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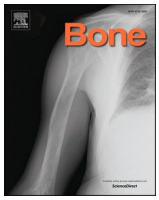

Full Length Article

Associations of global biomarkers of oxidative stress with osteoporosis, bone microstructure and bone turnover: Evidence from human and animal studies

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ABSTRACT

Purpose: Human evidence on the association between oxidative stress and osteoporosis is inconsistent. Fluorescent Oxidation Products (FlOPs) are global biomarkers of oxidative stress. We examined the associations of FlOPs (excitation/emission wavelengths 320/420 nm for FlOP_320, 360/420 nm for FlOP_360, and 400/475 nm for FlOP_400) with osteoporosis, bone microstructure, and bone turnover markers in humans and rats.
Methods: In humans, we conducted a 1:2 age, sex, hospital, and specimen-matched case-control study to test the association between FlOPs and osteoporosis diagnosed from dual-energy X-ray absorptiometry. In eight-week-old male Wistar rats, we administrated D-galactose and 0.9 % saline for 90 days in treatment and control groups (n = 8/group); micro-CT was used to determine bone microstructure.
Results: In humans, higher levels of FlOP_320 (OR for per 1 SD increase = 1.49, 95 % CI: 1.01–2.20) and FlOP_360 (OR for per 1 SD increase = 1.59, 95 % CI: 1.07–2.37) were associated with increased odds of osteoporosis. FlOP_400 were not associated with osteoporosis. D-galactose treated rats, as compared with control rats, showed higher levels of FlOP_320 and MDA, and lower P1NP levels during 90 days of experiment (all $P < 0.05$). The D-galactose group had lower trabecular bone volume fraction (0.07 ± 0.03 vs. 0.13 ± 0.05; $P = 0.008$) and volumetric BMD (225.4 ± 13.8 vs. 279.1 ± 33.2 mg HA/cm^3; $P = 0.001$) than the control group.
Conclusion: In conclusion, higher FlOP_320 levels were associated with increased odds of osteoporosis, impaired bone microstructure and decreased bone formation.

1. Introduction

Osteoporosis, a common metabolic bone disease, has become a critical public health issue worldwide. The prevalence of osteoporosis among women and men aged 50 years old or above are 35.3 % and 12.5 %, respectively [1]. Over the past three decades, the global burden of osteoporosis-related fractures has increased substantially [2]. The estimated incidence, prevalence, and years lived with disability (YLD) rates of hip fracture in patients aged over 55 years were 681.35/100, 000, 1191.39/100, 000 and 130.78/100,000, respectively [3]. The healthcare costs for patients with osteoporotic fractures are substantial in years 1–5 after fractures [4–6]. Osteoporosis and osteoporotic fracture are associated with greater risk of comorbidities, dependency, and death [2,7]. Within 1 year following a hip fracture, all-cause mortality ranged from 14.4 % to 28.3 % [8].

Oxidative stress may be involved in the development of osteoporosis via increased bone resorption and decreased bone formation [9,10]. *In vitro*, hydrogen peroxide (H_2O_2) increases the number and activity of osteoclasts in human marrow mononuclear cells [11]. H_2O_2 induces apoptosis of osteoblast cell lines [12]. *In vivo*, chronic exposure to

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advanced oxidation protein products (AOPP) promotes osteoclastogenesis and bone loss in mice, which can be alleviated by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor apocynin [13]. Animals treated with D-galactose (an inducer of oxidative stress) have decreased levels of osteoprotegerin (OPG) and impaired bone microstructure compared to controls [14,15].

Although basic research suggests that oxidative stress may play a role in the development of osteoporosis, evidence in humans is inconsistent. For example, higher malondialdehyde (MDA) levels were associated with lower bone mineral density (BMD) and higher risk of osteoporosis in some [16,17] but not other studies [18,19]. Baek et al. found that 8-hydroxy-2′-deoxyguanosine (8-OHdG) is inversely associated with BMD in postmenopausal women [11]. In contrast, Cervellati et al. did not observe a significant relationship between 8-OHdG and BMD among postmenopausal women [20]. Inconsistent results were also noted for AOPP and superoxide dismutase [21–23]. The reasons for these contradictory results are unclear, but may be related to the fact that MDA, AOPP, and 8-OHdG only reflect specific oxidative damage from lipids, protein, and DNA, respectively; and that using only one of these oxidative stress biomarkers may not be able to assess the overall impact of oxidative stress on bone health. In addition, it has been suggested that measurement of MDA is challenging from both analytical and biological perspectives [24]. For instance, Alajbeg et al. observed that the coefficient of variation (CV) for MDA was 16.6 % [25]. Assays for other oxidative stress biomarkers were also reported to be unreliable [25–27].

Fluorescent Oxidation Products (FlOPs), global markers of oxidative stress, reflect the oxidative damage of proteins, lipids, DNA, and carbohydrates [28,29]. Fluorescence measurement is suggested to be 10–100 times more sensitive than the colorimetric thiobarbituric acid reactive substances (TBARS) in the assay of MDA [29]. The inter-assay and intra-assay CVs for FlOP measurement were lower, at 3.3 % and 1.7 %, respectively. A prospective study suggested that higher levels of FlOPs measured at 320/420 nm (excitation/emission) wavelength are associated with increased risk of hip fracture in White postmenopausal women [30]. Among 164 Chinese males, FlOPs were negatively associated with hip BMD [31]. We therefore hypothesized that higher FlOP levels are associated with an increased odds of osteoporosis and bone microstructure. To test this hypothesis, we examined the associations of FlOPs with osteoporosis, bone microstructure and bone turnover using evidence from humans and rats, respectively.

2. Materials and methods

2.1. Human study

2.1.1. Study setting and participants

The participants for this study were recruited from the Department of Endocrinology in The Second Hospital of Jilin University and the Department of Osteoporosis and Treatment of FAW General Hospital of Jilin Province in China from June 2019 to September 2021. The former hospital is a major diagnosis and treatment institute for severe diseases (e.g., cancers and heart diseases) in Jilin province. The annual number of out- and inpatients is about 1.81 million and 0.11 million, respectively. The Department of Endocrinology has approximately 2000 visits for BMD measurements annually. The FAW General Hospital of Jilin Province has 30 clinical departments and 9 medical technology departments and has an annual outpatient and inpatient care of approximately 0.71 million and 39,000, respectively. The Department of Osteoporosis Diagnosis and Treatment is a major center for diagnosing and treating osteoporosis patients for the whole Jilin Province; its annual visit number for BMD measurements is about 10,000.

