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High-dose fish oil and antioxidants in Crohn's disease and the response of bone turnover: a randomised controlled trial

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Crohn's disease is associated with altered bone turnover that may be influenced by nutritional status, the systemic inflammatory response, cytokine production by circulating (peripheral blood) mononuclear cells (PBMC) and antioxidant micronutrient intake. High-dose fish oil is associated with reductions in disease relapse and inflammatory markers, and modulates PBMC function. The effect of fish oil plus antioxidants on bone turnover and PBMC function (the production of interferon-γ and prostaglandin E₂) in Crohn's disease was investigated in a randomised-controlled trial. Patients with currently or recently raised biochemical markers of inflammation (C-reactive protein $\geqq$ 6·9 mg/l or erythrocyte sedimentation rate $\geqq$ 18 mm/h) received fish oil (providing 2·7 g/d EPA and DHA) and antioxidants (vitamins A, C and E, and Se) (*n* 31) or placebo (*n* 30) for 24 weeks. Bone turnover was assessed by measuring the concentrations of urinary deoxypyridinoline (bone resorption) and serum osteocalcin (bone formation). Fish oil plus antioxidants were associated with increases in EPA, DHA Se in plasma (all $P < 0.01$), and with a reduction in interferon-γ production by mitogen-stimulated PBMC, which demonstrated a negative correlation with deoxypyridinoline/creatinine:osteocalcin ratio (r − 0·33, $P = 0.009$). There were no differences between the groups at 24 weeks in the response of deoxypyridinoline or osteocalcin or their ratio, or in nutritional status. Dietary supplementation in Crohn's disease with high intakes of EPA and DHA, as fish oil, plus antioxidants was associated with a modulated production of interferon-γ by PBMC but not altered indices of bone turnover.

Bone: Fish oil: Crohn's disease

Crohn's disease is a relapsing, inflammatory disease of the gastrointestinal tract that is associated with an increased risk of osteoporosis and low-impact fractures (Andreassen *et al.* 1997; De Vos *et al.* 1998; van Staa *et al.* 2003). The aetiology of altered bone metabolism in Crohn's disease is multifactorial but incompletely characterised. Proposed contributory factors include corticosteroid use, poor nutritional status and a reduced availability of vitamin D (Andreassen *et al.* 1997; De Vos *et al.* 1998; Habtezion *et al.* 2002). The influence of gastrointestinal inflammation on bone metabolism is unconfirmed, and the process by which this may occur is uncertain. There is evidence from a study of a small Crohn's disease cohort that higher rates of bone resorption may be associated with raised values of the laboratory markers of inflammation C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), and an altered production of the inflammatory mediators interferon-γ (IFN-γ) and prostaglandin (PG) E₂ by circulating peripheral blood mononuclear cells (PBMC) (Trebble *et al.* 2004*c*). Moreover, an altered production of cytokines by PBMC is associated with osteoporosis in postmenopausal women (Pietschmann *et al.* 2001). An increased production of CRP may be independently associated with altered cytokine production by PBMC (Wigmore *et al.* 1998, 2002), and in Crohn's disease, CRP and ESR are predictive of disease relapse (Brignola *et al.* 1986*a*; Belluzzi *et al.* 1996; Schreiber *et al.* 1999), correlate to TNF-α release by PBMC (Mazlam and Hodgson, 1992) and may indicate sub-clinical intestinal inflammation (Brignola *et al.* 1986*b*; Schreiber *et al.* 1999).

Fish oil is rich in the *n*-3 PUFA, EPA and DHA (British Nutrition Foundation, 1999). In high doses, dietary fish oil is associated with reductions in disease relapse rate and laboratory markers of inflammation (including ESR) in Crohn's disease (Belluzzi *et al.* 1996). Fish oil modulates PBMC function, including the production of IFN-γ in healthy subjects (Calder, 2001; Trebble *et al.* 2003*b*) and patients with Crohn's disease (Trebble *et al.* 2004*a*). EPA and DHA may, however, lead to increased lipid peroxidation and the production of free-radical oxidative moieties (Eritsland, 2000), with potentially detrimental effects on inflammatory activity in Crohn's disease patients (Simmonds &

Abbreviations: Cre, creatinine; CRP, C-reactive protein; DCAI, Chronic Disease Activity Index; DPD, deoxypyridinoline; ESR, erythrocyte sedimentation rate; IFN, interferon; PBMC, peripheral blood mononuclear cell; PG, prostaglandin.
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Rampton, 1993; Grisham, 1994) and on bone metabolism (Parhami, 2003). Dietary supplementation with antioxidants decreases this oxidative stress (Aghdassi *et al.* 2003).

The aims of this investigation were therefore to assess the effect of high-dose fish oil and antioxidant co-supplementation on bone metabolism and the PBMC production of inflammatory mediators in Crohn's disease patients with evidence of raised laboratory markers of inflammation.

