Published in final edited form as:

J Bone Miner Res. 2013 March ; 28(3): 505–515. doi:10.1002/jbmr.1772.

The association of red blood cell n-3 and n-6 fatty acids to dietary fatty acid intake, bone mineral density and hip fracture risk in The Women's Health Initiative

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Abstract

Omega-3 (n-3) and omega-6 (n-6) polyunsaturated fatty acids (PUFA) in red blood cells (RBC) are an objective indicator of PUFA status and may be related to hip fracture risk.

The primary objective of this study was to examine RBC PUFAs as predictors of hip fracture risk in postmenopausal women.

A nested case-control study (n=400 pairs) was completed within the Women's Health Initiative (WHI) using 201 incident hip fracture cases from the Bone Mineral Density (BMD) cohort, along with 199 additional incident hip fracture cases randomly selected from the WHI Observational Study. Cases were 1:1 matched on age, race, and hormone use with non-hip fracture controls. Stored baseline RBCs were analyzed for fatty acids using gas chromatography. After removing degraded samples, 324 matched pairs were included in statistical analyses. Stratified Cox proportional hazard models were constructed according to case-control pair status; risk of fracture was estimated for tertiles of RBC PUFA.

In adjusted hazard models, lower hip fracture risk was associated with higher RBC α -linolenic acid [Hazard ratio (HR) Tertile 3 (T3): 0.44; 95% CI: 0.23-0.85; *p* for linear trend 0.0154], eicosapentaenoic acid (HR T3: 0.46; 95% CI: 0.24-0.87; *p* for linear trend 0.0181) and total n-3 PUFAs (HR T3: 0.55; 95% CI: 0.30-1.01; *p* for linear trend 0.0492). Conversely, hip fracture nearly doubled with the highest RBC n-6/n-3 ratio (HR T3: 1.96; 95% CI: 1.03-3.70; *p* for linear trend 0.0399). RBC PUFAs were not associated with BMD. RBC PUFAs were indicative of dietary intake of marine n-3 PUFAs (Spearman's ρ =0.45, *p*<0.0001), total n-6 PUFAs (ρ =0.17, *p*<0.0001) and linoleic acid (ρ = 0.09, *p*<0.05).

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Disclosures : All authors state that they have no conflicts of interest.

Authors' roles: Study design: RJ and TO. Data extraction: TO. Laboratory analysis: TO and MB. Data analysis: BL. Data interpretation: RJ, SI, BL, MB, KJ, JWW and TO. Drafting manuscript: TO, BL and RJ. Revising manuscript: SI, BL, MB, KJ, JWW.

This trial was registered at clinicaltrials.gov as NCT00000611.

These results suggest that higher RBC α -linolenic acid, as well as eicosapentaenoic acid and total n-3 PUFAs, may predict lower hip fracture risk. Contrastingly, a higher RBC n-6/n-3 ratio may predict higher hip fracture risk in postmenopausal women.

Keywords

Hip fracture; polyunsaturated fatty acids; omega-3 (n-3) fatty acids; omega-6 (n-6) fatty acids; bone mineral density

Introduction

Postmenopausal osteoporosis is a major public health problem that contributes to an estimated 1.45 million fragility fractures, including 222,000 hip fractures annually in U.S. women. Hip fracture incidence is projected to increase 51% in the U.S. by 2025, with an increase in associated healthcare costs of 49% (1, 2). Determining the relation of various nutritional components to fracture outcomes is an important first step in developing dietary recommendations to decrease the burden of this disease. Recently, interest has arisen in the differential roles of polyunsaturated fatty acids (PUFAs) of the omega-3 (n-3) and omega-6 (n-6) families in altering inflammation that in turn, may modulate bone turnover. Chronic inflammation leads to uncoupling of bone formation and bone resorption, which is central to the pathogenesis of osteoporosis. The n-3 FAs produce eicosanoids that are generally less inflammatory than n-6 FA metabolites and lead to lipid mediators important in resolution of inflammation (3, 4). They also may act to decrease pro-inflammatory cytokines that impact critical regulators of bone turnover (5-8). Additionally, n-3 FAs may positively affect calcium absorption and excretion (9-12) and modulate transcription factors involved in regulation of bone turnover (8) and stem cell differentiation (13-15).

The relation of n-3 FA intake to bone mineral density (BMD) and fracture risk in animal models has been promising (9, 16, 17), but epidemiological research has yielded mixed results (18-21). Recently, investigators from the Framingham Osteoporosis Study reported that higher intakes of α -linolenic acid (ALA) in older men and women were associated with lower risk of hip fracture (21). In a Japanese population, fish consumption 3-4 times per week was also associated with a decreased risk of hip fracture (18). In contrast, researchers in the Cardiovascular Health Study reported that neither fish intake nor eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) intake were associated with risk for hip fracture (20). Additionally, in a recent analysis of Women's Health Initiative (WHI) participants, n-3 and n-6 FA intake measured by a food frequency questionnaire (FFQ) were not associated with hip fracture risk, but *total* fracture risk increased with higher n-3 intake from EPA +DHA and lower n-6 FA intake (22). The total fracture category included a number of different types of fracture, including hip fracture, which may respond differentially to PUFA exposure. In addition, the previous WHI analysis was limited by the reliability of nutrient intake data from a baseline FFQ and the lack of data on n-3 FA supplement usage by participants.

Analysis of PUFA levels in biological samples may more objectively assess the relationship of these fatty acids to skeletal outcomes. PUFAs have been frequently measured in various blood fractions (serum, plasma, red blood cells (RBCs)) and adipose tissue in an effort to predict disease outcomes (23-26). Of the blood fractions, RBCs have the longest half life and have been shown to reflect long-term dietary intake of marine n-3 FAs better than plasma in women (27). They also have the advantage of being less biologically variable within subjects and are less affected by recent n-3 FA intake than plasma (28, 29). When compared with adipose tissue, the marine n-3 FAs in RBCs are actually more strongly

correlated with self-reported dietary intake (30). Additionally, RBCs are easily obtained as opposed to adipose tissue biopsies. .

