and quality of frozen cod fillets [209,210]. Importantly, the freezing of cod fillets initiates a deterioration in flavor through rancidity, an undesirable fishy taste, and other off flavors that are believed to reflect the formation of low-molecular weight compounds from lipid oxidation and protein denaturation [208].

The cod powder used in Paper I was obtained from Seagarden AS and originated from freeze-dried cod fillets; it should be noted, moreover, that we don't have available data on storage conditions or FFA content in this powder or in the cod-containing diet in Paper I. However, analyses from the cod-containing Western diet in Paper II showed that the diet contained 2.55 mg FFA/g diet (in the unpublished results of this research). The cod fillets were provided in frozen conditions, but there are no data on how long the frozen cod fillets had been stored.

In Paper III, Experiment 1, the raw cod fillets were kept frozen for 12 weeks before being processed into powder. The frozen cod diet in Paper III contained 1.6 mg FFA/g diet, and FFA was not detected in the fresh cod diets. However, oxidation products in the cod fillets or cod-containing diets were not measured, but the FFA content indicated that the PL fraction had been hydrolyzed [206]. Hence, compounds from lipid oxidation and protein denaturation [208] could affect the taste of the cod-diet and so reduce feed intake. On a sidenote, FFAs are generally undesirable in food products and should be kept at a low level; the compositional specification for FFAs in crude fish oils is 2–5% [211]. Furthermore, Holm et al. [74] have performed a diet-preference test with either a lean seafood mixture or a lean meat mixture in Western diets, and they have observed no differences between the first choices of diets. However, after 6 hours with free access to both diets, the accumulative intake of the lean-meat-containing diet was higher than the accumulative intake of the lean-seafood-containing diet. After 12 weeks of feeding, the mice fed on lean seafood were less obese than the

lean-meat-fed mice [74]—although it should be noted that I have no available data on the storage conditions for the lean seafood used in this study.

In addition, in Paper II, I observed that the fat mass and liver weight in the pork-fed mice that were subjected to a 6% reduction in energy intake (pair-fed with cod-fed mice), was intermediate but not significantly different from either of the groups fed *ad libitum*. This suggests that decreased energy intake contributed but did not fully explain the anti-obesogenic effects of cod. Food restriction has been shown to produce favorable changes in metabolism [25]. On a sidenote, if the mice have lower feed intake due to off-flavors in the cod-diet, this might result in a restriction in the energy intake and could result in favorable metabolic changes in the mice.

Although the experiments present in Paper II and III were performed separately at different time points, Figure 7 illustrates that the freshness of cod in a Western diet might affect to some degree the energy intake and thereby obesity development. Overall, nutrients and foods that increase satiety are of great importance with regard to targeting and preventing at an early stage long-term weight gain. However, results from Paper I–III are inconsistent in terms of the role of cod in preventing diet-induced obesity and in reducing energy intake in mice. Results from Paper II and III could indicate that frozen cod has, compared to fresh cod, a higher capacity to attenuate obesity and to reduce feed intake. To investigate this possibility, experiments would be required that involve feeding mice diets containing cod that has been frozen for longer periods to systematically increase the FFA:PL ratio of lipids.

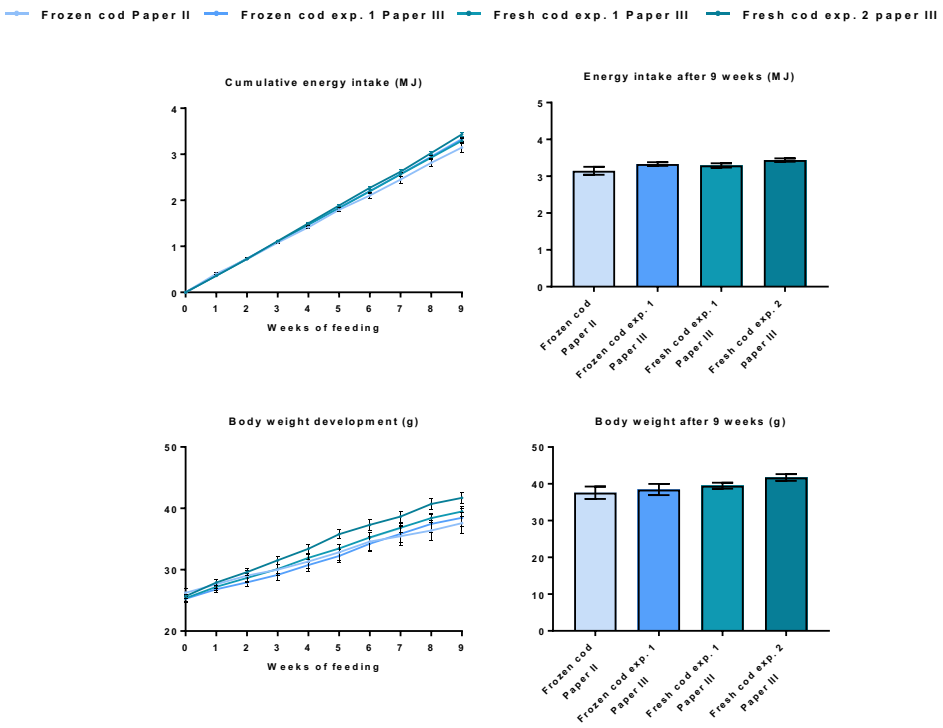

Figure 8: Body weight and energy intake after 9 weeks on a diet from mice that have been fed on cod-containing Western diets with frozen or fresh cod in Paper II and III.

The role of the protein source in the prevention of diet-induced obesity

A high protein:carbohydrate ratio is reported to prevent high-fat diet-induced obesity in rodents; however, these findings are largely based on studies that use casein or whey as the protein source [72]. In this research, we demonstrated that the type of dietary protein source is also of importance in preventing high-fat diet-induced obesity. Using a high-fat/high-protein diet in Paper, casein was the only protein source that completely protects against obesity in comparison to a low-fat diet, and the terrestrial proteins from meat, chicken, and pork promote fat mass gain to a greater extent than proteins from soya and cod (Paper I, Figure 1b–c). These findings are in agreement with results from the large prospective cohort study by Smith et al. (>120,000 participants) that showed that over time the intake of different protein-rich foods is associated with different body weight regulations. Dairy, plant-derived protein, and various seafood sources are

associated with protection against weight gain, whereas a high intake of meat protein predicts a higher weight gain. However, chicken without skin predicted weight loss in this study [78], while we observed that chicken without skin promoted obesity (Paper I, Figure 1). Importantly, the study also showed that protein-rich food and carbohydrates appear to interact in affecting how the protein sources influence body weight gain. This was not observed in Paper I, where lowering the protein:carbohydrate ratio increased high-fat-induced obesity, independent of whether casein or pork was used as a protein source (Paper I, Figure 2 a, b).

Fatty acid compostion in tissue and diet-induced obesity

The high-protein/high-fat diets in Paper I were balanced with corn-oil. Hence, the n-6:n-3 ratio was high in all diets, but the diets varied in the n-6:n-3 PUFA ratio due to the endogenous fat from the protein sources (Paper I, Table 3). However, fatty acid compositions in mouse tissue were not measured; hence, the results with regard to the relationship between n-6:n-3 PUFA and obesity from Paper II and III are discussed in this section.

The dietary intake of LA has increased dramatically during the 20th century [108] and is associated with the greater prevalence of obesity [106,107]. The Western diet provides a higher amount of n-6 PUFAs than n-3 PUFAs [9] and consequently increases the ratio of n-6 to n-3 PUFAs in membrane PLs [111]. A high intake of LA has been shown to increase the production of the endocannabinoids 2-AG and AEA, which are derived from ARA in membrane PLs. In C57BL/6j mice, diets containing 8 energy % as LA (the same amount of LA as the Western diet) have elevated AEA and 2-AG and have promoted adiposity [110,212]. The adipogenic effects of LA can be prevented by consuming sufficient amounts of EPA and DHA that reduce ARA in membrane PLs and thereby normalize the endocannabinoid tone [110]. The total content of EPA and DHA is 2.4 mg/g in raw fresh cod fillets and 3.3 mg/g in heated fresh cod fillets (based on the unpublished results from Paper III), but the majority of EPA and DHA are present in the polar fraction, at least in fresh cod (Paper III, Table S2) [168]. For mice fed on 3.4–5 mg/g PL-bound EPA and DHA in a high-fat diet, PL-bound EPA and DHA protected against adiposity and hepatic steatosis [176]. From

this we could expect that the supplemented EPA and DHA in a pork-containing Western diet probably would attenuate obesity development through a reduced n-6:n-3 ratio in polar tissue in mice that could, in turn, modulate the endocannabinoid profile.

In Paper II, we reported that, compared to pork-fed mice, cod-fed mice had a lower adiposity (Paper II, Figure 1) and lower levels of lipids in the liver (Paper II, Figure 2). This was accompanied by lower levels of ARA and higher levels of EPA and DHA in polar lipids in the liver (Paper II, Figure 3f) and in RBCs (Paper II, Table 4). Cod-fed mice had significantly lower levels of 2-AG and AEA in plasma compared to *ad libitum* pork-fed mice, while pork-fed mice that were peer fed with cod were intermediate in 2-AG (paper II, figure C). It has been shown that PL-bound n-3 PUFAs are more bioavailable and effective than TAG-bound n-3 PUFAs in preventing diet-induced obesity and that they improve the modulation of the endocannabinoid system activity [140-142], and it is well known, moreover, that endogenously produced ARA-derived endocannabinoids can stimulate obesity [110,194,212]. The majority of EPA and DHA in the cod-diet was present in the polar fraction, and we have suggested that the PL-bound n-3 PUFAs could attenuated adiposity in cod-fed mice somewhat through the modulation of the endocannabinoid profile.

With regard to the role of n-3 PUFAs in preventing obesity development, few long-term prospective cohort studies have examined the relationship between n-3 PUFA intake and body weight effects. Furthermore, results from these studies have also been conflicting [213,214]. An observational study in the US adult population of 19,916 individuals observed no relationship between n-3 PUFAs and BMI [215]. Still, it has been found that plasma LC n-3 PUFA levels were inversely related to BMI and waist circumference, and this suggests that a high plasma LF n-3 PUFA status might protect against obesity [216]. Furthermore, a number of studies in rodents have indicated that incorporating n-3 PUFA into high-fat obesogenic diets can attenuate increases in diet-induced obesity [110,125-132].

However, in Paper III, mice fed on fresh cod diets containing more PL-bound EPA and DHA (3.12 mg/g diet) than diets based on frozen cod (1.9 mg/g diet) gained more body

weight and fat mass in comparison to casein-fed mice. In addition, supplementing the mice with PL-bound EPA and DHA did not protect against but rather promoted obesity development in a pork-based diet. Thus, mice fed the pork-containing Western diet that provided the highest n-6:n-3 and ARA:EPA ratios in RBCs (4.08 and 21.7, respectively) had a lower development of obesity compared to pork-fed mice that had been supplemented with PL-bound n-3 PUFAs (1.85 and 4.9, respectively) (Paper III, Table 3). There were no significant differences in the n-6:n-3 PUFA ratio between fresh-cod, pork-fed mice that were supplemented with n-3 PUFAs as TAGs or PLs (Paper III, Table 3). Interestingly, mice supplemented with TAG-bound n-3 PUFAs were intermediate in the development of obesity, compared to mice supplemented with PL and to those not supplemented (Paper III, Figure 3). Furthermore, it has been suggested that PL-bound EPA and DHA are more efficiently incorporated into tissue PLs and seem to have higher capacity to affect endocannabinoid biosynthesis at much lower doses than TAG-bound EPA and DHA [139]. However, in Paper III, the PL-bound EPA and DHA were not incorporated into RBCs in a higher degree than TAG-bound EPA and DHA (Paper III, Table 3). It should be noted that endocannabinoids in plasma, liver, or adipose tissue were not measured in these experiments.

With regard to the increased adiposity in mice supplemented with PL-bound and, to a lesser extent, TAG-bound n-3 PUFAs, it has been shown that the inclusion of LC n−3 PUFA in a diet increased body weight but completely prevented the macrophage infiltration that is induced by a high-fat diet and additionally reduced inflammatory gene expression in diabetic mice, despite increased body weight gain [217]. In this study, male C57BL/KsJ-db/db diabetic mice and non-diabetic littermates (db/+) were fed high-fat diets for 6 weeks, which included 30% of calories from lard, safflower oil, or a diet where 40% of oil volume was replaced by TAG-bound EPA and DHA (25.1 mg LC n-3 PUFAs/g diet). Body weight was significantly higher in diabetic-mice that were fed the diet high in LC-n-3 PUFAs compared with the same diet rich in n-6 fatty acids. Importantly, an inflammatory response in WAT induced by a high-fat diet was prevented by the inclusion of LC n-3 PUFAs. In addition, weight gain only occurred in diabetic mice that were fed the high-fat diet; in non-diabetic mice, the safflower diet resulted in increased weight gain compared with a low-fat control diet, but the LC n-3

PUFA diet did not result in any greater weight gain. The increased weight gain in the diabetic mice that were fed the LC n-3 PUFA diet suggests that these fatty acids may exert quite different metabolic effects in diabetic mice [217].

Overall, in experiment 2 in Paper III, the opposite effects of what could be expected of n-3 PUFAs were observed: the highest n-6 to n-3 PUFAs in the diet provided the lowest development of obesity in pork-fed mice in a Western diet. Due to time limitations in Paper III, it was not possible to analyze endocannabinoids in plasma or other tissues in mice. This could have given important information as to how the EPA and DHA content in the pork-containing diet affected the endocannabinoid profile. Lastly, low-grade inflammation in WAT in the obese state has been associated with insulin resistance and the development of NAFLD [31]. Therefore, further investigations are needed to investigate the controversial results that PL-bound n-3 PUFAs increased obesity development in relation to inflammation in adipose tissue.

Fatty acid compostion in tissue and hepatic lipid accumulation

Obesity is strongly related to development of NAFLD [45]. In the obese state, lipids accumulate in the liver due to an imbalance in hepatic *de novo* lipogenesis and due to the transport of fatty acids from peripheral organs. Furthermore, this state is accompanied by a decreased fatty acid oxidation and by impaired secretion of lipids from the hepatocytes to the circulation and peripheral organs [218]. There is limited data available that investigates the influence of specific nutrients in the progress of intrahepatic lipid accumulation [219]. However, EPA and DHA have been shown to have potential beneficial effects in the progression of NAFLD. Marine n-3 PUFAs regulate hepatic lipid metabolism through several mechanisms; these include inhibiting SREBP-1 and ChREBP activity that stimulate *de novo* lipogenesis, and activating PPARs that increase hepatic fatty acid oxidation [138]. In addition, marine n-3 PUFAs released from liver PLs may also be converted to other n-3-derived lipid mediators such as endocannabinoids and eicosanoids that can potentially attenuate the development of both NAFLD and insulin resistance [111,220].

In line with this, in Paper II, cod-fed mice following the Western diet had lower liver mass, accumulated less lipids in the liver, and had a lower expression of the inflammation markers Cd68 and tumor necrosis factor (TNF) compared to *ad libitum* pork-fed mice. However, the pork-fed mice that were peer-fed with cod were intermediate in liver mass and with respect to the lipids in the liver, and they did not differ in inflammation markers from the cod-fed mice. The expression of genes involved in lipid uptake or of fibrotic markers was not different between the experimental groups. Cod-fed mice had lower levels of circulation of 2-AG and AEA in the plasma. It has been suggested that a high ratio of n-6 to n-3 PUFAs and the consequent ARA-derived eicosanoids and endocannabinoids 2-AG and AEA [111,138] may be important in the development of NAFLD. However, we were not able to detect AEA and 2-AG in the liver, while eicosanoids derived from ARA was detected, but they were not significantly different from each other (Paper II, Figure 4a)

In the three animal experiments from Paper II and III, mice fed on cod—regardless of the food storage condition (fresh or frozen)—had a reduced ARA:EPA and n-6:n-3 ratios in RBCs and/or hepatic polar lipids compared to mice that were fed on pork. Compared to pork-fed mice, hepatic lipid accumulation in cod-fed mice was significantly attenuated in Paper II (Figure 2). In addition, in Paper III, pork-fed mice but not frozen- or fresh-cod-fed mice had a significantly higher liver mass than casein-fed mice (Paper III, Figure 1k). However, this reduced liver mass and the accumulation of hepatic lipids in cod-fed mice was not observed in Paper III, when liver mass and lipids per liver in fresh-cod-fed and pork-fed mice were compared (Paper III Figure 3k–l). By using isocaloric and isolipidic diets with differing compositions of n-6 PUFAs or LC n-3 PUFA. It was shown that body and liver weight, the total lipid level, and abdominal fat deposits were significantly higher in mice fed on a n-6 PUFA diet compared to those fed on the n-3 PUFA diet. Analysis of the fatty acid profile in plasma and liver showed that mice on the n-6 diet had significantly higher ARA levels relative to mice on the LC n-3 PUFA diet. This was accompanied with a progression of hepatic steatosis [221].

Pork-fed mice supplemented with PL-bound n-3 PUFAs had as low a n-6:n-3 ratio in RBCs as cod-fed mice, but liver mass and lipids per liver were significantly higher than pork-fed mice that had not been supplemented (Paper III, Figure 3kl–l). Surprisingly, although mice supplemented with PL or TAG-bound n-3 PUFAs did not significantly differ in fat mass or liver weight, the mg lipids per liver was significantly higher in pork-fed mice supplemented with PL-bound n-3 PUFAs. The TAG-bound oil had a somewhat higher ratio of EPA:DHA in the neutral fraction (0.7) than PL-bound n-3 PUFA oil in the polar fraction (0.38) (Paper III, Supplementary Table, Table S8), which could affect the lipid accumulation in the liver. A major functional difference between EPA and DHA relates to their conversion to different eicosanoids, including pro-resolving mediators that may play important roles in the prevention of hepatic comorbidities associated with obesity [222].

The results from Paper III suggest that the accumulation of lipids in the liver was not associated with the n-6:n-3 or ARA:EPA ratio in RBCs in this experimental design. However, hepatic fatty acid compositions in the livers were not measured in Experiment II. However, EPA and DHA in RBC have been shown to reflect the PL composition of major organs in mice [146], and one could expect that the n-6:n-3 PUFA ratio in liver lipids in cod-fed mice and pork-fed mice supplemented with n-3 PUFAs was reduced. In line with this, reducing dietary n-6:n-3 levels in a high-fat diet lowered the ARA:EPA ratio in a dose-dependent manner but did not attenuate the development of hepatic lipid accumulation in C57BL/6J mice [223]. In addition, in human studies, obese subjects (BMI 43.8 ± 8.3) with NAFLD had significantly lower levels of DHA and EPA in liver PLs and, interestingly, not higher levels of total n-6 PUFAs in liver PLs when compared with lean control subjects [224]. Higher circulating levels of 2-AG and AEA in NAFLD patients compared with the levels in controls was independent of obesity. These findings therefore suggest an independent role of endocannabinoids in the pathogenesis of NAFLD [225]. Furthermore, the circulation of plasma PL n-6 PUFA LA levels was associated with a lower incidence of type II diabetes mellitus [226]. Overall, we did not observe an attenuated obesity development or reduced lipid content in mice that were fed on fresh cod or pork-fed mice

supplemented with n-3 PUFAs in Experiment II in Paper III, although these subjects had lower n-6:n-3 PUFA ratio in RBCs.

Mice as an experimental model for human nutrition

All study designs have their limitations, and it is important to collect evidence from a wide array of studies in an evidence-based hierarchy to understand differences in individual responsiveness to diets. Accordingly, one could undertake animal-based, experimental, mechanistic, and observational studies as well as both randomized and controlled trials in combination with genetic and epigenetic technics [227]. The obesity epidemic is aided by small weight gains that gradually add up over time, and this makes it challenging to study the prevention of long-term obesity development since weight gain occurs over many years [19]. Randomized controlled designs of long-term weight gain are difficult to perform, and thus data from observational studies gives important information. In addition, shorter durations for randomized control interventions can give important information that may be relevant for the long-term intake of different nutrients. Thus, as mice have a much shorter lifespan than humans, feeding experiments that use mice as an experimental model in a controlled environment give a unique opportunity to study how different food consumed over a longer time period influence pathways related to the organism as a whole and to specific outcomes in separate tissue. Additionally, mice are remarkably similar to humans in their genetic [228]. However, when a response is found in animal studies, the true relevance to humans is not known. The validity of an animal model as a predictor of human response depends on how closely the model resembles humans for the specific characteristics being investigated [229].

The C57BL/6J mice are broadly used in nutritional research due to their genetic as well as metabolic similarities to humans. Further, the C57BL/6J mice have a unique ability to develop obesity along with hyperinsulinemia and hyperglycemia in response to high-fat, high-sugar diets [154,155]. Furthermore, mice have been shown to be one of the species closest to humans in their fatty acid patterns [230], and the human adipose organ is similar to the visceral depots in mice, with the exception of epididymal fat,

which humans lack [231]. Therefore, mice provide a good model to study the pathophysiology of an obesity phenotype together with fatty acid patterns that are similar to humans. Additionally, important indications and results from animal studies may be used to plan and design human studies. Despite these advantages, when one interprets the human relevance of results from mice studies, precautions must be taken since most mice used in nutrition research are from an inbred strain with less genetic variety than humans. The inbreed strains provide the benefit of a high genetic resemblance with reduced variance, which, importantly, decreases the number of animals required for study.

Conclusions

Based on feeding experiments that use the obesity-prone C57BL/6J mice, we demonstrated in this dissertation that the use of various protein sources and also the interaction of these protein source with marine n-3 affect obesity and metabolic changes.

Paper I

The terrestrial proteins from meat, chicken, and pork promote fat mass gain to a greater extent than proteins from soya and cod. Exchanging pork with casein increases the maintenance of a β-adrenergic tone and a brown phenotype in iBAT in mice with high UCP1 expression (even at thermoneutrality). Thus, the source of dietary proteins is important in the obesity-protection potential of high-protein diets.

