Microalgal docosahexaenoic acid decreases plasma triacylglycerol in normolipidaemic vegetarians: a randomised trial

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Triacylglycerol (TG) lowering effects of *n*-3 long-chain PUFA (*n*-3 LCPUFA) have been repeatedly demonstrated, but studies investigating the individual effects of EPA or DHA on plasma TG and lipoproteins in man are rare. The effects of a new DHA-rich, almost EPA-free microalgae oil (*Ulkenia* sp.) on plasma lipids and several safety parameters were investigated in a double-blind, placebo-controlled, parallel design intervention study. Normolipidaemic vegetarians (eighty-seven females, twenty-seven males) consumed daily microalgae oil (0·94 g DHA/d) or olive oil (as placebo) for 8 weeks. DHA supplementation decreased plasma TG by 23 % from 1·08 (SEM 0·07) to 0·83 (SEM 0·04) mmol/l ($P<0.001$). Absolute TG decreases after DHA supplementation were inversely correlated to baseline TG concentrations ($r - 0.627$, $P<0.001$). Plasma total, LDL and HDL cholesterol increased significantly in the DHA group, resulting in lower TG:HDL cholesterol and unchanged LDL:HDL and total cholesterol:HDL cholesterol ratios. The intake of DHA-rich microalgae oil did not result in any physiologically relevant changes of safety and haemostatic factors. In conclusion, DHA-rich oil from microalgae *Ulkenia* sp. was well tolerated and can be considered a suitable vegetarian source of *n*-3 LCPUFA. Although DHA supplementation improved some CHD risk factors (plasma TG, TG:HDL cholesterol ratio), LDL cholesterol increased. Therefore, the overall effects of this intervention on CHD risk deserve further investigation.

Docosahexaenoic acid: Microalgae oil: Plasma lipids: Triacylglycerol

Circulating triacylglycerol (TG) levels in the fasting and postprandial states are associated with the severity and progression of atherosclerosis (Hodis, 1999) and are recognised as independent risk factors for CHD (Hokanson & Austin, 1996). A meta-analysis by Austin *et al.* (1998) suggested that after adjustment for HDL cholesterol and other risk factors, each 1 mM increase in TG is associated with a 14 % increase in CHD in men and a 37 % increase in women. Griffin (2001) concluded there is convincing evidence to show that even moderately raised plasma TG (>1.5 mM), which has a predicted frequency of between 25 and 30 % in middle-aged men and postmenopausal women, confer increased cardiovascular risk in otherwise normal, healthy individuals.

Long-chain *n*-3 PUFA (*n*-3 LCPUFA) reduce TG concentrations in man (Conquer & Holub, 1996; Davidson *et al.* 1997; Grimsgaard *et al.* 1997; Nelson *et al.* 1997; Stark & Holub, 2004; Nestel *et al.* 2002). In a meta-analysis of sixty-five studies, Harris (1997) concluded that an average dose of 4 g EPA and DHA per day results in a 25–30 % decrease of fasting TG in both normolipidaemic and hypertriacylglycerolaemic subjects. It has been assumed that the hypotriglyceridaemic effect of *n*-3 LCPUFA is mediated by several mechanisms such as increased hepatic fatty acid oxidation, inhibition of fatty acid and TG synthesis, and reduced assembly and secretion of VLDL TG (Nestel, 2000). Most studies of

n-3 LCPUFA have generally used oils containing mixtures of EPA and DHA in the range of 1–5 g/d, but studies investigating specifically the effects of one *n*-3 LCPUFA on TG and lipoproteins in man are rare.

Studies applying single-cell oil as DHA source, which contain only trace amounts of EPA and other *n*-3 fatty acids, reported a significant reduction in TG concentrations and an increase in HDL cholesterol levels at DHA intakes in the range of 1·25–2·5 g/d in normo- or hyperlipidaemic volunteers (Agren *et al.* 1996; Conquer & Holub, 1996; Davidson *et al.* 1997; Nelson *et al.* 1997), whereas other studies with DHA intakes of 0·7 g/d (Theobald *et al.* 2004) and 0·75–1·5 g/d (Conquer & Holub, 1998) did not find any significant effects on plasma TG and lipoproteins in normolipidaemic subjects.

Few studies have compared the effects of purified EPA and DHA (as ethyl esters), showing inconsistent results. In a study by Rambjor *et al.* (1996), supplementing normolipidaemic subjects with 3 g/d EPA or DHA for 3 weeks, EPA, but not DHA, had a TG-lowering effect. Other studies show that both EPA and DHA lower serum TG concentrations at intakes of 3·0–4·9 g/d (Grimsgaard *et al.* 1997; Mori *et al.* 2000; Nestel *et al.* 2002; Buckley *et al.* 2004), whereas HDL cholesterol concentrations tend to increase only with DHA supplementation (Rambjor *et al.* 1996; Grimsgaard *et al.* 1997; Mori *et al.* 2000). Future trials will be needed

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to determine minimum effective dosages of EPA and DHA over lengthy periods and to show reduction of CHD by intervention.

The aim of the present study was to investigate the effects of a relatively low dose of DHA (0·94 g/d, derived from microalgae *Ulkenia* sp.) on conventional cardiovascular risk factors (plasma total cholesterol, lipoproteins and TG) and on several safety parameters (e.g. haematology, biochemical markers of liver and cardiac functions, and certain haemostatic risk factors) in normolipidaemic vegetarians. Measures of total cholesterol:HDL cholesterol, LDL cholesterol:HDL cholesterol and TG:HDL cholesterol ratios were of particular interest as by some authors they are considered superior predictors of CHD compared with total and LDL cholesterol levels (Grover *et al.* 1994, 1995; Kinosian *et al.* 1994, 1995; Gaziano *et al.* 1997).