Thus based upon these data suggesting that RBC samples are useful surrogates reflecting PUFA exposure, and because hip fracture is associated with the greatest morbidity and mortality of all osteoporotic fractures, the primary goal of this study was to investigate n-3 and n-6 FAs in RBCs as predictors of hip fracture risk in postmenopausal women. Secondary goals were to (i) examine the association between major classes of fatty acids (saturated, monounsaturated and polyunsaturated) in RBCs and hip fracture risk, (ii) investigate the relationship between serial measures of BMD across varying levels of RBC n-3 and n-6 FAs, and (iii) examine the correlation of RBC FAs to self-reported dietary intake of FAs in this cohort.

Methods

Study population

The WHI is the largest prospective study of postmenopausal women to date focusing on prevention and control of common diseases, including osteoporotic fractures. Details of the study design and methods have been previously reported (31). Briefly, 161,808 ethnically diverse women between 50-79 years of age enrolled in WHI from 1993 to 1998. A total of 68,132 women joined at least one of the three randomized Clinical Trials (CT); a low fat dietary modification trial (DM) compared to usual diet, two placebo-controlled hormone therapy trials with estrogen alone or estrogen plus progestin and a calcium plus vitamin D supplement trial compared to placebo (32). Participants not eligible or not interested in the CT (n=93,676) were able to enroll in the Observational Study (OS). Information on demographics, health and medication history and life-style factors was collected using screening questionnaires and interviews at baseline enrollment. Women enrolled at three clinical centers (Birmingham, AL; Tucson/Phoenix, AZ; and Pittsburgh, PA) had bone mineral density (BMD) measurements of the hip, spine and total body using dual-energy x-ray absorptiometry. The study was reviewed and approved by the Human Subjects Review Committee at each of the 40 participating centers nationwide and all participants provided written informed consent.

Selection of case-control samples

This study used a nested case-control design within the WHI to examine the relation of RBC FAs to hip fracture risk in women with incident hip fracture versus matched (1:1) controls. The initial sample consisted of 400 case-control pairs. Eligible cases included all women with incident hip fracture as of August 15, 2008 who did not use bisphosphonates, selective estrogen receptor modulators or other bone-active medications (including calcitonin, raloxifene, tamoxifen, and teriparatide) at baseline, had at least 0.3 mL of stored RBCs available, and unless enrolled in the HT, reported their hormone use at baseline (no missing values). Personal hormone use (defined as prescription estrogen or estrogen plus progestin) was permitted. Potential controls included all women who had reported no baseline history of hip fracture after age 55, no incident hip fracture during WHI follow-up (as of August 15, 2008) and who did not meet any of the above exclusion criteria. Controls were matched on age (± 5 yrs) and race/ethnicity because of the strong association of these variables with fracture risk; geographic region by latitude to account for Vitamin D from potential sun exposure; length of follow-up to ensure equal probability of observing hip fracture during the study; and treatment group within the hormone trials (active hormone arm or placebo) or current personal use of hormone therapy at enrollment, because this exposure was shown to reduce risk of hip fracture by nearly 40% in WHI.

As of August 15, 2008, 2837 hip fractures had occurred in the WHI OS and CT, including 233 hip fractures in the bone mineral density (BMD) cohort. This cohort consisted of women (n=10,290) with baseline hip scans from three WHI clinical centers that measured serial BMD (33). In order to capture all women who had a hip fracture and BMD data, all eligible cases of hip fracture and matched controls were chosen from the WHI BMD cohort (n=201 pairs; 69 pairs were enrolled in one or more of the CTs and 132 pairs were in the OS). Remaining hip fracture cases were randomly selected and matched (n=199 pairs) to women from the non-BMD cohort within the WHI OS.

After screening RBC samples for degradation, 324 pairs were included in statistical analyses. Final sample size included 154 pairs from the BMD cohort and 170 pairs from the WHI OS.

Fracture and bone mineral density ascertainment

Data on incident fractures were collected by self-reported at annual WHI visits in the CT and by mailed questionnaire in the OS. Medical records were obtained for self-reported hip fractures. These cases were centrally adjudicated by medical record review by trained physicians. The central adjudication of hip fracture in WHI has been previously described (34). There was 96% agreement between central and local adjudication of hip fractures.

BMD measurements of the total body, total hip and lumbar spine (L2-L4) were obtained in women at three participating clinical centers (Birmingham, AL; Tucson/Phoenix, AZ; and Pittsburgh, PA) using dual-energy x-ray absorptiometry (QDR 2000, 2000+, or 4500W; Hologic Inc, Waltham, MA). BMD data collected by trained technicians using standard protocols (35) at the time of baseline randomization and annual visits 3 and 6 were used for this analysis. Ongoing WHI quality assurance programs included monitoring of spine and hip phantom scans, reviews of a random sample of all scans and flagging scans with specific problems. Interscanner variability between the three clinical centers was 4.8% for hip phantom scans tested over five consecutive days (36, 37).

Collection of covariates

A wide range of personal characteristics, health history, health habits, and medication use was collected by questionnaires, personal interviews and phone interviews at baseline and in some instances, at annual visits, in WHI. In addition to dietary data estimated by the baseline WHI Food Frequency Questionnaire (36), we extracted data from the WHI database on other significant predictors of hip fracture identified by Robbins *et al.* (38) such as height, weight, physical activity [metabolic equivalents (METs) in hours per week], fracture history after age 54, parental hip fracture, current smoking status, current corticosteroid use and treated diabetes.

Blood collection, storage and assay of biomarkers of hip fracture risk

Biospecimens including RBC were collected from WHI participants at the initial screening visit at baseline enrollment (1993-1998) according to established protocols (39). In summary, women were asked to fast for a period of 12 hours before all blood collection; take all regular medications except for hypoglycemics used to control diabetes; take no aspirin or non-steroidal anti-inflammatory drugs for 48 hours before the visit except those taken regularly; abstain from smoking for at least 1 hour before the blood draw; and refrain from vigorous physical activity for a period of at least 12 hours prior to the visit. Blood was drawn in a sitting position and processed locally according to standardized protocols. Biospecimens were labeled and stored at -70°C until shipment on dry ice to the central repository (McKesson Bioservices, Rockville, MD) where samples were held at -80°C for long-term storage.

