Oxidized fish oil does not influence established markers of oxidative stress in healthy humans. A randomized controlled trial.

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Abstract

Intake of fish oil reduces the risk of coronary heart disease (CHD) and CHD deaths. Marine omega-3 fatty acids are susceptible to oxidation, but to our knowledge the health effects of intake of oxidized fish oil have not previously been investigated in humans. The aim of the present study was to investigate markers of oxidative stress, lipid peroxidation and inflammation, and the level of plasma omega-3 fatty acids after intake of oxidized fish oil. In a double-blinded randomized controlled study healthy subjects (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 oxidized 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 week 0, 3 and 7. No significant changes between the different groups were observed with regard to urinary 8-iso-prostaglandin $F_{2\alpha}$, plasma levels of 4-hydroxy-2 hexenal (4-HHE), 4-hydroxy-2-nonenal (4-HNE), and α-tocopherol, serum hsCRP or activity of antioxidant enzymes in erythrocytes. Significant increase in plasma level of EPA+DHA was observed in both fish oil groups, but no significant difference between the fish oil groups was observed. No changes in a variety of *in vivo* markers of oxidative stress, lipid peroxidation or inflammation were observed after daily intake of oxidized fish oil for three or seven weeks, indicating that intake of oxidized fish oil may not have unfavourable short-term effects in healthy humans.

Introduction

Intake of fish and fish oil has been related to reduced risk of coronary heart disease (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 gram of marine omega-3 fatty acids (FA) eicosapentaenoic acid (EPA) and docosahexaenoic (DHA) per day. Omega-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 hypertriglyceridemia [2,3].

Long chain omega-3 polyunsaturated FA (LC PUFA) are susceptible to oxidation, and lipid peroxidation leads to 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 level of lipid peroxidation products in refined marine omega-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 meq/kg and 20, respectively [9]. High content of oxidation products (PV >10 meq/kg and/or AV >20) have been reported in omega-3 supplements available for consumers [10-13]. Whether intake of highly oxidized marine omega-3 oils is associated with unfavorable 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 defense system such as glutathione are involved in the detoxification of oxidized lipids, as are several antioxidants enzymes [16-18]. The understanding of the absorption of dietary lipid oxidation products in humans is limited and whether intake of oxidized 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 (TBARs) are methods common methods to measure oxidative stress in humans, but for several reasons these methods are considered inappropriate as reviewed elsewhere [14,23]. At present the 8-iso-prostaglandine-$F_{2\alpha}$ (8-iso$PGF_{2\alpha}$) is suggested as one of the most reliable markers to measure *in vivo* oxidative stress [14,24-26]. Elevated level 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 omega-3 FA

on these markers in healthy subjects are inconsistent [30-34]. We do not know of any existing studies investigating whether intake of oxidized fish oil may affect the level of 8-isoPGF$_{2\alpha}$ and CRP.

The 4-hydroxy-2-hexenal (4-HHE) and 4-hydoxy-2-nonenal (4-HNE) are secondary oxidation products derived from omega-3 and omega-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 intake of omega-3 FA or oxidized lipids is not well documented [36-42].

At present, human studies with the aim of investigating the health effects of intake of oxidized fish oil are lacking, still it has been suggested that regular consumption of oxidized encapsulated omega-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 oxidized fish oil on a variety of markers of oxidative stress, lipid peroxidation and inflammation, and the level of plasma omega-3 FA in healthy subjects in a seven weeks randomized controlled study.

Subjects and methods

Subjects

Healthy, non-smoking men and women aged 18-50 years with a stable body weight over the last three 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 mmol/l, triglycerides > 4 mmol/l, glucose > 6.0 mmol/l, C-reactive protein (CRP) > 10 mg/l, body mass index (BMI) ≥ 30 kg/m^2, hypertension (≥ 160/100), pregnancy and lactation. Those with serum level of thyroxin (TSH), 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 during the last three months. Use of lipid lowering and antihypertensive medications were not permitted. Iron supplementation was accepted among those with a regular use prior to inclusion in the study but was not to be taken concurrently (at the same meal) with the test capsules.

This 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.21924). Written informed consent for participation was obtained from all subjects. The study was also registered at www.clinicaltrial.gov (IDno. NCT01034423).

Study design

A seven-week double-blinded randomized controlled parallel-group study was conducted at Akershus University College from September to December 2009. Eighty-three subjects were screened for eligibility, 69 subjects were randomized, and 58 subjects received allocated interventions. Subjects lost during follow-up and number of subjects included in the statistical analysis is further outlined in the flowchart (Figure 1).

