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Short Communication

Supplementation with a low–moderate dose of *n*-3 long-chain PUFA has no short-term effect on bone resorption in human adults

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Abstract

Previous research suggests that *n*-3 PUFA may play a role in bone health. The present analysis aimed to investigate the impact of *n*-3 PUFA supplementation on bone resorption in adult men and women. Serum samples from 113 mild–moderately depressed individuals (twenty-six males and eighty-seven females, aged 18–67 years) randomised to receive 1·48 g EPA + DHA/d (*n* 53) or placebo (*n* 60) for 12 weeks as part of a large recent randomised controlled trial were assayed for *n*-3 PUFA status and a bone resorption marker, C-terminal cross-linking telopeptide of type 1 collagen (β-CTX). Regression analyses revealed that *n*-3 PUFA status following supplementation was associated with randomisation (placebo/*n*-3 PUFA) ($B = 3{\cdot}25$, 95 % CI 2·60, 3·91, $P < 0{\cdot}01$). However, β-CTX status following supplementation was not associated with randomisation ($B = -0{\cdot}01$, 95 % CI −0·03, 0·04). Change in β-CTX status was also not associated with change in *n*-3 PUFA status ($B = -0{\cdot}002$, 95 % CI −0·01, 0·01). These findings provide no evidence for an association between *n*-3 PUFA supplementation (1·48 g EPA + DHA/d) for 12 weeks and bone resorption in humans assessed by β-CTX, and suggest that *n*-3 PUFA supplementation may be unlikely to be of benefit in preventing bone loss.

Key words: ***n*-3 PUFA: Bone resorption: Telopeptide of type 1 collagen: Randomised controlled trials: Humans**

Long-chain PUFA, both from the *n*-3 and *n*-6 families, have been suggested to play an important role in bone metabolism and may represent a useful non-pharmacological means of ameliorating postmenopausal bone loss and risk of osteoporosis. *n*-3 and *n*-6 PUFA are precursors for several potent regulatory eicosanoids involved in bone metabolism including PG and leukotrienes. *n*-6 PUFA-derived eicosanoid signalling is known to be involved in the production of inflammatory cytokines such as IL-1, IL-6 and TNF-α, whereas *n*-3 PUFA can inhibit the production of these cytokines[1]. Inflammatory cytokines provide an important stimulus for osteoclastic bone resorption, and suppression of the production of these cytokines by *n*-3 PUFA may inhibit bone resorption and prevent bone loss[1].

Few studies have examined the role of these essential fatty acids, particularly *n*-3 PUFA, in skeletal metabolism in humans. Consistent with a protective effect of *n*-3 PUFA, in observational studies, serum *n*-3 PUFA concentrations were positively associated with total body and spine bone mineral density (BMD) in seventy-eight young men (mean age 22 years)[2], and a higher ratio of *n*-6:*n*-3 PUFA dietary intake was associated with a lower hip BMD in 1532 men and women aged 45–90 years[3]. In intervention studies, bone formation (as reflected by osteocalcin levels) was found to be enhanced following supplementation with fish oils (0·64 g EPA + 0·44 g DHA/d) compared with placebo for 16 weeks in forty older adults[4], bone resorption (as reflected by serum N-telopeptide of collagen cross-links) was found to be reduced

Abbreviations: β-CTX, C-terminal cross-linking telopeptide of type 1 collagen; BMD, bone mineral density; DASS, Depression, Anxiety and Stress Scale.

