ELSEVIER

Journal of Nutritional Biochemistry 14 (2003) 513–521

THE JOURNAL OF
Nutritional
Biochemistry

Dietary fish oil decreases C-reactive protein, interleukin-6, and triacylglycerol to HDL-cholesterol ratio in postmenopausal women on HRT

Irina Ciubotaru, Ye-Sun Lee, Rosemary C. Wander*

Department of Nutrition, The University of North Carolina at Greensboro, Greensboro, NC 27402, USA

Received 20 February 2003; received in revised form 20 May 2003; accepted 15 June 2003

Abstract

Background: Atherogenesis is a complex process involving both a low-grade inflammation and a disturbed lipid profile. Although dietary fish and fish oil improve the latter of these two risk factors, their impact on the former is less clear.

Objective: This study addressed the effect of supplementation with fish oil in doses achievable with diet on serum C-reactive protein (CRP), interleukin-6 (IL-6), and the lipid profile.

Methods and results: Thirty healthy subjects taking HRT were randomly divided into three groups and supplemented for five weeks with 14g/day safflower oil (SO), 7g/day of both safflower oil and fish oil (LFO), or 14g/day fish oil (HFO). Measurements included serum high-sensitivity CRP, IL-6 in plasma and in cell culture supernatant collected from 24-hr lipopolysaccharide (LPS)-stimulated whole blood, and lipid profile markers. CRP and IL-6 were adjusted for body mass index (BMI). Fish oil supplementation significantly decreased CRP and IL-6 compared to SO, with a greater effect in the LFO than HFO groups. Plasma triacylglycerol (TG) and the TG/HDL-C ratio were significantly lower in the HFO compared to the SO group.

Conclusions: These results suggest that dietary fish oil may decrease the risk for cardiovascular disease through the modulation of both plasma lipids and inflammatory markers in healthy postmenopausal women. © 2003 Elsevier Inc. All rights reserved.

Keywords: C-reactive protein; Interleukin-6; Triacylglycerol/HDL-cholesterol; Fish oil; HRT

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death in the United States. The main factor leading to CVD is atherosclerosis manifested at the coronary, cerebral, and peripheral level of the arterial system. The mechanism is a generalized cellular and humoral inflammatory response that leads to the formation of the atheromatous plaque [1]. Key participants in this process are the proinflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), IL-6, and acute phase reactants (APR) such as CRP. The potential role of CRP as a marker of subclinical atherosclerosis in identifying at-risk individuals has been studied in healthy women, healthy men, and elderly [2-5]. Postmenopausal women are considered at-risk individuals because of the imbalanced production of estrogens that renders them at risk for CVD [6]. HRT has been shown to increase CRP concentrations compared to non-users, and this effect is irrespective of the hormonal preparation [7]. Additional factors that may up-regulate CRP levels in healthy individuals are age [8], body mass index (BMI) [9], level of physical activity [10], smoking [11], alcohol consumption [12], and the polymorphism of genes associated with CRP production [13-15]. In addition to inflammation, serum lipids have been shown to play an important role in atherogenesis. After menopause changes in the lipid profile consist in increased triacylglycerol (TG), low density lipoprotein-cholesterol (LDL-C), and decreased high-density lipoprotein-cholesterol (HDL-C) concentrations, and constitute independent risk factors for CVD [6]. In postmenopausal women, HDL-C/TG ratio has emerged as a better predictor of myocardial infarction than the routinely used TC/HDL-C and LDL-C/HDL-C ratios [16].

Coldwater fish are rich in long chain polyunsaturated

* Corresponding author. Tel.: +1-336-256-0426; fax: +1-336-334-3009.

E-mail address: rcwander@uncg.edu (R.C. Wander).

0955-2863/03/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0955-2863(03)00101-3

fatty acids (PUFA) from the n-3 family, eicosapentaenoic acid (EPA, 20:5-n3) and docosahexaenoic acid (DHA, 22:6-n3). Consumption of fish or fish oil has been shown to have anti-inflammatory properties [17] and to improve the lipid profile as well [18, 19]. The intake of EPA and DHA used in these studies has ranged from a combined total of 1g, an amount that can be achieved through the diet, to 6 g and more, amounts that could not be obtained with foods. Additionally, only a limited number of studies have addressed the impact of dietary interventions with fish or fish oil on modification of risk factors for CVD in healthy postmenopausal women.

Consequently, the purpose of this study was to assess the dose-dependent effect of fish oil supplementation in amounts achievable by dietary modifications on CRP and IL-6 as markers of inflammation and on the lipid profile in postmenopausal women using HRT.

2. Methods and materials

Two hundred women were screened according to the following criteria: healthy, postmenopausal, taking HRT, non-smokers, not on special diets and willing to refrain from taking any supplements except vitamin D and calcium, from eating fish, and from taking medications that would interfere with study measurements (e.g., corticosteroids, thyroid hormones, and antihypertensive drugs). Postmenopausal status was defined as natural or surgery-induced amenorrhea for at least 12 months. Thirty women met the inclusion criteria and signed the informed consent. Subjects who were selected consumed approximately 30% kcal in their diet from fat and were willing to maintain the usual dietary habits for the duration of the study. Previous studies have shown that postmenopausal women do not modify the macronutrient intake of their diet significantly over a 9-month period [20]. Subjects were provided with scales (Ohaus Compact Scales, NJ) to weigh their food, and instructed on how to keep a 3-day diet record. The dietary information was analyzed using Food Processor Plus (version 7.1; ESHA, Salem, OR). A medical history was taken. Alcohol consumption and level of physical activity were determined. Weight, height, and blood pressure were measured, and blood was drawn for lipid and biochemical analyses.

