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The effect of dietary *n*-3 fatty acids on serum concentrations of C-reactive protein: a dose–response study

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C-reactive protein (CRP) is a sensitive marker for low-grade inflammation. Long-chain *n*-3 polyunsaturated fatty acids (PUFA) have anti-inflammatory effects. The objective of the present study was to investigate the effect on serum levels of CRP of *n*-3 PUFA at two different doses. We also investigated correlations between CRP and the cellular contents of PUFA. Sixty healthy volunteers (twenty-five women and thirty-five men) were randomly assigned to three treatment groups in a double-blind design. The subjects received a supplement of either 6·6 g *n*-3 PUFA/d, 2·0 g *n*-3 PUFA/d or placebo (olive oil) for 12 weeks. CRP was measured using a highly sensitive assay. The median serum CRP concentration was 0·78 mg/l. No significant correlations were found between CRP and the content of *n*-3 PUFA in granulocytes or platelets. Subjects receiving *n*-3 PUFA had a significant ($P<0·01$) increase in the cellular contents of 20 : 5*n*-3, 22 : 5*n*-3 and 22 : 6*n*-3, with the largest increase occurring in the group receiving 6·6 g PUFA/d. A significant ($P<0·01$) decrease in cellular content of 18 : 2*n*-6 and 20 : 4*n*-6 was observed simultaneously. Serum CRP concentrations, however, were unaffected by the PUFA-containing supplements. The present study shows that dietary supplementation with PUFA-containing supplements has no effect on serum concentrations of CRP, measured with a highly sensitive assay, in healthy subjects.

Fatty acids: Fish oil: Inflammation: C-reactive protein

C-reactive protein (CRP) is an acute-phase reactant produced in the liver in response to the cytokine interleukin 6 released from activated leucocytes (Castell *et al.* 1990). Infection, inflammatory disease, burns, trauma, surgery and tissue infarction cause changes in plasma CRP concentrations, which can increase more than 100-fold (Steel & Whitehead, 1994). In daily clinical practice CRP concentrations <10 mg/l are often considered as clinically insignificant. However, highly sensitive (hs) CRP assays are now available to detect CRP concentrations well below the lower limit of most assays used in clinical settings and, hence, biochemical signs of low-grade inflammation.

There is growing evidence that chronic low-grade inflammation has an important role in the initiation and progression of atherosclerosis (Ross, 1999). Therefore, a significant part of cardiovascular research in recent years has involved the measurement of hs-CRP. Slightly elevated baseline concentrations of CRP are predictive of a higher risk of future cardiovascular morbidity and mortality in apparently healthy people (Koenig *et al.* 1999), and it has been suggested that measurement of hs-CRP should be included in coronary risk assessment (Ridker *et al.* 1997; Ridker, 2001).

The marine *n*-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (20 : 5*n*-3) and docosahexaenoic acid (22 : 6*n*-3), found primarily in fish and fish oil, possess anti-inflammatory properties (Schmidt & Dyerberg, 1989; James *et al.* 2000; Calder, 2001), which may contribute to their cardioprotective effect (Schmidt, 1997). The objective of the present study was to examine the possible correlations between baseline CRP concentrations and the content of *n*-3 and *n*-6 fatty acids in granulocytes and platelets, and to investigate the effect of two different doses of *n*-3 PUFA supplementation on serum levels of CRP.

Subjects and methods

Subjects

Sixty healthy volunteers (twenty-five women and thirty-five men) were recruited from the medical staff, blood-bank employees and students in Aalborg, Denmark. None

Abbreviations: CRP, C-reactive protein; hs, highly sensitive; PUFA, polyunsaturated fatty acid.

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of the subjects had any known diseases or took any medication at the time of investigation. The mean age was 38 (range 21–57) years.

Study design

The present work is a substudy of a dose–response study focusing on *n*-3 PUFA and heart-rate variability (Christensen *et al.* 1999). The study design was double-blind and placebo-controlled with regard to the three dietary groups (see later). The protocol was approved by the regional Ethical Committee and written informed consent was obtained from all the subjects.

