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Association between Global Biomarkers of Oxidative Stress and Hip Fracture in Postmenopausal Women: A Prospective Study

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Abstract

Human studies suggest that oxidative stress is a risk factor for osteoporosis, but its relationship with fracture risk is poorly understood. The purpose of the present study was to investigate the association between biomarkers of oxidative stress and hip fracture in postmenopausal women. We conducted a prospective study in the Nurses' Health Study among 996 women aged 60 years or older at baseline blood collection in 1989–1990. Plasma fluorescent oxidation products (FLOPs) were measured at three excitation/emission wavelengths (360/420 nm named as FLOP_360; 320/420 nm named as FLOP_320 and 400/475 nm named as FLOP_400). FLOPs are generated from many different pathways (lipid, protein and DNA) and reflect a global oxidation burden. FLOP assay is 10–100 times more sensitive than measurement of malondialdehyde. We used Cox proportional hazards regression model to investigate the association between baseline plasma FLOPs and risk of hip fracture, adjusting for multiple hip fracture risk factors such as age, history of osteoporosis, history of hypertension, prior fracture and smoking status. Forty four hip fractures (4.4%) were identified during the follow-up (Maximum = 23 years). In the multivariable model, the hazard ratios (HR) of hip fracture in the second and third tertiles of FLOP_320 were 2.11 (95% confidence interval [CI] = 0.88–5.10) and 2.67 (95% CI = 1.14–6.27), respectively, in comparison with the lowest tertile, and the risk increased linearly with increasing FLOP_320 (P for trend = 0.021). Neither FLOP_360 nor FLOP_400 was significantly associated with risk of hip fracture (Tertile 3 versus tertile 1: HR = 0.70, 95% CI = 0.32–1.54, P for trend = 0.386 for FLOP_360; and HR = 0.88, 95% CI = 0.40–1.96, P for trend = 0.900 for FLOP_400). In this prospective study,

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higher plasma FLOP_320 was an independent risk factor for hip fracture. Our results need further confirmation.

Keywords

Fluorescent oxidation products; oxidative stress; hip fracture; postmenopausal women

Introduction

Oxidative stress is generated as a result of insufficient activity of the endogenous antioxidant defense system against reactive oxygen species (ROSs). Excessive ROSs, such as superoxide anion, hydrogen peroxide and peroxynitrites are able to exert oxidative damage to lipids, DNA, carbohydrate and protein. This oxidative damage is involved in the development of many diseases, including coronary heart disease, cancer and neurodegenerative diseases (1–6).

Whether oxidative stress is related to fracture risk is poorly understood. Experimental studies in cell and animal models suggested that oxidative stress is an important factor in the regulation of bone remodeling (7–9). The results have been extended by cross-sectional and case-control studies, in which oxidative stress characterized by high level of F2-isoprostanes in urine and low level of antioxidant enzymes in blood is associated with reduced bone mineral density and increased risk of osteoporosis in the elderly (10,11). Thus, further investigation on the prospective relationship between oxidative stress and fragility fracture is of great importance to better understand bone health.

The level of oxidative stress can be assessed by the fluorescent oxidation products (FLOPs) as shown in our previous work (4,5,12). ROSs interact with proteins, phospholipids and nucleic acids to form FLOPs which produce coloring products with characteristic fluorescence spectra and can be measured at three excitation/emission wavelengths (360/420 nm named as FLOP_360; 320/420 nm named as FLOP_320 and 400/475 nm named as FLOP_400) (4–6,12). FLOPs measured at different wavelengths reflect different oxidation products (see method section). Our previous studies have demonstrated that these three types of FLOPs have differential associations with coronary heart disease and breast cancers (4–6), and therefore, it is necessary to measure three of them, especially when we do not know which type of FLOPs is the best predictor for hip fracture. As compared to malondialdehyde (MDA) which is a specific marker for lipid oxidation, FLOPs are generated from many different pathways (lipid, protein and DNA) and reflect a global oxidation burden. FLOP assay is 10–100 times more sensitive than measurement of MDA via colorimetric thiobarbituric acid assay (13). In large human epidemiologic studies, we found that FLOPs are associated with several oxidative indicators (i.e. smoking, hypertension and reduced renal function) (4,5,12,14). Further, most ROSs have a very short half-life in cells (15), but we have found that the levels of FLOP_360, FLOP_320, FLOP_400 are stable in blood samples with delayed processing up to 48 hours at 4°C, stable for more than 10 years in plasma samples in liquid-nitrogen freezers, and highly reproducible over 1–2 year among the same individuals (4,14,16). The well-established lipid oxidation markers (e.g. F2-