The study was a double-blinded, placebo-controlled supplementation trial. The protocol was approved by the Institutional Review Board of The University of North Carolina at Greensboro. Subjects were randomly assigned to one of the three treatment groups, safflower oil (SO), low fish oil (LFO), or high fish oil (HFO). They consumed 14 one-gram capsules of an oil supplement daily for five weeks. The fatty acid profile of the oils was measured by gas chromatography, as reported below. Selected fatty acids provided by the daily supplement of the three oils are shown in Table 1. The amount of EPA and DHA can be obtained from approximately one and two servings, respectively, of Chinook salmon per day [21]. The SO was obtained in bulk from Arista Industries (Darien, CT). The fish oil was kindly provided in bulk by OmegaProtein (Houston, TX). The oils were encapsulated by Banner Pharmacaps, Inc. (High Point, NC). Vitamin E (courtesy of ADM Nutraceuticals, Decatur, IL) was added to the oils so that the amount of α, δ, and γ-tocopherol in the supplements matched for the three treatment groups. *Tert*-butylhydroquinone (TBHQ) (Eastman Chemical Company, Kingsport, TN) content of the oils was matched at 0.02%. The final vitamin E content of the oils was analyzed by normal phase high-pressure liquid chromatography (HPLC) [22]. They contained 0.556 mg α-tocopherol/g oil, 0.669 mg γ-tocopherol/g oil, and 0.171 mg δ-tocopherol/g oil. Lipid peroxidation in samples of each supplement was analyzed before and during the supplementation period. Neither the p-anisidine value nor the peroxide value increased during these times [23, 24]. Compliance was monitored by the changes in the plasma fatty acid profile and by the return of the empty supplement containers. The retention of subjects in the study was 100%.

Table 1
Selected fatty acids provided by supplements of 14 g/d SO, 7g/d SO and 7g/d FO (LFO), or 14g/fish oil (HFO)*

Fatty acid	SO	LFO	HFO
C16:0	1.41	1.76	2.11
C18:0	0.26	0.32	0.38
Σ SFA†	**1.68**	**2.51**	**3.38**
C18:1n-9	3.56	2.53	1.50
Σ MUFA‡	**3.56**	**3.37**	**3.16**
C18:2n-6	7.92	4.04	0.17
C18:3n-3	0.12	0.13	0.15
C20:5n-3	-	0.59	1.18
C22:6n-3	-	0.50	1.00
Σ PUFA§	**8.04**	**5.44**	**2.86**
Σ n-6•	7.92	4.11	0.30
Σ n-3¶	0.12	1.33	2.56

* Values are means ± SE (g/d).
† Σ SFA = C14:0 + C16:0 + C18:0 + C20:0 + C22:0 + C24:0.
‡ Σ MUFA = C14:1n-5 + C16:1n-7 + C18:1n-7 + C18:1n-9 + C20:1n-9 + C22:1n-9 + C22:1n-11.
§ Σ PUFA = C16:2n-4 + C16:4n-1 + C18:2n-6 + C18:3n-3 + C18:4n-3 + C20:2n-6 + C20:3n-6 + C20:4n-6 + C20:5n-3 + C22:5n-3 + C22:6n-3.
• Σ n-6 = C18:2n-6 + C20:2n-6 + C20:3n-6 + C20:4n-6.
¶ Σ n-3 = C18:3n-3 + C18:4n-3 + C20:5n-3 + C22:5n-3 + C22:6n3.

Venous blood was collected after an overnight fast of at least 12 h at the beginning of the study and after five weeks. The blood needed for plasma was collected in Na_2EDTA tubes (1.5 mg/mL plasma). Plasma and serum were separated at 1200 × g for 15 min at 4°C. Samples were immediately aliquoted and stored at −80°C or used within an hour of sampling. Whole blood for IL-6 measurement was collected on heparin, diluted (1:10) using RPMI 1640 medium, and stimulated with lipopolysaccharide (LPS), (20 μg/mL) for 24 h. Supernatant was stored at −80°C for subsequent analysis.

Plasma total cholesterol (TC) was determined enzymat-

ically as previously described [25], adapted for the use of microtiter plates. Triacylglycerol was measured using a modification of the method of McGowan et al. [26]. HDL-C was measured enzymatically after the precipitation of LDL-C and VLDL-C with phosphotungstic acid and $MgCl_2$ [27]. LDL-C was quantified using the formula of Friedewald et al. [28]. Each of the assays was performed using the appropriate Sigma Diagnostics kit (Sigma, St Louis, MO). The fatty acid profile of the supplements and plasma was analyzed by gas chromatography as previously described [29]. Briefly, lipids were extracted with chloroform methanol (vol 1:2) according to the method of Bligh and Dyer [30], and fatty acids were methylated using boron trifluoride in methanol. After methylation fatty acids methyl ester were measured by gas chromatography using heptadecanoic acid (Nu-Chek Prep, Elysian, MN) as an internal standard.

Serum CRP and plasma IL-6 were measured by a high sensitivity ELISA (Diagnostic System Laboratories, Inc., TX; R&D Systems, MN). IL-6 was also measured in the supernatant from LPS-stimulated whole blood using ELISA (R&D Systems).

Data are reported as means ± SE, unless stated otherwise. To minimize the variability among the subjects, difference scores (difference between final and initial values) [31] were calculated for each individual and appropriate statistics done with this new variable. Checking for ANOVA assumptions revealed that the distributions of serum CRP and plasma IL-6 were skewed to the right. Consequently, outliers were removed and data were logarithmically transformed [32, 33]. Using the box-plot method, outliers were identified and then removed, five for CRP, four for plasma IL-6, and one for the cell supernatant IL-6 analyses. For serum CRP and plasma IL-6 log-transformed data were used for all analyses [33]. Because BMI as an estimate of body fat has a proven impact on CRP and IL-6 levels, determination of significant differences in the log-transformed data was done by ANCOVA using BMI as a covariate [34]. We also adjusted supernatant IL-6 for BMI, assuming that the fat mass reflected by BMI imprints a specific cell phenotype that would be present *ex vivo*. Pearson correlation coefficient analysis was used to quantify associations between measured parameters. Significance was tested at the 0.05 level and the Tukey post hoc test was performed to detect the source of significance. All statistical analyses were performed with SPSS for Windows, v.10 (SPSS Inc). The effect size, representing the standardized difference between two treatments, was assessed by Cohen's *d* test. A small, medium, or large effect size was considered for a *d* equal to 0.2, 0.5, or 0.8, respectively [35].

