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Increased formation of 8-iso-prostaglandin $F_{2\alpha}$ is associated with altered bone metabolism and lower bone mass in hypercholesterolaemic subjects

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Abstract. Mangiafico RA, Malaponte G, Pennisi P, Li Volti G, Trovato G, Mangiafico M, Bevelacqua Y, Mazza F, Fiore CE (University of Catania, Catania; and University of Brescia, Brescia; Italy) Increased formation of 8-iso-prostaglandin $F_{2\alpha}$ is associated with altered bone metabolism and lower bone mass in hypercholesterolaemic subjects. *J Intern Med* 2007; **261**: 587–596.

Objectives. To investigate the relationship of 8-iso-prostaglandin (PG) $F_{2\alpha}$ levels, a reliable marker of *in vivo* oxidative stress and lipid peroxidation, with bone mineral density (BMD), bone turnover markers, osteoprotegerin (OPG) and receptor activator of nuclear factor-kappa B ligand (RANKL) in hypercholesterolaemia.

Design. Cross-sectional study

Setting. University hospital centre

Methods. Serum 8-iso-$PGF_{2\alpha}$ levels were measured in 173 hypercholesterolaemic subjects and in 152 age- and sex-matched normocholesterolaemic controls. Femoral neck and lumbar spine BMD, serum bone-specific alkaline phosphatase (BAP), osteocalcin (OC), OPG and RANKL levels, as well as urinary levels of C-terminal telopeptides of type I collagen (CTX-I), were also assessed.

Results. Hypercholesterolaemic subjects showed higher ($P < 0.0001$) serum 8-iso-$PGF_{2\alpha}$ levels than controls. They also had decreased ($P < 0.0001$) femoral neck and lumbar spine BMD, and lower ($P < 0.0001$) serum BAP and OC levels. No significant differences between hypercholesterolaemic and control subjects were found when comparing urinary CTX-I levels, or serum OPG and RANKL levels. In multivariate linear regression analysis, serum 8-iso-$PGF_{2\alpha}$ was the only negative predictor for femoral neck BMD and serum BAP and OC levels in hypercholesterolaemic subjects. No significant correlation (all $P > 0.25$) was present between serum 8-iso-$PGF_{2\alpha}$ levels and urinary CTX-I levels, or serum OPG and RANKL levels, in hypercholesterolaemic subjects.

Conclusions. We found an association between increased serum 8-iso-$PGF_{2\alpha}$ levels and lower bone mass and reduced serum BAP and OC concentrations in hypercholesterolaemic subjects. These results would suggest a possible role for oxidative stress in the development of lower bone mass in hypercholesterolaemia.

Keywords: bone density, hypercholesterolaemia, lipids, osteoporosis, oxidative stress.

Introduction

Increasing evidence suggests an age-independent association between osteoporosis and atherosclerosis [1–6], but the underlying mechanisms remain elusive. Recently, several lines of research have converged to indicate a possible link between hypercholesterolaemia, a major risk factor for atherosclerosis [7] and

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osteoporosis. Hypercholesterolaemia has been reported to be associated with low bone mass in a number of studies focused on postmenopausal or osteoporotic women [8–12]. The relationship between bone mass and lipid values has been shown to be negative for low-density lipoprotein (LDL) cholesterol [8, 12] and positive for high-density lipoprotein (HDL) cholesterol [8]. However, other studies found an opposite relationship between lipid profile and bone mineral density (BMD) [13], or no relationship at all [14].

Data from various observational studies [15–17], albeit not consistent [18, 19], suggested that the use of the lipid-lowering drugs statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) is associated with a reduced risk of fractures and increased bone mass. However, such association was not found in randomized controlled clinical trials of statins for cardiovascular disease [20, 21].

In vitro and animal studies showed that diet-induced hyperlipidaemia reduced bone density [22] by inhibiting osteoblastic [23, 24] and promoting osteoclastic differentiation [25, 26] via increased formation of lipid oxidation products. F_2-isoprostanes are stable prostaglandin (PG) F_2-like end products of lipid peroxidation and are formed *in vivo* from the nonenzymatic free radical-induced peroxidation of arachidonic acid, a ubiquitous polyunsaturated fatty acid [27]. F_2-isoprostanes, including 8-iso-$PGF_{2\alpha}$, are widely regarded as the best available biomarkers of *in vivo* oxidative stress and lipid peroxidation [28]. Other measures of oxidative stress, including malondialdehyde and lipid hydroperoxides, have been demonstrated to be of limited value *in vivo* because they lack sensitivity and/or specificity [28].

