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Randomized Control Trials

Changes in lipoprotein particle subclasses, standard lipids, and apolipoproteins after supplementation with n-3 or n-6 PUFAs in abdominal obesity: A randomized double-blind crossover study

Elise Grytten [a, d, 1], Johnny Laupsa-Borge [a, d, *, 1], Pavol Bohov [c], Bodil Bjørndal [c, 2], Elin Strand [c, 3], Jon Skorve [c], Jan Erik Nordrehaug [b, c], Rolf K. Berge [c], Espen Rostrup [b, 4], Gunnar Mellgren [a, d], Simon N. Dankel [a, d], Ottar K. Nygård [b, d]

[a] Hormone Laboratory, Department of Medical Biochemistry and Pharmacology, Haukeland University Hospital, 5021 Bergen, Norway
[b] Department of Heart Disease, Haukeland University Hospital, 5021 Bergen, Norway
[c] Department of Clinical Science, University of Bergen, 5020 Bergen, Norway
[d] Mohn Nutrition Research Laboratory, Department of Clinical Science, University of Bergen, 5020 Bergen, Norway

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SUMMARY

Background & aims: Marine-derived omega-3 (n-3) polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), lower circulating levels of triacylglycerols (TAGs), and the plant-derived omega-6 (n-6) PUFA linoleic acid (LA) may reduce cholesterol levels. Clinical studies on effects of these dietary or supplemental PUFAs on other blood fat fractions are few and have shown conflicting results. This study aimed to determine effects of high-dose supplemental n-3 (EPA + DHA) and n-6 (LA) PUFAs from high-quality oils on circulating lipoprotein subfractions and standard lipids (primary outcomes), as well as apolipoproteins, fatty acids, and glycemic control (secondary outcomes), in females and males with abdominal obesity.

Methods: This was a randomized double-blind crossover study with two 7-wk intervention periods separated by a 9-wk washout phase. Females (n = 16) were supplemented with 3 g/d of EPA + DHA (TAG fish oil) or 15 g/d of LA (safflower oil), while males (n = 23) received a dose of 4 g/d of EPA + DHA or 20 g/d of LA. In fasting blood samples, we investigated lipoprotein particle subclasses by nuclear magnetic resonance spectroscopy, as well as standard lipids, apolipoproteins, fatty acid profiles, and glucose and insulin. Data were analyzed by linear mixed-effects modeling with 'subjects' as the random factor.

Results: The difference between interventions in relative change scores was among the lipoprotein subfractions significant for total very-low-density lipoproteins (VLDLs) (n-3 vs. n-6: −38%* vs. +16%, $p < 0.001$; *: significant within-treatment change score), large VLDLs (−58%* vs. −0.91%, $p < 0.001$), small VLDLs (−57%* vs. +41%*, $p < 0.001$), total low-density lipoproteins (LDLs) (+5.8%* vs. −4.3%*, $p = 0.002$), large LDLs (+23%* vs. −2.1%, $p = 0.004$), total high-density lipoproteins (HDLs) (−6.0%* vs. +3.7%, $p < 0.001$), large HDLs (+11%* vs. −5.3%, $p = 0.001$), medium HDLs (−24%* vs. +6.2%, $p = 0.030$), and small HDLs (−9.9%* vs. +9.6%*, $p = 0.002$), and among standard lipids for TAGs (−16%* vs. −2.6%, $p = 0.014$), non-esterified fatty acids (−19%* vs. +5.5%, $p = 0.033$), and total cholesterol (−0.28% vs. −4.4%*, $p = 0.042$). A differential response in relative change scores was also found for apolipoprotein (apo)B (+0.40% vs. −6.0%*, $p = 0.008$), apoA-II (−6.0%* vs. +1.5%, $p = 0.001$), apoC-II (−11%* vs. −1.7%, $p = 0.025$), and apoE (+3.3% vs. −3.8%, $p = 0.028$).

Conclusions: High-dose supplementation of high-quality oils with n-3 (EPA + DHA) or n-6 (LA) PUFAs was followed by reductions in primarily TAG- or cholesterol-related markers, respectively. The responses after both interventions point to changes in the lipoprotein−lipid−apolipoprotein profile that have been

* Corresponding author. Centre for Nutrition, Haukelandsbakken 15, 5021 Bergen, Norway.
E-mail addresses: elise.grytten@uib.no (E. Grytten), johnny.laupsa-borge@uib.no (J. Laupsa-Borge), pavol.bohov@uib.no (P. Bohov), Bodil.Bjorndal@hvl.no (B. Bjørndal), elin.strand@helse-bergen.no (E. Strand), Jon.Skorve@uib.no (J. Skorve), jan.erik.nordrehaug@sus.no (J.E. Nordrehaug), Rolf.Berge@uib.no (R.K. Berge), espen.rostrup@me.com (E. Rostrup), gunnar.mellgren@uib.no (G. Mellgren), simon.dankel@uib.no (S.N. Dankel), ottar.kjell.nygard@helse-bergen.no (O.K. Nygård).
[1] Co-first authors and equally contributed to this paper.
[2] Present address: Department of Sports, Physical Activity and Food, Western Norway University of Applied Sciences, 5020 Bergen, Norway.
[3] Present address: Department of Immunology and Transfusion Medicine, Haukeland University Hospital, 5021 Bergen, Norway.
[4] Present address: Luzmon Medical AS, Oslo Science Park, Gaustadalléen 21, 0349 Oslo, Norway.

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associated with reduced cardiometabolic risk, also among people with TAG or LDL-C levels within the normal range.
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Abbreviations

AA	arachidonic acid
ALA	α-linolenic acid
ALAT	alanine aminotransferase
ALP	alkaline phosphatase
apo	apolipoprotein
ASAT	aspartate aminotransferase
B1	the first baseline visit/measurements before any intervention
CK	creatine kinase
CETP	cholesteryl ester transfer protein
CVD	cardiovascular disease
DGLA	dihomo-γ-linolenic acid
DHA	docosahexaenoic acid
DMBP	Department of Medical Biochemistry and Pharmacology at Haukeland University Hospital
DPA	docosapentaenoic acid
EPA	eicosapentaenoic acid
FAs	fatty acids
FC	free cholesterol
FFAs	free fatty acids
GLA	γ-linolenic acid
GT	γ-glutamyl transpeptidase
E%	energy percentage of total energy intake
HbA1c	glycated hemoglobin
HDLs	high-density lipoprotein particles
HDL-C	HDL cholesterol
HOMA2-IR	homeostasis model assessment of insulin resistance index 2
HOMA2-%B	homeostasis model assessment of beta-cell function index 2
HOMA2-%S	homeostasis model assessment of insulin sensitivity index 2
ICC	intraclass correlation coefficient
IDLs	intermediate-density lipoprotein particles
INCP	insulin C-peptide
LA	linoleic acid
LD	lactate dehydrogenase
LDLs	low-density lipoprotein particles
LDL-C	LDL cholesterol
Lp(a)	lipoprotein (a)
Lp-B	apoB-containing lipoproteins
LpL	lipoprotein lipase
NEFAs	non-esterified fatty acids
non-HDL-C	non-HDL cholesterol
n-3	omega-3
n-6	omega-6
PLs	phospholipids
PUFAs	polyunsaturated fatty acids
RBCMs	red blood cell membranes
RCT	randomized controlled trial
s%	sympercent
TAGs	triacylglycerols
TC	total cholesterol
TRFAs	trans fatty acids
TRLs	TAG-rich lipoproteins
TRL-C	TRL cholesterol
Tx	treatment
VLDLs	very-low-density lipoprotein particles
WC	waist circumference
wt%	weight percentage
95% BCa CI	95% bootstrapped (bias-corrected and accelerated) confidence interval

1. Introduction

Circulating levels of LDL cholesterol (LDL-C) are associated with risk of cardiovascular disease (CVD) [1,2], a leading cause of death worldwide [3]. However, the majority of clinical events are not prevented by substantial reductions in LDL-C levels alone [4,5]. Abnormalities in other components of the lipoprotein-lipid profile, which are often associated with obesity, particularly abdominal obesity [6], may partially account for the residual CVD risk [7,8]. Among these parameters are elevated blood levels of triacylglycerols (TAGs) and TAG-rich lipoproteins (TRLs) or their remnants [9]. Increasing CVD risk has been observed with increasing TAG levels within the widely accepted 'normal' range [10,11].

The concentrations of different lipoprotein subgroup particles, which vary in density, size, and composition of lipids and proteins, including apolipoproteins, have in recent years emerged as independent predictors of CVD comparable to or stronger than standard lipids [12–15]. Increased risk of CVD has been associated with elevated levels of large and medium VLDLs [9,16], total LDLs [12,16,17], and small, dense LDLs (sdLDLs) [16,18,19], as well as lower concentrations of HDLs [16,20,21]. In particular, the level of apolipoprotein (apo)B, a measure of the total number of atherogenic lipoprotein particles [22], is independently associated with CVD risk [23–25]. Moreover, apoB-containing lipoproteins (Lp-B) enriched with apoC-III, a potent modulator of TAG metabolism and cardiometabolic disease [26], are among the lipoprotein subfractions that have been most strongly related to CVD risk [27,28].

The composition of dietary fats modulates the lipoprotein, lipid, and apolipoprotein profiles [29–31]. Lower CVD risk has been associated with higher intakes or blood levels of marine-derived omega-3 (n-3) polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [32–35], and with higher intakes or circulating levels of plant-derived omega-6 (n-6) PUFAs, mainly linoleic acid (LA) [36–38]. This inverse relationship has been partially attributed to changes in blood

fat fractions [37,39], primarily reduced TAG levels after n-3 intake [29,40–43], and lower cholesterol levels after n-6 intake [30,31,44]. However, effects on CVD morbidity, mortality, and intermediate outcomes, including lipids and lipoproteins, have not been consistent across studies for n-3 [33,35,45–47], nor for n-6 [36,38,48–51], and it remains controversial whether dietary or supplemental PUFAs reduce cardiometabolic risk [44,47,52].

It is well established that increasing intakes or biomarker levels of marine n-3 PUFAs lower circulating concentrations of TAGs in a dose-dependent manner [29,40–42,53–59]. The effects have been less consistent for changes in other standard lipids and in lipoprotein profiles [54,59]. Notably, few clinical studies have investigated the effects of n-6 PUFAs alone on blood fat fractions [44,50], and existing evidence shows conflicting results [44,50,60–62]. How increased n-6 intakes affect lipoprotein and apolipoprotein profiles is particularly poorly documented. Among nine RCTs estimating effects of marine n-3 PUFAs on all the major lipoprotein subfractions [63–71], four of them specified a vegetable oil typically rich in LA as the comparator [64,66–68]. Only one of these published the fatty acid composition of the n-3 and n-6 oils [64], making it difficult to interpret results across studies. None reported any quality parameters, such as the content of oxidation products, which may interfere with treatment effects [72]. In the present study, we investigated effects of high-dose supplemental n-3 (3–4 g/d of EPA + DHA) and n-6 PUFAs (15–20 g/d of LA) from well-characterized (detailed fatty acid profile), high-quality (low oxidation) oils on fasting blood levels of lipoprotein subclass particles, standard lipids, and apolipoproteins, as well as on other CVD risk factors, in sedentary females and males with abdominal obesity. This is the first crossover trial comparing the effects of n-3 and n-6 PUFAs on all the major lipoprotein subfractions and related lipids and apolipoproteins in this population of high CVD and diabetes risk.

2. Materials and methods

2.1. Participants

People with abdominal obesity (waist circumference (WC) of ≥80 cm in females and ≥94 cm in males) and 30–70 years of age, sedentary (<2 h/wk of exercise at moderate to high intensity) but otherwise healthy, were recruited among respondents to newspaper advertisements and screened for eligibility from April to June 2015. The sex-specific cut-off points of WC were chosen according to the recommended thresholds for abdominal obesity reflecting high risk for CVD and diabetes, which are set at 80 cm and 94 cm in Caucasian females and males, respectively [73–75]. Excluded were individuals with diagnosed diabetes, severe psychiatric illness, or malabsorption disorders, together with those on regular medication that could influence study outcomes, including lipid-lowering and anti-hypertensive drugs, anticoagulants, antidepressants, and thyroid hormones. Regular use of other medications was also not permitted, but antibiotics, NSAIDs, antihistamines, diuretics, and hormone replacement therapy were accepted during the study if prescribed by a physician. Dietary supplements, including supplemental PUFAs other than the study products, were not allowed and had to be discontinued at least three months before the first baseline visit, except prescribed iron, calcium, and vitamin D in case of medical reasons. (Changes in prescribed medication and supplementation during the study are reported in the online Supporting Information, *Supplemental Text,* Results.) Other exclusion criteria were fasting serum levels of TAGs >5 mmol/L, cigarette smoking, alcohol or drug abuse, previous coronary interventions, previous bariatric surgery, pregnancy or lactation, blood donation within three months before baseline, scheduled hospitalization during the study, and pacemaker or implantable cardioverter defibrillator (ICD).

The study was conducted according to the guidelines in the Declaration of Helsinki, and the study protocol was reviewed and approved by the Regional Committee for Medical and Health Research Ethics (2014/2336/REK South-East). Study design, sample collection, and potential risks and benefits were carefully explained to each participant before they provided written informed consent.