Dietary supplements

The subjects were randomly assigned to one of three intervention groups. One group received a supplement of 6·6 g *n*-3 PUFA/d, containing approximately 3·0 g 20 : 5*n*-3 and 2·9 g 22 : 6*n*-3 (ten capsules of Pikasol® (a reesterified triacylglycerol, EPAX 5500); Pronova Biocare A/S, Sandefjord, Norway). The second group received 2·0 g *n*-3 PUFA (0·9 g 20 : 5*n*-3 and 0·8 g 22 : 6*n*-3 in three capsules of Pikasol®) and seven capsules of placebo (olive oil)/d. The third group received ten capsules of olive oil per d. One placebo capsule contained approximately 0·7 g olive oil. The subjects were randomised in blocks of ten and numbered consecutively. Treatment began at the time of allocation. The subjects took three capsules in the morning and seven capsules with the evening meal. The capsules were taken from two different boxes to maintain the blinding. The supplements were given daily for 12 weeks.

Blood sampling

Before and after 12 weeks of dietary supplementation, blood samples were drawn after an overnight fast of at least 10 h and immediately frozen at − 80°C for later analysis.

Fatty acid analysis

Granulocytes and platelets were isolated from whole blood, the lipids were extracted, and the fatty acids esterified as described earlier (Schmidt *et al.* 1991). The content of the different PUFA in granulocytes and platelets was measured using a Chrompack CP-9002 GC (Chrompack International, Middelburg, the Netherlands) and expressed as g/100 g total fatty acids.

Measurement of serum C-reactive protein

Measurement of CRP was performed with a BNII nephelometer (Dade Behring Marburg GmbH, Marburg, Germany), which employs fixed-time kinetic measurements at 840 nm. In this assay, polystyrene beads coated with monoclonal antibodies from mice bind CRP present in the serum samples, resulting in light-scattering aggregates proportional to the content of CRP. The particle enhancement makes the determination of values in the lower quartile of the reference range possible. The sensitivity of this assay (0·17 mg/l) is comparable with that of other hs-CRP assays such as ELISA (Macy *et al.* 1997). The calibration of the method ensures traceability to the primary reference material CRM 470. The method has been validated by others (Rifai *et al.* 1999). In our hands, the intra-run precision (CV) was estimated to 2·9 and 3·0 % at concentrations of 0·43 and 2·08 mg/l respectively.

Statistical analysis

The size of the study sample was based on an estimated SD 1·5 mg/l, type 1 error 5 % and type 2 error 20 %. Twenty subjects were required in each intervention group in order not to miss a 1·3 mg/l difference between the groups. Because the distribution of CRP values was skewed towards greater values, serum concentrations are presented as median values and interquartile ranges. The remaining variables are presented as mean values and standard deviations. Wilcoxon signed rank sum test was used to test for any differences within the groups before and after supplementation. Comparisons between groups were carried out using the Mann–Whitney U test or, when more than two groups were involved, by Kruskal–Wallis one-way ANOVA. Differences in nominal data were evaluated by the χ^2 test. Spearman's rank correlation test was used to assess relationships between variables. $P<0{\cdot}05$ (two-tailed) was considered statistically significant. The SPSS software package (version 7.0; SPSS, Chicago, IL, USA) was used for all the analyses.

Results

Before supplementation

The median serum CRP concentration was 0·78 (range 0·44–1·48) mg/l. CRP concentrations were <3 mg/l in 90 % of the subjects. There was no significant difference in CRP levels between men and women (median value 0·69 (interquartile range 0·36–1·08) *v.* 1·00 (interquartile range 0·50–1·90) mg/l, $P=0{\cdot}15$). In men, but not in women, CRP was positively correlated with the content of 18 : 2*n*-6 in granulocytes and of 16 : 0 in both granulocytes and in platelets. However, these correlations disappeared in a multivariate linear stepwise regression analysis. No significant correlations were found between CRP and the content of any of the other PUFA, including 18 : 1*n*-9, which olive oil is rich in (results not shown). CRP was positively correlated with BMI (Spearman's ρ 0·31, $P=0{\cdot}015$). The three intervention groups were comparable before supplementation with regard to age, sex, BMI and smoking status (Table 1), CRP concentrations (Table 2) and cellular content of fatty acids (Table 3).

Dietary supplementation

Serum CRP concentrations before and after the supplements are shown in Table 2. There were no statistically significant differences in CRP concentrations before and after supplementation in any of the three groups and no differences between groups (Table 2 and Fig. 1). When subjects with CRP >2 mg/l (n 11) were analysed separately, no difference with supplementation was found either.