It has been shown that enhanced formation of 8-iso-$PGF_{2\alpha}$ occurs in hypercholesterolaemic subjects [29, 30]. Moreover, urinary levels of 8-iso-$PGF_{2\alpha}$ have been reported to be negatively correlated with BMD at various sites in a sample from the general population [31]. However, no information is available from clinical studies on the possible relationship between F_2-isoprostanes and bone mass in male and female subjects with hypercholesterolaemia. It is also unknown whether F_2-isoprostanes are related to bone turnover markers and osteoclast regulatory factors osteoprotegerin (OPG) and receptor activator of nuclear factor-kappa B ligand (RANKL) in hypercholesterolaemia. Therefore, we assessed serum 8-iso-$PGF_{2\alpha}$ levels in relation to BMD in a group of untreated hypercholesterolaemic subjects. We also investigated the relationship of serum 8-iso-$PGF_{2\alpha}$ levels to two bone formation markers, serum bone-specific alkaline phosphatase (BAP) and osteocalcin (OC), one bone resorption marker, urinary C-terminal telopeptides of type I collagen, and serum levels of OPG and RANKL.

Materials and methods

Study population

A total of 173 ambulatory, well-functioning subjects with primary hypercholesterolaemia (aged 32–71 years; 83 males and 90 females) were consecutively recruited from the Lipid Clinic at our hospital between January and October 2005. Hypercholesterolaemia was defined as LDL cholesterol level >4.13 mmol L^{-1}. Exclusion criteria were secondary hyperlipidaemia owing to diabetes mellitus, renal, liver or thyroid diseases, and alcohol abuse. A total of 152 age- and sex-matched clinically healthy normocholesterolaemic subjects (aged 31–72 years; 75 males and 77 females) were selected from individuals receiving a medical check-up at our hospital and served as controls. Hypercholesterolaemic subjects were included in the study if they were not taking lipid-lowering drugs and were not on lipid-lowering diets. Patients with known hypertension or with cardiovascular disease (as assessed by clinical history, physical examination, and ECG) were excluded from the study. Patients who were taking drugs with established effect on bone turnover or were suffering from clinical disorders related to bone metabolism were also not enrolled in the study. None of the participants was a current smoker, and none was receiving vitamin or calcium supplements. All subjects completed a questionnaire that included age at menarche, postmenopausal status, lifestyle factors, medical history, and medication use. Physical activity was measured by the

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Paffenbarger Physical Activity Index [32]. The average daily calcium intake was determined by a quantitative food frequency questionnaire [33]. All participants underwent clinical examination, ECG, and measurements of BMD and biochemical parameters. The study protocol was approved by our institutional ethics committee in accordance to the Helsinki Declaration, and all study participants gave informed consent.

Measurement of bone mineral density

Areal BMD (g cm^{-2}; bone mineral content relative to projection area) was measured by dual-energy X-ray absorptiometry (Lunar DPXL, Lunar, Madison, WI, USA) at the lumbar spine (L_2–L_4) and the femoral neck. At these measurement sites, the precision of the method (coefficient of variation, CV) at our laboratory was 0.7% for the lumbar spine and 0.5% for the femoral neck.

Laboratory measurements

Venous blood samples and urine specimens were obtained after a overnight fast, between 9.00 and 9.30 a.m. Serum and urine aliquots were stored at −80 °C until analysis. The following measurements were carried out:

Total serum 8-iso-$PGF_{2\alpha}$ levels were measured by using a commercially available enzyme immunoassay (EIA) kit (Cayman Chemicals, Ann Arbor, MI, USA) according to the manufacturer's instructions. The detection limit of this assay was 5 pg mL^{-1}. The intra-assay and interassay CVs were <10%.