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in twenty-three middle-aged adults while consuming a diet rich in *n*-3 PUFA (6·5 % α-linolenic acid) for 6 weeks compared with an average American diet (0·8 % α-linolenic acid)[5] and BMD did not decrease following combined supplementation with 0·24 g EPA + 0·18 g DHA + 4·1 g *n*-6 PUFA/d plus Ca compared with a Ca supplement alone for 18 months in sixty-five older adults[6]. However, supplementation with a combined antioxidant and *n*-3 PUFA supplement (1·6 g EPA + 1·1 g DHA/d) for 24 weeks resulted in no change in bone turnover or bone resorption (as reflected by urinary excretion of free deoxypyridoxine) compared with placebo in sixty-one Crohn's patients[7], supplementation with 0·44 g fish oil plus Ca for 12 months was found to have no effect on BMD compared with Ca alone in eighty-five pre- and postmenopausal women[8] and supplementation with flaxseed (9 g α-linolenic acid/d) for 12 months was not found to prevent bone loss at the lumbar spine in 199 early postmenopausal women compared with placebo[9].

One possible explanation for these conflicting observations is that *n*-3 PUFA exert relatively modest effects on bone metabolism, making it difficult to detect changes after supplementation, and this may be a particular issue for indices such as BMD which are relatively insensitive to short-term change as compared with biochemical bone turnover markers[10]. In addition, measurements of bone resorption based on assays for urinary deoxypyridoxine have largely been superseded by more sensitive N-telopeptide of collagen cross links and C-terminal cross-linking telopeptide of type 1 collagen (β-CTX) assays[11], which have the added advantage that they can be used to measure serum/plasma samples.

The present analysis aimed to investigate the impact of *n*-3 PUFA supplementation on bone resorption using serum-based measures of bone resorption – the resorption marker, β-CTX. The null hypothesis being tested was that there would be no association between *n*-3 PUFA supplementation and bone resorption.

Methods

The analysis was conducted on data collected as part of a large randomised controlled trial investigating the impact of *n*-3 PUFA supplementation on depressed mood and cognitive function[12]. The trial was conducted in a community sample of 218 participants, randomised to receive either 1·48 g *n*-3 PUFA (0·63 g EPA + 0·85 g DHA)/d or olive oil placebo for 12 weeks. These levels of *n*-3 PUFA can be achieved through the consumption of appropriate foods in the diet. Participants were eligible for the study if they were suffering from mild–moderate depressed mood as reported in two screening questionnaires (depressed mood score of 10–24 on Depression, Anxiety and Stress Scales (DASS))[13], but were not receiving treatment for depressive disorders, were not severely ill and were low consumers of *n*-3 PUFA, as assessed using a specially designed FFQ[12]. Randomisation was stratified by sex (male/female), age (16–49/50 + years) and baseline depression score (DASS depression score 10–18/DASS depression score 19–24). *n*-3 PUFA supplement and placebo were provided throughout the study as identical capsules. Participants and researchers were blind to treatment allocation, and blinding was considered successful, as guesses of treatment allocation were no better than chance at the end of the study[12]. Full details of the trial methodology and all outcome measures related to depressed mood and cognitive function are provided elsewhere[12]. The trial was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human participants were approved by Southmead Local Research Ethics Committee, Bristol. Written informed consent was obtained from all participants.

n-3 PUFA status and β-CTX were assessed throughout the trial using fasting (overnight) venous blood samples collected before supplementation (week 0), after 4 weeks supplementation (week 4) and after 12 weeks supplementation (week 12). All blood samples were centrifuged within 1 h of collection, and 1 ml serum aliquots were subsequently stored at −80°C. Plasma concentrations of all fatty acids were assessed using GC. Total lipid was extracted from the plasma with chloroform and methanol (2:1, v/v). Fatty acid methyl esters were prepared by incubation of lipid with methanol containing 2 % (v/v) sulphuric acid at 50°C for 2 h and separated using a Hewlett Packard 6890 gas chromatograph (Agilent, Stockport, Cheshire, UK) equipped with a 30 m × 0·25 μm × 0·25 mm BPX-70 fused silica capillary column (SGE, Milton Keynes, Bucks, UK) and flame ionisation detection. The concentrations of individual fatty acids were determined by measurement of the peak area using ChemStation software (Agilent) and expressed as a proportion of total fatty acids[14]. Serum β-CTX was measured using an electrochemiluminescence immunoassay kit (Roche Diagnostic, Lewes, East Sussex, UK). In our laboratory, the β-CTX assay has an intra- and inter-assay CV of < 5 % across the working range of 0·01–6·0 μg/l.