isoprostanes and MDA) are not stable in blood samples that are not processed over 24 hours (16). Thus, Measurement of these FLOPs in plasma is a non-invasive and convenient approach to assess circulating oxidative stress in large epidemiologic studies.

We therefore hypothesized that elevated oxidative stress increases fracture risk in humans. The present prospective study sought to test the hypothesis by investigating the association between plasma levels of FLOP_360, FLOP_320, FLOP_400 and risk of hip fracture in a prospective study of postmenopausal women in the Nurse's Health Study.

Materials and Methods

Study setting and participants

The Nurses' Health Study (NHS) recruited 121,700 women ages 30 to 55 years in 1976 who responded to a mailed questionnaire on which they provided a medical history, personal characteristics and information on lifestyle factors (17). Follow-up questionnaires have been mailed every two years. In the NHS, blood samples were collected from 32,826 women through overnight mail in 1989–1990. The data for the present prospective study were derived from a selected group of women with FLOP measurements ($n = 1766$) who were previously sampled to prospectively examine the risk of breast cancer and coronary heart disease (4,6). The inclusion and exclusion criteria for identification of cases and controls in breast cancer (6) and coronary heart disease studies (4) were described previously. Briefly, in the breast cancer study (6), nearly all breast cancer cases (99%) between 2000 and 2006 were identified, and one control was selected randomly after matching for age, time and month of blood draw, fasting status, menopausal status and postmenopausal hormone use at the time of case diagnosis. In the coronary heart disease study (4), all coronary heart disease cases between 1989 and 2004 were identified, and two controls were selected randomly after matching for age, smoking and fasting status at the time of each case diagnosis. We used all original data of FLOP measurements from both studies in which we did not excluded any participants. All participants were free of cardiovascular diseases and cancers at the time of blood draw. We excluded the women in younger age (< 60 years) at baseline ($n = 770$) because most osteoporotic hip fractures (75%) occurred in women > 60 years. Thus, 996 generally healthy postmenopausal women were included in our final analyses. This investigation was approved by the Institutional Review Board of Brigham, Women's hospital and the University of Cincinnati.

Demographic data collection

Using the questionnaire closest to blood draw (1989–1990), we ascertained age, body weight, life style data (e.g. alcohol intake, physical activity, calcium intake, vitamin D, carotenoid intake and tobacco smoking), history of osteoporosis, history of hypertension and use of postmenopausal hormone therapy (HT). Body height was collected in 1976 questionnaire. Physical activity expressed as metabolic equivalent hours (MET-hours) was estimated from multiple individual activities (i.e., sitting, standing and walking) collected via 1988 questionnaire (18). All the other covariates were collected via 1990 questionnaire. Current and past smokers were defined as current and past tobacco use, respectively. Total calcium and vitamin D were intakes were calculated from food plus supplements (19,20).

Total carotenoid intake was the sum of α -carotene, β -carotene, β -cryptoxanthin, lycopene, and lutein/zeaxanthin (6). History of physician-diagnosed osteoporosis and hypertension were reported by the participants.

Blood collection

Blood samples were collected in heparin anticoagulant tubes (4,21). Then the tubes were placed on ice packs, stored in Styrofoam containers and returned to central laboratory by overnight courier. The blood samples were centrifuged, and plasma, packed erythrocytes and buffy coats were divided into aliquots for storage in liquid-nitrogen freezers (-130°C or colder) within 36 hours. Time since last meal was collected by questionnaire during blood collection.