Before undertaking the current study, a sample size calculation was performed based on pilot data from 4 osteoporosis cases and 8 controls. The average mean values of FlOP_320 in cases and controls were 367 ± 41 FI/ml and 331 ± 95 FI/ml, respectively. With a two-tailed significance level of $\alpha = 0.05$, statistical power of $1\text{-}\beta = 0.9$, the case-control ratio was set to 1:2, the required sample size for cases and controls was estimated to be 80 and 160, respectively.

We identified individuals aged ≥45 years old with complete and valid data on BMD measurements. We excluded individuals: 1) receiving medications known to affect bone metabolism (e.g., bisphosphonate, estrogen, and glucocorticoids); 2) with secondary osteoporosis (e.g., thyroid/parathyroid disease, chronic liver/kidney disease, type 1 diabetes mellitus, rheumatoid arthritis, and cancer); 3) with early (< 45 years old) menopause; and 4) with missing covariate data. This study was approved by the institutional review boards (IRBs) at The Second Hospital of Jilin University (IRB approval #: 2019–13) and FAW General Hospital of Jilin Province (IRB approval #: K2020–001-01). Written informed consent was obtained from all participants.

2.1.2. Ascertainment of cases and controls

BMD was measured with dual-energy X-ray absorptiometry (DXA) fan-beam bone densitometers (Discovery, Hologic Inc., Bedford, MA) at the two hospitals. All participants received lumbar spine BMD measurements, and 57 % had hip BMD measurements. Daily quality control for the densitometers was performed using a spine phantom. The CV for measurement was <1 %. As the DXA densitometer models at the two hospitals were the same (Discovery, Hologic), we did not perform the cross-calibration between the two DXA scanners. Osteoporosis cases were diagnosed if the T-score at the lumbar spine, femoral neck, or total hip was ≤ −2.5 [32]. The reference population for calculating lumbar spine and hip BMD T-scores used the mean and standard deviation (SD) for White females aged 20–29 years old from the manufacturer and the National Health and Nutrition Examination Survey (NHANES) III database [33,34]. The non-osteoporosis controls were individually matched with cases by age (± 5 years), sex, hospital, and specimen type (serum or plasma).

2.1.3. Blood collection in human study

Overnight fasting (> 8 h of fasting except for water) blood samples were collected with either heparin anticoagulant tubes or serum separation gel evacuated tubes (BD, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). These blood samples were stored on ice and transferred to the laboratory of Jilin University within 8 h. All blood samples were centrifuged at 3000 rpm for 10 min at 4 °C to obtain plasma or serum, and immediately stored at −80 °C until assay. Approximately two-thirds (64 %; $n = 168$) were serum samples, the others (36 %; $n = 93$) were plasma samples.

2.1.4. FlOP measurement

The procedures for measuring FlOP levels have been described in detail previously [31]. In brief, plasma or serum samples were extracted with ethanol/ether (3:1, *v*/v) and centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was added to a 96-well Microplate (96-well Black Flat Bottom Polystyrene High Bind Microplate 3925, Corning, USA) and measured by the fluorescent microplate reader (Cytation3 Cell Imaging Multi-Mode reader, BioTek, Vermont, USA). The excitation/emission wavelengths were 320/420 nm for FlOP_320, 360/420 nm for FlOP_360, and 400/475 nm for FlOP_400. FlOP_320 are formed when oxidation products (e.g., lipid hydroperoxides) react with DNA in the presence of metal and reducing agents (e.g., ascorbic acid). FlOP_360 represent lipid oxidation products that reacted with proteins, DNA, and carbohydrates in the presence of phospholipids. FlOP_400 reflect the interaction of MDA and phospholipids [28,29]. The fluorescence of FlOPs was expressed as relative fluorescent intensity units per milliliter (FI/ml). All FlOP levels were measured by the same technician in the laboratory of School of Public Health, Jilin University. The inter-assay CVs for FlOP_320, FlOP_360 and FlOP_400 were 3.3 %, 3.5 %, and 3.4 %, respectively; the intra-assay CV was 1.7 % for FlOP_320, 2.3 % for FlOP_360, and 2.6 % for FlOP_400. FlOP levels generally maintain a constant level over short-term (1.4 years) and long-term (10 years) periods [35]. The postprandial changes of FlOPs within 10 h were also

insignificant [36]; this indicates a small variation of FlOPs within a day. Based on a pilot study of 41 subjects, we found a high Pearson correlation ($r = 0.987$) between plasma and serum FlOP_320; the absolute difference between plasma and serum FlOP_320 was very low (mean difference = −0.62 FI/ml). Serum and plasma FlOP_360 ($r = 0.745$) and FlOP_400 ($r = 0.619$) were moderately correlated. Thus, plasma and serum FlOP_320 levels were used interchangeably; plasma and serum FlOP_360 and FlOP_400 levels were only analyzed together when specimen matching was considered.

2.1.5. Ascertainment of covariates

We used a structured questionnaire to collect participants' general information (e.g., age and sex), lifestyle habits (e.g., smoking and alcohol use), and medical history (e.g., hypertension and type 2 diabetes mellitus). Covariates for the statistical analyses were selected if they were potentially related to FlOPs and/or osteoporosis [28,37]. Smoking was defined as current or past tobacco use. A frequent alcohol user was defined as a participant who consumed ≥3 units of alcohol per day; one unit is equivalent to 8 g of pure alcohol. Physical activity was estimated from the frequency and duration of light, moderate, and vigorous physical activities and quantified as metabolic equivalent hours per week (Met-hours/week) [38]. A family history of osteoporosis, family history of kyphosis, individuals' diagnosis of hypertension, type 2 diabetes mellitus, and coronary heart disease were all self-reported. Personal history of fracture was defined as any self-reported fracture caused by low trauma (e.g., slipping and tripping from standing height). Self-reported height loss >3 cm refers to an individual's height loss >3 cm since the age of 40 years. Using a mechanical height and weight scale (RGZ120, Changzhou Wujin Weighing Apparatus Co., LTD., Jiangsu, China), we measured body height (with light clothing) to the nearest 0.5 cm, and body weight (without shoes) to the nearest 0.5 kg. BMI was calculated as body weight in kilograms divided by body height in meters squared (kg/m^2). Obesity was defined as BMI ≥ 28 kg/m^2 [39].

