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Lead induced differences in bone properties in osteocalcin +/+ and −/− female mice

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ABSTRACT

Lead (Pb) toxicity is a major health problem and bone is the major reservoir. Lead is detrimental to bone, affects bone remodeling and is associated with elderly fractures. Osteocalcin (OC) affects bone remodeling, improves fracture resistance and decreases with age and in some diseases. The effect of lead in osteocalcin depleted bone is unknown and of interest. We compared bone mineral properties of control and Pb exposed (from 2 to 6 months) femora from female adult C57BL6 OC+/+ and OC−/− mice using Fourier Transform Infrared Imaging (FTIRI), Micro-computed tomography (uCT), bone biomechanical measurements and serum turnover markers (P1NP, CTX). Lead significantly increased turnover in OC+/+ and in OC−/− bones producing increased total volume, area and marrow area/total area with decreased BV/TV compared to controls. The increased turnover decreased mineral/matrix vs. Oc+/+ and increased mineral/matrix and crystallinity vs. OC−/−. PbOC−/− had increased bone formation, cross-sectional area (Imin) and decreased collagen maturity compared OC−/− and PbOC+/+. Imbalanced turnover in PbOC−/− confirmed the role of osteocalcin as a coupler of formation and resorption. Bone strength and stiffness were reduced in OC−/− and PbOC−/− due to reduced material properties vs. OC+/+ and PbOC+/+ respectively. The PbOC−/− bones had increased area to compensate for weaker material properties but were not proportionally stronger for increased size. However, at low lead levels osteocalcin plays the major role in bone strength suggesting increased fracture risk in low Pb^{2+} exposed elderly could be due to reduced osteocalcin as well. Years of low lead exposure or higher blood lead levels may have an additional effect on bone strength.

1. Introduction

Lead exposure is an important public health problem in the U.S. and throughout the world with harmful effects reported in children and adults (Ignasiak et al., 2006; Campbell and Auinger, 2007; Gabler, n.d.). In addition to acute environmental exposure, past exposure can lead to chronic effects from the accumulation of lead in bone. Bone is the major reservoir of lead (Barry, 1981; Barry, 1975) where it is stored for decades (Brito et al., 2005; Skerfving and Nilsson, 1992), and can be released in blood and to other soft tissues causing harmful effects. Bone lead is a marker for past exposure and increases with age (Gamblin et al., 1994), placing the elderly at particular risk. Many individuals have sustained exposure to lead through both occupational and non-occupational sources during their lifetimes (Vig and Hu, 2000).

Lead is detrimental to the skeleton in epidemiological observations and in both in-vivo and in-vitro studies as well. Blood lead levels were associated with increased bone mineral density (Campbell et al., 2004) and inversely correlated with stature and chest circumference in children (Ignasiak et al., 2006; Schwartz et al., 1986; Shukla et al., 1989). Elevated blood lead was correlated with decreased bone mineral density

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(Campbell and Auinger, 2007; Potula et al., 2005; Nash et al., 2004), thinner cortices (Wong et al., 2015), increased bone resorption (Potula et al., 2005), and increased risk of fractures in older women (Khalil et al., 2008). In preclinical animal studies lead had detrimental effects on bone mineral properties (Monir et al., 2010; Beier et al., 2013; Escribano et al., 1997; Hamilton and O'Flaherty, 1994; Olchowik et al., 2014), altered bone turnover (Monir et al., 2010; Beier et al., 2013; Escribano et al., 1997; Beier et al., 2016; Hass, 1964; Brito et al., 2014), and impaired fracture healing (Carmouche et al., 2005). In-vitro lead stimulated bone resorption in osteoclasts (Miyahara et al., 1995), and suppressed intracellular signaling in chondrocytes (Zuscik et al., 2002). However, the role of noncollagenous bone proteins, in the detrimental effect of lead in bone is unclear.

Osteocalcin is a small (49 aa, 5850 MW) protein synthesized by osteoblasts and osteocytes and is one of the most abundant noncollagenous proteins in bone (Hauschka et al., 1975; Price et al., 1976). Osteocalcin production is stimulated by vitamin D (Lian et al., 1982). The structure of osteocalcin contains 3 γ-carboxyglutamic acid residues that can bind Ca^{2+} or Pb^{2+} in solution and in hydroxyapatite mineral. Both calcium and lead can induce a conformational change that increases the binding of osteocalcin to mineral hydroxyapatite in-vitro (Dowd et al., 2001; Dowd et al., 2003; Hoang et al., 2003).