Paper II

Exchanging pork with cod attenuates Western diet-induced obesity and hepatic lipid accumulation, and it decreases the n-6:n-3 PUFA ratio in liver lipids and RBCs. The data in this research demonstrates that the low content of EPA and DHA in the cod-containing Western diet is sufficient to replace ARA in RBCs and hepatic polar lipids and to modulate the endocannabinoid profile.

Paper III

The ability of cod to prevent Western diet-induced obesity seems to be dependent on the frozen storage conditions of the cod fillets. In addition, supplementation with PL-bound n-3 PUFAs to level of a fresh cod diet does not protect but rather promotes obesity development in mice fed on a pork-based diet. This needs further investigation as pork is a major component of the Western diet.

Future perspectives

Although the animal studies in Paper I–III have contributed to our understanding of how protein sources might influence diet-induced obesity and impact fatty acid composition tissue in mice, this research could be further developed by exploring new areas. Mice are a good experimental model for studying human nutrition, but it is possible that different results could occur in humans. Accordingly, results need to be verified in human subjects.

A relatively low dose of PL-bound n-3 PUFAs promoted obesity development in mice fed on a pork-based diet when compared to those given no supplementation. If similar effects of PL-bound n-3 PUFAs are found in humans, this is of great concern as pork is a major component in the Western diet pattern [108]. However, in diabetic mice, n-3 PUFAs have been shown to increase weight gain but also, importantly, to reduce inflammation in adipose tissue [217]. Therefore, it is necessary to investigate whether supplementing PL-bound n-3 PUFAs into a pork-containing Western diet reduce inflammation in adipose and liver tissue in mice. Moreover, since inflammation in adipose tissue promotes insulin resistance [232], it would be of great importance to measure insulin sensitivity. It would also be worth measuring the endocannabinoid profile from the mice in this experiment. The mice supplemented with n-3 PUFAs had a decreased n-6:n-3 ratio in their RBCs as compared to those without supplementation. Hence, it would be of great interest to further investigate whether the endocannabinoid profiles follow the obese state or the n-6:n-3 PUFA ratio in RBCs and polar membrane lipids. It is known that n-3 PUFAs accumulate in RBCs in a dose-response manner [233]. Therefore, it would be worth increasing the amount of PL-bound n-3 PUFAs in doses varying from 3–60 g/kg diet to observe whether the n-3 PUFA dose positively correlates to weight gain and how this effects the endocannabinoid profile.

Although marine n-3 PUFAs are considered beneficial in preventing of obesity and its comorbidities, their combination with other nutrients and background diets might influence the outcome in attenuating obesity. It has been shown that the anti-obesity effect of n-3 PUFAs is abrogated with both high-glycemic index carbohydrates [172]

and sucrose [187]. In the US diet, grains and sugar represent 22% and 17 % of the energy intake, respectively [108]. Based on these findings, it relevant to investigate further how the supplementation of n-3 PUFAs in a Western diet affects obesity in humans [234].

Furthermore, supplementation of n-3 PUFAs has been shown to prevent obesity development in rodents, but the protein sources used in these experiments were, in most cases, casein. If another study is conducted, it would be instructive to supplement a casein-based and cod-based diet with phospholipid bound-n-3 PUFAs to observe how the same amount of marine n-3 PUFAs affects the obese phenotype in sources other than pork, in a thermoneutral environment. As the human diet consists of multiple protein-rich foods, it would be well worth mixing different protein sources.

Further studies are also needed to confirm the possible dietary effects of frozen and fresh cod that this research observed. The reduced content of PLs and increased level of FFA in frozen cod indicates a lipid oxidation that might create an undesirable taste in the cod diet. It would be of great interest to measure oxidation products and protein denaturation in cod fillets to observe whether the levels of oxidized n-3 PUFAs or FFAs correlate with energy intake.

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Intake of a Western diet containing cod instead of pork alters fatty acid composition in tissue phospholipids and attenuates obesity and hepatic lipid accumulation in mice

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Abstract

The content of the marine n-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is far lower in lean than in fatty seafood. Cod filets contain less than 2 g fat per kg, whereof approximately 50% is EPA and DHA. However, a large fraction of these n-3 PUFAs is present in the phospholipid (PL) fraction and may have high bioavailability and capacity to change the endocannabinoid profile. Here we investigated whether exchanging meat from a lean terrestrial animal with cod in a background Western diet would alter the endocannabinoid tone in mice and thereby attenuate obesity development and hepatic lipid accumulation. Accordingly, we prepared iso-caloric diets with 15.1 energy (e) % protein, 39.1 e% fat and 45.8 e% carbohydrates using freeze-dried meat from cod filets or pork sirloins, and using a combination of soybean oil, corn oil, margarine, milk fat, and lard as the fat source. Compared with mice receiving diets containing pork, mice fed cod gained less adipose tissue mass and had a lower content of hepatic lipids. This was accompanied by a lower n-6 to n-3 ratio in liver PLs and in red blood cells (RBCs) in the mice. Furthermore, mice receiving the cod-containing diet had lower circulating levels of the two major endocannabinoids, N-arachidonoylethanolamine and 2-arachidonoylglycerol. Together, our data demonstrate that despite the relatively low content of n-3 PUFAs in cod fillets, the cod-containing diet could exert beneficial metabolic effects.

Keywords: Diet; Dietary lipids; Endocannabinoids; Fish oil; Phospholipids; Liver; Obesity

1. Introduction

The health beneficial effects of seafood, fatty fish in particular, have in general been attributed [1–6] to their content of marine n-3 polyunsaturated fatty acids (PUFAs). The ability of n-3 PUFAs to attenuate and reverse [7,8] obesity and hepatic steatosis in rodents is well documented. Additionally, treatment combining mild caloric restriction with n-3 PUFA supplementation is more effective than each treatment alone in protection against obesity development in mice [9]. This is in line with the study by Thorsdottir et al. demonstrating that 1.5 g of n-3 PUFAs per day, either as supplement or as 150 g of salmon 3 days per week, during eight weeks of caloric restriction significantly increased weight loss in young overweight adults [10].

To investigate the importance of the n-3 PUFA content in salmon, we recently performed a study where we replaced marine oils in salmon feed with vegetable oils. Replacement of fish oil with soybean oil, in particular, profoundly increased the n-6:n-3 ratio in fish filets and in red blood cells (RBCs) collected from mice consuming the salmon [11,12]. Of note, the increased n-6:n-3 ratio in these mice was accompanied with increased obesity, insulin resistance and hepatic steatosis [11–13]. However, the study by Thorsdottir et al. demonstrated that inclusion of lean fish in low energy diets was as efficient as inclusion of fatty fish or fish oil supplement in accentuating weight loss [10]. In C57BL/6 J mice, intake of lean seafood such as white crab meat, scallop and a mixture of cod and scallops has been demonstrated to attenuate obesity and hepatic steatosis induced by high fat, high sucrose feeding [14,15]. Furthermore, compared to a casein-based diet, intake of a cod-containing diet reduced hepatic triacylglycerol (TAG), plasma alanine aminotransferase and aspartate aminotransferase in type 2 diabetic KK-A^{y} mice [16]. Hence, exchanging meat from lean terrestrial animals with lean seafood may influence obesity development and hepatic steatosis. However, the possible importance

Abbreviations: AEA, *N*-arachidonoylethanolamine; 2-AG, 2-arachidonoylglycerol; ALT, alanine aminotransferase; ARA, arachidonic acid; CB1, cannabinoid receptor 1; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; LDH, lactate dehydrogenase; NAFLD, non-alcoholic fatty liver disease; PL, phospholipid; OH-butyrate, 3-hydroxy-butyrate; PUFAs, polyunsaturated fatty acids; RBCs, red blood cells; TAG, triacylglycerol.

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of the fatty acid composition in the lean seafood sources was not evaluated in these studies.

The total content of n-3 PUFAs is far lower in lean than in fatty seafood, but in lean seafood a large fraction of the fatty acids is present in the phospholipids (PLs) [17,18]. The bioavailability of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is higher when they are PL-bound than TAG-bound [19]. Both the anti-obesogenic and the anti-steatotic effects of PL-bound n-3 PUFAs are superior to TAG-bound n-3 PUFAs [20]. The high biological activity of PL-bound PUFAs is suggested to include effects mediated *via* the endocannabinoid signaling system [20,21]. Hence, intake of a Western diet in which lean meat from a terrestrial animal is replaced by lean seafood might be sufficient to modulate the endocannabinoid tone in mice and thereby attenuate development of obesity and hepatic steatosis.

2. Material and methods

2.1. Ethical statement

Animal handling and experiments were performed in accordance with the Norwegian Animal Research Authority (FOTS id.nr 5358), in compliance with the European convention for the protection of vertebrate animals used for experiments and other scientific purposes (Council of Europe, no. 123, Strasbourg, France, 1985).

2.2. Experimental diets

The experimental diets were based on the 5TJN, Western diet for rodents (Test Diet, USA). The cod and pork powders were prepared from filets of wild caught cod (Lerøy, Bergen, Norway) and pork sirloin (H. Bragstad A/S, Bergen, Norway) that had been heated in a steamer to a core temperature of 70 °C, freeze-dried and pulverized. In order to balance the macronutrient composition in the experimental diets, we measured total fat, nitrogen content as well as amino acid composition (Table S1) and fatty acid composition (Table S2) in the freeze-dried meat as described earlier [14]. The amount of freeze-dried meat required to achive 18 weight % (w%) protein was based on nitrogen measurements of the freeze-dried meat (crude protein, N × 5.6). Apart from supplemented cystein (3 g/kg), meat from cod and pork were used as the sole protein sources in the experimental diets, hence, the amino acid composition in the diets (Table S3) reflected the amino acid compositions measured in the freeze-dried pork and cod fillets. The freeze-dried cod and pork meat differed in fat content and had different fatty acid composition (Table S2). In particular, compared to pork meat, both absolute and relative levels of marine n-3 PUFA were higher and levels of n-6 PUFAs lower in cod meat. Hence, the n-6 to n-3 PUFA ratio was lower in cod meat than pork meat, 0.08 *versus* 6.37. Based on the amount of meat required to achieve 18 w% protein and the endogenous fat content, the amount of fat required to achieve 19.8 w% fat in both diets were calculated. The final diet recipes are shown in Table 1. As the amount of added fat by far exceeded the amount of endogenous fat in the freeze-dried meat, the relative differences in both n-3 PUFA and n-6 PUFAs were diluted (Table S4). Still, the n-6 to n-3 PUFA ratio was lower in the diet containing cod meat than the pork-based diet, 4.9 *versus* 9.3. The balanced Western diets were prepared using a Crypto Peerless EF20 blender and stored at −20 °C. Before use, we measured energy by bomb calorimetry and the total fat content of the diets as described earlier [14].

2.3. Animals

Fifty C57BL/6JBomTac male mice were obtained from Taconic at the age of 8 weeks (Ejby, Denmark). After one week of acclimatization on a low fat diet they were assigned to experimental groups (n=9) by body composition (see Section 2.4) and fed the experimental diets *ad libitum* for twelve weeks. A third group of mice fed the pork containing Western diet was pair fed with the group fed the cod containing Western diet, and therefore mildly calorie restricted. As a reference for expected growth and obesity development in C57BL/6 J mice, a reference group was fed a regular casein-based low-fat diet (ssniff EF R/M control (E15000–04), Germany). The mice were housed in individual cages at thermoneutrality (28–30 °C) with a 12 h light/dark cycle, fed three times a week and weighed once a week during the trial. After 12 weeks of feeding, fat mass and lean mass were measured (see Section 2.4). The mice were anesthetized using isoflurane (Isoba-vet, Schering-Plow, Denmark) and sacrificed by cardiac puncture. Liver and adipose tissue were quickly dissected out, weighed and snap-frozen in liquid nitrogen and stored at −80 °C before further analysis. Blood was collected in tubes containing EDTA, RBC and plasma were prepared by centrifugation and stored at −80 °C. Plasma for lipidomic analysis was prepared according to the procedure described in a separate section below.

2.4. Body composition of the mice

Whole body fat mass, lean mass and free water mass were determined in live conscious mice by noninvasive scanning using the Bruker Minispec LF50 Body Composition Analyzer mq 7.5 (Bruker Optik GmbH, Germany), which uses a time-domain magnetic resonance system as described elsewhere [22].

Table 1
Compositions of the diets

Component[a] (g/kg)	Low fat	Western diet	
	Casein	Cod	Pork
Cod	206.4	219.1[b]	
Pork			239.7[b]
Carbohydrates			
Corn starch		300.3	302.8
Dextrin	532.4	100.0	100.0
Sucrose	91.8	80.0	80.0
Fat			
Soybean oil		12.0	12.0
Corn oil	68.9	8.0	8.0
Milk fat		59.5	51.8
Lard		59.5	51.8
Margarine		59.5	51.8
Fat from protein source[c]		1.4	24.5
Cholesterol		1.5	1.5
Analyzed[d]			
Fat	71.0	188	189
Energy (kJ/g)	4.3	20.8	20.9

Abbreviations: AA; amino acids.
[a] All diets were supplemented with 0.01 g/kg t-Butylhydroquinone, 35 g/kg AIN93G mineral mix, 10 g/kg AIN93VX NCR95 compliant vitamin mix, 3 g/kg L-cystine, 2.5 g/kg choline bitartrate and 50 g/kg cellulose.
[b] The amount of freeze-dried meat powder added is based on measurements of nitrogen in protein powder. Crude protein concentration was calculated using the formula N*6.15 for casein and N*5.6 for cod and pork.
[c] The calculated contribution of fat present in the protein sources. Calculation is based on measurements of total lipid content in protein powder.
[d] Analyzed values represents mean of triplicate measurements.

2.5. Feed efficiency and apparent digestibility

Data collected during the first nine weeks of feeding was used to calculate feed efficiency as body mass gain per energy intake. The data for feed efficiency and body mass development are only reported until week 9, as both an insulin and glucose tolerance tests performed in the following weeks interfered with both feed intake and body mass gain. After 9 weeks of feeding, the mice were placed in new cages with the normal wood chip layer replaced by a paper lining, and 7 days feces was quantitatively collected, weighted and frozen at −80 °C until analyzed for nitrogen and total fat content. Based on feed intake and feces analyses apparent digestibility of fat and nitrogen was calculated as follows: 100 x [intake (mg) - fecal output (mg)/(intake (mg)].

2.6. Plasma analyses

MaxMat PL II analyzer (MAXMAT S.A., Montpellier, France) and conventional kits were used to measure lactate dehydrogenase (LDH), alanine aminotransferase (ALT), triacylglycerol (TAG) (MaxMat, France), HDL cholesterol (Dialab, Austria) and 3-hydroxy-butyrate (OH-butyrate) (Randox, United Kingdom). EDTA-plasma samples for analysis of oxylipins and endocannabinoids were prepared in methanol containing 1 μM butylated hydroxytoluene, (Sigma #47,168) and protease inhibitors; 1 μM soluble epoxide hydrolase inhibitor, (Cayman #10,007,927), 1 μM monoglycerol lipase inhibitor (Cayman #10,007,457), 1 μM omega-hydrolase inhibitor (Cayman #10,018) and 1 μM CYP450 inhibitor (Cayman #75,770). Analyses of oxylipins and endocannabinoids in plasma and liver were performed according to a published method [23]. Briefly, lipids were extracted using Strata-X SPE columns and analyzed with a UPLC system (UltiMate 3000 Binary RSLC System, Thermo) coupled to a Qtrap 5500 (AB-Sciex, Foster City, *CA*) mass spectrometer using multiple reaction monitoring.

2.7. Histology

Liver samples were prepared for histology from five mice in each group, selected based on a representative body mass close to the mean for each group. A part of the liver was fixed in 4% formaldehyde in 0.1 M phosphate buffer (PB) overnight. The tissue was rinsed once in PB, gradually dehydrated in increasing concentration of alcohol, cleared in xylene and embedded in paraffin blocks. Sectioning was performed at the Molecular Imaging Center (MIC) at the University of Bergen. Five μm thick sections were stained with Hematoxylin–Eosin for morphology investigations.

2.8. Real time qPCR

RNA was extracted by homogenization of liver tissue together with Trizol reagent (Invitrogen). The RNA quantity was evaluated using the NanoDrop ND-1000 UV–vis Spectrophotometer (NanoDrop Technologies), and RNA quality tested on a random selection

of samples by BioAnalyzer – RNA 6000 Nano (Agilent Technologies). Reverse transcription and real time qPCR analysis were performed as described [24] and mRNA expression normalized to the housekeeping gene *Calnexin*. Primer sequences are available on request.

2.9. Fatty acid composition measurements in neutral (NL) and polar lipids (PL)

Lipids were extracted from freeze-dried meat, diets, RBC and liver with chloroform-methanol (2:1, v/v). Extracts of diets and liver were evaporated to dryness and lipids recovered in chloroform to yield 50 mg lipid/ml. An aliquot of 10 mg lipid was subsequently applied to a solid-phase extraction column (Isolute, Biotage). Extracts of RBCs were evaporated to dryness and lipids recovered by three washings with 100 µl chloroform prior to application on the solid-phase column. Neutral lipids (NLs) were eluted with 10 ml chloroform-methanol (92:2, v/v) and polar lipids (PLs) were eluted with 10 ml methanol. The extracted lipids were filtered prior to saponification and methylation using 12% BF_3 in methanol. Methylated fatty acids were separated using a Trace gas chromatograph 2000 (Fison, Elmer, USA), equipped with a 50-m CP-sil 88

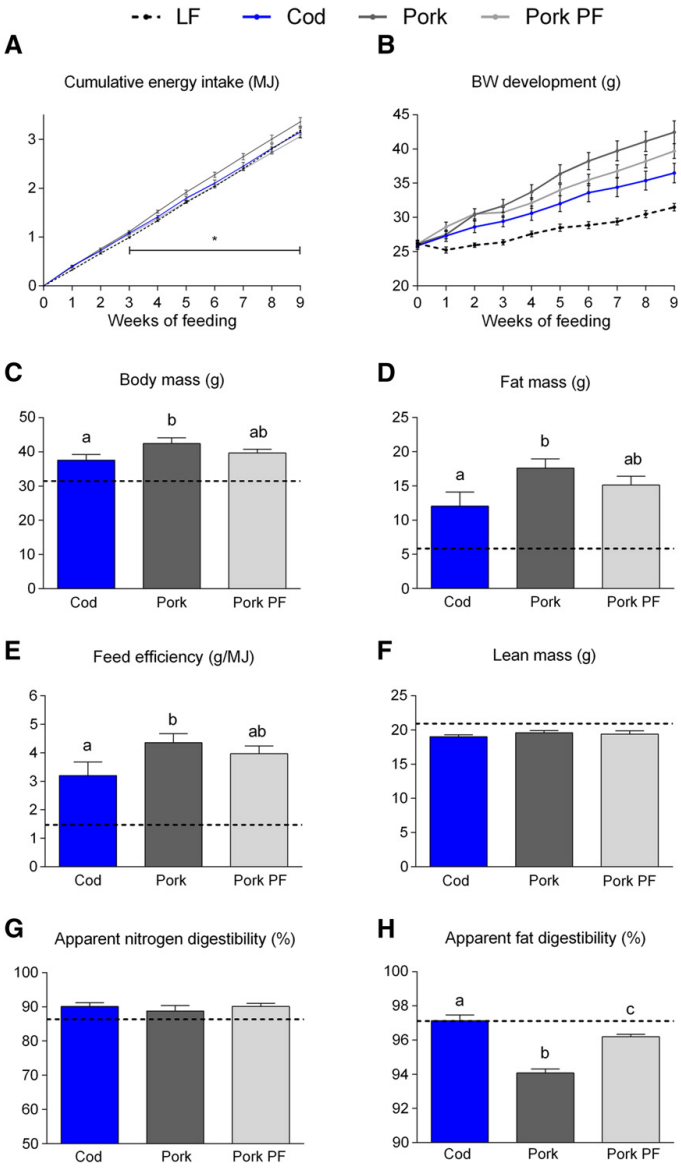

Fig. 1. Effect of Western diets with either cod or pork on body weight, feed efficiency and digestibility. Male C57BL/6 J mice were fed Western diets containing either cod or pork as protein source for 12 weeks. A separate group of mice receiving pork was pair-fed (Pork PF) with the group fed cod. A low fat fed reference group was also included and is shown as a dotted line. Feed intake was recorded continuously and (A) cumulative energy intake (MJ) determined and shown for the first 9 weeks of feeding. (B) Body weight (BW) development the first 9 weeks as well as (C) body mass and (D) fat mass, determined using nuclear magnetic resonance, after 12 weeks are presented. Based on energy intake and body mass (E) feed efficiency (g/MJ) was calculated. Body composition of (F) lean mass was measured after 12 weeks of feeding. Apparent (G) nitrogen and (H) fat digestibility (%) were calculated based on feed intake and feces collected in the 8th week of feeding. Data are presented as mean ± SEM (n = 8–9). Significant differences ($p<0.05$) between the groups are presented with different letters. * denotes significant differences between mice fed cod and pork *ad libitum* from week 3.

(Chromopack) fused silica capillary column (id: 0.32 mm) as described in detail earlier [18,25]. The fatty acids were identified by retention time using standard mixtures of methyl esters (Nu-Chek, Elyian, USA), and fatty acid composition (area %) was determined. All samples were integrated using the software Chromeleon® version 6.8. The fatty acids were quantified using the methyl ester of C19:0 (nonadecanoic acid) as an internal standard.

2.10. Statistics

Data throughout this paper are presented as mean ± SEM and were analyzed using one-way ANOVA followed by Fisher's LSD multiple comparison post-hoc test. For all analyses n = 9/group with the exception of n = 8 in the group fed cod, because one mouse in this group was excluded from the experiment due to an abnormal liver noticed during dissection. Cumulative energy intake was analyzed by repeated measurements ANOVA and Fisher's LSD post-hoc test. The LF group was included only as a reference group in this study and was thus not included in the statistical analysis. Differences between the group means were considered significant when $p < 0.05$, and this is presented by different letters in the figures and tables.

