Osteoporosis International (2019) 30:1071–1078
https://doi.org/10.1007/s00198-019-04881-3

ORIGINAL ARTICLE

Association of blood n-3 fatty acid with bone mass and bone marrow TRAP-5b in the elderly with and without hip fracture

B.-J. Kim[1] · H. J. Yoo[2] · S. J. Park[3] · M. K. Kwak[1] · S. H. Lee[1] · S. J. Kim[2] · M. W. Hamrick[4] · C. M. Isales[5] · S. H. Ahn[6] · J.-M. Koh[1]

Received: 20 September 2018 / Accepted: 27 January 2019 / Published online: 4 February 2019

Abstract

Summary The plasma n-3 fatty acid level was 26.2% lower in patients with osteoporotic hip fracture than in those with osteoarthritis. In all patients, n-3 fatty acid was positively associated with bone mineral density and inversely associated with tartrate-resistant acid phosphatase-5b level in bone marrow aspirates, reflecting the bone microenvironment.

Introduction Despite the potential beneficial role of n-3 fatty acid (FA) on bone metabolism, the specific mechanisms underlying these effects in humans remain unclear. Here, we assessed whether the plasma n-3 level, as an objective indicator of its status, is associated with osteoporosis-related phenotypes and bone-related markers in human bone marrow (BM) samples.

Methods This was a case-control and cross-sectional study conducted in a clinical unit. n-3 FA in the blood and bone biochemical markers in the BM aspirates were measured by gas chromatography/mass spectrometry and immunoassay, respectively. BM fluids were collected from 72 patients who underwent hip surgery because of either osteoporotic hip fracture (HF; $n = 28$) or osteoarthritis ($n = 44$).

Results After adjusting for confounders, patients with HF had 26.2% lower plasma n-3 levels than those with osteoarthritis ($P = 0.006$), and each standard deviation increment in plasma n-3 was associated with a multivariate-adjusted odds ratio of 0.40 for osteoporotic HF ($P = 0.010$). In multivariate analyses including all patients, a higher plasma n-3 level was associated with higher bone mass at the lumbar spine ($\beta = 0.615$, $P = 0.002$) and total femur ($\beta = 0.244$, $P = 0.045$). Interestingly, the plasma n-3 level was inversely associated with the tartrate-resistant acid phosphatase-5b level ($\beta = -0.633$, $P = 0.023$), but not with the bone-specific alkaline phosphatase level, in BM aspirates.

Conclusions These findings provide clinical evidence that n-3 FA is a potential inhibitor of osteoclastogenesis that favors human bone health.

Keywords Bone mass · Bone resorption · n-3 fatty acid · Osteoporotic fracture

B.-J. Kim and H.J. Yoo contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00198-019-04881-3) contains supplementary material, which is available to authorized users.

✉ B.-J. Kim
umkbj0825@amc.seoul.kr

[1] Division of Endocrinology and Metabolism, Asan Medical Center, University of Ulsan College of Medicine, 88 Olympic-ro 43-gil, Songpa-gu, Seoul 05505, South Korea

[2] Department of Convergence Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, South Korea

[3] Asan Institute for Life Sciences, Seoul, South Korea

[4] Department of Cellular Biology and Anatomy, Medical College of Georgia, Augusta University, Augusta, GA, USA

[5] Department of Orthopedic Surgery, Medical College of Georgia, Augusta University, Augusta, GA, USA

[6] Department of Endocrinology, Inha University School of Medicine, Incheon, South Korea

Introduction

The n-3 and n-6 fatty acids (FAs), which are subclasses of polyunsaturated FAs (PUFAs), have received attention because of their role in metabolic health. Although both n-3 and n-6 FAs are considered to be essential nutrients because they cannot be synthesized in mammals (including humans) and must be obtained from the diet, they are distinguished based on the location of the first double bond. Importantly, endogenous conversion between n-3 and n-6 FAs is impossible, and these molecules compete for incorporation into target tissues and metabolism by common enzymes because of their chemically similar structures [1, 2]. These characteristics of n-3 and n-6 FAs may contribute to their opposing health effects in humans. Specifically, n-3 FA shows beneficial effects against chronic metabolic diseases such as obesity, diabetes, and cardiovascular diseases, possibly through its anti-inflammatory and antithrombotic properties [3–5]. In contrast, the effects of n-6 FA on cardiometabolic outcomes are generally considered to be harmful; however, the evidence is relatively less clear [6, 7].

There is considerable evidence from in vitro and animal studies supporting the pivotal role of PUFAs, particularly n-3 FA, in bone metabolism [8–12]. However, several epidemiological studies assessing the association between dietary n-3 FA and fracture risk have yielded conflicting results, with some showing an inverse association and others showing no association [13–16]. Such inconsistencies may be attributable to the highly heterogeneous ingredients in dietary supplements such as fish oil, which contains many other bioactive components [17] that may also affect bone metabolism. The possible recall or misreporting bias of dietary questionnaires and limited accuracy also likely contributes to these discrepant results. Therefore, analyzing PUFA levels using reliable methods in biological samples would be more appropriate for objectively investigating the relationship of these FAs with human bone health. Furthermore, despite recognition of the potential roles of n-3 and n-6 FAs on bone, the detailed mechanisms underlying these effects have not been thoroughly examined in humans. As one of the efforts to clarify these issues, in this case-control and cross-sectional study, we assessed whether the plasma levels of n-3 and n-6 FAs and their ratio significantly differed between patients with osteoporotic hip fracture (HF) and osteoarthritis, as well as determined their associations with bone mass and bone biochemical markers measured in bone marrow (BM) samples in all patients.