Measurement of FIOPs

Assay procedure—Measurement of FIOPs was performed with previously described procedures (14). Briefly, plasma was extracted with ethanol/ether (3:1, v/v) and centrifuged at 3,000 rpm for 10 min at 4°C , after which supernatant was moved to cuvettes for fluorescence measurement. Fluorescence was determined with a fluorescent spectrophotometer. The excitation/emission wavelengths were 360/420 nm for FIOP_360, 320/420 nm for FIOP_320 and 400/475 nm for FIOP_400. The level of fluorescence was expressed as relative fluorescent intensity units per milliliter of plasma. FIOP_360 represents oxidation products that are generated from oxidized phospholipids or from lipid oxidation products reacting with proteins, DNA and carbohydrates in presence of phospholipids. FIOP_320 is formed when oxidation products such as lipid hydroperoxides, aldehydes, and ketones react with DNA in the presence of metals. Finally, FIOP_400 reflects the interaction between MDA, proteins and phospholipids (22). Because coefficient of variations (CVs) of FIOP measurements across batch of some samples were relatively high (18 to 20%) (6), FIOPs were adjusted for batch-to-batch variation using the method described by Rosner et al.(23). After recalibration, the within-run average CVs for FIOP measurements were $< 13\%$.

Assay stability in blood samples with delayed processing—The delay in processing blood samples up to 36 hours had minimal influence on the measurement of FIOPs. The overall intraclass correlation coefficients (ICCs) of FIOPs were all greater than 0.95 in the shorter- (0 to 24 hours) and longer-delayed processing (0 to 36 hours) (16).

Assay between- and within- person reproducibility—We conducted a pilot study in 40 NHS participants who donated two blood samples to assess the ICC of the between- and within- person variations of the FIOPs at an average of 1.4 years (range: 0.8–2.2 years) interval. After adjusting for fasting status, the ICC for repeated measurements over 1.4 year apart was 0.44 for FIOP_360, 0.55 for FIOP_320 and 0.70 for FIOP_400 (4). ICCs for FIOP_360, FIOP_320 and FIOP_400 over 10 years ranged from 0.14 to 0.30 (6). After classifying FIOP_360, FIOP_320 and FIOP_400 into tertiles, we found that 15%–27% of the 40 study participants changed FIOP tertiles over 1.4 years follow-up, and 53–57% of the 729 study participants changed FIOP tertiles over 10 years follow-up.

Fracture ascertainment

The circumstances and date of hip fracture were ascertained via the biennial questionnaires between 1989 and 2012. We included only the hip fractures incident after blood draw that were due to low or moderate trauma (e.g. tripping, slipping, falling from the height of a chair). Incidence of hip fracture was defined if it occurred after the time of blood draw, and was not due to high trauma events such as motor vehicle accidents, skiing and horseback riding. The validity of self-report hip fracture has been conducted in a previous study, in which all reported hip fractures (n=30) were confirmed via medical records (24). Prior fracture (a covariate) was defined as fractures ascertained before blood draw.

Statistical analysis

Baseline characteristics of women with and without hip fracture were compared by means (standard deviation, SD) or medians (inter-quartile range, IQR) for continuous variable, and percentages for categorical variable. We further examined the distribution of baseline characteristics according to the tertiles of FLOP_320, FLOP_360 and FLOP_400 in overall and fasting samples (< 8 hours).