2.2. Animal study

Eight-week-old male Wistar rats, with an average weight of 220 ± 20 g, were purchased from Beijing Huafukang Bioscience (Beijing, China). All rats were housed under specific pathogen-free (SPF) conditions in the Animal Experimental Center of the School of Public Health, Jilin University, fed with a standard chow diet, and provided purified water ad libitum. All rats were maintained under a 12-h light/12-h dark cycle. The ambient temperature was 22 ± 2 °C; the humidity was 40–60 %.

Rats were randomly allocated to the experimental group or control group ($n = 8$ for each group). The sample size for the animal study was estimated using the resource equation approach as three rats per group [40]. To ensure robust results and allow for potential death of rats, we increased the final sample size to eight rats per group. The rats were kept in polyacrylic cages with four rats per cage. The experimental group was treated with an intraperitoneal injection of 750 mg/kg/d D-galactose (Solarbio Science & Technology Co. Ltd., Beijing, China), and the control group received the same amount of 0.9 % normal saline. D-galactose-induced oxidative stress model is widely used in animals [41]. The 750 mg/kg/d dose was selected based on previous studies [14,15,42]. During the animal experiment, rat weights were measured once a week. The dose of D-gal intervention was adjusted using the most recent body weight measurement for each rat. We conducted the experiment over 90 days similar to previous studies [43]. D-galactose treatments were administered on 5 consecutive days per week followed by two rest days each week. The total period for this study was 90 days. We performed fasting tail-cut blood collections at 0, 30, and 60 days. At the end of 90 days of treatment, rats were anesthetized with an intraperitoneal injection of chloral hydrate. Subsequently, blood samples (plasma and serum) were collected via abdominal aorta puncture. Then all rats were sacrificed, their femurs were immediately removed, cleaned of soft tissue, and kept in 4 % paraformaldehyde tissue fixative until processing. At each time point, fasting (> 8 h of fasting except for water) blood samples were collected with heparin anticoagulant tubes and serum separation gel evacuated tubes (BD, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). All blood samples were stored at −80 °C until assay. Plasma samples were used for measuring oxidative stress biomarkers, while serum samples were used for measuring N-terminal lengthening peptide of type 1 collagen (P1NP) and C-terminal telopeptide of type I collagen (CTX). All experimental protocols were approved by the institutional review board (IRB) at The School of Public Health, Jilin University (IRB #: 2021-12-06).

2.2.1. Oxidative stress biomarkers measurements of rats

Plasma FlOP levels in rats were measured using the same methods as described previously. Plasma nitrotyrosine (NT), MDA, 8-OHdG, and pentosidine (PTD) were determined using the rat-specific enzyme-linked immunosorbent assay (ELISA) kits (Meimian Industrial Co., Ltd., Yancheng, Jiangsu, China) following the manufacturer's instructions. The estimated Pearson correlation (r) between the standard sample and measured sample was >0.95. The sensitivity limit of the assay was 2.5 nmol/L for NT, 0.0625 nmol/L for MDA, 0.75 ng/L for 8-OHdG, and 1.5 ng/L for PTD. The intra-assay and inter-assay CVs for NT, MDA, 8-OHdG, and PTD were < 10 % and < 12 %, respectively.

2.2.2. Bone turnover markers measurement of rats

Serum levels of P1NP and CTX were measured using the rat-specific ELISA kits in accordance with the manufacturer's instructions (Meimian Industrial Co., Ltd., Yancheng, Jiangsu, China). The estimated Pearson correlation (r) between the standard sample and measured sample was >0.95. The sensitivity limit of the assay was 0.05 μg/L for P1NP and 0.75 nmol/L for CTX. The intra-assay and inter-assay CVs for P1NP and CTX were all <10 % and < 12 %, respectively.

2.2.3. Bone microarchitecture measurements of rats

We used micro-computed tomography (Micro-CT, μCT 50, Scanco Medical AG, Bassersdorf, Zurich, Swizerland) to assess trabecular bone microstructure within the distal femoral metaphysis. The scanning parameters were X-ray bulb voltage of 70 kV, current of 114 μA, voxel size of 10 μm, and exposure time of 500 ms. The region of interest (ROI) was 1 mm below the growth plate of the distal femur, with a layer thickness of 2 mm, and extending to the proximal femur [44]. The images were processed via three-dimensional reconstruction software (μCT, Evaluation Program V6.6, Scanco Medical AG). The trabecular bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and volumetric BMD (vBMD) were analyzed by the Evaluation Program software (V6.6, Scanco Medical AG).

2.3. Statistical analysis

2.3.1. Human study

In the descriptive analysis, the characteristics of participants by osteoporosis status were compared using the Student's *t*-test for continuous variables or chi-square test or Fisher exact test for categorical variables. We used conditional logistic regression models to estimate the odds ratios (OR) and corresponding 95 % confidence intervals (CIs) for the associations between FlOPs (per 1 SD increase) and osteoporosis. We further treated FlOPs as a categorical variable (Tertile 1 [reference], 2, and 3) in the model. In these logistic regression models, we adjusted for BMI, smoking, physical activity, frequent alcohol use, height loss >3 cm, family history of kyphosis, and personal history of fracture. The selection of these covariates was based on the criterion of a statistically significant relationship with osteoporosis at $\alpha = 0.05$ in bivariate analyses; these adjusted covariates may also be potential confounders in this study. We included two-way interaction terms (i.e., FlOPs*BMI, FlOPs*smoking, FlOPs*physical activity, FlOPs*frequent

alcohol use, FlOPs* height loss >3 cm) to determine whether these factors moderate the FlOP-osteoporosis relationship. To examine whether postmenopausal status impacted the relationship between FlOPs and osteoporosis, we analyzed this association in women stratified by postmenopausal status. Results are reported as odds ratios (OR) with 95 % confidence intervals (95 % CI). To assess overall discrimination of FlOPs for predicting osteoporosis, the receiver operating characteristic curve (ROC) was used to estimate the area under the curve (AUC) after adjusting for covariates. We used scatter plots and Pearson correlations to assess the linear relationship between FlOPs and BMD at the lumbar spine, femoral neck, and total hip. In these correlation models, we adjusted for age, sex, BMI, smoking, frequent alcohol use, dairy or soy products intake, seafood intake, physical activity, use of calcium supplementation, hypertension, type 2 diabetes mellitus, height loss >3 cm, family history of kyphosis, and personal history of fracture. Results are reported as partial Pearson correlation coefficients (r) along with their *P* values.

