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Oxidised fish oil does not influence established markers of oxidative stress in healthy human subjects: a randomised controlled trial

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Abstract

Intake of fish oil reduces the risk of CHD and CHD deaths. Marine *n*-3 fatty acids (FA) are susceptible to oxidation, but to our knowledge, the health effects of intake of oxidised fish oil have not previously been investigated in human subjects. The aim of the present study was to investigate markers of oxidative stress, lipid peroxidation and inflammation, and the level of plasma *n*-3 FA after intake of oxidised fish oil. In a double-blinded randomised controlled study, healthy subjects (aged 18–50 years, *n* 54) were assigned into one of three groups receiving capsules containing either 8 g/d of fish oil (1·6 g/d EPA + DHA; *n* 17), 8 g/d of oxidised fish oil (1·6 g/d EPA + DHA; *n* 18) or 8 g/d of high-oleic sunflower oil (*n* 19). Fasting blood and morning spot urine samples were collected at weeks 0, 3 and 7. No significant changes between the different groups were observed with regard to urinary 8-iso-PGF$_{2\alpha}$; plasma levels of 4-hydroxy-2-hexenal, 4-hydroxy-2-nonenal and α-tocopherol; serum high sensitive C-reactive protein; or activity of antioxidant enzymes in erythrocytes. A significant increase in plasma level of EPA + DHA was observed in both fish oil groups, but no significant difference was observed between the fish oil groups. No changes in a variety of *in vivo* markers of oxidative stress, lipid peroxidation or inflammation were observed after daily intake of oxidised fish oil for 3 or 7 weeks, indicating that intake of oxidised fish oil may not have unfavourable short-term effects in healthy human subjects.

Key words: ***n*-3: Lipid peroxidation: Oxidative stress: Oxidised oil**

Intake of fish and fish oil has been related to a reduced risk of CHD and CHD deaths[1–3]. The recommended daily intake in primary prevention of CHD is at least two servings of fish per week, preferably fatty fish[2,3]. Such consumption is expected to provide about 0·5 g of marine *n*-3 fatty acids (FA) EPA and DHA per d. *n*-3 Supplements are recommended for those who do not include fish in their diet[3], and are used in clinical practice to prevent CHD and in the treatment of mild-to-moderate hypertriacylglycerolaemia[2,3].

Long-chain *n*-3 PUFA are susceptible to oxidation, and lipid peroxidation leads to the formation of a range of different oxidation products[4–8]. During the initial step of oxidation, primary oxidation products (hydroperoxides) are formed, and subsequently more stable secondary oxidation products are generated, such as aldehydes. The content of total primary and secondary oxidation products are measured as peroxide value (PV) and anisidine value (AV), respectively. Maximum acceptable levels of lipid peroxidation products in refined marine *n*-3 oils to be used for dietary supplements are defined by different monographs such as the European Pharmacopeia, which recommend that PV and AV should not exceed 10 and 20 mEq/kg, respectively[9]. High contents of oxidation products

Abbreviations: 4-HHE, 4-hydroxy-2-hexenal; 4-HNE, 4-hydoxy-2-nonenal; AV, anisidine value; CAT, catalase; CRP, C-reactive protein; E%, percentage of energy; FA, fatty acids; FO, 8 g/d of fish oil; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; HOSO, 8 g/d of high-oleic sunflower oil; oxFO, 8 g/d of oxidised fish oil; PV, peroxide value; tGSH, total glutathione.

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(PV > 10 mEq/kg and/or AV > 20) have been reported in *n*-3 supplements available for consumers[10–13]. Whether the intake of highly oxidised marine *n*-3 oils is associated with unfavourable health effects is unclear.

Oxidative stress is defined as an imbalance between oxidants and antioxidants, causing oxidative damage[14,15]. In healthy subjects, the endogenous antioxidant defence system, such as glutathione (GSH), are involved in the detoxification of oxidised lipids, as are several antioxidant enzymes[16–18]. Understanding of the absorption of dietary lipid oxidation products in human subjects is limited, and whether the intake of oxidised lipids can lead to oxidative stress and accumulation of oxidative damage is uncertain[16,19–22].

No single markers to determine *in vivo* lipid oxidation exist, and different methods to assess oxidative stress and lipid peroxidation have been suggested. Conjugated dienes and thiobarbituric acid-reactive substances are common methods to measure oxidative stress in human subjects, but for several reasons these methods are considered inappropriate, as reviewed elsewhere[14,23]. At present, 8-iso-PGF$_{2\alpha}$ is suggested as one of the most reliable markers to measure *in vivo* oxidative stress[14,24–26]. Elevated levels of 8-iso-PGF$_{2\alpha}$ and C-reactive protein (CRP) have been observed in a variety of oxidative stress-related diseases and during inflammation[24–29], but the effects of *n*-3 FA on these markers in healthy subjects are inconsistent[30–34]. We do not know of any existing studies investigating whether the intake of oxidised fish oil may affect the level of 8-iso-PGF$_{2\alpha}$ and CRP.

4-Hydroxy-2-hexenal (4-HHE) and 4-hydoxy-2-nonenal (4-HNE) are secondary oxidation products derived from *n*-3 and *n*-6 FA, respectively[4,6,8,35]. These aldehydes have been suggested as markers of *in vivo* lipid peroxidation, but whether these markers are affected by the intake of *n*-3 FA or oxidised lipids is not well documented[36–42].

At present, human studies with the aim of investigating health effects of the intake of oxidised fish oil are lacking, although it has been suggested that the regular consumption of oxidised encapsulated *n*-3 oils may lead to unfavourable health effects[8,14,43–45]. The aim of the present study was to investigate the effect of intake of oxidised fish oil on a variety of markers of oxidative stress, lipid peroxidation and inflammation, and the level of plasma *n*-3 FA in healthy subjects in a 7-week randomised controlled study.