Methods

Study design

This was a randomised, double-blind, placebo-controlled trial of the response to fish oil plus antioxidants in Crohn's disease. Randomisation was in permutated blocks of six, with patients stratified for current immunosuppressant use, gender and, in females, menopausal status. Randomisation codes were held by a designated pharmacist with no other direct involvement in the study.

Patients consumed 9 capsules/d fish oil (MaxEPA; Seven Seas Ltd., Hull, Humberside, UK) or placebo (olive oil, containing the MUFA oleic acid in addition to their habitual diet. The fish-oil supplement was equivalent to 2·7 g/d EPA and DHA (Table 1). Patients in the fish-oil group additionally received a compound antioxidant dietary supplement containing 200 μg Se, 3 mg Mn, 30 mg vitamin E as d-α-tocopheryl succinate, 450 μg vitamin A (300 μg retinol equivalents as retinol and 150 μg retinol equivalents as β-carotene) and 90 mg vitamin C as ascorbic acid. The placebo group received an identical capsule containing maltose and lactose (both antioxidant and placebo being supplied by Wassen International, Leatherhead, Surrey, UK).

Patients completed a questionnaire-based assessment of previous gastroenterological and medical history, and medical notes were reviewed for further information. Assessments were performed at baseline and at 8, 16 and 24 weeks, and included disease activity score, treatment compliance and side-effects, laboratory investigations and the issuing of fresh capsules. Compliance with the study was assessed by direct questioning and confirmed by an analysis of plasma phospholipid composition.

Subjects

The study was approved by the Southampton and South West Hampshire Joint Research Ethics Committee. Patients were identified using a database of Crohn's disease out-patients under active and recent follow-up at a large university hospital. All patients gave their informed consent. The diagnosis of Crohn's disease was based on endoscopic, histological or radiological findings, and patients with a disease distribution including small or large bowel or perianal disease were recruited.

Crohn's disease activity was assessed clinically using the Crohn's Disease Activity Index (CDAI; Best *et al.* 1976), a composite clinical disease score consisting of predetermined measures of symptoms (stool frequency, abdominal pain and general well-being), extra-intestinal manifestations of Crohn's disease, use of anti-diarrhoeals, presence of an abdominal mass, weight differential from predicted weight and haematocrit. Inflammatory activity was assessed by changes in the inflammatory markers CRP and ESR.

Criteria for inclusion in the study included laboratory markers of inflammation above the locally determined normal range for CRP concentration ($\geq 6·9$ mg/l) and/or ESR (≥ 18 mm/h) within the previous 4 weeks. Exclusion criteria included: severe disease (CDAI > 450); use of bisphosphonate, oral or intravenous corticosteroid medication within the previous 4 weeks; introduction of other immunosuppressant medication within the previous 8 weeks; awaiting or immediately post-surgery; use of nutritional support in any form; major small bowel intestinal resection, total or sub-total colectomy or features of short-bowel syndrome; inflammatory disease unrelated to Crohn's disease, hyperlipidaemia or diabetes mellitus, liver or renal impairment, cancer or any other serious acute medical condition; pregnancy or lactation; recent consumption of *n*-3 PUFA supplements; age under 18 years.

Plasma fatty acid measurement

To assess the fatty acid composition of plasma phosphatidylcholine, peripheral venous blood samples (30 ml) were taken into heparinised bottles from the antecubital fossa following an overnight fast. Blood was layered over 20 ml Histopaque (density 1·077 g/ml; Sigma Chemical Co., Poole, Dorset, UK) and centrifuged (720 ***g***) for 15 min at 20°C. The plasma layer was removed, stored at −70°C prior to analysis and analysed in batches. Internal standards (phosphatidylcholine 15:0/15:0 and phosphatidylethanolamine 17:0/17:0) were added to all samples prior to analysis.

Total plasma (1 ml) lipids were isolated by extraction with chloroform/methanol (2:1 vol/vol) containing 50 mg/l butylated hydroxytoluene. Plasma phosphatidylcholine was purified by solid-phase extraction on aminopropylsilica cartridges (Varian, Walton-on-Thames, Surrey, UK) and converted to methyl esters by incubation with methanol containing 2 % (vol/vol) sulphuric acid at 50°C for 18 h. Fatty acid methyl esters were separated, redissolved in hexane and analysed by capillary GC using a Hewlett Packard 5890 GC (Hewlett Packard, Stockport, Cheshire, UK) equipped with an HP7686 GC autosampler using a BPX-70 fused silica capillary column (50 m × 0·25 mm × 0·32 μm) with flame ionisation detection. Peaks were identified by retention times relative to standards. Fatty acids are reported as proportionate values (g/100 g total fatty acids). The co-efficient of variation was less than 5 % for determining fatty acid composition.