Table 1. Characteristics of the study participants before supplementation
(Mean values and standard deviations for twenty subjects per group)

	Placebo				2·0 g *n*-3 PUFA/d				6·6 g *n*-3 PUFA/d				Statistical significance
Treatment group...	Mean	SD	*n*	%	Mean	SD	*n*	%	Mean	SD	*n*	%	of effect
Age (years)	37	11			38	10			39	10			NS
Women			8	40			8	40			9	45	NS
Men			12	60			12	60			11	55	NS
BMI (kg/m^2)	24·0	2·7			25·1	2·9			24·6	10·1			NS
Current smoking (*n*)			5	20			6	20			5	20	NS

PUFA, polyunsaturated fatty acid.

Subjects receiving 2·0 or 6·6 g *n*-3 PUFA/d for 12 weeks had a significant ($P<0·01$) increase in cellular contents of 20 : 5*n*-3, 22 : 5*n*-3 and 22 : 6*n*-3, with the largest increase occurring in the group receiving 6·6 g PUFA/d. Significant ($P<0·01$) decreases in 18 : 2*n*-6 and 20 : 4*n*-6 were observed simultaneously in both granulocytes and platelets (Table 3).

Discussion

The median CRP concentrations in healthy populations are reported to be 1–2 mg/l (Rifai *et al.* 1999; Erlandsen & Randers, 2000). In healthy subjects, baseline CRP concentrations are not subject to diurnal variation (Meier-Ewert *et al.* 2001) and, furthermore, appear tightly regulated over long periods of time, with some individuals having consistently higher values than others (Macy *et al.* 1997). Thus, measurement of hs-CRP provides a stable measure of chronic low-grade inflammation.

The fatty-acid composition of cell membranes reflects the dietary intake of these fatty acids (Brown *et al.* 1991). In the Western diet, the *n*-6:*n*-3 PUFA ratio has increased greatly over the past century (Simopoulos, 1999). In the course of inflammatory activation the *n*-6 PUFA, arachidonic acid (20 : 4*n*-6), is released from membrane phospholipids and metabolised to highly proinflammatory eicosanoids such as leukotriene B_4 (Lee & Austen, 1986). When the intake of marine *n*-3 PUFA is high, eicosapentaenoic acid (20 : 5*n*-3) and docosahexaenoic acid (22 : 6*n*-3) partially replace arachidonic acid in the cell membranes, and eicosapentaenoic acid competes with arachidonic acid as a substrate for eicosanoids, leading to formation of the less active leucotriene B_5. The balance of *n*-6- and *n*-3-derived eicosanoids is therefore shifted towards a mixture with decreased inflammatory activity (James *et al.* 2000). Other mechanisms by which *n*-3 PUFA exert their anti-inflammatory properties include a reduced production of the major cytokine mediators of inflammation, interleukin 1 and tumour necrosis factor (Caughey *et al.* 1996), attenuation of leucocyte adhesion to the endothelium (De Caterina *et al.* 2000) and impaired leucocyte chemotaxis (Schmidt & Dyerberg, 1989).

In accordance with this, a beneficial effect of *n*-3 PUFA has been observed in patients with inflammatory diseases, such as rheumatoid arthritis (James & Cleland, 1997) and inflammatory bowel disease (Belluzzi *et al.* 2000).

The specific effect of dietary *n*-3 PUFA on CRP concentrations has scarcely been studied. Ernst *et al.* (1991) found that 3 weeks of supplementation with *n*-3 fatty acids reduced the concentrations of several acute-phase proteins in young healthy males and altered the pattern of change following exercise. CRP concentrations, however, remained below the detection limit of the only moderately sensitive assay used (2·8 mg/l).

In the present study, serum CRP levels were not reduced by 12 weeks of dietary supplementation with 2·0 or 6·6 g *n*-3 PUFA. Our present subjects were all apparently healthy and, accordingly, had low baseline concentrations of CRP. Little, if any, reduction of CRP was therefore to be expected.

Chan *et al.* (2002) recently reported 4·4-fold greater CRP concentrations in obese individuals compared with lean healthy controls. Atorvastatin significantly decreased CRP in the obese subjects, but this was not seen with PUFA given in doses of approximately 3·4 g/d. The authors suggested that the lack of effect of fish oil on CRP might be due to a small but deleterious effect of *n*-3 PUFA on insulin sensitivity, which would tend to stimulate CRP synthesis (Campos & Baumann, 1992) and counteract a supressive effect of *n*-3 PUFA on proinflammatory cytokines. Chan *et al.* (2002) used a dose of fish oil that was about 50 % that of our 6·6 g/d group, and the study went on for only 6 weeks compared with 12 weeks in our present study.