The main statistical model was Cox proportional hazards regression model, in which follow-up time was the time to hip fracture, death or last questionnaire follow-up from time of blood draw (median = 20.1 years; range = 0.1–22.8 years). We analyzed the association of FLOP_360, FLOP_320 and FLOP_400 separately with risk of hip fracture in the multivariate model. Covariates were defined if they are risk factors for fracture or may confound the association between oxidative stress and fracture. The covariates included age (continuous), history of hypertension (yes/no), body mass index (BMI; < 25, 25 and < 30, 30 and < 35, and ≥ 35 kg/m²), fasting status (< 8 and ≥ 8 hours), time and month of blood draw, alcohol intake (In quartiles: 0, > 0 and < 1, 1 and < 6.7, and ≥ 6.7 g/day), physical activity (In quartiles: < 5.2, 5.2 and < 12.7, 12.7 and < 25.9, and ≥ 25.9 MET-hours/week), total calcium intake (In quartiles: < 667, 667 and < 969, 969 and < 1347, and ≥ 1347 mg/day), total vitamin D intake (In quartiles: < 187, 187 and < 303, 303 and < 536, and ≥ 536 IU/day), history of osteoporosis (yes/no) and postmenopausal HT (yes/no). We tested for linear trend using the medians of FLOPs in each quartile as a continuous variable. We checked the proportional hazards assumption using Cox proportional hazards regression model and found that FLOP-hip fracture relationship did not interact with the time of follow-up. The association between FLOPs and hip fracture was also conducted in fasting samples (< 8 hours). When we found a significant association between FLOPs and hip fracture, we performed an interaction analysis between FLOPs and possible confounding factors (e.g. alcohol intake [< 1 and ≥ 1 g/day] and history of hypertension [yes/no]). To test the effect modification of total carotenoid intake, we analyzed the association between FLOPs (High versus low using median as cut-point) and hip fracture in the women with low and high total carotenoid intake (Using median as cut-point) separately. We derived data from two previous prospective studies which are nested case-control data of breast cancers (6) and coronary heart diseases (4). In the primary analysis, we did not exclude breast cancer and coronary heart disease cases in order to increase sample size and statistical power. To rule out the possibility that hip fracture incidence was due to breast cancers or coronary heart disease, we further analyzed the association between FLOPs and hip fracture in controls only

(free of coronary heart disease and breast cancer incidence). All analyses were performed with Statistical Analysis System (Version 9, SAS Institute Inc., Cary, NC).

Results

Baseline characteristics and plasma FLOPs of women with and without hip fracture

Forty four hip fractures (4.4%) were identified during the follow-up (Table 1). Women with hip fracture had higher proportion of hypertension than those without hip fracture. The median level of FLOP_320 was higher in women with hip fracture than in those without hip fracture. However, the median levels of FLOP_360 and FLOP_400 of women with and without hip fracture were not significantly different.

Baseline characteristics according to plasma FLOP levels

At baseline, higher levels of plasma FLOP_360, FLOP_320 and FLOP_400 were associated with greater proportion of current smokers (Table 2). Higher levels of plasma FLOP_360 and FLOP_320 were associated with lower proportion of women who had fasted 8 hours before blood draw. Higher levels of plasma FLOP_360 and FLOP_400 were associated with greater alcohol intake. Higher levels of FLOP_360 were correlated with lower BMI, greater proportion of history of hypertension. Higher levels of FLOP_320 were positively correlated with total vitamin D intake. Higher levels of FLOP_400 were associated with lower total calcium intake, total vitamin D intake and greater proportion of history of hypertension. Repeated analysis in fasting samples (< 8 hours) showed similar results as mentioned above.

Association between plasma FLOPs and hip fracture

In the multivariable model, the hazard ratios (HR) of hip fracture in the second and third tertiles of FLOP_320 were 2.11 (95% confidence interval [CI] = 0.88–5.10) and 2.67 (95% CI = 1.14–6.27), respectively, in comparison with the lowest tertile, and the risk increased linearly with increasing FLOP_320 (P for trend = 0.021) (Table 3). After stratifying our analysis by time of follow-up (<10 and ≥ 10 years), we found similar risk estimates of hip fracture in both time intervals as the overall association between FLOP_320 and hip fracture (data not shown). We did not find significant interaction of FLOP_320 with alcohol intake (P = 0.126) and history of hypertension (P = 0.854). When we analyzed the association between FLOP_320 and hip fracture in the women with low and high total carotenoid intake, the positive association between FLOP_320 (High versus low using median as cut-point) and hip fracture appeared to be stronger in women with low carotenoid intake (HR = 2.41, 95% CI = 0.98–5.92) than in those with high total carotenoid intake (HR = 1.81, 95% CI = 0.76–4.30). Neither FLOP_360 nor FLOP_400 was significantly associated with risk of hip fracture (Tertile 3 versus tertile 1: HR = 0.70, 95% CI = 0.32–1.54, P for trend = 0.386 for FLOP_360; and HR = 0.88, 95% CI = 0.40–1.96, P for trend = 0.900 for FLOP_400; Table 3).