The methods for determining cytokine production by PBMC have previously been described (Trebble *et al.* 2003*a* and 2004*a*). In brief, IFN-γ and PGE_2 synthesis by purified PBMC (1×10^6 cells/ml) was measured following incubation with and without the monocyte/macrophage stimulant lipopolysaccharide (PGE_2) or the T-cell stimulant concanavalin A (IFN-γ). IFN-γ concentrations were determined using EASIA ELISA kits (Biosource Europe SA, Nivelles, Belgium). PGE_2 concentrations were determined using NEOGEN ELISA kits (Neogen

Table 1. Total supplementary intake of fatty acids in the fish oil plus antioxidants (FOA) and placebo groups

	Fatty acid (g/d)							
	MA	PA	PoA	SA	OA	LA	EPA	DHA
FOA	0·63	1·44	0·9	0·41	1·28	0·41	1·62	1·08
Placebo	0	1·02	0·09	0·24	6·62	0·85	0	0

Saturated fatty acids: MA, myristic acid; PA, palmitic acid, SA, stearic acid.
MUFA: PoA, palmitoleic acid; OA, oleic acid.
n-6 PUFA: LA, linoleic acid.
n-3 PUFA: EPA; DHA.
For details of subjects and procedures, see p. 253.

Corporation, Lexington, KY, USA). The co-efficient of variation was less than 10 % for both cytokine and PG assays, and the limits of detection were 30 IU/l for IFN-γ.

Laboratory investigations and assessment of bone turnover

Full blood count, ESR, CRP, plasma micronutrient and vitamin D status were assessed in patients in the morning following an overnight fast, by standard methods of laboratory analysis.

Markers of bone turnover consisted of urinary deoxypyridinoline (DPD) and serum osteocalcin. DPD is a peptide derived from non-reducible pyridinium cross-links within mature collagen and is excreted in the urine following bone degradation, therefore being a marker of bone resorption (Clowes & Eastell, 2000). DPD was measured in urine samples collected as a second early morning void, following an overnight fast. Urinary DPD was measured by a heterogeneous competitive magnetic separation assay, a competitive immunoassay, and corrected for urinary creatinine (Cre) concentrations (DPD/Cre). The minimum detectable concentration was 0·9 nmol/l, and the normal ranges were 2·3–5·4 μM/M Cre in males and 3·0–7·0 μM/M Cre in females.

Osteocalcin is a non-collagenous protein synthesised by osteoblasts and incorporated within the bone matrix, and is a marker of bone formation (Clowes & Eastell, 2000). Osteocalcin was measured in serum extracted from non-heparinised, clotted blood samples taken in the morning following an overnight fast. The samples underwent centrifugation (720 *g* for 10 min at 4°C), and the extracted serum was analysed by a chemiluminescence assay (Nichols Institute Diagnostics Ltd., Heston, Merseyside, UK). The minimal detectable concentration was 0·5 ng/ml, and the normal ranges were 1·1–7·2 μg/l in males and 0·5–7·0 μg/l in females. All samples were stored at -70°C prior to analysis and analysed in batches using commercially available immunoassays. Values for DPD/Cre and osteocalcin were compared as the ratio DPD/Cre:osteocalcin (Crosbie *et al.* 1999; Takahashi *et al.* 2003).

Clinical and nutritional assessment

Clinical response was assessed by changes in CDAI scores from baseline values compared between the groups, as absolute values.

A semi-quantitative food-frequency questionnaire (Shaheen *et al.* 2001) was used to estimate habitual nutrient intake over the previous 12 months. Body composition was determined directly by anthropometry and indirectly by bioelectrical impedance. All measurements were made by a single investigator using standard methods and with the subject wearing only light clothes without shoes. Body height was measured to the nearest millimeter using a free-standing CMS Stadiometer (Chasmore Ltd, London, UK), and body weight was measured to the nearest 0·1 kg using electronic scales (Seca, Hamburg, Germany). BMI was calculated as body weight (kg) divided by height (m) squared.

Skinfold thickness was measured on the non-dominant side of the body in triplicate at four predetermined sites (identified using physical landmarks) with the skinfold parallel to the longitudinal axis of the body, using a single set of callipers (Holtain Ltd, Crymych, Dyfed, UK). The sites assessed were mid-biceps, mid-triceps (at the mid-point between the elbow and the acromium process with the arm hanging relaxed) and suprailiac and suprascapular points. The sum of the means for each site represented the value for skinfold thickness in each subject. Mid-arm circumference was measured on the non-dominant side using a flexible steel tape to the nearest millimeter; mid-arm muscle circumference was calculated as mid-arm circumference – (mean triceps skinfold thickness $\times \pi$). Bioelectric impedance was measured electronically (Biostat 1500, Bodystat, Douglas, Isle of Man, UK) between a set of four electrodes at predetermined points (identified relative to bony landmarks) on the dorsum of the hand and foot on the left side of the body, with the patient lying on the bed. All measurements were made in patients in the morning following an overnight fast.

Statistical analysis

Formal statistical advice was taken. The study was analysed as a proof of concept. The primary outcome variables, differences from baseline in the concentrations of urinary DPD/Cre, serum osteocalcin, and their ratio, and IFN-γ and PGE$_2$ production by PBMC were compared between groups. Secondary outcome variables included markers of nutritional status and clinical (CDAI) and laboratory (CRP and ESR) markers of disease activity. With a significance of level of 5 %, 80 % power and thirty-one subjects in each group, the study was powered to detect a 21 % reduction in DPD/Cre or a 36 % reduction in osteocalcin.