Subjects and methods

Subjects

Healthy, non-smoking men and women aged 18–50 years, with a stable body weight over the last 3 months (±5 %), were recruited among employees and students at Akershus University College from May to September 2009. Exclusion criteria were chronic illnesses and fasting serum level of total cholesterol >7·5 mM, TAG >4 mM, glucose >6·0 mM, CRP >10 mg/l, BMI ≥30 kg/m^2, hypertension (≥160/100 mmHg), pregnancy and lactation. Those with serum levels of thyroxine stimulating hormone, free T3 and free T4 above or below the normal reference ranges were also excluded. Thyroxin replacement therapy and contraceptives were accepted, provided a stable dose was administered during the last 3 months. Use of lipid-lowering and anti-hypertensive medications was not permitted. Fe supplementation was accepted among those with a regular use before inclusion in the study, but was not to be taken concurrently (at the same meal) with the test capsules.

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Regional Committee of Medical Ethics (approval no. 6.2008.2215) and by the Norwegian Social Science Data Services (approval no. 21 924). Written informed consent for participation was obtained from all subjects. The study was also registered at www.clinicaltrial.gov (ID no. NCT01034423).

Study design

A 7-week double-blinded, randomised, controlled parallel-group study was conducted at the Akershus University College from September to December 2009. A total of eighty-three subjects were screened for eligibility, sixty-nine subjects were randomised and fifty-eight subjects received allocated interventions. Subjects lost during follow-up and the number of subjects included in the statistical analysis are further outlined in the flowchart (Fig. 1).

At baseline, subjects were randomly assigned and stratified by sex into one of three intervention groups receiving sixteen capsules per d containing 8 g/d of fish oil (FO; *n* 19; 1·6 g/d EPA/DHA); 8 g/d of oxidised fish oil (oxFO; *n* 19; 1·6 g/d EPA/DHA); or 8 g/d of high-oleic sunflower oil (HOSO; *n* 20) for 7 weeks. The subjects were instructed to take the capsules with food (minimum two meals), and to store the capsule containers at 4°C during the study period. In the 4 weeks leading up to the baseline visit and during the intervention period, the subjects were not allowed to consume fish, fish products, marine *n*-3-enriched food or dietary supplements. The subjects received instructions on which food items to avoid and how to read the food labelling. At each visit, they were reminded by a clinical nutritionist to avoid marine *n*-3 FA products and to keep their weight stable. Fasting body weight was registered at all visits.

During the first 3 weeks of the intervention period, the subjects conducted a fully controlled isoenergetic diet. All foods to be consumed were distributed at the Akershus University College and the food items in the fully controlled diet period were vegetables (e.g. cucumbers, tomatoes, peppers and lettuce), fruits (e.g. oranges, bananas and grapes), juices (apple and orange), low-fat dairy products (milk (1·5 % fat) and yoghurt (0·1 % fat)), toppings (ham (<4 % fat), cheese (16 and 27 % fat), strawberry and raspberry jam and eggs), whole wheat bread (6 % fibre), crackers, chocolate and limited amounts of tea and coffee. Hot lunches (soup and pasta), dinner and desserts (crème brulée, chocolate pudding and mousse) were provided by Fjordland AS (Oslo, Norway). Dinners were delivered as vacuum-packed ready-made dishes. To achieve the individual energy level, deliveries were re-packed and vacuum packed again at Akershus University College. The diet was planned to provide

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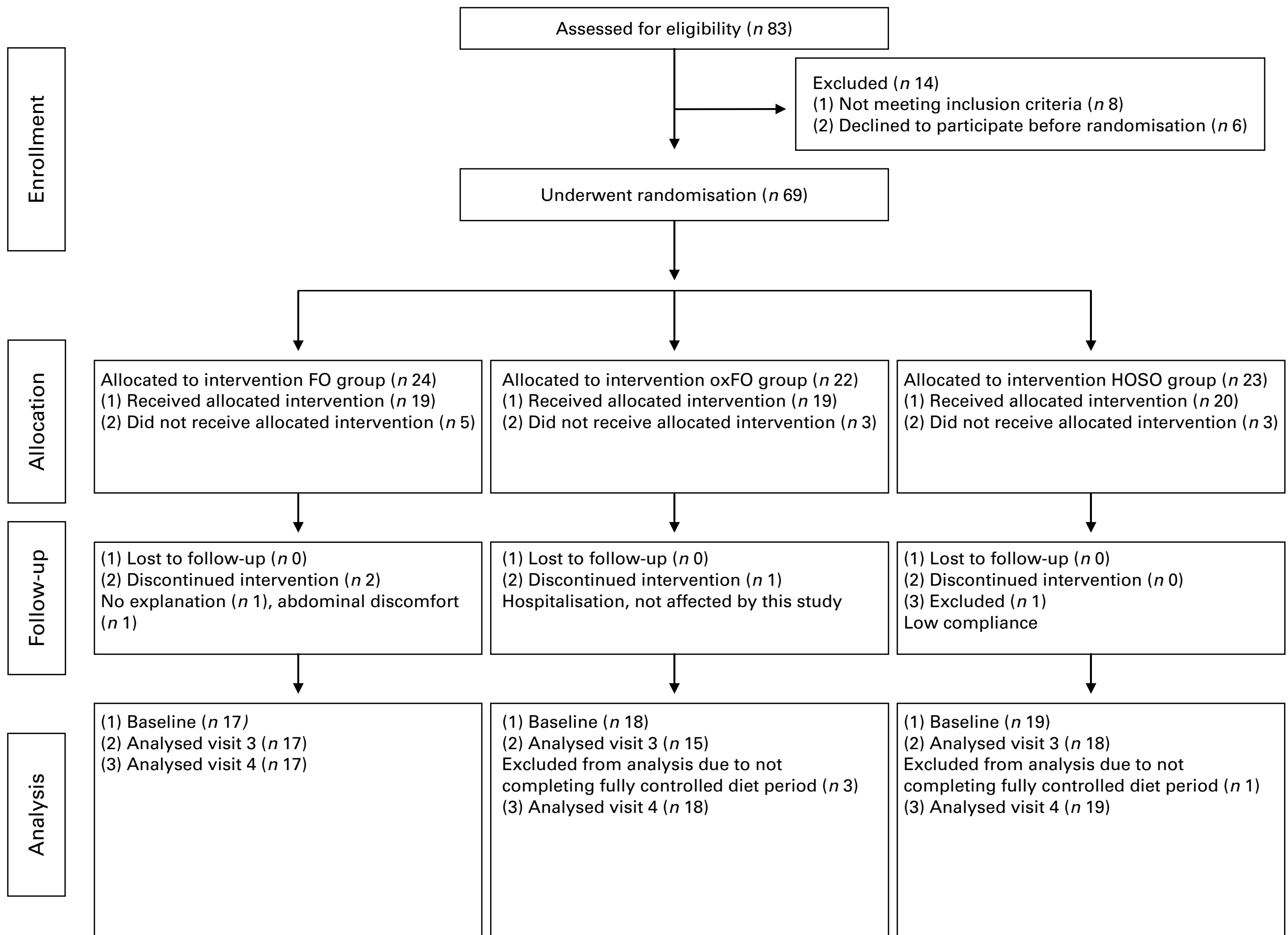