When we analyzed the relationship between plasma FLOPs and hip fractures in fasting samples only (< 8 hours; n = 597 subjects; n = 31 hip fractures), higher levels of FLOP_320 were associated with increased risk of hip fracture (Tertile 3 versus tertile 1: HR = 3.16, 95% CI = 1.18–8.46, P for trend = 0.018). Neither FLOP_360 nor FLOP_400 was associated

with risk of hip fracture in the fasting samples (Tertile 3 versus tertile 1: HR = 0.90, 95% CI = 0.32–2.54, *P* for trend = 0.849 for FLOP_360 and HR = 1.47, 95% CI = 0.57–3.77, *P* for trend = 0.523 for FLOP_400).

When we analyzed the relationship between plasma FLOPs and hip fractures in controls only (free of coronary heart disease and breast cancer incidence; *n* = 628 subjects; *n* = 31 hip fractures), FLOP_320 remained significantly associated with increased risk of hip fracture (Tertile 3 versus tertile 1: HR = 6.94, 95% CI = 1.88–25.54, *P* for trend = 0.005). Again, FLOP_360 and FLOP_400 were not associated with risk of hip fracture (Tertile 3 versus tertile 1: HR = 0.85, 95% CI = 0.32–2.28, *P* for trend = 0.566 for FLOP_360 and HR = 0.99, 95% CI = 0.36–2.77, *P* for trend = 0.548 for FLOP_400).

Discussion

To our knowledge, this is the first prospective study among postmenopausal women demonstrating that oxidative stress, quantified by FLOP_320, was a significant predictor for hip fracture. Further research is required to determine if FLOP_320 would be useful for prediction of hip fracture in a clinical setting.

Unlike FLOP_320, we did not find FLOP_360 or FLOP_400 to be independent risk factors for hip fracture. This is possibly due to the fact that FLOP_320 reflects oxidation products which are generated in presence of metals, whereas FLOP_360 and FLOP_400 can be generated without metals. With increased bone loss, heavy metals in the blood are commonly released from bones, which will promote the production of FLOP_320 in blood. Indeed, excessive ROSs increase bone loss by inhibiting bone formation and promoting bone resorption (7–9,25), and an increased level of heavy metals has been found to be associated with increased risk of osteoporosis and fractures (26–28). Thus, FLOP_320 is superior to other types of FLOPs in relation to bone loss and fracture as FLOP_320 reflects the coexisting effects of ROSs and heavy metals.

The present study has several strengths. This study has a long follow-up period. In addition, the prospective study design reduces the possibility of the influence from existing hip fracture.

A limitation of this study is that a single measurement of FLOPs may not accurately reflect the average levels of the biomarker over a prolonged period of time, especially after 10 years. However, we have assessed their reproducibility over approximately one-year period, and high ICCs suggest that this marker can be used as a marker for chronic exposure. Even though the ICCs of FLOP measurements across 10 years were relatively low, we still found significant association between plasma FLOP_320 at baseline and hip fracture. Our results suggest that past high FLOP_320 exposure may play significant role in the development of hip fracture occurs long after baseline exposure, even though FLOP_320 may not be consistently high in long term. Future studies with multiple and repeated measurements of FLOPs over several years interval are warranted to confirm our results. The number of hip fractures in women was relatively low, and the positive relationship between FLOP_320 and hip fracture was observed from multiple statistical tests and adjustments, which may cause

type I error. The study is also limited because the data on bone markers, bone mineral density and other fractures were not available.