BAP was measured by an enzyme-linked immunoabsorbent assay (ELISA) kit (Tandem-MP Ostase, Beckman Coulter, Fullerton, CA, USA). The sensitivity of the method was 0.7 μg L^{-1}. Within-run CV was 6.5% for low values, and 4.5% for high values.

OC was measured with a commercially available ELISA kit (N-MID Osteocalcin One Step) provided by Nordic Bioscience Diagnostics A/S, Herlev, Denmark. The sensitivity of the method was 0.5 ng mL^{-1}. Intra-assay CV was 3.4% for low values and 2.4% for high values.

Urinary CTX-I levels were measured by ELISA using reagents provided by Osteometer Bio Tech A/S (CrossLaps). The detection limit of this assay was 50 μg L^{-1}. Intra-assay and interassay CVs were, respectively, 5.7% and 9.4% for low values and 5.4% and 8.6% for very high values.

Serum concentration of OPG was determined by a sandwich ELISA (Osteoprotegerin, Immun Diagnostik, Bensheim, Germany). The detection limit of this assay system was 0.14 pmol L^{-1}. Intra-assay and interassay CVs were <10% at both low and high concentrations of OPG.

Serum levels of soluble RANKL were measured by EIA (sRANKL, Biomedica, Vienna, Austria). The detection limit of this assay system was 0.08 pmol L^{-1}. Intra-assay and interassay CVs were 5% and 9%, respectively.

Serum total cholesterol, HDL cholesterol and triglycerides were measured enzymatically using the Cobas Integra Roche analyser (Roche Diagnostics, Milan, Italy). LDL cholesterol was calculated using the Friedewald formula [34]. Serum calcium, phosphorus, and albumin were determined by colorimetric assays on the Cobas Integra Roche analyser (Roche Diagnostics). Serum calcium was adjusted for albumin concentration.

Statistical analysis

Continuous data are reported as mean ± standard deviation (SD) and categorical data as percentages. Males and females were analysed separately. Comparisons between groups were made by means of unpaired *t*-test or Mann–Whitney test (in the case of non-normal distribution) for continuous variables and by Fisher's Exact Test for categorical variables. Univariate relationships between 8-iso-$PGF_{2\alpha}$ levels and outcome variables amongst hypercholesterolaemic subjects were tested by Spearman's correlation coefficient. Outcome variables included femoral neck and

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lumbar spine BMD, serum levels of BAP, OC, OPG and RANKL, and urinary CTX-I levels. Multivariate linear regression analyses were performed to look for independent associations between 8-iso-PGF$_{2\alpha}$ levels (independent variable) and the outcome variables (dependent variables) that were found to have a statistical association ($P < 0.05$) with 8-iso-PGF$_{2\alpha}$ levels in the univariate analysis. In the models of multivariate analysis the following variables were also included as independent variables: age, body mass index (BMI), calcium intake, physical activity index, LDL cholesterol, HDL cholesterol, triglycerides, and for females, age at menarche and menopausal status. As total cholesterol and LDL cholesterol were highly correlated in both male and female hypercholesterolaemic subjects ($r = 0.90$, $P < 0.0001$), only LDL cholesterol was included in the models of multivariate analysis. We also tested the relationships between lipid parameters (total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides) and each of the outcome variables, as well as those between lipid parameters and 8-iso-PGF$_{2\alpha}$ levels. Univariate and multivariate analyses were also carried out in the whole hypercholesterolaemic population. In addition, correlation analyses were used to investigate the relationships between 8-iso-PGF$_{2\alpha}$ levels and BMD and the other outcome variables in the controls, both in the male and female subgroups and in the whole group. In multivariate analyses for the study groups as a whole, we also corrected for sex. In all analyses, a two-tailed P value <0.05 was considered significant. Statistical analysis was performed using GRAPHPAD INSTAT version 3.00 for Windows software (GraphPad Software, San Diego, CA, USA).

Results

The characteristics of the study population are shown in Table 1. Mean age, BMI, systolic and diastolic blood pressure values, daily calcium intake, estimated physical activity levels, as well as serum calcium and phosphorus were not significantly different comparing all the groups. By definition, serum total and LDL cholesterol levels were significantly ($P < 0.001$) higher in the hypercholesterolaemia group than in the control group, whereas HDL cholesterol and triglyceride concentrations were similar between all groups. There were no sex differences.