Fig. 1. Flow chart of the study. Number of subjects included, allocated to intervention, lost during follow-up with explanation for the dropouts, and number included in the statistical analysis after 3 and 7 weeks of intervention are shown. FO group, fish oil group; oxFO group, oxidised fish oil group; HOSO, high-oleic sunflower oil group.

(exclusive of capsules) 24 % of energy (E%) from fat, of which 8 E% from SFA, 5 E% from MUFA and 6 E% from PUFA. The protein content was 20 E%, and 57 E% was from carbohydrates, including 5 E% from added sugar. The fibre content was 39 g/d. One alcohol unit was allowed at two occasions during the period. During the last 4 weeks of the intervention period, the subjects continued to take the capsules, but returned to their habitual diet without consuming marine *n*-3 FA food items.

Blinding and randomisation

The study was blinded for the subjects and the study investigators by identical appearance of the different capsules and capsule containers; their contents were only identifiable by the ID numbers on the containers. Randomisation was performed by LINK Medical Research AS (Oslo, Norway), using Microsoft Excel and its random generator. The randomisation code was concealed from the study investigators until the statistical analyses were completed.

Compliance and side effects

Compliance was assessed by capsule count. The capsules were dispensed at baseline and after 3 weeks of intervention, and the subjects were instructed to deliver the unused capsules. We calculated the number of capsules used during the 7-week intervention, which was divided by the number of capsules scheduled for the intervention period[46]. The mean daily capsule count for each subject was expressed as a percentage, and subjects with compliance (mean daily capsule count) <70 % were excluded from the study. Average compliance was 96 (SD 6) % in the FO group (*n* 17), 100 (SD 3) % in the oxFO group (*n* 18) and 97 (SD 6) % in the HOSO group (*n* 19). The rate of reported side effects did not differ among the groups. In all, two subjects, one from each of the different fish oil groups, reported side effects (belching with fishy taste) and three subjects in the HOSO group reported side effects (constipation, urgent to stool and burping).

Study products

Refined and deodorised functional food-grade cod liver oil (*Gadidae* sp.) TINE EPADHA Oil 1200 was provided from TINE SA (Oslo, Norway), from which one batch was divided into two equal parts; one part (FO) was flushed with N_2 and stored at 4°C until encapsulation. The other part (oxFO) was

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oxidised by sparkling pure oxygen through the oil for 20 min twice a day for 21 d, at room temperature, then flushed with N_2 and stored at 4°C in dark until encapsulation. The level of antioxidants (tocopherols and rosemary extracts) was similar in all three study oils. The level of tocopherols was measured in the fish oil (before and after oxidation) and in the refined food-grade HOSO before encapsulation. Tocopherols and rosemary extracts were then added to obtain similar concentration in all three study oils. FO, oxFO and refined food-grade HOSO (AarhusKarlshamn AB, Malmø, Sweden) were encapsulated in 500 mg softgel capsules made of bovine gelatine (Eurocaps Limited, Wales, UK). All capsules were stored in closed containers at 4°C until the start of the study. Each fish oil capsule contained 45 mg EPA and 56 mg DHA (FO) and 46 mg EPA and 56 mg DHA (oxFO). The FA composition was measured in the oils after encapsulation, which is further outlined in Table 1.

Blood and urine sampling

The day before blood sampling, the subjects were told to refrain from alcohol consumption and vigorous physical activity; venous blood samples were drawn after an overnight fast (≥12 h). Serum was obtained from silica gel tubes (Becton Dickinson Vacutainer Systems, Plymouth, UK) and kept at room temperature for at least 30 min, until centrifugation (1500 ***g***, 12 min). Plasma was obtained from EDTA tubes (Becton Dickinson Vacutainer Systems), immediately placed on ice and centrifuged within 10 min (1500 ***g***, 4°C, 10 min). The N_2-flushed plasma samples were snap frozen and stored at −80°C until further analysis. Erythrocytes were isolated from plasma by centrifugation (1800 ***g***, 20°C, 20 min), and either stored at −80°C (for total GSH (tGSH) analysis) or diluted in water (1:1) and stored at −80°C until analysis (antioxidant enzyme activities). Morning spot urine samples were refrigerated (4°C) until delivered, and were immediately aliquoted and stored at −80°C until further analysis.