Materials and methods

Study participants and protocol

The study population consisted of consecutive patients aged 65 years or older who underwent hip surgery at the Department of Orthopedic Surgery, Asan Medical Center (Seoul, Korea) between November 2012 and December 2013. All patients had hip surgery because of either osteoporotic HF or osteoarthritis. Patients who had taken drugs that could affect bone metabolism for more than 6 months or within the previous 12 months before hip surgery, such as bisphosphonate, systemic glucocorticoids, or hormone replacement therapy, were excluded. Patients with diseases that may result in the development of secondary osteoporosis, such as hyperthyroidism or rheumatoid arthritis, were also excluded. Patients were excluded if they had a fever (oral temperature ≥ 38.0 °C) or abnormal findings on complete blood counts of leukocytes (< 4.0 or $> 10.0 \times 10^9$/L) or platelets (< 150 or $> 350 \times 10^9$/L). Abnormal liver or kidney functions were also considered to be exclusion factors. These criteria were used to rule out systemic illness. Finally, patients with fractures considered to be non-osteoporotic, such as those owing to motor vehicle accidents or falls from a standing height or higher, were excluded [18]. Among the 102 eligible participants not satisfying any of the detailed exclusion criteria, blood and BM samples were simultaneously collected during hip surgery from 72 patients after obtaining their consent. Consequently, 28 cases and 44 controls defined as patients with osteoporotic HF and with osteoarthritis, respectively, were finally enrolled in our study.

We obtained patient information using a questionnaire assessing smoking (current smoker), alcohol intake (≥ 3 U/day), and history of medication use, previous medical or surgical procedures, and reproductive status (including menstruation). We also collected information related to the history of previous osteoporotic fractures through an interviewer-assisted questionnaire.

This study was approved by the Institutional Review Board of Asan Medical Center and was conducted according to the Ethical Principles for Medical Research Involving Human Subjects as defined by the Helsinki Declaration.

Measurement of plasma n-3 and n-6 FAs

Fasting venous blood samples were obtained from all patients. After sample centrifugation at 3000 rpm for 5 min at 4 °C, we carefully collected the supernatants to exclude the cell components. All samples that exhibited hemolysis or clotting were discarded. To determine the concentrations of FAs, 50 μL plasma was combined and mixed well with 200 μL of a chloroform/methanol mixture (1:2, *v*/*v*) and 50 μL of an internal standard solution (0.1 mg/mL myristic acid-d_{14}). After centrifugation at 2000×*g* for 15 min, the lower organic phase was collected and dried. The dried sample was hydrolyzed with 200 μL of 0.5 M KOH in MeOH at 80 °C for 30 min, cooled to room temperature, and then reacted with 200 μL of 12% (*w*/*w*) BCl_3-MeOH (Sigma-Aldrich, St. Louis, MO, USA) at 60 °C for 30 min. Subsequently, 100 μL of H_2O

and 100 μL of hexane were added sequentially and mixed vigorously. After 5 min of rest, the upper lipid phase was collected and added to 20–30 mg of anhydrous sodium sulfate to remove traces of water, and the supernatant was subjected to gas chromatography/mass spectrometry analysis. FA methyl esters (Sigma-Aldrich) without derivatization were used to generate calibration curves. The concentration of n-3 FA was calculated by summing the eicosapentaenoic acid (C20:5 n-3) and docosahexaenoic acid (C22:6 n-3) contents, and the concentration of n-6 FA was calculated by summing the linoleic acid (C18:2 n-6), arachidonic acid (C20:4 n-6), and eicosatrienoic acid (C20:3 n-6) contents. The inter-assay coefficients of variation (CVs) for C18:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, and C22:6 n-3 were less than 5% with few exceptions, and all values were within the acceptable criterion of 15% for reproducible assays [19] (Supplemental Table 1).

Biochemical measurement in BM samples

BM fluids were collected during hip surgery. After sample centrifugation at 3000 rpm for 5 min at 4 °C, we carefully collected the supernatants to exclude the cell components. All samples showing hemolysis or clotting were discarded. The following bone biochemical markers were measured in BM samples with immunoassay kits: tartrate-resistant acid phosphatase 5b (TRAP-5b; Cat# SB-TR201A, Immunodiagnostic Systems, Scottsdale, AZ, USA), bone-specific alkaline phosphatase (BSALP; Cat# MBS262250, MyBioSource, San Diego, CA, USA), osteoprotegerin (OPG; Cat# ab100617, Abcam, Cambridge, MA, USA), and receptor activator of nuclear factor-κB ligand (RANKL; Cat# K1016, Immunodiagnostic Systems). The inter- and intra-assay CVs for each assay were as follows: TRAP-5b < 9% and < 9%, BSALP < 12% and < 8%, OPG < 12% and < 10%, and RANKL < 9.3% and < 3.5%, respectively.