2.2. Study design

This was a randomized double-blind two-period crossover study conducted at Haukeland University Hospital in Bergen, Norway, during a period of total 30 wk from May 2015 to March 2016. After a run-in period for 15 wk (from May/June 2015) without any dietary supplements, eligible participants were randomly assigned to one of two treatment sequences: supplementation with n-3 fatty acids (fish oil) in period one, followed by supplementation with n-6 fatty acids (safflower oil) in period two (sequence AB), or n-6 supplementation in period one, followed by n-3 supplementation in period two (sequence BA). Both intervention periods lasted for 7 wk and were separated by a 9-wk washout phase. The duration of the intervention periods was similar to that used in previous crossover trials (duration of 6 wk) measuring all major lipoprotein subfractions after PUFA interventions [66,70]. However, to reduce the risk of potential carryover effects, we included a longer washout phase than in these studies (duration of 2 wk or no washout) and the majority of other previous PUFA intervention trials with a crossover design (2–8 wk of washout) [76]. Clinical measurements and tissue sampling were conducted one day before treatment period one started (baseline visit 1, B1; September 2015), the day after 7 wk of the first intervention (follow-up visit 1, I1; November 2015), before treatment period two started (baseline visit 2, B2; January 2016), and after the second intervention (follow-up visit 2, I2; March 2016). Further details about the study design are presented in Fig. 1.

A 2 × 2 crossover design was chosen for this study because participants then act as their own matched control and thus allows treatment comparisons at the individual level, consequently reducing the influence of confounding covariates, yielding more efficient treatment comparisons, and requiring a smaller sample size. We did not expect a large dropout rate, and we believed that the 9-wk washout phase was sufficient to avoid that the effects of the first intervention persisted into the second period (carryover effect), which are some of the known challenges of a crossover design.

2.3. Randomization

Participants were at baseline allocated to the two different sequences of interventions by stratified randomization using sex as a stratum. An online software (Random Sequence Generator, www.random.org) was used to generate the true random number allocation sequence for each sex separately to ensure equal numbers of females across periods in each sequence, and the same for males. The randomization procedure was conducted by two external researchers who were otherwise not connected to the study. The randomization code was concealed from the study investigators until the statistical analyses were completed for all primary and most secondary outcomes.

2.4. Interventions

Both supplements were produced and sponsored by Pharmatech AS (Rolvsøy, Norway). The PUFAs were delivered in a liquid

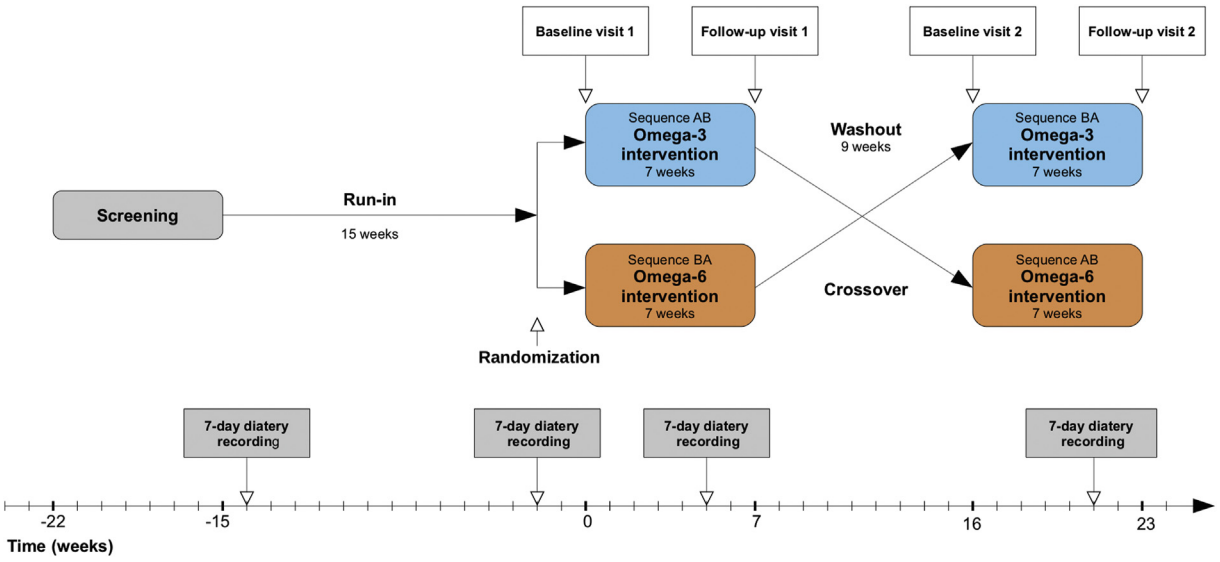

Fig. 1. Overview of the study. This was a randomized double-blind crossover study with two intervention periods of 7 wk separated by a 9-wk washout phase. Subjects were randomly assigned to intervention sequence AB or BA (A: n-3 intervention; B: n-6 intervention).

suspension consisting mainly of silicon dioxide and small amounts of mono- and diglycerides of fatty acids (magnesium stearates), and without water to reduce the rate of oxidation. The proportion of oil in this suspension was about 90%. No vitamins were added to the products.

The n-3 supplement was a hydrolyzed and re-esterified fish oil (TAG form) containing mainly EPA (C20:5n-3) and DHA (C22:6n-3), while the n-6 supplement was an organic, cold-pressed safflower oil containing mainly LA (C18:2n-6). Based on analyzed PUFA levels in these supplementation products, females should consume 5.6 mL/5.0 g (1.8 g EPA and 1.2 g DHA) and males 7.5 mL/6.7 g (2.4 g EPA and 1.6 g DHA) fish oil each day during the n-3 period to obtain a total dose with EPA + DHA of 3 and 4 g/d, respectively. In the n-6 period, the daily dose of safflower oil was for females 24.5 mL/22.6 g (15 g LA) and for males 32.7 mL/30.1 g (20 g LA). Different amounts of oils among females and males (a ratio of 3:4) allowed for similar intakes of EPA + DHA or LA per kg body weight or fat-free mass.

A supplemental dose of 3–4 g EPA + DHA was chosen based on previous studies showing that the effect on circulating levels of TAGs (the primary outcome used in the sample size analysis) is dose dependent [57,77] with a minimal effective dose of at least 2–4 g/d to significantly reduce (fasting) TAG levels among normo- to moderately hypertriacylglycerolemic individuals [41,43,78]. This daily dose was in accordance with scientific opinions from, e.g., the European Food Safety Authority [79] and the American Heart Association [10]. A fish oil in TAG form was used because of higher bioavailability than the ethyl ester form and less susceptibility to peroxidation than the free fatty acid (FFA) form [80].

The rationale for choosing a daily dose of 15–20 g LA was the intention to increase the total consumption of n-6 PUFAs (mainly LA) from a typically low dietary intake in Norway (~3.5 E%) [81] to a level in the upper range of the recommended intake of 5–10 E% [82,83], as well as observational data showing that the levels of dietary LA intake or circulating/tissue LA biomarkers are inversely associated with CVD risk in a dose–response manner [36,38]. Notably, we chose a supplemental approach to enable a double-blind design and because the therapeutic doses would be impossible to achieve by a dietary approach without significantly changing other dietary determinants not of interest in this study.

The total amount of supplement was divided into two daily dosages, one in the morning and one in the afternoon with meals, and the participants were instructed to take the supplement (using a syringe with dose marked) in each period from the morning after the baseline visit to the evening before the follow-up visit. A liquid form was selected instead of capsules as the amount of n-6 PUFAs implied many capsules daily, which could be challenging to swallow for many participants and thus influence their compliance with the intervention. Notably, the energy content in 5.0 and 6.7 g of fish oil is about 45 and 60 kcal, respectively, while 22.6 and 30.1 g of safflower oil provide 203 and 271 kcal extra. If the supplements, especially the n-6 oil, did not lead to a reduced intake of other ingredients in the diet, as we expected to happen, this would cause a theoretical difference in energy intake between the two intervention periods of 158 kcal and 211 kcal for females and males, respectively.

2.5. Composition and quality of supplements

The fatty acid (FA) compositions of the oils were analyzed by the Lipid Research Group at the University of Bergen (Supplemental Table 1), and these values were used in the calculations of PUFA intakes and in all reported analyses. To analyze the FA profiles in the supplements, 40–60 μg of oil dissolved in chloroform-methanol (1:1) was dosed into a glass vial with a Teflon-lined seal cap. Organic solvents were evaporated to dryness under a stream of nitrogen, and FA methyl esters were prepared and analyzed by gas–liquid chromatography as previously described [84]. We measured the relative content of total n-3 PUFAs in the fish oil to be 71.1 percentage by weight of the total fatty acid (TFA) content (g FA/100 g TFA; wt%), of which 62.1 wt% was EPA and DHA (38.6 wt% and 23.5 wt%, respectively; DHA/EPA ratio: 0.6). The content of LA was 1.1 wt%. The safflower oil contained 66.8 wt% of total n-6 PUFAs, which was almost exclusively LA (66.7 wt%), and 0.3 wt% EPA + DHA. The relative content of measured trans fatty acids (TRFAs) was 0.07 wt% and 0.05 wt% in the fish and safflower oils, respectively.

Analyses of primary and secondary oxidation products and FFAs, as well as acid values, were performed by Multilab Østfold AS (Rolvsøy, Norway) just before the first intervention period started

in September 2015 (Supplemental Table 2). These analyses indicated high-quality products at delivery. Participants were instructed to store the bottles with oil supplements at 4 °C in dark (i.e., the refrigerator) during the entire intervention periods to reduce the rate of lipid oxidation.

2.6. Blinding

The trial was blinded for the participants and the study investigators by equal appearance of the study products and the bottles containing these oils. Their contents were only identifiable by a randomly chosen ID on the bottles. To enable a double-blind design with the selected oils in a liquid form, the supplements were supplied in similar, dark bottles of polyethylene terephthalate. The taste was modified by adding 1.8% of a natural citrus aroma and 0.045% of steviol glycosides (E 960). Equal appearance was accomplished by using 0.85% calcium carbonate (E 170) and 0.2% turmeric (E100).

The participants received no information about which oil was given in which dosage or whether different dosages were tested for both supplements. The two external researchers who conducted the randomization, also packed the bottles with supplements, along with the necessary equipment and detailed instructions, in sealed bags marked with a three-digit code used only for this purpose to identify the participants. The bags were handed out to the participants after the measurements at B1 and B2. After study completion, we evaluated the blinding by asking the participants to guess which oil they had received in each period, and how confident they were about their assumptions on a four-response Likert item (a statement that the respondent is asked to evaluate in a survey): 0% (no clue), 25% (unsure), 75% (fairly sure), and 100% (totally sure).

2.7. Compliance and adverse effects

In a questionnaire, the participants were asked after two and 7 wk of intervention to rate their adherence to the correct dose of oil on a Likert item with five responses: 0% (never), 25% (occasionally), 50% (often), 75% (usually), and 100% (always). They also assessed their adherence to the timing of doses using the same Likert item. Good compliance was defined as taking at least 80% of the allocated supplements according to the instructions on both the amount of oil and timing of doses. These criteria were evaluated separately.

For each participant, we calculated the amount of oil (in ml) used during the 7-wk intervention period, i.e., the total amount of oil delivered at baseline minus leftover at follow-up measured by the participants (using a syringe with marked ml). To obtain a more objective measure of compliance than self-reported data on oil intakes [85], we measured the fasting levels of the supplemented PUFAs in red blood cell membranes (RBCMs) before and after the interventions.

In wk two and seven of each period, the participants completed a questionnaire including 20 potential adverse effects, such as bloating, constipation, diarrhea, dizziness, headache, heartburn, joint/muscle pain, nausea, palpitation, skin rash/itching, and stomach ache. The severity of each of these symptoms was assessed on a five-response Likert item: none, mild, moderate, severe, and very severe.

2.8. Dietary intake and physical activity

The participants were instructed to maintain their usual lifestyle, dietary habits, and physical activity level during the study. The habitual intake of foods and beverages was obtained by dietary recordings two times in the run-in period (baseline) and one time during both intervention periods (in wk five). Each recording was carried out for a period of seven consecutive days, from Monday to Sunday. The collection of dietary data was done by a self-developed application tailored to the project that allowed the electronic recording of all diet data via the iPhone, iPad, and iPod. The application was designed by the database program FileMaker Pro 12 Advanced (FileMaker, Inc., Santa Clara, CA, USA) and implemented in the app FileMaker Go 13. Beforehand, the participants were given detailed instructions on how to use the application and carry out the dietary recording, and they received practical assistance as needed.

The participants were questioned about their physical activity level at each baseline and follow-up visit using eight Likert responses for frequency (days per wk; score 0 to 7); five Likert responses for duration (<20 min, 20–30 min, 30–60 min, 1–2 h, or >2 h; score 1 to 5); and three Likert responses for intensity (low, medium, or high; score 1, 2 or 4, respectively). The physical activity level at work was a binominal Likert item (active or not; score 0 or 3). A total score for physical activity level was calculated from these responses, and the data were handled as Likert scale data and treated as interval data because of many possible numerical outcomes (range: 0–143). Notably, the tools to measure dietary intake and physical activity level were not validated before the study.

2.9. Study visits

Prior to each study visit, participants were instructed to fast for at least 10–12 h and avoid alcohol for at least 48 h. Besides, they were asked to not exercise the day before, as this may also affect circulating levels of lipids and lipoproteins [86]. In the morning between 07:30 and 11:30 a.m., venous blood samples were collected, and anthropometric variables were measured. The participants were also questioned about fasting status, alcohol intake, physical activity, stress level, use of medications and supplements, any health conditions, and compliance with the intervention since the last visit.

2.10. Anthropometrics

Body weight, height, and waist and hip circumferences were measured by standardized procedures, and body composition, i.e., body fat mass, body fat percent, visceral fat area, and fat-free mass, was further analyzed by using a bioelectrical impedance measurement system (InBody S10; InBody Co, Ltd., Seoul, South Korea) in a lying posture. The means of two measurements were calculated.