3. Results

The subjects were apparently healthy postmenopausal women taking HRT. The duration of hormone replacement therapy was at least a year at the time of study. Twenty subjects were taking estro-progestins, eight subjects were on unopposed estrogens, and two on a formula containing estrogen and methyl-testosterone. The use of different hormonal formulas was homogeneous among groups. The medical history excluded any infectious or inflammatory episodes during the three weeks before the study started, chronic inflammation, and cardiovascular pathology. Compliance of the subjects was excellent as indicated by the paucity of unused capsules that were returned and the increase in the EPA and DHA content of the plasma (Fig 1.) No significant differences were found among the three groups with regard to the baseline characteristics presented in Table 2. None of the participants was involved in intense physical activities. Subjects were non-smokers and free of unusual dietary habits. The dietary intake was calculated based on the 3-day records kept during the study. No significant differences were found among groups as shown in Table 3. The average daily energy intake was 6888 ± 1911 kJ, with 17 ± 4% from proteins, 51 ± 10% from carbohydrates, and 30 ± 7% from fat. Of the intake of energy from fat, 10 ± 3% was from saturated fat (SFA), 9 ± 3% from monounsaturated fatty acids (MUFA), and 5 ± 2% from PUFA). The intake of α-tocopherol was 4.4 ± 2.5 mg/day, lower than the currently recommended value of 15 mg/day [36]. The supplement added 7.8 mg α-tocopherol /day, resulting in a daily average intake approximately 81% of the recommended value. Alcohol consumption was not significantly different among groups at baseline and correlated with baseline concentrations of plasma IL-6, but not with serum CRP (data not shown).

There were no significant differences in plasma fatty acid concentration among the groups at baseline (data not shown). After 5-week supplementation there was no significant difference among groups with regard to SFA (P = 0.49), or MUFA (P = 0.83), either as the sum of the individual fatty acids (Fig. 1) or as individual fatty acids (data not shown). Although there were no significant differences among the concentration of the sum of the PUFA (P = 0.67) or the sum of the n-6 PUFA (P = 0.17) (Fig. 1), supplementation with fish oil led to a dose-dependent increase in plasma concentration of EPA and DHA (P = 0.000). The concentration of EPA increased approximately 380% in the LFO compared to the SO group (P = 0.003), 1000% in the HFO compared to the SO group (P = 0.000), and 145% in the HFO compared to the LFO group (P = 0.000). DHA increased 98% in the LFO compared to the SO group (P = 0.002), 176% in the HFO compared to the SO group (P = 0.000), and 40% in the HFO compared to the LFO group (P = 0.016). After 5-week supplementation there was no significant difference among groups with regard to arachidonic acid, (P = 0.23) (Fig. 1).

The initial and the difference score of plasma TC, HDL-C, LDL-C, TC/HDL-C, and LDL-C/HDL-C did not differ significantly among the three groups (Table 4). However, the difference score of plasma TG and the TG/HDL-C ratio were significantly lower (P = 0.011 and P = 0.007,

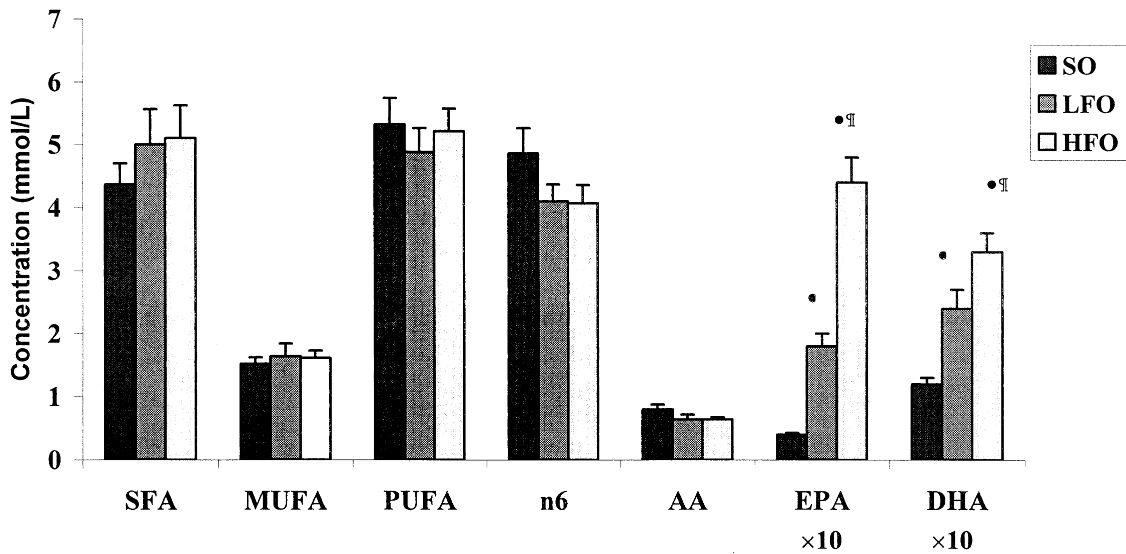

Fig. 1. Selected plasma fatty acids in postmenopausal women after 5-wk supplementation with SO, LFO, or HFO.
Values are means ± SE (mmol/L).
Σ SFA = C14:0 + C16:0 + C18:0 + C20:0 + C22:0 + C24:0.
Σ MUFA = C14:1n-5 + C16:1n-7 +C18:1n-7 + C18:1n-9 + C20:1n-9 + C22:1n-9 + C22:1n-11.
Σ PUFA = C16:2n-4 + C16:4n-1 + C18:2n-6 + C18:3n-3 + C18:3n-6 + C18:4n-3 + C20:2n-6 + C20:3n-6 + C20:4n-6 + C20:5n-3 + C22:5n-3 + C22:6n-3.
Σ n-6 = C18:2n-6 + C20:2n-6 + C20:3n-6 + C20:4n-6.
AA = arachidonic acid
P-value ≤ 0.05: • vs SO, ¶ vs LFO.

respectively) in the HFO compared to the SO group. There were no significant differences in these values between the SO and the LFO groups ($P = 0.43$ and $P = 0.30$, respectively) or between the LFO- and the HFO-supplemented groups ($P = 0.14$ and 0.15, respectively).