Measurement of bone mineral density

Areal bone mineral density (BMD; g/cm^2) was measured in the lumbar spine (L1–L4) and proximal femurs (femur neck and total femur) by dual-energy X-ray absorptiometry with a Lunar system (Prodigy, Madison, WI, USA), software version 9.30.044. The precision values of the equipment, presented as CVs, were 0.67% and 1.25% for the lumbar spine and femur neck, respectively, in 17 volunteers who were not enrolled in the study. Each volunteer underwent five scans on the same day, getting on and off the table between examinations. In addition to the absolute values, BMD was also expressed as the Z-score and T-score, which are defined as the number of standard deviations (SDs) above or below the mean for healthy subjects of the same sex and age and for a healthy 30-year-old adult, respectively.

Statistical analysis

All data are presented as the means ± SD or as numbers and percentages, unless otherwise specified. The baseline characteristics of the study population according to the status of osteoporotic HF and osteoarthritis were compared using Student's *t* tests for continuous variables and chi-square tests for categorical variables. The multivariate-adjusted least-square mean levels with 95% confidence intervals (CIs) of n-3 and n-6 FAs and their ratio in terms of the presence of osteoporotic HF were estimated and compared by analysis of covariance after adjusting for potential confounders. These confounding variables were selected based on their clinical relevance. Consequently, the base adjustment model included sex, age, and body mass index (BMI), while the multivariate adjustment model included sex, age, BMI, smoking status, alcohol intake, 25-hydroxyvitamin D_3 (25-OH-D_3), and history of previous osteoporotic fracture. To generate odds ratios (ORs) with 95% CIs for osteoporotic HF according to the plasma levels of n-3 and n-6 FAs and their ratio, we performed multiple logistic regression analyses after adjusting for confounding variables. To investigate the associations of plasma levels of n-3 and n-6 FAs and their ratio with BMD and bone-related markers measured in BM samples in all patients, we performed multiple linear regression analyses with the Enter method. All statistical analyses were performed using SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA). Differences were considered statistically significant at $P < 0.05$.

Results

The baseline characteristics of the study participants are shown in Table 1. Among the 28 cases with osteoporotic HF and 44 controls with osteoarthritis, 21 (75.0%) and 29 (65.9%) were women, respectively. The mean ages of cases and controls were 78.3 ± 9.1 years (range = 65–95 years) and 72.0 ± 5.3 years (range = 65–90 years), respectively. There were no significant differences in weight, height, BMI, smoking status, alcohol intake, history of osteoporotic fracture and myocardial infraction, and serum 25-OH-D_3 level between groups. The TRAP-5b level in BM aspirates was markedly higher in the cases than in the controls, whereas differences in the BSALP level and RANKL/OPG ratio in BM samples between groups were not significant. Not only absolute BMD values (Table 1) but also Z- and T-scores (Supplemental Table 2) at the lumbar spine and proximal femurs were significantly lower in patients with HF than in those without HF.

After adjusting for sex, age, and BMI, patients with osteoporotic HF had a 26.7% lower plasma n-3 level and 39.9% higher plasma n-6/n-3 ratio than those with osteoarthritis (Fig. 1). These differences remained statistically significant after additional adjustment for smoking status, alcohol intake,

Table 1 Baseline characteristics in patients with osteoporotic HF and osteoarthritis

Variables	Patients with HF ($n = 28$)	Patients with osteoarthritis ($n = 44$)	P
Sex, no. (%)			0.414
Female	21 (75.0)	29 (65.9)	
Male	7 (25.0)	15 (34.1)	
Age (years)	*78.3 ± 9.1*	*72.0 ± 5.3*	*0.002*
Weight (kg)	56.7 ± 11.7	57.9 ± 8.9	0.626
Height (cm)	155.0 ± 8.6	154.6 ± 8.1	0.838
Body mass index (kg/m^2)	23.6 ± 4.4	24.2 ± 3.1	0.498
Current smoker, no. (%)	4 (14.3)	7 (15.9)	0.852
Alcohol intake ≥ 3 U/day, no. (%)	3 (10.7)	8 (18.2)	0.391
Previous osteoporotic Fx, no. (%)	6 (21.4)	5 (11.4)	0.247
Hx of myocardial infraction, no. (%)	2 (7.1)	2 (4.5)	0.640
Serum 25-OH-D_3 (ng/mL)	18.3 ± 9.7	21.4 ± 11.0	0.229
Bone mineral density (g/cm^2)			
Lumbar spine	*0.818 ± 0.181*	*1.003 ± 0.236*	*0.002*
Femur neck	*0.644 ± 0.127*	*0.845 ± 0.192*	*< 0.001*
Total femur	*0.665 ± 0.132*	*0.850 ± 0.143*	*< 0.001*
Bone-related markers in BM samples			
TRAP-5b (U/L)	*4.15 ± 2.44*	*2.75 ± 1.79*	*0.008*
BSALP (ng/mL)	194.8 ± 49.3	201.6 ± 46.4	0.573
RANKL/OPG ratio	189.0 ± 766.9	55.5 ± 302.4	0.315
Plasma n-3 (μg/μL)	*0.253 ± 0.098*	*0.367 ± 0.134*	*< 0.001*
Plasma n-6 (μg/μL)	1.393 ± 0.280	1.329 ± 0.291	0.356
Plasma n-6/n-3 ratio	*6.24 ± 2.31*	*4.18 ± 1.91*	*< 0.001*