2.11. Biochemical variables

2.11.1. Fatty acid profiles in RBCMs

All blood samples were aliquoted and stored at −80 °C until the end of the study, when samples from all visits were analyzed at the same time. Fasting levels of 47 fatty acids (FAs) in RBCMs were measured by gas–liquid chromatography using the internal standard C21:0 as previously described [84]. The RBCMs were prepared from whole blood samples collected in EDTA-vacutainer tubes and frozen immediately at −80 °C. 1 mL of defrosted blood was centrifuged at 20,000 g in 30 min at 4 °C. Around 300 μl of the upper layer containing plasma and cytoplasm of lysed red blood cells were then removed. To wash the RBCMs and enhance lysing of remaining red blood cells, the lower layer was suspended in deionized water, shaken with a metal bead in a TissueLyser (Qiagen) at 25 Hz for 2 min, and incubated at ice for 10 min before centrifugation at 20,000*g* in 30 min at 4 °C. Washing and

centrifugation of the sediment were repeated two times, but without adding the metal bead. After the final removal of the supernatant, the pellet containing RBCMs was reconstituted in deionized water and immediately stored at −80 °C until analysis. The FA profile of RBCMs prepared by this method was comparable to the FA profile of RBCMs isolated from fresh blood samples using a procedure previously reported [87]. In the FA analyses, we used 20 μl of an aliquot of RBCMs, corresponding to 160 μl of blood, for the preparation of FA methyl esters of total lipids (μg FAs/ml blood). The total levels of analyzed n-3 and n-6 PUFAs are reported as the n-6/n-3 ratio, while the n-3 index was calculated as the sum of EPA and DHA in RBCMs and expressed as wt%. We also report the levels of other RBCM FAs measured as wt% to eliminate differences in RBCM amounts, which may vary between individual blood sampling, even in the same person. However, since there is no agreement on the choice of FA measure (absolute vs. relative) [85,88,89], data on the concentrations (μg/mL) of RBCM FAs are reported in the *Supporting Information*.

2.11.2. Lipoprotein particle subclasses

EDTA-plasma samples were shipped frozen on dry ice in June 2016 to LipoScience (now LabCorp, Inc., Raleigh, NC, US) for analyses, which were conducted within a month. Concentrations and sizes of lipoprotein particle subclasses, and a lipoprotein-based insulin resistance index (LP-IR), were determined by an automated nuclear magnetic resonance (NMR) spectroscopy assay according to the LipoProfile-3 algorithm [90,91]. Briefly, lipoprotein subclass particle concentrations (nmol/L for VLDLs, IDLs, and LDLs; μmol/L for HDLs) were calculated from the measured amplitudes of their spectroscopically distinct lipid methyl group NMR signals, and the mean VLDL, LDL, and HDL particle sizes (nm diameter) were obtained from a weighted average of each subclass diameter multiplied by its relative mass percentage derived from the NMR signal intensity. The following nine subfractions were measured by NMR (estimated ranges of particle diameter): large VLDLs (including chylomicrons if present; >60 nm), medium-sized (medium) VLDLs (42–60 nm), small VLDLs (29–42 nm), IDLs (23–29 nm), large LDLs (20.5–23.0 nm), small LDLs (18–20.5 nm), large HDLs (9.4–14 nm), medium HDLs (8.2–9.4 nm), and small HDLs (7.3–8.2 nm). Total VLDL and HDL particle concentrations were calculated as the sum of small, medium, and large subclass particle concentrations, and total LDL particle concentration is the sum of small LDL, large LDL, and IDL concentrations.

2.11.3. Lipids

We measured fasting serum TAGs, total cholesterol (TC), LDL-C, and HDL cholesterol (HDL-C) at the Department of Medical Biochemistry and Pharmacology (DMBP), Haukeland University Hospital, according to standardized procedures. Serum phospholipids (PLs), free cholesterol (FC), and non-esterified fatty acids (NEFAs) were analyzed on a Hitachi 917 Chemistry Analyzer (Boehringer Mannheim GmbH, Mannheim, Germany) using kits from DiaSys Diagnostic Systems GmbH (Holzheim, Germany). Non-HDL cholesterol (non-HDL-C) was calculated by subtracting HDL-C from TC, while TAG-rich lipoprotein cholesterol (TRL-C) was derived as non-HDL-C minus LDL-C.

2.11.4. Lipoprotein (a) and apolipoproteins

Serum concentrations of lipoprotein (a) [Lp(a)] and the apolipoproteins apoB and apoA-I were analyzed at DMBP. Serum levels of apoA-II, apoC-II, apoC-III, and apoE were measured using the MILLIPLEX MAP Human Apolipoprotein Magnetic Bead Panel – Cardiovascular Disease Multiplex Assay (APOMAG-62K; Merck Millipore, Billerica, MA, US) and detected by the Bio-Plex 200 System (Bio-Rad, Hercules, CA, US).

2.11.5. Indices of glycemic control and insulin sensitivity

Fasting serum glucose, insulin, and insulin C-peptide (INCP), as well as glycated hemoglobin (HbA1c) in whole blood, were analyzed at DMBP. The homeostasis model assessment (HOMA) is reported as an index of insulin sensitivity, and we used the HOMA2 calculator developed by the University of Oxford (http://www.dtu.ox.ac.uk/homacalculator/index.php) to estimate insulin resistance (homeostasis model assessment of insulin resistance index 2, HOMA2-IR), insulin sensitivity (HOMA2-%S), and beta-cell function (HOMA2-%B) based on the updated computerized model [92]. Fasting serum levels of glucose and INCP were used as input in these calculations. Another measure of insulin sensitivity, LP-IR, ranging from 0 (least) to 100 (most) insulin resistant, was calculated by LipoScience as a weighted combination of lipoprotein subclass and size parameters (large VLDLs, small LDLs, large HDLs, and VLDL, LDL, and HDL sizes) most closely associated with HOMA-IR [93].

2.11.6. Other biomarkers

Alanine aminotransferase (ALAT), albumin, alkaline phosphatase (ALP), aspartate aminotransferase (ASAT), bile, bilirubin, creatine kinase (CK), γ-glutamyl transpeptidase (GT), lactate dehydrogenase (LD), estrogen (17β-estradiol), and testosterone were analyzed at DMBP. Serum 25-OH vitamin D_3 was analyzed by liquid chromatography-tandem mass spectrometry at BEVITAL, Bergen (www.bevital.no).

2.12. Statistical analyses

The primary outcomes reported in this study are between-treatment differences for relative change scores (primary analysis) in fasting blood levels of NMR-measured lipoprotein subclass concentrations and sizes, blood lipids (TAGs, NEFAs, TC, LDL-C, and HDL-C), cholesterol levels calculated from these measures (non-HDL-C and TRL-C), and ratios between these variables. Related to the primary outcomes we also report changes in fasting serum levels of Lp(a), PLs, and FC. The results presented here are based on an intention-to-treat (ITT) analysis including all randomized participants (n = 39).

The sample size was initially derived from a calculation using a minimal expected between-treatment difference in one of the primary outcomes (TAGs) based on results from previous PUFA intervention trials [29,55,58,66,70]. With a power of 90%, a two-tailed nominal alpha level of 0.05, and an SD of 0.5, at least 33 subjects should be included to detect a minimal between-treatment difference in TAG levels of 0.4 mmol/L, corresponding to a relative difference of 15–25% in normo-to mildly hypertriacylglycerolemic individuals. We intended to include 40 participants to take into account a potential dropout rate of 15%. However, this sample size calculation was based on the difference between two independent means and the between-subject SD. Hence, it did not account for the crossover design by using an expected within-subject SD from paired measurements. A retrospective sample size analysis considering the AB/BA design and paired nature of the data estimated that at least 20 subjects should be included to detect a minimal between-treatment difference in relative TAG levels of 15% with a power of 90%, a two-tailed nominal alpha level of 0.05, and a within-subject SD of 21% [94].

Data are presented as raw unadjusted means (SDs), geometric means (1 SD ranges), or mean score differences (±absolute/relative effect estimates [95% CIs]) as specified elsewhere. The geometric SD ranges used in descriptive statistics were calculated by dividing and multiplying the geometric means with the geometric SD factors to obtain the lower and upper limits, respectively [95]. The distribution of data points from different measurements are shown by

violin and error bar plots in Supplemental Figs. 1–4. All inferential tests were two-tailed with a nominal alpha level of 0.05.

The statistical analyses were conducted with R v3.6.1 (https://www.r-project.org), and data transformation and exploration were done by using the *tidyverse* packages (https://tidyverse.tidyverse.org). To assess if the blinding of participants was successful, we used the *chisq.test* function in the *stats* package v3.6.1 to conduct a Pearson's chi-squared test. To explore relationships between different variables, we conducted bivariate and partial Pearson's correlation analyses by using the *pcor.test* function in the *RVAideMemoire* package v0.9–73, and in these analyses, we obtained 95% CIs from a bootstrapping procedure (bias-corrected and accelerated, BCa) using 2000 replicates. Linear regression modeling was done with the *lm* function in the *stats* package. All plots were made by the *ggplot2* package v3.3.0 and several of its extensions, e.g., the *ggupset* package v0.3.0. The *TwoSampleCrossOver.NIS* function in the *TrialSize* package v1.4 was used to conduct the retrospective sample size analysis.

2.12.1. Primary analysis

Study outcomes were analyzed by linear mixed-effects models (LMEMs) with 'subjects' as the random factor. The mixed modeling was performed with the *lme* function in the *nlme* package v3.1–140. In the primary analysis, we used a design-driven approach with pre-defined fixed effects ('treatment', 'time', 'sex', 'period', 'treatment × time', 'treatment × sex'), random effects (random intercepts and slopes for 'time'), and correlation structure (general unstructured) in the primary period-adjusted mixed model. Sex was used as a stratum in the randomization of the participants and should, therefore, be included in the model to give valid inference [96]. In case of heterogeneity, the model included a variance structure allowing for different variances per stratum of 'treatment' and/or 'time'. The correlation structure was simplified to compound symmetry if the model did not converge.

Results within and between periods were analyzed by extending the fixed effects structure with the three-way interaction 'treatment × period × time' and its daughter terms, and by including random slopes for 'period' in the random part of the model (the maximal mixed model). The categorical main terms in the models ('treatment', 'period', 'time', and 'sex') were defined by orthogonal sum coding in planned comparisons showing absolute or relative within- and between-treatment differences from baseline to follow-up (change scores). In the between-treatment comparisons, the n-6 intervention was defined as the reference group if not otherwise specified (i.e., n-3 vs. n-6).

As most biological variables fit a log-normal (multiplicative) distribution equally well or better than a normal (additive) distribution [95], which was also the case for most outcome variables in the present study, we transformed by natural logarithm the values before the analyses of responses in relative terms. Relative within-treatment changes from baseline to follow-up and between-treatment differences are reported in the main text and tables as percentages calculated from the regression coefficients (i.e., the average of log-ratios) by the formula $100 \times (\exp^{estimate} - 1)$. In the figures, however, we show results in relative terms as sympercents (s%), which are additive and symmetric percentage differences on the 100 $\log_e$ scale [97]. This relative measure is calculated as the difference between the natural logs of two numbers multiplied by 100, i.e.,$100 \times \ln(a) - 100 \times \ln(b)$, making it straightforward to present and interpret without back transformation. This approach is useful when analyzing positive valued continuous outcome data, which are often positively skewed, and it avoids the problems of asymmetry and non-additivity when using the conventional percentage difference. The sympercent may be different from the percentage derived from the average of log-ratios, which is calculated from the sympercent by using the formula $100 \times (\exp^{(s\%/100)} - 1)$. Notably, relative changes shown as sympercents in graphical presentations depict the exact same pattern as to when illustrated by, e.g., log2 fold changes.

As part of the model validation procedure, the Shapiro–Wilk test for normality, the D'Agostino test for skewness, and graphical tools (boxplots, quantile–quantile plots, histograms) were used to assess the distribution of standardized residuals. In cases of clear non-normality or extreme skewness, we conducted separate analyses with rank transformed values and report p-values if the nominal significance differed from the primary analyses of log-transformed values. See the Supporting Information (Supplemental Text, *Materials and Methods*) for further details about the mixed modeling and model validation procedure.

2.12.2. Secondary analyses

Although we did not expect carryover effects after the 9-wk washout phase, we could not rule out this possibility. Because of this, we conducted a secondary analysis of between-treatment differences in follow-up scores adjusted for pre-treatment values from B1 and the main effect of 'period' by an ANCOVA mixed model including appropriate fixed terms ('treatment', 'sex', 'period', and 'B1') and random effects (random intercepts only). In this model, we controlled for B1 only since potential carryover effects would affect most the baseline measurements after the washout period.

In separate models, we also adjusted for specific period level factors (covariates that can differ during the trial) one by one. Total energy intake and BMI were included due to different amounts of oils between the interventions. The sex hormones estrogen and testosterone were measured and controlled for because these steroids are known to affect the metabolism of n-3 and n-6 PUFAs [98], and these hormone levels may differ across periods. Vitamin D_3 was also adjusted for due to possible effects on blood lipids, such as LDL-C [99], and its often varying tissue concentrations between the seasons. A fully adjusted model included all of these covariates along with the main term 'period'. All continuous covariates were centered around the mean.

2.12.3. Adjustment for multiplicity

Raw p-values were adjusted for multiple testing by controlling the false discovery rate with the Benjamini and Hochberg method when all primary and secondary variables reported in the current study were analyzed at the same time. The distribution of raw p-values from this joint analysis was used to determine the critical value (q-value). However, adjusted p-values should be interpreted with caution because a general adjustment method for mixed modeling of repeated measurements has not yet been developed due to difficulties associated with the correlation structure, which has to be taken into account [100].