Determination of fatty acid composition of red blood cells

RBC fatty acids were extracted and derivatized according to the method of Harris *et al.* (40). Fatty acid methyl esters (FAMES) were extracted in hexane and analyzed by a gas chromatograph (Shimadzu) equipped with a flame ionization detector and a 30-m Omegawax capillary column (Supelco Chromatography Products) using a 3:1 split and standard conditions as described by Belury (41). FAs were identified by comparing their retention times with those of a standard mixture of FAs. Data are reported as g/100 g fat or % fatty acid/total detected FAs. A duplicate RBC sample was included in each batch of FAMES run on the gas chromatograph in the Belury lab at The Ohio State University for quality control purposes. Mean CVs from duplicate pairs were 2.01% for 18:2n-6 (LA), 1.55% for 20:4n-6 (AA), 7.40% for 18:3n-3 (ALA), 9.78% for 20:5n-3 (42) and 4.30% for 22:6n-3 (DHA). All other CVs were below 10% with the exception of 20:2n-6 and 18:3n-6, which were very near the reliable detection limit of 0.1 mean area%.

Because RBC PUFAs may be unstable at varying temperatures(43, 44), and samples had been stored for over 10 years, subjected to one freeze/thaw cycle and shipped on dry ice prior to chromatographic analysis, we employed a screening step to detect potential sample degradation and exclude these samples from further analysis. This screening criteria (polyunsaturated/saturated FA ratio (P/S) of <0.74) was based on the mean (P/S = 1.0) of over 1500 RBC samples from women in the Framingham Heart Study with a similar age profile as WHI women (45). Of our 800 samples, 3 had an inadequate amount of RBCs to perform the assay, 116 had a P/S ratio <0.74, and 33 were matched to a sample that was inadequate or had a P/S ratio below the screening threshold. Therefore, 152 samples were excluded, leaving 648 samples (324 pairs) in the final statistical analysis.

Statistical Analysis

Stratified Cox proportional hazard models were used to account for the matched structure. RBC FA values were categorized by tertiles and the hazard ratio with 95 percent confidence intervals for hip fracture was estimated according to RBC levels of n-3 FA, n-6 FA and the n-6/n-3 ratio. The time to hip fracture was the primary outcome variable, with PUFA levels as predictors, controlling for potential confounding factors as identified by Robbins *et al* (38), including parental history of fracture, personal fracture after age 54 years, treated diabetes, current corticosteroid use, current smoking status, physical activity in metabolic equivalents (METs) per week, height (cm) and weight (kg). After constructing the above models based on significant predictors of hip fracture in WHI (38), associations were then examined with the inclusion of additional potential confounders : alcohol consumption (servings/week), total energy intake (kcal/d), total calcium (from diet + supplements in mg/d), total vitamin D (from diet + supplements in IU/d), and current multivitamin use (yes or no). Linear mixed models were built with PUFA levels as predictors and hip BMD as the primary outcome to accommodate repeated BMD measures at years 1, 3, 6. This analysis only included a smaller subset of our original cohort due to missing data and was not based on matched pairs, so we controlled for potential confounders of age, race and HT use in the regression. We also tested for trend over tertiles of FA at each year by including an interaction term between time (year) and tertiles of FA in the model. Spearman's correlation coefficients were calculated to estimate the association of RBC FAs and FA data from the WHI food frequency questionnaire (FFQ).

Results

Descriptive Data

Descriptive characteristics of the 324 case-control pairs included in statistical analyses are shown in Table 1. Approximately 27% of women in this analysis either reported hormone

therapy use at baseline or were randomized to treatment in the HT trial. Cases reported significantly more fractures after age 54, weighed significantly less and had lower baseline total hip BMD than controls. They also tended to be taller ($p = 0.06$) and use more corticosteroids ($p = 0.08$) than controls. Based upon baseline FFQ data, the dietary intake of energy, protein, carbohydrate, and total fat was similar for cases and controls. In addition, there were no differences noted in calcium and vitamin D intake. These nutrient consumption levels are typical of US women over the age of 50, with the exception of vitamin D intake, which was lower in this sample of WHI women than reported in NHANES 2007-08 (42). Although fatty acid intake did not differ significantly by case-control status, the saturated fat intake trended higher in hip fracture cases ($p=0.0557$). Over 99.9% of total n-6 FA intake was derived from LA, therefore, due to rounding, values appear to be the same for these two categories of FAs in Table 1 and 2.

RBC FAs and correlation with dietary FAs

The relative abundance of RBC FAs was assessed by gas chromatography and compared to dietary intake of FAs estimated from the WHI FFQ (Table 2). SFA was the largest class of FAs in RBCs (~43.91%), while MUFA contributed ~15.02% and PUFA provided ~41.07% of total FAs. The vast majority of RBC PUFA could be attributed to n-6 FAs. The long chain n-3 FAs in this cohort were low, as is typical in Western populations (24). DHA was the n-3 FA found in greatest quantity (mean 3.68 $\pm$ 1.09%) in RBCs. The n-3 Index of women in this analysis ranged from 1.82 to 11.67% with a mean of approximately 4.2 $\pm$ 1.3%. There were no significant differences in individual or total n-3 FAs or n-6 FAs in RBC samples based on case-control status (data not shown).

FA exposure as assessed by self-reported FFQ was not significantly correlated with RBC SFA and MUFA. In contrast, total RBC PUFAs were weakly correlated with dietary intake ($\rho=0.09$, p 0.0206), as were total n-6 FAs and linoleic acid ($\rho=0.17$, p <0.0001 and $\rho=0.09$, p 0.0201, respectively). Stronger associations were found between intake of marine n-3 FAs and RBC n-3 FAs. Individual EPA and DHA in RBCs were significantly associated with dietary intake ($\rho=0.38$, p <0.0001 and $\rho=0.43$, p <0.0001 respectively). The magnitude of association was strongest between FFQ marine n-3 FAs (EPA+DHA) and the n-3 index ($\rho=0.45$, p <0.0001).