Values are presented as the mean ± standard deviation unless otherwise specified. Italicized values are statistically significant

HF, hip fracture; *Fx*, fracture; *25-OH-D_3*, 25-hydroxyvitamin D_3; *Hx*, history; *BM*, bone marrow; *TRAP*, tartrate-resistant acid phosphatase; *BSALP*, bone-specific alkaline phosphatase; *RANKL*, receptor activator of nuclear factor-κB ligand; *OPG*, osteoprotegerin

previous osteoporotic fracture, and serum 25-OH-D_3 level. However, the plasma n-6 level was not significantly different between patients with and without osteoporotic HF in any of the adjustment models.

In the base adjustment model, the ORs per SD increment in plasma n-3 level and per increment in the plasma n-6/n-3 ratio for osteoporotic HF were 0.39 and 1.51, respectively (Table 2). Even after adjusting for all potential confounders, each SD increment in the plasma n-3 level and each increment in the plasma n-6/n-3 ratio were associated with multivariate-adjusted ORs of 0.40 and 1.53 for osteoporotic HF, respectively. However, the OR for HF according to the plasma n-6 level was not statistically significant.

Multiple linear regression analyses were performed to examine the association of plasma levels of n-3 and n-6 FAs and their ratio with BMD (Table 3). After adjusting for sex, age, and BMI, the plasma n-3 level was positively associated with the BMD values at the lumbar spine and total femur, and the significance at these sites remained even after adjustment for all potential confounders. In both the base and multivariate adjustment models, the plasma n-6/n-3 ratio was inversely correlated with the BMD values at the lumbar spine and total femur. Additionally, a higher n-6/n-3 ratio tended to be associated with a lower femur neck BMD, although it did not reach statistical significance. However, no correlations were found between plasma n-6 level and BMD values at any site, regardless of the adjustment model used. When we adopted the Z- (Supplemental Table 3) or T-score (data not shown), rather than the BMD value, as the dependent variable in multiple linear regression analyses, the associations of the plasma n-3 FA level and n-6/n-3 ratio with bone mass parameters were still significant.

Finally, to elucidate the possible mechanisms explaining the effects of n-6 and n-3 FAs in human bone metabolism, we investigated their associations with the levels of TRAP-5b and BSALP, as well as the RANKL/OPG ratio in BM samples (Table 4). After adjusting for sex, age, and BMI, the plasma n-3 level and plasma n-6/n-3 ratio were inversely and positively associated with the TRAP-5b level in BM aspirates, respectively, and these significant associations persisted after considering all potential confounders. However, there was no