2.12.4. Within-subject variability and correlation

To help with the planning of future crossover studies [101], we also report within-subject SDs and Pearson's *r*s for correlations between follow-up scores in the two intervention periods. The within-subject SD was obtained from the LMEM output as the residual SD (sigma).

3. Results

3.1. Study participants

A total of 232 females and males were pre-screened for participation in the study, of which 69 attended the screening visit, 56 met the inclusion criteria, and 40 accepted the invitation to participate (Fig. 2). In total, 38 participants completed the study,

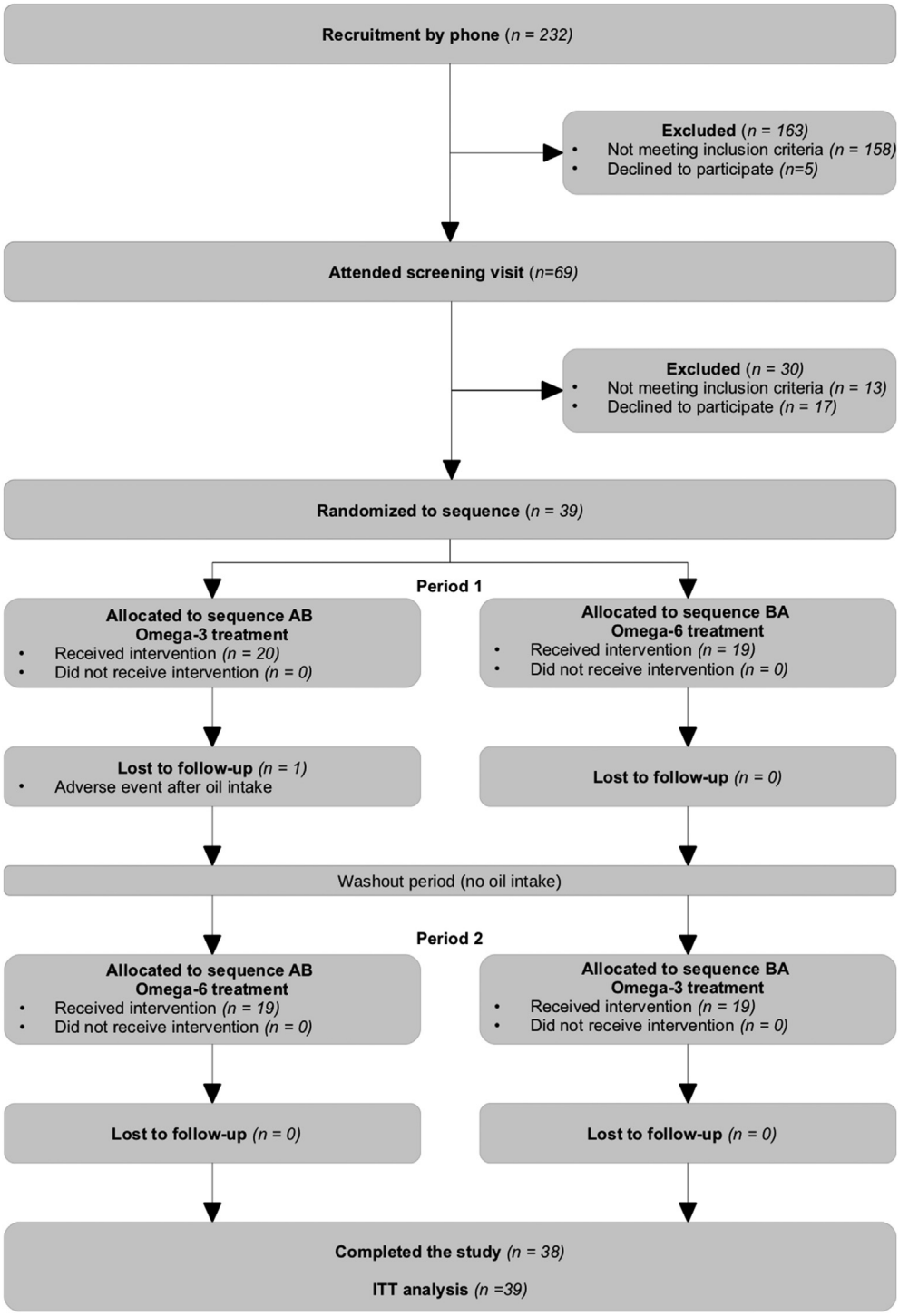

Fig. 2. Flow diagram of the study participants. The diagram shows the numbers of participants screened, included, allocated to sequences, and analyzed in the present study (CONSORT 2019 format [101]). 40 accepted the invitation to participate in the study, but one male dropped out for personal reasons before the first baseline visit and randomization. Another male, assigned to sequence AB, was lost to follow-up after one month in the first period due to adverse events of nausea after intake of the n-3 oil.

while the 39 randomized individuals, 16 females and 23 males, were included in the final intention-to-treat analysis.

The study participants were at baseline middle-aged (56 [SD 9.3] years) and had abdominal obesity (BMI: 28.5 [4.5] in females, 29.8 [3.8] in males; WC: 100 [11.2] cm in females, 107 [8.7] cm in males), and they were normolipidemic (n = 7; all males) or hyperlipidemic (n = 32) (Fig. 3), according to the American College of Cardiology/American Heart Association guidelines [10,102]. Pre-treatment clinical characteristics of the subjects randomly assigned to intervention sequence AB or BA (A: n-3; B: n-6) are shown by sequence and period in Table 1 (geometric means for biochemical variables) and Supplemental Table 3 (arithmetic means for biochemical variables). All participants had at B1 an n-3 index in RBCMs higher than 4 wt%. According to proposed n-3 index risk

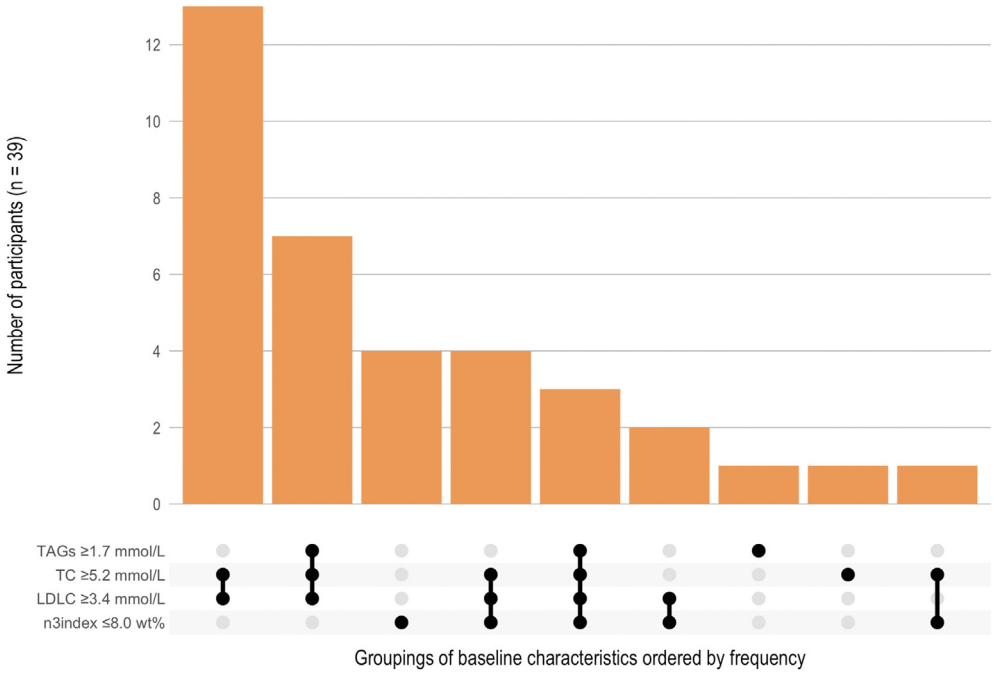

Fig. 3. Lipid levels at baseline. The upset plot shows the co-occurrence of hyperlipidemic traits at baseline. The circulating lipid levels for borderline high hyperlipidemic individuals are according to the American College of Cardiology/American Heart Association guidelines [10,102]. Abbreviations: LDLC, LDL cholesterol; n3index, n-3 index (weight percent of EPA and DHA in red blood cell membranes); TAGs, triacylglycerols; TC, total cholesterol. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

zones [103], 15 and 24 participants were at intermediate (4–8 wt%) and low (>8 wt%) risk, respectively. In our sample, the relatively high pre-treatment n-3 indexes did not correlate significantly (Pearson's *r* [95% BCa CI]: −0.026 [−0.35, 0.30], p = 0.877) with the TAG levels at B1, of which we measured <1.13 mmol/L in 17 participants, ≥1.7 mmol/L in 11 participants, and ≥2.3 mmol/L in 3 participants. Of note, the mean B1 level of LA in RBCMs was similar to the wt% of EPA + DHA and lower compared to circulating levels of LA measured as wt% in erythrocyte phospholipids in seven prospective observational studies [38]. Thus, our sample was characterized by a relatively high EPA + DHA/LA ratio in blood.

3.2. Oil intake, compliance, and blinding

Based on the fatty acid compositions of the oils (Supplemental Table 1) and the self-reported data on oil intakes (Supplemental Table 4), we calculated that the average intake of EPA and DHA per 100 kg body weight was 4.63 (SD 1.14) g/d in females and 4.21 (0.91) g/d in males (females vs. males [95% CI]: 0.40 [−0.08 to 0.87], p = 0.279; from linear regression). During the n-6 period, the average intake of LA was 17.2 (3.46) g/d in females and 18.4 (3.06) g/d in males when normalized to 100 kg body weight (females vs. males [95% CI]: −1.24 g/d [−2.77, 2.82], p = 0.282). The participants reported overall good compliance (>80%) throughout both periods (Supplemental Table 4), and none of them were excluded from the final analysis due to low adherence. Responses to the questionnaire that asked which intervention the participants assumed that they were given in each period indicated successful blinding (Pearson's chi-squared test: $x^2 = 1.31$, p = 0.253) (Supplemental Table 5).

3.3. Dietary intake and physical activity level

The changes from baseline in total dietary and supplemental energy intake differed significantly between the interventions (n-3 vs. n-6 [95% CI]: −163 kcal [−287, −38.6], p = 0.011) (Table 2), which is explained by a higher caloric intake from the supplemental n-6 oil and lack of a similar compensatory decrease in dietary energy intake. The energy percentage (E%) of fat also increased significantly with the n-6 supplementation, and a concurrent decrease in protein intake was recorded. The changes in these nutrients were significantly less during the n-3 intervention. Notably, the intakes of MUFAs, SFAs, and trans fatty acids did not change significantly with any treatment. Besides, we found no significant differences in the recorded intakes of total energy and macronutrients (E%) between the two baseline measurements during the run-in period, and dietary intakes of energy and macronutrients did not change significantly from baseline to follow-up visits (data not shown).

During n-6 supplementation, the total intake of PUFAs increased from a recorded baseline level of 5.95 E% (lower limit of the recommended intake) to 11.0 E% (upper limit of the recommended intake), while the total intake of n-6 PUFAs increased almost two-fold (from 3.60 E% at baseline to 9.35 E% at follow-up), as did the total intake of n-3 PUFAs with n-3 supplementation (from 1.10 E% to 2.91 E%) (Table 2). This led to a two-fold increase in the n-6/n-3 ratio from 3.71 at baseline to 11.4 at follow-up after n-6 supplementation. For the pooled sample, this ratio correlated modestly at baseline with the n-6/n-3 ratio measured in RBCMs (Pearson's *r* [95% BCa CI]: 0.46 [0.13, 0.67], p = 0.004), while the correlation was stronger at follow-up (0.78 [0.70, 0.84], p < 0.001) and between relative change scores (0.87 [0.80, 0.91], p < 0.001).

The Likert scale data on physical activity level showed no significant changes in mean total scores from baseline to follow-up within treatment period one (absolute change [95% CI]: +0.27 [−1.35, 1.89], p = 0.741) and period two (+1.12 [−1.75, 3.98], p = 0.441), during the washout phase (−0.77, [−3.57, 2.02], p = 0.585), or between baseline measurements (B2 vs. B1 [95% CI]: −0.50 [−2.79, 1.79], p = 0.666).