Our knowledge of the 3-Gla form of osteocalcin and its role in bone and bone abnormalities is limited. In-vivo, the 3-Gla osteocalcin is mainly bound to bone mineral, with lower concentrations in the serum (Hauschka et al., 1989; McKee et al., 1993). Earlier studies demonstrated osteocalcin affected bone mineral formation and mineral crystal growth in solution (Hunter et al., 1996; Romberg et al., 1986). It was also involved in the recruitment, differentiation, and maturation of osteoclasts (Lian et al., 1984; Liggett et al., 2004; Ishida and Amano, 2004). Studies in the osteocalcin-depleted knock-out mouse demonstrated osteocalcin inhibited bone formation (Ducy et al., 1996; Berezovska et al., 2019) and suggested that osteocalcin affected mineral maturity and bone remodeling (Boskey et al., 1998). More recently, using the knock-out mouse on a C57BL6 background, osteocalcin enhanced bone strength (Berezovska et al., 2019; Moriishi et al., 2020) and fracture resistance (Poundarik et al., 2012) as well as regulated mineral crystal size (Berezovska et al., 2019; Poundarik et al., 2018).

The role of osteocalcin in lead toxicity is unknown. Lead is detrimental to bone, increases bone turnover (Monir et al., 2010) and is associated with fractures in the elderly (Khalil et al., 2008). Osteocalcin is beneficial to bone. It affects bone remodeling and is involved in bone strength and fracture resistance. Because osteocalcin decreases with age (Hauschka et al., 1989; Ingram et al., 1994; Gundberg et al., 1983) and in some diseases (diabetes) (Hauschka et al., 1989; Okazaki et al., 1997; Sanches et al., 2017), the effect of lead on osteocalcin-depleted mineral is of interest. It is also of interest to study the effect of lead on a different genotype. This study investigated the effect of lead on bone mineral properties in the presence and absence of osteocalcin. Comparisons were made between mineral from control and lead-exposed adult female wild-type mice and those of age- and genetic background-matched female osteocalcin knock-out mice treated similarly. Biomarkers for bone formation and resorption were measured in serum. Fourier Transform Infrared Imaging (FTIRI) was used to measure detailed mineral properties such as mineral/matrix ratio and bone crystal size and perfection. Bone mineral density and geometry were investigated using quantitative microcomputed tomography (microCT) and whole bone strength was measured in three-point bending.

2. Experimental

2.1. Animals

Osteocalcin deficient mice were provided by Gerard Karsenty, on a mixed 129/B6 background, as described (Ducy et al., 1996). We generated congenic strains by back-crossing to a C57BL/6J (B6) background for 10 generations using animals purchased from Jackson Laboratories (Bar Harbor, Maine). Genotypes were identified by PCR using DNA extracted from a 3-mm tail specimen using specific oligonucleotide primers for osteocalcin and the neo insert. Two-month-old female, background matched wild-type and osteocalcin knockout mice (n = 10–11/group) were fed a normal diet and exposed to either 200 ppm lead acetate (treatment) or sodium acetate (control) in the drinking water continuously from 2 to 6 months and then euthanized. Lead was administered during the late adolescence and then bones were collected and analyzed from an adult age (6 months). Reports have indicated cortical peak bone density and maturity is reached by 4–6 months in C57BL6 mice (Buie et al., 2008; Somerville et al., 2004) so the results are relevant to mature mineralized bone. Four groups were analyzed: wild-type control (OC+/+), osteocalcin knock-out (OC−/−), lead-exposed wild-type (PbOC+/+) and lead-exposed osteocalcin knock-out (PbOC−/−) mice.

Blood lead was measured using atomic absorption (AA) spectroscopy of blood samples collected via periorbital blood collection. Serum was obtained from a portion of the blood samples for bone biomarker measurements. At euthanasia, the right and left femora from each animal were dissected and cleaned of soft tissue. One femur was used for FTIR imaging and the second femur was used for microcomputed tomography (microCT) and mechanical measurements. Bone lead, calcium/phosphate ratios and mineral bound osteocalcin measurements were measured on the marrow-depleted tibial diaphysis using AA and a radioimmunoassay, respectively.