3. Results

3.1. Exchanging pork with cod attenuates obesity

Here we aimed to investigate whether exchanging meat from a terrestrial animal with meat from a lean fish attenuates obesity development and modulates the endocannabinoid tone. We freeze-dried meat from cod filets and pork sirloins and prepared Western diets with 39.1 e% fat using a combination of soybean oil, corn oil, milk fat, lard and margarine, matching the 5TJN, Western diet for rodents, Test Diet (Table 1). To investigate the effect of these Western diets containing cod or pork on obesity development, we fed C57BL/6 J mice *ad libitum*. We have earlier observed that energy intake is lower in crab and scallop fed mice than in chicken fed mice, suggesting that seafood protein may reduce feed intake [14]. Accordingly, we included a second group of pork-fed mice that were pair-fed (pork-PF) with the group of mice fed cod (Fig. 1A). Energy intake was 6% lower in *ad libitum* cod fed mice than in pork fed mice, and hence, the pair-fed pork mice were mildly energy restricted. As references for expected growth and obesity development in these mice, a reference group was fed a regular casein-based low-fat diet.

Compared with mice fed the pork containing diet *ad libitum*, mice fed the cod containing diet gained significantly less body mass and fat mass (Fig. 1B, C and D). Body mass and fat mass in the energy restricted pork-fed group were in between and not significantly different from either of the *ad libitum* fed groups (Fig. 1C and D). Of note, feed efficiency in the *ad libitum* pork fed mice was significantly higher than that of cod fed mice (Fig. 1E). The lean body masses were comparable (Fig. 1F), and hence, feed efficiency reflected total body mass and total fat mass. To exclude the possibility that the observed differences in feed efficiency were a consequence of different digestibility, we measured apparent digestibility of nitrogen and fat. The apparent digestibility of nitrogen was similar in all mice (Fig. 1G), whereas the apparent digestibility of fat was higher in the cod fed than in the pork fed mice (Fig. 1H). Thus, the reduced fat mass in cod fed mice could not be explained by lower digestibility.

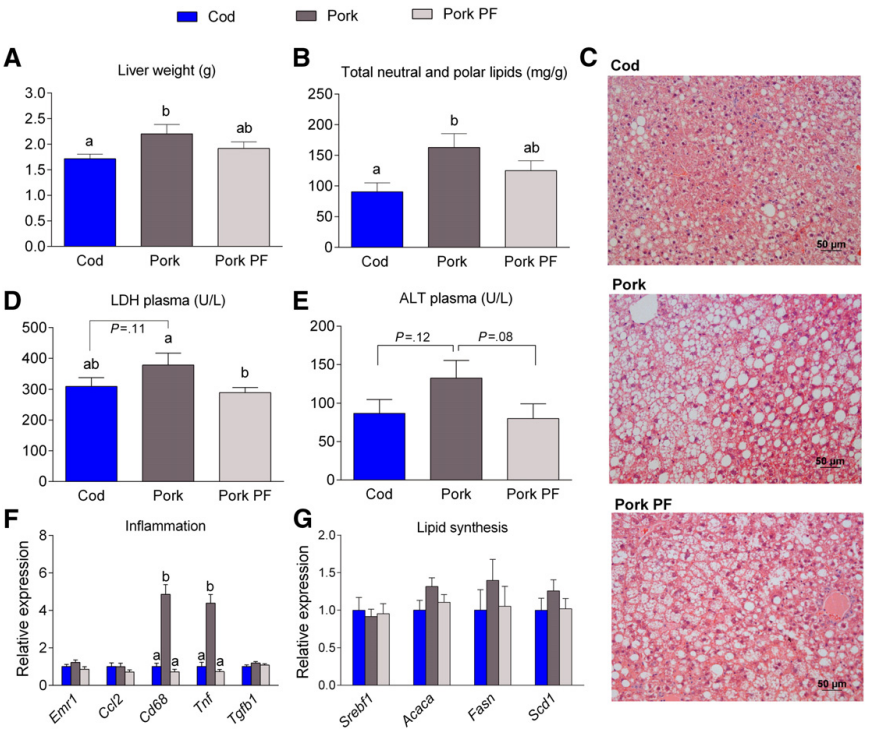

Fig. 2. Effect of Western diets with cod or pork on lipid accumulation and inflammation in liver. Male C57BL/6 J mice were fed Western diets containing either cod or pork. A separate group of mice receiving pork was pair-fed (Pork PF) with the group fed cod. After 12 weeks of feeding, the mice were sacrificed and livers dissected out and (A) weighed before lipids were extracted, separated in the NL and PL fraction and (B) quantified. A sample from the liver of each mouse was also prepared for histology, stained with hematoxylin and eosin and (C) a micrograph (20x) of one representative mouse in each group is presented. Plasma was prepared from EDTA blood and levels of circulating (D) LDH and (E) ALT were determined using Maxmat. RNA was isolated from liver samples using Trizol, cDNA prepared and relative mRNA expression of genes related to (F) inflammation and (G) lipid synthesis is shown. Data are presented as mean ± SEM (n = 8–9). Significant differences ($p<0.05$) between the groups are marked with different letters.

3.2. Exchanging pork with cod attenuates hepatic lipid accumulation

Mice fed the Western diets containing cod had lower liver mass and accumulated less fat in the liver than mice fed the Western diets containing pork *ad libitum* (Fig. 2A and B). The higher hepatic lipid accumulation was further visualized by histological analyses demonstrating large lipid droplets in pork fed mice (Fig. 2C). Accumulation of hepatic fat in the energy restricted pork-fed mice was not significantly different from either of the *ad libitum* fed groups (Fig. 2B). Plasma levels of lactate dehydrogenase (LDH) and alanine aminotransferase (ALT) in *ad libitum* fed mice reflected hepatic fat accumulation, but were not significantly different (Fig. 2D and E). In line with this, expression of fibrotic markers, such as collagen, type 1, alpha 1 (*Col1a1*) and matrix metallopeptidase 2 and 9 (*Mmp2* and *Mmp9*) did not differ significantly (not shown). Measurements of expression levels of inflammatory markers, however, demonstrated that exchanging pork with cod attenuated expression of *Cd68* and tumor necrosis factor (*Tnf*) (Fig. 2F). Increased hepatic lipid accumulation arises from an imbalance between lipid disposal (secretion, oxidation) and availability (uptake and synthesis). Hence, to investigate the mechanism by which cod attenuated hepatic lipid accumulation we measured the expression of genes involved in lipoprotein secretion, fatty acid oxidation, fatty acid uptake and *de novo* fatty acid synthesis. Expression levels of genes involved in lipoprotein secretion and fatty acid oxidation were not significantly different between cod and pork fed mice (Fig. S1 A). In keeping with this, plasma levels of triacylglycerol, HDL-cholesterol and 3-hydroxy-butyrate were not affected by exchange of the dietary protein source (Fig. S1C, D and E). Thus, lipid disposal appeared to be unaffected.

Expression of genes involved in lipid uptake was not affected by the meat source (Fig. S1B). The increased accumulation of saturated and mono-unsaturated fatty acid in the liver of pork fed mice (Table 2) did not reflect the dietary level (Table S4) suggesting that these fatty acids originated from de novo syntheses rather than uptake. Expression levels of acetyl-CoA carboxylase alpha (*Acaca*), fatty acid synthase (*Fasn*) and stearoyl-Coenzyme A desaturase 1 (*Scd1*) followed the same pattern with a tendency towards higher expression in mice fed the pork-based diet *ad libitum* compared to the cod fed mice (Fig. 2G).

3.3. Exchanging pork with cod decreases n-6:n-3 PUFA ratio in red blood cells (RBCs) and liver lipids

The fat content in both Western diets comprised more than 90% neutral lipids (Fig. 3A). Hence, the fatty acid composition in the neutral lipid fraction would dominate the dietary intake. Accordingly, although 49% of the fatty acids in cod meat comprise the marine omega-3 fatty acids, EPA and DHA (Fig. 3B and Table S2), the relative proportions of EPA and DHA in the cod containing diet were only, 0.6 and 1.3%, respectively (Table S4). Less than 10% of the dietary fat was in the form of phospholipids (PLs) (Fig. 3A). However, this fraction was strongly dominated by the marine n-3 PUFAs, EPA and DHA, in the cod containing diets and by the n-6 PUFAs, linoleic acid (LA) and arachidonic acid (ARA), in the pork based diet (Fig. 3B and Table 3).

To investigate the spillover effect in the mice receiving the diets we measured fatty acid composition in RBCs and in neutral and polar liver lipids. We observed higher amounts of EPA and DHA in RBCs collected from cod fed mice than pork fed mice (Table 4). Furthermore, lower amounts of n-6 PUFAs, in particular ARA were found in RBCs collected from cod fed mice compared to RBCs from pork fed mice (Table 4). Despite a relatively low amount of n-3 PUFAs in the cod diet and comparable amounts of n-6 PUFAs, higher levels of n-3 and lower levels of n-6 PUFAs were observed in RBCs collected from cod fed mice (Table 4). We also observed higher amounts of EPA and DHA in both neutral and polar hepatic lipids in mice fed cod than in mice fed pork (Fig. 3C and D). Importantly, this was accompanied by a lower n-6:n-3 PUFA as well as ARA:EPA ratio (Table 4). Of note, the relative amounts of LA were high, 19.8 and 19.9%, in the cod and pork containing diets, respectively (Table S4), and LA accumulated mainly in neutral liver lipids (Fig. 3E). By contrast, the relative amounts of dietary ARA were low, 0.2% and 0.6%, in the cod and pork containing diets, respectively (Table S4), and ARA accumulated to a higher level in the polar lipids (Fig. 3F). The amounts of both LA and ARA were higher in neutral liver lipids in pork than in cod fed mice (Fig. 3E and F), and the level of ARA, but not LA, was also higher in polar liver lipids in pork than in cod fed mice (Fig. 3E and F). Non-alcoholic fatty liver disease (NAFLD) is associated with increased hepatic levels of saturated and monounsaturated fat [26]. In line with attenuated hepatic lipid accumulation in cod fed mice, these mice accumulated significantly less saturated and monounsaturated fatty acids in hepatic neutral lipids than mice fed pork (Fig. 3G and H and Table 4).

Table 2
Fatty acid compositions in neutral and polar lipid fractions isolated from mouse liver

Experimental diets	Cod	Pork	Pork PF
Neutral lipid fraction			
Fatty acid (mg/g)			
Sum SFA	17 ± 3^{a}	36 ± 6^{b}	26 ± 4^{b}
Sum MUFA	40 ± 15^{a}	88 ± 14^{b}	65 ± 10^{ab}
OA 18:1n-9	29 ± 6^{a}	63 ± 10^{b}	47 ± 7^{ab}
LA 18:2n-6	10 ± 2^{a}	18 ± 2^{b}	13 ± 2^{ab}
ARA 20:4n-6	0.35 ± 0.05^{a}	1.1 ± 0.2^{b}	0.7 ± 0.1^{c}
Sum n-6	11 ± 2^{a}	20 ± 3^{b}	15 ± 2^{ab}
ALA 18:3n-3	0.7 ± 0.1	0.8 ± 0.1	0.6 ± 0.1
EPA 20:5n-3	0.6 ± 0.1^{a}	0.19 ± 0.02^{b}	0.15 ± 0.02^{b}
DHA 22:6n-3	3.1 ± 0.5^{a}	0.9 ± 0.1^{b}	0.6 ± 0.1^{b}
Sum n-3	5.2 ± 0.9^{a}	2.6 ± 0.3^{b}	1.8 ± 0.2^{b}
Sum identified FAs	74 ± 14^{a}	148 ± 23^{b}	109 ± 16^{ab}
n-6:n-3 ratio	2.3 ± 0.2^{a}	7.8 ± 0.4^{b}	8.1 ± 0.3^{b}
ARA:EPA ratio	0.7 ± 0.2^{a}	5.4 ± 0.3^{b}	5.0 ± 0.5^{b}
Polar lipid fraction			
Sum SFA	5.8 ± 0.2^{a}	4.9 ± 0.2^{b}	5.3 ± 0.2^{ab}
Sum MUFA	2.4 ± 0.2	2.4 ± 0.1	2.6 ± 0.1
OA 18:1n-9	1.62 ± 0.07	1.5 ± 0.1	1.7 ± 0.1
LA 18:2n-6	2.9 ± 0.2^{a}	2.2 ± 0.2^{b}	2.6 ± 0.2^{ab}
ARA 20:4n-6	1.8 ± 0.1^{a}	2.8 ± 0.2^{b}	3.1 ± 0.2^{b}
Sum n-6	5.2 ± 0.3^{a}	5.6 ± 0.4^{ab}	6.3 ± 0.4^{b}
ALA 18:3n-3	0.011 ± 0.003	0.006 ± 0.003	0.004 ± 0.002
EPA 20:5n-3	0.56 ± 0.05^{a}	0.078 ± 0.007^{b}	0.08 ± 0.01^{b}
DHA 22:6n-3	2.5 ± 0.1^{a}	1.3 ± 0.1^{b}	1.4 ± 0.1^{b}
Sum n-3	3.2 ± 0.2^{a}	1.5 ± 0.1^{b}	1.6 ± 0.1^{b}
Sum identified FAs	16.6 ± 0.8	14.5 ± 0.8	16.0 ± 0.8
n-6:n-3 ratio	1.60 ± 0.03^{a}	3.7 ± 0.1^{b}	3.9 ± 0.1^{b}
ARA:EPA ratio	3.2 ± 0.1^{a}	37 ± 2^{b}	36 ± 3^{b}

The values represent mean ± SEM and indicate mg fatty acid/g liver. Significant differences ($p<0.05$) between the groups are marked with different letters.
Abbreviations: SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, OA; oleic acid, LA; linoleic acid, ARA; arachidonic acid, ALA; α-linolenic acid, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid, FAs; fatty acids.

3.4. Exchanging pork with cod reduces plasma levels of 2-arachidonoylglycerol (2-AG) and N-arachidonoylethanolamine (AEA)

Both ARA and n-3 PUFAs released from liver PLs may be converted into lipid mediators, such as endocannabinoids and eicosanoids that potentially affect development of NAFLD. In line with the concomitantly lower accumulation of ARA and higher accumulation of EPA and DHA in hepatic PLs, plasma levels of COX/LOX products using ARA as a substrate, such as 15-HETE, 5-HETE and 6-keto-PGF1α tended to be higher in pork than in cod fed mice (Fig. 4A). At the same time, COX/LOX products using n-3 PUFAs as a substrate, such as 14-HDoHE, 17-HDoHE and 10,17-diHDHA tended to be lower (Fig. 4B). Furthermore, plasma levels of the two major endocannabinoids produced from ARA, 2-AG and AEA were significantly higher in pork than in cod fed mice,

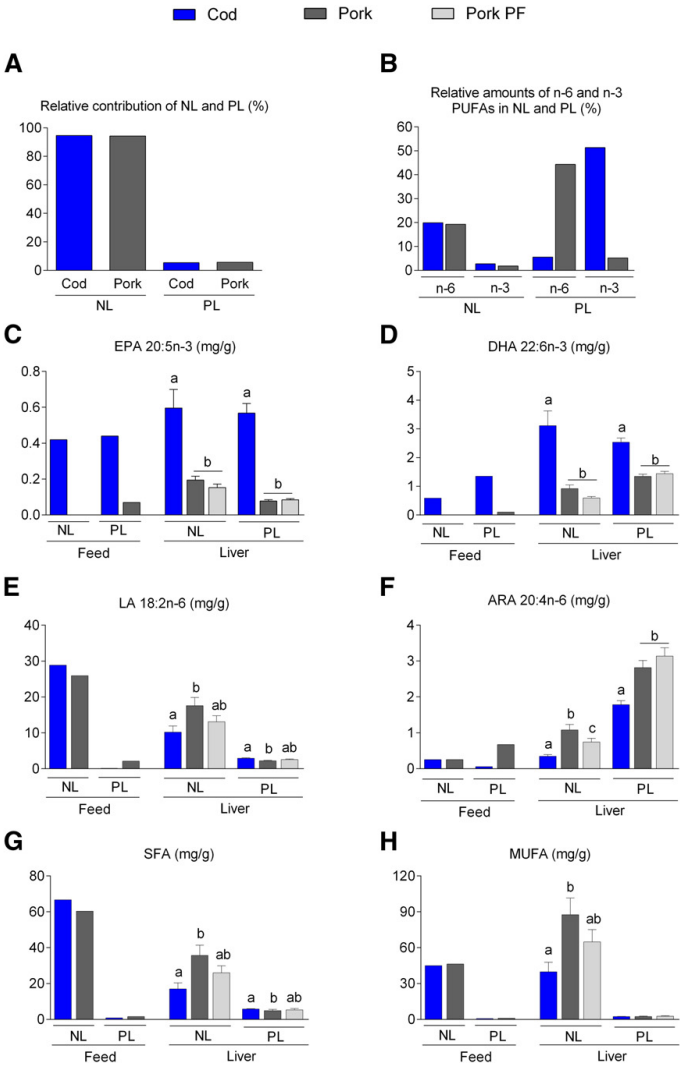

Fig. 3. Effect of cod and pork on fatty acid composition in the Western diets and liver from the mice fed the diets. Filets of cod and pork were freeze-dried, homogenized and used as protein source in Western diets. Lipids from a sample of each diet were extracted and separated into NL and PL fractions prior to quantification and determination of fatty acid composition. (A) The relative contribution of fatty acids in the NL and PL-fraction. (B) The relative amounts of n-6 and n-3 PUFAs in the NL and PL-fraction. Male C57BL/6 J mice were fed the Western diets containing either cod or pork for 12 weeks. A separate group of mice receiving pork was pair-fed (Pork PF) with the group fed cod. After sacrificing the mice, fat from the liver was extracted and the fatty acids (C) EPA, (D) DHA, (E) linoleic acid (LA), (F) arachidonic acid (ARA) and (G) saturated fatty acids (SFA) and (H) mono-unsaturated fatty acids (MUFA) were measured in the NL and PL fractions in the Western diets and in liver for comparison. Data from the liver represent mean ± SEM (n = 8–9). Significant differences ($p<0.05$) between the groups are marked with different letters.

whereas docosahexaenoyl ethanolamide (DHEA) produced from DHA was significantly lower (Fig. 4C). Together, our data demonstrate that a low proportion of n-3 PUFAs in cod filets used in the diet is sufficient to replace ARA in RBCs and hepatic phospholipids in mice and thereby modulate the endocannabinoid profile.

4. Discussion

In this study we demonstrate that intake of a Western diet containing cod instead of pork alters fatty acid composition in tissue phospholipids and attenuates obesity and hepatic lipid accumulation in mice. Although pork and cod are both lean meat sources, they differ with respect to fatty acid composition and distribution of fatty acids between TAGs and PLs. Thus, despite a low content of n-3 PUFAs in cod fillets, mice fed a Western diet containing cod exhibited a marked increase in the levels of EPA and DHA at the expense of ARA in RBCs and hepatic PLs in comparison with mice fed a Western diet containing pork. This was accompanied by a modulation of the endocannabinoid profile, attenuated obesity development and reduced accumulation of hepatic lipids.

Table 3
Fatty acid compositions in neutral and polar lipid fractions isolated from feed

	Neutral lipid fraction				Polar lipid fraction			
	Cod		Pork		Cod		Pork	
Fatty acid	mg/g	(%)	mg/g	(%)	mg/g	(%)	mg/g	(%)
Sum SFA	66.7	(46.0)	60.4	(44.4)	0.9	(25.0)	1.6	(27.6)
OA 18:1n-9	38.8	(31.0)	40.0	(29.0)	0.3	(8.5)	0.8	(11.8)
Sum MUFA	44.9	(26.8)	46.4	(33.7)	0.6	(17.7)	1.0	(15.2)
LA 18:2n-6	28.9	(19.9)	26.0	(19.1)	0.1	(2.8)	2.1	(34.5)
ARA 20:4n-6	0.2	(0.1)	0.3	(0.2)	0.06	(1.7)	0.7	(12.1)
Sum n-6	29.4	(20.3)	26.6	(19.6)	0.2	(5.6)	2.9	(50.0)
ALA 18:3n-3	2.6	(1.8)	2.3	(1.7)	0.01	(0.3)	0.07	(1.21)
EPA 20:5n-3	0.4	(0.3)	<0.01	(n.d)	0.4	(11.1)	0.07	(1.21)
DHA 22:6n-3	0.6	(0.4)	<0.01	(n.d)	1.4	(38.9)	0.1	(1.7)
Sum n-3	4.0	(2.8)	2.5	(1.8)	1.9	(52.8)	0.3	(5.2)
Sum identified FAs	145	(100.0)	136	(100.0)	3.6	(100.0)	5.9	(100.0)
n-6:n-3 ratio	7.3		10.6		0.1		9.7	
ARA:EPA ratio	0.5		n.d		0.2		10.0	

All values represent mean of triplicate measurements and indicate mg fatty acid/g diet. The values in within parentheses represent mol% of the fatty acids.
Abbreviations: SFA; saturated fatty acids, OA; oleic acid, MUFA; monounsaturated fatty acids, LA; linoleic acid, ARA; arachidonic acid, ALA; α-linolenic acid, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid, FAs; fatty acids.

The added fat in both Western diets used in this experiment was a combination of soybean oil, corn oil, milk fat, lard and margarine, comprising more than 90% of the neutral lipids. Hence, the fatty acid profile in neutral lipids dominated the total fatty acid composition in the diets. The amount of EPA and DHA in the Western diet containing cod was 3 mg/g, a dose far below those used in studies examining the anti-obesogenic and anti-steatotic effect of n-3 PUFAs where doses ranging from 60 up to 350 mg/g have been used [1–8]. Less than 10% of the dietary fat was in the form of PLs, in which the n-3 PUFA levels were higher and n-6 PUFA levels lower in the cod containing diet than in the pork containing diet. In line with the reported higher bioavailability of PL-bound relative to TAG-bound EPA and DHA [19], we observed markedly different n-6:n-3 ratios in RBCs and liver collected from cod and pork fed mice with the n-6:n-3 ratio being significantly reduced in the PL-fraction from cod fed mice. The striking increase in the intake of n-6 PUFA accompanied with reduced intake of n-3 PUFAs is a potential human health concern [27–29]. Thus, the observation that n-3 PUFAs from lean seafood is able to replace ARA at relatively low doses warrants further investigations in humans.