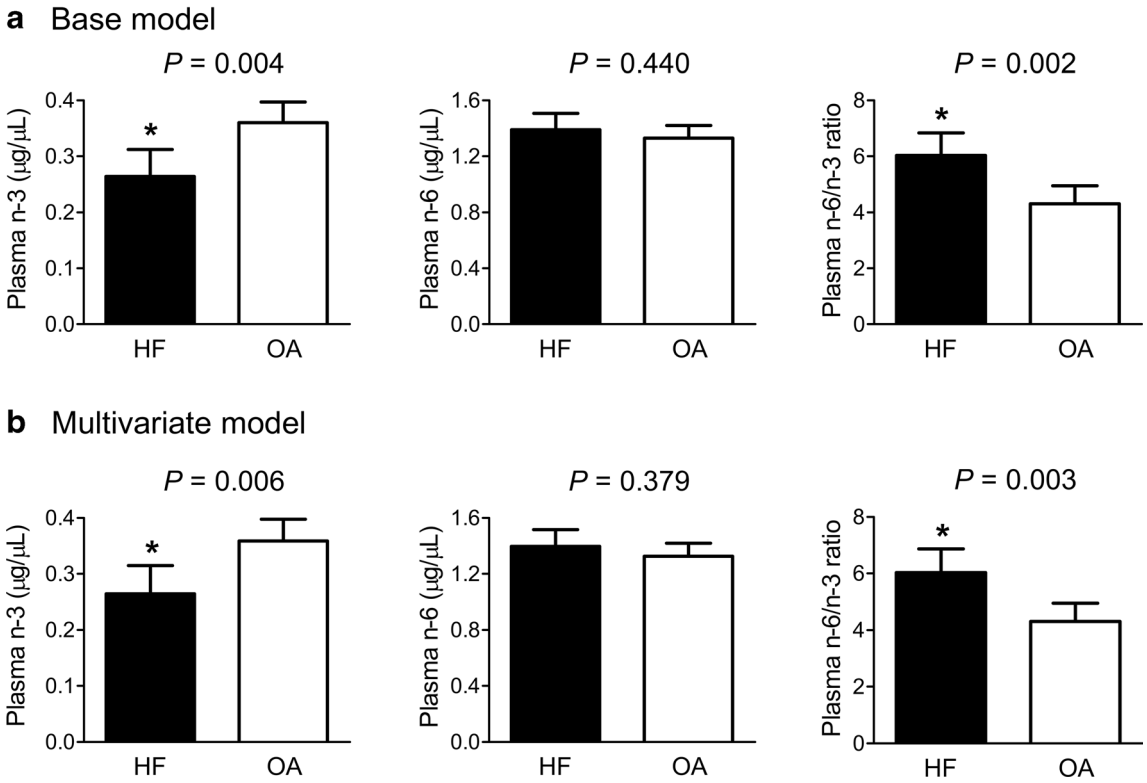

Fig. 1 Differences in the plasma levels of n-3 and n-6 fatty acids and their ratio according to the osteoporotic hip fracture and osteoarthritis status. After adjusting for confounders, the estimated means with 95% confidence intervals were generated and compared by analysis of covariance (ANCOVA). *Statistically significantly different from the control by ANCOVA. Base model (**a**): adjusted for sex, age, and body mass index. Multivariate model (**b**): adjusted for sex, age, body mass index, smoking status, alcohol intake, 25-hydroxyvitamin D_3, and history of previous osteoporotic fracture. HF, hip fracture; OA, osteoarthritis

correlation between the plasma n-6 level and TRAP-5b level in BM samples. Furthermore, neither the plasma levels of n-6 and n-3 FAs nor the plasma n-6/n-3 ratio was associated with the BSALP level and RANKL/OPG ratio in BM aspirates.

Although the major strength of this study is that we used BM samples to analyze bone biochemical markers, we also investigated the associations between plasma n-3 and bone turnover markers in the peripheral blood. After adjusting for confounders, the plasma n-3 level was inversely correlated with the serum C-terminal telopeptide of type I collagen level, a bone resorption marker (regression coefficient = − 0.249, $P = 0.037$), but not with the serum osteocalcin level, a bone formation marker ($P = 0.327$).

Discussion

Among Korean patients from whom both blood and BM samples were collected during hip surgery, we found that a higher plasma n-3 level was significantly associated with a lower OR for osteoporotic HF and with a higher bone mass after statistical adjustment of confounders. Importantly, the plasma n-3 level was inversely related to the TRAP-5b level, an osteoclast differentiation marker, in BM aspirates, reflecting the bone microenvironment. To the best of our knowledge, no previous study has simultaneously used both blood and BM samples in terms of analyses of PUFAs and bone health in humans. Moreover, this is the first study to provide clinical evidence

Table 2 The odds ratios for osteoporotic hip fracture according to plasma levels of n-3 and n-6 fatty acids and their ratio

Adjustment model	OR (95% CIs), per SD increment in plasma n-3 level	*P*	OR (95% CIs), per SD increment in plasma n-6 level	*P*	OR (95% CIs), per increment in plasma n-6/n-3 ratio	*P*
Base	*0.39 (0.20–0.78)*	*0.008*	1.23 (0.73–2.08)	0.433	*1.51 (1.13–2.03)*	*0.005*
Multivariate	*0.40 (0.20–0.80)*	*0.010*	1.28 (0.73–2.22)	0.389	*1.53 (1.14–2.08)*	*0.006*

Italicized values are statistically significant. Base model: adjusted for sex, age, and body mass index. Multivariate model: adjusted for sex, age, body mass index, smoking status, alcohol intake, 25-hydroxyvitamin D_3, and history of previous osteoporotic fracture

OR, odds ratio; *CI*, confidence interval; *SD*, standard deviation

Table 3 The association of plasma levels of n-3 and n-6 fatty acids and their ratio with bone mineral density

	Plasma n-3 level				Plasma n-6 level				Plasma n-6/n-3 ratio			
	β	SE	*β*	*P*	β	SE	*β*	*P*	β	SE	*β*	*P*
Base model												
Lumbar spine BMD	*0.588*	*0.198*	*0.339*	*0.005*	−0.038	0.104	−0.043	0.713	*−0.033*	*0.011*	*−0.342*	*0.004*
Femur neck BMD	0.199	0.152	0.136	0.196	−0.015	0.067	−0.022	0.825	−0.017	0.009	−0.198	0.062
Total femur BMD	*0.246*	*0.116*	*0.199*	*0.038*	−0.031	0.052	−0.054	0.557	*−0.017*	*0.007*	*−0.236*	*0.015*
Multivariate model												
Lumbar spine BMD	*0.615*	*0.193*	*0.354*	*0.002*	−0.019	0.103	−0.021	0.854	*−0.035*	*0.011*	*−0.359*	*0.002*
Femur neck BMD	0.199	0.156	0.136	0.208	−0.010	0.069	−0.014	0.889	−0.017	0.009	−0.203	0.062
Total femur BMD	*0.244*	*0.119*	*0.197*	*0.045*	−0.036	0.053	−0.062	0.504	*−0.018*	*0.007*	*−0.253*	*0.010*

Italicized values are statistically significant. Base model: adjusted for sex, age, and body mass index. Multivariate model: adjusted for sex, age, body mass index, smoking status, alcohol intake, 25-hydroxyvitamin D_3, and history of previous osteoporotic fracture

β, unstandardized regression coefficient; *SE*, standard error; *β*, standardized regression coefficient

that n-3 FA may play a beneficial role as an inhibitor of osteoclastogenesis on human bone metabolism, which may contribute to decreasing osteoporotic fractures.