Hazard ratios for hip fracture risk based on RBC FAs

Hazard ratios for hip fracture by tertiles of RBC FAs with multivariate adjustment for risk factors per Robbins *et al* (38) are reported in Table 3. No significant associations were found between RBC total SFA, MUFA or PUFA and risk of hip fracture. However, there was a significant inverse linear association between hip fracture risk and total n-3 FAs in RBCs (p for linear trend 0.0492). When examining individual n-3 FAs, there was a 56% lower relative risk of hip fracture with highest RBC ALA [Tertile(T) 3 HR: 0.44; 95% CI: 0.23-0.85; p for linear trend 0.0154], and a 54% lower hip fracture risk with highest EPA levels (T3 HR: 0.46; 95% CI : 0.24-0.87; p for linear trend 0.0181) compared to T1. Neither DHA nor the n-3 Index was significantly associated with risk of fracture. In contrast, hip fracture risk nearly doubled in women in the highest tertile of the n-6/n-3 FA ratio (HR T3:1.96; 95% CI: 1.03-3.70; p for linear trend 0.0399). Because the n-6/n-3 FA ratio in RBCs primarily reflects the ratio of AA to EPA and DHA, we further examined the relation of the AA/EPA+DHA ratio to hip fracture risk. Similar to the n-6/n-3 FA ratio, a higher AA/EPA+DHA ratio produced higher HR for hip fracture, but the association was not significant (T3 HR: 1.692; 95% CI: 0.86-3.31; p for linear trend 0.1242). Although the direction of association between total n-6 FAs, AA and hip fracture was toward harm (T3 HR: 1.32; 95% CI: 0.72-2.42; p for linear trend 0.3820), there was no significant relation of either total n-6 FAs or AA with hip fracture. There was an inverse direction of association between LA and

hip fracture risk, but again, this was not statistically significant (T3 HR: 0.77; 95% CI: 0.40-1.49; p for linear trend 0.5140). Inclusion of additional potential confounders (alcohol consumption, total energy intake, total calcium intake, total vitamin D intake, and multivitamin use) in the model produced similar results. Additionally, these covariates were not previously identified as significant predictors of hip fracture in WHI participants (38); therefore, we did not include them in the final Cox models.

RBC FAs and BMD

RBC FAs and serial BMD at baseline, year 3 and year 6 of follow-up were available in 229 women in this case-control study. All participants in this sub-group were white and none reported hormone therapy use. Average total hip BMD was 0.73 (95% CI: 0.71, 0.75) g/cm² for cases and 0.80 (95% CI: 0.78, 0.83) g/cm² for controls at time of baseline measurement. There was no relationship of RBC n-3 or n-6 FAs to baseline BMD. After adjustment for important covariates, percent change in total hip BMD over 6 years of follow-up was not associated with total n-3 FAs (Figure 1A) or total n-6 FAs (Figure 1B) in RBCs. Likewise, no associations were found between any of the individual n-3 FAs, individual n-6 FAs or the n-6/n-3 ratio and percent change in BMD over course of follow-up (data not shown). Inclusion of additional covariates (alcohol consumption, total energy intake, total calcium intake, total vitamin D intake, and multivitamin use) in the model did not substantially change the results.

Discussion

This is the first study to examine the relation of RBC PUFAs to hip fracture risk in postmenopausal women. RBC ALA, EPA, and total n-3 FAs were significantly inversely associated with risk of hip fracture in these WHI participants. Conversely, women with an n-6/n-3 FA ratio in the highest tertile had nearly twice the risk of hip fracture compared to those in the lowest tertile.

In order to study the association of dietary fatty acids to chronic diseases such as osteoporosis, it is necessary to have reliable measures of fatty acid exposure. Biospecimens such as RBCs may provide a more definitive marker of n-3 FA consumption over several months than self-reported FFQ data. In this analysis, RBC PUFAs were a significant, though weak, indicator of dietary intake of total PUFA, total n-6 FAs and linoleic acid. RBC ALA was not correlated with ALA estimated from the WHI FFQ. However, n-3 FAs from marine sources (EPA and DHA), which may be more easily identified by self-report, were significantly correlated with RBC EPA and DHA. This confirms previous reports that RBC FAs are a suitable biomarker of marine n-3 FA intake (46-48). The strongest association that we observed was between the n-3 Index and dietary intake of EPA+DHA. The mean n-3 Index in this WHI cohort was $4.12 \pm 1.3\%$. This is slightly lower than the mean n-3 Index of $4.9 \pm 2.1\%$ reported in a sample of 163 younger US men and women (mean age 48 ± 15 yr) who were not taking n-3 FA supplements (47). This is consistent with the likelihood that few women in this WHI cohort were taking n-3 FA supplements at the time of the baseline blood draw. Low levels of long chain n-3 FAs in these WHI participants could have made it difficult to detect additional beneficial skeletal associations, if they indeed existed. An optimal level of n-3 FA in RBCs to protect bone is not known.

In this nested case-control study within WHI, there was no significant relation of total RBC n-6 FAs to hip fracture. Nevertheless, it is interesting to note that the direction of association was toward increased risk and this appears to be primarily driven by RBC AA. In contrast, a recent analysis from the Framingham Osteoporosis Study suggests that higher AA in plasma phosphatidyl choline (49) may be protective in terms of hip fracture risk in older women and men (50). There are several differences between the Framingham study and WHI which may

have impacted our results. First, the WHI cohort is composed of postmenopausal women, while the recent Framingham analysis included both older women and men. Second Framingham researchers used plasma PC to estimate PUFA exposure. Plasma PC is indicative of PUFA intake over a few days to weeks prior to collection and is more readily affected by intake within the 24 hours prior to collection than RBCs (51). As we were interested however in long-term exposure, we chose to analyze RBC PUFAs because they better reflect PUFA status over a period of several months with less biological variability than plasma PUFAs (24, 29). Finally, in light of the very low EPA concentrations in most plasma PC samples from Framingham, it is a possibility that sample degradation impacted results; but without knowing individual fatty acid content, it is difficult to assess sample integrity. In our study, we excluded RBC specimens that did not meet our screening criteria for sample degradation prior to statistical analysis. A few other studies have examined dietary intake of n-6 FAs, primarily from LA, and fracture risk. In a smaller case-control study in Spain, where dietary intake of total n-6 FAs was higher than in US women, n-6 FA intake 18 g/d was associated with over a three-fold increased risk of fragility fractures (52). However, dietary intake of n-6 FAs, almost exclusively from LA, was not associated with hip fracture risk in previous WHI analyses, but low n-6 FA consumption (<10% of energy) was actually associated with increased risk of total fractures (22). Similarly, in this analysis, the lowest tertile of RBC LA produced the highest HR for hip fracture, though there was no significant linear trend across tertiles. Any relation of RBC LA to hip fracture risk may require a larger sample size to detect a relationship.

