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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 x 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

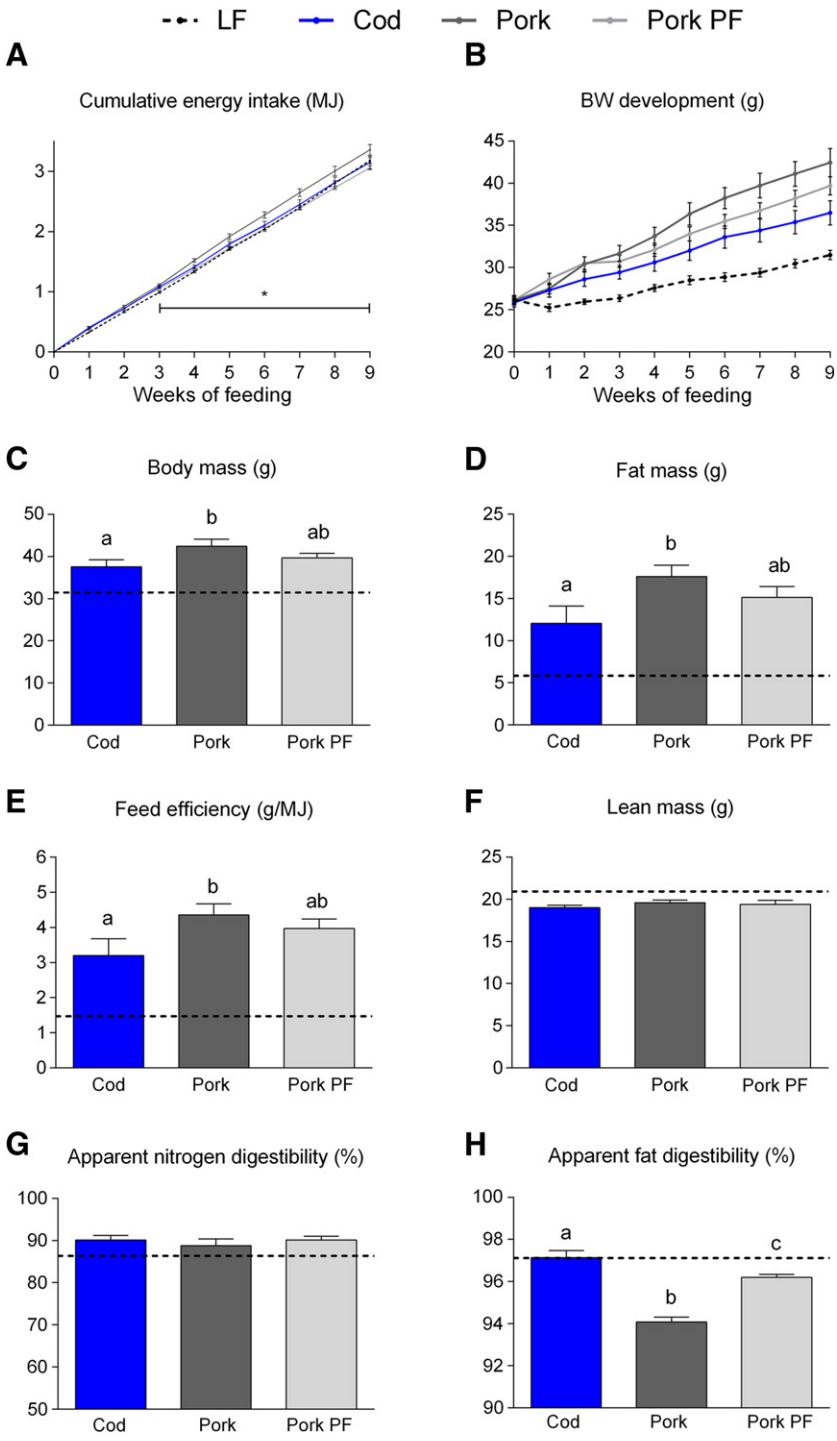

Fig. 1. Effect of Western diets with either cod or pork on body weight, feed efficiency and digestibility. Male C57BL/6 J mice were fed Western diets containing either cod or pork as protein source for 12 weeks. A separate group of mice receiving pork was pair-fed (Pork PF) with the group fed cod. A 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.