2.2. Bone biomarker measurements

The bone formation marker P1NP and the bone resorption marker CTX were assayed in serum collected at euthanasia from control and lead-exposed mice. Mineral-bound osteocalcin was extracted from bone and measured with an in-house equilibrium radioimmunoassay (Gundberg et al., 1984). Serum P1NP and CTX (RatLaps) were assayed by rodent specific kits (Immunodiagnostic Systems Inc., Fountain Hills, AZ) (Hale et al., 2007; Garnero et al., 2003).

2.3. Histomorphometry

Mouse femora were processed and embedded in polymethyl methacrylate. Five micron thick calcified sections were placed on silane-coated slides, deplasticized and stained with toluidine blue. Static Histomorphometry was performed on the endocortical surface of bones from wild-type and osteocalcin knock-out mice at a magnification of ×10. Measurements of the number of osteoblasts and osteoclasts per bone perimeter were obtained.

2.4. Microcomputed tomography (microCT)

MicroCT was used to provide information on 3-D cortical bone structure. Femora were scanned in PBS (Scanco μ-CT 35 system, Scanco Medical, Basserdorf, Switzerland) with a 15 μm voxel size, 55 kVp, 0.36 degree rotation step (360 degree angular range) and a 400 ms per view exposure. The region of interest for the cortical bone consisted of a 0.47 mm segment beginning inferior to the third trochanter and extending toward the distal end of the bone and it was analyzed with thresholding at 0.4 g/cm^3. Three-dimensional reconstruction and image viewing were performed with Scanco system software (HP, DECwindows Motif 1.6). The following parameters were measured for cortical bone: bone mineral density (BMD), total, bone and marrow areas, bone-area-to-total-area ratio (BA/TA), cortical thickness, marrow-area-to-total-area ratio, and minimum and maximum area moment of inertia (I_{min}, I_{max}). The region of interest for the proximal bone was comprised of 1.05 mm of bone in the proximal femur from the lower trochanter to just proximal to the third trochanter. The parameters measured for proximal trabecular bone were: Total volume (TV), bone volume (BV), bone volume/total volume (BV/TV), connectivity density (CD), BMD, trabecular number, thickness and spacing (Tb. N., Tb.TH., and Tb.Sp respectively).

2.5. Whole bone mechanical testing

The whole bone bending stiffness and strength were measured by loading the femora to failure in three-point bending in a servohydraulic testing system (MiniBionix 858, MTS Systems, Eden Prairie, MN and MLP 25 load cell, Transducer Techniques, Temecullah CA). Each femur was positioned with the posterior surface on the supports (7.1 mm span). The load was applied to the anterior surface at a constant rate of 0.1 mm/s. The bones were oriented so that the test was done in the anterior posterior plane which corresponded to I_{min}.

Load and displacement data were sampled at 20 Hz. Maximum and failure moments, bending stiffness and displacement at maximum and failure loads were determined. The bending stiffness (EI) is the slope of the force vs. displacement curve. Adjusting the bending stiffness and maximum bending moment for cross-sectional geometric contributions to strength with I_{min} allows the relative contributions of material properties and bone size to be understood. If there was a significant difference in stiffness and/or maximum bending moment the parameter was normalized by the moment of inertia corresponding to the plane of bending testing (I_{min}). If the difference in the parameter between the two groups is removed by this normalization then the effect was due to a difference in size or geometry and not material properties.

2.6. Fourier-transform infrared microspectroscopy (FTIRM)

To measure mineral and matrix composition by spectroscopy, femora were cleaned of soft tissue, processed and embedded in polymethylmethacrylate (PMMA) according to standard protocol (Gourion-Arsiquaud et al., 2008). Spectral images were collected at a 4 cm^{-1} spectral resolution and ~7 μm spatial resolution from 2 μm thick longitudinal sections mounted on infrared windows (Spotlight 400 Imaging system, Perkin Elmer Instruments, Shelton, CT USA). Background spectra were collected under identical conditions from clear Ba_2F windows and subtracted from sample data by instrumental software. IR spectra were collected from three areas (~500 μm × 500 μm) of cortical bone per sample. After acquisition, all spectra were normalized to the PMMA peak at 1728 cm^{-1}, spectral contribution of PMMA embedding media was subtracted, and spectra were baseline-corrected using ISYS Chemical Imaging Software (Malvern, Worcestershire, UK). Spectroscopic parameters collected included: mineral-to-matrix ratio, crystallinity and collagen maturity were calculated (Boskey and Mendelsohn, 2005). The mineral-to-(collagen)-matrix ratio was the integrated area ratio of the ν_1 ν_3 PO_4 band (900–1200 cm^{-1})/amide I band (1590–1712 cm^{-1}), the mineral crystallinity parameter corresponds to the crystallite size and perfection as determined by x-ray diffraction and is calculated from the intensity ratios of subbands at 1030 cm^{-1} (stoichiometric apatite) and 1020 cm^{-1} (nonstoichiometric apatite) (Mendelsohn et al., 1999). The collagen maturity parameter is the ratio of nonreducible (mature) to reducible (immature) collagen cross-links, which is expressed as the intensity ratio of 1660 cm^{-1}/1690 cm^{-1} (Paschalis et al., 2001). The acid phosphate content in the mineral is measured from the peak height ratio of 1128/1096 (Spevak et al., 2013). The results for each parameter were expressed as a histogram describing the pixel distribution giving the mean value of the distribution and associated color coded images were generated at the same time by ISYS.