Total RBC n-3 FAs were significantly inversely associated with risk of hip fracture in these postmenopausal women. A similar significant inverse linear trend was noted for individual RBC ALA and EPA. Women in the highest tertile of RBC ALA had a 56% lower risk of hip fracture than women in the lowest tertile. To our knowledge, no other study has examined the relationship of red blood cell levels of n-3 FAs to hip fracture risk, but higher dietary intake of ALA in older men and women in the Framingham Osteoporosis Study was associated with a 54% decreased risk of hip fracture (21). In contrast, previous analyses of hip fracture risk in WHI women using FFQ data found no association of self-reported ALA intake to hip fracture (22), which may not be unexpected given the lack of correlation between dietary ALA estimated from the WHI FFQ and RBC ALA in this cohort. ALA consumption may be more difficult to detect using FFQ than marine n-3 FAs because plant oils high in ALA are often used as ingredients in other foods and may not be easily recognized by participants or readily detected by a nutrient database. In addition, even supplementation with fairly large doses of ALA (14 g/d) resulted in only modest increases in plasma ALA, presumably related to the rapid catabolism of ALA to carbon dioxide for energy and the conversion of up to 21% of ALA to EPA in women (53).

Women with highest RBC EPA in this case-control study had a 54% lower risk of hip fracture. RBC EPA may be directly impacted by intake of marine sources of EPA and indirectly impacted by the metabolism of ALA to EPA. The extent of ALA conversion to EPA is not only affected by ALA consumption, but also by the balance of n-6 and n-3 FA intake and genetic variability between individuals (53-55). Although EPA and DHA are commonly consumed together in marine foods and DHA makes up the vast majority of n-3 FAs in RBCs, we found no significant association between RBC DHA or the n-3 index and hip fracture risk, which suggests that the conversion of ALA to EPA, though small, may be an important factor in the lower hip fracture risk noted with higher RBC EPA. DHA may be retro-converted to EPA, but this appears to make a minimal contribution to plasma EPA in individuals consuming normal dietary amounts of DHA (56), as were the women in this study. RBC EPA represents the interplay of genetics, metabolism and environmental exposure to foods high in n-3 and n-6 FAs. This may partially explain why other observational studies that have used only self-reported food intake to examine individual

EPA or EPA+DHA consumption have found no association with hip fracture risk (21, 52, 57). Similarly, in our previous WHI analysis using FFQ data, we detected no risk or benefit of n-3 or n-6 FA consumption in relation to hip fracture, however higher EPA+DHA and lower n-6 FAs were both associated with higher *total* fracture risk (22). This may have been related to the decreased precision of PUFAs measured by FFQ or a differential effect of n-3 and n-6 FAs at some of the skeletal sites included in the more heterogeneous total fracture phenotype. In the future, it may be valuable to examine the relationship of PUFA status to fracture risk at various skeletal sites.

It has been proposed that the balance between n-3 and n-6 FAs may impact skeletal health more than individual classes of PUFAs (17, 58, 59). For this reason, we examined the RBC n-6/n-3 FA ratio. Risk of hip fracture nearly doubled in women with the highest RBC n-6/n-3 FA ratio compared to those in the lowest tertile. The few studies that have used self-reported dietary intake to examine the relation of the n-6/n-3 ratio to hip fracture have found no significant associations (21, 22). This may be related to differences in the major PUFAs that contribute to the *dietary* ratio versus the *RBC* ratio. In previous WHI analyses, the dietary n-6/n-3 FA ratio was primarily driven by the 18 carbon PUFAs, LA (~99% of n-6 FA intake) and ALA (~90% of n-3 FA intake) (22). As is typical of RBC FA samples from other Western population groups (60), AA made up ~45% and LA ~39% of n-6 FA in RBCs in this study, while the very long chain n-3 FAs (EPA, DPA and DHA) comprised ~96% of the total n-3 FAs in RBCs. Thus, the dietary ratio primarily reflects the proportion of the 18 carbon, mostly plant-based PUFAs, while the RBC ratio is primarily driven by the balance of the very long chain 20-22 carbon PUFAs derived from metabolism or animal and marine sources. To investigate this further, we examined the association of the AA/EPA+DHA ratio in RBCs to hip fracture risk and did indeed observe results similar to those seen for the total n-6/n-3 FA ratio in RBCs; namely higher values for either ratio were associated with increased risk of hip fracture, but the linear trend for AA/EPA+DHA did not reach statistical significance. No relationship was found between total RBC PUFA, MUFA or SFA and risk of hip fracture. Previously in a larger WHI cohort, a significantly higher relative risk of hip fracture was found with higher self-reported SFA intake (22). It is not surprising that results obtained from dietary intake of SFA and RBC SFA may differ because blood levels of SFA are often a weak or inconsistent biomarker of dietary intake (60). RBC SFA did not correlate with SFA intake in this case-control study or other analyses from the Nurses' Health Study (27). This may be related to desaturation of some SFAs to MUFAs and endogenous synthesis of SFA from excess dietary carbohydrate (61).