Subjects and methods

Subjects and study design

One hundred and fourteen free-living, apparently healthy vegetarians aged 18–43 years (eighty-seven females, twenty-seven males) were recruited in the Munich area via posters displayed in health food shops and on the university campus, and through personal contacts. Inclusion criteria included adherence to a vegetarian diet for at least one year (no meat, less than one fish meal per month), age ≥ 18 years and BMI between 18 and 25 kg/m². Exclusion criteria were an intake of medication with known influence on the lipid metabolism during the last 3 months, intake of n-3 fatty acid supplements, presence of metabolic, cardiovascular, renal or neurological diseases, or pregnancy and lactation. The study was approved by the ethics committee of the Bavarian Board of Physicians. Written informed consent was obtained from all subjects. Participants received a financial compensation of 200 Euro each for their participation in the study.

The study was conducted between June and November 2003 as a randomised double-blind, placebo-controlled intervention study with two parallel groups. The subjects consumed 2·28 g daily of either DHA-rich oil from microalgae *Ulkenia* sp. providing 0·94 g DHA, or the same amount of olive oil (as placebo) for 8 weeks. They were randomly assigned to one of the intervention groups with stratification for gender. There was no difference between the groups with respect to age, BMI, blood pressure (BP), heart rate, duration of vegetarian diet, proportion of non-smokers and gender (Table 1). Before the first visit, the subjects had to complete a questionnaire, which included a survey on medications, metabolic diseases, CVD, dietary supplements, frequency of fish and egg consumption, and a 3 d dietary record (two weekdays and one weekend day). During the intervention, the subjects noted side-effects, signs of illness, intake of medication and the number of capsules not consumed. At the end of the intervention period, they recorded their diet again for 3 d. At baseline and after 56–60 d of intervention, fasted blood samples were obtained and body weight, height, BP and heart rate were measured. Telephone interviews were performed every 2 weeks of the intervention period to monitor study compliance, side-effects and intercurrent disease. Compliance was assessed by counting leftover capsules and calculated as the percentage of the prescribed capsules taken.

Study oils

Each DHA capsule contained 571 mg oil derived from microalgae *Ulkenia* sp. (Nutrinova® DHA; Nutrinova GmbH, Frankfurt/Main, Germany). Nutrinova® DHA is a highly concentrated, TG-based oil, containing at least 43 g DHA/100 g total fatty acids (wt%) and almost no EPA. Matching placebo capsules contained 562 mg olive oil, which is free of n-3 LCPUFA. The fatty acid composition of the two study oils as determined during the intervention period is given in Table 2. To ensure that the treatments had a similar antioxidant content, each oil contained 1000 ppm mixed natural tocopherols (equals 2·2–2·3 mg mixed natural tocopherols per day). The volunteers were required to take one capsule each during breakfast and dinner and two capsules during lunch (a total of four capsules per day). Capsules were stored refrigerated or at room temperature in a dry, dark place.

Table 1. Baseline characteristics of the subjects

	DHA (*n* 59)		Placebo (*n* 55)		
	Mean	SD	Mean	SD	P*†
No. of female/male subjects	44/15		43/12		0·667
Age (years)	25·7	5·4	26·1	5·8	0·902
BMI (kg/m²)	21·4	1·8	21·2	2·0	0·532
Blood pressure (mmHg)					
Systolic	98	10	96	8	0·571
Diastolic	67	9	67	7	0·733
Heart rate (beats/min)	67	9	67	7	0·870
Years on a vegetarian diet	9·8	5·5	8·8	4·8	0·442
Non-smokers (%)	75		80		0·512

DHA, docosahexaenoic acid.

* The between-group differences at baseline were analysed using Student's unpaired *t* test (BMI) or Mann-Whitney *U* test (age, systolic and diastolic blood pressure, heart rate, years on a vegetarian diet).

† For bivariate tabular analysis the χ^2 test was used (gender ratio, number of non-smokers).

Table 2. Major fatty acids of microalgae and placebo oil (g/100 g fatty acids)

	Microalgae oil (*n* 12)		Placebo oil (*n* 12)	
	Mean	SD	Mean	SD
SFA	39·99	0·25	14·62	0·98
18:1*n*-9	0·52	0·03	75·86	0·76
18:1*n*-7	0·08	0·01	2·37	0·04
18:2*n*-6	1·21	0·08	5·32	0·14
18:3*n*-6	0·22	0·01	ND	
20:4*n*-6	0·09	0·01	ND	
22:5*n*-6	9·70	0·14	ND	
18:3*n*-3	0·11	0·01	0·68	0·02
20:5*n*-3	0·29	0·01	ND	
22:5*n*-3	0·09	0·01	ND	
22:6*n*-3	46·13	0·20	ND	

ND, not detected; SFA, saturated fatty acids.

Measurements, blood sampling and storage

All anthropometric measurements followed standardised procedures. Subjects were weighed before and after the intervention; height was measured only at entry. Seated BP was determined using a stethoscope and a standard sphygmomanometer. Heart rate was measured for 15 s at the wrist.

Venous blood samples were collected from an antecubital vein of the forearm into EDTA-, citrate- and lithium heparin-containing tubes (Sarstedt, Nümbrecht, Germany) as well as special tubes for platelet function analysis (containing sodium citrate, 1:10) after an overnight fast. For factor VII, von Willebrand factor and PAI-1 analyses, citrated blood, and for vitamin E and fatty acid analyses, EDTA blood was centrifuged at $1000\,g$ for 7 min at room temperature within 2 h. The sub-samples of plasma were stored at $-80°C$ and analysed within 12 months of storage; these parameters for each subject were analysed in a single batch at the end of the study. The other biochemical parameters were analysed on the day of blood drawing with routine methods in the clinical chemistry laboratories of the University of Munich hospital.