2.3.2. Animal study

The body weight, FlOPs, NT, MDA, 8-OHdG, PTD, P1NP, CTX, and trabecular bone parameters for D-galactose and control groups were presented as mean ± standard deviation (SD) at 0, 30, 60, and 90 days. We used two-way repeated measures ANOVA to assess differences in groups and the impact of group (control or D-galactose group), time, and group by time interaction on the changes in FlOPs, NT, MDA, 8-OHdG, PTD, P1NP, and CTX levels over 90 days. In the case of a statistically significant interaction between time and group, a post-hoc Bonferroni comparison was performed to test for significant difference between groups [45]. We used Student's *t*-test to compare the difference in trabecular bone parameters between D-galactose group and control group at 90 days. We also estimated the Pearson correlations for FlOPs with NT, MDA, 8-OHdG and PTD. All analyses were performed using the SPSS (version 24.0, IBM SPSS Inc., Chicago, IL) or R (version: 4.0.0; R Foundation for Statistical Computing, Vienna, Austria) statistical software.

3. Results

3.1. Human study

Initially, we identified 706 individuals (60 % were males). After excluding 27 ineligible participants and matching, we identified 87 cases and 174 controls in the final study population (Supplemental Fig. 1). The mean age was 55.6 years (SD = 6.8 years) for controls and 56.4 years (SD = 6.7 years) for cases and 55.2 % were females for both case and control groups (Table 1). Compared to controls, cases tended to be thinner, had higher percentages of smokers, frequent alcohol users, and less physical activity, and had higher percentages of height loss >3 cm, family history of kyphosis, and personal history of fracture.

After adjusting for multiple clinical covariates, higher levels of FlOP_320 (OR for per 1 SD increase = 1.49, 95 % confidence interval [CI]: 1.01–2.20; Table 2) and FlOP_360 (OR for per 1 SD increase = 1.59, 95 % CI: 1.07–2.37) were associated with increased odds of osteoporosis. FlOP_400 were not associated with osteoporosis (OR for per 1 SD increase = 1.16, 95 % CI: 0.82–1.63). Similar results were observed when FlOP_320 was categorized into tertiles (OR for tertile 3 vs. tertile 1: 2.63, 95 % CI: 1.02–6.81; *P* for trend = 0.039). Specimen type, BMI, smoking, physical activity, frequent alcohol use, and height loss >3 cm did not modify the FlOPs-osteoporosis relationship (all *P* for interaction >0.05). Similar trends of relationships of FlOP_320 and FlOP_360 with osteoporosis was observed in postmenopausal or non-postmenopausal women (Supplemental Table 1); postmenopausal status did not modify these relationships (*P* for interaction >0.05).

After adjusting for covariates, the AUC of FlOP_320, FlOP_360, and FlOP_400 with osteoporosis was 0.742 (95 % CI: 0.678–0.806), 0.756 (95 % CI: 0.692–0.819), and 0.738 (95 % CI: 0.674–0.801), respectively.

Table 1
Characteristics of cases and controls.

Variables	Controls (*n* = 174)	Cases (*n* = 87)	P
Age (years), mean (SD)	55.6 (6.8)	56.4 (6.7)	0.387
Female (n, %)	96 (55.2)	48 (55.2)	1.000
Body mass index (kg/m^2), mean (SD)	24.8 (3.0)	23.3 (3.2)	0.002
Smoking (n, %)	33 (19.0)	26 (29.9)	0.047
Frequent alcohol users (n, %)	20 (11.5)	22 (25.3)	0.004
Dairy or soy products intake ≥3 times per week (n, %)	69 (39.7)	27 (31.0)	0.173
Seafood intake ≥2 times per week (n, %)	44 (25.3)	21 (24.1)	0.840
Physical activity (MET-hours/week), median (IQR)	13.2 (6.6, 20.1)	6.6 (6.6, 20.1)	0.004
Use of calcium supplementation (n, %)	36 (20.7)	11 (12.6)	0.111
Hypertension (n, %)	36 (20.7)	14 (16.1)	0.374
Type 2 diabetes mellitus (n, %)	28 (16.1)	13 (14.9)	0.810
Coronary heart disease (n, %)[a]	5 (2.9)	3 (3.5)	1.000
Height loss >3 cm (n, %)	26 (14.9)	25 (28.7)	0.008
Family history of kyphosis (n, %)	17 (9.8)	16 (18.4)	0.048
Personal history of fracture (n, %)	11 (6.3)	12 (13.8)	0.045
FlOP_320 (FI/ml), median (IQR)	122.2 (111.4, 137.8)	148.9 (128.5, 115.7)	0.010
FlOP_360 (FI/ml), median (IQR)	109.3 (101.8, 122.7)	116.2 (107.3, 129.8)	0.005
FlOP_400 (FI/ml), median (IQR)	34.7 (30.6, 39.8)	35.9 (32.9, 40.1)	0.095
BMD (g/cm^2), mean (SD)			
Lumbar spine	0.96 (0.11)	0.73 (0.07)	< 0.001
Femoral neck	0.73 (0.09)	0.59 (0.08)	<0.001
Total hip	0.90 (0.11)	0.72 (0.09)	<0.001
T-scores (SD)			
Lumbar spine	−0.73 (0.98)	−2.81 (0.64)	<0.001
Femoral neck	−1.04 (0.74)	−2.23 (0.62)	<0.001
Total hip	−0.36 (0.88)	−1.80 (0.70)	<0.001

Note: SD: standard deviation; IQR: interquartile range; MET: Metabolic equivalent of task. [a]Chi-squared test with Yates' continuity correction.

Correlation analysis revealed that serum FlOP_360 was negatively associated with lumbar spine BMD (*P* = 0.035; Supplemental Fig. 3). We also noted negative correlations of BMD with FlOP_320 and FlOP_400, although the correlation coefficients were not statistically significant (all *P* > 0.05; Supplemental Figs. 2 and 4).