CRP serum concentrations, both untransformed data and log-transformed data adjusted for BMI, are presented in Table 5. When using the log-transformed data adjusted for BMI, highly significant differences were observed among the groups at baseline ($P = 0.000$), after the 5-week supplementation ($P = 0.004$), and for the difference score ($P = 0.000$). For the initial values, the post-hoc analysis showed significant differences between the HFO and SO groups ($P = 0.000$) and between the HFO and LFO groups ($P = 0.002$), but not between LFO and SO groups ($P = 0.34$). After the 5-week supplementation CRP concentrations in serum were significantly lower in the LFO group compared to the SO group ($P = 0.01$) or to the HFO group ($P = 0.006$). There was no significant difference between SO and HFO groups ($P = 0.9$). However, when the difference scores were compared, the decrease in CRP concentration was significantly greater in the LFO and HFO groups compared to the SO group ($P = 0.000$ and 0.004, respectively) and the LFO compared to HFO groups ($P = 0.015$). CRP serum concentrations were also compared using log-transformed data not adjusted for BMI (data not shown). A

Table 2
Baseline characteristics of the subjects

Characteristics	SO	LFO	HFO
N	10	10	10
Age	60 ± 6	60 ± 7	60 ± 3
BMI (kg/m^2)	23 ± 3	15 ± 3	26 ± 3
SBP (mmHg)	128 ± 2	131 ± 1	136 ± 1
DBP (mmHg)	79 ± 1	73 ± 4	85 ± 8
Total cholesterol (mmol/L)	5.9 ± 0.8	5.5 ± 0.8	5.7 ± 0.7
LDL-cholesterol (mmol/L)	3.5 ± 0.8	3 ± 0.6	3.3 ± 0.6
HDL-cholesterol (mmol/L)	1.8 ± 0.5	1.9 ± 0.5	1.7 ± 0.3
Triacylglycerol (mmol/L)	1.3 ± 0.6	1.2 ± 0.6	1.6 ± 0.3

Values are means ± SD. Conversion factors used to transform cholesterol and triacylglycerol from mmol/L to mg/dL are 38.6 and 88.5, respectively.

BMI-body mass index, SBP-systolic blood pressure, DBP-diastolic blood pressure.

Table 3
The average daily intake

	SO	LFO	HFO
Energy (kJ)	6755 ± 788	7008 ± 2728	6905 ± 1911
Protein (% energy)	17 ± 5	16 ± 3	16 ± 4
Carbohydrate (% energy)	54 ± 8	53 ± 13	49 ± 10
Fat (% energy)	30 ± 8	30 ± 9	31 ± 7
SFA	10 ± 3	10 ± 3	11 ± 3
MUFA	8 ± 3	9 ± 3	9 ± 2
PUFA	5 ± 3	5 ± 2	4 ± 2
Cholesterol (mmol/L)	7 ± 3	5 ± 2	6 ± 3

Values are means ± SD. Conversion factors used to transform cholesterol from mmol/L to mg/dL are 38.6.

Table 4
Baseline values (I) and the difference score (F-I) in the plasma lipid profile of postmenopausal women after 5-wk supplementation with SO, LFO, and HFO*

	SO n = 10		LFO n = 10		HFO n = 10	
	I	F-I	I	F-I	I	I
TC (mmol/L)	5.56 ± 0.24	0.04 ± 0.14	5.36 ± 0.21	0.01 ± 0.18	5.81 ± 0.27	−0.15 ± 0.13
LDL-C (mmol/L)	3.43 ± 0.30	−0.20 ± 0.22	2.97 ± 0.19	−0.11 ± 0.17	3.48 ± 0.22	−0.11 ± 0.10
HDL-C (mmol/L)	1.74 ± 0.08	0.01 ± 0.06	1.76 ± 0.15	0.11 ± 0.06	1.64 ± 0.11	0.14 ± 0.06
TG (mmol/L)•	0.95 ± 0.11	0.20 ± 0.09	1.11 ± 0.21	−0.04 ± 0.09	1.52 ± 0.16	−0.40 ± 0.16†
TC/HDL-C•	3.7 ± 0.7	−0.3 ± 0.4	3.2 ± 0.4	−0.3 ± 0.2	3.7 ± 0.2	−0.4 ± 0.1
LDL-C/HDL-C	2.4 ± 0.7	−0.48 ± 0.50	1.8 ± 0.3	−0.11 ± 0.16	2.2 ± 0.2	−0.3 ± 0.1
TG-HDL-C	0.54 ± 0.06	0.13 ± 0.07	0.71 ± 0.17	−0.08 ± 0.05	0.99 ± 0.13	−0.34 ± 0.12†

* Data are reported as mean ± SE.
• TG and TG/HDL-C are calculated in the plasma from 26 subjects (SO = 8, LFO = 8, HFO = 10).
Significant differences among groups are depicted by vs † SO (TG:P ≤ 0.05, TG/HDL-C: P ≤ 0.01).

significant difference was observed among groups (P = 0.03) when difference scores were compared. The subsequent post-hoc analysis detected a significant difference between the SO and LFO groups (P = 0.023), but not between the SO and HFO groups (P = 0.29) or the LFO and HFO groups (P = 0.41).