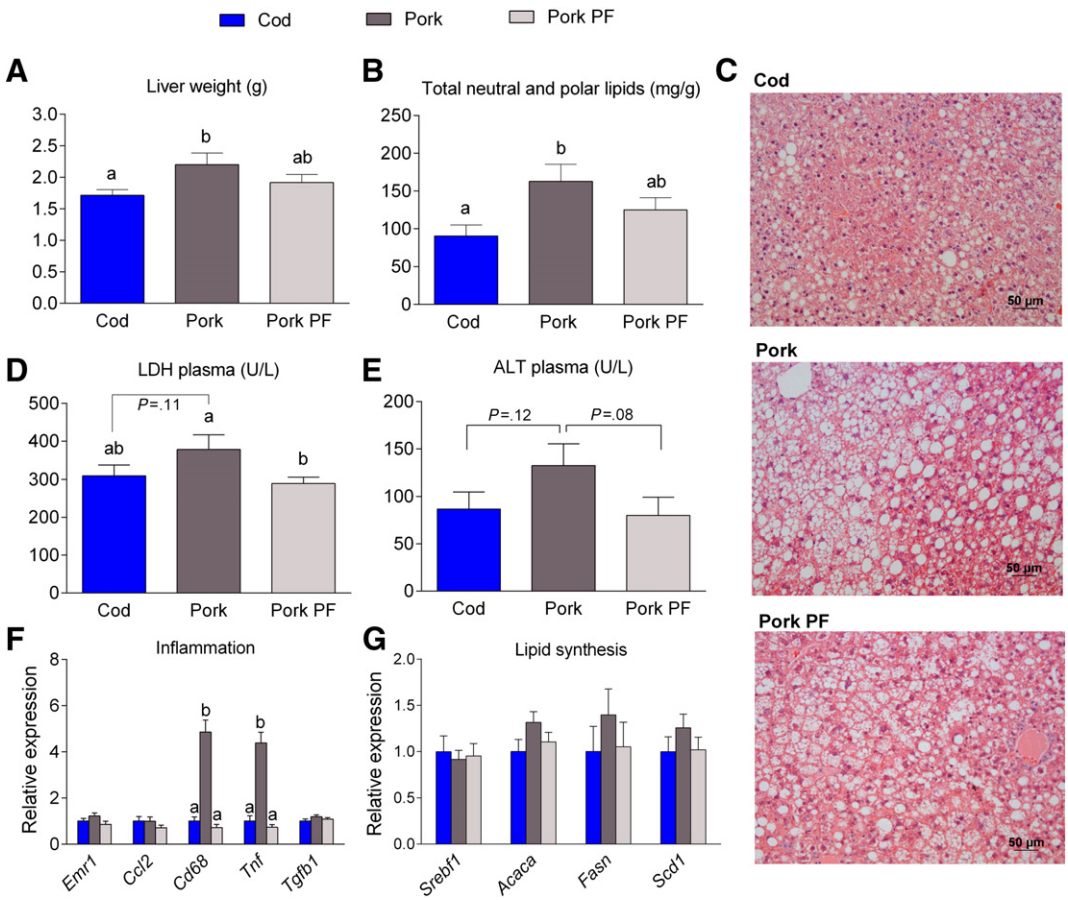

Fig. 2. Effect of Western diets with cod or pork on lipid accumulation and inflammation in liver. Male C57BL/6 J mice were fed Western diets containing either cod or pork. A separate group of mice receiving pork was pair-fed (Pork PF) with the group fed cod. After 12 weeks of feeding, the mice were sacrificed 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,