2.7. Statistical methods

Data was collected for a number of parameters for each mouse using 3 different techniques on cortical bone, one technique on serum, histomorphometry and one technique on trabecular bone. There were correlations between the dependent variables within each technique so to reduce type I error we calculated 6 separate MANOVAs (one for dependent variables within each technique). We conducted model diagnostics for each MANOVA using a combination of statistical tests (i.e. Levene's test within each group) and visual inspection using multivariate normality plots (chi^2 quantiles against Mahalanobis $distance^2$) using the statistics program Jamovi. Using this strategy, the assumption of normality was reasonably met for all MANOVAS.

We ran 6 MANOVAS, using the statistics program SPSS, where the data was modeled using a two-way Multivariate Analysis of Variance. The multivariate tests assessed whether the effects of the independent variables (osteocalcin, lead and/or interaction of the two) differed across dependent variables. We report Pillai's trace, for determining a significant main effect or interaction effect, which is robust against violation of assumptions. If the multivariate tests showed significant effects of independent variables then univariate tests assessed the effect of the independent variables (osteocalcin and/or lead and/or interaction) on each dependent variable separately. If univariate tests showed a significant main effect of either Pb^{2+} and/or osteocalcin the estimated marginal means for each dependent variable were presented and the p-values for the effect. In cases where univariate tests showed statistically significant interactions, we analyzed the interaction effects using planned contrasts with simple effects coding (i.e., simple effects contrasts). The simple effects contrasts tested whether the effect of lead depended on osteocalcin level and whether the effect of osteocalcin depended on the level of lead. The model estimated means from these univariate analyses are presented in tables and graphs with standard error of the mean. Differences for each measured parameter are estimated to be statistically significant when $p \leq 0.05$.

3. Results

3.1. Whole-blood Pb^{2+} and bone Pb^{2+} and Ca^{2+} measurements

Groups of wild-type (OC+/+) and osteocalcin knock-out (OC−/−) mice were exposed to lead in the drinking water for four months, resulting in environmentally relevant blood lead concentrations (Hwang et al., 2001; Kalahasthi and Barman, 2018; Keller et al., 2017). The average blood lead concentration was not different between lead-exposed wild-type and lead-exposed knock-out mice (18.0 ± 2.8 μg/dL (n = 10) and 14.3 ± 6.7 μg/dL (n = 9) for PbOC+/+ and PbOC−/− respectively) with blood lead concentrations of <0.20 for the respective controls that were given equivalent concentrations of sodium acetate. The bone lead concentration was not different between the PbOC+/+ and PbOC−/− mice (115.2 ± 19.6 and 102.9 ± 36 μg/g bone, respectively) with mean bone lead measurements of <1.3 μg/g bone for both controls. Bone calcium concentration with lead treatment was not different compared to the respective controls in either genotype (wild-type: 347 ± 19 and 338 ± 4 mg Ca/g ash; knockout: 351 ± 6 and 354 ± 8 mg Ca/g ash). Bone calcium concentration was not different between the OC−/− and OC+/+ controls (354 ± 8 and 338 ± 4 mg Ca/g ash respectively) nor the PbOC+/+ versus the PbOC−/− mineral (347 ± 19 vs. 351 ± 6).