Routine laboratory analysis

Fasting serum high-sensitive CRP, total cholesterol, LDL-cholesterol, HDL-cholesterol, TAG, glucose, thyroxine-stimulating hormone, free T3, free T4, alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transferase and alkaline phosphatase were measured by standard methods at a routine laboratory (Fürst Medical Laboratory, Oslo, Norway).

tGSH in erythrocytes and urinary 8-iso-$PGF_{2\alpha}$ in morning spot urine samples were determined by Vitas AS (Oslo, Norway). tGSH was measured using a kit provided by Bio Rad Laboratories GmbH (München, Germany) validated for GSH, as described elsewhere[47]. Urinary 8-iso-$PGF_{2\alpha}$ was analysed by liquid chromatography with negative electrospray ionisation coupled to tandem mass spectrometric detection (liquid chromatography/MS/MS), according to Bastani *et al.*[48]. Urinary levels of 8-iso-$PGF_{2\alpha}$ are presented as the ratio 8-iso-$PGF_{2\alpha}$:creatinine.

Determination of hydoxy-nonenal and hydroxy-hexenal in plasma

A method based on Luo *et al.*[49] was further developed and in-house validated for use on human plasma by Nofima (Ås, Norway). ^{2}H-labelled 4-HNE was added to 3–500 μl plasma and the sample was directly derivatised in two steps to generate the *O*-pentafluorobenzyl-oxime-trimethylsilyl derivatives of the *syn*- and *anti*-stereoisomers of the respective 4-hydroxy-alkenals before GC–MS analysis – trimethylchlorosilane. An Agilent 7890A gas chromatograph interfaced with a 5975C mass-selective detector (Agilent Technologies, Little Falls, DE, USA) was used. The *O*-pentafluorobenzyl-oxime-trimethylsilyl derivatives were injected splitless and separated on a HP-5MS-fused silica capillary column (30 m × 0·25 mm × 0·25 μm) using helium as carrier gas at a flow rate of 1 ml/min. The oven temperature was programmed from 50°C (1 min) at 10°C/min to 240°C (0 min) followed by 20°C/min to 300°C (5 min). Derivatised aldehydes were

Table 1. Characterisation of the encapsulated oil

	Fish oil	Oxidised fish oil	High oleic sunflower oil
Fatty acid			
SFA (g/100 g)	15·9	15·8	6·6
MUFA (g/100 g)	46·5	46·3	75·6
PUFA (g/100 g)	28·3	28·4	8·8
n-3 Fatty acids			
EPA (20 : 5*n*-3) (g/100 g)	9·0	9·1	0
DHA (22 : 6*n*-3) (g/100 g)	11·1	11·2	0
DPA (22 : 5*n*-3) (g/100 g)	1·1	1·1	0
ALA (18 : 3*n*-3) (g/100 g)	0·8	0·8	0·3
Oxidation level			
PV (mEq/kg)	4	18	4
AV	3	9	3
Totox	11	45	11
Volatile oxidation products of *n*-3 fatty acid			
Pentanal (μg/100 g)	6·2	137·8	1·1
1-Penten-3-ol (μg/100 g)	12·8	132·4	0·1

DPA, docosapentaenoic acid; ALA, α-linolenic acid; PV, peroxide value; AV, anisidine value.

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measured in negative ion chemical ionisation mode. Ion source temperature was 230°C, with electron ionisation energy of 100 eV and methane as reagent gas. Stereoisomer peaks with the highest intensity (*anti-*) were monitored at *m/z* 291 (4-HHE) corresponding to $(M - C_2F_4 - H_2O)^-$, at *m/z* 283 (4-HNE) corresponding to $(M - CF_2 - H_2O)^-$ and quantification was done by measuring *m/z* 286 corresponding to $(M - CF_2 - H_2O)^-$ of the ^{2}H-labelled 4-HNE internal standard. CV of measured replicated samples was $<8\%$.

Determination of α-tocopherol in plasma

Plasma (500 μl) was added to chloroform–methanol (5 ml; 2:1) and α-tocopherol acetate in chloroform–butylated hydroxytoluene as internal standard, then mixed (5 min) and distilled water (1 ml) was added before centrifugation (1000 rpm, 4°C, 10 min). The organic phase was evaporated under N_2 at 33°C, re-dissolved in *n*-heptane–butylated hydroxytoluene (500 μl) and transferred to HPLC vial. A normal-phase HPLC method was used as described elsewhere[50]. Plasma concentration of α-tocopherol is normalised to the α-tocopherol to total lipid concentration (α-tocopherol/total cholesterol + TAG), expressed as α-tocopherol:plasma total lipid ratio. CV were $<5\%$.

Erythrocyte antioxidant enzyme activities

Activity of the antioxidant enzymes GSH reductase (GR), GSH peroxidase (GPx) and catalase (CAT) were spectrophotometrically assayed in erythrocyte lysates on a Cobas Mira S analyser (Triolab, Brøndby, Denmark) according to Wheeler *et al.*[51]. All measures of enzyme activities were related to the amount of Hb in the sample. Hb content was determined using a commercially available kit (Randox, Ardmore, UK). Samples from each subject were analysed in the same batch in random order and a control sample was analysed for every fifteenth sample. CV were $<7\%$.

Fatty acids analysis

Plasma lipids were extracted by use of the Bligh and Dyer method[52]. FA in Bligh and Dyer extract and oils were derivatised and analysed as methyl esters on a GC (HP 6890) equipped with a BPX-70 column, 60 m × 0·25 mm inner diameter, 0·25-μm film (SGE Analytical Science Private Limited, Melbourne, Australia). The temperature program started at 70°C for 1 min, increased with 30°C/min to 170°C, with 1·5°C/min to 200°C and with 3°C/min to 220°C, with a final hold time of 5 min. Peaks were integrated with HP GC ChemStation software (rev. B.01.01; Agilent Technologies, Palo Alto, CA, USA) and identified by use of external standards. CV were $<5\%$. The concentration of the individual FA was expressed in percentage of total FA.