At baseline subjects were randomly assigned and stratified by gender into one of three intervention groups receiving 16 capsules/day containing either: 8 g/d of fish oil (FO) (n=19) (1.6 g/d EPA/DHA), 8 g/d of oxidized fish oil (oxFO) (n=19) (1.6 g/d EPA/DHA) or 8 g/d of high oleic sunflower oil (HOSO) (n=20) for seven weeks. The subjects were instructed to take the capsules with food (minimum 2 meals), and to store the capsule containers at 4ºC during the study period. In the four weeks leading up to the baseline visit and during the intervention period the subjects were not allowed to consume fish, fish products, marine omega-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 omega-3 FA products and to keep the weight stable. Fasting body weight was registered at all visits.

During the first three weeks of the intervention period the subjects conducted a fully controlled isocaloric diet. All foods to be consumed were distributed at Akershus University College and the food items in the fully controlled diet period were vegetables (e.g cucumbers, tomatoes, peppers, lettuce), fruits (e.g. oranges, bananas, grapes), juices (apple- and orange), low fat dairy products (milk (1.5% fat), yoghurt (0.1% fat)), toppings: ham (< 4% fat), cheese (16 and 27% fat), strawberry and raspberry jam and eggs, whole wheat bread (6% fiber), 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 calorie level, deliveries were re-packed and vacuum packed again at Akershus University College. The diet was planned to provide (exclusive of capsules) 24 E% from fat of which 8 E% from saturated FA, 5 E% from monounsaturated FA and 6 E% from polyunsaturated FA. 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 four weeks of the intervention period the subjects continued to take the capsules, but returned to their habitual diet without consuming marine omega-3 FA food items.

Blinding and randomization

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. Randomization was performed by LINK Medical Research AS, Oslo, Norway using Microsoft Excel and its random generator. The randomization 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 was dispensed at baseline and after three weeks of intervention, and the subjects were instructed to deliver the unused capsules. We calculated number of capsules used during the seven weeks intervention, which was divided by the number of capsules scheduled for the intervention period [46]. The mean daily capsule count (MDCC) for each subject was expressed in per cent, and subjects with compliance (MDCC) < 70% were excluded from the study. Average compliance was 96 ± 6% in the FO group (n=17), 100 ± 3% in the oxFO group (n=18) and 97 ± 6% in the HOSO group

(n=19). The rate of reported side effects did not differ among the groups. 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 deodorized 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 4ºC until encapsulation. The other part (oxFO) was oxidized by sparkling pure oxygen through the oil for 20 minutes twice a day for 21 days, at room temperature, then flushed with N_2 and stored at 4ºC 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 prior to 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 Ltd, Wales, UK). All capsules were stored in closed containers at 4ºC until study start. Each fish oil capsule contained 45 mg EPA and 56 mg DHA (FO) and 46 mg EPA and 56 mg DHA (oxFO). The fatty acid composition was measured in the oils after encapsulation, which is further outlined in Table 1.

Blood and urine sampling

The day prior to 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 hours). Serum was obtained from silica gel tubes (Becton Dickinson vacutainer, Plymouth, UK) and kept at room temperature for at least 30 minutes, until centrifugation (1500*g*, 12 min.). Plasma was obtained from EDTA tubes (BD vacutainer, Plymouth, UK), immediately placed on ice and centrifuged within 10 minutes (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*, 20ºC, 20 min.), and either stored at -80ºC (for total glutathione analysis), or diluted in H_2O (1:1) and stored at -80ºC until analysis (antioxidant enzyme activities). Morning spot urine samples were to be refrigerated (4ºC) until delivered, and were immediately aliquoted and stored at -80ºC until further analysis.

197

198 ***Routine laboratory analysis***

199 Fasting serum hsCRP, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides,
200 glucose, TSH, free T3, free T4, alanine-aminotransferase (ALT), aspartame- aminotransferase
201 (AST), gamma-glutamyl transferase (G-GT) and alkaline phosphatase (ALP) were measured
202 by standard methods at a routine laboratory (Fürst Medical Laboratory, Oslo, Norway).
203 Total glutathione (tGSH) in erythrocytes and urinary 8-iso-$PGF_{2\alpha}$ in morning spot urine
204 samples were determined by Vitas AS, Oslo, Norway. tGSH was measured using a kit
205 provided by Bio Rad (Laboratories GmbH, Munchen, Germany) validated for GSH as
206 described elsewhere [47]. Urinary 8-iso-$PGF_{2\alpha}$ was analyzed by liquid chromatography with
207 negative electrospray ionization (ESI) coupled to tandem mass spectrometric detection
208 (LC/MS/MS) according to Bastani et al.[48]. Urinary levels of 8-iso-$PGF_{2\alpha}$ are presented as the
209 ratio 8-iso-$PGF_{2\alpha}$/ creatinine.