Table 4
Fatty acid compositions in red blood cells

Experimental diets	Cod	Pork	Pork PF
Fatty acid (mg/g)			
Sum SFA	1.33 ± 0.03	1.20 ± 0.03	1.20 ± 0.04
Sum MUFA	0.49 ± 0.02	0.51 ± 0.01	0.51 ± 0.02
LA 18:2n-6	0.42 ± 0.01^{a}	0.34 ± 0.01^{b}	0.35 ± 0.01^{b}
ARA 20:4n-6	0.265 ± 0.009^{a}	0.53 ± 0.01^{b}	0.55 ± 0.02^{b}
Sum n-6	0.75 ± 0.02^{a}	0.87 ± 0.03^{b}	0.90 ± 0.03^{b}
ALA 18:3n-3	0.0011 ± 0.0008	0.002 ± 0.001	0.002 ± 0.001
EPA 20:5n-3	0.075 ± 0.003^{a}	0.012 ± 0.001^{b}	0.012 ± 0.001^{b}
DHA 22:6n-3	0.299 ± 0.009^{a}	0.169 ± 0.004^{b}	0.176 ± 0.007^{b}
Sum n-3	0.41 ± 0.01^{a}	0.216 ± 0.006^{b}	0.223 ± 0.008^{b}
Sum identified FAs	2.98 ± 0.08	2.9 ± 0.2	3.0 ± 0.2
n-6:n-3 ratio	1.81 ± 0.04^{a}	4.64 ± 0.03^{b}	4.64 ± 0.07^{b}
ARA:EPA	3.5 ± 0.2^{a}	43.4 ± 1.6^{b}	46.4 ± 1.5^{b}

The values represent mean ± SD and indicate mg fatty acid/g red blood cells. Significant differences ($p<0.05$) between the groups are marked with different letters.
Abbreviations: SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, LA; linoleic acid, ARA; arachidonic acid, ALA; α-linolenic acid, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid, FAs; fatty acids.

The anti-obesogenic effect of PL-bound n-3 PUFAs is reported to be superior to TAG-bound n-3 PUFAs, and the high biological activity of PL-bound PUFAs is suggested to be mediated *via* the endocannabinoid signaling system [20,21]. Competition between n-3 PUFAs and ARA for incorporation into PLs reduces substrate availability for syntheses of the two major endogenous endocannabinoids 2-AG and AEA and increases substrate availability for formation of the EPA and DHA derived endocannabinoids, eicosapentaenoyl ethanolamide (EPEA) and DHEA, respectively [5,13,20,30]. In line with this, it is likely that the lower circulating levels of 2-AG and AEA and higher circulating levels of DHEA in cod compared to pork fed mice were directly related to the n-6:n-3 ratio in the liver PL-fraction. Given the importance of the cannabinoid receptor CB1 in diet-induced obesity [31–33], modulation of the endocannabinoid tone represents a mechanism by which an exchange of pork with cod may attenuate development of obesity.

The importance of the cannabinoid receptor CB1 in satiety and energy intake is well described [31,33–35]. In keeping with this, the lower circulating levels of 2-AG and AEA may explain the reduced cumulative energy intake. Hence, cod may attenuate obesity development *via* reduced energy intake mediated by a reduced endocannabinoid tone. Still, feed efficiency was significantly lower in cod fed mice than *ad libitum* pork fed mice. Furthermore, as fat mass in pork fed mice that were pair-fed were in between cod and *ad libitum* pork fed mice other mechanisms may be involved. Mechanisms not directly related to reduced energy intake may, however, still be related to a reduced endocannabinoid tone as treatment of mice with the CB1 antagonist, Rimonabant, leads to a transient reduction in feed-intake, whereas weight loss is sustained [32]. Moreover, pair-feeding experiments have demonstrated that the reduced weight gain in aging CB1-KO mice is not solely due to reduced energy-intake [33].

Expression of the hepatic CB1 receptor has also been demonstrated to be required for diet-induced steatosis [36]. Our finding that mice fed the pork containing diet with a high content of PL n-6 PUFAs have increased levels of circulating 2-AG and AEA is in line with the observation that treatment of mice with the CB1-agonist HU210 led to increased hepatic lipogenesis [35]. Thus, the lower hepatic lipid accumulation in cod than pork fed mice may also relate to a reduced endocannabinoid tone. Worth noting, the superior anti-steatotic effect of PL-bound n-3 PUFAs compared to TAG-bound n-3 PUFAs is suggested to be mediated *via* a pronounced effect of PL-bound n-3 PUFAs on the endocannabinoid signaling system [20,21]. Still, other mechanisms may occur. Of note, non-alcoholic fatty liver disease is associated with increased hepatic levels of saturated and monounsaturated fat [26]. In this study, the observed higher accumulation of saturated and monounsaturated fatty acids in the liver of pork than cod fed mice did not reflect the dietary level. In keeping with a tendency towards higher expression of *Acaca*, *Fasn* and *Scd1* in *ad libitum* pork fed mice, compared with cod fed mice, this is in line with the well described ability of fish oil and n-3 PUFAs to suppress expression of genes involved in lipogenesis [37]. Further, n-3 PUFAs are suggested to reduce hepatic accumulation of TAG by increasing fatty acid oxidation and suppressing TAG formation. However, we did not observe any significant changes in expression of genes related to these processes.

Cod contains a higher proportion of taurine and glycine than pork. Both taurine and glycine have been reported to reduce fat mass and steatosis in rodents [38–40]. Thus, we cannot exclude the possibility that the observed reduction in fat mass and hepatic lipid accumulation in cod, compared with pork fed mice, at least in part, also is related to the differences in amino acid compositions. The gut microbiome has been demonstrated to be affected in mice fed a Western diet based on lean seafood mixture when compared to lean meat mix [41]. This also suggests that we cannot rule out an impact of the gut microbiome in this study between the mice fed cod and pork.

Taken together, our data demonstrate that the amount of n-3 PUFAs in cod filets is sufficient to replace ARA with EPA and DHA in

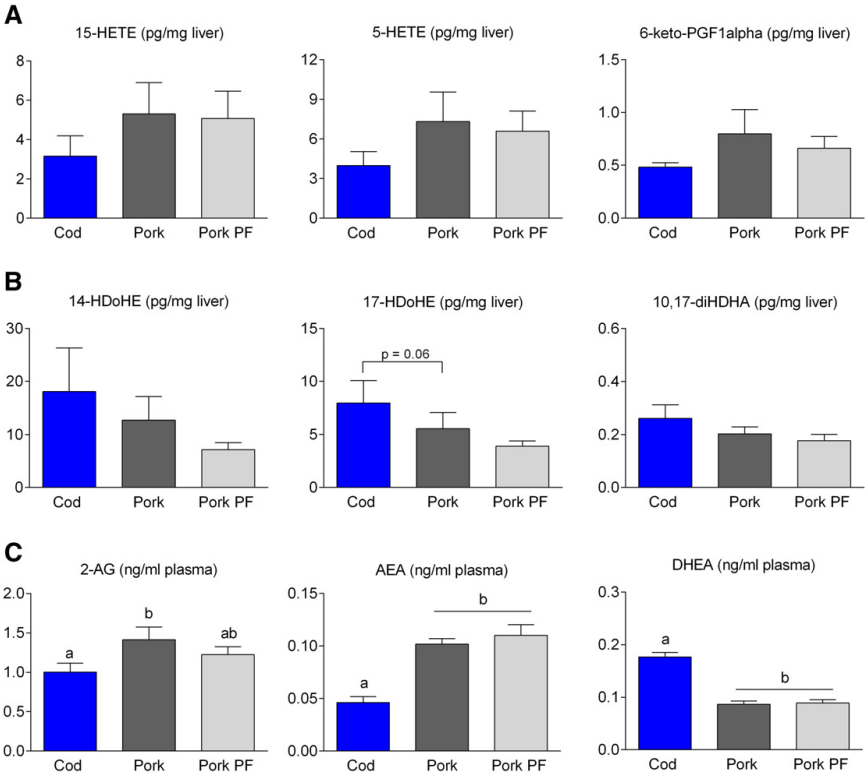

Fig. 4. Effect of Western diets with cod or pork on eicosanoids in liver and endocannabinoids in plasma. Male C57BL/6 J mice were fed Western diets containing either cod or pork for 12 weeks before they were sacrificed. A separate group of mice receiving pork was pair-fed (Pork PF) with the group fed cod. Plasma was prepared in methanol with butylated hydroxytoluene and protease inhibitors, and the livers dissected out. Eicosanoid products in liver derived from (A) ARA and (B) DHA and endocannabinoids (C) in the plasma were extracted using Strata-X SPE columns and analyzed with UPLC. Data are presented as mean ± SEM (n = 8–9). Significant differences ($p<0.05$) between the groups are marked with different letters.

RBCs and hepatic phospholipids in mice, and thereby modulate the endocannabinoid profile. Given the importance of the cannabinoid receptor CB1 in satiety and energy intake as well as development of diet-induced obesity and steatosis, this represents a mechanism by which an exchange of pork with cod in a Western diet may attenuate obesity development and hepatic lipid accumulation.

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Supplementary Tables:

Supplementary Table S1. Amino acid profile in the protein sources

Amino acid	Freeze-dried cod fillet		Freeze-dries pork sirloin	
	mg/g	(%)	mg/g	(%)
Indispensable				
Histidine	16.6	(2.0)	26.6	(3.3)
Isoleucine	39.0	(4.7)	40.9	(5.0)
Leucine	69.0	(8.3)	68.5	(8.4)
Lysine	85.7	(10.3)	81.5	(10.0)
Methionine	27.0	(3.3)	27.0	(3.3)
Phenylalanine	32.7	(3.9)	32.3	(4.0)
Threonine	37.8	(4.5)	37.4	(4.6)
Tryptophan	10.5	(1.3)	11.2	(1.4)
Valine	42.7	(5.1)	42.6	(5.2)
Dispensable				
Alanine	52.4	(6.3)	49.3	(6.0)
Arginine	49.5	(5.9)	48.0	(5.9)
Aspartate/Aspargine	92.7	(11.1)	84.7	(10.4)
Cysteine	5.7	(0.7)	10.2	(1.2)
Glutamate/Glutamine	137.6	(16.5)	133.3	(16.3)
Glycine	37.2	(4.5)	33.3	(4.1)
Proline	29.2	(3.5)	30.8	(3.8)
Serine	37.8	(4.5)	31.8	(3.9)
Tyrosine	28.4	(3.4)	26.6	(3.3)
Sum indispensable	361	(43)	368	(45)
Sum dispensable	471	(57)	448	(55)
Sum BCAA[1]	150	(18)	152	(19)
Sum amino acids	832	(100)	816	(100)
Taurine	4.0		< 0.6	

All values represent mean of triplicate measurements.

[1]Sum BCAA represents the sum of the branched chain amino acids, Leucine, Isoleucine and Valine.

Supplementary Table S2. Fatty acid compositions in the protein powders

	Freeze-dried cod fillet		**Freeze-dried pork sirloin**	
Fatty acid	mg/g	(%)	mg/g	(%)
Sum SFA	4.18	(23.4)	37.10	(37.3)
Sum MUFA	4.31	(24.1)	47.40	(47.7)
LA 18:2n-6	0.27	(1.5)	11.61	(11.7)
ARA 20:4n-6	0.31	(1.7)	1.73	(1.7)
Sum n-6	0.58	(3.2)	13.34	(13.4)
ALA 18:3n-3	0.06	(0.3)	0.84	(0.8)
EPA 20:5n-3	2.65	(14.8)	0.28	(0.3)
DHA 22:6n-3	6.07	(34.0)	0.49	(0.5)
Sum n-3	8.78	(49.2)	1.61	(1.6)
Sum FA	17.85	(100.0)	99.50	(100.0)
n-6:n-3 ratio	0.08		6.37	
ARA:EPA ratio	0.12		6.18	

All values represent mean of triplicate measurements.

Abbreviations: SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, LA; linoleic acid, ARA; arachidonic acid, ALA; α-linolenic acid, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid, FA; fatty acids

Supplementary Table S3. Amino acid profile in the experimental diets.

	Western diet			
	Cod		Pork	
Amino acid	mg/g	(%)	mg/g	(%)
Indispensable				
Histidine	3.6	(1.9)	6.6	(3.3)
Isoleucine	9.1	(4.7)	9.7	(4.8)
Leucine	15.9	(8.2)	16.6	(8.3)
Lysine	20.3	(10.5)	19.2	(9.6)
Methionine	7.3	(3.8)	6.9	(3.5)
Phenylalanine	7.1	(3.7)	8.1	(4.0)
Threonine	8.4	(4.4)	9.2	(4.6)
Tryptophan	2.1	(1.1)	2.5	(1.3)
Valine	9.8	(5.1)	10.1	(5.1)
Dispensable				
Alanine	12.1	(6.2)	11.8	(5.9)
Arginine	10.6	(5.5)	11.4	(5.7)
Aspartate/Aspargine	21.1	(10.9)	20.2	(10.1)
Cysteine	5.3	(2.7)	6.3	(3.1)
Glutamate/Glutamine	31.8	(16.4)	32.1	(16.0)
Glycine	8.3	(4.3)	8.1	(4.1)
Proline	6.8	(3.5)	7.5	(3.7)
Serine	8.4	(4.3)	7.9	(4.0)
Tyrosine	5.3	(2.8)	5.7	(2.9)
Sum indispensable	80	(41)	82	(41)
Sum dispensable	113	(59)	118	(59)
Sum BCAA[1]	35	(18)	36	(18)
Sum amino acids	193	(100)	200	(100)
Taurine	0.8		< 0.6	

All values represent mean of triplicate measurements.

[1]Sum BCAA represents the sum of the branched chain amino acids, Leucine, Isoleucine and Valine.

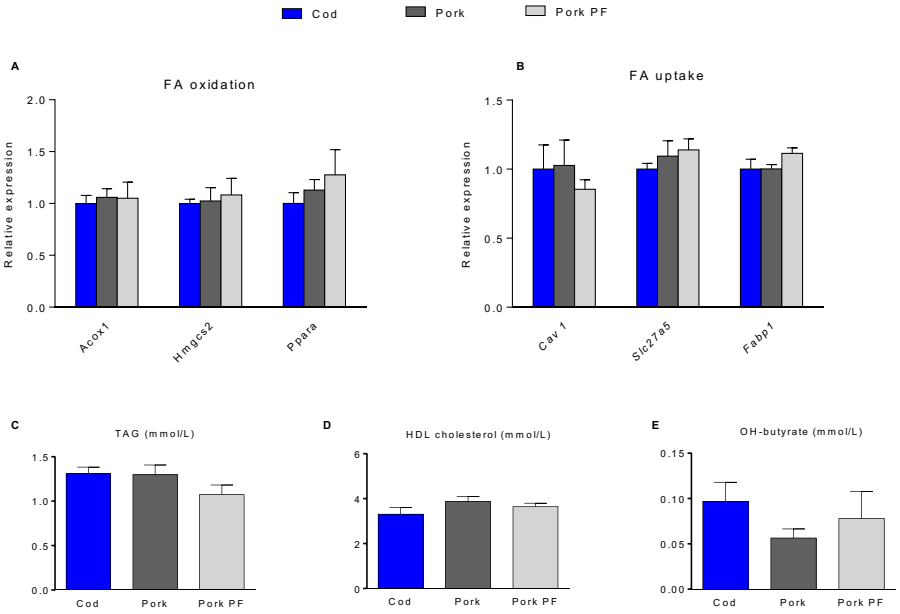
Supplementary table S4. Fatty acid compositions in the experimental diets

	Western diet			
	Cod		Pork	
Fatty acid	mg/g	(%)	mg/g	(%)
Sum SFA	71.3	(45.2)	70.9	(43.9)
Sum MUFA	49.1	(31.1)	55.0	(34.0)
LA 18:2n-6	31.2	(19.8)	32.1	(19.9)
ARA 20:4n-6	0.3	(0.2)	0.6	(0.4)
Sum n-6	31.5	(20.0)	32.7	(20.3)
ALA 18:3n-3	2.9	(1.8)	2.9	(1.8)
EPA 20:5n-3	0.9	(0.6)	0.1	(0.0)
DHA 22:6n-3	2.1	(1.3)	0.0	(0.0)
Sum n-3	5.9	(3.7)	3.0	(1.8)
Sum FA	157.8	(100.0)	161.6	(100.0)
n-6:n-3 ratio	4.9		9.3	
ARA:EPA ratio	0.3		8.7	

All values represent mean of triplicate measurements.

Abbreviations: SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, LA; linoleic acid, ARA; arachidonic acid, ALA; α-linolenic acid, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid, FA; fatty acids

Supplementary Figures:



Supplementary Figure S I: Male C57BL/6J mice were fed Western diets containing either cod or pork for 12 weeks before they were sacrificed and liver and plasma collected. A separate group of mice receiving pork was pair-fed (Pork PF) with the group fed cod. RNA was isolated from liver and relative mRNA expression of marker genes for (A) fatty acid (FA) oxidation and (B) FA uptake was determined using Real time qPCR. The metabolites (C) triacylglycerol (TAG), (D) HDL cholesterol and (E) 3-hydroxy-butyrate (OH-butyrate) were quantified in plasma by Maxmat. Data from the liver and plasma represent mean ± SEM (n=8-9).

III

nutrients

MDPI

Article

Effects of Frozen Storage on Phospholipid Content in Atlantic Cod Fillets and the Influence on Diet-Induced Obesity in Mice

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Abstract: A large fraction of the *n*-3 polyunsaturated fatty acids (PUFAs) in cod fillet is present in the form of phospholipids (PLs). Freezing initiates hydrolysis of the PLs present in the fillet. Here, we compared the effects of Western diets based on frozen cod, fresh cod or pork with a diet based on casein in male C57BL/6J mice fed for 12 weeks at thermoneutrality. Diets based on fresh cod contained more PL-bound *n*-3 PUFAs (3.12 mg/g diet) than diets based on frozen cod (1.9 mg/g diet). Mice fed diets containing pork and fresh cod, but not frozen cod, gained more body and fat mass than casein-fed mice. Additionally, the bioavailability of *n*-3 PUFAs present in the cod fillets was not influenced by storage conditions. In a second experiment, diets with pork as the protein source were supplemented with *n*-3 PUFAs in the form of PL or triacylglycerol (TAG) to match the levels of the diet containing fresh cod. Adding PL-bound, but not TAG-bound, *n*-3 PUFAs, to the pork-based diet increased body and fat mass gain. Thus, supplementation with PL-bound *n*-3 PUFAs did not protect against, but rather promoted, obesity development in mice fed a pork-based diet.

Keywords: obesity; Western diet; cod; pork; free fatty acids; phospholipids; triacylglycerol; *n*-3 polyunsaturated fatty acids; mice

1. Introduction

The changing of dietary patterns represents a tool to curb the development of obesity and type 2 diabetes [1]. Epidemiological studies have also indicated that the intake of dairy and plant-derived protein as well as protein from various seafood sources is associated with protection against obesity development, whereas a high intake of meat protein predicts higher weight gain [1,2]. In line with these findings, C57BL/6J mice fed a Western diet (40% of energy as fat, 44% of energy as carbohydrates and 16% of energy as protein) containing a mixture of lean seafood had lower adiposity than mice fed a Western diet containing a mixture of skinless chicken breast fillet, pork tenderloin and beef sirloin [3]. Furthermore, compared with low fat-fed mice, mice fed lean meat exhibited increased levels of fasting blood glucose, fasting plasma insulin and liver lipids. The energy intake was reduced by 8% when the mice were fed lean seafood, and this was further accompanied by a reduced feed efficiency. Exchanging meat from lean pork sirloins with cod fillets in diets with a similar composition of macronutrients led to attenuated obesity development accompanied with a 6% lower feed intake and a significantly reduced feed efficiency [4]. Further, the analysis of a second group of pork-fed mice

that were pair-fed with the cod-fed mice corroborated the finding that cod, only in part, attenuates obesity development via a reduced feed intake [4].

In rat studies, the intake of both cod and soy proteins provided in a low-fat diet improved peripheral insulin sensitivity compared with rats fed a casein-based, low fat diet [5]. In a follow up study with high fat/high sucrose diets, it was demonstrated that in the absence of any effect on adipose tissue mass, feeding rats cod protein prevented the development of insulin resistance in skeletal muscle [6]. The diets in the latter study contained 67% energy as fat, 18% energy as sucrose and 15% energy as protein.

Obesity development and energy intake in chicken and cod-fed mice were similar when C57BL/6J mice were fed chicken breast fillets or cod fillets in diets based on the same high fat/high sucrose diet [7] used by Lavigne et al. [6]. However, the intake of high fat/high protein diets with cod fillets as the protein source led to decreased obesity development compared with mice fed protein from chicken breast fillet or pork sirloin, but the cod-fed mice had greater adipose tissue mass than mice fed casein or soy as protein sources [8]. In the latter experiment, glucose tolerance and insulin sensitivity mirrored fat mass. Hence, data on the effect of cod intake on the development of obesity and regulation of glucose homeostasis has varied between experiments, possibly reflecting differences in specific experimental conditions.

In the abovementioned rat studies by Lavigne et al. [5,6], diethyl ether was used to remove the small amount of endogenous fat present in the cod fillets, whereas in our previous mice experiments, freeze-dried cod fillets were used [3,4,7,8]. Intake of a Western diet containing freeze-dried cod fillets led to increased accumulation of the marine *n*-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in both liver and red blood cells (RBCs) in mice [4]. A large fraction of the marine *n*-3 PUFAs in cod fillets is present in the form of phospholipids (PLs) [9], and it has been reported that the bioavailability as well as the anti-obesogenic and anti-steatotic effects of PL-bound *n*-3 PUFAs are superior to triacylglycerol (TAG)-bound *n*-3 PUFAs [10,11]. Hence, we have previously suggested that despite a low fat content, PL-bound *n*-3 PUFAs may mediate some dietary effects of cod [4].