Baseline variables were compared between the fish oil plus antioxidants and placebo groups by the two-sample *t* test for continuous variables and the test for categorical variables. Statistical comparisons of outcome variables in the fish oil plus antioxidants and placebo groups were performed using analysis of covariance adjusted for factors that may have confounded the outcome analysis. These factors consisted of differences in the baseline values of the respective variables, the use of corticosteroids by patients during the study, and gender and menopausal status. There was a retrospective analysis of differences between groups in changes from baseline to intermediate time points in markers of bone turnover. Non-parametric variables were log-transformed prior to analysis. Correlations between change in IFN-γ and PGE$_2$ with changes in osteocalcin, DPD/Cre and the ratio DPD/Cre:osteocalcin were performed using Pearson's correlations with log-transformation of non-parametric data.

Analyses were performed using SPSS for Windows (SPSS Inc., Chicago, IL, USA). To account for multiple comparisons, two-sided significance levels of 1 % were adopted for secondary outcomes, except for baseline comparisons.

Results

Subjects

A total of seventy-seven patients were recruited and randomised to fish oil plus antioxidants or placebo. Sixteen patients were excluded from the outcome analysis. These included six patients who withdrew from the study (two in the fish oil plus antioxidants group, and four in the placebo group), one patient who was receiving bisphosphonate treatment and nine patients who were retrospectively identified with laboratory markers of inflammation (ESR or CRP) below the threshold for recruitment. There were no major side-effects of treatment with either fish oil plus antioxidants or placebo. Six patients (19·4 %) in the fish oil plus antioxidants group and seven (23·3 %) in the placebo group required oral corticosteroids owing to increased disease activity during the intervention period.

The baseline characteristics of the remaining Crohn's disease patients in the fish oil plus antioxidants (*n* 31) and placebo

(n 30) groups are shown (Table 2). There were no significant differences between groups (data not shown).

Fatty acid and antioxidant composition in plasma

There were no significant differences at baseline between the fish oil plus antioxidants and placebo groups in the dietary intake of n-3 PUFA or n-6 PUFA (Table 3). However, a trend towards a higher intake of monounsaturated fat ($P=0.085$) was noted in the fish oil plus antioxidants group. There were no significant differences between groups in the dietary intakes of carotene, retinol, Se, vitamin C or vitamin E. There were no significant differences at baseline between the fish oil plus antioxidant and placebo groups in mean estimated vitamin D intake (3·4 (SD 1·6) mg/d *v.* 3·9 (SD 1·3) mg/d; $P=0.760$) or mean plasma level (22·3 (SD 10·4) μg/l *v.* 21·9 (SD 9·8) μg/l; $P=0.873$).

There were no significant differences between the groups at baseline in the proportions of EPA ($P=0.802$) or DHA ($P=0.672$) in plasma phosphatidylcholine. Fish oil plus antioxidants resulted in a significant increase over baseline in the proportions of EPA and DHA in plasma phosphatidylcholine at 24 weeks compared with placebo (Table 4). Fish oil plus antioxidants were associated with a lower oleic acid, but this reflected a decrease in the intervention group and only a small increase in the placebo group.

There were no significant differences between groups at baseline in the plasma levels of vitamin E ($P=0.869$), vitamin A ($P=0.244$), Se ($P=0.861$), Zn ($P=0.439$) or Cu ($P=0.884$). Fish oil plus antioxidants were associated with an increase in the plasma concentration of Se and a trend towards an increase in vitamin E above baseline, compared with the placebo group (Table 4). There were no differences in the response of plasma Cu or vitamin A.

Table 2. Baseline characteristics of patients with Crohn's disease in the fish oil plus antioxidants and placebo groups

	Fish oil plus antioxidants (n 31)	Placebo (n 30)
Gender		
Male	11	8
Female		
Pre-menopausal	13	14
Post-menopausal	7	8
Mean age (years)	45·4 (SD 13·1)	39·6 (SD 13·1)
Mean disease duration (years)	10·0 (SD 8·5)	10·3 (SD 9·8)
Disease site		
Small bowel	8	6
Large bowel	8	12
Small and large bowel	14	12
Perianal only	1	–
Previous intestinal resection	10	6
Current drug history		
Azathioprine/ methotrexate	10	6
5-ASA	4	3
Rheumatological history		
Osteopenia	1	1
Osteoporosis	2	1
Fractures	7	12
Current smoking	10	12
Mean activity score	5·5	5·4

Interferon-γ and prostaglandin E₂ production by stimulated peripheral blood mononuclear cells

Differences in the production of IFN-γ and PGE$_2$ from baseline to 24 weeks were compared between groups. Fish oil and antioxidants were associated with significant decreases in the production of IFN-γ ($P=0.017$) and PGE$_2$ ($P=0.012$) (Trebble *et al.* 2004*a*).