Hip fracture is regarded as the most severe manifestation of osteoporotic fracture, because it is associated with substantial cost, as well as higher risk of disability, comorbidities and mortality than any other fractures (29–33). Current fracture risk assessment is far from optimal by using traditional risk factors such as age and osteoporosis (34). If our findings are confirmed in other studies, adding this marker into existing fracture assessment model may improve the prediction of hip fracture in postmenopausal women.

In summary, we found an independent and positive relationship between FLOP_320 and risk of hip fracture among postmenopausal women. FLOP_360 and FLOP_400 were not the predictors for risk of hip fracture. Since this is the first prospective study examining the relationship of oxidative stress with fracture risk, our findings warrant further investigation and validation. In addition, the underlying mechanisms for FLOP_320 as a risk factor for hip fracture merit additional studies.

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Table 1

Baseline characteristics and fluorescent oxidation products of postmenopausal women with and without hip fracture (N=996)

	Hip fracture (N=44)	Non-hip fracture (N=952)	P value
Age (years)	64.6 (2.6)	64.4 (2.5)	0.604
Body mass index (kg/m ²)	25.3 (4.4)	25.7 (4.6)	0.578
Alcohol intake (g/day) *	1.7 (0, 5.4)	1.0 (0, 6.7)	0.746
Physical activity (MET-hours/week) *	12.7 (5.4, 21.5)	10.4 (4.0, 22.2)	0.533
Total calcium intake (mg/day) *	874 (657, 1400)	976 (668, 1343)	0.333
Total vitamin D intake (IU/day) *	471 (207, 641)	299 (186, 533)	0.197
Total carotenoid intake (1000 IU/day) *	10.0 (6.5, 12.8)	9.0 (6.2, 12.8)	0.518
Fasting status (< 8 hours; n, %)	32 (73%)	718 (75%)	0.616
History of osteoporosis (n, %)	3 (6.8%)	49 (5.2%)	0.626
History of hypertension (n, %)	17 (39%)	239 (25%)	0.045
Smokers (n, %)			
Current smokers	6 (14%)	167 (18%)	0.504
Past smokers	16 (36%)	379 (40%)	0.648
Hormone therapy (n, %) [£]	16 (36%)	360 (38%)	0.846
Prior fracture (n, %)	1 (2.3%)	33 (3.5%)	0.670
Oxidative stress biomarkers (FI/ml)			
FIOP_360 *	206 (187, 271)	216 (177, 286)	0.123
FIOP_320 *	491 (351, 1307)	402 (300, 664)	0.031
FIOP_400 *	56.8 (48.7, 79.3)	61.1 (49.4, 81.1)	0.355

Variables with normal distribution are shown in mean (standard deviation), unless otherwise specified.

* Variables with skew distribution are shown in median (inter-quartile range).

Abbreviations: FIOP = Fluorescent oxidation products, FI = Fluorescent intensity units, METs = Metabolic equivalents.

[£] Postmenopausal hormone therapy.

Table 2

Baseline characteristics according to the tertiles of fluorescent oxidation products