In the present study, there were two potential outliers (1016.8 and 1045.3 FI/ml) in FlOP_320 and one potential outlier (315 FI/ml) in FlOP_360. The mean level of FlOP_320 and FlOP_360 were 146.0 ± 107.6 FI/ml and 116.6 ± 22.3 FI/ml, respectively. However, when we removed these potential outliers, the partial Pearson correlation coefficients (r) and *P* values for FlOPs and BMD changed only slightly. For example, the partial r between FlOP_320 and lumbar spine BMD changed from −0.078 to −0.059, while the *P* value changed from 0.221 to 0.360.

3.2. Animal study

Body weight increased in both control and D-galactose groups over time (30, 60, and 90 day measurements). There was no significant differences in body weight between the two groups at any time point (all *P* > 0.05, Supplemental Fig. 5).

D-galactose treated rats, as compared with control rats, showed higher levels of FlOP_320 (*P* = 0.014; Fig. 1A) and MDA (*P* < 0.001; Fig. 1E) during 90 days of experiment; these differences were more obvious for 30 days. The overall trends of NT, MDA, 8-OHdG, and PTD with time of the experiment in D-galactose group were generally similar to that of FlOP_320 (Fig. 1A, D-G).

Overall, serum P1NP levels in D-galactose group were lower than control group (*P* = 0.02; Supplemental Fig. 6). Serum levels of CTX were not significantly different between two groups (*P* = 0.485). There were

Table 2
Association between FlOPs and osteoporosis: multivariable conditional logistic regression analyses ($n = 261$).

Variables	FlOP Range (Fl/ml)	Unadjusted Model		Adjusted Model[a]	
		OR (95 % CI)	P	OR (95 % CI)[a]	P
FlOP_320 (Fl/ml) Continuous model:					
FlOP_320 (per 1-SD increase)		1.36 (0.99, 1.87)	0.059	1.49 (1.01, 2.20)	0.046
Categorical model:					
Tertile 1	< 116.7	Reference		Reference	
Tertile 2	116.7–134.5	1.34 (0.66, 2.72)	0.425	1.28 (0.56, 2.91)	0.564
Tertile 3	≥ 134.6	2.44 (1.12, 5.28)	0.024	2.63 (1.02, 6.81)	0.046
P for trend across tertiles			0.020		0.039
FlOP_360 (Fl/ml) Continuous model:					
FlOP_360 (per 1-SD increase)		1.63 (1.16, 2.28)	0.005	1.59 (1.07, 2.37)	0.022
Categorical model:					
Tertile 1	< 106.3	Reference		Reference	
Tertile 2	116.3–120.7	1.97 (0.98, 3.98)	0.057	1.96 (0.86, 4.47)	0.109
Tertile 3	≥ 120.8	1.94 (0.94, 4.00)	0.074	1.88 (0.80, 4.42)	0.150
P for trend across tertiles			0.114		0.208
FlOP_400 (Fl/ml) Continuous model:					
FlOP_400 (per 1-SD increase)		1.30 (0.97, 1.74)	0.082	1.16 (0.82, 1.63)	0.396
Categorical model:					
Tertile 1	< 32.8	Reference		Reference	
Tertile 2	32.8–38.4	3.65 (1.65, 8.08)	0.001	3.46 (1.35, 8.86)	0.010
Tertile 3	≥ 38.5	3.23 (1.38, 7.57)	0.007	2.22 (0.85, 5.78)	0.103
P for trend across tertiles			0.026		0.265

[a] ORs were adjusted for body mass index, smoking, frequent alcohol use, physical activity, height loss >3 cm, family history of kyphosis, and personal history of fracture. OR = odds ratio; CI = confidence interval.

inverted "U"-shaped trends in P1NP and CTX levels with time in both groups.

At 90 days, the BV/TV and volumetric BMD of rats in the D-galactose group were significantly lower compared to the control group (Fig. 2A and E); other micro-CT results including Tb.N, Tb.Th, and Tb.Sp were not different between groups (Fig. 2B-D). The three-dimensional images of trabecular bone suggested that D-galactose group had thinner trabecular bone and reduced trabecular bone connection than control group (Fig. 3).

The Pearson correlation coefficients for FlOPs with traditional oxidative stress biomarkers are presented in Supplemental Table 2. We did not find statistically significant correlations between FlOPs and traditional oxidative stress biomarkers, except for the negative correlation between FlOP_400 and PTD.

4. Discussion

In the present study, higher levels of FlOP_320 and FlOP_360 were associated with increased odds of osteoporosis in humans. D-galactose-treated rats, as compared with control rats, showed higher levels of FlOP_320 and MDA, and lower P1NP levels throughout 90 days of the experiment. Furthermore, rats in D-galactose group had lower BV/TV and volumetric BMD than control group.

To the best of our knowledge, this is the first study examining the associations of FlOPs with osteoporosis, bone microstructure, and bone turnover. The association between FlOP_320 and osteoporosis is partly in line with two previous studies, in which higher levels of FlOP_320 were associated with lower hip BMD in Chinese male veterans and increased risk of hip fracture in White postmenopausal women [30,31]. Similar results were also found in a previous study in which FlOP_320, but not FlOP_360 or FlOP_400, were associated with hip fracture [30]. Since FlOPs measured at different wavelengths reflect different oxidation products [28,29], it is reasonable to speculate that the impact of oxidative stress on bone health is different across various oxidation products. This is partly in line with results reported by Wu et al., which concluded that lumbar spine BMD was negatively associated with AOPP but not MDA in postmenopausal women [18]. We also found that serum FlOP_360 were negatively associated with lumbar spine BMD. FlOP_320 levels were not associated with lower BMD at the lumbar spine, femoral neck, and total hip, this is not consistent with our previous study [31]. The null associations of hip BMD with FlOPs may be due to the fact that 43 % of individuals only had lumbar spine BMD measurements, but not hip BMD. However, almost all the FlOP-BMD correlations showed a negative trend in the present study. These findings are generally consistent with the association between FlOPs and osteoporosis in our study.

D-galactose is a well-accepted way to induce global oxidative stress in vivo [46]. The primary mechanism for this is that excess D-galactose can be oxidized to the aldehydes and hydroperoxide under the catalyzation of galactose oxidase, leading to increased levels of reactive oxygen species (ROS) [41]. Previous studies found that traditional oxidative stress biomarkers (e.g., MDA) are increased in D-galactose-treated rats [15,47]. Similar results were also found in our study. Moreover, rats treated with D-galactose combined with antioxidants had lower MDA levels compared to D-galactose treated group [15]. This proved that we have built a successful oxidative stress model using D-galactose.