RBC PUFAs were not associated with baseline BMD or change in BMD in this cohort. In contrast, a recent case-control study of postmenopausal Korean women, who consume much larger quantities of fish than American women, found that EPA, DHA and total n-3 FAs in RBCs were correlated with higher BMD of the femoral neck, while a higher n-6/n-3 FA ratio was correlated with lower femoral neck BMD (62). In research using serum phospholipids to investigate associations with BMD in young men, the n-6/n-3 FA ratio was inversely associated with spine BMD, but no relation was found with hip BMD (23). Additionally, researchers in the Rancho-Bernardo study found that a higher dietary n-6/n-3 FA ratio was associated with lower BMD at the hip in older adults (59). However, the impact of the n-6 and n-3 FAs on BMD and risk of osteoporosis remains unclear. Moon *et al.* recently reported that despite positive correlations between RBC n-3 FAs and femoral neck BMD, RBC n-3 FAs did not significantly reduce risk of osteoporosis in postmenopausal Korean women, and risk of osteoporosis was actually lower with higher RBC LA and total PUFA (62). In contrast, data from the Framingham Osteoporosis Study suggest that higher plasma LA is associated with lower femoral neck BMD in women. Additionally, women with higher plasma PC DHA concentrations had greater losses of femoral neck BMD over a four year period, while men with the highest DHA appeared to be

protected from this BMD loss (50). In another analysis of Framingham data, *dietary* intake of LA in older women tended to be associated with femoral neck BMD loss (p for trend <0.06) and higher EPA+DHA coupled with higher AA intake was associated with higher femoral neck BMD (63). We did not observe any significant relationships with RBC PUFAs and BMD in the subset of women who had BMD measured in our analysis.

Strengths and Limitations

The primary strengths of this study are the use of an objective biospecimen reflecting n-3 and n-6 FA status, the quality of the hip fracture phenotype and the availability of data on multiple covariates contributing to fracture risk. Additionally, mean n-3 FA levels were typical of older US women and controls were not specifically selected for fracture risk, which may make the results of this study more broadly generalizable to postmenopausal women in the US.

There were also several limitations of this research. First, this was an observational study using a nested case-control design within the prospective WHI cohort. Second, prolonged storage and shipment of RBC samples could have contributed to oxidation of fatty acids in RBCs and results may have differed if fresh blood samples would have been available. However, large epidemiological studies have used RBC samples frozen for as long as 12 years to assess FA status (27, 64) and previous research has shown that FAs in RBCs were virtually unchanged from initial analysis after storage at -80°C for at least four years (44). Additionally, we employed a screening procedure to detect potential sample degradation, retaining only those samples that met our most restrictive criteria. As a result, women with potentially degraded RBC samples along with their matched pair were excluded from statistical analysis. These exclusions, as well as missing values for some covariates used in adjusted models, decreased the power of the study. Nevertheless, we had adequate power to detect significant associations between total n-3 FAs, ALA, EPA and hip fracture. Third, RBC samples were collected at the baseline visit and may not have reflected the changing intake of n-3 FA food sources and supplements over the course of WHI. However, estimates of n-3 supplement use in the US near the beginning of WHI (2000-2002) were relatively low, ranging from 3.8-7.5% of the population (65, 66). Usage did not appear to change substantially over a period of 3-4 years based on surveys of older women taken between 2005-2006 (67).

Conclusions

These results suggest that higher RBC total n-3 FAs may predict lower risk of hip fracture. This beneficial association was noted with either ALA or EPA, but not DHA. Whether this reflects conversion of ALA to EPA requires further research. It may be especially important to investigate the mechanisms behind these relationships in light of the relatively large amount of ALA consumed in Western diets compared to the marine n-3 FAs and the potential impact on skeletal health that increasing RBC ALA or EPA may have on vulnerable groups such as postmenopausal women.

Acknowledgments

The authors would like to thank Ms. Rachel Cole for her valuable direction and supervision of fatty acid analysis and gas chromatography, Ms. Rachel Press for her excellent assistance with fatty acid sample processing, and Mr. Keding Hua for his help in data extraction. We would also like to express our gratitude to Dr. William Harris for sharing his expertise and insights on red blood cell fatty acid assays.

The authors would like to acknowledge the following WHI investigators:

Program Office: (National Heart, Lung, and Blood Institute, Bethesda, Maryland) Jacques Rossouw, Shari Ludlam, Joan McGowan, Leslie Ford, and Nancy Geller.

Clinical Coordinating Center: (Fred Hutchinson Cancer Research Center, Seattle, WA)

Ross Prentice, Garnet Anderson, Andrea LaCroix, Charles L. Kooperberg, Lesley Tinker; (Medical Research Labs, Highland Heights, KY) Evan Stein; (University of California at San Francisco, San Francisco, CA) Steven Cummings.

Clinical Centers: (Albert Einstein College of Medicine, Bronx, NY) Sylvia Wassertheil-Smoller; (Baylor College of Medicine, Houston, TX) Haleh Sangi-Haghpeykar; (Brigham and Women's Hospital, Harvard Medical School, Boston, MA) JoAnn E. Manson; (Brown University, Providence, RI) Charles B. Eaton; (Emory University, Atlanta, GA) Lawrence S. Phillips; (Fred Hutchinson Cancer Research Center, Seattle, WA) Shirley Beresford; (George Washington University Medical Center, Washington, DC) Lisa Martin; (Los Angeles Biomedical Research Institute at Harbor- UCLA Medical Center, Torrance, CA) Rowan Chlebowski; (Kaiser Permanente Center for Health Research, Portland, OR) Erin LeBlanc; (Kaiser Permanente Division of Research, Oakland, CA) Bette Caan; (Medical College of Wisconsin, Milwaukee, WI) Jane Morley Kotchen; (MedStar Research Institute/Howard University, Washington, DC) Barbara V. Howard; (Northwestern University, Chicago/Evanston, IL) Linda Van Horn; (Rush Medical Center, Chicago, IL) Henry Black; (Stanford Prevention Research Center, Stanford, CA) Marcia L. Stefanick; (State University of New York at Stony Brook, Stony Brook, NY) Dorothy Lane; (The Ohio State University, Columbus, OH) Rebecca Jackson; (University of Alabama at Birmingham, Birmingham, AL) Cora E. Lewis; (University of Arizona, Tucson/Phoenix, AZ) Cynthia A. Thomson; (University at Buffalo, Buffalo, NY) Jean Wactawski-Wende; (University of California at Davis, Sacramento, CA) John Robbins; (University of California at Irvine, CA) F. Allan Hubbell; (University of California at Los Angeles, Los Angeles, CA) Lauren Nathan; (University of California at San Diego, LaJolla/Chula Vista, CA) Robert D. Langer; (University of Cincinnati, Cincinnati, OH) Margery Gass; (University of Florida, Gainesville/Jacksonville, FL) Marian Limacher; (University of Hawaii, Honolulu, HI) J. David Curb; (University of Iowa, Iowa City/Davenport, IA) Robert Wallace; (University of Massachusetts/Fallon Clinic, Worcester, MA) Judith Ockene; (University of Medicine and Dentistry of New Jersey, Newark, NJ) Norman Lasser; (University of Miami, Miami, FL) Mary Jo O'Sullivan; (University of Minnesota, Minneapolis, MN) Karen Margolis; (University of Nevada, Reno, NV) Robert Brunner; (University of North Carolina, Chapel Hill, NC) Gerardo Heiss; (University of Pittsburgh, Pittsburgh, PA) Lewis Kuller; (University of Tennessee Health Science Center, Memphis, TN) Karen C. Johnson; (University of Texas Health Science Center, San Antonio, TX) Robert Brzyski; (University of Wisconsin, Madison, WI) Gloria E. Sarto; (Wake Forest University School of Medicine, Winston-Salem, NC) Mara Vitolins; (Wayne State University School of Medicine/Hutzel Hospital, Detroit, MI) Michael S. Simon.