3.2. Microcomputed tomography and biomechanical measurements

MicroCT scans were collected on control and lead treated femora from OC+/+ and OC−/− mice and important parameters are shown in Table 1. Univariate tests showed that for the femoral diaphysis lead treatment produced a significant effect on total volume, BV/TV, total area and marrow area/total area. Lead significantly decreased BV/TV and significantly increased total volume, total area and marrow area/total area in all lead exposed femora (PbOC+/+ and PbOC−/−) regardless of the presence of osteocalcin. Cortical thickness was not significantly different between any of the groups. A significant interaction between Pb^{2+} and osteocalcin was shown for bone mineral density and I_{min}. The interaction effects were analyzed with simple effects tests. The simple effect of lead on BMD in the presence of osteocalcin is not

Table 1
The MANOVA analysis reported a significant main effect of Pb^{2+} (Pillai's trace $p < 0.001$), a significant effect of osteocalcin (Pillai's trace $p = 0.003$) and a significant interaction effect between Pb^{2+} and osteocalcin (Pillai's trace $p \leq 0.038$). Univariate tests showed a significant main effect of Pb^{2+} for the variables in the table. The model estimated means and significance levels from the univariate analyses are presented.

Parameter	$-Pb^{2+}$	$+Pb^{2+}$	Significance
Total volume (mm^3)	0.766 ± 0.008	0.814 ± 0.008	<0.001
BV/TV	0.440 ± 0.004	0.421 ± 0.004	<0.001
Total area (mm^2)	1.758 ± 0.018	1.873 ± 0.018	<0.001
Marrow area/total area	0.560 ± 0.004	0.579 ± 0.004	<0.001
Cortical thickness (mm)	0.187 ± 0.002	0.184 ± 0.002	0.198
Sample size (N)	20	19	

Parameter	OC+/+	PbOC+/+	OC−/−	PbOC−/−
BMD (mg/cm^3)	1089 ± 4.7^{a}	1089 ± 5.2	$1067 \pm 5.2^{a,b}$	1101 ± 4.9^{b}
I_{min} (mm^4)	0.110 ± 0.003	0.114 ± 0.004^{c}	0.110 ± 0.004^{b}	$0.129 \pm 0.003^{b,c}$
Sample size (N)	11	9	9	10

Univariate analysis found a significant interaction effect between lead and osteocalcin for the parameters in the table. The results of simple effects analysis for lead and for osteocalcin are shown where groups with the same letter exhibit significant differences (a: $p < 0.003$, b: $p < 0.001$, c: $p < 0.009$).

significant but the effect of lead in the absence of osteocalcin is significant. Lead significantly increased bone mineral density in the PbOC−/− vs. OC−/− bones ($p < 0.001$). It can also be interpreted that the simple effect of osteocalcin on BMD is significant only in the absence of lead. The bone mineral density was significantly decreased in OC−/− vs. OC+/+ mineral ($p < 0.003$) as previously reported (Berezovska et al., 2019).

The simple effect of lead on I_{min} in the presence of osteocalcin was not significant while in the absence of osteocalcin it was significant. The I_{min} parameter was significantly increased in PbOC−/− vs. OC−/− ($p < 0.001$). It is also shown that the significant effect of osteocalcin on I_{min} is not significant in the absence of lead but is significant in the presence of lead. Lead significantly increased I_{min} in PbOC−/− vs. PbOC+/+ mineral ($p < 0.009$).

MicroCt parameters were measured for proximal trabecular bone. Data tables showing the parameters are reported in the Supplementary data section (S1 Supplementary data). Univariate tests showed lead significantly increased total volume in all lead exposed trabecular bone (PbOC+/+ and PbOC−/− vs. OC+/+ and OC−/−). Trabecular bones containing osteocalcin (OC+/+ and PbOC+/+) had significantly higher bone mineral density and trabecular spacing and significantly decreased trabecular number compared to osteocalcin knock-out bones (OC−/− and PbOC−/−). A significant interaction effect was found between lead and osteocalcin for bone volume to total volume (BV/TV) and connectivity density (CD). The simple effects of lead on BV/TV and CD in the presence of osteocalcin were not significant but were significant in its absence. Lead significantly reduced BV/TV ($p < 0.002$) and connectivity density in ($p < 0.002$) in the absence of osteocalcin. There were significant simple effects of osteocalcin in the absence of lead for BV/TV and CD but in the presence of lead the simple effects were not significant. As previously reported BV/TV and CD were significantly increased in the OC−/− vs. OC+/+ bone ($p \leq 0.001$) (Berezovska et al., 2019).