Markers of bone formation and resorption

There were no significant differences at baseline in DPD/Cre ($P=0.556$), osteocalcin ($P=0.339$) or the ratio DPD/Cre:osteocalcin ($P=0.568$) between the fish oil plus antioxidants and the placebo groups. There were no significant differences between groups in the response of absolute values for DPD/Cre ($P=0.290$), osteocalcin ($P=0.562$) or the ratio DPD/Cre:osteocalcin ($P=0.400$) between baseline and 24 weeks (Table 5). A retrospective analysis was undertaken of intermediate time points. There were no differences between groups in the response of DPD/Cre from baseline at 8 ($P=0.542$) or 16 ($P=0.460$) weeks. The level of osteocalcin was lower in the fish oil plus antioxidant compared with the placebo group at 8 weeks ($P=0.035$) but not at 16 weeks ($P=0.372$). The response of the ratio of DPD/Cre:osteocalcin was similar between groups between baseline and 8 weeks ($P=0.063$) but was higher in the fish oil plus antioxidants group at 16 weeks ($P=0.032$).

Changes in IFN-γ production between baseline and 24 weeks (n 61) were significantly correlated with changes in the DPD/Cre:osteocalcin ratio (r -0.33, $P=0.009$). This correlation was strongest within the fish oil plus antioxidants group (r -0.47, $P=0.008$). The correlations with DPD/Cre and osteocalcin did not reach statistical significance (r -0.12, $P=0.38$ and r 0.20, $P=0.14$, respectively). There were no significant correlations between changes in PGE$_2$ production between baseline and 24 weeks and changes in DPD/Cre (r -0.24, $P=0.06$), osteocalcin (r -0.36, $P=0.78$) and DPD/Cre:osteocalcin ratio (r -0.21, $P=0.10$).

Clinical and inflammatory disease activity and nutritional status

There were no significant differences between the fish oil plus antioxidants and placebo groups at baseline in terms of CRP ($P=0.233$), ESR ($P=0.486$) or CDAI ($P=0.515$). There were also no significant differences in the response of absolute values for CDAI ($P=0.459$), CRP ($P=0.994$) or ESR ($P=0.283$) to fish oil plus antioxidants and placebo at 24 weeks compared with baseline values.

At baseline, there was a trend towards a higher BMI ($P=0.059$) and higher mid-arm muscle circumference ($P=0.015$) in the fish oil plus antioxidants group, but there were no differences between groups in percentage fat mass ($P=0.352$), percentage lean mass ($P=0.354$) or skinfold thickness ($P=0.244$). Fish oil plus antioxidants was associated with a trend towards a reduction in mid-arm muscle circumference from baseline values at 24 weeks compared with placebo, but there were no significant differences in BMI, skinfold thickness, percentage fat mass or percentage lean mass (Table 6). There were no differences between groups at intermediate time points.

Table 3. Habitual dietary intake of fat and antioxidants in the fish oil plus antioxidants and placebo groups (determined by food-frequency questionnaire) and composition of the fish oil and antioxidants dietary supplement

(Mean values and standard deviations for thirty-one subjects per group)

	Fish oil plus antioxidants		Placebo			Supplement composition		
	Mean	SD	Mean	SD	P^*	Fish oil	Placebo	RNI
MUFA (g/d)	26·3	10·6	22·2	7·2	0·085	1·3	6·6	
n-6 PUFA (g/d)	11·5	6·4	9·5	4·2	0·145	0·41	0·85	10·2†
n-3 PUFA (g/d)	1·75	0·76	1·48	0·76	0·169	2·7	0	1·8†
Vitamin A‡ (μg/d)	1120	642	849	428	0·058	450	0	700
Se (μg/d)	67	31	66	32	0·815	200	0	75
Vitamin C (mg/d)	136	88	115	65	0·296	90	0	40
vitamin E (mg/d)	8·9	4·2	7·5	3·8	0·205	30	0	5−7§

RNI, Reference Nutritional Intake (UK values; Department of Health, 1991).

* Two-sample t test.

† Estimated average UK intake (British Nutrition Foundation, 1999).

‡ Total vitamin A (retinol equivalents) = retinol + (carotene ÷ 6).

§ Estimated average UK requirement (UK values; Department of Health, 1991).

For details of subjects and procedures, see p. 253.

Discussion

The current study was a randomised-controlled trial of high-dose fish oil and antioxidants in Crohn's disease. Subjects demonstrated current or recent raised levels of laboratory markers of inflammation, and groups were compared for the response of metabolic markers of bone turnover and production of the inflammatory mediators IFN-γ and PGE$_2$ by PBMC. Factors that may have confounded the response of bone turnover, including nutritional status and clinical and biochemical markers of disease activity, were also investigated.