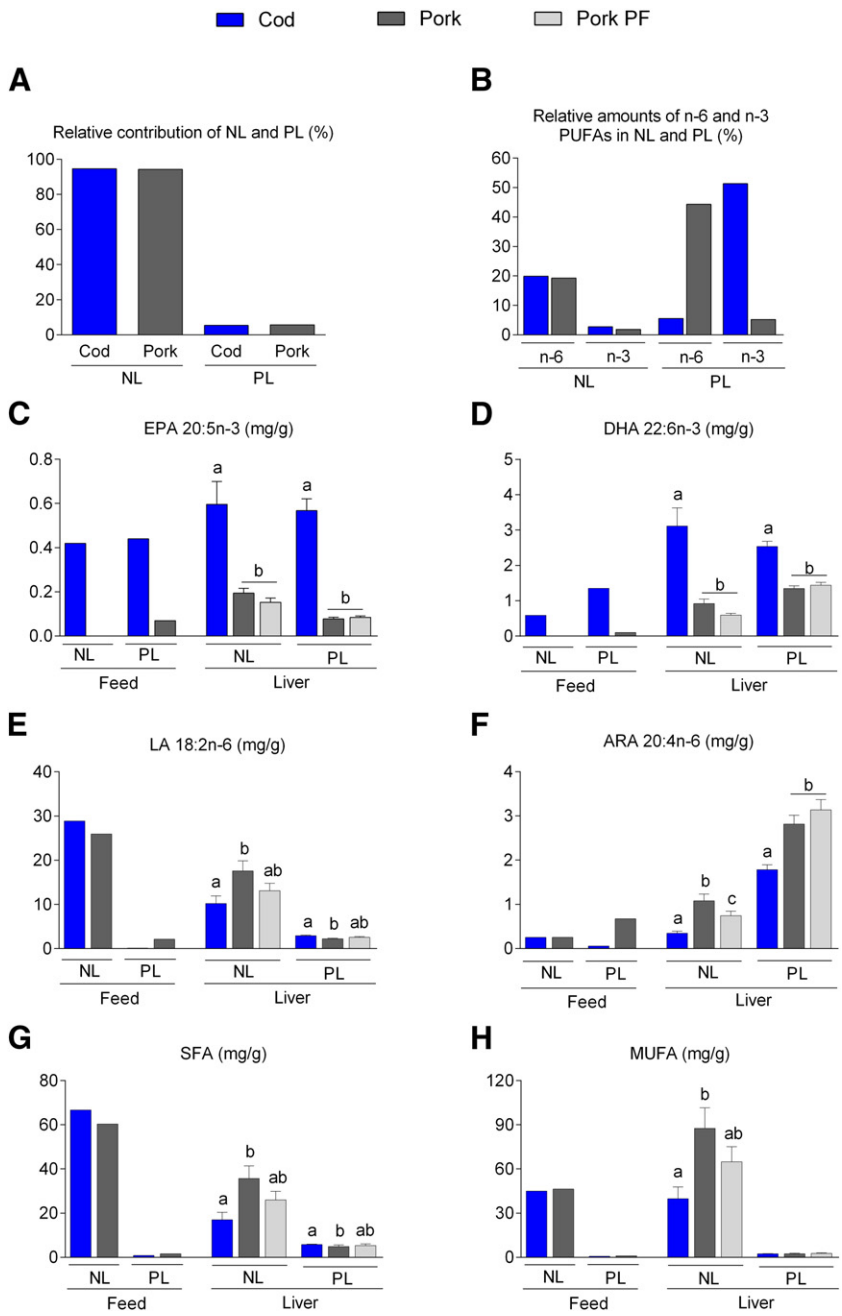

Fig. 3. Effect of cod and pork on fatty acid composition in the Western diets and liver from the mice fed the diets. Filets of cod and pork were freeze-dried, homogenized and used as protein source in Western diets. Lipids from a sample of each diet were extracted and separated into NL and PL fractions 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