Oxidised lipids in study products and food items

The PV and AV were measured in the oils after encapsulation using methods according to AOCS Official Method Cd 8-53 and Cd 18-90, respectively. Total antioxidant value (Totox) was calculated as Totox = 2PV + AV (Table 1). A random sample of food items considered prone to oxidation was stored at −80°C until further analysis. Contents of volatile oxidation products in encapsulated oil and food items were analysed by dynamic headspace/GC–MS, as described by Olsen *et al.*[53], with small modifications in the method. A measure of 20 g of homogenised food samples or 2 g oil were heated to 70°C and purged with 100 ml/min N_2 through a Drechsel head for 30 min. Volatiles were adsorbed on Tenax GR (mesh size 60/80; Alltech Associates, Inc., Deerfield, IL, USA). Trapped compounds were desorbed at 250°C for 5 min in a Markes Unity/Ultra TD automatic desorber (Markes International Limited, Llantisant, UK) and transferred to an Agilent 6890 GC System (Agilent, Palo Alto, CA, USA) with an Agilent 5973 mass-selective detector operated in electron impact mode at 70 eV. CV were $<10\%$. The concentrations of the individual volatiles was calculated as μg/g sample based on the internal standards. The food analysis showed that the food items used in the fully controlled diet period contributed to no or only minor levels of volatile oxidation products (data not shown). The concentrations of volatile *n*-3-derived oxidation products in the capsules, pentanal and 1-penten-3-ol, are shown in Table 1.

Statistics

Sample size was calculated using an expected change in plasma *n*-3 FA from baseline to the end of the study of 1·7 (SD 1·2) %. The level of significance was set to 5 % (two-sided) and the power to 80 %. A total of thirty-nine subjects were required in the present study, but a high dropout rate was expected, and it was considered necessary to include a total of seventy-five subjects (twenty-five per arm). Data are presented as mean values and standard deviations or as medians (25th–75th percentiles). Differences between the randomisation groups were analysed at baseline and after 3 and 7 weeks (baseline-adjusted values) of intervention. Data were analysed by one-way ANOVA when normally distributed or by the non-parametric Kruskal–Wallis test when not normally distributed. Bonferroni *post hoc* analysis was performed when the ANOVA analysis was significant. Changes within groups after 7 weeks of intervention were analysed by one-sample *t* test and Wilcoxon signed-rank test when appropriate. Correlation analysis was performed using Spearman's rank-order correlation. All analyses were performed using SPSS for Windows (version 18.0; SPSS, Inc., Chicago, IL, USA).

Results

In the present study, fifty-four healthy subjects (thirty-nine women and fifteen men) participated (Fig. 1). The subjects were 27 (SD 7) years of age, with a mean BMI within the normal range (22·6 (SD 2·6) kg/m^2). At baseline, no significant differences in age, BMI, serum level of glucose, lipids (total cholesterol, LDL-cholesterol, HDL-cholesterol and TAG) or in serum markers of liver enzyme activity (aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transferase and

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alkaline phosphatase) between the different randomisation groups were observed (Table 2). After 7 weeks of intervention, the serum level of lipids, markers of the liver enzyme activity and BMI were not significantly changed between or within the different groups (data not shown).

Plasma 4-hydroxy-2-hexenal and 4-hydoxy-2-nonenal

The median plasma 4-HHE and 4-HNE concentrations at baseline were 3·7 (1·6–5·0) and 3·9 (2·6–5·3) ng/ml, respectively (*n* 54), and no significant differences between the different groups were observed. Changes in plasma 4-HHE and 4-HNE were not significantly different between the randomisation groups after 3 or 7 weeks of intervention. Data after 7 weeks of intervention are presented in Table 3 (data after 3 weeks are not shown). Within the groups, a significant reduction in 4-HNE was only observed after 7 weeks of intervention in the FO group, when compared to baseline ($P=0·03$).

Urinary 8-iso-PGF$_{2\alpha}$

The median baseline level of urine 8-iso-PGF$_{2\alpha}$ per mg creatinine in morning spot urine samples was 281 (170–370) pg/mg (*n* 54). The baseline level of 8-iso-PGF$_{2\alpha}$ per mg creatinine was not significantly different between the groups, and no significant change within or between the different groups was observed during the intervention period. Data after 7 weeks are shown in Table 3 (data after 3 weeks are not shown).

Antioxidant defence system

Median plasma concentrations of α-tocoferol and tGSH at baseline were 22·8 (18·9–27·3) μM (*n* 54) and 1·5 (1·2–1·7) mM (*n* 53), respectively. The median baseline levels of the enzymatic activity of GR, GPx and CAT were 7·8 (7·0–8·8) U/g Hb, 114 (106–124) U/g Hb and 10·1 (8·9–10·6) U/g Hb (*n* 53), respectively. For each of the antioxidant defence markers measured in the present study, we found no significant differences across the randomisation groups at baseline or within and between the groups after 3 (data not shown) or 7 weeks of intervention (Table 3).

Serum high-sensitive C-reactive protein

Median baseline serum level of high sensitive C-reactive protein was 0·7 (0·3–1·7) mg/l (*n* 51), and no significant difference between the groups was observed. No significant changes within or between the randomisation groups were observed after 3 (data not shown) or 7 weeks of intervention (Table 3).

Plasma fatty acids

At baseline, there were no significant differences between the three different groups in the plasma levels of α-linolenic acid, EPA, docosapentaenoic acid, DHA or arachidonic acid (Table 4). Plasma linolenic acid was, however, significantly higher at baseline in the FO group compared to the oxFO and HOSO groups (Table 4). After 3 and 7 weeks of intervention, the plasma level of EPA, docosapentaenoic acid and DHA were significantly increased in both fish oil groups compared to the HOSO group, but no significant difference in EPA, docosapentaenoic acid and DHA between the FO and oxFO groups was observed. After 3 weeks of intervention, plasma level of arachidonic acid was significantly reduced in the FO group compared to the HOSO group ($P=0·01$), and after 7 weeks of intervention, arachidonic acid was significantly reduced in both fish oil groups compared to the HOSO group (Table 4). In both fish oil groups, the *n*-6:*n*-3 ratio was significantly reduced from approximately 10:1 to 5:1 after 7 weeks, regardless of the quality of the oil. The ratio