High-dose fish oil and antioxidants were not associated with effects on markers of bone turnover at trial completion, i.e. 24 weeks, but there was a trend towards lower osteocalcin levels, reflecting a decrease in bone formation, at intermediate points and an increase in DPD/Cre:osteocalcin ratio (reflecting bone resorption to formation). This was associated with a lower production of IFN-γ and PGE$_2$ by PBMC. Changes in IFN-γ production correlated weakly but negatively with the DPD/Cre:osteocalcin ratio. There were no significant effects on nutritional status, or on clinical or biochemical markers of disease activity.

The habitual dietary intakes of n-3 and n-6 PUFA amongst patients in the fish oil plus antioxidants and placebo groups were consistent with estimated mean values for the UK population of 1·8 and 10·2 g/d, respectively (British Nutrition Foundation, 1999). The supplementary intake of EPA and DHA in the current study represented an increase of more than ten times the estimated habitual intake in the general UK population and is similar to the supplementary intake used by Belluzzi et al. (1996) to demonstrate biochemical and clinical anti-inflammatory

Table 4. Fatty acid composition (g/100 g total fatty acids) of plasma phosphatidylcholine (PC) and plasma antioxidant concentrations from patients with Crohn's disease at baseline and following dietary supplementation with fish oil plus antioxidants (FOA) or placebo (PI) at 24 weeks

(Mean values and standard deviations for thirty-one subjects per group)

			Week						
			Baseline		24				
			Fatty acid		Fatty acid				
			Mean	SD	Mean	SD	Mean	95 % CI	P^*
Plasma PC (g/100 g total fatty acid)	EPA	FOA	1·22	0·67	4·65	2·46	3·59	2·63, 4·55	<0·001
		PI	1·18	0·73	1·14	0·85			
	DHA	FOA	3·49	1·29	6·54	1·95	2·97	2·14, 3·81	<0·001
		PI	3·63	1·32	3·61	1·33			
	OA	FOA	11·3	1·1	10·2	1·5	−1·65	−2·33, −0·97	<0·001
		PI	11·2	2·0	11·7	1·8			
Se (μmol/l)		FOA	0·90	0·19	1·26	0·33	0·39	0·26, 0·512	
		PI	0·91	0·18	0·87	0·20			<0·001
Cu (μmol/l)		FOA	22·2	2·7	20·3	7·0	−1·80	−4·58, 0·98	0·199
		PI	22·4	6·4	22·3	8·5			
Vitamin A (μmol/l)		FOA	1·8	0·64	1·63	0·50	0·144	−0·13, 0·41	0·286
		PI	2·02	0·82	1·90	0·44			
Vitamin E (μmol/l)		FOA	29·4	7·9	30·2	8·4	2·85	−0·08, 5·8	0·056
		PI	29·0	9·4	28·2	7·2			

OA, oleic acid.

* Analysis of covariance, differences between fish oil plus antioxidants and placebo groups in change from baseline to 24 weeks, adjusted for corticosteroid use, gender and menopausal status.

For details of subjects and procedures, see p. 253.

Table 5. Bone markers, deoxypyridinoline (creatinine corrected) (DPD/Cre) (bone resorption) and osteocalcin (bone formation) in patients with Crohn's disease at baseline and following dietary supplementation with fish oil plus antioxidants (FOA) or placebo (Pl)

(Mean values and standard deviations for thirty-one subjects per group)

| | | Week | | | | | | | | | | |
| | | Baseline | | 8 | | 16 | | 24 | | | | |
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	95 % CI	P*
DPD/Cre (μM/M Cre)	FOA	7·6	2·7	7·9	4·9	7·3	2·6	7·5	3·3	0·69	−0·60, 1·98	0·290
	Pl	7·9	2·4	7·5	2·5	7·3	2·9	7·0	2·5			
Osteocalcin (ng/ml)	FOA	5·5	2·6	4·9	2·0	4·7	2·3	4·7	2·0	−0·19	−0·82, 0·45	0·562
	Pl	6·2	3·1	5·9	2·3	5·5	2·4	5·3	2·6			
DPD/Cre:osteocalcin†	FOA	0·17	0·31	0·20	0·26	0·23	0·28	0·22	0·30	0·04	−0·05, 0·14	0·400
	Pl	0·13	0·24	0·12	0·23	0·13	0·23	0·14	0·26			

* Analysis of covariance, differences between fish oil plus antioxidants and placebo groups in change from baseline to 24 weeks, adjusted for corticosteroid use, gender and menopausal status.

† Ratio log-transformed.

For details of subjects and procedures, see p. 253.

effects in Crohn's disease. In the current study, fish oil was administered with a high intake of nutritional antioxidants to decrease the potential hazards of a large rise in the dietary load of long-chain *n*-3 PUFA. This included Se and vitamins A and C equivalent to 50 % or more of the Reference Nutritional Intakes (Department of Health, 1991), and of vitamin E equivalent to four times the estimated average requirement in the UK population (Table 3). The fish oil and antioxidants supplement was associated with significantly higher plasma concentrations of EPA, DHA and Se, and a trend towards a higher plasma concentration of vitamin E.