Funding: The project described was supported by Award Number TL1RR025753 and UL1RR025755 from the National Center for Research Resources. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center for Research Resources or the National Institutes of Health.

The WHI program is funded by the National Heart, Lung, and Blood Institute, National Institutes of Health, U.S. Department of Health and Human Services through contracts N01WH22110, 24152, 32100-2, 32105-6, 32108-9, 32111-13, 32115, 32118-32119, 32122, 42107-26, 42129-32, and 44221.

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Figure 1.

Panel A. Percent change in Total Hip BMD over 6 years by tertiles (T) of baseline RBC total n-3 fatty acids ¹⁻³

Panel B. Percent change in Total Hip BMD over 6 years by tertiles (T) of baseline RBC total n-6 fatty acids ¹⁻³

¹n=229 ²SE=Standard Error ³Adjusted for age, geographical region by latitude, height, weight, corticosteroid use, treated diabetes, smoking. Race=Caucasian, HT=0

Table 1

Baseline characteristics and dietary variables of hip fracture cases and controls from the Women's Health Initiative Observational Study and Clinical Trials

	Women with hip fracture (Cases)	Women without hip fracture (Controls)	
<i>Characteristic</i>	<i>n=324</i> ¹	<i>n=324</i>	<i>p</i> ²
Age at screening (y) ³	69.13 [68.41, 69.86]	69.18 [68.47, 69.90]	0.4748
Caucasian (n { % })	304 {94.70%}	304 {94.70%}	1.000
Parental fracture (n { % }) ⁴	125 {43.55%}	120 {41.81%}	0.6670
Fracture after age 54 years(n { % }) ⁴	69 {29.36%}	26 {11.06%}	<0.0001
Treated diabetes (n { % })	17 {5.25%}	11 {3.4%}	0.2393
Current Corticosteroid use (n { % })	9 {2.78%}	3 {0.93%}	0.0833
Current smoker (n { % }) ⁴	21 {6.73%}	14 {4.49%}	0.2230
Very good - excellent general health (n { % }) ⁴	130 {48.33%}	149 {55.39%}	0.1045
Physical Activity (MET hours/wk)	12.11[10.77, 13.45]	13.13 [11.58, 14.69]	0.3013
Height (cm)	161.97 [161.14, 162.80]	160.90 [160.16, 161.65]	0.0643
Weight (kg)	67.94 [66.31, 69.58]	70.94 [69.04, 72.83]	0.0133
Total Hip BMD (g/cm ²) ⁴	0.73 [0.71, 0.75]	0.80 [0.78, 0.83]	<0.0001
<i>Dietary Variable</i>			
Energy intake (kcal/d) ⁵	1544 (717)	1517 (617)	0.5909
Carbohydrate intake (g/d)	193 (87)	195 (79)	0.7009
Protein intake (g/d)	63 (29)	63 (29)	0.9080
Fat intake (g/d)	57 (37)	54 (30)	0.1278
Saturated fat	20 (14)	18 (11)	0.0557
Monounsaturated fat	22 (14)	20 (11)	0.1582
Polyunsaturated fat ⁶	12 (8)	11 (6)	0.4410
n-3 fatty acids	1.34(0.83)	1.26 (0.71)	0.3026
ALA	1.22 (0.80)	1.14 (0.67)	0.1995
EPA + DHA	0.10 (0.09)	0.11 (0.11)	0.1603
n-6 fatty acids	10.15 (6.98)	9.77 (5.59)	0.4442
LA	10.15 (6.98)	9.77 (5.59)	0.4440
AA	0.08 (0.06)	0.08 (0.06)	0.6639
Calcium intake (mg/d) ⁷	1154 (698)	1206 (687)	0.3214
Vitamin D intake (mcg/d) ⁷	9.38 (9.32)	9.71 (7.06)	0.6201

¹ n=324 pairs unless otherwise noted

² p values are from paired t-tests for continuous variables and from McNemar's test for dichotomous variables

³ Mean [95% Confidence Intervals] all such

⁴Total n less than 324 pairs due to missing covariate data

⁵Mean (std dev) all such values

⁶(ALA) alpha-linolenic acid, (EPA) eicosapentaenoic acid, (DHA) docosahexaenoic acid, (LA) linoleic acid, (AA) arachidonic acid

⁷Diet plus supplement intake

Table 2

Means and Spearman's correlation coefficients between red blood cell fatty acids and dietary fatty acid intake of 648 women in WHI OS and CT