Statistical analysis

Effects of *n*-3 PUFA supplementation on *n*-3 PUFA status and β-CTX were investigated using linear regression, where *n*-3 PUFA/β-CTX status was predicted by (1) randomisation (placebo/*n*-3 PUFA), (2) randomisation, sex (male/female), age (continuous variable) and baseline DASS depression score (continuous variable) and (3) where baseline β-CTX (continuous variable) and baseline *n*-3 PUFA status (continuous variable) were also included in the model. *n*-6 PUFA status and *n*-3:*n*-6 PUFA ratio were also investigated alongside analyses on *n*-3 PUFA status due to likely inter-relations. All analyses were conducted twice, once for data at week 12 and once for data at week 4. Analyses were not conducted using data from weeks 12 and

4 together, due to multicollinearity. Associations between change in *n*-3 PUFA status and change in β-CTX were also investigated using separate linear regression analyses. Change in *n*-3 PUFA status co-varies with randomisation and *n*-3 PUFA status at baseline, so this could not be included in previous analyses. All analyses were also repeated using EPA status and DHA status separately in place of *n*-3 PUFA status and using separate groups of men and women above and below 50 years of age. The number of participants in all main analyses allows differences between groups following supplementation (placebo/*n*-3 PUFA) of 0·4 SD to be detected at 90 % power, and the number of participants in subgroup analyses allows a difference between groups of 0·5 SD to be detected at this level of power. A cut-off value of $P=0·05$ was used to define statistical significance.

Results

Of the 190 participants who completed the trial, 113 participants provided data for *n*-3 PUFA status and β-CTX at all three time points, fifty-three were randomised to receive supplementation with *n*-3 PUFA, sixty were randomised to receive placebo. Of those who received *n*-3 PUFA, twelve (23 %) were male, forty (75 %) were under the age of 50 years and thirty-four (64 %) were mildly depressed. Of those who received placebo, fourteen (23 %) were male, forty-four (73 %) were under the age of 50 years and forty-four (73 %) were mildly depressed. There were no differences between these distributions ($\chi^2 = 1·16$; $P>0·05$). Neither the use of bone-active drugs such as bisphosphonates and hormone replacement therapy nor menopausal status was recorded as part of the study. Using age as a proxy measure for menopausal status in the group who received *n*-3 PUFA, twenty-nine (71 %) women were under the age of 50 years, twelve (29 %) women were 50 years or older. In the group who received placebo, thirty-three (72 %) women were under the age of 50 years, thirteen (28 %) women were 50 years or older. There were also no differences between the groups in other factors that may also affect bone metabolism – height, weight, smoking status or alcohol intake (largest $t(84) = 1·10$; $P=0·28$). Participants who provided data at all three time points were lighter in body weight than those who provided data at fewer time points ($t(182) = 2·00$; $P=0·05$) (most plausibly due to the difficulty in extracting blood from heavier participants), but no other differences were found, particularly with respect to *n*-3 PUFA status or β-CTX (largest $t(191) = 0·66$; $P=0·51$).

n-3 PUFA status and β-CTX at weeks 0, 4 and 12 for individuals receiving *n*-3 PUFA and placebo are shown in Table 1. *n*-3 PUFA levels were similar in the two groups at baseline ($t(111) = 0·05$; $P=0·83$). While the levels remained similar at subsequent time points for the placebo group ($F(2,118) = 2·21$; $P=0·12$), there was an increase by over 150 % in the *n*-3 PUFA group ($F(2,104) = 137·09$; $P<0·01$). The levels of *n*-6 PUFA and *n*-3:*n*-6 PUFA ratios (similar in the two groups at baseline (largest $t(111) = 0·93$; $P=0·74$)) also showed related changes (placebo group: largest $F(2,118) = 2·30$; $P=0·11$; *n*-3 PUFA group: smallest $F(2,104) = 7·23$; $P<0·01$). The levels of β-CTX were similar in the two groups at baseline ($t(111) = 1·92$; $P=0·06$), and within each group, β-CTX values remained similar at all three time points (largest $F(2,104) = 1·36$; $P=0·26$).