The choice of placebo in the current study was olive oil, which contains MUFA in the form of oleic acid. The immunological response to dietary supplementation with oleic acid has been investigated in a number of studies (Yaqoob, 1998), but these have not demonstrated a significant effect in human subjects (Yaqoob, 1998), including the production of cytokines by T-cells (Granato *et al.*, 2000). Yaqoob (1998) explains the lack of immune effect of dietary supplementation with oleic acid as a consequence of the high level of monounsaturated fat in the habitual Western diet. In the current study, the total estimated intake of

MUFA consumed by the patients in their habitual diet and supplements was approximately 28 g/d in both the intervention and placebo groups. Differences were noted between groups in the plasma concentrations of oleic acid. However, this principally reflected a reduction in the fish oil plus antioxidant group rather than an absolute increase in the placebo group.

To our knowledge, this is the first study in the literature to investigate the response of bone turnover in Crohn's disease to high-dose fish oil and antioxidants. Markers of bone turnover, DPD/Cre and osteocalcin, are dynamic measurements of bone resorption and formation, with a predictive power for fracture risk (Clowes & Eastell, 2000). In comparison, the assessment of bone mass is an 'historical' measurement reflecting the accumulative effects of bone resorption and formation, and is affected by both pre-morbid and co-morbid factors. Changes in bone mass occur slowly, and therefore prolonged periods of intervention are required to confirm the benefit of a therapy experimentally.

Although a number of studies in ovariectomised rodents have demonstrated a positive effect of dietary supplementation with fish oil on bone mass and strength, with reductions in markers of bone resorption (Sakaguchi *et al.* 1994; Schlemmer *et al.*

Table 6. Nutritional markers in patients with Crohn's disease at baseline and following dietary supplementation with fish oil plus antioxidants (FOA) or placebo (Pl)

(Mean values and standard deviations for thirty-one subjects per group)

| | | Week | | | | | | | | | | |
| | | Baseline | | 8 | | 16 | | 24 | | | | |
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	95 % CI	P*
BMI (kg/m²)	FOA	27·8	6·7	27·2	5·8	27·5	5·7	27·7	5·9	−0·035	−0·84, 0·77	0·930
	Pl	25·1	2·9	25·5	2·9	25·5	3·1	25·4	3·0			
Weight (kg)	FOA	73·6	14·1	73·3	14·5	73·8	14·4	74·3	14·4	0·257	−1·82, 1·31	0·743
	Pl	69·1	10·9	70·8	11·0	70·6	10·9	70·1	11·1			
Fat mass (%)	FOA	33·3	11·2	32·6	9·9	33·1	10·8	32·9	10·7	0·935	−0·76, 2·63	0·275
	Pl	30·9	8·7	31·8	9·0	31·3	8·9	30·5	9·4			
Lean mass (%)	FOA	66·6	11·2	67·3	9·9	66·9	10·8	66·4	10·7	−0·934	−2·63, 0·76	0·275
	Pl	69·1	8·7	68·2	9·0	68·3	8·9	69·5	9·4			
Mid-arm muscle circumference (mm)	FOA	24·7	3·2	24·8	3·4	24·6	3·2	24·4	3·3	−0·85	−1·58, −0·12	0·023
	Pl	22·6	2·7	23·1	2·6	23·3	2·9	23·5	2·5			
Mean skinfold thickness (mm)	FOA	20·8	8·6	20·3	8·4	20·2	8·8	20·6	8·9	0·084	−1·70, 1·86	0·924
	Pl	18·6	4·9	19·4	5·6	18·5	5·7	18·2	5·9			

* Analysis of covariance, differences between fish oil plus antioxidants and placebo groups in change from baseline to 24 weeks, adjusted for corticosteroid use, gender and menopausal status.

For details of subjects and procedures, see p. 253.

1999; Fernandes *et al.* 2003; Sun *et al.* 2003; Green *et al.* 2004), Poulsen and Kruger (2004) noted that high-dose EPA may be associated with a reduction in bone density. There are conflicting reports of the effects of fish oil on bone mass in healthy, post-menopausal women. Kruger *et al.* (1998) demonstrated an improvement in bone mass following dietary supplementation with fish oil (0·42 g/d EPA and DHA) and evening primrose oil (containing γ-linoleic acid) compared with placebo. However, this contrasted with the findings from Bassey *et al.* (2000) using a similar *n*-3 PUFA dose. There are insufficient data in the literature to determine the response of markers of bone turnover to fish oil. In a small study of hyperlipidaemic, post-menopausal women, dietary supplementation with 1·8 g/d EPA, as an ethyl ester, was associated with trends towards an increase in serum bone alkaline phosphatase, reflecting bone formation, but there were no significant effects on bone formation or resorption (Terano, 2001).