Analytical methods

Three-day dietary records were entered into the computer program Prodi version 4·5 LE 2003 (Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, Germany). Nutrient intake was calculated based on the German Nutrient Data Base BLS, version II.3 (BgVV, Berlin, Germany).

Fatty acids from the two study oils, plasma phospholipids as well as erythrocyte phosphatidylcholine, phosphatidylethanolamine and total lipids were analysed by capillary GLC (Geppert *et al.* 2005).

TG, total and HDL cholesterol concentrations were determined in lithium heparin plasma by standard enzymatic methods on a Cobas Integra 800 automated sample processor (Roche, Mannheim, Germany) with the appropriate reagent systems. LDL cholesterol concentrations were calculated using the Friedewald formula (Friedewald *et al.* 1972).

Platelet function analysis was performed within 2 h of blood collection in citrated whole blood using a Platelet Function Analyser (PFA-100; Dade Behring, Darmstadt, Germany). Membranes pre-coated with collagen/epinephrine or collagen/adenosine diphosphate were used to stimulate platelet aggregation. Prothrombin time, partial thromboplastin time and fibrinogen were measured in citrated plasma using an automated analyser (Amelung Amax CS-190; Trinity Biotech, Darmstadt, Germany) with commercially available kits (Dade Behring, Marburg, Germany). The concentration of d-dimers was determined in citrated plasma on a Roche Hitachi autoanalyser (Roche) with the appropriate test kit. PAI-1 activities were measured using the Coatest PAI-1 assay kit (Chromogenix-Instrumentation Laboratory, Milan, Italy). Factor VII was measured using an ACL 9000 automated analyser (Instrumentation Laboratory, Milan, Italy) and factor VII-deficient plasma (HemosIL, Factor VII-deficient plasma; Instrumentation Laboratory). Von Willebrand factor analysis was performed on a Behring Coagulation System (BCS; Dade Behring) with a commercial test kit (IL Test™ Von Willebrand Faktor; Instrumentation Laboratory).

Full blood cell counts were performed in EDTA blood on a coulter analyser (Beckmann Coulter Micro Diff II, Krefeld, Germany). Bilirubin, creatinine, γ-glutamyl transpeptidase, alanine aminotransferase, aspartate aminotransferase, cholinesterase, creatine kinase, lactate dehydrogenase, uric acid, total protein, C-reactive protein and glucose were measured in lithium heparin plasma on a Roche Hitachi 912 automated sample processor (Roche) with the appropriate reagent systems.

For α-tocopherol analysis, 50 μl internal standard (retinyl acetate in ethanol–butylated hydroxytoluene) and 500 μl precipitation solution (Immundiagnostik AG, Bensheim, Germany) were added to 250 μl plasma and the tube was vortexed for 15 s. Hexane (1 ml) was added, the tube was vortexed for 30 s and centrifuged at $1500\,g$ for 5 min. This step was performed twice. The hexane extracts were combined, evaporated under nitrogen and redissolved in 100 μl mobile phase, which consisted of acetonitril–tetrahydrofuran–methanol–ammonium acetate solution (684:220:68:28, by vol.; Hess *et al.* 1991). The α-tocopherol content of the extracts was quantified by HPLC using a RP 18 analytical column (LiChroCART 250-3, LiChrospher 100, RP18 (5 μm); Merck, Darmstadt, Germany) and UV-visible detection at 292 nm. Intra- and inter-assay CV were below 4 %.

Statistical methods

All statistical analyses were done using the Statistical Package for the Social Sciences, version 12.0 (SPSS Inc., Chicago, IL, USA). After checking for the normal distribution of the data, any differences between the groups at baseline or the impact of treatment on the absolute changes (week 8 minus week 0) in each treatment group were tested for significance using Student's unpaired *t* test for normally distributed variables or the Mann–Whitney *U* test for variables not normally distributed. Within each of the two groups, changes from 0 to 8 weeks were determined by Student's *t* test for dependent samples and Wilcoxon non-parametric test, respectively. Correlations between parameters were estimated by computing Pearson's correlation coefficient in the case of normally distributed values and Spearman's Rho correlation coefficient in the case of other distributions, respectively. For bivariate tabular analysis the χ^2 test was used. In cases of expected values smaller than 5, a Fisher exact test was used instead. Unless otherwise noted, results are given as mean values with their standard errors. $P < 0·05$ was considered significant.

Results

Two of the 114 subjects recruited into the study dropped out during the intervention period: one subject in the placebo group came down with a renal colic, which was considered unrelated to the dietary supplement, and the contact with one subject of the DHA group was lost. Five individuals in the DHA group and one in the placebo group were excluded from the analyses. The reasons for exclusions were suspected hypertriacylglycerolaemia (basal TG > 2·5 mmol/l, *n* 2), poor compliance with study protocol (*n* 2) and diarrhoea/vomitus for more than 6 d of the intervention period (*n* 2, one subject from each group). Thus, 106 subjects are included in the present analysis.

Compliance and side-effects

The median number of days in the study was 56 (range 56–60 d) in both groups. Compliance as judged by capsule count was 98 (SD 2) % for the DHA group and 99 (SD 2) % for the placebo group, with no significant between-group difference. Side-effects were reported in the DHA group by 11 % and in the placebo group by 8 % of the subjects (Table 3) including gastro-intestinal upsets (flatulence, pain, diarrhoea, belching) and minor skin reactions (acne). Side-effects were evenly distributed between DHA and placebo group.

Diet, body weight, blood pressure and heart rate

Baseline intakes of EPA + DHA (median, 5th and 95th percentile in parentheses) were 23 (0, 121) mg/d in the DHA group and 23 (3, 118) mg/d in the placebo group and did not change in either group during intervention (Geppert *et al.* 2005).