Although epidemiologic research has been performed to assess the potential role of PUFAs, mainly estimated using a food frequency questionnaire (FFQ), on human bone health [13–16, 20], the results are largely inconsistent, which is most likely due to the methodological limitations of the FFQ. Therefore, there has been an increasing need for identification of a reliable FA biomarker reflecting dietary or supplementary FA intake. Analysis of PUFAs in the adipose tissue can serve this purpose well, with estimates of the half-life of FAs ranging from 6 months to 2 years [21, 22]. However, this invasive method requires biopsy and thus is unrealistic for clinical use. Measurement of PUFAs in the plasma and red blood cells (RBCs) may be another alternative, as blood collection is relatively simple and blood samples can be allocated for other biochemical analyses [23]. Although the optimal biomarker for reflecting usual FA intake remains controversial, measurement of FAs in both plasma and RBCs is regarded as an objective measure of FAs available to peripheral tissues [23–25]. However, the preparation of washed RBCs exceeds the cost of isolating the plasma, and thus, the measurement of FAs in the plasma may be a more clinically feasible approach for routine practice.

Table 4 The association of plasma levels of n-3 and n-6 fatty acids and their ratio with bone-related markers measured in bone marrow aspirates

	Plasma n-3 level				Plasma n-6 level				Plasma n-6/n-3 ratio			
	β	SE	*β*	*P*	β	SE	*β*	*P*	β	SE	*β*	*P*
Base model												
BM aspirates												
TRAP-5b	*−0.699*	*0.266*	*−0.327*	*0.011*	0.025	0.122	0.026	0.838	*0.042*	*0.016*	*0.337*	*0.009*
BSALP	−0.056	0.100	−0.074	0.576	−0.044	0.043	−0.126	0.316	0.003	0.006	0.078	0.564
RANKL/OPG ratio	−1.992	1.239	−0.211	0.113	−0.222	0.552	−0.051	0.689	0.111	0.073	0.202	0.135
Multivariate model												
BM aspirates												
TRAP-5b	*−0.633*	*0.272*	*−0.296*	*0.023*	0.037	0.124	0.037	0.768	*0.039*	*0.016*	*0.315*	*0.018*
BSALP	−0.030	0.101	−0.040	0.763	−0.039	0.044	−0.114	0.371	0.002	0.006	0.035	0.799
RANKL/OPG ratio	−2.188	1.282	−0.232	0.093	−0.257	0.572	−0.059	0.655	0.121	0.076	0.221	0.117

The levels of TRAP-5b and BSALP and RANKL/OPG ratio were log-transformed because of their skewed distributions. Italicized values are statistically significant. Base model: adjusted for sex, age, and body mass index. Multivariate model: adjusted for sex, age, body mass index, smoking status, alcohol intake, 25-hydroxyvitamin D_3, and history of previous osteoporotic fracture

β, unstandardized regression coefficient; *SE*, standard error; *β*, standardized regression coefficient; *BM*, bone marrow; *TRAP*, tartrate-resistant acid phosphatase; *BSALP*, bone-specific alkaline phosphatase; *RANKL*, receptor activator of nuclear factor-κB ligand; *OPG*, osteoprotegerin

A literature search revealed two clinical studies that investigated the relationship of the plasma or RBC n-3 FA level with fracture. In a recent longitudinal study of 1438 participants, Harris et al. [26] reported that a greater plasma n-3 FA concentration was associated with a lower osteoporotic fracture risk in older adults, particularly in men. A similar result was obtained in the Women's Health Initiative study showing that a higher RBC n-3 FA level may predict a lower hip fracture risk [27]. Furthermore, we found that the plasma n-3 level was significantly lower in patients with osteoporotic HF and positively associated with BMD. Collectively, these results consistently indicate a positive correlation between the n-3 FA level and human osteoporosis-related phenotypes.

There are several possible explanations for the role of n-3 FA in bone metabolism, such as the modulation of calcium absorption, prostaglandin synthesis, and lipid oxidation [8]. Moreover, in vitro and animal studies indicated that n-3 FA can directly act to both increase bone formation and decrease bone resorption [8, 9, 28]. However, the specific mechanisms by which n-3 FA positively affects human bone health remain unclear. In the present study, we demonstrated that a higher plasma n-3 level was associated with a lower TRAP-5b level, but not BSALP level, in BM samples. Furthermore, the statistical significance of the inverse correlation between the plasma n-3 FA level and RANKL/OPG ratio in BM aspirates was marginal in the multivariate model. Taken together, these results suggest that the main effects of n-3 FA in human bone metabolism could be mediated through the direct inhibition of osteoclastogenesis and bone resorption rather than the increase of bone formation or the regulation of RANKL or OPG secretion from osteoblasts.

In our efforts to further elucidate the potential role of n-3 FA in human bone, we measured the levels of TRAP-5b, BSALP, RANKL, and OPG in the BM samples rather than in the peripheral blood. Drake et al. [29] compared the correlation between the levels of these markers in the peripheral blood and BM fluids in humans. Despite the strong relationship between circulating and BM levels of sclerostin, the associations between the blood and BM levels of TRAP-5b, BSALP, and OPG were considerably weaker. Even RANKL was not detectable in the peripheral blood of most samples. Consequently, measurement of these bone markers in the peripheral blood may not accurately represent the bone microenvironment and thus may be inappropriate in a human mechanism study related to bone. We believe that the use of BM samples in this clinical study is the major strength of our study.