Concentrations of IL-6 in plasma and in the supernatant of LPS-stimulated whole blood are given in Table 6. When using the log-transformed data adjusted for BMI, significant differences in plasma IL-6 concentration were observed among the groups at baseline (P = 0.000), after the 5-week supplementation (P = 0.05), and for the difference score (P = 0.000). For the initial values, the post-hoc analysis showed significant differences between the HFO and SO groups (P = 0.000) and between the HFO and LFO groups (P = 0.000), and a tendency to significance in the LFO compared the SO groups (P = 0.06). After the 5-week supplementation IL-6 concentrations decreased in all three groups. The IL-6 concentration in plasma was significantly lower in the LFO group compared to the HFO group (P = 0.04). There was no significant difference between the SO and either the LFO (P = 0.8) or the HFO groups (P = 0.11). However, when the difference scores were compared, the decrease in plasma IL-6 concentration was significantly greater in the LFO and SO groups compared to the HFO group (P = 0.000 and 0.000, respectively), and also significantly greater in LFO compared to SO (P = 0.03). IL-6 plasma concentrations were also compared using log-transformed data not adjusted for BMI (data not shown). No significant differences were observed among groups (P = 0.77) when difference scores were compared.

The effect of the treatment on IL-6 was assessed also by measuring the production of the cytokine in whole blood stimulated with LPS. As these data were not skewed, log-transformation was not necessary. There were no significant differences among the groups (data not shown). However, adjusting for BMI revealed significant differences among the groups after the 5-week supplementation (P = 0.04), and for the difference score (P = 0.000), but not at baseline (P = 0.3). After the 5-week supplementation IL-6 concentration in the supernatant of the LPS-stimulated whole blood was significantly lower in the LFO group compared to the SO group (P = 0.05). There was no significant difference between the HFO and either the LFO (P = 0.12) or the SO groups (P = 0.93). However, when the difference scores were compared, the decrease in IL-6 concentration was significantly greater in the LFO compared to either the SO (P = 0.000) or the HFO (P = 0.000) groups, but not significantly different in the SO compared to the HFO (P = 0.45) groups.

The effect size for the difference score for serum CRP and plasma IL-6 concentrations was calculated on untransformed data after removing the outliers and reported as Cohen's d (Table 7). For CRP, there was a medium effect size of the treatment in both the LFO (d = 0.54) and the HFO (d = 0.55) groups compared to the SO group. When HFO was compared to the LFO group the effect size did not reach the conventional threshold of 0.20 for a "small" effect (d = 0.10). For plasma IL-6, on the other hand, the largest effect size was when the LFO group was compared to the

Table 5
Concentration of serum CRP in postmenopausal women before and after 5-wk supplementation with SO, LFO, and HFO

	SO (n = 9)	LFO (n = 8)	HFO (n = 8)
Initial (I)			
Log-transformed*	0.62 ± 0.03	0.67 ± 0.04	0.84 ± 0.02‡§
Untransformed (mg/L)†	(6.29 ± 1.76)	(6.27 ± 2.05)	(7.97 ± 1.48)
Final (F)			
Log-transformed	0.71 ± 0.06	0.42 ± 0.08‡	0.75 ± 0.04§
Untransformed (mg/L)†	(7.29 ± 1.63)	(5.57 ± 2.66)	(7.03 ± 1.47)
Difference (F-I)			
Log-transformed	0.09 ± 0.03	−0.24 ± 0.04‡	−0.09 ± 0.02‡§
Untransformed (mg/L)†	(0.99 ± 1.27)	(−0.69 ± 0.73)	(−0.94 ± 1.09

* Values are means ± SE of the log-transformed data adjusted for BMI (ANCOVA).
† Values are means ± SE of the untransformed data.
P-value ≤ 0.05; ‡ vs SO, § vs LFO.

Table 6
IL-6 concentration in the plasma and in the supernatant of LPS-stimulated whole blood in postmenopausal women before and after 5-wk supplementation with SO, LFO, and HFO

	SO	LFO	HFO
Plasma	(n = 10)	(n = 10)	(n = 6)
Initial (I)			
Log-transformed/BMI*	0.15 ± 0.01	0.19 ± 0.01	0.06 ± 0.01‡§
Untransformed (pg/mL)†	(1.63 ± 0.31)	(1.74 ± 0.31)	(1.20 ± 0.12)
Final (F)			
Log-transformed/BMI*	0.09 ± 0.01	0.10 ± 0.02	0.03 ± 0.01§
Untransformed (pg/mL)†	(1.50 ± 0.27)	(1.36 ± 0.15)	(1.14 ± 0.17)
Difference (F-I)			
Log-transformed/BMI*	−0.06 ± 0.01	−0.09 ± 0.01‡	−0.03 ± 0.01‡§
Untransformed (pg/mL)†	(−0.13 ± 0.30)	(−0.38 ± 0.23)	(−0.05 ± 0.10)
Supernatant	(n = 10)	(n = 10)	(n = 9)
Initial (I)			
(pg/mL)•	15.13 ± 0.33	14.39 ± 0.40	14.76 ± 0.26
Final (F)			
(pg/mL)•	14.13 ± 0.41	12.66 ± 0.50‡	13.90 ± 0.26
Difference (F-I)			
(pg/mL)•	−1.00 ± 0.08	−1.72 ± 0.10‡	−0.85 ± 0.06§

* Values are mean ± SE of the log-transformed data adjusted for BMI (ANCOVA).
† Values are means ± SE of the data prior to any transformation and adjustment for BMI.
• Values are means ± SE of the data adjusted for BMI.
P-value ≤0.05; ‡ vs SO, § vs LFO.

HFO group ($d = 0.60$). A small-to-medium effect size was noted when the SO group was compared to the LFO group ($d = 0.29$). The comparison between the SO and the HFO groups elicited an effect size below the threshold of 0.20 for a "small" effect ($d = 0.11$).