Body weight, BMI, BP and heart rate did not differ between DHA and placebo group at baseline and changes from baseline were not significantly different between the two groups. An examination of within-group changes demonstrated a slight but significant increase of systolic BP in the placebo group. In the DHA group we observed the same trend, but the change did not reach significance ($P = 0.066$). Body weight and BMI increased significantly by 0.5 kg or 0.2 kg/m^2 in the placebo group.

Plasma and erythrocyte fatty acids

Fatty acid compositions of erythrocyte total lipids, phosphatidylcholine and phosphatidylethanolamine as well as plasma phospholipids were not different between groups at baseline and changed negligibly in the placebo group (phospholipid data are shown in Table 4). After DHA supplementation, no change or little increase was observed for saturated fatty acids (16:0 and 18:0). The MUFA 18:1n-9 decreased significantly in all measured fractions relative to baseline. Microalgae oil supplementation resulted in significant increases of 22:5n-6, EPA and DHA levels and significant decreases of 18:2n-6, 20:4n-6 and 22:5n-3 levels in all measured fractions relative to baseline (Geppert *et al.* 2005).

Plasma triacylglycerol and lipoproteins

Measured lipid metabolism parameters were not different between the two groups at study entry and did not change

Table 3. Reported side-effects of the intervention (number and %)*

	DHA (n 53)		Placebo (n 53)	
	n	%	n	%
Total side-effects	6	11·3	4	7·5
Skin reactions	–		1	1·9
Flatulence	3	5·7	2	3·8
Stomach ache	2	3·8	1	1·9
Diarrhoea	–		2	3·8
Belching	2	3·8	1	1·9

* Side-effects were evenly distributed between DHA and placebo group ($P > 0.05$).

in the placebo group (Table 5). A significant 23 % decrease ($P < 0.001$) in plasma TG concentrations from 1·08 to 0·83 mmol/l was found in the DHA group. Absolute TG changes from baseline were significantly different between the DHA group (−0·25 mmol/l, mean) and the placebo group (−0·00 mmol/l). TG changes (absolute and percentage) after DHA supplementation were inversely correlated with TG concentrations at baseline (Fig. 1). After DHA supplementation, observed TG concentrations did not exceed 1·5 mmol/l, whereas after placebo intervention the values were distributed beyond 2·5 mmol/l. The number of subjects with plasma TG levels > 1·5 mmol/l did not differ between the DHA and placebo group at week 0 (17 % *v.* 15 %), but after the intervention the frequency was significantly lower in the DHA-supplemented group compared to the placebo group (0 % *v.* 21 %).

Plasma total, LDL and HDL cholesterol increased significantly in the DHA group; the changes from baseline in these parameters were significantly different between DHA and placebo group (Table 5). We found a negative correlation between absolute changes in TG and in HDL concentrations after DHA supplementation (r −0·308, $P = 0.025$). In the DHA group, there were no correlations between individual DHA changes in plasma/erythrocyte and changes in plasma TG, total, LDL and HDL cholesterol concentrations (data not shown).

The ratio of TG to HDL cholesterol was significantly reduced from 0·75 (SEM 0·08) to 0·51 (SEM 0·04) in the DHA group ($P < 0.001$) and remained unchanged in the placebo group; these changes from baseline were significantly different between the two goups. Total cholesterol:HDL cholesterol and LDL cholesterol:HDL cholesterol ratios were not affected by intervention.

Vitamin E, haematology and blood chemistry

Absolute α-tocopherol levels as well as α-tocopherol levels adjusted for cholesterol, TG and the sum of cholesterol and TG (α-tocopherol levels divided by the sum of TG and/or total cholesterol) were not different between the two groups at study entry and did not change in the placebo group. There were no changes in α-tocopherol levels and lipid-adjusted concentrations of α-tocopherol after DHA supplementation (Table 5). Cholesterol-adjusted concentrations of α-tocopherol decreased and TG-adjusted concentrations of α-tocopherol increased significantly after DHA intake; the changes from baseline in these parameters were significantly different between DHA and placebo groups.

The supplementation of DHA-rich oil from microalgae *Ulkenia* sp. did not result in any physiologically relevant changes in haematology and blood chemistry (data not shown). Incidence of liver (γ-glutamyl transpeptidase, alanine aminotransferase, aspartate aminotransferase, cholinesterase) and cardiac enzymes (creatine kinase, lactate dehydrogenase) out of the laboratory's reference range did not differ either at baseline or at week 8 between the DHA and placebo groups.

Discussion

Our present results show that a supplementation with 0·94 g/d DHA for 8 weeks significantly lowered TG in normolipidaemic

Table 4. Fatty acid composition of plasma phospholipids at weeks 0 and 8 (g/100 g fatty acid)*

	DHA group (n 53)					Placebo group (n 53)				
	Week 0		Week 8			Week 0		Week 8		
	Mean	SEM	Mean	SEM	P†	Mean	SEM	Mean	SEM	P†
16:0	28.2	0.2	28.4	0.2	0.129	28.0	0.3	27.8	0.3	0.373
18:0	12.1	0.2	12.0	0.2	0.679	12.1	0.2	12.1	0.2	0.980
18:1n-9	10.4	0.2	9.1	0.2	<0.001	10.3	0.2	10.5	0.2	0.490
18:2n-6	22.1	0.4	20.7	0.4	<0.001	23.0	0.4	22.9	0.4	0.712
20:4n-6	8.9	0.2	8.0	0.2	<0.001	8.9	0.2	9.0	0.2	0.674
22:5n-6	0.36	0.02	0.66	0.02	<0.001	0.34	0.02	0.34	0.02	0.777
20:5n-3	0.58	0.03	0.77	0.03	<0.001	0.57	0.04	0.52	0.03	0.062
22:5n-3	0.91	0.04	0.56	0.02	<0.001	0.85	0.04	0.85	0.04	0.865
22:6n-3	2.8	0.1	7.3	0.2	<0.001	2.6	0.1	2.5	0.1	0.677

* For details of procedures, see pp. 780–781.
† The within-group differences from 0 to 8 weeks were analysed using Student's paired t test (all parameters other than DHA) and Wilcoxon non-parametric test (DHA).