As noted above, the existing literature suggested that FlOPs are 10–100 times more sensitive than MDA in assessing oxidative stress [29]. Human studies also reported that FlOPs are associated with oxidative stress-related diseases such as coronary heart disease, kidney diseases, and cancer [35,48,49]. FlOPs are not correlated with C-reactive protein [35], indicating it is a specific marker of oxidative stress. In our animal study, we also found that FlOPs can detect oxidative damage induced by D-galactose (a global oxidative stress inducer) earlier than PTD and NT. In summary, FlOPs are likely to be sensitive and specific markers of global oxidative stress.

In our study, compared with control rats, rats treated with D-galactose had increased FlOP_320 levels during 90 days of experiment; this increase was more obvious for 30 days. Higher FlOPs in the D-galactose group than in the control group between 30 days are partially consistent with previous studies, in which continuous injections of D-galactose for 4–8 weeks induced higher levels of MDA [47,50]. We observed higher levels of FlOP_320 at 30 days, and higher MDA levels at 30 and 60 days in the D-galactose group than in controls. There was no significant difference in levels of 8-OHdG and PTD throughout 90 days of the experiment. These findings suggest that traditional oxidative stress biomarkers, such as 8-OHdG and PTD, might not be as sensitive as FlOPs in dynamically evaluating oxidative stress in rats. Thus, measuring a single traditional oxidative stress biomarker may not be able to assess the overall impact of oxidative stress on bone health.

In rats, BV/TV and volumetric BMD in the D-galactose group were lower than in control group at 90 days. This is consistent with the findings in our human study. Yu et al. [14] and Imerb et al. [50] also

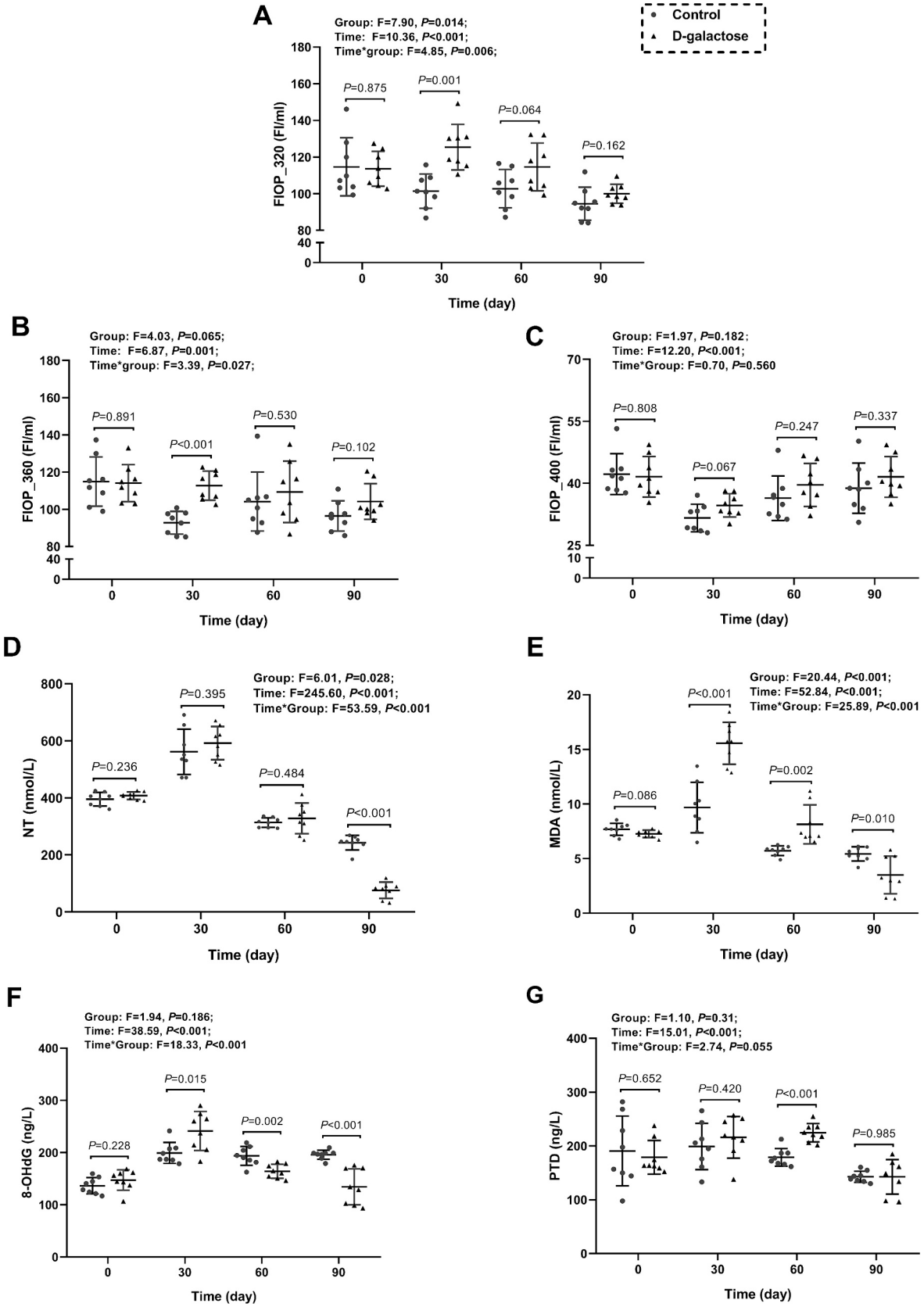

Fig. 1. Changes in FlOPs and traditional oxidative stress biomarkers in the D-galactose and control groups over time.
A-C: FlOP levels of rats at the indicated time points. D-G: Plasma levels of traditional oxidative stress-related biomarkers at the indicated time points. Error bars represent mean ± standard deviation. n = 8 rats per group. The main effects of group and time, and their two-way interaction were analyzed by two-way ANOVA with repeated measures.