210

211 ***Determination of HNE and HHE in plasma***

212 A method based on Luo et al.[49] was further developed and in-house validated for use on
213 human plasma by Nofima, Ås, Norway. Deuterated $D_3$4-HNE was added 3-500 µl plasma and
214 the sample was directly derivatized in two steps to generate the *O*-pentafluorobenzyl-oxime-
215 trimethylsilyl (PFB-TMS) derivatives of the syn- and anti-stereoisomers of the respective 4-
216 hydroxy-alkenals prior to GC-MS analysis: trimethylchlorosilane. An Agilent 7890A gas
217 chromatograph interfaced with a 5975C mass selective detector (Agilent Technologies, Little
218 Falls, DE, USA) was used. The PFB-TMS derivatives were injected splitless and separated on
219 a HP-5MS fused silica capillary column (30mx0.25mmx0.25 µm) using helium as carrier gas
220 at a flow rate of 1ml/min. The oven temperature was programmed from 50°C (1 min.) at
221 10°C/min. to 240°C (0 min.) followed by 20°C/min. to 300°C (5 min.). Derivatized aldehydes
222 were measured in negative ion chemical ionisation mode. Ion source temperature was 230°C,
223 with electron ionization energy of 100 eV with methane as reagent gas. Stereoisomer peaks
224 with the highest intensity (anti-) were monitored at m/z 291 (4-HHE) corresponding to [M-
225 C_2F_4-H2O]$^-$, m/z 283 (4-HNE) corresponding to [M-CF_2-H_2O]$^-$ and quantification was done
226 by measuring m/z 286 corresponding to [M-CF_2-H_2O]$^-$ of the deuterated 4-HNE-D_3 internal
227 standard. Variation of coefficients of measured replicated samples was < 8%.

228

229 ***Determination of α-tocopherol in plasma***

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

Erythrocyte antioxidant enzyme activities

Activity of the antioxidant enzymes glutathione reductase (GR), glutathione 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 haemoglobin (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 15th sample. Coefficients of variation were <7%.

Fatty acids analysis

Plasma lipids were extracted by use of the Bligh and Dyer (B&D) method [52]. Fatty acids in B&D extract and oils were derivatized and analyzed as methyl esters on a GC (HP 6890) equipped with a BPX-70 column, 60m* 0.25mm i.d, 0.25 µm film (SGE Analytical Science Pty Ltd, Austria). The temperature program started at 70°C for 1 min, increased with 30°C min^{-1} to 170°C, 1.5°C min^{-1} to 200°C and 3°C min^{-1} to 220°C with a final hold time of 5 minutes. Peaks were integrated with HP GC ChemStation software (rev. A.05.02) and identified by use of external standards. Coefficients of variation were < 5%. The concentration of the individual fatty acids was expressed in % of total fatty acids.

Oxidized 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. Totox were calculated as Totox=2PV+AV (Table 1). A random sample of food items considered prone to oxidation was stored at -80ºC until further analysis. Content of volatile oxidation products in encapsulated oil and food items were analyzed by Dynamic headspace/GC-MS as described

by Olsen et al. with small modifications of the method [53]. 20 g homogenized food samples or 2 g oil were heated to 70 °C and purged with 100 mL/min. nitrogen through a Drechsel-head for 30 minutes. 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 minutes in a Markes Unity/Ultra TD automatic desorber (Markes International ltd, Llantisant, England) and transferred to an Agilent 6890 GC System (Agilent, Palo Alto, CA, USA) with an Agilent 5973 Mass selective detector operated in electron impact (EI) mode at 70 eV. Coefficients of variation were < 10%. The concentration of the individual volatiles was calculated as µg per gram 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 omega-3 FA from baseline to end of study of 1.7% (SD 1.2). The level of significance was set to 5% (two-sided) and the power 80%. A total of 39 subjects were required in this study, but a high dropout rate was expected and it was considered necessary to include a total of 75 subjects (25 per arm). Data are presented as mean (± SD) or median (25-75 percentiles). Differences between the randomization groups were analyzed at baseline and after three and seven weeks (baseline-adjusted values) of intervention. Data was analyzed 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 seven weeks of intervention were analyzed 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 (version18.0).