Significant positive correlations were found at baseline between CRP concentration and plasma IL-6 concentration (0.462, $P = 0.01$), TG concentration (0.623, $P = 0.000$), and TG/HDL-C ratio (0.577, $P = 0.001$).

4. Discussion

Although CVD is often associated with men, after menopause the risk for CVD is similar in both genders [37]. For many years HRT was considered beneficial to postmenopausal women due to improving their lipid profile, as well as osteoporosis outcome, memory and mood, and hot flashes. A recent report [38] revealed that for HRT users' risks outweigh the benefits and HRT can lead to fatal cardiovascular events and breast and uterine cancers. Some of the predictive factors for cardiovascular pathology aggravated during HRT are CRP and IL-6 [39, 40]. It has been documented that there is a significant difference regarding these markers between women taking hormones and non-users, but no difference among HRT and estrogen replacement therapy (ERT) users [40]. In addition to inflammation markers, TG concentration may be particularly important for postmenopausal women taking HRT, being 38% higher in women using HRT compared to those not using HRT [41]. Also HDL-C may be lowered by HRT [42, 43]. It is reasonable to think that many postmenopausal women on HRT will remain on this therapy, so complementary measures should be taken to decrease the aforementioned risks. Results of the present study show that fish oil supplementation can improve some of the lipid and inflammatory risk factors for CVD in postmenopausal women taking HRT.

Research in the n-3 PUFA area [41, 44] has consistently shown that consumption of fish and fish oils lowers serum TG concentrations in humans. In addition, Stark et al. [41] showed that a supplement of 4.0 g/day EPA and DHA reduced the ratio of TG/HDL-C ratio, a CVD-risk factor shown to be a better predictor of myocardial infarction than the more commonly used ratios of TC/ HDL-C or LDL-C/ HDL-C [16] in postmenopausal women. In a recent meta-analysis, Austin et al. [45] found that a 1 mmol/L-rise in plasma TG was associated with a 32% increase in CVD risk in men and 76% increase in women. Supplementation with 14 g/day fish oil in the present study led to a significant

Table 7
Cohen's d as a measure of the effect size

	CRP* (n = 25)	IL-6† (n = 26)
SO/LFO	0.54	0.29
SO/HFO	0.55	0.11
HFO/LFO	0.10	0.60

Cohen's d is calculated based on mean and SD using data prior to any transformation.
* CRP in serum; † IL-6 in plasma.

decrease in the TG concentration and TG/HDL-C ratio in the HFO compared to the SO groups. This change in TG suggests that the risk of CVD may be decreased by as much as 30%. The parallel decrease of the TG/HDL-C ratio with fish oil supplementation strengthens the significance of this observation and provides a basis for the use of this ratio in assessing the risk for CVD in future clinical trials.

Fish oil supplementation significantly decreased serum CRP concentration compared to control, with a greater effect in the LFO compared to the HFO group. Only the lower dose of fish oil decreased IL-6 concentrations, either in plasma or the supernatant from LPS-stimulated whole blood.

Taking into consideration the fact that the sample size was small, ranging from 6 to 10 subjects in each group, additional information was needed to reflect the clinical significance of the results found in the present study. Therefore, besides the *P*-values (statistical significance), the effect sizes (practical significance) (Table 7) were calculated [46]. For the difference score of serum CRP concentration the comparison of both doses of fish oil to the placebo oil yielded medium effect sizes as well as statistically significant differences, suggesting a possible clinical significance of these results. However, for the difference score of the plasma IL-6 concentration, although the values measured in all three groups were statistically different, a medium-to-large effect size was determined only for the LFO to HFO comparison. This suggests that the two doses of fish oil have clinical significance for plasma IL-6, but not serum CRP concentrations.

Research on the effect of fish oil supplementation on CRP and IL-6 is limited. Therefore, it is difficult to make definitive statements about this relationship and the underlying mechanism. Ernst *et al*. [47] supplemented healthy males for 3 weeks with highly purified n-3 PUFA (1.75g EPA +1.05g DHA) and measured changes in acute-phase proteins induced by exercise in association with supplementation. The absence of a high-sensitivity assay for CRP at that time made it impossible to assess the effect of n-3 PUFA, since the protein levels were undetectable. A series of studies have been done in subjects with cancer cachexia who were supplemented with various doses of n-3 PUFA. As an example, Wigmore *et al*. [48] reported a significant decrease in serum CRP concentration after 4-week supplementation with doses of EPA (97.5% EPA+2.5% DHA) ranging from 1 to 6g/day in patients with pancreatic cancer. The supplementation significantly decreased the production of IL-6 by LPS-stimulated peripheral blood mononuclear cells isolated from subjects, but had no significant effect on plasma IL-6 concentration. Madsen *et al*. [49] studied the effect of fish consumption on CRP concentrations in patients with clinical suspicion of coronary artery disease. The DHA content of granulocyte membranes inversely correlated with serum CRP concentrations but there was no correlation between the EPA content of granulocytes and CRP concentrations. In a recent study, Chan *et al*. [50] supplemented obese men with 4g/day Omacor™ (45% EPA +39% DHA) alone or in association with atorvastatin for 6 weeks. At the end of this interval there was a significant decrease in plasma CRP and IL-6 concentrations in the atorvastatin + Omacor™ group, but not in the fish oil alone. Collectively, these studies indicate that the role of fish oil on CRP and IL-6 is poorly understood.

The mechanisms by which n-3 PUFA may regulate CRP and IL-6 are unclear. An aspect suggested by the current study is that the eicosanoid pathway may not be the major regulator of these proteins, since fish oil supplementation did not significantly change plasma concentration of arachidonic acid, the major precursor of eicosanoids. However, the n-3 fatty acids may target other proteins that are involved in the modulation of CRP and IL-6, such as IL-1, TNF-α, HNF-4, PPARα, and other transcription factors.