Table 2. Comparison of the randomisation groups at baseline (*n* 54)*
(Mean values and standard deviations, medians and 25–75th percentiles)

	Fish oil		Oxidised fish oil		High-oleic sunflower oil		
	Baseline		Baseline		Baseline		
	Mean/median	SD/25–75 percentile	Mean/median	SD/25–75 percentile	Mean/median	SD/25–75 percentile	*P*
Male/female (*n*)	5/12		5/13		5/14		
Age (years)†	25	23–32	22	21–28	25	22–31	0·32
BMI (kg/m^2)	22·1	2·5	22·2	1·7	23·5	3·1	0·20
TC (mM)	4·6	0·8	4·7	0·9	4·9	0·8	0·57
LDL-C (mM)	2·5	0·8	2·7	0·8	2·7	0·6	0·63
HDL-C (mM)	1·5	0·3	1·4	0·4	1·5	0·4	0·88
TAG (mM)†	0·8	0·7–0·9	0·9	0·5–1·5	1	0·6–1·2	0·77
Glucose (mM)	4·6	0·3	4·8	0·4	4·8	0·5	0·27
AST (U/l)†	21	19–23	20	17–22	22	19–23	0·61
ALT (U/l)†	19	16–25	16	13–20	18	15–25	0·61
G-GT (U/l)†	15	13–25	16	12–20	16	12–20	0·30
ALP (U/l)†	64	58–92	53	48–70	60	51–68	0·30

TC, total cholesterol; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase; G-GT, γ-glutamyl transferase; ALP, alkaline phosphatase.
* Differences between the groups were calculated using one-way ANOVA test or the Kruskal–Wallis test. *P*-values $< 0·05$ were considered significant.
† Values are medians and 25–75th percentiles.

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Table 3. Markers of lipid peroxidation, oxidative stress and inflammation at baseline and after 7 weeks intervention (*n* 54)†

(Medians and 25–75th percentiles)

	Fish oil				Oxidised fish oil				High-oleic sunflower oil					
	Baseline		End of study		Baseline		End of study		Baseline		End of study			
Variables	Median	25–75th percentile	Median	25–75th percentile	Median	25–75th percentile	Median	25–75th percentile	Median	25–75th percentile	Median	25–75th percentile	*P*‡	*P*§
Plasma 4-hydroxy-2-hexenal (ng/ml)	3·0	1·6–3·8	2·2	1·6–3·6	3·7	1·9–5·0	3·1‖	1·9–5·1	4·3	1·6–5·5	3·5¶	2·1–4·8	0·86	0·54
Plasma 4-hydroxy-2-nonenal (ng/ml)	3·4	2·4–2·2	3·3*	2·7–4·5	4·4	3·7–4·2	4·3‖	1·3–4·9	3·9	2·6–6·3	3·3¶	2·3–4·3	0·54	0·47
Urine 8-iso-PGF$_{2\alpha}$ (pg/mg creatinine)	288	225–339	239	156–320	280	194–381	248	171–307	237	149–360	280	103–414	0·77	0·15
Plasma α-tocopherol/plasma total lipids	4·0	3·6–4·6	4·0	3·6–4·5	4·0	3·5–4·3	4·0	3·5–4·3	3·9	3·7–4·2	4·1	3·7–4·3	0·72	0·67
Erythrocyte tGSH (mM)	1·3‖	1·0–1·7	1·4‖	1·1–1·6	1·5	1·3–1·7	1·7	1·4–2·0	1·6	1·2–1·7	1·3	1·2–1·9	0·45	0·44
Erythrocyte GR (U/g Hb)	7·3‖	6·8–8·6	7·5‖	6·8–8·5	8·3	7·4–9·0	8·2**	7·1–8·5	7·8	7·2–9·2	8·1	7·5–8·8	0·5	0·58
Erythrocyte GPx (U/g Hb)	120‖	115–124	118‖	113–126	110	105–124	109	96–121	113	101–122	111	103–120	0·09	0·54
Erythrocyte CAT (U/g Hb)	10·3‖	9·2–10·6	9·7‖	9·3–10·1	9·3	8·5–10·7	9·6**	8·7–10·0	10·0	8·9–10·4	9·4**	9·2–10·3	0·81	0·7
Serum hsCRP (mg/l)††	0·5‖	0·2–1·2	0·8‖	0·2–1·3	0·6**	0·3–1·4	0·7	0·3–1·7	1·0¶	0·5–2·7	1·2¶	0·6–3·1	0·13	0·68

tGSH, total glutathione; GR, glutathione reductase; GPx, glutathione peroxidase; CAT, catalase; hsCRP, high-sensitive C-reactive protein.

* Values were significantly different between baseline and the end of the study in the fish oil group ($P<0·05$).

† Significance was calculated between the different randomisation groups at baseline, and after 7 weeks using baseline-adjusted values. Analysis was performed by the Kruskal–Wallis test. *P*-values $<0·05$ were considered significant.

‡ Between groups at baseline.

§ Comparing change from baseline to the end of the study between groups.

‖ *n* 16.

¶ *n* 18.

** *n* 17.

†† Subjects with hsCRP $>$ 10 mg/l was excluded from the statistical analysis of hsCRP since $>$10 mg/l coincided with symptoms known to affect hsCRP.