If the dietary effects of cod are mediated via PL-bound *n*-3 PUFAs, the storage time of the fillets may be of importance. Cod has a short commercial catch season, spanning four months in the winter, and hence, freezing is a broadly used method for preservation to ensure the availability of cod throughout the low season [12]. It is known that frozen storage of cod fillets initiates enzymatic hydrolysis of the PLs present in the fillets [13,14], resulting in increased levels of free fatty acids (FFAs) in the frozen fillets with time [15,16]. In addition to a changed PL:FFA ratio, lipid oxidation and ice crystal formation have been shown to play important roles in protein denaturation and consequently, change the texture and quality of frozen cod fillets [17,18].

Considering the variable outcomes reported regarding the link between dietary cod intake and obesity development, and a possible role of cod freshness, i.e., the PL content of the cod, we here aimed to investigate whether frozen storage of cod influences the bioavailability of *n*-3 PUFAs as well as the ability of cod to modulate energy intake, development of obesity, and steatosis in obesity-prone C57BL/6J mice. We also investigated whether PL-bound *n*-3 PUFAs supplemented at a level comparable to that in fresh cod are able to affect the development of obesity and steatosis in mice fed Western diets.

2. Materials and Methods

2.1. Ethical Statement

The animal experiments were approved by the Norwegian Animal Research Authority (Norwegian Food Safety Authority; FOTS id.nr 7882). Animal care and handling were performed in accordance with national and international guidelines (Regulation on the use of animals in research,

Ministry of Agriculture and Food, 1 July 2015; according to Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010).

2.2. Animal Studies

Two animal studies were performed. In both experiments, male C57BL/6J BomTac mice, 8 weeks of age at arrival, with an average body weight of 24.9 ± 0.1 g, average fat mass of 2.46 ± 0.06 g and average lean mass of 19.0 ± 0.1 g, were obtained from Taconic (Ejby, Denmark). The mice were housed (one animal per cage) in individually ventilated cages (IVC) in a thermoneutral environment (29 ± 1 °C) with 50% relative humidity and on a 12:12 h light-dark cycle. After one week of acclimatization to a low fat diet (based on the Ssniff EF R/M control diet (E15000-04), Soest, Germany), the mice were divided into feeding groups based on measurements of body weight and relative fat and lean mass. Five mice continued with the low fat diet to monitor normal weight and health development. The mice had free access to water and were fed their respective diets *ad libitum*. In both experiments, fresh water was provided twice per week, and fresh feed was provided three times per week. Body weight was recorded once per week and feed intake was recorded three times per week. In both experiments, the mice were sacrificed by cardiac puncture under isoflurane anesthesia (Isoba vet, Schering-Plough A/S, Farum, Denmark) after 12 weeks of feeding. Blood was collected from the heart into tubes containing EDTA. The RBC fraction was prepared by centrifugation (1500 G, 15 min, 4 °C) and stored at −80 °C until further analyses. Liver and adipose tissues were dissected out, weighed, snap-frozen in liquid nitrogen and stored at −80 °C until further analyses.

2.3. Lipid Class Composition

The lipid class composition of raw and heated fresh cod fillets, freeze dried powders, diets and mouse livers was measured using high-performance thin-layer chromatography (HPTLC), as described by Jordal et al. [19]. Lipids were extracted from the samples by adding 20× the amount of sample (*v*/*w*) of chloroform:methanol (2:1) with 0.01% BHT. After extraction of lipids, the samples were filtered, taken to dryness, and diluted in chloroform with 0.01% BHT, to obtain a final concentration of 5 mg/mL. One microliter of the solution was applied to a 20 × 10 cm HPTLC Silica 60 plate (Merck, Darmstadt, Germany) that had been pre-run in chloroform and activated at 110 °C for 30 min. The plates were developed to 48 mm in a polar solution of chloroform, isopropanol, methyl acetate, methanol and 0.25% (*w*/*v*) aqueous KCl (25:25:25:10:9, by volume) to separate polar from neutral lipid classes running at the solvent front. After drying, the plates were fully developed in isohexane, diethyl ether and acetic acid (80:20:1.5, by volume) to separate neutral lipids and cholesterol. Lipid classes were visualized by charring at 160 °C for 15 min after development in 3% copper acetate (*w*/*v*) in 8% (*v*/*v*) phosphoric acid for 10 s and identified by comparison with commercially available standards. Lipid classes were quantified using a densitometer (CAMAG TLC Scanner 3, CAMAG, Muttenz, Switzerland) and calculated using an integrator (winCATS Planar Chromatography Manager, Version 1.4.2, CAMAG, Muttenz, Switzerland). Finally, quantitative determination of lipid classes (mg lipid class/g tissue) was performed by establishing standard equations for each lipid class within a linear range of area, in addition to including a standard mixture of all the lipid classes at each HPTLC plate for corrections of between plate variations. The limit of quantification was 0.01 mg lipids/g sample.

2.4. Fatty Acid Composition in the Polar and Neutral Lipid Fractions

The FA composition of RBCs and the FA composition in the polar and neutral lipid fractions of raw and heated fresh cod fillets, freeze-dried powders, diets and mouse livers were measured, as described by Liisberg et al. [4]. Lipids were extracted by adding 20× the amount of sample (*v*:*w*) of chloroform:methanol (2:1). After filtration of the extract, solvents were evaporated, and the residue was dissolved in 2% methanol in chloroform and separated into polar and neutral fractions using solid phase extraction (SPE). The SPE cartridge (Biotage Isolute SI 500 mg/10 mL, Uppsala, Sweden) was conditioned with 5 mL of hexane. The sample was then loaded and eluted with 10 mL 2%

methanol in chloroform, and the neutral fraction was collected. Then, 15 mL of methanol was added, and the fraction was collected as the polar lipids. Methyl ester of C19:0 (nonadecanoic acid) was added to each fraction/sample as an internal standard, before saponifying the lipid samples with NaOH and methylating the FAs using 12% BF_3 in methanol. The quantity of FAs in each fraction was determined by gas chromatography coupled with a flame ionization detector, identified by retention time using standard mixtures of methyl esters (Nu-Chek-Prep, Elysian, MN, USA) and quantified towards the internal standard under the conditions previously described by Torstensen et al. [20], based on Lie et al. [9]. The limit of quantification was 0.01 mg FA/g per sample.

2.5. Experimental Diets

Western and low fat diets were prepared to match the macronutrient composition used in an earlier study [4]. We used casein powder (C8654 SIGMA, Merck, Darmstadt, Germany), fillets from fresh, wild-caught Atlantic cod (Lerøy Alfheim AS, Bergen, Norway) and fresh pork sirloins (H. Bragstad A/S, Bergen, Norway) as protein sources. Fresh cod fillets and pork sirloins were prepared as powders, as described by Liisberg et al. [4]. In all diets, the amounts of casein powder and pulverized freeze-dried cod fillets and pork sirloins added to achieve 200 g crude protein/kg diet were calculated from measurements of nitrogen content in the powders, determined by the Dumas method using a Leco FP 628 nitrogen analyzer (Leco Corporation Svenska AB, Täby, Sweden). The nitrogen to protein conversion factors used for the calculation of crude protein in the diets were N × 6.15 for casein and N × 5.6 for cod and pork [21]. Based on measurements of endogenous fat in the protein powders (described in the experiments), we balanced the diets with equal parts of margarine, lard and milk fat to achieve 180 g fat/kg diet. The diets were blended with a Crypto Peerless EF20 blender and analyzed for gross energy by bomb calorimetry (Parr Instrument, Moline, IL, USA).

Experiment 1: Half of a batch of raw fresh cod fillets and a whole batch of fresh pork sirloins were heated in a steamer to a core temperature of 70 °C, freeze-dried to >97% dryness, homogenized to powder and stored at −20 °C until use (12 weeks). The raw, fresh cod fillets were analyzed before and after heating to determine the lipid class composition (Table S1) and FA composition in the polar and neutral lipid fractions (Table S2). The remaining batch of raw, fresh cod fillets was stored at −20 °C to initiate enzymatic hydrolysis of the PLs, which ought to increase the FFA content in the raw cod fillets during freezing [13,14]. After 12 weeks of storage, the frozen cod fillets were thawed overnight at 4 °C, and prepared as powder, as described above. The lipid class compositions of the powders of frozen and fresh cod are shown in Table 1, and the FA compositions of the polar and neutral lipid fractions of the powders are shown in Table S3. The compositions of the diets are shown in Table S4, and the FA compositions of the polar and neutral lipid fractions in the frozen cod, fresh cod and pork-containing diets are shown in Table S5.

Experiment 2: Fillets of fresh cod and fresh pork sirloins were prepared as powders, as described above. The lipid class composition of the fresh cod powder is shown in Table S6, and the FA compositions of the polar and neutral lipid fractions are shown in Table S7. We aimed to investigate whether PL-bound EPA+DHA influenced obesity development and hepatic lipid accumulation in mice fed Western diets. Hence, the amount of EPA+DHA present in the fresh cod Western diet, 2.8 mg EPA+DHA/g diet (Table 2), was added to a Western diet containing pork. Equal parts of margarine, milk fat and lard were replaced with *n*-3 PUFA oils, either as PL-bound *n*-3 PUFAs extracted from herring roe diluted in soybean oil (pork *n*-3 PL diet), prepared by Innolipid AS (Aalesund, Norway), or TAG-bound *n*-3 PUFAs from cod liver oil (pork *n*-3 TAG diet) (Möller's cod liver oil, Orkla Health, Oslo, Norway). The FA compositions of the polar and neutral lipid fractions of the oils are presented in Table S8. One Western diet containing pork was not supplemented with *n*-3 PUFAs (pork diet). The compositions of the diets are shown in Table S9, and the FA compositions of the polar and neutral lipid fractions of the Western diets are shown in Table 2.

Table 1. Lipid class composition in freeze-dried fresh and frozen cod fillets.

	Freeze-Dried Frozen Cod Fillets		Freeze-Dried Fresh Cod Fillets	
Lipid Class	**mg/g**	**%**	**mg/g**	**%**
PC	18.80 ± 0.3	42.0	36.5 ± 0.8	57.9
PE	5.1 ± 2.2	11.5	14.2 ± 0.3	22.6
PI	<0.01	<0.01	0.48 ± 0.05	0.76
PS	0.12 ± 0.02	0.27	0.86 ± 0.07	1.4
LPC	2.43 ± 0.03	5.43	2.4 ± 0.1	3.9
SM	0.66 ± 0.02	1.48	0.81 ± 0.03	1.28
CL	0.145 ± 0.005	0.33	0.64 ± 0.06	1.0
Sum polar lipids	27.3 ± 0.5	61.0	56.0 ± 0.7	88.8
FFA	15.1 ± 0.2	33.72	4.30 ± 0.07	6.8
CHOL	2.36 ± 0.03	5.27	2.64 ± 0.04	4.20
TAG	<0.01	<0.01	0.11 ± 0.03	0.18
DAG	<0.01	<0.01	<0.01	<0.01
CE	<0.01	<0.01	<0.01	<0.01
Sum neutral lipids	17.4 ± 0.2	39.0	7.1 ± 0.1	11.2
Sum lipids	44.7 ± 0.7		63.0 ± 0.7	
Polar lipid:FFA ratio	1.809 ± 0.008		13.0 ± 0.2	

Results are presented as means ± SEMs of three samples and indicate mg lipids/g and percent lipid class of total lipids in the freeze-dried frozen and fresh cod fillets. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; LPC, lysophosphatidylcholine; SM, sphingomyelin; CL, cardiolipin; FFA, free fatty acids; CHOL, cholesterol; TAG, triacylglycerol; DAG, diacylglycerol; CE, cholesteryl ester.

Table 2. Fatty acid composition in the polar and neutral lipid fractions isolated from Western diets.

Fatty Acid (mg/g)	**Fresh Cod**	**Pork**	**Pork *n*-3 TAG**	**Pork *n*-3 PL**
Polar lipid fraction				
Sum SFA	1.20 ± 0.02	0.76 ± 0.01	1.64 ± 0.07	2.12 ± 0.08
Sum MUFA	0.73 ± 0.03	0.474 ± 0.007	1.20 ± 0.06	1.014 ± 0.006
LA 18:2*n*-6	0.100 ± 0.009	0.76 ± 0.01	1.07 ± 0.04	0.91 ± 0.03
ARA 20:4*n*-6	0.097 ± 0.002	0.236 ± 0.005	0.257 ± 0.009	0.312 ± 0.008
Sum *n*-6	0.22 ± 0.01	1.06 ± 0.02	1.42 ± 0.05	1.23 ± 0.04
ALA 18:3*n*-3	0.020 ± 0.001	0.0240 ± 0.0001	0.067 ± 0.003	0.037 ± 0.006
EPA 20:5*n*-3	0.690 ± 0.007	0.02763 ± 0.0004	0.043 ± 0.003	0.74 ± 0.06
DHA 22:6*n*-3	1.78 ± 0.01	0.015 ± 0.001	0.037 ± 0.003	2.0 ± 0.1
Sum EPA+DHA	2.47 ± 0.02	0.043 ± 0.001	0.080 ± 0.005	2.7 ± 0.2
Sum *n*-3	2.61 ± 0.03	0.130 ± 0.002	0.22 ± 0.01	2.8 ± 0.2
Sum identified FAs	4.8 ± 0.1	2.44 ± 0.04	4.5 ± 0.2	7.2 ± 0.3
n-6:*n*-3 ratio	0.085 ± 0.005	8.2 ± 0.2	6.4 ± 0.1	0.43 ± 0.03
EPA:DHA ratio	0.388 ± 0.001	1.80 ± 0.05	2.2 ± 0.3	0.376 ± 0.008
ARA:EPA ratio	0.14 ± 0.001	8.53 ± 0.04	6.0 ± 0.3	0.43 ± 0.04
Neutral lipid fraction				
Sum SFA	76 ± 1	76 ± 2	71 ± 2	72 ± 0.5
Sum MUFA	58 ± 2	60 ± 2	63 ± 2	57.7 ± 0.8
LA 18:2*n*-6	24 ± 1	23.1 ± 0.4	22.1 ± 0.7	22.5 ± 0.5
ARA 20:4*n*-6	0.188 ± 0.007	0.302 ± 0.009	0.36 ± 0.02	0.32 ± 0.02
Sum *n*-6	24 ± 1	23.7 ± 0.4	23.0 ± 0.8	23.0 ± 0.5
ALA 18:3*n*-3	4.0 ± 0.2	3.8 ± 0.2	3.7 ± 0.1	3.86 ± 0.04
EPA 20:5*n*-3	0.15 ± 0.01	<0.01	1.17 ± 0.06	0.15 ± 0.01
DHA 22:6*n*-3	0.16 ± 0.01	<0.01	1.64 ± 0.09	0.096 ± 0.004
Sum EPA+DHA	0.31 ± 0.01	<0.01	2.8 ± 0.2	0.24 ± 0.01
Sum *n*-3	4.7 ± 0.2	4.1 ± 0.2	7.3 ± 0.3	4.51 ± 0.05
Sum identified FAs	164 ± 5	163 ± 4	164 ± 4	157 ± 2
n-6:*n*-3 ratio	5.2 ± 0.1	5.8 ± 0.3	3.2 ± 0.2	5.00 ± 0.06
EPA:DHA ratio	0.93 ± 0.15	*	0.725 ± 0.007	1.6 ± 0.1
ARA:EPA ratio	1.3 ± 0.1	*	0.31 ± 0.03	2.1 ± 0.1

Results are presented as means ± SEMs of three samples and indicate mg FAs in the polar and neutral lipid fractions/g Western diet. * not possible to calculate; EPA and DHA levels are under the limit of quantification (<0.01 mg/g). Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; LA, linoleic acid; ARA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; FAs, fatty acids.

2.6. Body Composition of the Mice

Free water, lean and fat mass were measured in live mice using a Bruker minispec LF50 Body composition Analyzer mq 7.5 (Bruker Optik GmBh, Ettlingen, Germany) as described by Halldorsdottir et al. [22].

2.7. Apparent Digestibility of Nitrogen and Fat

In both experiments, after six weeks of feeding, the mice were placed in cages with standard wood bedding for one week. All feces were collected from the cages, weighed and frozen at −20 °C until analysis. Nitrogen content in feces was determined with the Dumas method, as described above, and the total fat content in feces samples was determined gravimetrically after extraction with organic solvents before and after acidic hydrolysis, as described previously [7]. The apparent digestibilities of nitrogen and fat were calculated as follows: 100 × (intake (mg) − feces output (mg)/(intake (mg)).

2.8. Oral Glucose Tolerance Test

An oral glucose tolerance test (OGTT) was performed in Experiment 1 after 11 weeks of experimental feeding. Fasting blood glucose was measured in Experiment 2 after 10 weeks of experimental feeding. After 6 h of fasting, the mice were given 3 mg of glucose/g of lean mass by gavage. Blood was collected from the tail veins of conscious mice, and blood glucose was measured using a glucometer (Contour® Next, Ascensia Diabetes care Holdings AG, Basel, Switzerland). Blood glucose was measured in the fasting state (0), and again at 15, 30, 60 and 120 min after the glucose injection [23]. Plasma was collected in the fasting state (0), and again after 15, 30, and 120 min following the glucose injection. An Ultra Sensitive Mouse Insulin ELISA Kit (Crystal chem (Europe) catalog# 90080, Zaandam, The Netherlands) was used according to the producer's manual to quantify the plasma insulin collected during the OGTT. The incremental area under the curve (iAUC) for the OGTT was calculated using the fomula iAUC = AUC − (basal glucose × 120 min).

2.9. Statistics

Mice fed a low fat diet were only used as a reference for normal weight and health development, and thus, were not included in the statistical analyses. All data are presented as means ± SEMs. The homogeneity of variance in the data was established by Bartlett's test, and the data were compared between groups using one-way ANOVA followed by Fisher's multiple comparison post hoc tests. In Experiment 1, Western diet groups were compared with a reference group fed a casein-based Western diet. The reference group was not included in the bioavailability measurements of FAs in liver lipids and RBCs; hence, data were compared between the experimental Western diet groups. In Experiment 2, data were compared between all groups of Western diet-fed mice. In both experiments, cumulative energy intake was analyzed by repeated measures ANOVA and Fisher's LSD multiple comparison post hoc tests. Group means were considered to be statistically different at $p < 0.05$. Statistical analyses were performed using Graph Pad Prism version 7.01 (GraphPad software, La Jolla, CA, USA).

3. Results

3.1. Frozen Storage of Cod Fillets Decreased the Polar Lipid:FFA Ratio

Raw fresh cod fillets contained 5.7 mg lipids/g (Table S1). Heating the fillets in a steamer to a core temperature of 70 °C led to a loss of water and FFAs, and the heated fillets contained 10.5 mg lipids/g. The relative levels of FFAs were reduced from 12.7 to 6.3% upon heating, but the relative amounts of either polar or neutral lipids did not change (Table S1). The contents of EPA and DHA in the polar and neutral lipid fractions were 55.4% and 49.2%, respectively, in the raw fillets, and heating did not significantly influence the EPA and DHA contents (Table S2). As expected, the relative amounts of FFAs were higher in freeze-dried frozen cod fillets than in freeze-dried fresh fillets, whereas the relative

polar lipid levels were lower (Table 1). Consequently, storage of the cod fillets prior to heating and freeze-drying decreased the polar lipid:FFA ratio (Table 1). The relative proportions of EPA and DHA in both the polar and the neutral lipid fractions were comparable in powders from freeze-dried fresh and frozen cod, respectively (Table S3).

The energy contents in the diets (Table S4) were confirmed using bomb calorimetry and were 20.38 ± 0.06 kJ/g in the Western diets, and 18.51 ± 0.03 kJ/g in the low fat reference diet. FFAs at the level above the limit of quantification were only present in the Western diet containing frozen cod (1.6 mg FFAs/g diet). We determined the contribution of endogenous fat from the protein sources in the diets to the FA composition in both the polar and neutral lipid fractions extracted from the diets (Table S5). Due to the dominant contribution from the added milk fat, margarine, and lard, the most pronounced differences between the diets were observed in the polar fractions. Still, the contents of the marine *n*-3 PUFAs, EPA and DHA were higher in both fractions extracted from the diets containing cod compared to the pork-containing diet. Due to the relatively high levels of *n*-3 PUFA alpha-linolenic acid (ALA) in the neutral lipid fractions extracted from all Western diets, the difference in the *n*-6:*n*-3 ratio between the pork and cod diets was far more pronounced in the polar lipid fraction than in the neutral fraction. Still, the neutral lipid fraction of the frozen cod-based diet also contained higher amounts of total EPA and DHA compared to the fresh cod-based diet: 1.2% versus 0.52%. However, in the polar lipid fraction, the sum of EPA and DHA represented as much as 39% and 45% of the total identified FAs from diets containing frozen and fresh cod, respectively.

3.2. Fresh Cod, But Not Frozen Cod, Is More Obesogenic than Casein in a Western Diet

To investigate if prolonged storage of fresh cod at −20 °C influences the ability of cod to attenuate obesity and steatosis development, C57BL/6J mice were fed Western diets containing frozen or fresh cod for 12 weeks. For comparison, mice were fed a low fat diet or a Western diet using casein as the protein source. Compared with mice fed a casein-based Western diet, mice fed pork and fresh cod, but not frozen cod, gained significantly more weight after 9 weeks (Figure 1a,b). Similarly, compared with mice fed casein, fat mass was higher in mice fed pork and fresh cod, but not frozen cod (Figure 1c), while lean body mass was comparable in mice fed either of the diets (Figure 1d). After 12 weeks, epididymal white adipose tissue (eWAT) mass was significantly higher in pork-fed mice, but not in frozen or fresh cod fed mice when compared to mice fed casein (Figure 1e). Of note, the mass of the perirenal/retroperitoneal white adipose tissue (p/r WAT) depot in mice fed fresh cod, but not frozen cod, was borderline ($p = 0.057$) higher than that of casein-fed mice (Figure 1e). Mice fed frozen cod, fresh cod, and pork were comparable to casein-fed mice in relation to the inguinal white adipose tissue mass (iWAT; Figure 1e).