The significant positive correlations that were found at baseline between serum CRP concentration and plasma IL-6, TG, and TG/HDL-C ratio suggest that the approach in preventing CVD should entail both lipid and inflammation risk factors. The present study suggests that fish oil supplementation in amounts achievable by dietary modifications could reverse some of the undesirable effects of HRT by lowering the TG/HDL ratio, and plasma TG, serum CRP, and IL-6 concentrations in healthy postmenopausal women. Subjects took a 14 g supplement of oil daily for five weeks. When they were taking the high fish oil supplement they would have to eat six oz of Chinook salmon, tuna, or mackerel each day. This is the size of a serving one usually receives at a restaurant. Similarly those women taking the low fish oil supplement would have to eat three oz of fish daily. This is the serving size recommended by the U.S. Department of Agriculture. Since most Americans do not usually eat fish daily, to be sure that the changes that we saw can be produced by dietary modification, the study needs to be confirmed in a population where fish is consumed less frequently.

Acknowledgments

We are grateful for the help of Dr. Robin Hopkins, Cissy Geigerman, and Karishma Fernandes during the supplementation period, and to Dr. Kenneth Gruber for the help on statistical analyses. This study was supported by the National Research Initiative Competitive Grants Program (Grant # 99-35200-7784) from the US Department of Agriculture, and the Linus Pauling Institute at Oregon State University, Corvallis, OR.

References

[1] Ross R. Atherosclerosis - an inflammatory disease. N Engl J Med 1999;340(2):115–26.

[2] Ridker PM, Buring JE, Shih H, Hennekens CH. Prospective study of C- reactive protein and the risk of future cardiovascular events among apparently healthy women. Circulation 1998;98(8):731–3.