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Table 4. Plasma fatty acids (*n* 54)*
(Mean values and standard deviations)

	Fish oil				Oxidised fish oil				High-oleic sunflower oil					
	Plasma (%wt)				Plasma (%wt)				Plasma (%wt)					
	Baseline		End of study		Baseline		End of study		Baseline		End of study			
Fatty acid	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	*P*†	*P*‡
18 : 2*n*-6 (LA)	32·7	3·4	29·3	4·4	28·2	4·5	28	2·6	28·9	5·0	29·1	3·8	0·01§	0·01‖
20 : 4*n*-6 (AA)	6·3	1·1	5·7	0·8	6·3	1·0	5·7	0·8	6·2	1·7	6·4	1·4	0·97	0·02¶
Sum *n*-6 fatty acid	39·0	3·1	35·0	0·5	34·5	4·8	33·7	2·9	35·1	5·7	35·4	4·2	0·01**	0·01††
18 : 3*n*-3 (ALA)	0·6	0·2	0·5	0·1	0·6	0·2	0·5	0·1	0·6	0·2	0·5	0·1	1·0	0·98
20 : 5*n*-3 (EPA)	0·7	0·2	2·5	0·8	0·6	0·3	2·6	1·0	0·6	0·2	0·7	0·3	0·97	<0·001‡‡
22 : 5*n*-3 (DPA)	0·5	0·1	0·7	0·2	0·5	0·1	0·7	0·1	0·5	0·1	0·5	0·1	0·99	<0·001‡‡
22 : 6*n*-3 (DHA)	2·2	0·5	3·8	0·9	2·0	0·6	3·9	0·8	2·2	0·5	2·2	0·5	0·46	<0·001‡‡
Sum *n*-3 fatty acid	4·0	0·7	7·5	1·8	3·7	0·9	7·7	1·9	4	0·8	3·9	0·8	0·66	<0·001‡‡
n-6LC:*n*-3LC§§	10·2	2·5	5·1	2·1	9·7	2·5	4·8	1·8	9·1	1·6	9·5	2·0	0·3	<0·001‡‡

LA, linolenic acid; AA, arachidonic acid; ALA, α-linolenic acid; DPA, docosapentaenoic acid; LC, long chain.
* Analysis was performed by one-way ANOVA using *post hoc* Bonferroni test when significant. *P*-values <0·05 were considered significant.
† Between groups at baseline.
‡ Comparing change from baseline to the end of the study between groups.
§ Fish oil group *v.* oxidised fish oil group (*P*=0·01) and fish oil group *v.* high-oleic sunflower oil group (*P*=0·03).
‖ Fish oil group *v.* oxidised fish oil group (*P*=0·03) and fish oil group *v.* high-oleic sunflower oil group (*P*=0·008).
¶ Fish oil group *v.* high-oleic sunflower oil group (*P*=0·04) and oxidised fish oil group *v.* high-oleic sunflower oil group (*P*=0·04).
** Fish oil group *v.* high-oleic sunflower oil group (*P*=0·02).
†† Fish oil group *v.* high-oleic sunflower oil group (*P*=0·03) and fish oil group is borderline significant compared to oxidised fish oil group (*P*=0·05).
‡‡ Fish oil group v. high-oleic sunflower oil group (*P*<0·001) and oxidised fish oil group v. high-oleic sunflower oil group (*P*<0·001).
§§ *n*-6LC = 18 : 2*n*-6 + 20 : 4*n*-6; *n*-3LC = 18 : 3*n*-3 + 20 : 5*n*-3 + 22 : 5*n*-3 + 22 : 6*n*-3.

remained unchanged (9:1) during the intervention period in the HOSO group (Table 4).

Correlations

In order to investigate whether the plasma level of 4-HHE was related to the plasma level of *n*-3 FA (sum EPA and DHA in percentage of total FA) at baseline and after 7 weeks of intervention, correlation analyses were performed. No correlations between plasma 4-HHE and plasma level of EPA/DHA were observed at baseline (*n* 54; *R* 0·19, *P* = 0·18) or after 7 weeks among those receiving fish oil (FO and oxFO; *n* 33; *R* −0·19, *P*=0·29).

Discussion

In the present study, we have shown that a daily intake of 8 g of oxidised *n*-3 fish oil (PV 18 mEq/kg oil) did not influence a variety of *in vivo* markers of oxidative stress, lipid peroxidation and inflammation in healthy subjects after 3 or 7 weeks of intervention. The present results do not support the hypothesis that a regular intake of oxidised marine *n*-3 oil has unfavourable health effects in healthy subjects.

Use of *n*-3 capsules are associated with beneficial health effects such as a reduced risk of CHD and CHD deaths[33,54,55]. However, it has been suggested that a high intake of *n*-3 long-chain PUFA could increase *in vivo* lipid peroxidation[8,44,56,57]. The present study does not confirm that the intake of either fish oil or oxidised fish oil affects reliable and established markers of oxidative stress and inflammation such as urinary 8-iso-$PGF_{2\alpha}$ and circulating levels of CRP in healthy human subjects. These results are in accordance with previous *n*-3 FA intervention studies, which have not been able to demonstrate a significant change in 8-iso-$PGF_{2\alpha}$ measured in plasma and spot urine in healthy subjects[32,58,59]. The results are, however, in contrast to studies showing decreased urinary concentration of 8-iso-$PGF_{2\alpha}$ after intake of *n*-3 FA, when measured in 24 h urine collection[33,34,58]. Whether this discrepancy is due to differences in the sampling for the isoprostane measurements is difficult to conclude, as there are also differences in the doses of *n*-3 FA administered in the studies, the duration of the studies and the study populations (patients *v.* healthy subjects). In the present study, we cannot rule out the possibility that measurements of 8-iso-$PGF_{2\alpha}$ in 24 h urine samples would have generated different results. However, we have measured a range of antioxidant and oxidative stress markers (e.g. α-tocopherol, tGSH, CAT activity and GPx activity), in addition to isoprostanes, all showing corresponding results (no effects after *n*-3 supplementation; with similar results for fish oil and oxidised fish oil), which strengthens our findings.