After 6 weeks of feeding, a reduced energy intake was observed in mice fed frozen and fresh cod, but not in pork-fed mice, compared to casein-fed mice (Figure 1f). Compared with casein-fed mice, body mass gained per unit of energy intake and fat mass gained per unit of energy intake were higher in mice fed frozen cod, fresh cod and pork (Figure 1g,h). The apparent digestibility of fat was significantly higher in mice fed frozen cod and pork and tended ($p = 0.07$) to be higher in mice fed fresh cod compared to casein-fed mice (Figure 1i). The apparent digestibility of nitrogen was significantly higher in all mice fed cod and pork compared to casein-fed mice (Figure 1j).

The liver mass was significantly higher (22%; $p < 0.05$) in pork-fed mice compared to casein-fed mice. The liver mass was borderline ($p = 0.05$) higher in mice fed frozen cod compared to casein-fed mice, while the liver mass of fresh cod fed mice was not statistically different from casein-fed mice (Figure 1k). No differences in glucose tolerance, fasting plasma insulin or fasting blood glucose were observed among groups (Figure S1). Hence, Western diets containing fresh cod, but not frozen cod, appear to be more obesogenic than a Western diet based on casein as the protein source.

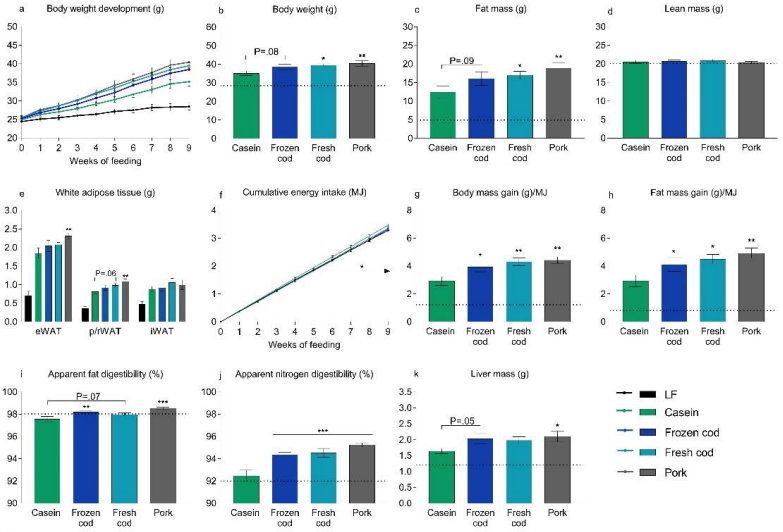

Figure 1. Effects of Western diets with different protein sources on body composition, energy intake and tissue weights. Male C57BL/6J mice were fed Western diets containing casein, frozen cod, fresh cod or pork as protein sources for 12 weeks. As a reference, a group of low fat (LF)-fed mice (n = 5) was also included and is shown as a dotted line. (**a**) Body weight development was measured and is shown for the first 9 weeks of feeding. (**b**) Body weight was measured and (**c**) fat mass and (**d**) lean mass were determined using nuclear magnetic resonance after 9 weeks of feeding. (**e**) Epididymal white adipose tissue (eWAT), inguinal white adipose tissue (iWAT) and perirenal/retroperitoneal white adipose tissue (p/r WAT) were dissected out after 12 weeks of feeding, and their masses were recorded; (**f**) Feed intake was recorded continuously, and cumulative energy intake (MJ) was determined and is shown for the first 9 weeks of feeding. The arrow indicates a significantly ($p < 0.05$) decreased energy intake in mice fed frozen cod and fresh cod compared to mice fed casein. (**g**) After 9 weeks of feeding, body mass gained per energy unit consumed and (**h**) fat mass gained per energy unit consumed were calculated; Apparent (**i**) fat and (**j**) nitrogen digestibility (%) were calculated based on feed intake and feces collected in the 6th week of feeding. (**k**) Livers were dissected out and weighed after 12 weeks of feeding. Data are presented as means ± SEMs (n = 10) and were analyzed using one-way ANOVA followed by Fisher's LSD post hoc tests. Cumulative energy intake was analyzed by repeated measures ANOVA and Fisher's LSD post hoc tests. *, ** and *** represent significant different from the Western diet containing casein at $p < 0.05$, $p < 0.01$ and $p < 0.001$ levels, respectively.

3.3. Bioavailability of n-3 PUFAs in Frozen and Fresh Cod Diets

To investigate if the bioavailability of EPA and DHA was influenced by frozen storage of the cod fillets, we measured the FA composition in RBCs and livers collected from the mice. We found that the EPA and DHA contents of the frozen and fresh cod containing diets were similar in the Western diets (3.7 mg/g EPA+DHA vs. 3.87 mg/g EPA+DHA, respectively), but distributed differently between the polar and neutral lipid fractions. The frozen cod diet contained 0.44 mg/g EPA and 1.49 mg/g DHA in the polar lipid fraction and 0.68 mg/g EPA and 1.10 mg/g DHA in the neutral lipid fraction (Figure 2a,b and Table S5). In comparison, the fresh cod diet contained 0.81 mg/g EPA and 2.32 mg/g DHA in the polar lipid fraction and 0.35 mg/g EPA and 0.40 mg/g DHA were present in the neutral lipid fraction (Figure 2a,b and Table S5). Hence, when raw fresh cod fillets were stored at −20 °C for 12 weeks prior to preparation of powders, lower proportions of EPA and DHA were present in the polar lipid fraction and higher proportions were found in the neutral lipid fraction compared to the diet prepared using fresh cod.

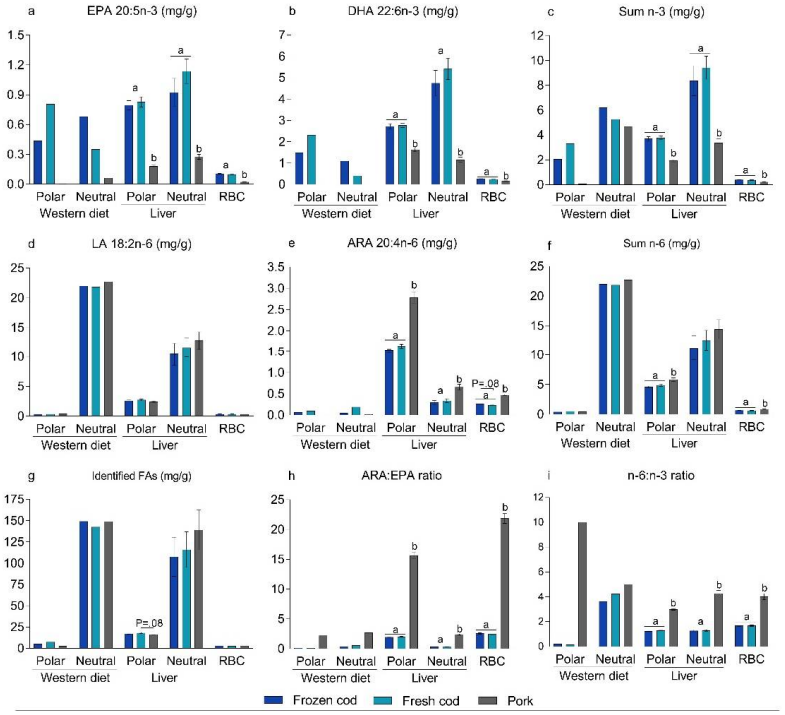

Figure 2. Fatty acid (FA) composition in polar and neutral lipid fractions in Western diets based on frozen cod, fresh cod, and pork as protein sources, and in mouse livers and red blood cells (RBCs) following intake of the different diets. Data from the Western diets represent the means of three samples, and data from the livers and RBCs represent means ± SEMs (n = 10). Results indicate mg FAs in the polar and neutral lipid fractions/g Western diet and livers, and mg FAs/g RBCs. Lipids from Western diets and livers were extracted and separated into polar and neutral lipid fractions. The FA compositions of the fractions from the Western diets and livers as well as the FA compositions of extracted lipids from RBC were quantification and determined; (**a**) eicosapentaenoic acid (EPA); (**b**) docosahexaenoic acid (DHA); (**c**) sum *n*-3; (**d**) linoleic acid (LA); (**e**) arachidonic acid (ARA); (**f**) sum *n*-6; (**g**) identified fatty acids (FAs); (**h**) arachidonic acid: eicosapentaenoic acid ratio (ARA:EPA); (**i**) *n*-6:*n*-3 ratio. Data from livers and RBCs were analyzed using one-way ANOVA followed by Fisher's LSD post hoc tests. Different letters denote statistical significance ($p \leq 0.05$) between the groups.

Compared to pork-fed mice, mice fed frozen and fresh cod had significantly higher levels of EPA, DHA, and sum *n*-3, and lower levels of ARA in hepatic polar and neutral lipids and in RBCs (Figure 2a–c,e and Tables S10 and S11). However, the levels of EPA, DHA, sum *n*-3, and ARA were similar in RBCs and liver lipids of mice fed frozen cod and fresh cod, suggesting that the bioavailability of *n*-3 PUFAs present in the cod fillets was not influenced by the storage conditions. In the present study, despite a higher content of PL-bound *n*-3 PUFAs, a Western diet containing fresh cod, but not frozen cod, appeared to be more obesogenic than a casein-based Western diet, suggesting that PL-bound *n*-3 PUFAs did not mediate an anti-obesogenic effect. To the contrary, our results indicate that the PL-bound *n*-3 PUFAs present in a Western diet may rather increase obesity development.

3.4. Supplementation with n-3 PUFAs in Pork-Based Western Diets

To further investigate the effect of PL-bound *n*-3 PUFAs in a Western diet on energy intake, obesity development, and hepatic steatosis, new fresh cod fillets were obtained. After heating and

freeze-drying, the fresh cod fillet powder contained 39 mg lipids/g powder, and the relative levels of polar lipids and FFA were 94% and 1%, respectively (Table S6). The freeze dried fresh cod powder was blended into a Western diet. The polar lipid fraction of the diet contained 2.47 mg EPA+DHA/g diet (Table 2). To investigate whether PL-bound *n*-3 PUFAs increase obesity development in mice fed Western diets, we also prepared diets using pork, with or without addition of the same amount of PL-bound *n*-3 PUFAs as found in the fresh cod diet. The pork-containing diet supplemented with PL-bound *n*-3 PUFAs (pork *n*-3 PL diet) contained 2.7 mg EPA+DHA/g diet in the polar lipid fraction. The EPA:DHA ratios were similar in the fresh cod diet (0.39) and the pork *n*-3 PL diet (0.38) (Table 2).

We also supplemented a pork diet with TAG-bound *n*-3 PUFAs (pork *n*-3 TAG diet), with an EPA:DHA ratio similar to the pork *n*-3 PL diet, as a control for the bioavailability of PL-bound EPA+DHA. As we were unable to obtain TAG-bound *n*-3 PUFAs with an EPA:DHA ratio similar to cod fillets and PL-bound *n*-3 PUFAs, the EPA:DHA ratio was somewhat higher in the pork *n*-3 TAG diet (0.725) than in the other diets. However, FA analyses demonstrated that the pork *n*-3 TAG diet contained 2.8 mg/g EPA+DHA in the neutral lipid fraction (Table 2), and EPA+DHA levels were hence comparable.

3.5. Adding PL-Bound n-3 PUFAs to a Western Diet Containing Pork Promoted Obesity in Mice

The Western diets containing fresh cod or pork supplemented with either TAG or PL-bound *n*-3 PUFAs containing similar amounts of *n*-3 PUFAs and a Western diet containing pork without supplementation were fed to C57Bl/6 mice for 12 weeks. After 9 weeks of feeding, intake of the pork diet with the PL-bound, but not TAG-bound, *n*-3 PUFAs resulted in a higher average body weight and fat mass compared to mice fed pork without *n*-3 PUFAs (Figure 3a–c). Fresh cod and unsupplemented pork-fed mice did not differ in body weight or fat mass (Figure 3a–c). Mice fed the pork *n*-3 PL diet tended to have a higher average iWAT mass compared to fresh cod-fed mice ($p = 0.07$) and pork-fed mice ($p = 0.09$) (Figure 3e). However, there were no significant differences in the white adipose tissue mass of mice fed any of the Western diets (Figure 3e). After 9 weeks of feeding, lean mass and energy intake were similar between all Western diet-fed mice (Figure 3d,f).

Calculating body and fat mass gain relative to energy intake after 9 weeks of feeding revealed that mice fed the pork *n*-3 PL diet gained significantly more body and fat mass per unit of energy intake compared to intake of both the fresh cod- and pork-containing diets (Figure 3g,h). Mice fed pork supplemented with TAG-bound EPA+DHA had in between values. However, the increased body and fat mass gain per unit of energy intake did not appear to be caused by higher fat or nitrogen digestibility in mice fed the pork *n*-3 PL diet (Figure 3i,j). Furthermore, compared to mice fed the pork diet with no supplementation of EPA+DHA, the intake of PL-bound *n*-3 PUFAs, but not TAG-bound *n*-3 PUFAs, led to increased liver mass (Figure 3k) and increased hepatic accumulation of lipids and cholesterol in the liver (Figure 3l). Additionally, neutral lipids and TAGs were borderline higher in the livers of pork *n*-3 PL-fed mice than pork *n*-3 TAG-fed mice ($p = 0.05$) and pork *n*-3 TAG-fed mice ($p = 0.06$). Hepatic lipid accumulation was similar between fresh cod, pork and pork *n*-3 TAG-fed mice. No differences in fasting blood glucose were observed between groups (Figure S2). Hence, supplementation of PL-bound *n*-3 PUFAs to a pork diet did not protect against, but rather promoted, obesity and hepatic lipid accumulation in mice fed Western diets.

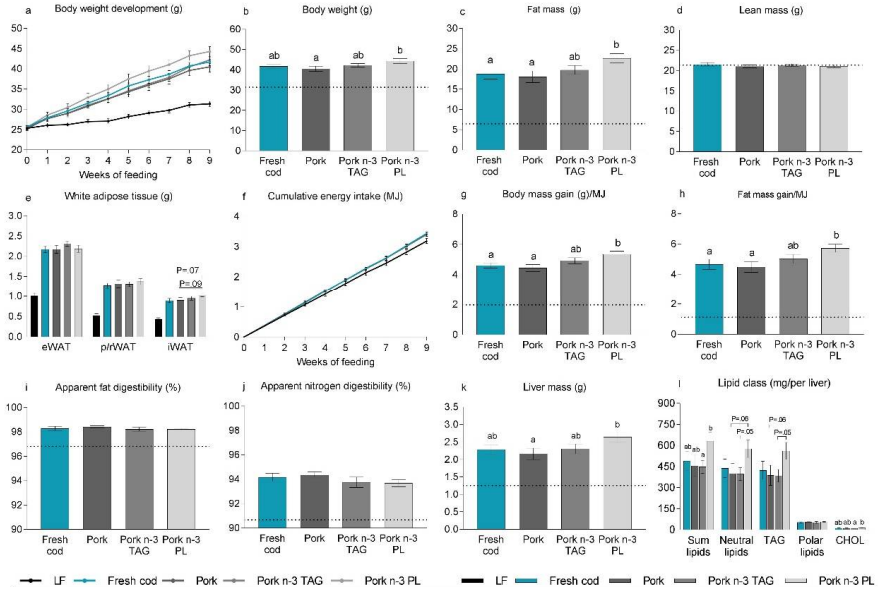

Figure 3. Effects of pork-containing Western diets with added *n*-3 PUFAs to match the level of *n*-3 PUFAs in a Western diet containing fresh cod. Male C57BL/6J mice were fed Western diets containing either fresh cod or fresh pork as a protein source for 12 weeks. The two pork diets were supplemented with either TAG-bound (pork *n*-3 TAG) or PL-bound (pork *n*-3 PL) EPA+DHA at levels matching the content of the fresh cod-containing diet. As reference, a group of low fat (LF)-fed mice ($n = 5$) was included and is shown as a dotted line. (**a**) Body weight development was determined and is shown for the first 9 weeks of feeding. (**b**) Body weight was measured and (**c**) fat and (**d**) lean mass were determined using nuclear magnetic resonance after 9 weeks of feeding. (**e**) Epididymal white adipose tissues (eWAT), inguinal white adipose tissues (iWAT) and perirenal/retroperitoneal white adipose tissues (p/r WAT) were dissected out after 12 weeks of feeding and their masses were recorded. (**f**) Feed intake was recorded continuously, and cumulative energy intake (MJ) was determined and is shown for the first 9 weeks of feeding; (**g**) After 9 weeks of feeding, the amount of body mass gained per energy unit consumed and (**h**) fat mass gained per energy unit consumed were calculated; Apparent (**i**) fat and (**j**) nitrogen digestibilities (%) were calculated based on feed intake and feces collected during the 6th week of feeding; (**k**) After 12 weeks of feeding, livers were dissected out and weighed (**l**). Lipids were extracted from livers ($n = 7$) and total lipids, neutral lipids, triacylglycerol (TAG), polar lipids and cholesterol (CHOL) were quantified, and results are presents as mg lipids per liver (mg lipids/g liver × liver weight (g)). Data are presented as means ± SEMs ($n = 14$–15), except for the lipid class analysis ($n = 7$) and were analyzed using one-way ANOVA followed by Fisher's LSD post hoc tests. Cumulative energy intake was analyzed by repeated measures ANOVA and Fisher's LSD post hoc tests. Different letters denote statistical significance ($p \leq 0.05$) between the groups.

3.6. Bioavailability of PL-Bound n-3 PUFAs

To evaluate the bioavailability of the dietary *n*-3 PUFAs, EPA and DHA, we analyzed the FA compositions of RBCs collected from the mice. There were no differences in the levels of EPA+DHA in RBCs collected from mice fed fresh cod, pork *n*-3 PL or pork *n*-3 TAG diets (Table 3). The ARA:EPA ratio was significantly higher in pork *n*-3 PL-fed mice compared to pork *n*-3 TAG and fresh cod-fed mice. Supplementation of *n*-3 PUFA bound to TAG or PL had the same ability to increase the levels of EPA+DHA and to decrease the ratio of *n*-6:*n*-3 PUFAs in RBCs. Hence, the bioavailability of TAG and PL-bound *n*-3 PUFAs supplemented to a pork-based diet were similar. Further, pork-fed mice

had the highest *n*-6:*n*-3 ratio in their RBCs and additionally, had lower body weights and fat and liver masses compared to mice fed pork supplemented with *n*-3 PUFAs as TAG or PL. Thus, a decreased *n*-6:*n*-3 ratio in RBCs did not protect against obesity. Hence, we conclude that supplementation of a pork-based diet with *n*-3 PUFAs at relatively low levels did not protect against obesity, but rather increased the development of diet-induced obesity.

Table 3. Fatty acid composition in red blood cells.

Fatty Acid (mg/g)	Fresh Cod	Pork	Pork *n*-3 TAG	Pork *n*-3 PL
Sum SFA	1.56 ± 0.06	1.51 ± 0.04	1.47 ± 0.05	1.48 ± 0.04
Sum MUFA	0.69 ± 0.03	0.70 ± 0.02	0.67 ± 0.01	0.65 ± 0.02
LA 18:2*n*-6	0.48 ± 0.03	0.46 ± 0.02	0.44 ± 0.03	0.43 ± 0.02
ARA 20:4*n*-6	0.41 ± 0.01 ^{a}	0.66 ± 0.01 ^{b}	0.45 ± 0.01 ^{ac}	0.464 ± 0.009 ^{c}
Sum *n*-6	0.99 ± 0.05 ^{a}	1.27 ± 0.03 ^{b}	0.97 ± 0.03 ^{a}	0.99 ± 0.02 ^{a}
ALA 18:3*n*-3	<0.01	<0.01	<0.01	<0.01
EPA 20:5*n*-3	0.119 ± 0.005 ^{a}	0.031 ± 0.001 ^{b}	0.127 ± 0.004 ^{a}	0.095 ± 0.002 ^{c}
DHA 22:6*n*-3	0.39 ± 0.02 ^{a}	0.224 ± 0.007 ^{b}	0.354 ± 0.009 ^{c}	0.381 ± 0.007 ^{ac}
Sum EPA+DHA	0.51 ± 0.02 ^{a}	0.255 ± 0.008 ^{b}	0.48 ± 0.01 ^{a}	0.477 ± 0.008 ^{a}
Sum *n*-3	0.57 ± 0.02 ^{a}	0.313 ± 0.008 ^{b}	0.55 ± 0.01 ^{a}	0.535 ± 0.009 ^{a}
Sum identified FAs	3.8 ± 0.1	3.80 ± 0.09	3.7 ± 0.1	3.66 ± 0.08
n-6:*n*-3 ratio	1.73 ± 0.03 ^{a}	4.08 ± 0.09 ^{b}	1.76 ± 0.03 ^{a}	1.85 ± 0.04 ^{a}
ARA:EPA ratio	3.49 ± 0.09 ^{a}	21.7 ± 0.8 ^{b}	3.6 ± 0.1 ^{a}	4.9 ± 0.2 ^{c}

Results are presented as means ± SEMs (n = 10) and indicate mg FAs/g RBCs. Data were analyzed using one-way ANOVA followed by Fisher's LSD post hoc tests. Different letters denote statistical significance ($p \leq 0.05$) between the groups. Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; LA, linoleic acid; ARA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; FAs, fatty acids; RBCs, red blood cells.

4. Discussion

The present study in mice provides surprising evidence that frozen storage of cod modulates the ability of cod intake to attenuate obesity development when incorporated into a Western diet. Cod is a lean fish, but marine *n*-3 PUFAs comprise a large fraction of the FAs in muscle PLs. It has been reported that the bioavailability as well as the anti-obesogenic and anti-steatotic effects of PL-bound *n*-3 PUFAs are superior to those of TAG-bound *n*-3 PUFAs [11]. Previously, we showed that in comparison to pork-fed mice, mice fed frozen cod exhibit diminished development of obesity and steatosis, accompanied with a lower *n*-6 to *n*-3 ratio in RBC and liver PLs as well as lower circulating levels of the two major ARA-derived endocannabinoids: *N*-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) [4]. Hence, we suggested that the PL-bound *n*-3 PUFAs may, at least in part, mediate the effects of a cod-containing diet on metabolism [4].