Table 1
Baseline characteristics by sequence and period.[a]

Characteristics	Pooled	Sequence AB		Sequence BA	
	Baseline 1 (n = 39)	Period 1 (n = 20)	Period 2 (n = 19)	Period 1 (n = 19)	Period 2 (n = 19)
Male (%)[b]	23 (59)	12 (60)	11 (58)	11 (58)	11 (58)
Age, years	55.6 (9.3)	57.0 (10.0)		54.2 (8.5)	
BMI, kg/m^2	29.2 (4.11)	29.5 (4.23)	29.8 (4.48)	28.9 (4.07)	29.2 (4.20)
Waist circumference, cm	104 (10.2)	104 (10.8)	103 (10.7)	104 (9.89)	103 (10.3)
Visceral fat area, cm^2	163 (61.6)	159 (64.2)	171 (68.4)	167 (60.3)	171 (49.0)
Energy intake, kcal/d	2224 (529)	2259 (451)	2281 (477)	2190 (608)	2085 (649)
Carbohydrate, E%	37.4 (7.04)	38.6 (7.07)	39.8 (6.98)	36.2 (6.98)	38.2 (8.09)
Protein, E%	17.4 (3.67)	16.4 (2.53)	16.3 (2.11)	18.4 (4.37)	19.0 (3.80)
Fat, E%	38.8 (6.10)	38.3 (4.54)	37.4 (4.87)	39.2 (7.45)	37.6 (7.05)
SFAs, E%	14.4 (3.62)	13.8 (2.94)	14.0 (3.11)	14.9 (4.20)	14.5 (3.47)
MUFAs, E%	12.9 (2.99)	12.4 (2.73)	12.6 (2.35)	13.4 (3.21)	13.4 (2.90)
PUFAs, E%	5.95 (1.92)	6.06 (2.23)	5.65 (1.62)	5.83 (1.61)	5.01 (1.32)
Physical activity score	9.79 (8.66)	9.74 (9.29)	7.58 (8.20)	9.84 (8.25)	11.0 (9.44)
n-3 index, wt%, RBCMs	8.47 (7.14, 10.1)	8.57 (7.02, 10.5)	9.77 (8.41, 11.3)	8.37 (7.29, 9.61)	8.38 (7.03, 9.99)
LA, wt%, RBCMs	8.76 (8.06, 9.52)	8.81 (8.08, 9.60)	8.67 (7.82, 9.62)	8.71 (8.02, 9.46)	8.68 (8.03, 9.38)
Total VLDLs, nmol/L	42.7 (22.5, 81.0)	43.0 (23.0, 80.5)	44.6 (28.5, 69.7)	42.3 (21.5, 82.9)	45.3 (26.1, 78.5)
Large VLDLs, nmol/L	2.77 (0.83, 9.23)	2.85 (0.82, 9.93)	4.20 (1.21, 14.6)	2.69 (0.82, 8.82)	3.29 (1.00, 10.9)
Small VLDLs, nmol/L	20.1 (9.84, 41.0)	17.6 (8.48, 36.5)	17.3 (6.10, 49.0)	23.3 (11.7, 46.3)	21.3 (8.96, 50.5)
Total LDLs, nmol/L	1323 (1052, 1663)	1297 (1019, 1651)	1268 (939, 1712)	1350 (1084, 1681)	1361 (1039, 1783)
Large LDLs, nmol/L	488 (336, 710)	488 (328, 726)	444 (262, 751)	489 (341, 702)	507 (350, 735)
Small LDLs, nmol/L	450 (190, 1067)	413 (166, 1026)	429 (149, 1236)	493 (216, 1125)	538 (241, 1199)
Total HDLs, μmol/L	30.1 (26.0, 34.7)	30.2 (26.0, 35.0)	30.9 (26.6, 35.9)	30.0 (25.9, 34.6)	29.5 (25.4, 34.3)
Large HDLs, μmol/L	5.93 (3.30, 10.7)	5.77 (2.94, 11.3)	7.18 (4.15, 12.4)	6.09 (3.72, 9.98)	6.40 (3.93, 10.4)
Small HDLs, μmol/L	16.9 (12.7, 22.6)	17.4 (13.7, 22.2)	14.0 (8.93, 22.1)	16.4 (11.7, 22.9)	15.9 (13.7, 18.4)
Lp(a), mg/L	99.6 (37.6, 264)	90.6 (35.4, 232)	86.4 (33.0, 226)	110 (39.4, 307)	107 (38.9, 296)
TAGs, mmol/L	1.30 (0.84, 2.00)	1.32 (0.86, 2.04)	1.34 (0.83, 2.16)	1.28 (0.82, 1.98)	1.25 (0.75, 2.06)
NEFAs, mmol/L	0.49 (0.28, 0.88)	0.56 (0.34, 0.90)	0.42 (0.26, 0.68)	0.43 (0.22, 0.82)	0.49 (0.31, 0.78)
TC, mmol/L	5.66 (4.85, 6.62)	5.64 (4.92, 6.48)	5.77 (4.87, 6.83)	5.69 (4.76, 6.79)	5.76 (4.87, 6.83)
LDL-C, mmol/L	3.83 (3.14, 4.66)	3.78 (3.15, 4.54)	3.89 (3.11, 4.87)	3.87 (3.12, 4.81)	4.05 (3.32, 4.95)
HDL-C, mmol/L	1.38 (1.02, 1.87)	1.40 (1.01, 1.93)	1.44 (1.01, 2.06)	1.36 (1.03, 1.81)	1.36 (1.02, 1.80)
non-HDL-C, mmol/L	4.20 (3.41, 5.16)	4.14 (3.38, 5.08)	4.18 (3.26, 5.37)	4.26 (3.44, 5.28)	4.34 (3.50, 5.38)
ApoB, g/L	1.08 (0.91, 1.29)	1.06 (0.90, 1.25)	1.09 (0.89, 1.33)	1.11 (0.92, 1.34)	1.13 (0.94, 1.36)
ApoA-I, g/L	1.48 (1.26, 1.74)	1.49 (1.26, 1.74)	1.50 (1.25, 1.80)	1.48 (1.25, 1.74)	1.45 (1.23, 1.70)
HOMA2-IR	1.53 (1.03, 2.30)	1.51 (0.95, 2.41)	1.90 (1.34, 2.69)	1.55 (1.10, 2.19)	1.59 (1.03, 2.43)

Abbreviations: Apo, apolipoprotein; E%, energy percentage of total energy intake; HDLs, HDL particles; HDL-C, HDL cholesterol; HOMA2-IR, homeostasis model assessment of insulin resistance index 2 (computer model); LA, linoleic acid (C18:2n6); LDLs, LDL particles; LDL-C, LDL cholesterol; Lp(a), lipoprotein (a); MUFAs; monounsaturated fatty acids; n-3, omega-3 PUFAs; n-6, omega-6 PUFAs; NEFAs, non-esterified fatty acids; non-HDL-C, non-HDL cholesterol; PUFAs; polyunsaturated fatty acids; RBCMs, red blood cell membranes; SFAs; saturated fatty acids; TAGs, triacylglycerols; TC, total cholesterol; VLDLs, VLDL particles; wt%, weight percentage of total fatty acids.

[a] Values are geometric means (1 SD ranges) and arithmetic means (SDs) of fasting blood levels and other measurements, respectively, at baseline in period 1 (before randomization) and period 2 (after the washout phase) of each sequence. Pooled values at the first baseline visit before any intervention are also shown. Sequence AB and BA received the n-3 and n-6 intervention first, respectively. One participant was lost to follow-up after one month during the first intervention period. The n-3 index is the total sum of EPA (C20:5n-3) and DHA (C22:6n-3) measured in RBCMs (wt%).[c]

[b] Values are numbers (%) of males in each sequence and period.

[c] Arithmetic means (SDs) of biochemical variables are shown in Supplemental Table 3.

3.4. Fatty acid profiles in RBCMs as a measure of compliance

The observed within-treatment changes and between-treatment differences in the RBCM fatty acid profiles indicated good compliance with the interventions (Table 3, Supplemental Tables 6 and 7). The n-6/n-3 ratio of RBCMs decreased on average by 40.2% (−1.07) after the n-3 intervention and increased by 10.8% (+0.20) after n-6 supplementation (Table 3, Supplemental Table 7). On the other hand, the n-3 index increased by 48.4% (+4.14 wt%) and decreased by 5.25% (−0.44 wt%) after the n-3 and n-6 treatments, respectively, from a relatively high mean level of 8.50 (SD 1.37) wt% at B1. In all participants, the n-3 index increased and achieved a level of ≥8 wt% after n-3 supplementation, of whom 13 achieved a level of ≥12 wt%.

The RBCM levels of EPA and DHA expressed as wt% increased by 227% (+2.65 wt%, +18.0 μg/mL) and 17.7% (+1.24 wt%, +8.24 μg/mL), respectively, after the n-3 intervention, and decreased by 16.8% (−0.49 wt%, −4.12 μg/mL) and 2.99% (−0.22 wt%, −3.96 μg/mL) after n-6 supplementation (Table 3, Supplemental Table 7). Furthermore, LA wt% in RBCMs decreased by 18.2% (−1.58 wt%, −11.8 μg/mL) after n-3 and increased by 13.4% (+1.16 wt%, +8.90 μg/mL) after n-6.

Notably, we found a period-specific response in most of the reported FA measures for the n-6 intervention, but not for n-3 (Supplemental Tables 8 and 9). The relative changes in the period with n-6 treatment that followed the n-3 intervention in sequence AB, were significantly greater than in the period with n-6 treatment that preceded the n-3 intervention in sequence BA, except for ALA, LA, and GLA. Importantly, the changes in RBCM FAs after n-3 supplementation in sequence AB did not fully rebound to the pre-treatment levels after the washout phase and consequently affected these FA levels in RBCMs into the next intervention period (Supplemental Text, *Results*; Supplemental Fig. 5), which may be related to the significant differences in between-period responses observed in most FA parameters after n-6 supplementation (Supplemental Tables 8 and 9).

3.5. Primary outcomes

3.5.1. Lipoprotein particle subclasses

The period-adjusted mixed modeling showed that the difference between interventions in relative changes from baseline to follow-up was significant for total (n-3 vs. n-6: −38.2% [−15.7 nmol/L] vs. +16.3% [+4.25 nmol/L], $p < 0.001$), large (−58.1%

Table 2
Recorded intakes of energy and macronutrients (E%) at baseline and during the intervention periods.[a]

Variable and treatment	Baseline (n = 38)[b]	Follow-up (n = 35)[c]	Absolute change[d]	Time[e]	Tx × time[f]
Energy, diet, kcal[g]					0.886
n-3	2224 (529)	2144 (563)	−71.4 (−178, 35.6)	0.189	
n-6		2147 (516)	−62.5 (−170, 45.2)	0.253	
Energy, total, kcal[g]					0.011
n-3	2224 (529)	2197 (555)	−6.75 (−113, 99.8)	0.900	
n-6		2366 (532)	+156 (50.2, 262)	0.004	
Carbohydrate, E%					0.055
n-3	37.4 (7.04)	38.0 (7.49)	+0.86 (−1.07, 2.78)	0.380	
n-6		36.6 (7.40)	−0.88 (−2.79, 1.04)	0.366	
Protein, E%					0.001
n-3	17.4 (3.67)	17.1 (3.07)	−0.25 (−1.17, 0.67)	0.588	
n-6		15.7 (2.79)	−1.74 (−2.65, −0.83)	<0.001	
Fat, E%					<0.001
n-3	38.8 (6.10)	39.4 (5.83)	+0.53 (−1.47, 2.53)	0.602	
n-6		42.8 (5.86)	+4.22 (2.22, 6.21)	<0.001	
Fiber, E%					0.135
n-3	1.80 (0.43)	1.80 (0.46)	−0.020 (−0.16, 0.12)	0.770	
n-6		1.69 (0.42)	−0.12 (−0.26, 0.014)	0.077	
Added sugar, E%					0.762
n-3	4.92 (3.58)	4.74 (3.34)	+0.042 (−0.76, 0.84)	0.918	
n-6		4.68 (3.30)	−0.068 (−0.86, 0.73)	0.865	
Alcohol, E%					0.336
n-3	4.71 (4.29)	3.82 (3.65)	−1.03 (−2.11, 0.057)	0.063	
n-6		3.29 (3.38)	−1.47 (−2.54, −0.39)	0.008	
SFAs, E%					0.141
n-3	14.4 (3.62)	14.1 (2.75)	−0.21 (−1.28, 0.86)	0.696	
n-6		13.4 (3.21)	−0.91 (−1.98, 0.16)	0.095	
MUFAs, E%					0.233
n-3	12.9 (2.99)	12.8 (2.99)	−0.033 (−1.03, 0.96)	0.947	
n-6		13.2 (2.48)	+0.50 (−0.50, 1.49)	0.323	
PUFAs, E%					<0.001
n-3	5.95 (1.92)	6.88 (1.45)	+0.88 (0.15, 1.62)	0.019	
n-6		11.0 (1.88)	+5.14 (4.42, 5.88)	<0.001	
TRFAs, E%					0.331
n-3	0.31 (0.16)	0.32 (0.14)	+0.002 (−0.054, 0.058)	0.947	
n-6		0.29 (0.14)	−0.025 (−0.080, 0.031)	0.382	
n-3 PUFAs, E%					<0.001
n-3	1.10 (0.55)	2.91 (0.84)	+1.81 (1.56, 2.05)	<0.001	
n-6		0.95 (0.36)	−0.16 (−0.40, 0.085)	0.197	
n-6 PUFAs, E%					<0.001
n-3	3.60 (1.25)	3.42 (0.99)	−0.20 (−0.73, 0.32)	0.444	
n-6		9.35 (1.64)	+5.76 (5.24, 6.29)	<0.001	
n-6/n-3 ratio					<0.001
n-3	3.71 (1.44)	1.26 (0.50)	−2.49 (−3.61, −1.37)	<0.001	
n-6		11.4 (4.52)	+7.64 (6.53, 8.75)	<0.001	

Abbreviations: E%, energy percentage of total energy intake; LMEM, linear mixed-effects model; n-3, omega-3 PUFAs; n-6, omega-6 PUFAs; TRFAs, trans fatty acids; Tx, treatment.
[a] Pooled period data of total dietary and supplemental intakes of energy and macronutrients were analyzed with LMEMs (see main text).
[b] Values are arithmetic means (SDs) of dietary intakes recorded two times during seven consecutive days in the run-in period (baseline).
[c] Values are arithmetic means (SDs) of total dietary and supplemental intakes recorded over seven consecutive days during both intervention periods (in wk five). One participant was lost to follow-up during the first intervention period in sequence AB.
[d] Absolute changes from baseline to follow-up as mean change scores (95% CIs) from LMEMs.
[e] P-values (from LMEMs) for absolute changes from baseline to follow-up within treatments (time effects).
[f] P-values (from LMEMs) for absolute changes from baseline to follow-up between treatments (group differences in time effects).
[g] Energy, diet: recorded energy intake from the diet only. Energy, total: total energy intake from the diet and supplementation products.