[3] Ridker PM, Hennekens CH, Buring JE, Rifai N. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. N Engl J Med 2000;342(12):836–43.
[4] Ridker, PM, Cushman, M, Stampfer, MJ, Tracy, RP, Hennekens, CH. Inflammation, aspirin and the risk of cardiovascular disease in apparently healthy men [published erratum appears in N Engl J Med 1997 Jul 31;337(5):356]. N Engl J Med 1997;336(14):973–9.
[5] Tracy RP, Lemaitre RN, Psaty BM, Ives DG, Evans RW, Cushman M, Meilahn EN, Kuller LH. Relationship of C-reactive protein to risk of cardiovascular disease in the elderly. Results from the cardiovascular Health Study and the Rural Health Promotion Project. Arterioscler Thromb Vasc Biol 1997;1(7):1121–7.
[6] Knopp RH. Risk factors for coronary artery disease in women. Am J Cardiol 2002;89(12 suppl):28E–35E.
[7] Ridker PM, Hennekens CH, Rifai N, Buring JE, Manson JE. Hormone replacement therapy and increased plasma concentration of C-reactive protein. Circulation 1999;100:713–6.
[8] Hutchinson WL, Koenig W, Frochlich M, Sund M, Lowe GDO, Pepys MB. Immunoradiometric assay of circulating C-reactive protein: age related values in adult general population. Clin Chem 2000; 46(7):934–38.
[9] Heilbronn LK, Clifton PM. C-reactive protein and coronary artery disease: influence of obesity, caloric restriction and weight loss. J Nutr Biochem 2002;13(6):316–21.
[10] Taaffe DR, Harris TB, Ferrucci L, Rowe J, Seeman TE. Cross-sectional and prospective relationships of interleukin-6 and C-reactive protein with physical performance in elderly persons: MacArthur Studies of successful aging. J Geront 2000;55A(12):M709–M715.
[11] Das I. Raised C-reactive protein levels in serum from smokers. Clin Chim Acta 1985;153(1):9–13.
[12] Imhof A, Froehlich M, Brenner H, Boeing H, Pepys MB, Koenig W. Effect of alcohol consumption on systemic markers of inflammation. Lancet 2001;357:763–7.
[13] Vickers MA, Green FR, Terry C, Mayosi BM, Julier C, Lathrop M, Ratcliffe PJ, Watkins HC, Keavney B. Genotype at the promoter polymorphism of the interleukin-6 gene is associated with baseline levels of plasma C-reactive protein. Cardiovasc Res 2002;53(4):1029–34.
[14] Berger P, McConnell JP, Nunn M, Kornman KS, Sorrell J, Stephenson K, Duff GW. C-reactive protein levels are influenced by common IL-1 gene variations. Cytokine 2002;17(4):171–4.
[15] Zee RY, Ridker PM. Polymorphism in the human C-reactive protein (CRP) gene, plasma concentrations of CRP, and the risk of future arterial thrombosis. Atherosclerosis 2002;162(1):217–9.
[16] Sharrett AR, Sorlie PD, Chambless LE, Folsom AR, Hutchinson RG. Relative importance of various risk factors for asymptomatic carotid atherosclerosis versus coronary heart disease incidence: the Artherosclerosis Risk in Communities Study. Am J Epidemiol 1999;149(9):843–52.
[17] Barber MD. Cancer cachexia and its treatment with fish-oil-enriched nutritional supplementation. Nutrition 2001;17(9):751–5.
[18] Schmidt EB, Varming K, Ernst E, Madsen P, Dyeberg J. Dose response studies on the effect of n-3 polyunsaturated fatty acids on lipids and haemostasis. Thromb Haemost 1990;63(1):1–5.
[19] GISSI Prevenzione Investigators. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: the results of the GISSI Prevenzione trial. Lancet 1999;354:447–55.
[20] Wander RC, Du SH. Oxidation of plasma proteins is not increased after supplementation with eicosapentaenoic and docosahexaenoic acids. Am J Clin Nutr 2000;7(2):731–7.
[21] Wander RC, Patton BD. Comparison of three species of fish consumed as part of a Western diet: effects on platelet fatty acids and function, homeostasis, and production of tromboxane. Am J Clin Nutr 1991;54(2):326–33.
[22] Higdon JV, Liu J, Du SH, Morrow JD, Ames BN, Wander RC. Supplementation of postmenopausal women with fish oil rich in eicosapentaenoic acid and docosahexaenoic acid is not associated with greater in vivo lipid peroxidation compared with oils rich in oleate and linoleate as assessed by plasma malondialdehyde and F(2)-isoprostanes. Am J Clin Nutr 2000;72(3):714–22.
[23] El-Hamdy AH, Perkins EG. High performance reversed phase chromatography of natural triglyceride mixtures: critical pair separation. J Am Oil Chem Soc 1991;5(8):867–72.
[24] American Oil Chemists' Society. Method Cd-8-53. In Official Methods and Recommended Practices. American Oil Chemists' Society, Champaign, IL 1988; 8:–53.
[25] Allain CC, Poon LS, Cahn CS, Richmond W, Fu PC. Enzymatic determination of total serum cholesterol. Clin Chem 1974;20(4):470–5.
[26] McGowan MW, Artiss JD, Strandbergh DR, Zak B. A peroxidase-coupled method for colorimetric determination of serum triglycerides. Clin Chem 1983;29(3):538–42.
[27] Marz W, Gross W. Evaluation of a phosphotungstic acid/MgCl2 precipitation and quantitative lipoprotein electrophoresis assay. Clin Chem 1986;160(1):1–18.
[28] Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972;18(6):499–502.
[29] Song J, Wander RC. Effects of dietary selenium and fish oil (Max-EPA) on arachidonic acid metabolism and hemostatic function in rats). J Nutr 1991;12(1):284–92.
[30] Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Phys 1995;3(7):911–7.
[31] Jamieson J. Dealing with baseline differences: two principles and two dilemmas. Int J Psychophysiol 1999;31(2):155–61.
[32] Fry, J. Biological data analysis. A Practical Approach/ edited by John C. Fry. Oxford; New York: IRL Press at Oxford University Press, 1993 [chapter 1].
[33] Zar JH. Biostatistical analysis, 3rd ed. New Jersey: Prentice Hall, 1996, chapter 13.
[34] Bermudez EA, Rifai N, Buring J, Manson JE, Ridker PM. Interrelationships among circulating interleukin-6, C-reactive protein, and traditional cardiovascular risk factors in women. Arterioscler Thromb Vasc Biol 2002;22(10):1668–73.
[35] Cohen J. Statistical power analysis for the behavioral sciences. 2nd ed. Hillsdale, NY: Lawrence Erlbaum Associates, 1988. p. 40.
[36] Krauss RM, Eckel RH, Howard B, Appel LJ, Daniels SR, Deckelbaum RJ, Erdman JW Jr, Kris-Etherton P, Goldberg IJ, Kotchen TA, Lichtenstein AH, Mitch WE, Mullis R, Robinson K, Wylie-Rosett J, St Jeor S, Suttie J, Tribble DL, Bazzarre TL. AHA Dietary Guidelines: revision 2000 a statement for healthcare professionals from the Nutrition Committee of the American Heart Association. Circulation 2000;102(18):2284–99.
[37] Colditz GA, Willett WC, Stampfer MJ, Rosner B, Speizer FE, Hennekens CH. Menopause and the risk of coronary heart disease in women. N Engl J Med 1987;316(18):1105–10.
[38] Writing Group for the Women's Health Initiative Investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. JAMA 2002;288(3):321–33.
[39] Herrington DM, Brosnihan KB, Pusser BE, Seely EW, Ridker PM, Rifai N, MacLean DB. Differential effects of E and droloxifene on C-reactive protein and other markers of inflammation in healthy postmenopausal women. J Clin Endocrinol Metab 2001;86(9):4216–22.
[40] Ridker PM, Hennekens CH, Rifai N, Buring JE, Manson JE. Hormone replacement therapy and increased plasma concentration of C-reactive protein. Circulation 1999;10:713–16.
[41] Stark KD, Park EJ, Maines VA, Holub BJ. Effect of a fish-oil concentrate on serum lipids in postmenopausal women receiving and not receiving hormone replacement therapy in a placebo-contralled, double-blind trial. Am J Clin Nutr 2000;72(2):389–94.
[42] Goudev A, Georgiev D, Koycheva N, Manasiev N, Kyurkchiev S. Effects of low dose hormone replacement therapy on markers of inflammation in postmenopausal women. Maturitas 2002;30(1):43–9.

[43] Ranta V, Oksanen H, Arrenbrecht S, Ylikorkala O. National differences in lipid response to postmenopausal hormone replacement therapy. Maturitas 2002;42(4):259–65.
[44] Harris WS. n-3 fatty acids and serum lipoproteins: human studies. Am J Clin Nutr 1997;65:1645S–1654S.
[45] Austin MA, Hokanson JE, Edwards KL. Hypertriglyceridemia as a cardiovascular risk factor. Am J Cardiol 1998;81(4A):7B–12B.
[46] Thompson B. "Statistical", "Practical", and "Clinical:" How many kinds of significance do counselors need to consider? J Counseling Development 2002;8:64–71.
[47] Ernst E, Saradeth T, Achhammer G. n-3 fatty acids and acute-phase proteins. Eur J Clin Invest 1991;21(1):77–82.
[48] Wigmore SJ, Fearon KC, Ross JA. Modulation of human hepatocyte acute phase protein production in vitro by n-3 and n-6 polyunsaturated fatty acids. Ann Surg 1997;225(1):103–11.
[49] Madsen T, Skou HA, Hansen VE, Fog L, Christensen JH, Toft E, Schmidt EB. C-reactive protein, dietary n-3 fatty acids, and the extent of coronary artery disease. Am J Cardiol 2001;88(10):1139–42.
[50] Chan DC, Watts GF, Barrett PH, Beilin LJ, Mori TA. Effect of atorvastatin and fish oil on plasma high-sensitivity C-reactive protein concentrations in individuals with visceral obesity. Clin Chem 2002;48(6 Pt 1):877–83.