Table 5. Plasma lipids, lipoproteins and α-tocopherol at weeks 0 and 8*

	DHA group (n 53)					Placebo group (n 53)					
	Week 0		Week 8			Week 0		Week 8			
	Mean	SEM	Mean	SEM	P†	Mean	SEM	Mean	SEM	P†	P‡
TG (mmol/l)	1.08	0.07	0.83	0.04	<0.001	1.07	0.06	1.07	0.07	0.977	0.034
Total cholesterol (mmol/l)	4.58	0.13	4.85	0.14	0.001	4.72	0.13	4.69	0.12	0.623	0.004
LDL cholesterol (mmol/l)	2.45	0.10	2.71	0.11	<0.001	2.56	0.11	2.54	0.10	0.764	0.003
HDL cholesterol (mmol/l)	1.65	0.06	1.77	0.06	0.002	1.67	0.06	1.66	0.06	0.487	0.002
LDL cholesterol:HDL cholesterol	1.58	0.07	1.64	0.08	0.180	1.64	0.09	1.65	0.09	0.798	0.441
Total cholesterol:HDL cholesterol	2.92	0.10	2.87	0.09	0.485	2.95	0.10	2.97	0.11	0.790	0.486
TG:HDL cholesterol	0.75	0.08	0.51	0.04	<0.001	0.69	0.05	0.70	0.06	0.733	0.022
α-Tocopherol (μmol/l)	20.7	0.5	20.3	0.5	0.193	21.2	0.6	21.1	0.6	0.761	0.510
α-Tocopherol:total cholesterol (μmol/mmol)	4.58	0.09	4.24	0.09	<0.001	4.55	0.09	4.55	0.09	0.924	0.001
α-Tocopherol:TG (μmol/mmol)	22.9	1.4	27.2	1.4	0.001	23.1	1.3	22.9	1.2	0.830	0.008
α-Tocopherol:(total cholesterol + TG) (μmol/mmol)	3.70	0.08	3.61	0.08	0.143	3.72	0.07	3.72	0.08	0.931	0.281

TG, triacylglycerol.
* For details of procedures, see pp. 780–781.
† The within-group differences from 0 to 8 weeks were analysed using Student's paired t test (all parameters other than TG:HDL cholesterol) and Wilcoxon non-parametric test (TG:HDL cholesterol).
‡ The between-group differences in the absolute changes from baseline at week 8 were analysed using Student's unpaired t test (all parameters other than TG and TG:HDL cholesterol) or Mann–Whitney U test (TG and TG:HDL cholesterol).

(basal TG < 2.5 mM) subjects. Previous studies with single-cell oil sources of DHA reported a significant reduction in TG concentrations and an increase in HDL cholesterol levels with DHA intakes in the range 1.6–2.4 g/d in normolipidaemic volunteers (Agren *et al.* 1996; Conquer & Holub, 1996; Nelson *et al.* 1997), whereas other studies with DHA intakes of 0.7 g/d (Theobald *et al.* 2004) and 0.75–1.5 g/d (Conquer & Holub, 1998) did not find any significant effects on plasma TG and lipoproteins. In persons with combined hyperlipidaemia, significant reductions in TG and increases in LDL and HDL cholesterol concentrations were observed after supplementation with 1.25 or 2.5 g/d DHA (Davidson *et al.* 1997). n-3 LCPUFA lower plasma TG concentrations by several mechanisms such as increased hepatic fatty acid oxidation, inhibition of fatty acid and TG synthesis, and depressed assembly and secretion of VLDL TG (Nestel, 2000). The TG-lowering effect of DHA is greater in subjects with higher initial TG concentrations (Harris, 1997). We found a similar relationship in normolipidae-

mic subjects, in whom absolute and percentage TG changes were negatively correlated with baseline TG concentrations.

HDL cholesterol concentrations increased after 8 weeks of DHA supplementation. Plasma TG and HDL cholesterol of all subjects at baseline were inversely correlated ($r - 0.207$, $P = 0.033$), and absolute changes in TG correlated inversely with HDL concentrations after DHA supplementation ($r - 0.308$, $P = 0.025$). The mechanisms by which DHA supplementation increases HDL cholesterol are not known, but may be related to alterations in lipid transfer protein activity (Abbey *et al.* 1990). A decrease of cholesteryl ester transfer protein activity would reduce the exchange from HDL cholesterol ester and VLDL TG, resulting in larger, more cholesterol-rich HDL cholesterol particles. Indeed, other studies applying purified DHA (3.6 and 4.9 g/d, respectively) observed a decrease in the apoA1:HDL cholesterol ratio (Grimsgaard *et al.* 1997; Buckley *et al.* 2004). Their findings suggest an effect of DHA supplementation on HDL particle size with
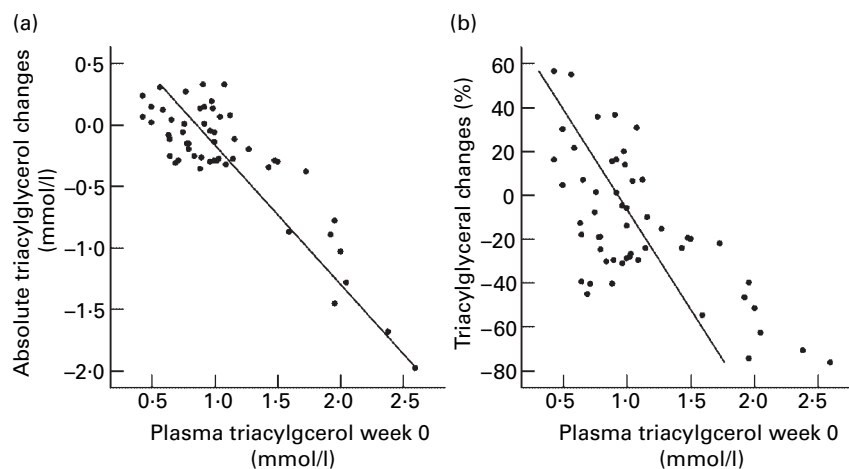
Fig. 1. Correlations between plasma triacylglycerol levels at week 0 and (a) absolute triacylglycerol changes (n 53, r −0·627, $P<0·001$) and (b) percentage triacylglycerol changes (n 53, r −0·534, $P<0·001$) after 8 weeks of supplementation (Spearman's Rho correlation coefficients). For details of procedures, see pp. 780−781.