Fatty Acid ^I	FFQ FA Mean (g/d)	SD	RBC FA Mean (Area%)	SD	Spearman's rho	<i>p</i>
SFA	18.92	12.59	43.9109	1.91	0.01	<i>0.9404</i>
MUFA	20.86	12.81	15.0210	1.27	-0.05	<i>0.1692</i>
PUFA	11.34	7.08	41.0681	2.04	0.09	<i>0.0206</i>
Total n-3	1.30	0.77	6.5440	1.50	0.02	<i>0.5727</i>
ALA	1.18	0.74	0.2558	0.08	0.04	<i>0.3497</i>
EPA	0.04	0.04	0.5208	0.30	0.38	<i><0.0001</i>
DHA	0.07	0.07	3.6783	1.09	0.43	<i><0.0001</i>
EPA+DHA	0.10	0.10	4.1991	1.30	0.45	<i><0.0001</i>
Total n-6	9.96	6.32	34.5240	2.26	0.17	<i><0.0001</i>
LA	9.96	6.32	13.2959	2.14	0.09	<i>0.0201</i>
AA	0.08	0.06	15.6235	1.53	0.08	<i>0.0517</i>

^I(SFA) saturated fatty acids, (MUFA) monounsaturated fatty acids, (PUFA) polyunsaturated fatty acids, (ALA) alpha-linolenic acid, (EPA) (42) eicosapentaenoic acid, (DHA) docosahexaenoic acid, (LA) linoleic acid, (AA) arachidonic acid

Table 3Multivariate-adjusted hazard ratios for risk of hip fracture based on tertiles of fatty acids in red blood cells¹⁻⁴

RBC Fatty Acids	Tertile 1 [Range] ⁵	Tertile 2 [Range]	Tertile 3 [Range]	p for trend
Total SFA ⁶	[37.25 - 43.02]	[43.03 - 44.70]	[44.71 - 49.21]	
HR (95% CI)	1.00	0.71(0.40-1.26)	1.23(0.63-2.40)	0.6047
Total MUFA	[12.08 - 14.39]	[14.40 - 15.43]	[15.44 - 19.21]	
HR (95% CI)	1.00	0.73(0.39-1.38)	0.55(0.28-1.07)	0.0768
Total PUFA	[35.19 - 40.30]	[40.31 - 42.14]	[42.15 - 47.18]	
HR (95% CI)	1.00	1.04(0.56-1.92)	1.25(0.66-2.35)	0.7530
Total n-3 FA ⁷	[3.51 - 5.84]	[5.85 - 6.85]	[6.86 - 16.44]	
HR (95% CI)	1.00	0.85(0.47-1.55)	0.55(0.30-1.01)	0.0492
ALA	[0.08 - 0.21]	[0.22 - 0.27]	[0.28 - 0.93]	
HR (95% CI)	1.00	0.75(0.42-1.32)	0.44(0.23-0.85)*	0.0154
EPA	[0.04 - 0.37]	[0.38 - 0.53]	[0.54 - 3.85]	
HR (95% CI)	1.00	0.51(0.28-0.92)*	0.46(0.24-0.87)*	0.0181
DHA	[1.59 - 3.13]	[3.14 - 3.93]	[3.94 - 7.81]	
HR (95% CI)	1.00	0.65(0.36-1.18)	0.64(0.35-1.19)	0.1675
EPA+DHA (n-3 Index)	[1.82 - 3.53]	[3.54 - 4.44]	[4.45 - 11.67]	
HR (95% CI)	1.00	0.72(0.40-1.30)	0.65(0.36-1.18)	0.1598
Total n-6 FA ⁸	[24.39 - 33.63]	[33.64 - 35.58]	[35.59 - 40.78]	
HR (95% CI)	1.00	0.90(0.51-1.59)	1.32(0.72-2.42)	0.3820
LA	[8.05 - 12.27]	[12.28 - 13.92]	[13.93 - 23.31]	
HR (95% CI)	1.00	0.65(0.36-1.15)	0.77(0.40-1.49)	0.5140
AA	[10.66 - 15.03]	[15.04 - 16.37]	[16.38 - 20.38]	
HR (95% CI)	1.00	1.06(0.59-1.89)	1.47(0.78-2.77)	0.2466
n-6/n-3 FA ratio	[1.48 - 5.00]	[5.01 - 6.07]	[6.08 - 10.59]	
HR (95% CI)	1.00	1.28(0.71-2.30)	1.96(1.03-3.70)*	0.0399

¹ Hazard ratios (HR) are obtained from Cox Proportional Hazards models. *p*-values are from tests for linear trend.² n=255 pairs³ Pairs matched on age, race/ethnicity, randomization to hormone therapy or self-reported hormone use and geographical region by latitude.⁴ Adjusted for parental history of fracture, self-reported fracture after age 54 yr, height, weight, exercise, corticosteroid use, treated diabetes, current smoking.⁵ Tertile ranges based on % total fatty acids⁶ (SFA) saturated fatty acids, (MUFA) monounsaturated fatty acids, (PUFA) polyunsaturated fatty acids. Percent fatty acids from total of the following RBC fatty acids: arachidonic acid, alpha linolenic acid, docosatetraenoic acid, docosahexaenoic acid, docosapentaenoic acid n-3, eicosapentaenoic acid, eicosadienoic acid, eicosatrienoic acid, gamma linolenic acid, linoleic acid, myristic acid, oleic acid, palmitic acid, palmitoleic acid, stearic acid.

⁷Total n-3 fatty acids: alpha linolenic acid (ALA), docosahexaenoic acid (DHA), docosapentaenoic acid n-3, eicosapentaenoic acid (EPA), as percent fatty acids from total of the following RBC fatty acids: arachidonic acid, alpha linolenic acid, docosatetraenoic acid, docosahexaenoic acid, docosapentaenoic acid n-3, eicosapentaenoic acid, eicosadienoic acid, eicosatrienoic acid, gamma linolenic acid, linoleic acid, myristic acid, oleic acid, palmitic acid, palmitoleic acid, stearic acid.

⁸Total n-6 fatty acids: arachidonic acid (AA), docosatetraenoic acid, docsapentaenoic acid, eicosadienoic acid, eicosatrienoic acid, gamma linolenic acid, linoleic acid (LA).

*
 $p < 0.05$ relative to Tertile 1.