Although a possible role for n-3 FA in bone metabolism has long been recognized, the specific receptor involved in these effects was only recently identified. An animal study using genetically engineered mice revealed that free fatty acid receptor 4 (FFA4; also known as GPR120) mediates the anti-osteoporotic effects of n-3 FA [28]. However, from a clinical perspective, the amount of n-3 FA–enriched fish oils that would have to be consumed to sustain chronic agonism of FFA4 is likely too high to be practical in daily life [30, 31]. Therefore, the identification of FFA4 as a central mediator of the effects of n-3 FAs on human metabolism bypasses the need to ingest large amounts of n-3 FA supplements by providing the possibility of developing high-affinity small-molecular agonists of FFA4.

Several potential limitations should be considered when interpreting our data. Importantly, because this was a case-control and cross-sectional study, we could not determine whether a causal relationship exists between the plasma n-3 FA level and osteoporosis-related phenotypes. Second, because of the difficulties in obtaining BM samples, our sample size was relatively small and thus the conclusion may be less convincing; however, as described above, this is a unique cohort from which blood and BM samples were simultaneously collected. Third, supplementary and dietary FA intakes were not evaluated in this study. The lack of this information could be a confounding factor in terms of blood FA levels, because patients with osteoarthritis may perhaps be consuming more supplementation such as fish oil. Fourth, bone turnover markers, including TRAP-5b, can remain elevated up to 4 months after an acute fracture, and thus, this may be a confounding factor, especially in patients with HF. Future longitudinal studies would be important to understand the dynamic role of TRAP-5b in these fractures. Finally, our study population consisted of patients who visited a referral hospital and therefore may not be representative of the general population and may have resulted in selection bias.

In conclusion, we demonstrated that the plasma n-3 level was lower in patients with osteoporotic HF than in those with osteoarthritis and that a higher circulating n-3 FA level was correlated with a higher BMD and lower level of TRAP-5b, but not BSALP, in BM aspirates. These results suggest that the positive association of the n-3 FA level with bone phenotypes may be mainly explained by the decrease in osteoclastogenesis and bone resorption in humans.

Funding information This study was supported by a grant from the Bio & Medical Technology Development Program of the National Research Foundation, funded by the Korean government, MSIP (2016M3A9E8941329).

Compliance with ethical standards This study was approved by the Institutional Review Board of Asan Medical Center and was conducted according to the Ethical Principles for Medical Research Involving Human Subjects as defined by the Helsinki Declaration.

Informed consent All participants in this study provided informed consent.

Conflicts of interest None.