a shift towards the larger, more cholesterol-rich HDL-2 particle. Other investigators observed an increased HDL-2:HDL-3 cholesterol ratio (Agren *et al.* 1996; Rambjor *et al.* 1996) or increased HDL-2 cholesterol (Mori *et al.* 2000) after DHA supplementation.

In the present study, total and LDL cholesterol levels increased after DHA intake. Other studies did not detect any significant changes in total cholesterol after supplementation with 0·75−4·9 g/d DHA (Rambjor *et al.* 1996; Grimsgaard *et al.* 1997; Conquer & Holub, 1998; Nestel *et al.* 2002; Buckley *et al.* 2004). Inconsistent effects of DHA on LDL cholesterol levels were reported in previous studies; some investigators found a LDL cholesterol-raising effect (Davidson *et al.* 1997; Mori *et al.* 2000; Theobald *et al.* 2004) of DHA or no effect on LDL cholesterol levels (Conquer & Holub, 1996, 1998; Rambjor *et al.* 1996; Grimsgaard *et al.* 1997; Nelson *et al.* 1997; Nestel *et al.* 2002; Buckley *et al.* 2004). The repeatedly observed increase in plasma LDL cholesterol after DHA/fish oil supplementation must be due to either decreased clearance of LDL or increased production of LDL. The limited amount of TG available for packaging into VLDL after supplementation with *n*-3 LCPUFA could result in smaller VLDL particles that contain less TG. Small VLDL are more readily converted to LDL, which may cause increased LDL cholesterol (Griffin, 2001). However, previous studies did not find increases in smaller VLDL subspecies with *n*-3 fatty acid supplementation (Inagaki & Harris, 1990; Lu *et al.* 1999). Lu *et al.* (1999) suggested that *n*-3 fatty acids could enhance the conversion of VLDL to LDL via increased inherent susceptibility of VLDL particles to lipolysis and/or increased *in vivo* lipolytic activity.

Decreased clearance of LDL might be due to decreases in LDL receptor binding activity or reduced LDL receptor expression. Animal experiments feeding fish oil with DHA to hamsters and primates showed decreased receptor-mediated clearance of LDL cholesterol (Surette *et al.* 1992; Schectman *et al.* 1996). Binding of LDL to the LDL receptor decreased because of an altered LDL structure and a reduced affinity of LDL for its receptor. Lindsey *et al.* (1992) observed an *in vitro* depression of LDL receptor activity and expression

in human hepatoma HepG2 cells after supplementation with 3·6 g/d EPA and 2·9 g/d DHA for only 2 weeks. Whether *n*-3 fatty acids alter LDL receptor activity or expression in man is not known, but LDL kinetic studies showed no decrease in fractional catabolic rate relative to a high saturated fat diet (Illingworth *et al.* 1984) or a vegetable diet (Fisher *et al.* 1998), providing no evidence for a change in LDL receptor activity in man. Further research is needed to clarify the reasons for the increase in LDL cholesterol with moderate intakes of DHA and the possible consequences for CHD risk in normolipidaemic subjects.

Kinosian *et al.* (1994, 1995) proposed that changes in total cholesterol:HDL cholesterol and LDL:HDL cholesterol ratios are better predictors of risk for CHD than changes in total or LDL cholesterol alone. In our present study, the total cholesterol:HDL cholesterol ratio as well as the LDL:HDL cholesterol remained unchanged in both intervention groups, and the TG:HDL cholesterol ratio was significantly lower after DHA supplementation. The ratio of TG to HDL cholesterol is a predictor of the risk of myocardial infarction (Gaziano *et al.* 1997). Stark & Holub (2004) also reported a significant decrease of TG:HDL cholesterol after supplementation with 2·8 g/d DHA over 4 weeks in postmenopausal women. Some studies found decreased ratios of total:HDL cholesterol (Conquer & Holub, 1996; Grimsgaard *et al.* 1997) and LDL:HDL cholesterol (Conquer & Holub, 1996) after supplementation with 1·6−3·6 g/d DHA or no changes in LDL:HDL or total:HDL cholesterol ratios with DHA intakes of 0·7−2·8 g/d (Stark & Holub, 2004; Conquer & Holub, 1998).

In the present study, heart rate and diastolic BP were not affected by DHA supplementation. Similar results were shown previously with DHA intakes of 0·7 and 3·0 g/d (Conquer & Holub, 1998; Nestel *et al.* 2002). Mori *et al.* (1999) reported a decrease of systolic and diastolic BP after DHA supplementation in men with otherwise normal BP, whereas Grimsgaard *et al.* (1998) did not detect a reduction of BP in comparable subjects. Both groups observed a significant influence of DHA on heart rate in healthy men. Very clear effects in respect to *n*-3 LCPUFA on BP control have been observed in hypertensive patients (Knapp & FitzGerald, 1989; Bonaa *et al.* 1990; Lungershausen *et al.* 1994; Toft *et al.* 1995), but these investigators used

extremely large amounts of *n*-3 fatty acids ranging from 3·4 to 15 g/d. In the present study, systolic and diastolic BP at baseline were very low (DHA group, 98/67 mmHg; placebo group, 96/67 mmHg); therefore, a further decrease of BP could not be expected. The observed increase of systolic BP in the placebo group and the same trend in the DHA group may be caused by a systematic change in BP measurement or by environmental influences.