	Tertile 1	Tertile 2	Tertile 3	P for trend
FIOP_360				
Range (FI/ml)	< 192	192; < 255	255	---
N	332	331	333	---
Age (years)	64.3 (2.5)	64.3 (2.4)	64.6 (2.5)	0.189
Body mass index (kg/m ²)	26.6 (4.9)	25.4 (4.4)	25.0 (4.5)	< 0.001
Alcohol intake (g/day)*	0 (0, 3.5)	1.5 (0, 8.3)	1.5 (0, 8.3)	< 0.001
Physical activity (MET-hours/week)*	10.2 (3.9, 20.2)	10.2 (4.0, 23.5)	11.8 (4.0, 24.6)	0.054
Total calcium intake (mg/day)*	975 (675, 1327)	963 (679, 1338)	997 (650, 1411)	0.207
Total vitamin D intake (IU/day)*	286 (184, 514)	303 (197, 549)	324 (170, 540)	0.203
Total carotenoid intake (1000 IU/day)*	8.5 (6.1, 12.1)	9.5 (6.6, 13.4)	9.0 (6.1, 12.8)	0.632
Fasting status (8 hours; n, %)	265 (80%)	258 (78%)	227 (68%)	0.020
History of osteoporosis (n, %)	18 (5.4%)	17 (5.1%)	17 (5.1%)	0.874
History of hypertension (n, %)	77 (23%)	72 (22%)	107 (32%)	0.002
Current smokers (n, %)	24 (7%)	54 (16%)	95 (29%)	< 0.001
Past smokers (n, %)	120 (36%)	146 (44%)	129 (39%)	0.840
Hormone therapy (n, %) [‡]	112 (34%)	130 (39%)	134 (40%)	0.128
Prior fracture (n, %)	8 (2.4%)	15 (4.5%)	11 (3.3%)	0.761
FIOP_320				
Range (FI/ml)	< 335	335; < 546	546	---
N	331	331	334	---
Age (years)	64.6 (2.5)	64.1 (2.5)	64.5 (2.5)	0.006
Body mass index (kg/m ²)	25.9 (4.4)	25.6 (4.7)	25.4 (4.7)	0.396
Alcohol intake (g/day)*	0.9 (0, 5.5)	0.9 (0, 6.7)	1.1 (0, 7.6)	0.208
Physical activity (MET-hours/week)*	10.3 (5.2, 20.7)	11.6 (3.5, 24.0)	10.4 (3.9, 22.4)	0.164
Total calcium intake (mg/day)*	951 (679, 1314)	930 (664, 1317)	1021 (669, 1415)	0.983
Total vitamin D intake (IU/day)*	271 (179, 475)	302 (192, 533)	348 (193, 590)	0.034

	Tertile 1	Tertile 2	Tertile 3	P for trend
Total carotenoid intake (1000 IU/day)*	8.5 (6.1, 12.4)	9.3 (6.6, 13.2)	9.2 (6.5, 12.8)	0.626
Fasting status (8 hours; n, %)	284 (86%)	242 (73%)	224 (67%)	0.002
History of osteoporosis (n, %)	14 (4.2%)	18 (5.4%)	20 (6.0%)	0.469
History of hypertension (n, %)	72 (22%)	78 (24%)	106 (32%)	0.578
Current smokers (n, %)	28 (8%)	68 (21%)	77 (23%)	< 0.001
Past smokers (n, %)	139 (42%)	133 (40%)	123 (37%)	0.636
Hormone therapy (n, %) [£]	123 (37%)	125 (38%)	128 (38%)	0.873
Prior fracture (n, %)	8 (2.4%)	9 (2.7%)	17 (5.1%)	0.806
FIOP_400				
Range (FI/ml)	< 52.9	52.9; < 72.9	72.9	---
N	333	332	331	---
Age (years)	64.5 (2.5)	64.2 (2.5)	64.4 (2.5)	0.620
Body mass index (kg/m ²)	26.2 (5.0)	25.6 (4.4)	25.2 (4.5)	0.062
Alcohol intake (g/day)*	0.9 (0, 3.5)	1.1 (0, 6.7)	1.5 (0, 11.0)	< 0.001
Physical activity (MET-hours/week)*	10.7 (4.0, 20.4)	10.2 (3.9, 21.6)	11.7 (4.0, 25.0)	0.263
Total calcium intake (mg/day)*	1045 (691, 1353)	954 (665, 1357)	924 (641, 1313)	0.046
Total vitamin D intake (IU/day)*	313 (203, 535)	303 (182, 548)	293 (181, 529)	0.015
Total carotenoid intake (1000 IU/day)*	9.5 (6.6, 14.1)	9.0 (6.1, 12.8)	8.5 (6.1, 11.9)	0.842
Fasting status (8 hours; n, %)	275 (83%)	238 (72%)	237 (72%)	0.155
History osteoporosis (n, %)	16 (4.8%)	16 (4.8%)	20 (6.0%)	0.152
History of hypertension (n, %)	78 (23%)	73 (22%)	105 (32%)	0.001
Current smokers (n, %)	10 (3%)	44 (13%)	119 (36%)	< 0.001
Past smokers (n, %)	129 (39%)	146 (44%)	120 (36%)	0.210
Hormone therapy (n, %) [£]	134 (40%)	120 (36%)	122 (37%)	0.739
Prior fracture (n, %)	16 (4.8%)	11 (3.3%)	7 (2.1%)	0.980