Table 2. Serum C-reactive protein values in the three treatment groups before and after 12 weeks of dietary supplementation*
(Median values and interquartile ranges for twenty subjects per group)

Treatment group...	Placebo		2·0 g *n*-3 PUFA/d		6·6 g *n*-3 PUFA/d	
Time (weeks)...	0	12	0	12	0	12
Median	0·67	0·63	0·69	0·67	1·07	0·88
Interquartile range	0·30–1·54	0·36–1·01	0·41–1·21	0·26–2·81	0·67–2·36	0·54–1·95

PUFA, polyunsaturated fatty acid.
* For details of subjects, supplements and procedures, see Table 1 and p. 518.

Table 3. Content of fatty acids (g/100 g total fatty acids) in granulocyte and platelet membranes in the three treatment groups before and after 12 weeks of supplementation§ (Mean values and standard deviations for twenty subjects per group)

Treatment group…	Placebo						2·0 g *n*-3 PUFA/d						6·6 g *n*-3 PUFA/d					
Time (weeks)…	0		12		Difference‖		0		12		Difference‖		0		12		Difference‖	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Granulocytes																		
16 : 0	11·72	1·11	11·58	0·93	0·14	0·71	11·72	0·65	11·87	0·79	−0·15	0·56	11·80	0·83	12·09	0·87*	−0·29	0·55
18 : 0	16·97	0·78	16·69	1·25	0·28	0·93	17·00	0·71	16·95	0·57	0·05	0·61	17·20	0·78	17·21	0·58	−0·01	0·69
18 : 1*n*-9	31·43	1·63	32·31	1·91*	−0·88	1·50	31·69	1·43	32·83	1·49**	−1·14	1·39	31·31	1·82	32·12	1·34*	−0·82	1·50
18 : 2*n*-6	10·09	1·01	9·81	0·69	0·27	0·94	10·53	1·41	10·10	1·13	0·43	1·25	10·53	1·28	9·61	1·02**	0·93	1·07
18 : 3*n*-3	0·12	0·06	0·13	0·06	−0·01	0·05	0·14	0·08	0·14	0·07	−0·00	0·04	0·16	0·09	0·13	0·06**	0·03	0·05
20 : 4*n*-6	13·37	1·37	13·40	1·49	−0·03	0·70	12·49	1·21	11·28	1·08**	1·21	0·86†	12·47	1·51	9·76	1·43**	2·71	1·22‡
20 : 5*n*-3	0·51	0·23	0·55	0·39	−0·04	0·28	0·60	0·48	1·85	0·83**	−1·25	0·57††	0·58	0·19	4·08	1·05**	−3·50	1·03‡
22 : 5*n*-3	1·65	0·53	1·67	0·57	−0·02	0·39	1·46	0·29	2·39	0·48**	−0·92	0·43††	1·66	0·60	3·71	1·06**	−2·04	0·68‡
22 : 6*n*-3	1·53	0·39	1·51	0·47	0·02	0·36	1·46	0·45	1·75	0·59**	−0·29	0·36††	1·58	0·52	2·15	0·41**	−0·57	0·37‡
Platelets																		
16 : 0	15·54	1·52	15·38	0·88	0·16	1·35	15·60	1·22	15·95	1·26	−0·35	0·83	15·54	0·69	15·84	0·86	−0·30	0·66
18 : 0	19·04	0·69	19·11	0·94	−0·07	0·73	19·15	0·76	18·95	0·81	0·20	0·42	19·22	0·73	18·66	0·73*	0·44	0·82
18 : 1*n*-9	18·35	0·78	18·68	0·79	−0·33	0·82	17·93	0·85	19·09	0·82**	−1·16	0·81††	18·26	0·67	19·51	0·87**	−1·25	0·53‡
18 : 2*n*-6	5·85	0·63	5·59	0·38*	0·26	0·48	5·97	0·80	5·62	0·63*	0·36	0·56	6·04	0·63	5·25	0·52**	0·79	0·61‡
18 : 3*n*-3	0·14	0·07	0·14	0·07	−0·01	0·04	0·14	0·09	0·15	0·08	−0·01	0·06	0·15	0·08	0·12	0·08*	0·03	0·05
20 : 4*n*-6	26·14	1·60	26·33	1·37	−0·19	1·49	26·04	1·63	24·07	1·28**	1·97	1·05††	25·63	0·96	21·14	1·95**	4·49	1·59‡
20 : 5*n*-3	0·73	0·27	0·74	0·39	−0·01	0·23	0·75	0·26	2·02	0·59**	−1·27	0·54††	0·87	0·32	4·68	1·37**	−3·82	1·28‡
22 : 5*n*-3	1·80	0·48	1·87	0·47	−0·07	0·24	1·79	0·37	2·33	0·38**	−0·54	0·25††	1·94	0·36	3·26	0·38**	−1·32	0·44‡
22 : 6*n*-3	2·43	0·45	2·36	0·60	0·06	0·39	2·46	0·42	2·82	0·38**	−0·36	0·32††	2·68	0·47	3·60	0·48**	−0·92	0·43‡