Serum 8-iso-PGF$_{2\alpha}$ levels were significantly higher in the hypercholesterolaemic subjects than in the respective controls, with no differences between sexes (178.0 ± 126.0 and 28.5 ± 12.2 pg mL^{-1} in male hypercholesterolaemic and control subjects, respectively, $P < 0.0001$; 181.5 ± 122.0 and 30.0 ± 13.0 pg mL^{-1} in female hypercholesterolaemic and control subjects, respectively, $P < 0.0001$). Mean BMD at the femoral neck (0.78 ± 0.14 and 1.00 ± 0.04 g cm^{-2} in male hypercholesterolaemic and control subjects, respectively, $P < 0.0001$; 0.77 ± 0.14 and 0.99 ± 0.07 g cm^{-2} in female hypercholesterolaemic and control subjects, respectively, $P < 0.0001$) and lumbar spine (1.02 ± 0.13 and 1.16 ± 0.02 g cm^{-2} in male hypercholesterolaemic and control subjects, respectively, $P < 0.0001$; 1.05 ± 0.17 and 1.16 ± 0.04 g cm^{-2} in female hypercholesterolaemic and control subjects, respectively, $P < 0.0001$) were similarly reduced in both male and female subjects with hypercholesterolaemia when compared with the respective controls (Table 1).

There were no significant differences in serum levels of OPG and RANKL, and in urinary levels of CTX-I between all groups (Table 2). Serum BAP and OC levels were similarly lower ($P < 0.0001$) in male (8.2 ± 2.0 μg L^{-1} and 19.9 ± 3.2 ng mL^{-1}, respectively) and female (8.0 ± 1.8 μg L^{-1} and 19.8 ± 5.3 ng mL^{-1}, respectively) subjects with hypercholesterolaemia when compared with male (13.4 ± 1.6 μg L^{-1} and 27.0 ± 3.7 ng mL^{-1}, respectively) and female (13.6 ± 0.7 μg L^{-1} and 28 ± 3.1 ng mL^{-1}, respectively) controls (Table 2).

In univariate analysis for male and female hypercholesterolaemic subjects, serum 8-iso-PGF$_{2\alpha}$ levels were significantly negatively correlated with femoral neck BMD in both males ($r = -0.27$, $P = 0.01$) and females ($r = -0.29$, $P = 0.004$). A significant negative correlation between serum iso-PGF$_{2\alpha}$ levels and serum BAP ($r = -0.35$, $P = 0.001$, in males; $r = -0.32$, $P = 0.002$, in females) and OC ($r = -0.33$, $P = 0.001$, in males; $r = -0.34$,

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Table 1 Characteristics of hypercholesterolaemic and control subjects