References

1. Simopoulos AP (1999) Essential fatty acids in health and chronic disease. Am J Clin Nutr 70:560s–569s
2. Wang L, Manson JE, Rautiainen S, Gaziano JM, Buring JE, Tsai MY, Sesso HD (2016) A prospective study of erythrocyte polyunsaturated fatty acid, weight gain, and risk of becoming overweight or obese in middle-aged and older women. Eur J Nutr 55:687–697
3. Lorente-Cebrian S, Costa AG, Navas-Carretero S, Zabala M, Martinez JA, Moreno-Aliaga MJ (2013) Role of omega-3 fatty acids in obesity, metabolic syndrome, and cardiovascular diseases: a review of the evidence. J Physiol Biochem 69:633–651
4. Saravanan P, Davidson NC, Schmidt EB, Calder PC (2010) Cardiovascular effects of marine omega-3 fatty acids. Lancet (London, England) 376:540–550
5. Simopoulos AP (2008) The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Exp Biol Med (Maywood) 233:674–688
6. Ramsden CE, Zamora D, Leelarthaepin B, Majchrzak-Hong SF, Faurot KR, Suchindran CM, Ringel A, Davis JM, Hibbeln JR (2013) Use of dietary linoleic acid for secondary prevention of coronary heart disease and death: evaluation of recovered data from the Sydney Diet Heart Study and updated meta-analysis. BMJ (Clin Res Ed) 346:e8707
7. Patterson E, Wall R, Fitzgerald GF, Ross RP, Stanton C (2012) Health implications of high dietary omega-6 polyunsaturated fatty acids. J Nutr Metabol 2012:539426
8. Watkins BA, Li Y, Lippman HE, Seifert MF (2001) Omega-3 polyunsaturated fatty acids and skeletal health. Exp Biol Med (Maywood) 226:485–497
9. Watkins BA, Lippman HE, Le Bouteiller L, Li Y, Seifert MF (2001) Bioactive fatty acids: role in bone biology and bone cell function. Prog Lipid Res 40:125–148
10. Watkins BA, Li Y, Seifert MF (2006) Dietary ratio of n-6/n-3 PUFAs and docosahexaenoic acid: actions on bone mineral and serum biomarkers in ovariectomized rats. J Nutr Biochem 17: 282–289
11. Shen CL, Yeh JK, Rasty J, Li Y, Watkins BA (2006) Protective effect of dietary long-chain n-3 polyunsaturated fatty acids on bone loss in gonad-intact middle-aged male rats. Br J Nutr 95:462–468
12. Banu J, Bhattacharya A, Rahman M, Kang JX, Fernandes G (2010) Endogenously produced n-3 fatty acids protect against ovariectomy induced bone loss in fat-1 transgenic mice. J Bone Miner Metab 28: 617–626
13. Suzuki T, Yoshida H, Hashimoto T, Yoshimura N, Fujiwara S, Fukunaga M, Nakamura T, Yoh K, Inoue T, Hosoi T, Orimo H (1997) Case-control study of risk factors for hip fractures in the Japanese elderly by a Mediterranean Osteoporosis Study (MEDOS) questionnaire. Bone 21:461–467
14. Virtanen JK, Mozaffarian D, Cauley JA, Mukamal KJ, Robbins J, Siscovick DS (2010) Fish consumption, bone mineral density, and risk of hip fracture among older adults: the cardiovascular health study. J Bone Min Res 25:1972–1979
15. Farina EK, Kiel DP, Roubenoff R, Schaefer EJ, Cupples LA, Tucker KL (2011) Dietary intakes of arachidonic acid and alpha-linolenic acid are associated with reduced risk of hip fracture in older adults. J Nutr 141:1146–1153
16. Orchard TS, Cauley JA, Frank GC, Neuhouser ML, Robinson JG, Snetselaar L, Tylavsky F, Wactawski-Wende J, Young AM, Lu B, Jackson RD (2010) Fatty acid consumption and risk of fracture in the Women's Health Initiative. Am J Clin Nutr 92:1452–1460
17. Simopoulos AP (1998) Redefining dietary reference values and food safety. World Rev Nutr Diet 83:219–222
18. Langsetmo L, Shikany JM, Cawthon PM, Cauley JA, Taylor BC, Vo TN, Bauer DC, Orwoll ES, Schousboe JT, Ensrud KE (2017) The association between protein intake by source and osteoporotic fracture in older men: a prospective cohort study. J Bone Min Res 32:592–600
19. (2001) Guidance for Industry: Bioanalytical Method Validation. In US Department of Health and Human Services FaDA, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM) (ed)
20. Virtanen JK, Mozaffarian D, Willett WC, Feskanich D (2012) Dietary intake of polyunsaturated fatty acids and risk of hip fracture in men and women. Osteoporos Int 23:2615–2624
21. Beynen AC, Hermus RJ, Hautvast JG (1980) A mathematical relationship between the fatty acid composition of the diet and that of the adipose tissue in man. Am J Clin Nutr 33:81–85
22. Strawford A, Antelo F, Christiansen M, Hellerstein MK (2004) Adipose tissue triglyceride turnover, de novo lipogenesis, and cell proliferation in humans measured with 2H2O. Am J Phys Endocrinol Metab 286:E577–E588
23. Hodson L, Eyles HC, McLachlan KJ, Bell ML, Green TJ, Skeaff CM (2014) Plasma and erythrocyte fatty acids reflect intakes of saturated and n-6 PUFA within a similar time frame. J Nutr 144: 33–41
24. Hodson L, Skeaff CM, Fielding BA (2008) Fatty acid composition of adipose tissue and blood in humans and its use as a biomarker of dietary intake. Prog Lipid Res 47:348–380
25. Serra-Majem L, Nissensohn M, Overby NC, Fekete K (2012) Dietary methods and biomarkers of omega 3 fatty acids: a systematic review. Br J Nutr 107(Suppl 2):S64–S76
26. Harris TB, Song X, Reinders I, Lang TF, Garcia ME, Siggeirsdottir K, Sigurdsson S, Gudnason V, Eiriksdottir G, Sigurdsson G, Steingrimsdottir L, Aspelund T, Brouwer IA, Murphy RA (2015) Plasma phospholipid fatty acids and fish-oil consumption in relation to osteoporotic fracture risk in older adults: the Age, Gene/Environment Susceptibility Study. Am J Clin Nutr 101:947–955
27. Orchard TS, Ing SW, Lu B, Belury MA, Johnson K, Wactawski-Wende J, Jackson RD (2013) The association of red blood cell n-3 and n-6 fatty acids with bone mineral density and hip fracture risk in the women's health initiative. J Bone Miner Res 28:505–515
28. Ahn SH, Park SY, Baek JE, Lee SY, Baek WY, Lee SY, Lee YS, Yoo HJ, Kim H, Lee SH, Im DS, Lee SK, Kim BJ, Koh JM (2016) Free fatty acid receptor 4 (GPR120) stimulates bone formation and suppresses bone resorption in the presence of elevated n-3 fatty acid levels. Endocrinology 157:2621–2635
29. Drake MT, Srinivasan B, Modder UI, Peterson JM, McCready LK, Riggs BL, Dwyer D, Stolina M, Kostenuik P, Khosla S (2010) Effects of parathyroid hormone treatment on circulating sclerostin levels in postmenopausal women. J Clin Endocrinol Metab 95: 5056–5062
30. Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, Li P, Lu WJ, Watkins SM, Olefsky JM (2010) GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell 142:687–698
31. Greenhill C (2014) Diabetes: Gpr120 agonist has anti-inflammatory and insulin-sensitizing effects. Nat Rev Endocrinol 10:510