[−2.76 nmol/L] vs. −0.91% [−2.22 nmol/L], p < 0.001), and small (−57.0% [−8.27 nmol/L] vs. +40.7% [+4.60 nmol/L], p < 0.001) VLDLs (Fig. 4A–E, Supplemental Tables 10 and 11). A large but non-significant difference was found for medium VLDLs (−23.1% [−2.66 nmol/L] vs. +2.76% [−1.20 nmol/L], p = 0.163), and the relative change scores in mean VLDL size differed non-significantly between the treatments (−0.77% [−0.48 nm] vs. −5.00% [−3.12 nm], p = 0.235). These differences arose from significant reductions in total, large, and small VLDLs following the n-3 intervention, while small VLDLs increased after n-6 supplementation, contributing to the significant decrease in mean VLDL size following this intervention.

A differential response in relative change scores was also found for total (n-3 vs. n-6: +5.76% [+79.3 nmol/L] vs. −4.25% [−59.3 nmol/L], p = 0.002) and large (+22.9% [+121 nmol/L] vs. −2.12% [−12.0 nmol/L], p = 0.004) LDLs (Fig. 4F–J, Supplemental Tables 10 and 11). Here, we observed in total LDLs a significant increase after n-3 and a decrease after n-6, while large LDLs changed significantly only after n-3 supplementation. We found no significant between-treatment difference for IDLs (−59.8% [−92.2 nmol/L] vs. −44.8% [−97.9 nmol/L], p = 0.258) after large reductions following both interventions, neither for small LDLs (+2.34% [+44.4 nmol/L] vs. +4.04% [+29.1 nmol/L], p = 0.886), nor for LDL size (−0.36% [−0.080 nm] vs. −0.71%

Table 3
Relative changes in RBCM fatty acid levels after 7 wk of supplementation with n-3 or n-6 PUFAs.[a]

Variable and treatment	Baseline (n = 39)[b]	Follow-up (n = 38)[b]	Relative change[c]	Time[d]	Tx × time[e]
n-6/n-3 ratio					<0.001
n-3	2.43 (1.97, 2.99)	1.45 (1.22, 1.73)	−40.2 (−42.1, −38.2)	<0.001	
n-6	2.20 (1.80, 2.69)	2.57 (2.17, 3.03)	+10.8 (7.18, 14.5)	<0.001	
n-3 index, wt%					<0.001
n-3	8.48 (7.04, 10.2)	12.5 (11.0, 14.3)	+48.4 (44.1, 52.9)	<0.001	
n-6	9.04 (7.69, 10.6)	8.33 (7.18, 9.66)	−5.25 (−8.03, −2.38)	0.001	
ALA, wt%					0.019
n-3	0.18 (0.14, 0.24)	0.15 (0.12, 0.19)	−16.1 (−21.5, −10.3)	<0.001	
n-6	0.19 (0.15, 0.24)	0.14 (0.12, 0.17)	−24.3 (−29.2, −19.1)	<0.001	
EPA, wt%					<0.001
n-3	1.33 (0.91, 1.94)	4.14 (3.25, 5.26)	+227 (198, 258)	<0.001	
n-6	1.66 (1.19, 2.31)	1.22 (0.90, 1.63)	−16.8 (−22.3, −10.9)	<0.001	
DPA, wt%					<0.001
n-3	3.00 (2.74, 3.29)	3.88 (3.61, 4.18)	+29.0 (26.6, 31.4)	<0.001	
n-6	3.30 (2.92, 3.72)	3.03 (2.78, 3.30)	−4.74 (−6.52, −2.94)	<0.001	
DHA, wt%					<0.001
n-3	7.11 (6.08, 8.32)	8.36 (7.56, 9.24)	+17.7 (14.8, 20.7)	<0.001	
n-6	7.34 (6.42, 8.39)	7.09 (6.21, 8.09)	−2.99 (−4.76, −1.18)	0.001	
LA, wt%					<0.001
n-3	8.74 (8.06, 9.49)	7.21 (6.45, 8.06)	−18.2 (−20.1, −16.2)	<0.001	
n-6	8.69 (7.92, 9.54)	9.90 (9.14, 10.7)	+13.4 (10.8, 16.2)	<0.001	
GLA, wt%					<0.001
n-3	0.050 (0.037, 0.068)	0.029 (0.021, 0.040)	−43.8 (−48.5, −38.6)	<0.001	
n-6	0.049 (0.036, 0.066)	0.051 (0.038, 0.068)	+1.38 (−7.19, 10.7)	0.758	
DGLA, wt%					<0.001
n-3	1.59 (1.35, 1.87)	1.16 (0.97, 1.38)	−27.1 (−29.3, −24.8)	<0.001	
n-6	1.56 (1.33, 1.84)	1.60 (1.36, 1.87)	+1.76 (−1.32, 4.94)	0.262	
AA, wt%					<0.001
n-3	15.3 (13.8, 16.9)	13.5 (12.3, 14.8)	−11.5 (−12.6, −10.4)	<0.001	
n-6	14.8 (13.3, 16.3)	15.2 (13.9, 16.5)	+1.17 (−0.071, 2.42)	0.064	

Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; LA, linoleic acid; LMEM, linear mixed-effects model; n-3, omega-3 PUFAs; n-6, omega-6 PUFAs; PUFAs, polyunsaturated fatty acids; RBCMs, red blood cell membranes; Tx, treatment; wt%, weight percentage of total fatty acids.

[a] Pooled period data of fasting RBCM fatty acid levels (measured as wt%) were analyzed with LMEMs adjusted for the main effect of period. Values were transformed by the natural logarithm before the analyses. The n-6/n-3 ratio was calculated from the total levels of n-6 and n-3 PUFAs. The n-3 index is the total sum of EPA and DHA measured in RBCMs (wt%).

[b] Values are geometric means (1 SD ranges) of fasting levels at baseline and follow-up.[f]

[c] Relative changes from baseline to follow-up as percentages (95% CIs) calculated from LMEM estimates: $\% = (\exp^{estimate} - 1) \times 100$.[f]

[d] P-values (from LMEMs) for relative changes from baseline to follow-up within treatments (time effects).

[e] P-values (from LMEMs) for relative changes from baseline to follow-up between treatments (group differences in time effects).

[f] Arithmetic means (SDs) and absolute change scores (95% CIs) from mixed modeling of untransformed data are shown in Supplemental Table 7.

[−0.16 nm], p = 0.343) after a significant reduction following the n-6 supplementation.

Moreover, a significant between-treatment difference in relative change scores was observed for total (n-3 vs. n-6: −5.97% [−1.83 μmol/L] vs. +3.73% [+1.25 μmol/L], p < 0.001), large (+10.5% [+0.84 μmol/L] vs. −5.32% [−0.20 μmol/L], p = 0.001), medium (−24.4% [−0.96 μmol/L] vs. +6.21% [+0.31 μmol/L], p = 0.030), and small (−9.86% [−1.45 μmol/L] vs. +9.55% [+1.24 μmol/L], p = 0.002) HDLs (Fig. 4K–O, Supplemental Tables 10 and 11). After the n-3 intervention, the mixed modeling showed significant reductions in total, medium, and small HDLs, and a higher concentration of large HDLs, while small HDLs increased after n-6 supplementation. The redistribution of HDL subfractions translated into a significant between-treatment difference in mean HDL size (+1.22% [+0.12 nm] vs. −1.17% [−0.11 nm], p < 0.001) after a significant increase following the n-3 supplementation and a decrease after the n-6 intervention.

Looking at the intervention periods separately (Supplemental Tables 12 and 13), we found a period-specific response in relative change scores for medium HDLs (P1 vs. P2: +6.49% [+0.39 μmol/L] vs. −50.1% [−2.94 μmol/L], p = 0.001) and small HDLs (−23.4% [−3.29 μmol/L] vs. −1.57% [+0.10 μmol/L], p = 0.014) after the n-3 intervention, and for large VLDLs (+49.0% [+0.69 nmol/L] vs. −29.6% [−3.03 nmol/L], p = 0.011), mean HDL-P size (+0.032% [+0.005 nm] vs. −2.02% [−0.20 nm], p = 0.018), and Lp(a) (−14.2% [−23.3 mg/L] vs. +3.21% [−0.44 mg/L], p = 0.009) after n-6 supplementation.

3.5.2. Standard lipids

Regarding Lp(a) and standard blood lipids, our period-adjusted mixed modeling showed a significant between-treatment difference in relative changes from baseline to follow-up for TAGs (n-3 vs. n-6: −15.7% [−0.26 mmol/L] vs. −2.60% [−0.12 mmol/L], p = 0.014), NEFAs (−18.6% [−0.091 mmol/L] vs. +5.46% [+0.028 mmol/L], p = 0.033), and TC (−0.28% [−0.007 mmol/L] vs. −4.44% [−0.25 mmol/L], p = 0.042), and also for the lipid ratio TAGs/HDL-C (−20.3% [−0.29] vs. −4.42% [−0.16], p = 0.006) (Fig. 5A–J, Supplemental Tables 10 and 11). Non-significant differences were found for LDL-C (−0.84% [−0.024 mmol/L] vs. −5.84% [−0.23 mmol/L], p = 0.067), HDL-C (+5.44% [+0.071 mmol/L] vs. +2.44% [+0.036 mmol/L], p = 0.219), non-HDL-C (−2.23% [−0.091 mmol/L] vs. −7.07% [−0.30 mmol/L], p = 0.059), TRL-C (−17.5% [−0.071 mmol/L] vs. −19.3% [−0.084 mmol/L], p = 0.844), and the TC/HDL-C ratio (−5.93% [−0.28] vs. −6.95% [−0.33], p = 0.684), as well as for PLs (−1.87% [−0.054 mmol/L] vs. −2.23% [−0.070 mmol/L], p = 0.871), FC (−0.52% [−0.003 mmol/L] vs. −2.93% [−0.046 mmol/L], p = 0.305), and Lp(a) (+0.36% [−1.48 mg/L] vs. −8.89% [−15.5 mg/L], p = 0.067). The n-3

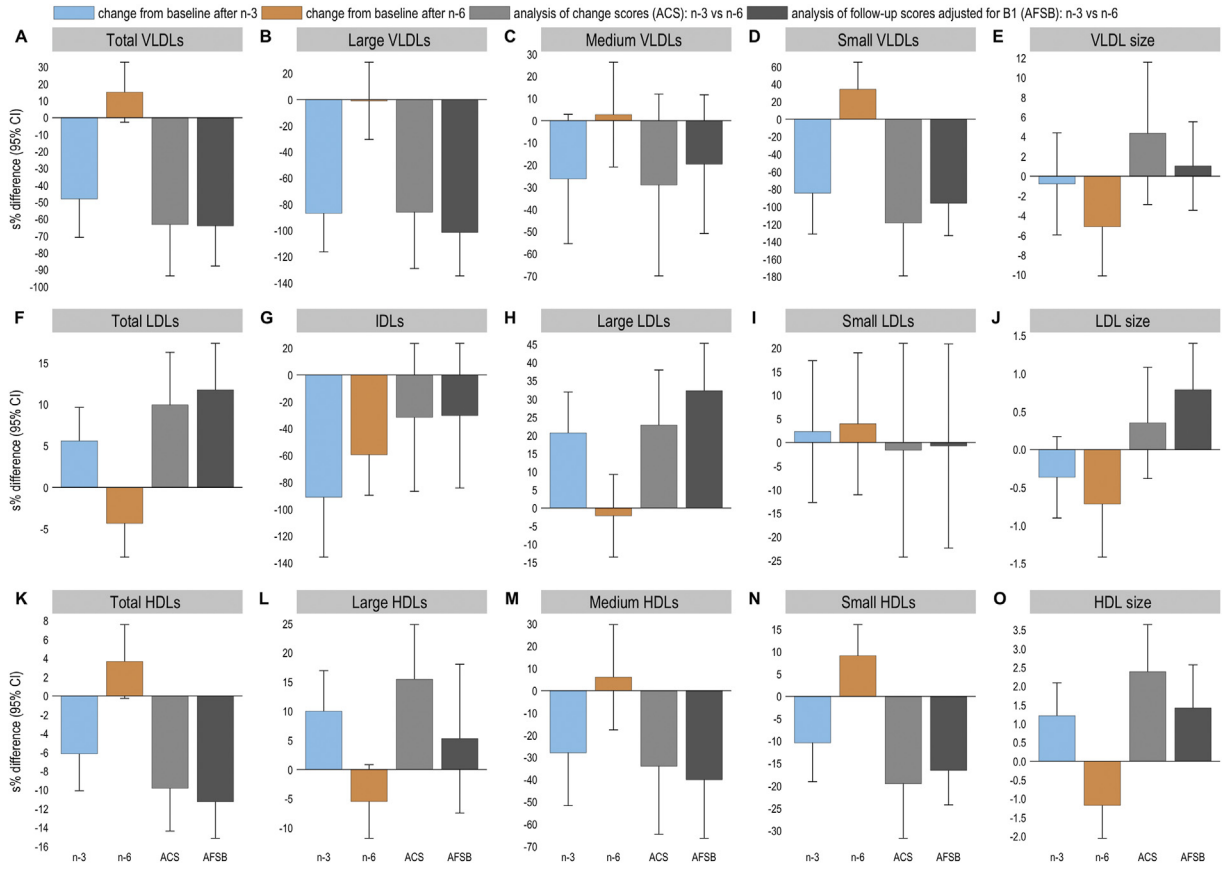

Fig. 4. Differences between treatments in period-adjusted change and follow-up scores for lipoprotein subfractions after 7 wk of supplementation with n-3 or n-6 PUFAs. The bar plots show relative changes from baseline to follow-up and between-treatment differences in change and follow-up scores for lipoprotein subclass particles and sizes. VLDL and LDL particles were measured in nmol/L, HDL particles in μmol/L, and particle sizes in nm. Pooled period data were analyzed with LMEMs adjusted for overall period effects (see main text). Before the analysis, values were transformed by the natural logarithm and multiplied by 100 to show within-group changes and between-group differences as additive, symmetric percentages (sympercents; see main text). Error bars represent 95% confidence intervals. Relative within-treatment changes from baseline to follow-up after n-3 supplementation are shown in blue bars, and after n-6 supplementation in orange bars. Between-treatment differences in relative change scores are shown by light grey bars (primary analysis), while the dark grey bars display relative between-treatment differences in follow-up scores adjusted for B1 (secondary analysis). Number of participants included: n = 39 at baseline in P1; n = 38 at follow-up in P1; n = 38 at baseline in P2; n = 38 at follow-up in P2. Abbreviations: ACS, analysis of change scores; AFSB, analysis of follow-up scores adjusted for pre-treatment values from the first baseline measurements (B1); Apo, apolipoprotein; HDL, HDL particles; IDLs, intermediate-density lipoprotein particles; LDLs, LDL particles; LMEM, linear mixed-effects model; n-3, omega-3 PUFAs; n-6, omega-6 PUFAs; P1, the first intervention period; P2, the second intervention period; s%, sympercent; VLDLs, VLDL particles. Total VLDLs and large VLDLs also include chylomicrons if present. One influential outlier was excluded from the final analyses of medium HDLs.

intervention was followed by significant reductions in TAGs, NEFAs, and TRL-C, and in the lipid ratios TAG/HDL-C and TC/HDL-C, while HDL-C increased significantly. On the other hand, we found after n-6 supplementation lower levels of TC, LDL-C, non-HDL-C, and TRL-C, and also a decrease in the TC/HDL-C ratio and the Lp(a) level.