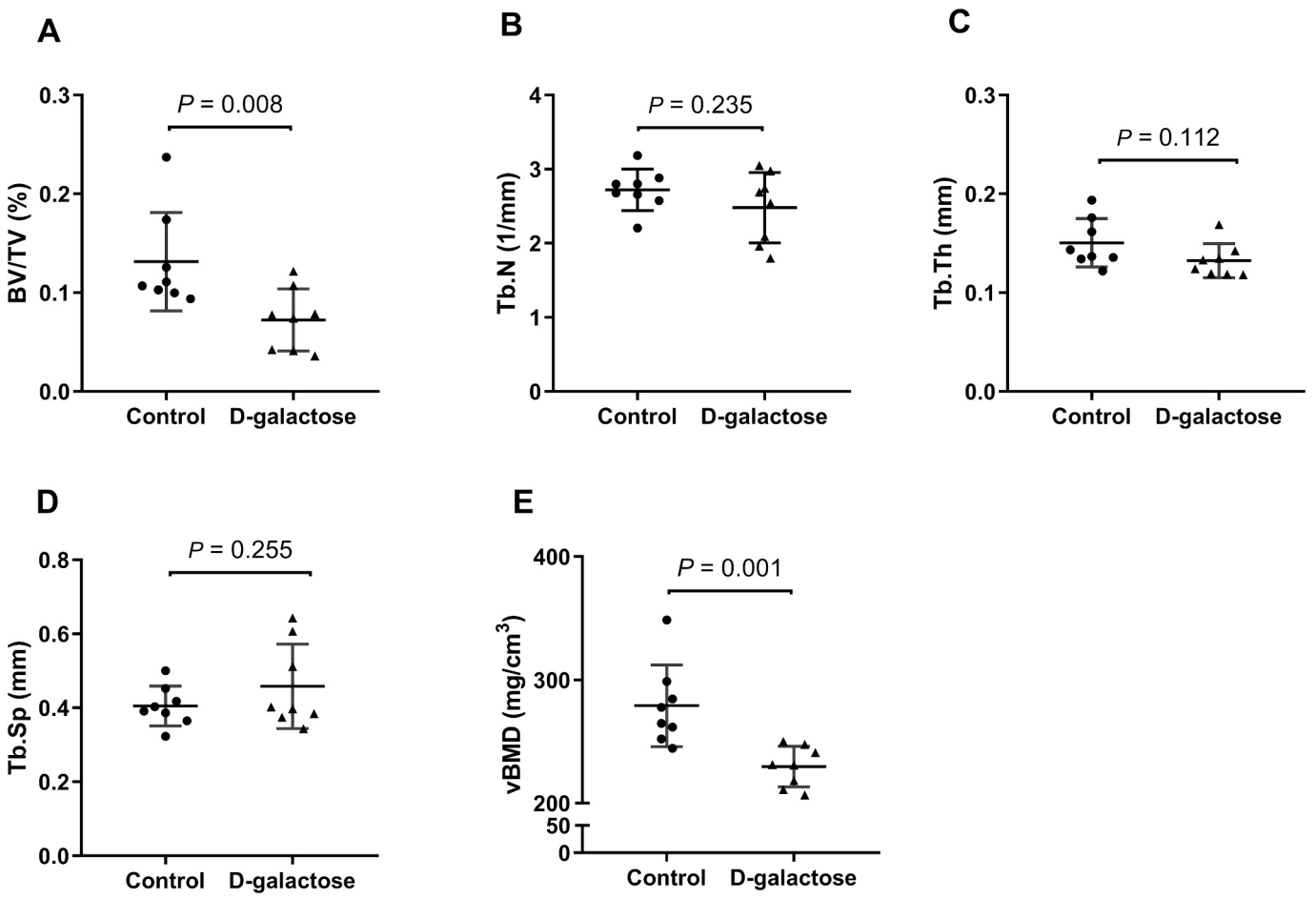

Fig. 2. Micro-CT results in the D-galactose and control groups.
A: Trabecular bone volume fraction (BV/TV). **B:** Trabecular number (Tb.N). **C:** Trabecular thickness (Tb.Th). **D:** Trabecular separation (Tb.Sp). **E:** Volumetric bone mineral density (vBMD). **F:** Three-dimensional images.
A-E: Error bars represent mean ± standard deviation. n = 8 rats per group. The differences in BV/TV, Tb.N, Tb.Th, Tb.Sp, and vBMD between D-galactose and control group were examined by Student's *t*-test.

found lower trabecular bone volume fraction and volumetric BMD in D-galactose group as compared to control group.

In our study, P1NP levels were lower in the D-galactose group than that in the control group during 90 days of experiment. This finding is partly in line with a prior study, in which P1NP was lower on a normal diet with D-galactose group than on a normal diet with vehicle [50]. In our study, the CTX results were generally parallel with P1NP results. This is likely due to the coupling effect between bone formation and bone resorption [51]. We therefore speculate that D-galactose-induced oxidative stress mainly plays a role in decreasing bone formation rather than bone resorption. This was supported by other studies which found lower levels of bone formation markers (e.g., OPG and bone-specific alkaline phosphatase) in D-galactose-treated rats [15,52]. Excessive accumulation of D-galactose can be converted into advanced glycation end products (AGEs) via the Maillard reaction. The accumulation of AGEs inhibits the activity of osteoblasts, resulting in decreased bone formation [53]. When it comes to molecular signaling pathways involved, accumulating evidence suggests that higher levels of oxidative stress induce osteoblast apoptosis and inhibit the differentiation of osteoblasts through the canonical Wnt/β-catenin, extracellular signal-regulated kinase (ERK), and mitogen-activated protein kinase signaling pathway [12,54–56]. Furthermore, oxidative stress induced by high glucose promotes the proliferation of osteoblasts via the PI3/Akt pathway [57]. Although *in vivo* and *in vitro* evidence suggests that accumulated ROS can also stimulate the proliferation and differentiation of osteoclasts [11,58], however, we did not observe an increase in bone resorption marker in the D-galactose group. Such finding warrants further study.

Based on the results from our animal study, except for the negative relationship between FlOP_400 and PTD, FlOPs were not associated with traditional oxidative stress biomarkers. The underlying reasons for causing this remain unclear. However, FlOPs are likely to be formed in the final stage of the oxidation reactions [59], while traditional markers of oxidative stress may be produced in early stage of oxidation process.