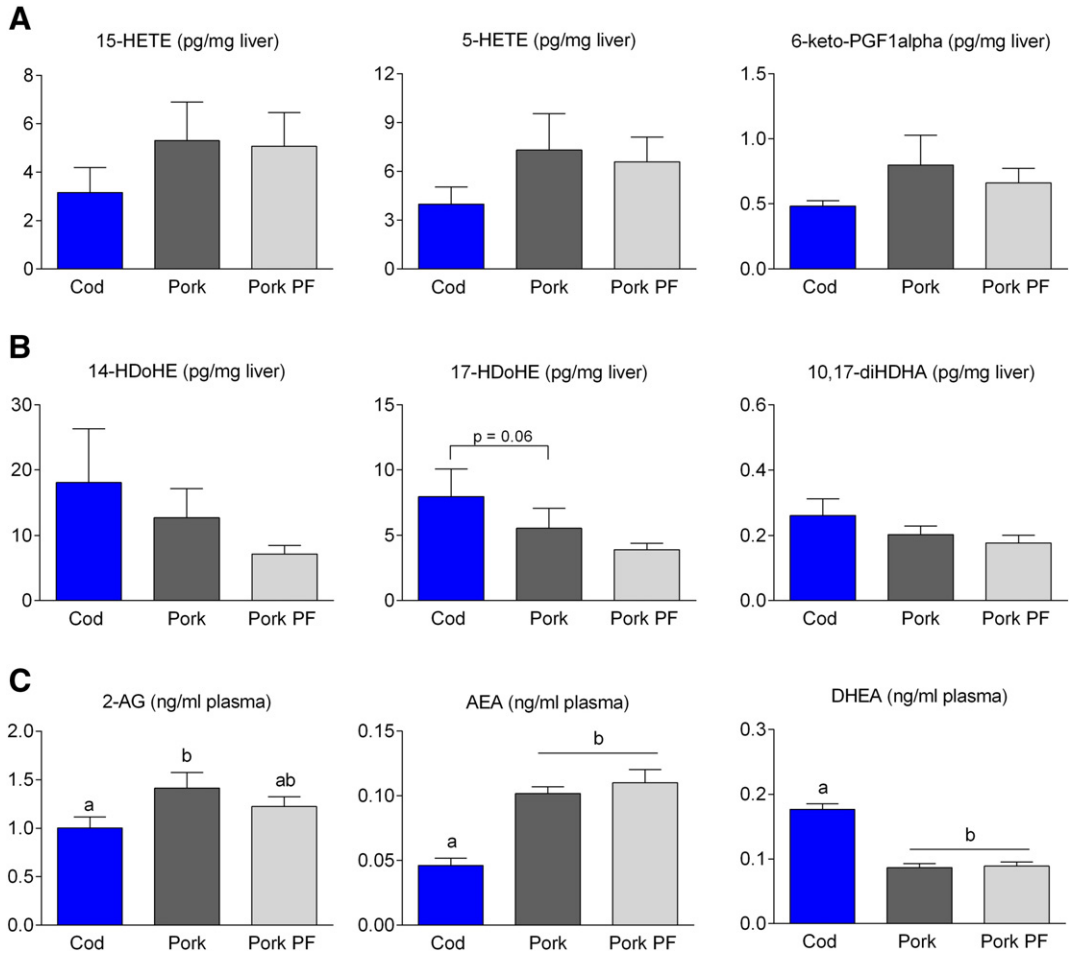

Fig. 4. Effect of Western diets with cod or pork on eicosanoids in liver and endocannabinoids in plasma. Male C57BL/6 J mice were fed Western diets containing either cod or pork for 12 weeks before they were sacrificed. A separate group of mice receiving pork was pair-fed (Pork PF) with the group fed 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.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2016.03.014.

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