Biomechanical properties were measured on cortical bone in the four groups. A significant main effect of osteocalcin was found for all biomechanical parameters, regardless of lead concentration (Table 2). Cortical bones containing osteocalcin (OC+/+ and PbOC+/+) had significantly increased stiffness and a significantly higher maximum bending moment verses bones without osteocalcin (OC−/− and PbOC−/−).

Table 2
The MANOVA gave a significant main effect for osteocalcin (Pillai's trace $p = 0.001$) Mechanical properties of femurs tested in three-point bending to failure are shown below. The model estimated means and the significance from the univariate analyses are presented in the table. (OC+/+ refers to OC+/+ and PbOC+/+, OC−/− refers to OC−/− and PbOC−/−.)

Parameter	OC+/+	OC−/−	Significance
Bending stiffness (EI) (N-mm^2)	959 ± 41	831 ± 41	$p = 0.033$
Size-adjusted bending stiffness (EI/I_{min}) (N/mm^2)	8529 ± 337	6947 ± 336	$p = 0.002$
Maximum bending moment (M_{max}) (N mm)	37.6 ± 0.9	34.1 ± 0.9	$p = 0.010$
Size adjusted maximum bending moment (M_{max}/I_{min}) (N/mm^3)	338 ± 8.0	287 ± 7.9	$p < 0.001$
Sample size (N)	20	20	

To determine whether differences in bending stiffness or maximum bending moment were due to bone geometry (size) or tissue material properties, the parameters were normalized by the moment of inertia corresponding to the plane of bending testing (I_{min}). Cortical bones containing osteocalcin (OC+/+ and PbOc+/+) had significantly higher size adjusted bending stiffness and size adjusted maximum bending moment as compared to bones without osteocalcin (OC−/− and PbOC−/−). Based on these size adjusted results, the decreased stiffness and maximum bending moment in the OC−/− and PbOC −/− cortical tissue is due to a difference in material properties in the knock-out mineral rather than cortical geometry.

3.3. Fourier Transform Infrared Imaging

Fourier Transform Infrared Imaging (FTIRI) data were collected on the cortices of the four different bone groups (Figs. 1 and 2). Representative color-coded images of control and lead treated cortical parameters in the OC+/+ and OC−/− mice are shown in Fig. 1a and b with plotted values in Fig. 2a and b. Results from multivariate tests are in the figure captions. Univariate tests showed a significant interaction effect between lead and osteocalcin for all four FTIR imaging parameters. Each interaction effect was analyzed using planned contrasts simple effects tests.

The simple effect of lead on mineral to matrix ratio in the presence and absence of osteocalcin is significant. The effect of lead depends on the osteocalcin level. Lead significantly reduced the mineral to matrix ratio in the presence of osteocalcin ($p < 0.009$) and significantly increased the ratio in the absence of osteocalcin ($p < 0.021$). The interaction could also be interpreted as the simple effect of osteocalcin on the mineral to matrix ratio in the absence of lead is significant but in the presence of lead is not significant. Comparing the OC+/+ to the OC−/− mineral showed that the OC−/− bones had a reduced ($p < 0.001$) mineral-to-matrix ratio as reported previously (Berezovska et al., 2019).

The crystallinity parameter is related to bone crystal size and perfection. The simple effect of lead on crystallinity was not significant in the presence of osteocalcin and but was significant in the absence of osteocalcin. Crystallinity was significantly increased in PbOC−/− vs. OC−/− ($p < 0.021$). The effect of lead depended on the osteocalcin level. Another interpretation is that the simple effect of osteocalcin on crystallinity in the absence of lead was significant while in the presence of lead the effect is insignificant. Bone crystallinity was significantly reduced in the OC−/− bone as compared to OC+/+ ($p < 0.009$) as previously reported (Berezovska et al., 2019).