A period-specific response in relative change scores was found after the n-6 treatment for TAGs (P1 vs. P2: +9.26% [+0.10 mmol/L] vs. −10.8% [−0.24 mmol/L], p = 0.021), PLs (+2.06% [+0.054 mmol/L] vs. −5.96% [−0.18 mmol/L], p = 0.005), and FC (+0.90% [+0.011 mmol/L] vs. −7.04% [−0.12 mmol/L], p = 0.011) (Supplemental Tables 12 and 13). Accordingly, we observed for TAGs, as we did for large, TAG-rich VLDLs, a significant between-treatment difference in relative change scores after the first period, but not after the second period.

Taken together, our results showed among the primary outcomes significantly greater reductions in circulating levels of TAG-rich lipoproteins (TRLs), TAGs, and NEFAs after n-3, and in TC after n-6, relative to the other supplement. Notably, both large and small VLDL subclasses were significantly reduced after the n-3 intervention, while small VLDLs increased after n-6. Although large, cholesterol-rich LDLs increased significantly after n-3 supplementation compared to n-6, we found no significant between-treatment difference in relative change scores for LDL-C or non-HDL-C. Furthermore, the distribution of all HDL subclasses changed differently after the interventions, leading to a significantly larger mean HDL size after n-3 supplementation relative to n-6, while we found no significant between-treatment difference in relative change scores for HDL-C. Large variations in individual responses were observed for all lipoprotein subclasses and standard lipids, and the mean levels after the first intervention period did not fully rebound to the pre-treatment levels after the washout phase for all of these variables (Supplemental Figs. 6 and 7). Findings from analyses of rank-transformed values and adjustments of multiplicity are presented in Supporting Information (Supplemental Text, *Results*).

3.6. Secondary outcomes

3.6.1. Apolipoprotein profile

Fasting serum levels of apolipoproteins showed significantly different between-treatment changes from baseline to follow-up in relative terms for apoB (n-3 vs. n-6: +0.40% [+0.005 g/L] vs. −6.04% [−0.066 g/L], p = 0.008), apoA-II (−6.03% [−0.027 g/L] vs. +1.53% [+0.008 g/L], p = 0.001), apoC-II (−11.0% [−0.026 g/L] vs. −1.74%

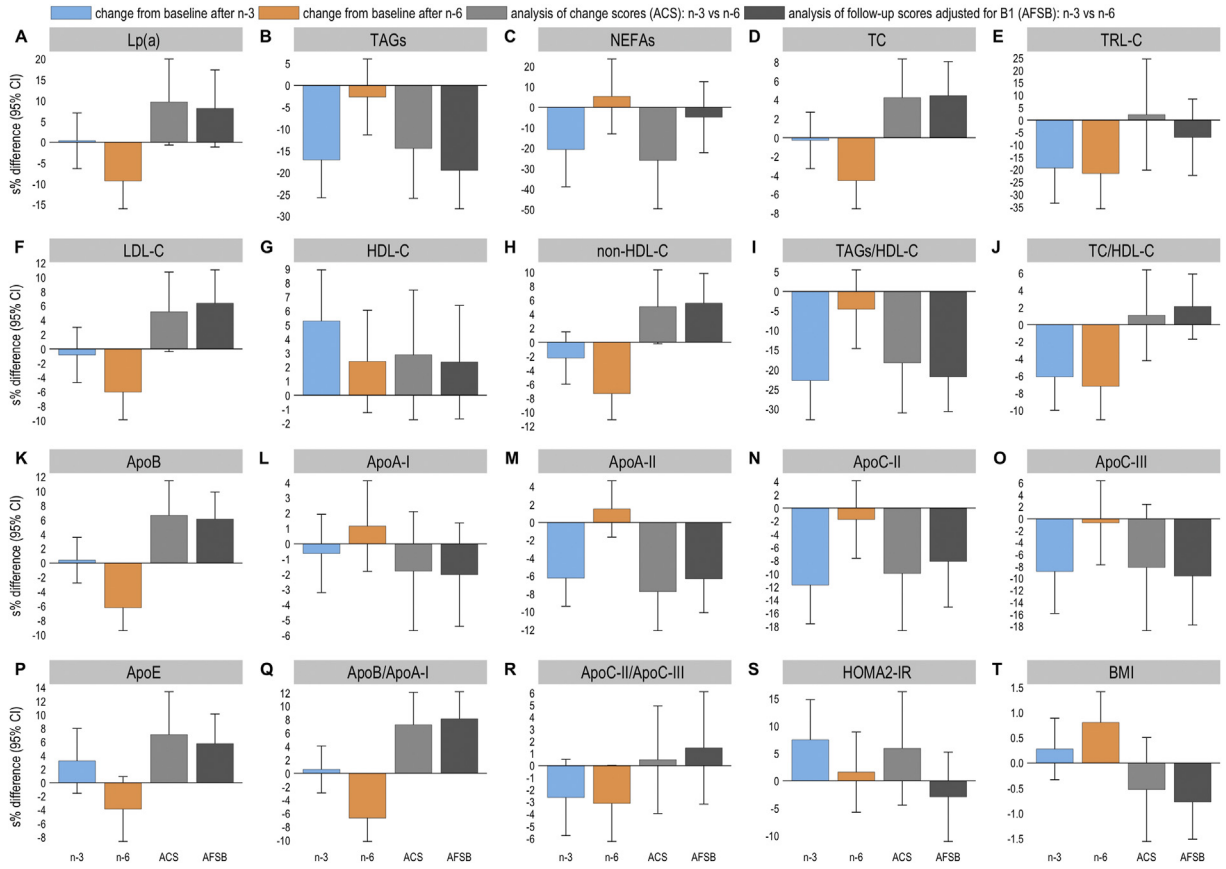

Fig. 5. Differences between treatments in period-adjusted change and follow-up scores for lipoprotein (a), blood lipids, and apolipoproteins after 7 wk of supplementation with n-3 or n-6 PUFAs. The bar plots show relative changes from baseline to follow-up and between-treatment differences in change and follow-up scores for lipoprotein (a) (A), blood lipids (B–J), and apolipoproteins (K–R), as well as for HOMA2-IR (S) and BMI (T). Blood lipids were measured in mmol/L and apolipoproteins in g/L. Abbreviations: Apo, apolipoprotein; HDL-C, HDL cholesterol; HOMA2-IR, homeostasis model assessment of insulin resistance index 2 (computer model); LDL-C, LDL cholesterol; Lp(a), lipoprotein (a); NEFAs, non-esterified fatty acids; non-HDL-C, non-HDL cholesterol; TAGs, triacylglycerols; TC, total cholesterol; TRL-C, TAG-rich lipoprotein cholesterol. See further explanation and abbreviations in Fig. 4.

[−0.007 g/L], p = 0.025), and apoE (+3.28% [+0.003 g/L] vs. −3.79% [−0.004 g/L], p = 0.028), as well as for the ratio apoB/apoA-I (+0.58% [+0.002] vs. −6.45% [−0.051], p = 0.004) (Fig. 5K–R, Supplemental Tables 10 and 11). We found no significant difference between treatments for apoA-I (−0.63% [−0.012 g/L] vs. +1.17% [+0.016 g/L], p = 0.364) and apoC-III (−8.45% [−0.028 g/L] vs. −0.67% [−0.007 g/L], p = 0.129), and for the apoC-II/apoC-III ratio (−2.59% [−0.013] vs. −3.06% [−0.018], p = 0.828). The mixed modeling showed that the n-3 intervention was followed by significantly lower serum levels of apoA-II, apoC-II, and apoC-III, while we observed after n-6 supplementation reductions in apoB and the apoB/apoA-I ratio. Significant period-specific responses in relative terms were found for the n-6 treatment in apoC-II (P1 vs. P2: +5.54% [+0.009 g/L] vs. −9.04% [−0.022 g/L], p = 0.011), apoC-III (+11.7% [+0.031 g/L] vs. −9.22% [−0.028 g/L], p = 0.002), and apoE (+2.93% [+0.002 g/L] vs. −8.93% [−0.009 g/L], p = 0.010) (Supplemental Tables 12 and 13). In several of these variables we also observed that the concentrations did not fully rebound to the pre-treatment levels after the washout phase (Supplemental Fig. 8).

3.6.2. Glycemic control and insulin sensitivity

The relative change scores were non-significantly different between treatments for all indices of glycemic control and insulin sensitivity, including glucose (n-3 vs. n-6: +1.24% [+0.075 mmol/L] vs. +1.27% [+0.066 mmol/L], p = 0.983), insulin (+10.1% [+0.95 mU/L] vs. +1.70% [+0.45 mU/L], p = 0.282), INCP (+7.35% [+0.048 nmol/L] vs. +0.92% [+0.009 nmol/L], p = 0.220), and HOMA2-IR (+7.80% [+0.11] vs. +1.60% [+0.031], p = 0.260) (Supplemental Tables 14 and 15, Fig. 5S for HOMA2-IR). The mixed modeling showed a significant change over time in relative terms after the n-3 intervention for fasting INCP, HOMA2-IR, and HOMA2-%S, but not for glucose, HbA1c, insulin, or LP-IR. For n-6 supplementation, these variables changed on average in the same direction as for n-3, but to a lesser degree and statistically non-significant. Notably, we found period-specific responses in most markers of glycemic control and insulin sensitivity after the n-6 intervention. These period differences were, however, statistically significant in relative terms only for glucose (P1 vs. P2: +4.43% [+0.23 mmol/L] vs. −1.33% [−0.078 mmol/L], p = 0.030) (Supplemental Tables 16 and 17).

3.6.3. BMI and body composition

The recorded difference in total energy intake between interventions (Table 2) could potentially lead to different changes in anthropometric measurements, such as body weight or fat accumulation, which could indirectly affect other outcome measures. However, in our period-adjusted analysis, we found trivial or small and non-significant group differences in absolute and relative change scores for all of the anthropometric variables measured (Supplemental Tables 14 and 15, Fig. 5T for BMI). Notably, we observed after n-6 supplementation a small but statistically significant increase in mean body weight (0.81% [+0.68 kg], p = 0.010) and BMI (+0.81% [+0.23 kg/m^2], p = 0.010), while body fat mass

increased significantly following both the n-3 (+1.84% [+0.44 kg], p = 0.046) and n-6 (+1.84% [+0.59 kg], p = 0.046) interventions. A period-specific response was not observed among anthropometric variables, except for visceral fat area after the n-6 intervention, which increased non-significantly in the first period and decreased non-significantly in the second period (P1 vs. P2: +4.44% [+5.68 cm^2] vs. −6.24% [−7.05 cm^2], p = 0.014) (Supplemental Tables 16 and 17).

3.7. Secondary analyses

Results from the secondary analyses of baseline- and period-adjusted follow-up scores are summarized in Supplemental Table 18 and also graphically presented in Figs. 4 and 5 to enable direct comparisons of the primary and secondary analyses of between-treatment differences. The secondary analyses supported overall the results from the primary analyses of change scores by showing nearly the same pattern of between-treatment differences, characterized by significantly greater reductions in TAG-related markers after n-3 supplementation and in cholesterol-related measures after n-6 supplementation, compared to the other intervention.

Findings from covariate-adjusted mixed models are summarized in the Supporting Information (Supplemental Text, *Results*). The mixed modeling did not indicate significant carryover effects for any of the primary lipoprotein and lipid measures (Supplemental Text, *Results*).

3.8. Liver markers

Among the circulating safety markers related to liver function, we observed a significant difference between treatments in relative change scores only for ALP (n-3 vs. n-6: −4.18% [−2.79 U/L] vs. +0.81% [+0.24 U/L], p = 0.041) after a significant decrease following the n-3 intervention and a non-significant increase after n-6 supplementation (Supplemental Tables 14 and 15). Additionally, ALAT showed a significant increase (+10.5% [+3.68 U/L], p = 0.013) after n-3 supplementation.