	Hypercholesterolaemic subjects ($n = 173$)		Controls ($n = 152$)	
Characteristic	Male	Female	Male	Female
Number (%)	83 (47.9)	90 (52.0)	75 (49.3)	77 (50.6)
Age (years)	52.2 ± 10.0	52.3 ± 12.0	53.4 ± 10.7	52.7 ± 11.5
Body mass index (kg m^{-2})	26.6 ± 2.2	26.5 ± 2.3	26.1 ± 1.2	26.6 ± 1.6
Age at menarche (years)	–	13.1 ± 1.0	–	12.9 ± 0.8
Postmenopausal status, n (%)	–	43 (47.7)	–	36 (46.7)
Systolic blood pressure (mmHg)	122.6 ± 4.7	121 ± 6.5	121.8 ± 6.0	122 ± 6.6
Diastolic blood pressure (mmHg)	72.8 ± 4.4	73.6 ± 5.0	74.0 ± 3.6	74.5 ± 3.1
Daily calcium intake (mg)	852.2 ± 194.8	857.7 ± 239.2	834.9 ± 291.9	831.4 ± 288.8
Physical activity index (kcal week^{-1})	825.9 ± 142.1	821.1 ± 144.5	846.9 ± 208.5	836.1 ± 166.9
Total cholesterol (mmol L^{-1})	7.22 ± 0.71*	7.20 ± 0.74*	4.93 ± 0.24	5.01 ± 0.28
LDL cholesterol (mmol L^{-1})	5.36 ± 0.58*	5.34 ± 0.61*	3.07 ± 0.18	3.12 ± 0.19
HDL cholesterol (mmol L^{-1})	1.31 ± 0.23	1.29 ± 0.25	1.32 ± 0.14	1.35.3 ± 0.17
Triglycerides (mmol L^{-1})	1.14 ± 0.01	1.26 ± 0.55	1.15 ± 0.16	1.15 ± 0.35
Calcium (mmol L^{-1})	2.32 ± 0.07	2.34 ± 0.08	2.31 ± 0.02	2.33 ± 0.06
Phosphorus (mmol L^{-1})	1.07 ± 0.08	1.08 ± 0.09	1.08 ± 0.02	1.07 ± 0.07
8-iso-PGF$_{2\alpha}$ (pg mL^{-1})	178.0 ± 126.0*	181.5 ± 122.0*	28.5 ± 12.2	30.0 ± 13.0
Femoral neck BMD (g cm^{-2})	0.78 ± 0.14*	0.77 ± 0.14*	1.00 ± 0.04	0.99 ± 0.07
Lumbar spine BMD (g cm^{-2})	1.02 ± 0.13*	1.05 ± 0.17*	1.16 ± 0.02	1.16 ± 0.04

Values are reported as mean ± standard deviation unless otherwise indicated. LDL, low-density lipoprotein; HDL, high-density lipoprotein; PGF$_{2\alpha}$, prostaglandin F$_{2\alpha}$; BMD, bone mineral density. *$P < 0.0001$ vs. controls.

Table 2 Serum levels of OPG, RANKL, BAP, OC and urinary CTX-I levels in hypercholesterolaemic ($n = 173$) and control ($n = 152$) subjects

	Hypercholesterolaemic subjects		Controls	
Variable	Male	Female	Male	Female
OPG (pmol L^{-1})	3.6 ± 0.9	3.7 ± 1.1	3.7 ± 1.0	3.6 ± 0.9
RANKL (pmol L^{-1})	0.6 ± 0.2	0.5 ± 0.3	0.5 ± 0.2	0.5 ± 0.2
BAP (μg L^{-1})	8.2 ± 2.0*	8.0 ± 1.8*	13.4 ± 1.6	13.6 ± 0.7
OC (ng mL^{-1})	19.9 ± 3.2*	19.8 ± 5.3*	27.0 ± 3.7	28 ± 3.1
Urinary CTX-I (μg mmol^{-1} creatinine)	259.8 ± 31.6	270.2 ± 46.9	262.9 ± 42.5	257.2 ± 31.3

Values are reported as mean ± standard deviation. OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-kappa B ligand; BAP, bone-specific alkaline phosphatase; OC, osteocalcin; CTX-I, C-terminal telopeptides of type I collagen. *$P < 0.0001$ vs. controls.

$P = 0.0008$, in females) concentrations was also found. In multivariate linear regression analysis for male and female hypercholesterolaemic subjects, serum 8-iso-PGF$_{2\alpha}$ was the only significant negative predictor for femoral neck BMD and serum BAP and OC levels in males and females (Tables 3–5). In both male and female hypercholesterolaemic subjects, there were no correlations (all $P > 0.25$) between serum 8-iso-PGF$_{2\alpha}$ levels and lumbar spine BMD, as well as between serum 8-iso-PGF$_{2\alpha}$ levels and serum OPG and RANKL concentrations, or urinary CTX-I levels. No correlation (all $P > 0.20$) was found between lipid values and femoral neck or lumbar spine BMD, as well as between lipid values and serum BAP, OC, OPG and RANKL concentrations, or urinary CTX-I levels. There was also no significant correlation (all $P > 0.10$) between lipid parameters and 8-iso-PGF$_{2\alpha}$ levels. Similar results were obtained when univariate

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Table 3 Multivariate linear regression analysis of the association between serum 8-iso-PGF$_{2\alpha}$ levels and femoral neck BMD in male and female hypercholesterolaemic subjects ($n = 173$)