PUFA, polyunsaturated fatty acid.
Mean values were significantly different from those before supplementation at time 0: *$P<0·05$, **$P<0·01$.
Mean values were significantly different from those of the placebo group: †$P<0·05$, ††$P<0·01$.
Mean values are significantly different from those of the placebo group and the 2·0 g PUFA/d group: ‡$P<0·01$.
§ For details of subjects, supplements and procedures, see Table 1 and p. 518.
‖ Difference = value at week 0 − value at week 12.

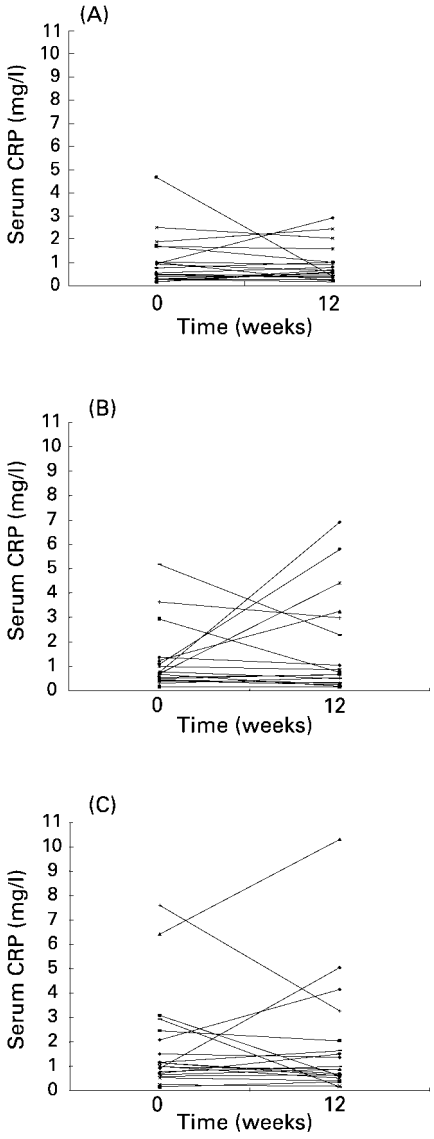

Fig. 1. Serum C-reactive protein (CRP) concentrations at baseline and after 12 weeks of dietary supplementation with placebo (A), 2·0 g *n*-3 polyunsaturated fatty acids/d (B) or 6·6 g *n*-3 polyunsaturated fatty acids/d (C). For details of subjects, supplements and procedures, see Table 1 and p. 518.

There is, however, some evidence that dietary supplementation with *n*-3 PUFA might have an effect on elevated CRP concentrations. In a placebo-controlled intervention study, a statistically significant decrease in CRP from 21 to 17 mg/l was seen in patients with rheumatoid arthritis treated with 3·6 g *n*-3 PUFA/d for 12 weeks, while CRP concentrations were unaltered among controls (Nielsen *et al.* 1992).

In a recent study involving 269 patients referred for coronary angiography due to clinical suspicion of coronary artery disease, we found that CRP concentrations were correlated independently and inversely with the content of 22 : 6*n*-3 in granulocyte membranes (Madsen *et al.* 2001). It remains to be tested whether dietary *n*-3 PUFA supplementation actively lowers CRP concentrations in patients with stable coronary artery disease, who often have slightly elevated CRP (Tataru *et al.* 2000). In patients with acute coronary syndrome the inflammatory system is highly activated and this indicates a worse prognosis (Toss *et al.* 1997; Biasucci *et al.* 1999). It would be of particular interest to investigate the effect of *n*-3 PUFA on CRP concentrations and outcome in these high-risk patients.

In conclusion, 12 weeks of supplementation with *n*-3 PUFA had no effect on CRP in healthy subjects with low baseline CRP levels. However, it remains to be investigated whether intervention with *n*-3 PUFA has any effect on CRP and outcome in subjects with CHD and high CRP levels.

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