The levels of PLs are higher in fresh, than in stored, frozen cod fillets, as frozen storage leads to enzymatic hydrolysis of PLs [13,14], and accordingly, we expected that the intake of fresh cod fillets would attenuate obesity development more efficiently than the intake of cod fillets that had been stored at −20 °C. As anticipated, the levels of PLs decreased during frozen storage, and hence, although diets prepared with frozen cod contained similar total amounts of EPA+DHA to diets prepared from fresh cod, a lower proportion of *n*-3 PUFAs was present in the polar lipid fraction in the diets prepared with frozen cod. However, this did not translate into lower bioavailability. Further, mice fed a Western diet containing fresh cod, but not frozen cod, gained more fat mass than casein-fed mice. This indicates that a high proportion of EPA+DHA in PLs accentuates, rather than attenuates, obesity. In line with this, we observed that supplementing a pork-containing diet with the same amount of *n*-3 PUFAs present in fresh cod in the form of PLs, but not as TAGs, increased weight gain and fat mass in mice after 9 weeks.

In contrasting to our results, previous studies have reported a superior effect of *n*-3 PUFAs in the form of PL. Some [24–27], but not all [11,28] used krill oil as the single source for PL-bound *n*-3 PUFAs. Krill oil is extracted from Antarctic krill (*Euphausia superba*), where 30–65% of the FAs are incorporated into PLs, mainly phosphatidylcholine [29]. Also, krill oil contains choline, which is a conditionally

essential nutrient, and is also important for the transport of lipids [30]. Moreover, krill oil contains vitamin E, which may protect the unsaturated bounds in PUFAs from oxidation and the biological membranes from lipid peroxidation [31], as well as astaxanthin, a powerful antioxidant, which has been linked to the prevention and reversion of diet-induced insulin resistance and steatohepatitis in mice [32]. While the EPA:DHA ratio in krill oil is 1.8 [33], the EPA:DHA ratio in the polar fraction of the freeze-dried fresh cod has a ratio of 0.34 (Table S3), and PLs extracted from herring roe have a EPA:DHA ratio of 0.38 (Table S8). EPA enriched PLs have been demonstrated to attenuate high fat diet-induced obesity and hyperlipidemia more efficiently than DHA-enriched PLs [34]. Additionally, EPA and DHA are metabolized differently in rat livers [35]. A major functional difference between EPA and DHA relates to their conversion to different eicosanoids, including pro-resolving mediators that may play important roles in the development of different metabolic disorders [36]. Furthermore, it has been demonstrated that EPA is the hypotriacylglycerolemic component of fish oil [37]. Hence, our study cannot be directly compared with studies using krill oil.

Using PLs from marine fish, Rossmeisl et al. [11] demonstrated that the superior anti-obesogenic effect of PL-bound, compared to TAG-bound, *n*-3 PUFAs was related to higher bioavailability [11]. In agreement with this, earlier studies from our laboratory have demonstrated that increased *n*-3:*n*-6 ratios in fish fillets and in PLs from livers and RBCs collected from mice consuming fish-containing diets, are associated with reduced obesity [4,38–40]. However, in the present study, the dietary *n*-3 PL-bound PUFAs were not incorporated into mice RBCs to a higher degree than TAG-bound *n*-3 PUFAs. Of note, in line with our results, a critical review published in 2014 concluded that there was no evidence for greater bioavailability of *n*-3 PUFAs from PLs compared with TAGs [41]. Surprisingly, we did not observe an anti-obesogenic effect of PL-bound *n*-3 PUFAs, but rather, a weak obesity promoting effect, which may relate to the housing temperature and the use of pork-based diets.

To our knowledge, we are the first to investigate how PL-bound *n*-3 PUFAs affect the obesity phenotype of mice fed Western diets using a pork-based background diet in a thermoneutral environment. Housing mice under thermoneutrality is claimed to be an advantageous step towards aligning murine energy metabolism to human energy metabolism [42], but possible effects of *n*-3 PUFAs on energy expenditure in brown or brown-like adipocytes [43,44] may be masked. At ambient temperatures below 30 °C, higher expression of the uncoupling protein 1 (UCP1) allows energy to be dissipated in the form of heat, and its expression is positively correlated with metabolic inefficiency and low energy efficiency [45]. UCP1-KO as well as fatty acid elongase-2 (Elovl2)-KO mice require housing under thermoneutrality to reveal the obesity phenotype [46,47]. Cold-exposure and even ambient housing temperatures of 20–22 °C require higher food intakes to meet the increased energy demand for thermogenesis [42,45], and a reduction in the ambient temperature has been demonstrated to attenuate high fat diet-induced obesity [48]. Performing experiments at a temperature at which the capacity for thermogenesis is reduced may have contributed to the increased obesity development in the present study. Along this line, it is also important to note that in the experiments in which an anti-obesogenic effect of PL-bound *n*-3 PUFA was observed, the background diets were based on casein [11,49].

We have previously reported that, at least in mice fed high protein/high fat diets, a characteristic brown adipocyte morphology of interscapular brown adipose tissue is dependent on the protein source [8]. Casein stands out as the most efficient protein source for preserving the brown adipocyte morphology, whereas the intake of proteins from pork and chicken leads to a more white-like adipocyte morphology. Hence, the thermogenic capacity may be further reduced by using proteins from pork instead of casein in the background diet. Therefore, we cannot exclude the possibility that both the housing temperature and the use of pork protein may have contributed to the unexpected obesogenic effect of PL-bound *n*-3 PUFAs.

Results from an earlier study showed that mice fed frozen cod in a Western diet had 6% lower energy intakes compared to mice fed pork *ad libitum*, and additionally, mice fed frozen cod were leaner than pork-fed mice [4]. Compared to mice fed casein in the first experiment, we observed

lower energy intakes in both frozen and fresh cod-fed mice, but not pork-fed mice after 6 weeks of feeding. In the second experiment, mice fed fresh cod and pork were compared, and we observed no differences in energy intake or diet-induced obesity between the mice. The freeze-dried fresh cod powder in the first experiment was stored at −20 °C for 12 weeks prior to be blended into a Western diet. Although the fresh cod powder was freeze-dried to >97% dryness before storage, we cannot rule out that the 12 weeks storage may have affected the powder. In contrast, the fresh cod powder in the second experiment was blended directly into the diet after preparation with minimum storage at −20 °C and was fed to the mice. It has been shown that storage of cod fillets at −23 °C leads to increased development of off flavors, affecting the quality of cod fillets [50]. The freezing of cod fillets initiates deterioration in flavor through rancidity, an undesirable fishy taste and other off flavors, believed to reflect the formation of low-molecular weight compounds from lipid oxidation and protein denaturation [51]. Hence, we cannot exclude the possibility that differences in the frozen storage time led to the observed differences in energy intake. To investigate this possibility, feeding mice diets containing cod frozen for longer periods to systematically increase the FFA:PL ratio of lipids would be required.

An important limitation in the study design is that we were unable to obtain *n*-3 PUFAs extracted from cod fillets, but used PL-oil extracted from herring roe. Still, the EPA:DHA ratio was similar to that of the fresh cod powder, and we supplemented the pork-containing diet with the same amount of *n*-3 PUFAs as found in the fresh cod diet. Another limitation is that we did not analyze other compounds that may have affected the quality of the frozen cod fillets. It would be of interest to investigate whether the accumulation of compounds from protein denaturation and lipid oxidation products during frozen storage is related to diet-induced obesity and energy intake in mice fed frozen cod fillets. It would further be of interest to investigate how PL-bound *n*-3 PUFAs vs. TAG-bound *n*-3 PUFAs affect obesity development when combined with other protein sources than pork.

In conclusion, compared with mice fed a casein-based Western diet, mice fed fresh cod, but not frozen cod with a reduced PL content, showed increased obesity development. Furthermore, the intake of a pork-containing diet with PL-bound, but not TAG-bound *n*-3 PUFAs, led to significantly average higher body weight and fat mass compared to the intake of a pork-based diet that was not supplemented with *n*-3 PUFAs. The present study points to a possible surprising effect of frozen storage of cod in relation to metabolism and obesity development and raises an interesting question as to whether similar effects of intake of fresh cod versus frozen cod may be observed in humans.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/10/6/695/s1; Figure S1: Effects of Western diets with different protein sources on glucose tolerance and glucose-stimulated insulin secretion in male C57BL/6J after 11 weeks on the experimental diets. As reference, a low fat fed group ($n = 5$) was included and is shown as a dotted line. (a) An oral glucose tolerance test (OGTT) was performed on mice fasted for 6 h. Blood glucose levels were recorded before (0) and at 15, 30, 60 and 120 min after oral administration of glucose (3 mg glucose/g lean mass). (b) Blood glucose area under the curve (AUC) and (c) incremental blood glucose area under the curve (iAUC) were calculated. (d) Blood was collected and plasma prepared for insulin measured before (0) and at 15, 30 and 120 min after oral administration of glucose. (e) Measurements of 6 h fasted blood glucose (f) 15 min, (g) 30 min, (h) and 120 min after the oral administration of glucose. (i) 6 h fasted plasma insulin (j) and plasma insulin levels at 15 min, (k) 30 min, (l) and 120 min after the oral administration of glucose. Data are presented as mean ± SEM ($n = 10$) and were analyses using one-way ANOVA followed by Fisher's LSD post hoc test. Different letters denote statistical significance ($p \leq 0.05$) between the groups; Figure S2: Effects of Western diets on fasting blood glucose levels after 10 weeks on experimental diets. Male C57BL/6J mice were fed Western diets containing either, fresh cod (fresh cod) or fresh pork (pork) as protein sources for 12 weeks, two pork diets was added n-3 PUFAs to the level of the fresh cod containing diet either as TAG-bound (pork n-3 TAG) or PL-bound (pork n-3 PL) bound n-3 EPA+DHA. As reference, a low fat fed group ($n = 5$) was included and is shown as a dotted line. Data are presented as mean ± SEM ($n = 14$–15) and were analyses using one-way ANOVA followed by Fisher's LSD post hoc test. Different letters denote statistical significance ($p \leq 0.05$) between the groups; Table S1: Lipid class composition in raw or heated fresh cod fillets; Table S2: Fatty acid composition in the polar and neutral lipid fractions isolated from raw or heated fresh cod fillets; Table S3: Fatty acid composition in the polar and neutral lipid fractions isolated from freeze dried fresh and frozen cod fillets; Table S4: Compositions of the diets in experiment 1; Table S5: Fatty acid composition in the polar and neutral lipid fractions isolated from Western diets; Table S6: Lipid class composition in freeze dried fresh cod fillets; Table S7: Fatty acid compositions in the polar and neutral lipid fractions isolated from freeze dried fresh cod fillets; Table S8: Fatty acid compositions in the polar and neutral lipid fractions isolated from soybean

oil, PL-soybean oil and cod liver oil; Table S9: Compositions of the diets in experiment 2; Table S10: Fatty acid composition in the polar and neutral lipid fractions isolated from mouse liver; Table S11: Fatty acid composition in red blood cells.

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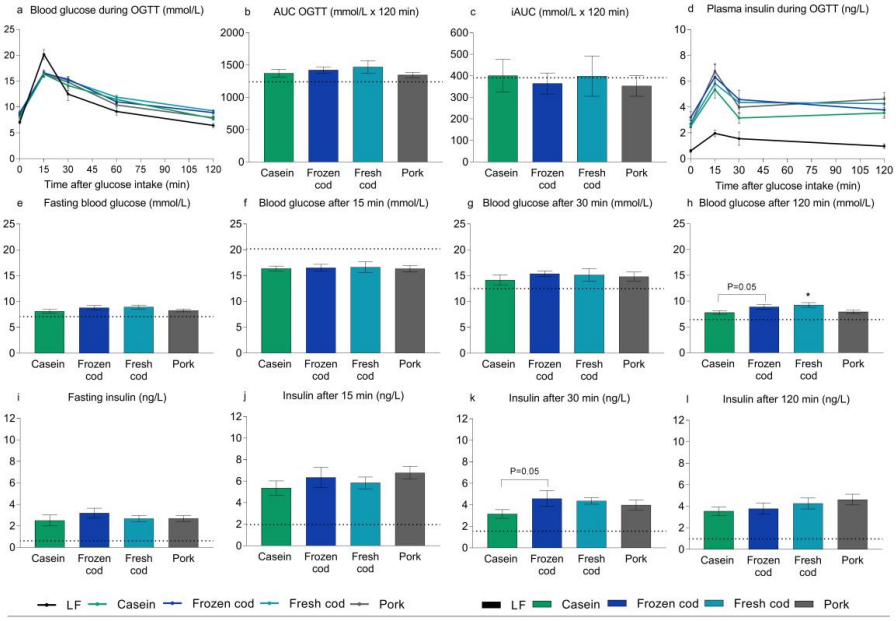

Figure S1. Effects of Western diets with different protein sources on glucose tolerance and glucose-stimulated insulin secretion in male C57BL/6J after 11 weeks on the experimental diets. As reference, a low fat fed group (n=5) was included and is shown as a dotted line. (**a**) An oral glucose tolerance test (OGTT) was performed on mice fasted for 6 h. Blood glucose levels were recorded before (0) and at 15, 30, 60 and 120 minutes after oral administration of glucose (3 mg glucose/g lean mass). (**b**) Blood glucose area under the curve (AUC) and (**c**) incremental blood glucose area under the curve (iAUC) were calculated. (**d**) Blood was collected and plasma prepared for insulin measured before (0) and at 15, 30 and 120 minutes after oral administration of glucose. (**e**) Measurements of 6 h fasted blood glucose (**f**) 15 min, (**g**) 30 min, (h) and 120 min after the oral administration of glucose. (**i**) 6 h fasted plasma insulin (**j**) and plasma insulin levels at 15 min, (**k**) 30 min, (**l**) and 120 min after the oral administration of glucose. Data are presented as mean ± SEM (n=10) and were analyses using one-way ANOVA followed by Fisher's LSD post hoc test. Different letters denote statistical significance (P=<0.05) between the groups.

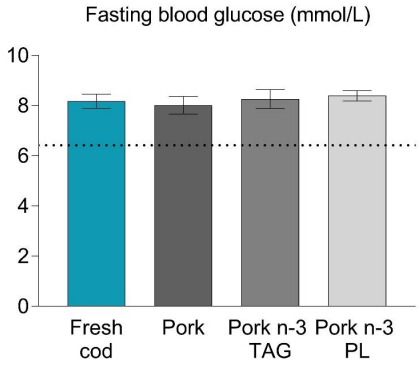

Figure S2. Effects of Western diets on fasting blood glucose levels after 10 weeks on experimental diets. Male C57BL/6J mice were fed Western diets containing either, fresh cod (fresh cod) or fresh pork (pork) as protein sources for 12 weeks, two pork diets was added n-3 PUFAs to the level of the fresh cod containing diet either as TAG-bound (pork n-3 TAG) or PL-bound (pork n-3 PL) bound n-3 EPA+DHA. As reference, a low fat fed group (n=5) was included and is shown as a dotted line. Data are presented as mean ± SEM (n=14-15) and were analyses using one-way ANOVA followed by Fisher's LSD post hoc test. Different letters denote statistical significance (P=<0.05) between the groups.

Table S1. Lipid class composition in raw or heated fresh cod fillets

	Raw fresh cod fillets		**Heated fresh cod fillets**	
Lipid class	**mg/g**	**%**	**mg/g**	**%**
PC	2.90 ± 0.04	51	5.25 ± 0.09	50.1
PE	1.06 ± 0.06	18.7	2.05 ± 0.03	19.5
PI	0.21 ± 0.01	3.6	0.461 ± 0.007	4.39
PS	0.07 ± 0.03	1.2	0.362 ± 0.009	3.45
LPC	0.28 ± 0.01	5.0	0.27 ± 0.02	2.5
SM	0.171 ± 0.007	3.0	0.335 ± 0.008	3.19
CL	0.033 ± 0.003	0.576	0.080 ± 0.007	0.76
Sum polar lipids	4.7 ± 0.1	83	8.80 ± 0.03	83.9
FFA	0.72 ± 0.03	12.7	0.66 ± 0.04	6.3
CHOL	0.48 ± 0.03	8.4	0.81 ± 0.03	7.8
TAG	0.02 ± 0.01	0.3	0.16 ± 0.02	1.5
DAG	<0.01	<0.01	<0.01	<0.01
CE	<0.01	<0.01	<0.01	<0.01
Sum neutral lipids	1.0 ± 0.3	17	1.69 ± 0.03	16.1
Sum Lipids	5.7 ± 0.4		10.49 ± 0.02	
Polar lipids:FFA ratio	6.57 ± 0.02		13.3 ± 0.7	

Results are presented as mean ± SEM of three samples and indicate mg lipids/g and percent lipid class of total lipids in the raw or heated fresh cod fillets Abbreviations: PC; Phosphatidylcholine, PE; Phosphatidylethanolamine, PI; Phosphatidylinositol, PS; Phosphatidylserin, LPC; Lysophosphatidylcholine, SM; Sphingomyelin , CL; Cardiolipin, FFA; Free fatty acid, CHOL; Cholesterol, TAG; Triacylglycerol, DAG; Diacylglycerol, CE; Cholesteryl ester.

Table S2. Fatty acid composition in the polar and neutral lipid fractions isolated from raw or heated fresh cod fillets

	Raw fresh cod fillets		Heated fresh cod fillets	
Fatty acid	**mg/g**	**%**	**mg/g**	**%**
Polar lipid fraction				
Sum SFA	0.63 ± 0.08	24.41	1.05 ± 0.08	24.77
Sum MUFA	0.37 ± 0.05	14.2	0.54 ± 0.04	12.785
LA 18:2n-6	0.024 ± 0.003	0.95	0.036 ± 0.002	0.86
ARA 20:4n-6	0.050 ± 0.006	1.93	0.085 ± 0.006	2.01
Sum n-6	0.09 ± 0.01	3.55	0.15 ± 0.01	3.49
ALA 18:3n-3	<0.01	<0.01	<0.01	<0.01
EPA 20:5n-3	0.39 ± 0.05	15.4	0.61 ± 0.05	14.37
DHA 22:6n-3	1.0 ± 0.1	40.0	1.8 ± 0.1	42.31
Sum EPA + DHA	1.4 ± 0.2	55.4	2.4 ± 0.2	56.67
Sum n-3	1.5 ± 0.2	57.8	2.5 ± 0.2	58.88
Sum identified FAs	2.6 ± 0.3		4.2 ± 0.3	
n-6:n-3 ratio	0.062 ± 0.001		0.0592 ± 0.0004	
EPA:DHA	0.385 ± 0.005		0.3396	
ARA:EPA ratio	0.1255 ± 0.0004		0.1400 ± 0.0008	
Neutral lipid fraction				
Sum SFA	0.19 ± 0.03	24.5	0.18 ± 0.01	22.8
Sum MUFA	0.15 ± 0.02	19.7	0.18 ± 0.02	22.8
LA 18:2n-6	<0.01	<0.01	<0.01	<0.01
ARA 20:4n-6	0.012 ± 0.002	1.60	0.016 ± 0.001	1.988
Sum n-6	0.024 ± 0.004	3.17	0.029 ± 0.002	3.65
ALA 18:3n-3	<0.01	<0.01	<0.01	<0.01
EPA 20:5n-3	0.15 ± 0.02	19.2	0.156 ± 0.010	19.5
DHA 22:6n-3	0.23 ± 0.04	30.0	0.22 ± 0.02	27.47
Sum EPA + DHA	0.38 ± 0.06	49.2	0.38 ± 0.03	47.0
Sum n-3	0.40 ± 0.07	52.5	0.40 ± 0.03	50.6
Sum identified FAs	0.8 ± 0.1		0.80 ± 0.06	
n-6:n-3 ratio	0.060 ± 0.001		0.0722 ± 0.0009	
EPA:DHA	0.64 ± 0.01		0.710 ± 0.005	
ARA:EPA ratio	0.084 ± 0.002		0.1019 ± 0.0006	

Results are presented as mean ± SEM of three samples and indicate mg FAs/g and percent FAs of total identified FAs in the raw or heated fresh cod fillets. Abbreviations: SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, LA; linoleic acid, ARA; arachidonic acid, ALA; alpha-linolenic acid, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid, FAs; fatty acids.

Table S3. Fatty acid composition in the polar and neutral lipid fractions isolated from freeze dried fresh and frozen cod fillets

	Freeze dried frozen cod		Freeze dried fresh cod	
Fatty acid	**mg/g**	**%**	**mg/g**	**%**
Polar lipid fraction				
Sum SFA	4.1 ± 0.7	25.1	4.8 ± 0.7	25.5
Sum MUFA	2.3 ± 0.3	13.9	2.6 ± 0.4	13.8
LA 18:2n-6	0.14 ± 0.02	0.884	0.17 ± 0.03	0.89
ARA 20:4n-6	0.29 ± 0.04	1.78	0.33 ± 0.04	1.75
Sum n-6	0.52 ± 0.07	3.21	0.61 ± 0.06	3.3
ALA 18:3n-3	0.022 ± 0.003	0.137	0.027 ± 0.005	0.143
EPA 20:5n-3	2.2 ± 0.4	13.4	2.7 ± 0.4	14.0
DHA 22:6n-3	7 ± 1	42.5	8 ± 1	41.4
Sum EPA + DHA	9 ± 1	55.87	10 ± 1	55.4
Sum n-3	9 ± 2	57.79	11 ± 1	57.3
Sum identified FAs	16 ± 3		19 ± 3	
n-6:n-3 ratio	0.055 ± 0.001		0.058 ± 0.002	
EPA:DHA ratio	0.32 ± 0.02		0.34 ± 0.02	
ARA:EPA ratio	0.133 ± 0.008		0.125 ± 0.007	
Neutral lipid fraction				
Sum SFA	2.5 ± 0.6	23.5	2.0 ± 0.5	23.1
Sum MUFA	1.8 ± 0.3	18	1.6 ± 0.2	20
LA 18:2n-6	0.12 ± 0.02	1.15	0.10 ± 0.02	1.17
ARA 20:4n-6	0.23 ± 0.05	2.20	0.19 ± 0.05	2.19
Sum n-6	0.41 ± 0.09	3.83	0.34 ± 0.09	3.9
ALA 18:3n-3	0.025 ± 0.004	0.24	0.024 ± 0.002	0.29
EPA 20:5n-3	2.2 ± 0.4	21.3	1.8 ± 0.4	21.9
DHA 22:6n-3	3.3 ± 0.8	31	2.6 ± 0.8	28.8
Sum EPA + DHA	6 ± 1	52	4 ± 1	51
Sum n-3	6 ± 1	55	5 ± 1	53
Sum identified FAs	11 ± 2		9 ± 2	
n-6:n-3 ratio	0.0701 ± 0.0009		0.073 ± 0.003	
EPA:DHA ratio	0.71 ± 0.08		0.77 ± 0.08	
ARA:EPA ratio	0.104 ± 0.007		0.101 ± 0.007	

Results are presented as mean ± SEM of three samples and indicate mg FAs /g and percent FAs of sum identified FAs in the freeze dried frozen or fresh cod fillets. Abbreviations: SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, LA; linoleic acid, ARA; arachidonic acid, ALA; alpha-linolenic acid, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid, FAs; fatty acids.