Results

In this study 54 healthy subjects (39 women and 15 men) participated (Figure 1). The subjects were 27 ± 7 years of age, with a mean BMI within the normal range (22.6 ± 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 triglycerides) or in serum markers of liver enzyme activity (AST, ALT, G-GT and ALP) between the different randomization groups were observed (Table 2). After seven 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-HHE and 4-HNE

The median plasma 4-HHE and 4-HNE concentrations at baseline were 3.7 ng/ml (1.6-5.0 ng/ml) and 3.9 ng/ml (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 randomization groups after three or seven weeks of intervention. Data after seven weeks of intervention are presented in Table 3 (data after three weeks are not shown). Within the groups a significant reduction in 4-HNE was only observed after seven weeks of intervention in the FO group, when compared to baseline (P=0.03).

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

Median baseline level of urine 8-iso-PGF$_{2\alpha}$/mg creatinine in morning spot urine samples was 281 pg/mg (170-370 pg/mg) (n=54). The baseline level of 8-iso-PGF$_{2\alpha}$/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 seven weeks are shown in Table 3 (data after three weeks are not shown).

Antioxidant defence system

Median plasma concentrations of α-tocoferol and tGSH at baseline were 22.8 µmol/l (18.9-27.3 µmol/l) (n=54) and 1.5 mM (1.2-1.7 mM) (n=53), respectively. The median baseline levels of the enzymatic activity of glutathione reductase (GR), glutathione peroxidase (GPx) and catalase (CAT) were 7.8 U/g Hb (7.0 – 8.8 U/g Hb), 114 U/g Hb (106-124 U/g Hb) and 10.1 U/g Hb (8.9-10.6 U/g Hb) (n=53), respectively. For each of the antioxidant defence markers measured in this study we found no significant differences across the randomization

groups at baseline or within and between the groups after three (data not shown) or seven weeks of intervention (Table 3).

Serum hs-CRP

Median baseline serum level of hsCRP was 0.7 mg/l (0.3-1.7 mg/l) (n=51), and no significant difference between the groups was observed. No significant changes within or between the randomization groups were observed after three (data not shown) or seven 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 alpha linolenic acid (ALA), EPA, docosapentaenoic acid (DPA), DHA or arachidonic acid (AA) (Table 4). Plasma linolenic acid (LA) was however significantly higher at baseline in the FO group compared to the oxFO and HOSO groups (Table 4). After three and seven weeks of intervention the plasma level of EPA, DPA and DHA were significantly increased in both fish oil groups compared to the HOSO group, but no significant difference in EPA, DPA and DHA between the FO and oxFO groups was observed. After three weeks of intervention plasma level of AA was significantly reduced in the FO group compared to the HOSO group (P=0.01), and after seven weeks of intervention AA was significantly reduced in both fish oil groups compared to the HOSO group (Table 4). In both fish oil groups the omega-6/omega-3 ratio was significantly reduced from approximately 10:1 to 5:1 after seven weeks, regardless of the quality of the oil. The ratio 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 omega-3 FA (sum EPA and DHA in per cent of total FA) at baseline and after seven 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 seven 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 oxidized omega-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 three or seven weeks of intervention. Our results do not support the hypothesis that a regular intake of oxidized marine omega-3 oil has unfavourable health effects in healthy subjects.

Use of omega-3 capsules are associated with beneficial health effects such as reduced risk of CHD and CHD deaths [33,54,55]. However, it has been suggested that high intake of omega-3 LC PUFA could increase *in vivo* lipid peroxidation [8,44,56,57]. The present study does not confirm that intake of either fish oil or oxidized fish oil affects reliable and established markers of oxidative stress and inflammation, such as urinary 8-iso-$PGF_{2\alpha}$ and circulating level of CRP in healthy humans. These results are in accordance with previous omega-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 omega-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 since there are also differences in the doses of n-3 fatty acids administered in the studies, the duration of the studies and the study populations (patients versus 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, catalase 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 omega-3 on the circulating level of CRP is inconsistent and most of the studies observe that intake of omega-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 randomized controlled trial with omega-3 FA. In the present study a daily intake of omega-3 FA (~0.7 g EPA and ~0.9 g DHA per day) regardless of the oil quality does not seem to affect lipid peroxidation or oxidative stress in healthy subjects. Nor did we observe any significant correlation between plasma 4-HHE and the plasma level of EPA+DHA before or after the intervention period. Thus our results are in contrast to a