Abbreviations: FIOP = Fluorescent oxidation products, FI = Fluorescent intensity units, MET's = Metabolic equivalent hours.

[£]Postmenopausal hormone therapy.

Variables with normal distribution are shown in mean (standard deviation), unless otherwise specified.

* Variables with skew distribution are shown in median (inter-quartile range).

Table 3

Association between plasma fluorescent oxidation products and risk of hip fracture

	Tertile 1	Tertile 2	Tertile 3	P for trend
FIOP_360				
Range (FI/ml)	< 192	192; < 255	255	---
Median (FI/ml)	163	215	338	---
Incidence of hip fracture (n, %)	15 (4.5%)	17 (5.1%)	12 (3.6%)	---
Adjusted for age	1 (ref)	1.13 (0.56, 2.26)	0.85 (0.40, 1.81)	0.638
Adjusted for age and hypertension	1 (ref)	1.16 (0.58, 2.32)	0.79 (0.37, 1.69)	0.540
Adjusted for multiple risk factors*	1 (ref)	1.03 (0.50, 2.14)	0.70 (0.32, 1.54)	0.386
FIOP_320				
Range (FI/ml)	< 335	335; < 546	546	---
Median (FI/ml)	270	404	1337	---
Incidence of hip fracture (n, %)	9 (2.7%)	15 (4.5%)	20 (6.0%)	---
Adjusted for age	1 (ref)	1.81 (0.79, 4.14)	2.51 (1.14, 5.52)	0.012
Adjusted for age and hypertension	1 (ref)	1.78 (0.78, 4.06)	2.35 (1.07, 5.18)	0.015
Adjusted for multiple risk factors*	1 (ref)	2.11 (0.88, 5.10)	2.67 (1.14, 6.27)	0.021
FIOP_400				
Range (FI/ml)	< 52.9	52.9; < 72.9	72.9	---
Median (FI/ml)	45.6	61.0	93.4	---
Incidence of hip fracture (n, %)	15 (4.5%)	17 (5.1%)	12 (3.6%)	---
Adjusted for age	1 (ref)	1.12 (0.56, 2.25)	0.89 (0.42, 1.91)	0.973
Adjusted for age and hypertension	1 (ref)	1.15 (0.57, 2.30)	0.84 (0.39, 1.81)	0.875
Adjusted for multiple risk factors*	1 (ref)	1.21 (0.58, 2.52)	0.88 (0.40, 1.96)	0.900

Values are hazard ratios (95% confidence interval), unless otherwise specified. FIOP = Fluorescent oxidation products, FI = Fluorescent intensity units.

* Risk factors included age (continuous), history of hypertension (yes/no), body mass index (< 25, 25 and < 30, 30 and < 35, and 35 kg/m²), fasting status (< 8 and 8 hours), time and month of blood draw, alcohol intake (In quartiles: 0, > 0 and < 1, 1 and < 6.7, and 6.7 g/day), physical activity (In quartiles: < 5.2, 5.2 and < 12.7, 12.7 and < 25.9, and 25.9 MET-hours/week), total calcium intake (In quartiles: < 667, 667 and < 969, 969 and < 1347, and 1347 mg/day), total vitamin D intake (In quartiles: < 187, 187 and < 303, 303 and < 536, and 536 IU/day), history of osteoporosis (yes/no) and postmenopausal hormone therapy (yes/no).