In regression analyses, *n*-3 PUFA status at week 12 was associated with randomisation (placebo/*n*-3 PUFA) in all three models (final model: $B = 3·25$, 95 % CI 2·60, 3·91), as was *n*-3:*n*-6 PUFA ratio (final model: $B = 0·09$, 95 % CI 0·07, 0·12), although *n*-6 PUFA status was not (final model: $B = -0·73$, 95 % CI −2·34, 0·89). β-CTX at week 12 was not associated with randomisation (final model: $B = -0·01$, 95 % CI −0·03, 0·04). *n*-3 PUFA status at week 12 was also associated with baseline *n*-3 PUFA ($B = 0·54$, 95 % CI 0·26, 0·82), and β-CTX at week 12 was associated with baseline β-CTX ($B = 0·87$, 95 % CI 0·77, 0·96). Change in β-CTX from week 0 to week 12 was not associated with change in *n*-3 PUFA status ($B = -0·002$, 95 % CI −0·01, 0·01) or change in *n*-3:*n*-6 PUFA ratio ($B = -0·06$, 95 % CI −0·28, 0·16). Similar patterns of results were also found for *n*-3 PUFA status, *n*-3:*n*-6 PUFA ratios, β-CTX status, change in *n*-3 PUFA status, change in *n*-3:*n*-6 PUFA ratios and change in β-CTX status at week 4 (data not shown). Similar results were

Table 1. Mean concentrations of *n*-3 PUFA, *n*-6 PUFA, *n*-3:*n*-6 PUFA ratio and C-terminal cross-linking telopeptide of type 1 collagen (β-CTX) in both *n*-3 PUFA-supplemented and placebo groups at weeks 0, 4 and 12

(Mean values and standard deviations)

		Week 0		Week 4		Week 12	
Group	Variable	Mean	SD	Mean	SD	Mean	SD
n-3 PUFA (*n* 53)	*n*-3 PUFA (% total fatty acids)	4·6*	1·2	8·1*	2·0	8·3*	1·9
	n-6 PUFA (% total fatty acids)	37·2*	4·3	35·9*	4·0	35·7*	4·1
	n-3 PUFA:*n*-6 PUFA ratio	0·1*	0·03	0·2*	0·07	0·2*	0·06
	β-CTX (μg/l)	0·27	0·19	0·26	0·16	0·28	0·18
Placebo (*n* 60)	*n*-3 PUFA (% total fatty acids)	4·6	1·2	4·9	1·4	5·1	1·7
	n-6 PUFA (% total fatty acids)	37·3	4·5	37·1	4·4	37·1	4·7
	n-3 PUFA:*n*-6 PUFA ratio	0·1	0·04	0·1	0·04	0·1	0·06
	β-CTX (μg/l)	0·34	0·18	0·34	0·18	0·34	0·19

* Mean values were statistically different over time ($P<0·05$).

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also found in all analyses where EPA status and DHA status were investigated separately, and where men and women above and below the age of 50 years were analysed separately (data not shown).