The mechanism by which localised gastrointestinal inflammation may alter bone metabolism is uncertain. There is evidence that mononuclear cells may influence osteocyte differentiation and function through cytokine release and the expression of specific membrane ligands (Horowitz *et al.* 2001; Katagiri & Takahashi, 2002; Theill *et al.* 2002), that the interaction between activated T-cells and osteoclasts may have similarities to the interactions between activated T-cells and monocytes (Theill *et al.* 2002), possibly reflecting the origin of osteoclasts and monocyte/macrophages from similar progenitor cell lines (Miyamoto *et al.* 2001), and that activated T-cells demonstrate the capacity to induce osteoclastogenesis from circulating monocytes (Kotake *et al.* 2001). Activated T-cells directly promote osteoclast differentiation and function via expression of the membrane protein RANK ligand (Takayanagi *et al.* 2002), which interacts with RANK on osteoclast precursor cells (Horwood *et al.* 1999; Kotake *et al.* 2001).

Fernandes *et al.* (2003) suggest that the beneficial effects of fish oil on bone metabolism in ovariectomised rodents may occur because of inhibition of activation of the RANK ligand, possibly through a modulation of the release of pro-inflammatory cytokines including TNF-α. An alternative explanation may, however, involve IFN-γ release by activated T-cells, which inhibits osteoclast activation via the RANK–RANK ligand system as a counter-regulatory mechanism to prevent uncontrolled bone resorption during the inflammatory response (Arron & Choi, 2000).

The production of IFN-γ by circulating PBMC is lower in individuals with Crohn's disease than in healthy subjects (Miura & Hiwatashi, 1985; Mutchnick *et al.* 1988; Sasaki *et al.* 1992; Trebble *et al.* 2004*b*); this may be associated with increased rates of bone resorption in patients with raised inflammatory markers (Trebble *et al.* 2004*c*). Therefore, the mechanism by which altered bone metabolism occurs in Crohn's disease may be a consequence of attenuated IFN-γ release by activated T-cells, with a consequential reduction in osteoclast inhibition during the inflammatory response (i.e. associated with raised laboratory markers of inflammation). Dietary supplementation with fish oil may be associated with increased IFN-γ production by PBMC in healthy subjects (Trebble *et al.* 2003*b*) and was therefore considered to be an intervention with the potential to modulate bone turnover therapeutically in Crohn's disease.

In the current study, however, the intervention (equivalent to 2·7 g/d EPA and DHA in addition to antioxidants) was associated with an unpredicted decrease, rather than an increase, in the production of IFN-γ by activated T-cells. This was associated with a trend towards an increase in the DPD/Cre:osteocalcin ratio. There was, however, a significant negative correlation between change in IFN-γ and the change in DPD/Cre:osteocalcin ratio consistent with the hypothesis that IFN-γ plays an important regulatory role in bone metabolism during the inflammatory response.

The failure to demonstrate a significant beneficial effect of fish oil on bone metabolism in patients with Crohn's disease is disappointing and may reflect a number of factors. First, the decrease in IFN-γ production was unexpected and may have been due to the dose of fish oil used. In healthy subjects, low-to-moderate supplementary intakes of fish oil (less than 2 g/d EPA and DHA) lead to a higher production of IFN-γ (Trebble *et al.* 2003*b*). However, high-dose intakes of fish oil (more than 4 g/d EPA and DHA) are associated with inhibition of IL-2 (Endres *et al.*, 1993), a T-helper 1-associated cytokine (consistent with IFN-γ); an explanation for the non-linear dose response of fish oil (EPA and DHA) on the PBMC production of cytokines is described elsewhere (Trebble *et al.* 2003*a*).

Second, the antioxidant formulation contained vitamin A, which may be associated with reduced rates of bone formation and increased resorption (Barker & Blumsohn, 2003), although there are no direct data from intervention studies to support this.

Third, the therapeutic modification of bone metabolism in Crohn's disease may require reduced gastrointestinal or systemic inflammation, which may alter PBMC function as a secondary or associated effect. The findings of the current study failed to demonstrate an effect of the intervention on any clinical or laboratory markers of inflammation and contrast with the findings of a study by Belluzzi *et al.* (1996) that involved fish oil alone. The Belluzzi study, however, had a different trial design, including recruitment criteria, intervention and study end points. In particular, our subjects were more generalisable to an out-patient population in that we recruited patients who demonstrated a range of both clinical and biochemical markers of disease activity, including those with clinically active disease.

Fourth, anthropometry and bioelectric impedance suggested that the Crohn's disease cohort in the current study did not suffer with altered nutritional status, which may be required for a therapeutic response to fish oil and antioxidants, or other nutritional interventions.

There are clinical implications to be drawn from the results of this study. Dietary supplementation with high-dose fish oil and antioxidants failed to exert a therapeutic effect on bone turnover in an out-patient population of adult patients with Crohn's disease with mild-to-moderate disease activity. Our data support an important role for IFN-γ in bone metabolism in patients with Crohn's disease and a non-linear association between the supplementary intake of *n*-3 PUFA and IFN-γ production by PBMC. The response of bone metabolism in Crohn's disease to low-to-moderate intakes of fish oil, which may lead to a contrasting effect on PBMC function, remains uncertain and warrants further investigation.

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