previous study where the plasma level of 4-HHE was significantly increased after intake of capsules containing high compared to low doses of DHA (0.8 g/d and 1.6 g/d vs 0.2 g/d and 0.6 g/d, respectively), whilst 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 omega-3 FA or oxidized 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 glutathione peroxidase (GPx) and glutathione reductase (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 homeostasis of aldehydes, such as 4-HNE, and it has been suggested that these secondary oxidation products could inactivate the antioxidant enzyme activity [63,64]. Results from clinical trials with omega-3 FA in patients with oxidative stress related diseases are however inconclusive. In some studies the antioxidant enzyme activity is increased whilst in others it is decreased, and in some studies the activity remains unchanged after omega-3 FA supplementation [65-69]. GSH, GPx and catalase are suggested to play a pivotal role in detoxification of hydroperoxides. We find 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 three and seven weeks of intervention. The relative change in plasma EPA and DHA after daily intake of 1.6 g omega-3 FA confirms the findings of other omega-3 supplementation studies [70-72]. This clearly shows that the content of hydroperoxides in fish oil supplement, even with a PV that exceeds the European Pharmacopeia for marine omega-3 oils, does not apparently influence the plasma level of omega-3 FA. The current understanding of how dietary oxidized lipids are absorbed in humans is limited [16,17,21,22,73,74]. It has been suggested that the gastrointestinal tract act as a barrier against oxidized lipids and that hydroperoxides from the diet are not transferred into the circulatory system [64,74,75]; we do not know whether the oxidized lipids from the oxidized fish oil were eliminated in the gastro intestinal 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 oxidized vegetable oils (~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 have not been able to detect any increase in secondary oxidation products (4-HHE, 4-HNE, 8-iso-$PGF_{2\alpha}$) after three or seven weeks of intervention nor in chylomicrons after intake of one dose with 9 g oxidized fish oil (unpublished data). Our findings contrast with previously published human [45,77-79,81-83] and animal studies [75,84-86] investigating the effects of oxidized oil. The discrepancy among the human studies could at least partially be explained by type of oil (vegetable vs fish oil), variation in the contribution of hydroperoxides and by the study design (postprandial studies vs intervention study). The relevance of animal studies in predicting effects in humans remains controversial, as the results from animal studies are not always reproduced in humans. In the present study, we did not observe any changes in serum triglycerides. The triglyceride lowering effect from omega-3 FA has been shown to be dependent on the dose and the baseline triglyceride 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 seven weeks had no effect on triglyceride levels in a healthy non-hyperlipidemic population with baseline levels of triglycerides ≤ 1.0 mmol/L[59]. Our study has several strengths such as the study design; using a blinded randomized controlled study design and a three weeks 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 this study were most likely not affected by any compliance issue. The fish oils used in our study was provided from one single batch and the use of highly specific and sensitive methods (GC, GC-MS, 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. Also, 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 omega-3 supplements available to the customers is uncertain.

To our knowledge this is the first human study investigating the health effects of intake of oxidized omega-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 intake of 8 g highly oxidized fish oil per day for three and seven weeks. Accumulation of lipid peroxidation products has been associated with the pathogenesis of inflammation and

458 oxidative stress related diseases [14,21,22], but in the present study the oxidative stress status
459 remained unchanged. The relatively short duration of our study does not allow us to conclude
460 that long term intake of oxidized omega-3 supplements do not have unfavourable health
461 effects. Whether these results are applicable for other marine oils remains uncertain and to
462 what degree our results are valid in subjects with elevated level of inflammation and/or
463 oxidative stress needs to be further investigated in larger studies before firm conclusions can
464 be drawn.
465

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Conflicts of interest: Inger Ottestad, Gjermund Vogt, Kjetil Retterstøl, Mari C. Myhrstad, John-Erik Haugen, Astrid Nilsson, Gitte Ravn-Haren, Lene F. Andersen, Kirsten B. Holven and Stine M. Ulven have "no conflicts of interest", or any financial or personal interest. Kirsti W. Brønner is clinical nutritionist/Project manager and Berit Nordvi is research manager on ingredients in TINE SA R&D Center, Norway. They have no financial interest.

The study was registered at www.clinicaltrials.gov, IDno. NCT01034423

Designed research (project conception, development of overall research plan, and study oversight): IO, GV, AN, KR, KWB, BN, LFA, MCM, KBH, SMU
Conducted research (hands-on conduct of the experiments and data collection): IO, GV, JEH, AN, KR, KBH, MCM, GRH, SMU
Provided essential reagents or provided essential materials (applies to authors who contributed by providing animals, constructs, databases, etc., necessary for the research): GV, AN, KWB, BN
Analyzed data or performed statistical analysis: IO, KBH, SMU
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