3.9. Adverse events

The questionnaire about symptoms typically related to changes in dietary or supplemental intake showed that the participants most frequently reported regurgitation, rumbling in stomach/intestines, bloating/distension, heartburn, diarrhea, and/or acute stomach ache (Supplemental Table 19). Overall, the severity was experienced to be mild or moderate, and adverse events occurred more often during the n-6 than the n-3 intervention.

3.10. Within-subject variability and correlation

Within-subject SDs and Pearson's *r*s for correlations between follow-up scores in the two intervention periods are presented in Supplemental Table 20 for all primary and some secondary outcomes.

4. Discussion

We conducted the first crossover trial comparing the effects of n-3 (high EPA + DHA) and n-6 (high LA) PUFAs on the concentrations and sizes of all major lipoprotein particle subclasses and related lipids and apolipoproteins in a high-risk population of inactive adults with abdominal obesity and various degrees of dyslipidemia. Our findings showed that marine-based n-3 PUFAs (mainly EPA + DHA) primarily lowered fasting blood levels of TAG-related markers, while plant-based n-6 PUFAs (mainly LA) primarily reduced cholesterol-related measures, compared to the other intervention. These changes after n-3 and n-6 supplementation have been associated with reduced cardiometabolic risk [1,9,16], partly via differential mechanisms [2,10].

Cardiometabolic effects of supplemental PUFAs are largely mediated through changes in the FA composition of different tissues, and circulating FA levels can reflect such changes [103], as well as adherence to the interventions [85]. The responses in the RBCM fatty acid profile after the high-dose supplementation with EPA + DHA, showing that the content of DHA increased much less than EPA, are in line with previous n-3 PUFA intervention trials [104–109]. These results may be partly explained by retroconversion of DHA to EPA [104,110], a rate of DHA utilization that matches DHA synthesis [109], and/or a feedback inhibition of EPA metabolism resulting in an accumulation of EPA (from ALA) following DHA supplementation [109,111]. Moreover, our observation that RBCM AA did not increase significantly with higher LA intake is consistent with previous research demonstrating that a dose response between dietary LA intakes and tissue AA levels does not exist among individuals consuming a Western-type diet containing LA over a wide range of E% [112–114]. This is probably due to limited enzymatic conversion of LA to AA, estimated to be 0.3–0.6% in stable isotope tracer studies [115], and not because of tissue AA saturation [114].

Among 10 other RCTs reporting NMR-measured concentrations and sizes of all major lipoprotein particle subclasses after treatment with n-3 or n-6 PUFAs [63–71,116], none showed overall the same pattern (signs and magnitudes) of changes across the lipoprotein subfractions. Of note, these RCTs were highly heterogeneous in study design, with supplemental doses of n-3 PUFAs ranging from 2 to 4 g/d of EPA and/or DHA (DHA/EPA ratio between 0.3 and 1.7). The only consistent findings across these studies were lower concentrations of large VLDLs and reduced mean VLDL sizes following EPA and/or DHA interventions compared to placebos containing highly variable oil types and LA quantities. In the present study, we also observed a significant reduction in large VLDLs (and chylomicrons when present) after n-3 supplementation, and the effect size was comparable to the previous RCTs with similar supplemental doses (3–4 g EPA + DHA/d) [65–67,71,117]. However, we found no change in mean VLDL size, partly because of a concurrent reduction in small particles, as observed in only one of the other RCTs [66]. Of note, none of the other n-3 intervention trials used fish oil in TAG form compared to a vegetable oil rich in LA, as in the present study.

Increased intake of marine, long-chained n-3 PUFAs primarily inhibits hepatic synthesis and secretion of larger, TAG-rich VLDLs, and secondarily improves VLDL clearance and enhances VLDL conversion to IDLs and LDLs [40,53,58], as well as increasing chylomicron TAG clearance [118], thereby lowering circulating TAG levels. Tracer kinetic studies have consistently shown that 3–4 g EPA/DHA per day induces a ~30% reduction in hepatic VLDL-TAG production [58], which likely explains most of the ~30% (range 16–45%) decrease in plasma TAG levels observed in numerous clinical trials [29,55]. It is also well established that intake of n-3 PUFAs lowers TAG concentrations in a dose-dependent manner and relative to pre-treatment levels [41,57,59]. Thus, the modest TAG reduction (−16%) observed in the present study may be partly explained by the relatively low VLDL and TAG concentrations at baseline, possibly related to the high pre-treatment n-3 index, which is typically observed in Scandinavia [119].

NEFAs from adipose tissues contribute the largest fraction of FAs to hepatic VLDL-TAG production in both the fasted and fed states in different metabolic conditions [120], and it has been proposed that the TAG lowering effect of n-3 PUFAs is best explained by an effect

on the NEFA pool [58]. Consistent with this, we observed a significant reduction in NEFAs after n-3 supplementation that may be large enough to explain most of the reduction in TAGs [58,120]. The significant increase in fasting insulin levels following this intervention may also have affected the NEFA flux and hepatic output of TRLs, as insulin inhibits lipolysis in adipose tissues [121]. Accordingly, we found that relative changes in NEFAs and insulin were inversely related (data not shown).

Moreover, n-3 PUFAs may increase the expression and activity of lipoprotein lipase (LpL) [118,122], the intravascular lipolytic enzyme that hydrolyzes TAGs in chylomicrons and VLDLs [123], forming smaller and less TAG-rich remnant particles, including smaller VLDLs and IDLs [124,125]. Thus, both reduced hepatic secretion and increased lipolytic conversion of TRLs may explain the lower levels of these particles after n-3 supplementation in the present study. The large reductions in small VLDLs and IDLs probably resulted partly from lipolytic conversion to even smaller particles [10]. Besides, medium-sized lipoproteins normally have a greater affinity for hepatic apoB/apoE receptors and are, therefore, more effectively cleared from the circulation than larger or smaller particles [124–126].

The significant increase we found after the n-3 intervention in large LDLs, which are overall much less strongly related to CVD risk than sdLDLs [2,127], has been observed in other RCTs [63,65–68,71], but not all [64,69,70]. This elevation may be partly explained by the fewer VLDLs secreted from the liver and enhanced hydrolysis of those particles that are secreted, of which a higher proportion may be smaller, denser, and less TAG-rich [124,128], resulting in greater conversion to IDLs and LDLs and formation of larger, more buoyant, and cholesterol-rich LDLs [56,124,126]. Yet, the cholesterol-increasing effect reported by several investigators [55,129], especially when supplementing with DHA [42], was not found in the present study, probably partly due to the large concurrent reduction in IDLs.

Although animal studies have shown that n-3 PUFAs, especially DHA, increase the plasma level and activity of cholesteryl ester transfer protein (CETP) [130], the shift in HDL (and LDL) subclass particles we observed after n-3 supplementation indicates reduced activity of CETP [131,132], probably related to the lower TRL and TAG levels [124,126,133]. Lowered CETP activity decreases the exchange of cholesterol from HDLs for TAGs from VLDLs [131,132], and consequently increases larger, cholesterol-rich HDLs and lowers TAG-rich HDLs, that otherwise have their TAGs hydrolyzed by hepatic lipase to form smaller HDL subfractions [133]. Accordingly, we observed higher HDL-C, despite the reduction in total HDLs, partly because of elevation in large HDLs and decrease in small HDLs, which is consistent with some previous reports [63,66].

The significant reductions observed after n-3 supplementation in apoC-II, an important cofactor for LpL, and apoC-III, an inhibitor of LpL activity and, more importantly, hepatic clearance of lipoproteins [134], which also has been reported by others [65,67,76], likely reflect a smaller pool of circulating lipoprotein carriers, primarily chylomicron remnants, large VLDLs, IDLs, and HDLs. Besides, the apoC-III concentration in serum and on lipoprotein particles may be lowered by direct effects of n-3 PUFAs and insulin on hepatic apoC-III production [134]. Overall, this indicates a reduction in apoC–III–enriched Lp-B, which are among the most atherogenic particles [27,28]. The significant reduction after n-3 supplementation in apoA-II, which primarily resides on medium-sized and small HDLs [135], was probably related to the lower concentrations of these particles and possibly also of VLDLs and IDLs, of which small subpopulations contain apoA-II [136].

Experimental data have demonstrated that n-6 PUFAs (LA) reduce circulating VLDL levels by increasing VLDL lipolysis and uptake [137]. This is consistent with a previous PUFA intervention trial [68], but not with several other reports [64,66,67,116], and it was not evident in the present study, showing trivial changes in TAGs and TRLs and a significant increase in small VLDLs after n-6 supplementation. The large decrease in IDLs also after this intervention, which correlated significantly with the reduction in total LDLs (data not shown), may be related to a reduced LDL apoB-100 production rate with increased LA intake [138], as well as high affinity of IDLs for hepatic apoB/apoE receptors [124,125]. The redistribution of LDL subfractions after the n-6 treatment probably led to the significant reduction in mean LDL size, as well as in LDL-C, and the change in total serum level of apoB indicates fewer potentially atherogenic particles in circulation [22], including less Lp(a), which has been independently associated with CVD [139,140].

According to a recent extensive systematic review of RCTs assessing cardiometabolic effects of n-6 fats in the long term [50], higher n-6 intake was associated with a small reduction in serum TC (10 trials) but insignificant changes in TAGs (5 trials), LDL-C (2 trials), and HDL-C (4 trials). Moreover, several short-term RCTs have demonstrated that plant oils rich in n-6 PUFAs reduce the levels of TC and LDL-C, while the effects on HDL-C were insignificant or unclear [44,60,61]. Notably, a recent systematic review and network meta-analysis comparing the effects of oils and solid fats on blood lipids found that safflower oil, used in our study, was most effective for decreasing TC and LDL-C compared to rapeseed, sunflower, and soybean oils [62]. The findings from this analysis and the short-term RCTs are consistent with the current study.

Among the strengths of the present work are the detailed dietary information, use of high-quality supplemental products in high doses, well-characterized fatty acid composition and measures of oxidative quality of both oils, and detailed analyses of fatty acid profiles in blood. Notably, it has been shown that high-quality versus oxidized fish oils have different effects on lipoprotein-lipid profiles [72], emphasizing the importance of reporting quality parameters of supplemental oils, which has been lacking in similar RCTs [63–71,116]. Moreover, using a high-quality n-6-rich oil with low levels of oxidation products and TRFAs may be an important factor to provide a cardioprotective effect. Previous n-6 PUFA intervention trials have used n-6-rich oils or other fatty foods from different sources, often with unknown levels of oxidation products and TRFAs. Different concentrations across studies of such substances, contained originally in the products and/or formed during different cooking processes [141,142], may partly explain some of the controversies regarding LAs cardioprotective effects [49,143–146].

Limitations of the present work include short treatment duration and an increased risk of false positives with a large number of statistical tests. Additionally, we recruited a heterogeneous group of participants regarding the degree of pre-treatment dyslipidemia and insulin resistance, and sex-dependent effects may have contributed to outcome heterogeneity. Notably, this study was conducted in a population from Western Norway with high pre-treatment levels of circulating n-3 PUFAs (EPA + DHA) and relatively low levels of n-6 PUFAs (LA), which is typically observed in Nordic countries [38,119]. This FA composition probably reflects the dietary habits in Norway, including a higher proportion of fatty fish compared with many other countries [147], as well as a typically low intake of n-6 PUFAs [81], and may partly explain that the majority of participants had normal or low pre-treatment levels of TAGs and TRLs. However, previous studies have reported higher CVD risk with increasing TAG levels within what is considered a 'normal' range [11].

Another important issue is the presence of potential carryover effects, since n-3 supplementation in the first intervention period affected the RBCM FA levels into the next period, indicating a too

short washout phase, although it was much longer than in previous crossover trials measuring all major lipoprotein subfractions after PUFA interventions [66,70]. Therefore, we have reported results for each intervention in each period, as recommended for crossover studies [101], and conducted a secondary analysis of between-treatment differences in follow-up scores, which are less biased by potential carryover effects than change scores [101,148–150]. However, in the absence of a 3 × 3 crossover design including a placebo control intervention assumed to have neutral effects on the primary outcomes, a challenge described elsewhere [47,71,151,152], we were not able to provide placebo-adjusted treatment effect estimates for both the n-3 and n-6 interventions.

5. Conclusions

Supplementation with marine-derived n-3 PUFAs (mainly EPA + DHA) was followed by reduced levels of TAGs and TRLs, a shift in LDLs and HDLs towards larger and more cholesterol-rich particles, and lower apoC-III concentration. After treatment with plant-derived n-6 PUFAs (mainly LA), on the other hand, we observed less Lp-B and lower cholesterol levels in conjunction with a reduction in IDLs and total LDLs, as well as Lp(a). The responses after both interventions point to changes in the lipoprotein–lipid–apolipoprotein profile that have been associated with reduced cardiometabolic risk, also among people with TAG or LDL-C levels within the normal range. Our study supports that physicians should encourage high-risk patients with abdominal obesity and low consumption levels of n-3 and/or n-6 PUFAs to increase the intake of high-quality oils and/or other foods containing these fatty acids.

Data sharing

Data described in the manuscript and analytic codes will be made available upon request.

Ethical committee

Approved by Regional Committees for Medical and Health Research (REK), Ref: 2014/2336/REK South-East.

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Author contributions

EG, JLB, JS, JEN, RKB, ER, and OKN designed the research project; EG, JLB, PB, BB, ES, and ER conducted research; JLB analyzed data and performed the statistical analyses; EG and JLB wrote the manuscript; EG, JLB, GM, SND, and OKN revised the manuscript; EG, JLB, GM, SND, and OKN had primary responsibility for the final content; all authors read and approved the final manuscript; ER, GM, SND, and OKN supervised the trial; and JLB developed the electronic app for dietary recording on iPhone/iPad/iPod.

Conflict of interest

All the authors declare to have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2021.03.040.

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