	β	SE	P
Males			
8-iso-PGF$_{2\alpha}$	−0.0003	0.0001	0.003
Age	−0.002	0.001	0.12
BMI	0.001	0.006	0.10
LDL cholesterol	0.001	0.007	0.09
HDL cholesterol	−0.001	0.001	0.30
Triglycerides	−0.0001	0.001	0.88
Calcium intake	−3.75	7.64	0.62
Physical activity index	4.86	0.0001	0.96
Females			
8-iso-PGF$_{2\alpha}$	−0.0005	0.0001	<0.0001
Age	0.005	0.002	0.07
BMI	0.002	0.006	0.74
LDL cholesterol	0.0009	0.0006	0.13
HDL cholesterol	−0.0007	0.001	0.63
Triglycerides	−0.0002	0.0002	0.46
Calcium intake	7.75	6.10	0.20
Physical activity index	−2.45	9.92	0.98
Age at menarche	−0.006	0.01	0.63
Postmenopausal status	−0.06	0.06	0.30

β, regression coefficient; SE, standard error; PGF$_{2\alpha}$, prostaglandin F$_{2\alpha}$; BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Table 4 Multivariate linear regression analysis of the association between serum 8-iso-PGF$_{2\alpha}$ and bone-specific alkaline phosphatase levels in male and female hypercholesterolaemic subjects ($n = 173$)

	β	SE	P
Males			
8-iso-PGF$_{2\alpha}$	−0.005	0.001	0.001
Age	−0.02	0.02	0.26
BMI	0.02	0.10	0.82
LDL cholesterol	−0.006	0.01	0.58
HDL cholesterol	0.03	0.02	0.17
Triglycerides	0.02	0.01	0.14
Calcium intake	1.89	0.75	0.08
Physical activity index	−0.0003	0.001	0.79
Females			
8-iso-PGF$_{2\alpha}$	−0.004	0.001	0.004
Age	−0.04	0.03	0.21
BMI	0.04	0.08	0.59
LDL cholesterol	−0.003	0.008	0.67
HDL cholesterol	0.03	0.02	0.09
Triglycerides	0.003	0.003	0.42
Calcium intake	0.0003	0.0007	0.69
Physical activity index	0.0009	0.001	0.45
Age at menarche	0.13	0.19	0.49
Postmenopausal status	1.42	0.81	0.08

β, regression coefficient; SE, standard error; PGF$_{2\alpha}$, prostaglandin F$_{2\alpha}$; BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

and multivariate analyses were carried out in the whole hypercholesterolaemic population. Correlation analyses between 8-iso-PGF$_{2\alpha}$ and BMD and the other outcome variables in male and female control subjects showed similar results as those of hypercholesterolaemic subjects. In univariate analysis, there was a significant negative correlation between serum 8-iso-PGF$_{2\alpha}$ levels and femoral neck BMD ($r = -0.25$, $P = 0.02$, in males; $r = -0.26$, $P = 0.01$, in females) and serum BAP ($r = -0.32$, $P = 0.004$, in males; $r = -0.31$, $P = 0.002$, in females) and OC ($r = -0.26$, $P = 0.02$,in males; $r = -0.27$, $P = 0.01$, in females) concentrations. In multivariate linear regression analysis, only serum iso-PGF$_{2\alpha}$ was independently negatively associated with femoral neck BMD ($\beta = -0.001$, SE $= 0.0004$, $P = 0.003$, in males; $\beta = -0.001$, SE $= 0.0006$, $P = 0.01$, in females) and serum BAP ($\beta = -0.03$, SE $= 0.01$, $P = 0.03$, in males; $\beta = -0.02$, SE $= 0.007$, $P = 0.0008$, in females) and OC ($\beta = -0.08$, SE $= 0.02$, $P = 0.02$, in males; $\beta = -0.06$, SE $= 0.02$, $P = 0.02$, in females) levels. Univariate and multivariate correlation analyses in the whole control group yielded results similar to those from the male and female subgroups.