Previous studies also show that the effect of *n*-3 on the circulating level of CRP is inconsistent, and most of the studies observe that intake of *n*-3 FA does not affect CRP[30,31,60]. It has been suggested that 4-HHE and 4-HNE could be used as markers of *in vivo* lipid peroxidation[37,38,42], but to our knowledge these markers have not previously been investigated in a randomised controlled trial with *n*-3 FA. In the present study, a daily intake of *n*-3 FA (approximately 0·7 g EPA and approximately 0·9 g DHA/d) regardless of the oil quality does not seem to affect lipid peroxidation or oxidative stress in healthy subjects. We also did not observe any significant correlation between plasma 4-HHE and the plasma level of EPA + DHA before or after the intervention period. Thus,

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the present results are in contrast to a previous study in which the plasma level of 4-HHE was significantly increased after intake of capsules containing high compared to low doses of DHA (0·8 and 1·6 g/d *v.* 0·2 and 0·6 g/d, respectively), while 4-HNE remained unchanged[41]. Whether 4-HHE is a reliable marker of *in vivo* lipid peroxidation and whether the plasma level can be affected by intake of *n*-3 FA or oxidised lipids is still uncertain and needs to be further investigated.

GSH is the most important endogenous cellular antioxidant with the ability to degrade both primary and secondary lipid oxidation products, and a high capacity of GSH provides optimal activity of GPx and GR[16–18]. Plasma level of GSH and the activity of endogenous antioxidants in erythrocytes have been used to assess changes in oxidative stress status in dietary intervention studies and in diseases linked to oxidative stress[16,47,61,62]. Furthermore, cell studies have demonstrated that GST and GPx are regulators of the homoeostasis of aldehydes, such as 4-HNE, and it has been suggested that these secondary oxidation products could inactivate antioxidant enzyme activity[63,64]. Results from clinical trials with *n*-3 FA in patients with oxidative stress-related diseases are, however, inconclusive. In some studies, antioxidant enzyme activity is increased, whereas in others it is decreased, and in some studies the activity remains unchanged after *n*-3 FA supplementation[65–69]. GSH, GPx and CAT are suggested to play a pivotal role in detoxification of hydroperoxides. We found no significant changes in plasma level of tGSH and α-tocoferol or in the enzymatic activity of GR, GPx and CAT. These findings indicate that the daily intake of FO or oxFO oil did not affect the *in vivo* antioxidant defence system. In the present study, we show that the plasma levels of EPA and DHA in both fish oil groups were significantly increased; the increased levels remained stable after 3 and 7 weeks of intervention. The relative change in plasma EPA and DHA after daily intake of 1·6 g *n*-3 FA confirms the findings of other *n*-3 supplementation studies[70–72]. This clearly shows that the content of hydroperoxides in fish oil supplements, even with a PV that exceeds the European Pharmacopeia for marine *n*-3 oils, does not apparently influence the plasma level of *n*-3 FA. The present understanding of how dietary oxidised lipids are absorbed in human subjects is limited[16,17,21,22,73,74]. It has been suggested that the gastrointestinal tract acts as a barrier against oxidised lipids and that hydroperoxides from the diet are not transferred into the circulatory system[64,74,75]; we do not know whether the oxidised lipids from the oxidised fish oil were eliminated in the gastrointestinal tract and prevented from reaching the circulatory system. Results from animal and cell studies demonstrate that hydroperoxides are converted into secondary oxidation products during the digestion process, and that these components are being absorbed, at least partially[75,76]. Human studies have demonstrated that intake of high doses of oxidised vegetable oils (approximately 50–100 g) alters the endothelial function and increases postprandial level of lipid peroxides in plasma and chylomicrons[77–82]. In the present study, however, we were not able to detect any increase in secondary oxidation products (4-HHE, 4-HNE or 8-iso-PGF$_{2\alpha}$) after 3 or 7 weeks of intervention, nor in chylomicrons after intake of one dose with 9 g of oxidised fish oil (I Ottestad, unpublished results). Our findings contrast with previously published human[45,77–79,81–83] and animal studies[75,84–86] investigating the effects of oxidised oil. The discrepancy among the human studies could, at least partially, be explained by type of oil (vegetable *v.* fish oil), variation in the contribution of hydroperoxides and by the study design (postprandial studies *v.* intervention study). The relevance of animal studies in predicting effects in human subjects remains controversial, as the results from animal studies are not always reproduced in human subjects. In the present study, we did not observe any changes in serum TAG. The TAG-lowering effect from *n*-3 FA has been shown to be dependent on the dose and on baseline TAG level[60]. We have previously shown, in line with the present results, that an intake of 0·9 g EPA + DHA given as fish oil for 7 weeks had no effect on TAG levels in a healthy non-hyperlipidaemic population with baseline levels of TAG ≤1·0 mM[59]. The present study has several strengths such as the study design; using a blinded randomised controlled study design; and a 3-week fully controlled diet period. Moreover, a high compliance was determined by capsule count, which was supported by the significant increase in plasma level of EPA and DHA after intake of fish oil. Given the few and discrete side effects reported, the capsules seemed to be well tolerated and the results in the present study were most probably not affected by any compliance issue. The fish oils used in the present study was provided from one single batch, and the use of highly specific and sensitive methods (GC, GC–MS and HPLC) to measure several biological markers strengthens our findings further. Among the limitations of the present study are the relative short intervention and the fact that the required sample size was estimated on the expected change in plasma EPA + DHA. In addition, whether the composition of the primary and secondary oxidation products generated during the oxidation process at our laboratory reflects the content of oxidation products that could be formed in *n*-3 supplements available to the customers is uncertain.

To our knowledge, this is the first human study investigating the health effects of intake of oxidised *n*-3 oil. The present study shows that a variety of *in vivo* markers of oxidative stress, lipid peroxidation and inflammation are not significantly affected in healthy subjects after the intake of 8 g of highly oxidised fish oil per day for 3 and 7 weeks. Accumulation of lipid peroxidation products has been associated with the pathogenesis of inflammation and oxidative stress-related diseases[14,21,22], but in the present study the oxidative stress status remained unchanged. The relatively short duration of the present study does not allow us to conclude that a long-term intake of oxidised *n*-3 supplements does not have unfavourable health effects. Whether these results are applicable for other marine oils remains uncertain and to what degree the present results are valid in subjects with elevated levels of inflammation and/or oxidative stress needs to be further investigated in larger studies before firm conclusions can be drawn.

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