Our study has several strengths and weaknesses. The individually 1:2 matched case-control study design reduced the potential confounding effects of age, sex, sample source, and specimen type. In addition, human and animal studies offer complementary ways to assess the associations of FlOPs with osteoporosis and bone microstructure. Limitations should be considered when interpreting the results of the present study. Firstly, due to the cross-sectional nature of the human study, we cannot assume a causal relationship between FlOPs and osteoporosis. However, our animal study may partially address the temporal relationship between FlOP_320 and osteoporosis. Secondly, approximately 43 % of participants had lumbar spine BMD measurements, but not hip BMD. However, osteoporosis is more commonly identified from lumbar spine than hip BMD [60]. The percentage of patients with osteoporosis at the hip, but not at the lumbar spine, is relatively low [61]. Third, since the human study population was hospital-based, it may not generalize to the general population.

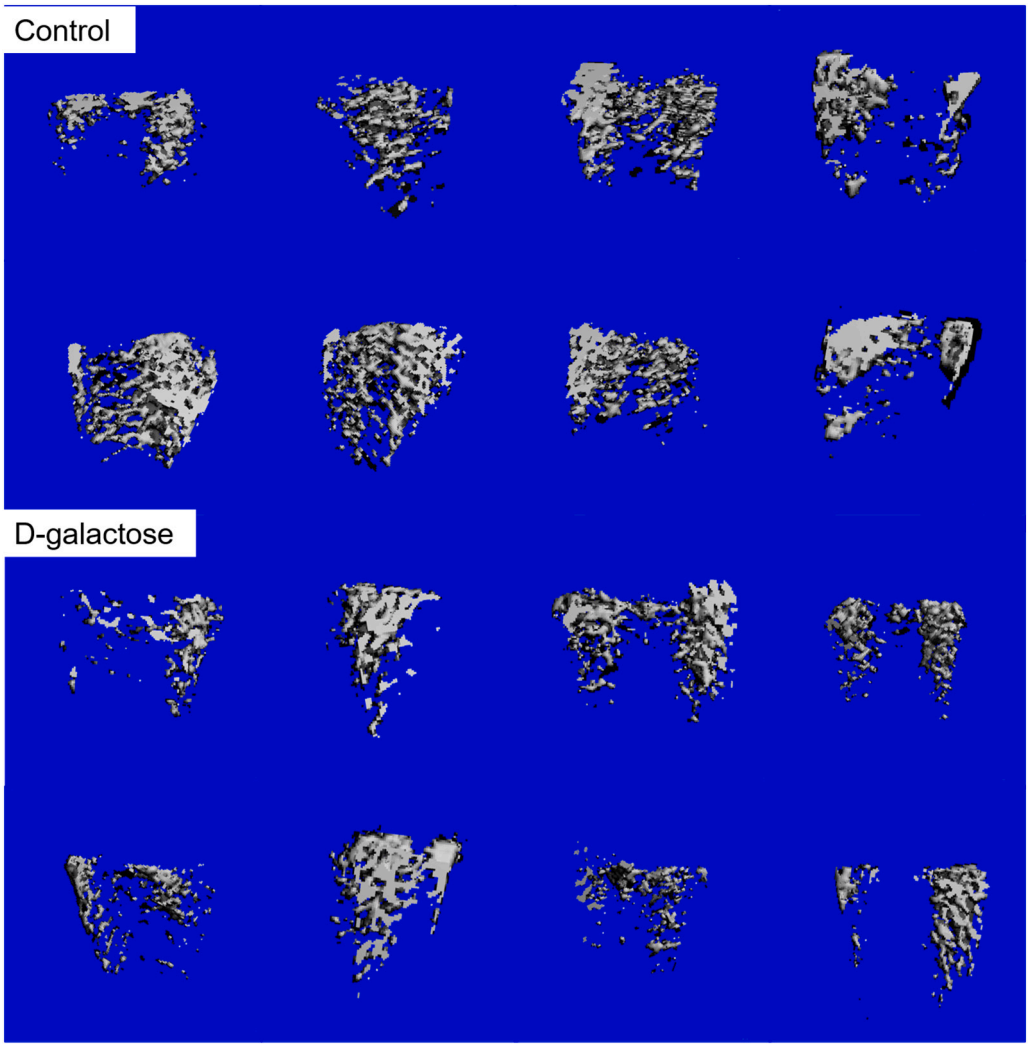

Fig. 3. The three-dimensional image of the femoral trabecular bone in the D-galactose and control groups.
Scale bar: 1.0 mm.

Finally, we only measured traditional biomarkers of oxidative stress (i.e., MDA and 8-OHdG) in animal study, but not in human study. Whether humans and animals have comparable results remain unclear. One potential limitation of our animal study is that we only measured bone microstructure at the terminal point.

Accumulating evidence suggests that oxidative stress is linked to osteoporosis [9,11,18]. However, there is still no consensus on the ideal oxidative stress-related biomarker for assessing the risk of osteoporosis. Compared to traditional oxidative stress-related markers that only capture a portion of oxidative damage (e.g., MDA and AOPP), FlOPs reflect the global level of oxidation. The present study, combining human and animal data, observed significant associations of higher FlOP levels with increased odds of osteoporosis and impaired bone microstructure. If our findings are confirmed in prospective epidemiologic studies, FlOPs may be a better oxidative stress biomarker than traditional oxidative stress biomarkers (e.g., NT, MDA, and PTD) for assessing the impact of oxidative stress on bone health.

5. Conclusions

In summary, the present study demonstrates that higher FlOP_320 levels are associated with increased odds of osteoporosis, impaired bone microstructure, and decreased bone formation.

CRediT authorship contribution statement

Xue Shen: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mengmeng Zhang:** Writing – review & editing, Methodology, Investigation. **Hanqing Cai:** Methodology, Investigation. **William D. Leslie:** Writing – review & editing. **Lisa M. Lix:** Writing – review & editing. **Depeng Jiang:** Writing – review & editing. **Lijie Feng:** Methodology, Investigation. **Haitao Cheng:** Funding acquisition. **Xianbao Shi:** Writing – review & editing. **Yuzhong Gao:** Writing – review & editing. **Shuman Yang:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

The dataset used or analyzed in the present study are available from the corresponding author and required to be approved by the institutional review board (IRB) of Jilin University, The Second Hospital of Jilin University, and FAW General Hospital of Jilin Province.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2024.117077.

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