Discussion

These results provide no evidence of an association between *n*-3 PUFA supplementation (1·48 g/d EPA + DHA) for a period of 12 weeks and bone resorption as assessed using β-CTX. Habitual *n*-3 PUFA intake of the study population was similar to that of the UK population based on *n*-3 PUFA concentrations measured at baseline[15], the *n*-3 PUFA supplement led to a 150 % increase in *n*-3 PUFA concentration compared with baseline by the end of the 12-week study period, and a 12-week period has previously been suggested as adequate for the incorporation of *n*-3 PUFA into cell membranes[16]. In our laboratory, the β-CTX assay is very precise with low CV across the measuring range and is one of the most sensitive biochemical estimates of bone resorption currently available[11], and was used here in fasting serum samples collected at the same time each day[10]. The trend towards lower β-CTX levels in the *n*-3 PUFA supplementation group at baseline, also if anything, would have biased the present study in favour of finding an association between *n*-3 PUFA supplementation and reduced β-CTX levels. The lack of association between *n*-3 PUFA supplementation and bone resorption in the present study, therefore, provides good evidence against the hypothesis that *n*-3 PUFA supplementation at 1·48 g/d for 12 weeks affects bone resorption in humans, and suggests that *n*-3 PUFA supplementation over the short term may be unlikely to be of benefit in preventing bone loss.

This result is consistent with those of other studies where *n*-3 PUFA supplementation for 24 weeks was not associated with bone turnover markers[7], and where *n*-3 PUFA supplementation for 12 months or more was found to have no influence on BMD[8,9]. Rather than influencing bone resorption, there is some evidence from animal studies that *n*-3 PUFA may stimulate bone formation possibly through the effects on PG synthesis[15]. As well as leading to an increase in BMD, such an action might be expected to 'uncouple' bone formation from resorption, leading to an increase in formation relative to resorption. Since formation markers were not analysed, we were unable to address such a mode of action in the present investigation.

The present results differ from those of Griel *et al.*[5] who reported a decrease in bone resorption in association with the consumption of a diet enriched with *n*-3 PUFA. Observational studies in humans, as well as evidence from studies based on animal models, have likewise suggested that *n*-3 PUFA may suppress bone resorption and inhibit bone loss[1]. It is conceivable that a weak inhibitory effect of *n*-3 PUFA supplementation was missed in the present study because this can be more marked in certain groups, such as postmenopausal women or older adults, in whom baseline rates of bone turnover tend to be higher and responses to anti-resorptive therapy are easier to detect. However, in sensitivity analyses here in women above and below 50 years of age, there was again no suggestion of any decrease in β-CTX following *n*-3 PUFA supplementation in any subgroup.

Other differences between studies, however, also exist. Differences between participants may extend beyond menopausal status and age to include differences in sex, dietary history or medication history[7,10,17,18]. Different measures of bone resorption and bone turnover have already been mentioned. Differences in *n*-3 PUFA supplementation may also be crucial. α-Linolenic acid, EPA and DHA, while all *n*-3 PUFA, are thought to have many differing roles and actions in the body, which may or may not be linked to skeletal metabolism[16–18]. Differences in *n*-3 PUFA dose, duration and supplement intake may also have effects[17,18], as may additional *n*-6 PUFA supplementation or corresponding *n*-6 PUFA levels[18]. Further study in this area is clearly needed to establish the impact of all these effects.

The analyses here are limited by the use of only one bone resorption marker, β-CTX, and the limited time frame over which supplementation was undertaken. The β-CTX marker is precise and provides a reliable and valid measurement of bone resorption. Formation markers, such as osteocalcin and amino terminal N-telopeptide of collagen cross-links, however, would also have provided valuable evidence on bone turnover. The time period of supplementation was selected based on primary outcome measures of the supplementation trial[12], and although 12 weeks is thought to be adequate to assess changes in bone turnover over the short term[15], extrapolation from the present results to issues of longer-term bone health may be inappropriate. Investigation of the effects of *n*-3 PUFA supplementation for longer periods and/or at higher doses on both bone formation and bone resorption markers are therefore needed before an effect of these agents on bone metabolism can be excluded.

In summary, no evidence for an association between *n*-3 PUFA supplementation and bone resorption was found. We conclude that *n*-3 PUFA supplementation for 12 weeks at the level used in the present study (1·48 EPA + DHA g/d) has no effect on bone resorption in humans. These findings suggest that *n*-3 PUFA supplementation at 1·48 g/d may not be beneficial in preventing bone loss and the risk of sustaining osteoporotic fractures in the longer term, but more work is needed here.

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