Sanders & Hinds (1992) reported that plasma α-tocopherol concentrations fell below the normal range during a period of fish oil supplementation (2·1 g DHA and 0·8 g EPA per day), suggesting that fish oil increases the requirement for antioxidants. No significant changes in plasma α-tocopherol were evident following fish oil treatment providing 21 mg/d total tocopherol of which 16 mg was α-tocopherol (Leigh-Firbank *et al.* 2002). In the present study, a total of 2·2–2·3 mg/d mixed natural tocopherols was provided by the olive oil and microalgae oil capsules. No changes of α-tocopherol levels and lipid-adjusted α-tocopherol concentrations were observed after intervention in both groups, suggesting that 2·3 mg mixed natural tocopherols are sufficient to maintain α-tocopherol levels at a DHA intake of 0·94 g/d.

For safety and tolerance evaluation, side-effects were reported and several haematology and biochemistry parameters were analysed. The supplementation of DHA-rich oil from microalgae *Ulkenia* sp. did not result in any physiologically relevant changes of safety and haemostatic factors. Reported side-effects were equally distributed between DHA and placebo groups.

In conclusion, 8-week supplementation with DHA-rich microalgae oil was associated with improvements in some CHD risk factors (plasma TG, TG:HDL cholesterol ratio), but others, notably LDL cholesterol, worsened. Therefore, the overall effects of this treatment on CHD risk are unclear and should be further investigated.

Acknowledgements

References

Abbey M, Clifton P, Kestin M, Belling B & Nestel P (1990) Effect of fish oil on lipoproteins, lecithin:cholesterol acyltransferase, and lipid transfer protein activity in humans. *Arteriosclerosis* **10**, 85–94.

Agren JJ, Hanninen O, Julkunen A, Fogelholm L, Vidgren H, Schwab U, Pynnonen O & Uusitupa M (1996) Fish diet, fish oil and docosahexaenoic acid rich oil lower fasting and postprandial plasma lipid levels. *Eur J Clin Nutr* **50**, 765–771.

Austin MA, Hokanson JE & Edwards KL (1998) Hypertriglyceridemia as a cardiovascular risk factor. *Am J Cardiol* **81**, 7B–12B.

Bonaa KH, Bjerve KS, Straume B, Gram IT & Thelle D (1990) Effect of eicosapentaenoic and docosahexaenoic acids on blood pressure in hypertension. A population-based intervention trial from the Tromso study. *N Engl J Med* **322**, 795–801.

Buckley R, Shewring B, Turner R, Yaqoob P & Minihane AM (2004) Circulating triacylglycerol and apoE levels in response to EPA and docosahexaenoic acid supplementation in adult human subjects. *Br J Nutr* **92**, 477–483.

Conquer JA & Holub BJ (1996) Supplementation with an algae source of docosahexaenoic acid increases (n-3) fatty acid status and alters selected risk factors for heart disease in vegetarian subjects. *J Nutr* **126**, 3032–3039.

Conquer JA & Holub BJ (1998) Effect of supplementation with different doses of DHA on the levels of circulating DHA as non-esterified fatty acid in subjects of Asian Indian background. *J Lipid Res* **39**, 286–292.

Davidson MH, Maki KC, Kalkowski J, Schaefer EJ, Torri SA & Drennan KB (1997) Effects of docosahexaenoic acid on serum lipoproteins in patients with combined hyperlipidemia: a randomized, double-blind, placebo-controlled trial. *J Am Coll Nutr* **16**, 236–243.

Fisher WR, Zech LA & Stacpoole PW (1998) Apolipoprotein B metabolism in hypertriglyceridemic diabetic patients administered either a fish oil- or vegetable oil-enriched diet. *J Lipid Res* **39**, 388–401.

Friedewald WT, Levy RI & Fredrickson DS (1972) Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* **18**, 499–502.

Gaziano JM, Hennekens CH, O'Donnell CJ, Breslow JL & Buring JE (1997) Fasting triglycerides, high-density lipoprotein, and risk of myocardial infarction. *Circulation* **96**, 2520–2525.

Geppert J, Kraft V, Demmelmair H & Koletzko B (2005) Docosahexaenoic acid supplementation in vegetarians effectively increases omega-3 index: a randomized trial. *Lipids* **40**, 870–814.

Griffin BA (2001) The effect of n-3 fatty acids on low density lipoprotein subfractions. *Lipids* **36**, S91–S97.

Grimsgaard S, Bonaa KH, Hansen JB & Myhre ES (1998) Effects of highly purified eicosapentaenoic acid and docosahexaenoic acid on hemodynamics in humans. *Am J Clin Nutr* **68**, 52–59.

Grimsgaard S, Bonaa KH, Hansen JB & Nordoy A (1997) Highly purified eicosapentaenoic acid and docosahexaenoic acid in humans have similar triacylglycerol-lowering effects but divergent effects on serum fatty acids. *Am J Clin Nutr* **66**, 649–659.

Grover SA, Coupal L & Hu XP (1995) Identifying adults at increased risk of coronary disease. How well do the current cholesterol guidelines work? *JAMA* **274**, 801–806.

Grover SA, Palmer CS & Coupal L (1994) Serum lipid screening to identify high-risk individuals for coronary death. The results of the Lipid Research Clinics prevalence cohort. *Arch Intern Med* **154**, 679–684.