Discussion

The present study shows that increased serum 8-iso-PGF$_{2\alpha}$ was the only factor found to be independently and negatively related to BMD at the femoral neck in both male and female hypercholesterolaemic subjects. Higher concentrations of 8-iso-PGF$_{2\alpha}$ were also the

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Table 5 Multivariate linear regression analysis of the association between serum 8-iso-$PGF_{2\alpha}$ and osteocalcin levels in male and female hypercholesterolaemic subjects ($n = 173$)

	β	SE	P
Males			
8-iso-$PGF_{2\alpha}$	−0.009	0.002	0.002
Age	−0.008	0.03	0.81
BMI	−0.07	0.15	0.62
LDL cholesterol	0.0009	0.01	0.95
HDL cholesterol	0.03	0.04	0.33
Triglycerides	−0.01	0.03	0.55
Calcium intake	−0.001	0.001	0.55
Physical activity index	−0.005	0.002	0.09
Females			
8-iso-$PGF_{2\alpha}$	−0.01	0.004	0.006
Age	0.003	0.10	0.97
BMI	0.15	0.23	0.50
LDL cholesterol	−0.01	0.02	0.53
HDL cholesterol	0.08	0.05	0.14
Triglycerides	−0.001	0.01	0.90
Calcium intake	−0.002	0.002	0.27
Physical activity index	0.01	0.003	0.08
Age at menarche	−0.31	0.55	0.57
Postmenopausal status	−0.52	2.36	0.82

β, regression coefficient; SE, standard error; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

only significant and independent predictor for lower serum concentrations of BAP and OC in both sexes. Our findings are the first observation that enhanced formation of 8-iso-$PGF_{2\alpha}$, a reliable marker of *in vivo* oxidative stress and lipid peroxidation, is associated with lower bone density and decreased levels of peripheral blood markers of bone formation such as BAP and OC in hypercholesterolaemia.

These results confirm findings from previous studies [29, 30, 35] that hypercholesterolaemia is associated with increased formation of F_2-isoprostanes. In this study, in agreement with the report by Roberts and Morrow [35], we did not see any correlation between lipid parameters and serum 8-iso-$PGF_{2\alpha}$ levels. Conversely, our data differ from those reported by Davì *et al.* [29] and Reilly *et al.* [30], who found a positive correlation between urinary excretion of 8-iso-$PGF_{2\alpha}$ and LDL cholesterol levels. Our findings and those by Roberts and Morrow [36] would suggest that the mechanisms for the increased levels of F_2-isoprostanes in hypercholesterolaemic subjects may not be simply related to the presence of more lipid, i.e. arachidonic acid substrate. It has been hypothesized that enhanced oxidative stress associated with hypercholesterolaemia could result from the activation of the metabolism of the arachidonic acid pathway, which, in turn, seems to be associated with increased NAD(P)H oxidase activity [36]. Alternatively, intracellular metabolism of mevalonate could enhance oxidative stress via protein isoprenylation and subsequent production of proinflammatory and pro-oxidant cytokines such as tumour necrosis factor-α [36]. However, the underlying basis for the increase in oxidative stress seen in hypercholesterolaemia remains poorly defined.

The results of our study extend previous findings in hyperlipidaemic postmenopausal women [11,12] by showing lower femoral neck and lumbar spine BMD also in male hypercholesterolaemic subjects. Consistent with the study by Orozco [11], we were not able to find any correlation between lipid parameters and BMD. This finding would seem to suggest that serum cholesterol is not a direct pathogenic factor in the development of lower bone mass in this patient population. Additionally, in line with one previous study [10], no correlations between lipid parameters and biochemical markers of bone turnover could be demonstrated that could have indicated a direct influence of lipids on bone metabolism. In contrast, our observation that serum 8-iso-$PGF_{2\alpha}$ was the only independent negative predictor for BMD and serum BAP and OC would seem to indicate that oxidative stress, rather than serum cholesterol levels, is directly responsible for the adverse effects of hypercholesterolaemia on bone.