Table S4. Compositions of the diets in experiment 1

Components (g/kg diet)	Casein	Frozen cod	Fresh cod	Pork	Low fat
Casein	222.0				222.0
Freeze dried frozen cod fillets		235.9			
Freeze dried fresh cod fillets			242.9		
Freeze dried pork sirloins				241.5	
Corn starch	296.0	292.6	290.4	294.9	
Dextrin from potato starch	100.0	100.0	100.0	100.0	507.5
Sucrose	80.0	80.0	80.0	80.0	100.0
Soybean oil	12.0	12.0	12.0	12.0	
Corn oil	8.0	8.0	8.0	8.0	70.0
Milk fat	60.0	56.5	54.9	53.9	
Lard	60.0	56.5	54.9	53.9	
Margarine	60.0	56.5	54.9	53.9	
Cholesterol	1.5	1.5	1.5	1.5	
Lipids from freeze dried frozen cod fillets		10.6			
Lipids from freeze dried fresh cod fillets			15.3		
Lipids from freeze dried pork sirloins				18.4	
Analyzed					
Energy (KJ/g diet)	20.52 ± 0.01	20.28 ± 0.28	20.2 ± 0.2	20.5 ± 0.1	18.51 ± 0.03

All diets were supplemented with 0.014 g/kg t-Butylhydroquinone, 35 g/kg AIN93G mineral mix, 10 g/kg AIN93VX NCR95 compliant vitamin mix, 3g/kg L-cystine, 2.5g/kg choline bitartrate and 50g/kg cellulose. The amount of casein, freeze dried frozen and fresh cod fillets and pork sirloins added is based on measurements of nitrogen in the protein powders. Crude protein concentration was calculated using the formula N*6.15 for casein and N*5.60 for cod fillets and pork. The calculated contribution of lipids present in the protein sources is based on measurements of total lipid content in the protein powders measured with HPTLC. Analyzed values represents mean ± SEM of three samples.

Table S5. Fatty acid composition in the polar and neutral lipid fractions isolated from Western diets

	Frozen cod		Fresh cod		Pork	
Fatty acid	**mg/g**	**%**	**mg/g**	**%**	**mg/g**	**%**
Polar lipid fraction						
Sum SFA	1.65 ± 0.03	31.5	2.20 ± 0.07	29.4	1.22 ± 0.04	45.9
Sum MUFA	1.12 ± 0.03	21.4	1.39 ± 0.06	18.6	0.91 ± 0.02	34.2
LA 18:2n-6	0.32 ± 0.02	6.0	0.36 ± 0.03	4.7	0.430 ± 0.008	16.2
ARA 20:4n-6	0.065 ± 0.002	1.25	0.099 ± 0.001	1.35	<0.01	<0.01
Sum n-6	0.41 ± 0.02	7.9	0.49 ± 0.03	6.5	0.448 ± 0.009	16.8
ALA 18:3n-3	0.054 ± 0.004	1.04	0.064 ± 0.004	0.83	0.06 ± 0.03	2
EPA 20:5n-3	0.44 ± 0.02	8.4	0.805 ± 0.002	11.0	<0.01	0.13
DHA 22:6n-3	1.49 ± 0.08	28	2.315 ± 0.008	31.9	<0.01	<0.01
Sum EPA+DHA	1.9 ± 0.1	37	3.12 ± 0.01	42.8	<0.01	<0.01
Sum n-3	2.1 ± 0.1	39	3.31 ± 0.01	45.4	0.07 ± 0.03	3
Sum identified FAs	5.23 ± 0.02		7.4 ± 0.2		2.66 ± 0.03	
n-6:n-3 ratio	0.20 ± 0.02		0.149 ± 0.008		10 ± 6	
EPA:DHA ratio	0.294 ± 0.005		0.3475 ± 0.0006		*	
ARA:EPA ratio	0.150 ± 0.005		0.123 ± 0.002		2.2 ± 1.0	
Neutral lipid fraction						
Sum SFA	66.0 ± 0.5	44.2	63.25 ± 0.05	44.44	67.0 ± 0.5	44.79
Sum MUFA	54.3 ± 0.2	36.37	51.37 ± 0.07	35.95	54.5 ± 0.6	36.44
LA 18:2n-6	22.02 ± 0.03	14.75	21.85 ± 0.02	15.5	22.7 ± 0.1	15.17
ARA 20:4n-6	0.044 ± 0.008	0.029	0.192 ± 0.006	0.03	0.025 ± 0.005	0.017
Sum n-6	22.62 ± 0.04	15.1	22.4222 ± 0.0004	15.80	23.2 ± 0.1	15.55
ALA 18:3n-3	3.94 ± 0.02	2.639	4.04 ± 0.03	2.86	4.21 ± 0.02	2.82
EPA 20:5n-3	0.68 ± 0.04	0.46	0.35 ± 0.03	0.24	0.064 ± 0.004	0.043
DHA 22:6n-3	1.10 ± 0.07	0.74	0.40 ± 0.02	0.26	<0.01	<0.01
Sum EPA+DHA	1.8 ± 0.1	1.19	0.75 ± 0.05	0.50	0.076 ± 0.009	0.051
Sum n-3	6.2 ± 0.1	4.18	5.26 ± 0.08	3.65	4.65 ± 0.05	3.11
Sum identified FAs	149.3 ± 0.7		142.5 ± 0.2		149 ± 1	
n-6:n-3 ratio	3.63 ± 0.09		4.26 ± 0.06		5.00 ± 0.06	
EPA:DHA ratio	0.617 ± 0.005		0.87 ± 0.03		*	
ARA:EPA ratio	0.36 ± 0.03		0.56 ± 0.07		2.7 ± 0.2	

Results are presented as mean ± SEM of three samples, with exception of the fresh cod diet that contained two samples. Results indicate mg FA in the polar and neutral lipid fractions/g Western diet. *not possible to calculate; EPA and DHA levels are under limit of quantification (<0.01 mg/g). Abbreviations: SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, LA; linoleic acid, ARA; arachidonic acid, ALA; alpha-linolenic acid, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid, FAs; fatty acids.

Table S6. Lipid class composition in freeze dried fresh cod fillets

	Freeze-dried fresh cod fillets	
Lipid class	**mg/g**	**%**
PC	27.4 ± 0.2	71
PE	7.0 ± 0.5	18.0
PI	<0.01	<0.01
PS	<0.01	<0.01
LPC	1.5 ± 0.1	3.7
SM	0.55 ± 0.08	1.4
CL	<0.01	<0.01
Sum polar lipids	36.5 ± 0.8	94
FFA	0.6 ± 0.4	1
CHOL	1.9 ± 0.7	5
TAG	<0.01	<0.01
DAG	<0.01	<0.01
CE	<0.01	<0.01
Sum neutral lipids	2 ± 1	6
Sum Lipids	39 ± 2	
Polar lipid:FFA ratio	88 ± 61	

Results are presented as mean ± SEM of three samples and indicate mg lipids/g and percent lipid class of total lipids in freeze dried fresh cod fillets. Abbreviations: PC; Phosphatidylcholine, PE; Phosphatidylethanolamine, PI; Phosphatidylinositol, PS; Phosphatidylserin, LPC; Lysophosphatidylcholine, SM; Sphingomyelin , CL; Cardiolipin, FFA; Free fatty acid, CHOL; Cholesterol, TAG; Triacylglycerol, DAG; Diacylglycerol, CE; Cholesteryl ester.

Table S7. Fatty acid compositions in the polar and neutral lipid fractions isolated from freeze dried fresh cod fillets

	Freeze dried fresh cod fillets	
Fatty acid	**mg/g**	**%**
Polar lipid fraction		
Sum SFA	4.6 ± 0.1	24.3
Sum MUFA	2.51 ± 0.02	13.4
LA 18:2n-6	0.245 ± 0.004	1.31
ARA 20:4n-6	0.392 ± 0.007	2.088
Sum n-6	0.75 ± 0.01	3.99
ALA 18:3n-3	0.074 ± 0.004	0.40
EPA 20:5n-3	3.2 ± 0.2	17.0
DHA 22:6n-3	7.2 ± 0.1	38.4
Sum EPA+DHA	10.4 ± 0.2	55.4
Sum n-3	10.9 ± 0.2	58.3
Sum identified FAs	18.8 ± 0.3	
n-6:n-3 ratio	0.0685 ± 0.0009	
EPA:DHA ratio	0.44 ± 0.03	
ARA:EPA ratio	0.06 ± 0.03	
Neutral lipid fraction		
Sum SFA	0.9 ± 0.2	24
Sum MUFA	1.0 ± 0.2	26
LA 18:2n-6	0.06 ± 0.01	1.56
ARA 20:4n-6	0.09 ± 0.02	2.127
Sum n-6	0.17 ± 0.04	4.13
ALA 18:3n-3	0.025 ± 0.005	0.63
EPA 20:5n-3	0.64 ± 0.09	16
DHA 22:6n-3	1.2 ± 0.5	26
Sum EPA+DHA	1.8 ± 0.6	43
Sum n-3	2.0 ± 0.6	46
Sum identified FAs	4 ± 1	
n-6:n-3 ratio	0.090 ± 0.005	
EPA:DHA ratio	0.7 ± 0.1	
ARA:EPA ratio	0.21 ± 0.03	

Results are presented as mean ± SEM of three samples and indicate mg FA/g and percent FA of sum identified FAs in the freeze dried fresh cod fillets. Abbreviations: SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, LA; linoleic acid, ARA; arachidonic acid, ALA; alpha-linolenic acid, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid, FAs; fatty acids.

Table S8. Fatty acid compositions in the polar and neutral lipid fractions isolated from soybean oil, PL-soybean oil and cod liver oil

Fatty acid (mg/g)	Soybean oil	PL-soybean oil	Cod liver oil
Polar lipid fraction			
Sum SFA	1.2 ± 0.1	75 ± 4	8 ± 1
Sum MUFA	2.2 ± 0.3	32 ± 1	5.2 ± 0.4
LA 18:2n-6	4.3 ± 0.5	1.7 ± 0.1	<0.01
ARA 20:4n-6	<0.01	1.33 ± 0.07	<0.01
Sum n-6	4.3 ± 0.5	3.6 ± 0.2	<0.01
ALA 18:3n-3	0.44 ± 0.05	0.89 ± 0.08	<0.01
EPA 20:5n-3	<0.01	44 ± 5	1.01 ± 0.08
DHA 22:6n-3	<0.01	114 ± 8	1.4 ± 0.1
Sum EPA+DHA	<0.01	158 ± 12	2.4 ± 0.2
Sum n-3	0.45 ± 0.05	165 ± 13	2.4 ± 0.2
Sum identified FAs	8 ± 1	277 ± 18	15 ± 2
n-6:n-3 ratio	9.48 ± 0.05	0.022 ± 0.001	*
EPA:DHA ratio	*	0.38 ± 0.01	0.737 ± 0.006
ARA:EPA ratio	*	0.031 ± 0.002	*
Neutral lipid fraction			
Sum SFA	137.8 ± 0.8	84.8 ± 0.6	147.6 ± 0.7
Sum MUFA	259 ± 2	163 ± 1	445.9 ± 1.7
LA 18:2n-6	493 ± 3	262 ± 2	19.5 ± 0.2
ARA 20:4n-6	<0.01	0.34 ± 0.02	3.95 ± 0.04
Sum n-6	493 ± 3	262 ± 2	28.0 ± 0.3
ALA 18:3n-3	51.6 ± 0.5	28.5 ± 0.3	7.73 ± 0.06
EPA 20:5n-3	0.46 ± 0.01	4.40 ± 0.06	78.6 ± 0.3
DHA 22:6n-3	<0.01	5.51 ± 0.09	113.4 ± 0.4
Sum EPA+DHA	0.46 ± 0.01	9.9 ± 0.2	192.1 ± 0.7
Sum n-3	52.3 ± 0.5	41.4 ± 0.5	247 ± 1
Sum identified FAs	943 ± 6	551 ± 5	871 ± 4
n-6:n-3 ratio	9.43 ± 0.03	6.33 ± 0.02	0.113 ± 0.001
EPA:DHA ratio	*	0.798 ± 0.003	0.6931 ± 0.0004
ARA:EPA ratio	*	0.078 ± 0.006	0.0503 ± 0.0004

Results are presented as mean ± SEM of three samples and indicate mg FA/g oil. . *not possible to calculate; EPA and DHA levels are under limit of quantification (<0.01 mg/g). Abbreviations: SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, LA; linoleic acid, ARA; arachidonic acid, ALA; alpha-linolenic acid, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid, FAs; fatty acids, PL; phospholipid.

Table S9. Compositions of the diets in experiment 2

Components (g/kg diet)	Fresh cod	Pork	Pork n-3 TAG	Pork n-3 PL	Low fat
Freeze dried fresh cod fillets	241.9				
Freeze dried pork sirloins		240.6	240.6	240.6	
Casein					217.5
Corn starch	281.6	288.0	288.0	288.0	
Dextrin from potato starch	100.0	100.0	100.0	100.0	512.0
Sucrose	80.0	80.0	80.0	80.0	100.0
Soybean oil	12.0	12.0	12.0	2.6*	
Corn oil	8.0	8.0	8.0	8.0	70.0
Milk fat	58.2	56.5	51.3	53.3	
Lard	58.2	56.5	51.3	53.3	
Margarine	58.2	56.5	51.3	53.3	
Cod liver oil			15.4		
PL-soybean oil				18.7	
FAs from freeze dried cod loins	5.5				
FAs from freeze dried pork sirloins		10.6	10.6	10.6	
Cholesterol	1.5	1.5	1.5	1.5	
Analyzed					
Energy (KJ/g diet)	20.6 ± 0.1	21.17 ± 0.03	21.17 ± 0.03	21.07 ± 0.08	18.60 ± 0.01

All diets were supplemented with 0.014 g/kg t-Butylhydroquinone, 35 g/kg AIN93G mineral mix, 10 g/kg AIN93VX NCR95 compliant vitamin mix, 3g/kg L-cystine, 2.5g/kg choline bitartrate and 50g/kg cellulose. The amount of fresh freeze dried fresh cod fillets, freeze dried pork sirloins and casein added is based on measurements of nitrogen in the protein powders. Crude protein concentration was calculated using the formula N*6.15 for casein and N*5.60 for cod fillets and pork. The calculated contribution of fatty acids (FAs) present in the protein sources is based on measurements of FA in the polar and neutral lipid fractions from the protein powders measured with GC-FID. Analyzed values represents mean ± SEM of three samples. *The PL-soybean oil contributed with 9.4 g soybean oil/kg diet. Hence, the final content of soybean oil in the Pork n-3 PL diet was 12.0 g/diet. Abbreviations: FAs; Fatty acids, PL; phospholipids.

Table S10. Fatty acid composition in the polar and neutral lipid fractions isolated from mouse liver

Fatty acid (mg/g)	Frozen cod	Fresh cod	Pork
Polar lipid fraction			
Sum SFA	6.2 ± 0.2 ^{ab}	6.5 ± 0.2 ^{a}	5.7 ± 0.3 ^{b}
Sum MUFA	2.73 ± 0.06	2.8 ± 0.1	2.7 ± 0.1
LA 18:2n-6	2.6 ± 0.1	2.7 ± 0.1	2.4 ± 0.2
ARA 20:4n-6	1.52 ± 0.04 ^{a}	1.63 ± 0.05 ^{a}	2.8 ± 0.1 ^{b}
Sum n-6	4.6 ± 0.2 ^{a}	4.9 ± 0.2 ^{a}	5.8 ± 0.3 ^{b}
ALA 18:3n-3	0.033 ± 0.002 ^{a}	0.033 ± 0.003 ^{a}	0.023 ± 0.002 ^{b}
EPA 20:5n-3	0.79 ± 0.04 ^{a}	0.83 ± 0.05 ^{a}	0.18 ± 0.01 ^{b}
DHA 22:6n-3	2.7 ± 0.1 ^{a}	2.77 ± 0.09 ^{a}	1.62 ± 0.07 ^{b}
Sum EPA+DHA	3.5 ± 0.1 ^{a}	3.6 ± 0.1 ^{a}	1.80 ± 0.08 ^{b}
Sum n-3	3.7 ± 0.1 ^{a}	3.8 ± 0.1 ^{a}	1.93 ± 0.09 ^{b}
Sum identified FAs	17.2 ± 0.6	18.0 ± 0.7	16.2 ± 0.7
n-6:n-3 ratio	1.24 ± 0.02 ^{a}	1.29 ± 0.02 ^{a}	2.99 ± 0.06 ^{b}
ARA:EPA ratio	1.95 ± 0.08 ^{a}	2.01 ± 0.08 ^{a}	15.6 ± 0.6 ^{b}
Neutral lipid fraction			
Sum SFA	24 ± 5	27 ± 5	33 ± 6
Sum MUFA	63 ± 15	67 ± 13	88 ± 16
LA 18:2n-6	10 ± 2	12 ± 2	13 ± 1
ARA 20:4n-6	0.29 ± 0.05 ^{a}	0.34 ± 0.04 ^{a}	0.66 ± 0.06 ^{b}
Sum n-6	11 ± 2	12 ± 2	14 ± 2
ALA 18:3n-3	1.4 ± 0.3	1.5 ± 0.2	1.2 ± 0.1
EPA 20:5n-3	0.9 ± 0.1 ^{a}	1.1 ± 0.1 ^{a}	0.28 ± 0.03 ^{b}
DHA 22:6n-3	4.7 ± 0.6 ^{a}	5.4 ± 0.5 ^{a}	1.2 ± 0.1 ^{b}
Sum EPA+DHA	5.7 ± 0.7 ^{a}	6.5 ± 0.6 ^{a}	1.4 ± 0.1 ^{b}
Sum n-3	8 ± 1 ^{a}	9.4 ± 0.9 ^{a}	3.4 ± 0.3 ^{b}
Sum identified FAs	108 ± 23	116 ± 21	139 ± 23
n-6:n-3 ratio	1.28 ± 0.09 ^{a}	1.30 ± 0.06 ^{a}	4.3 ± 0.2 ^{b}
ARA:EPA ratio	0.32 ± 0.02 ^{a}	0.30 ± 0.02 ^{a}	2.41 ± 0.09 ^{b}

Results are presented as mean ± SEM (n=10) and indicate mg FAs in the polar and neutral lipid fractions/g liver. Results are analyzed using one-way ANOVA followed by Fisher's LSD post hoc test. Significant differences ($P<0.05$) between the groups are marked with different letters. Abbreviations: SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, LA; linoleic acid, ARA; arachidonic acid, ALA; alpha-linolenic acid, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid, FAs; fatty acids.

Table S11. Fatty acid composition in red blood cells

Fatty acid (mg/g)	Frozen cod	Fresh cod	Pork
Sum SFA	1.27 ± 0.04	1.23 ± 0.07	1.26 ± 0.05
Sum MUFA	0.54 ± 0.02	0.53 ± 0.03	0.55 ± 0.02
LA 18:2n-6	0.35 ± 0.01	0.33 ± 0.02	0.32 ± 0.02
ARA 20:4n-6	0.264 ± 0.006 [a]	0.24 ± 0.01 [a]	0.47 ± 0.01 [b]
Sum n-6	0.66 ± 0.01 [a]	0.61 ± 0.04 [a]	0.87 ± 0.02 [b]
ALA 18:3n-3	<0.01	<0.01	<0.01
EPA 20:5n-3	0.104 ± 0.004 [a]	0.097 ± 0.006 [a]	0.022 ± 0.001 [b]
DHA 22:6n-3	0.264 ± 0.007 [a]	0.24 ± 0.02 [a]	0.164 ± 0.009 [b]
Sum EPA+DHA	0.37 ± 0.01 [a]	0.34 ± 0.02 [a]	0.19 ± 0.01 [b]
Sum n-3	0.40 ± 0.01 [a]	0.37 ± 0.02 [a]	0.22 ± 0.01 [b]
Sum identified FAs	2.87 ± 0.07	2.7 ± 0.2	2.91 ± 0.09
n-6:n-3 ratio	1.67 ± 0.03 [a]	1.68 ± 0.06 [a]	4.0 ± 0.2 [b]
ARA:EPA ratio	2.6 ± 0.1 [a]	2.43 ± 0.07 [a]	21.9 ± 0.8 [b]

Results are presented as mean ± SEM and indicate mg FA/g red blood cells. Data were analyzed using one-way ANOVA followed by Fisher's LSD post hoc test. Different letters denote statistical significance (P=<0.05) between the groups. Abbreviations: SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, LA; linoleic acid, ARA; arachidonic acid, ALA; alpha-linolenic acid, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid, FAs; fatty acids.

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