Harris WS (1997) N-3 fatty acids and serum lipoproteins: human studies. *Am J Clin Nutr* **65**, 1645S–1654S.

Hess D, Keller HE, Oberlin B, Bonfanti R & Schuep W (1991) Simultaneous determination of retinol, tocopherols, carotenes and lycopene in plasma by means of high-performance liquid chromatography on reversed phase. *Int J Vit Nutr Res* **61**, 232–238.

Hodis HN (1999) Triglyceride-rich lipoprotein remnant particles and risk of atherosclerosis. *Circulation* **99**, 2852–2854.

Hokanson JE & Austin MA (1996) Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: a meta-analysis of population-based prospective studies. *J Cardiovasc Risk* **3**, 213–219.

Illingworth DR, Harris WS & Connor WE (1984) Inhibition of low density lipoprotein synthesis by dietary omega-3 fatty acids in humans. *Arteriosclerosis* **4**, 270–275.

Inagaki M & Harris WS (1990) Changes in lipoprotein composition in hypertriglyceridemic patients taking cholesterol-free fish oil supplements. *Atherosclerosis* **82**, 237–246.

Kinosian B, Glick H & Garland G (1994) Cholesterol and coronary heart disease: predicting risks by levels and ratios. *Ann Intern Med* **121**, 641–647.

Kinosian B, Glick H, Preiss L & Puder KL (1995) Cholesterol and coronary heart disease: predicting risks in men by changes in levels and ratios. *J Invest Med* **43**, 443–450.

Knapp HR & FitzGerald GA (1989) The antihypertensive effects of fish oil. A controlled study of polyunsaturated fatty acid supplements in essential hypertension. *N Engl J Med* **320**, 1037–1043.

Leigh-Firbank EC, Minihane AM, Leake DS, Wright JW, Murphy MC, Griffin BA & Williams CM (2002) Eicosapentaenoic acid and docosahexaenoic acid from fish oils: differential associations with lipid responses. *Br J Nutr* **87**, 435–445.

Lindsey S, Pronczuk A & Hayes KC (1992) Low density lipoprotein from humans supplemented with n-3 fatty acids depresses both LDL receptor activity and LDLr mRNA abundance in HepG2 cells. *J Lipid Res* **33**, 647–658.

Lu G, Windsor SL & Harris WS (1999) Omega-3 fatty acids alter lipoprotein subfraction distributions and the in vitro conversion of very low density lipoproteins to low density lipoproteins. *J Nutr Biochem* **10**, 151–158.

Lungershausen YK, Abbey M, Nestel PJ & Howe PRC (1994) Reduction of blood pressure and plasma triglycerides by omega-3 fatty acids in treated hypertensives. *J Hypertens* **12**, 1041–1045.

Mori TA, Bao DQ, Burke V, Puddey IB & Beilin LJ (1999) Docosahexaenoic acid but not eicosapentaenoic acid lowers ambulatory blood pressure and heart rate in humans. *Hypertension* **34**, 253–260.

Mori TA, Burke V, Puddey IB, Watts GF, O'Neal DN, Best JD & Beilin LJ (2000) Purified eicosapentaenoic and docosahexaenoic acids have differential effects on serum lipids and lipoproteins, LDL particle size, glucose, and insulin in mildly hyperlipidemic men. *Am J Clin Nutr* **71**, 1085–1094.

Nelson GJ, Schmidt PC, Bartolini GL, Kelley DS & Kyle D (1997) The effect of dietary docosahexaenoic acid on plasma lipoproteins and tissue fatty acid composition in humans. *Lipids* **32**, 1137–1146.

Nestel PJ (2000) Fish oil and cardiovascular disease: lipids and arterial function. *Am J Clin Nutr* **71**, 228–231.

Nestel P, Shige H, Pomeroy M, Cehun M, Abbey M & Raederstorff D (2002) The n-3 fatty acids eicosapentaenoic acid and docosahexaenoic acid increase systemic arterial compliance in humans. *Am J Clin Nutr* **76**, 326–330.

Rambjor GS, Walen AI, Windsor SL & Harris WS (1996) Eicosapentaenoic acid is primarily responsible for hypotriglyceridemic effect of fish oil in humans. *Lipids* **31**, S45–S49.

Sanders TAB & Hinds A (1992) The influence of a fish oil high in docosahexaenoic acid on plasma lipoprotein and vitamin E concentrations and haemostatic function in healthy male volunteers. *Br J Nutr* **68**, 163–173.

Schectman G, Boerboom LE, Hannah J, Howard BV, Mueller RA & Kissebah AH (1996) Dietary fish oil decreases low-density-lipoprotein clearance in nonhuman primates. *Am J Clin Nutr* **64**, 215–221.

Stark KD & Holub BJ (2004) Differential eicosapentaenoic acid elevations and altered cardiovascular disease risk factor responses after supplementation with docosahexaenoic acid in postmenopausal women receiving and not receiving hormone replacement therapy. *Am J Clin Nutr* **79**, 765–773.

Surette ME, Whelan J, Lu GP, Broughton KS & Kinsella JE (1992) Dependence on dietary cholesterol for n-3 polyunsaturated fatty acid-induced changes in plasma cholesterol in the Syrian hamster. *J Lipid Res* **33**, 263–271.

Theobald HE, Chowienczyk PJ, Whittall R, Humphries SE & Sanders TAB (2004) LDL cholesterol-raising effect of low-dose docosahexaenoic acid in middle-aged men and women. *Am J Clin Nutr* **79**, 558–563.

Toft I, Bonaa KH, Ingebretsen OC, Nordoy A & Jenssen T (1995) Effects of n-3 polyunsaturated fatty acids on glucose homeostasis and blood pressure in essential hypertension: a randomized, controlled trial. *Ann Intern Med* **123**, 911–918.