In this study, both femoral neck and lumbar spine BMD were reduced in hypercholesterolaemic subjects. However, unlike femoral neck BMD, lumbar spine BMD was not correlated with serum 8-iso-$PGF_{2\alpha}$ levels. A possible explanation for this discrepancy is that osteoarthritis, which is associated with an increased

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BMD, occurs quite often in the spine in older adults, whereas the femoral neck is less often involved [37]. This may result in an overestimation of BMD measured at lumbar spine and may have concealed a relation between lumbar spine BMD and serum 8-iso-$PGF_{2\alpha}$ in hypercholesterolaemic subjects.

Correlation analyses between 8-iso-$PGF_{2\alpha}$ and BMD and the other outcome variables in the control group showed results similar as those of hypercholesterolaemic group, thus confirming the independent negative relationship between 8-iso-$PGF_{2\alpha}$ and BMD and bone formation markers, even in a population with a smaller range in serum 8-iso-$PGF_{2\alpha}$ levels.

In the present study, BAP and OC levels were reduced in hypercholesterolaemic subjects, and this finding may indicate that osteoblastic activity is inhibited in hypercholesterolaemia. The observation of a relationship between serum 8-iso-$PGF_{2\alpha}$ and decreases in bone mass and serum BAP and OC concentrations would suggest that enhanced oxidative stress, as measured by serum 8-iso-$PGF_{2\alpha}$ levels, may have a negative impact on bone mass in hypercholesterolaemic subjects possibly via reduced bone formation. This suggestion is supported by *in vitro* and animal studies showing that lipid oxidation products may inhibit osteoblastic differentiation of preosteoblasts, alkaline phosphatase activity [23, 24] and OC expression [38], and increase adipogenic and reduce osteogenic differentiation of marrow stromal cells [24]. Oxidative stress has also been shown to inhibit *in vitro* the differentiation of osteoblastic cells, with antioxidants counteracting this effect [39]. Furthermore, animal studies demonstrated that antioxidant vitamin E protects against cellular lipid peroxidation and has beneficial effects on bone mass [40]. Potential involvement of oxidative stress in osteoporosis is also consistent with previous evidence from some [41–43], but not all [44], epidemiological studies that suggested that higher dietary antioxidant intake has a protective role on bone health.

Based on an assumption that serum and urine levels of bone markers, and serum OPG and RANKL concentrations track tissue levels, this study shows no evidence that upregulation of bone resorption stimulating mechanisms takes part in the pathophysiologic processes mediating bone loss in hypercholesterolaemic subjects.

No statistically significant difference in urinary CTX-I levels between hypercholesterolaemic and control subjects was observed, thus suggesting that osteoclastic activity is not enhanced in hypercholesterolaemia. This finding and the lack of a correlation between serum 8-iso-$PGF_{2\alpha}$ and urinary CTX-I do not support the hypothesis from experimental research that bioactive products of lipid oxidation [25, 26] and oxidative stress [45] may promote bone resorptive potential in hypercholesterolaemic subjects.

No increased activation of the RANKL/RANK system was found in hypercholesterolaemic subjects, a finding indicating that this osteoclast regulatory system [46] is not involved in the development of bone loss associated with hypercholesterolaemia. Based on our findings, where serum 8-iso-$PGF_{2\alpha}$ was not associated with OPG and RANKL levels, it is unlikely that the relationship between oxidative stress and bone in hypercholesterolaemia is mediated through the OPG/RANKL signalling pathway.

Taken together, these findings would suggest that increased oxidative stress associated with hypercholesterolaemia may adversely influence bone metabolism by uncoupling bone formation (decreased) from bone resorption (unchanged), which may result in a loss of bone mass. However, the mechanisms underlying selective effects of oxidative stress on bone formation over bone resorption remain to be elucidated.

In conclusion, our findings show an association between increased serum 8-iso-$PGF_{2\alpha}$ levels and reduced bone density in hypercholesterolaemic subjects. Increased serum 8-iso-$PGF_{2\alpha}$ also appears to be associated with reduced serum concentrations of the bone formation markers BAP and OC. These results would suggest that enhanced oxidative stress might play a role in the development of lower bone mass associated with hypercholesterolaemia possibly via reduced bone formation. Additional studies are

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however needed to investigate the association between oxidative stress and lower bone mass in hypercholesterolaemia and to define precisely the underlying mechanisms.

Conflict of interest statement

All authors have no conflicts of interest.

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