Acid phosphate (HPO_4^{2-}) is elevated in areas with new mineral deposition (Fig. 1c). The simple effect of lead on acid phosphate in the presence or absence of osteocalcin is not significant. The simple effect of osteocalcin on acid phosphate level is only significant in the absence of lead ($p < 0.001$). Acid phosphate is significantly increased in the OC−/− mineral as compared to OC+/+. The significant increase in acid

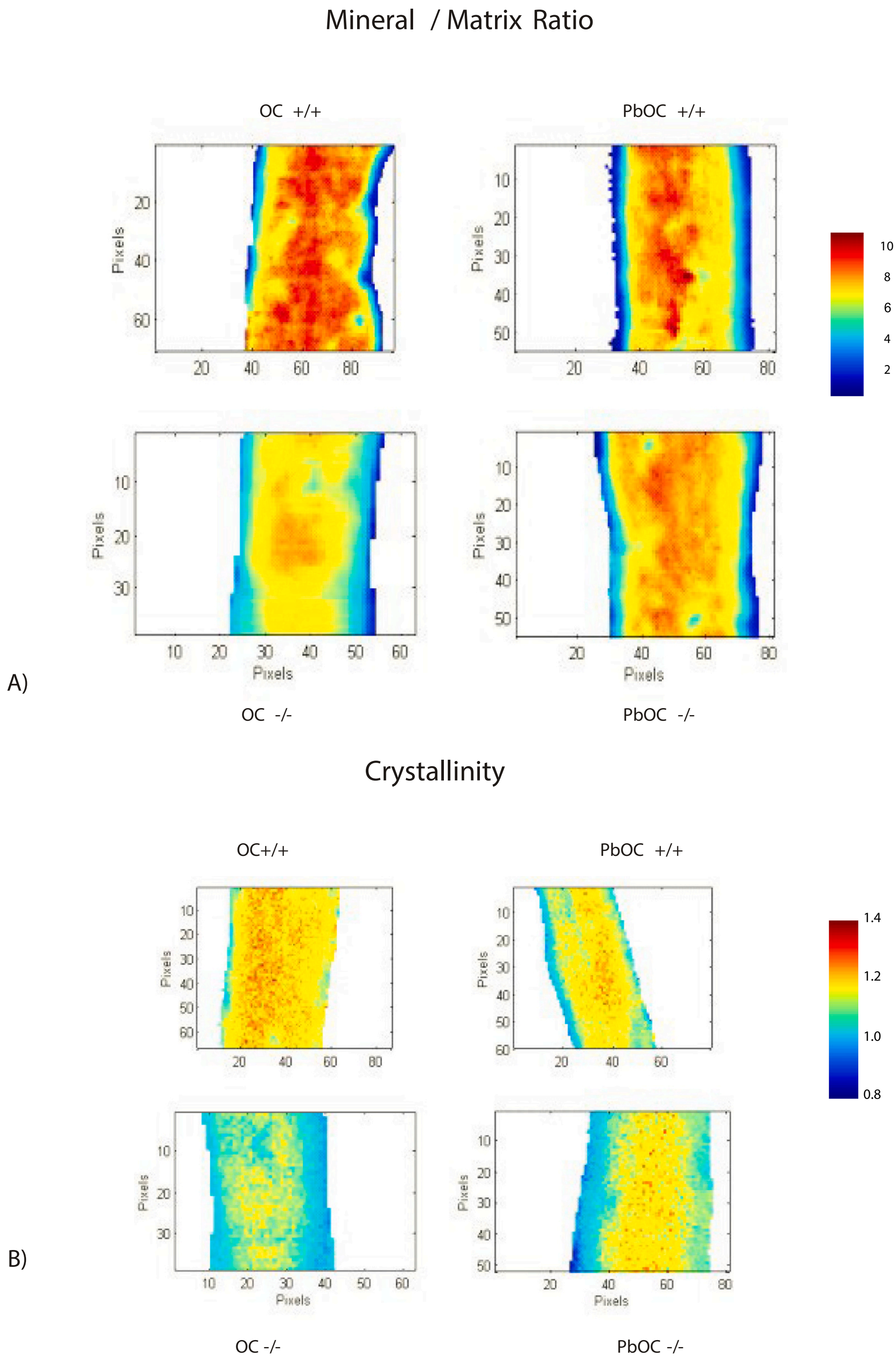


Fig. 1. A and B: FTIR imaging data of the cortical mineral to collagen matrix ratio and bone crystallinity.
C and D: FTIR imaging data of cortical acid phosphate and collagen crosslink maturity. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

phosphate indicates an increase in new bone formation in the OC−/− as compared to the OC+/+ (Berezovska et al., 2019).

The collagen maturity parameter is the ratio of the mature non-reducible crosslinks to the immature reducible crosslinks. The simple effect of lead on collagen maturity in the presence of osteocalcin is not significant but in the absence of osteocalcin it is significant. For collagen maturity PbOC−/− is significantly reduced compared to OC−/− ($p < 0.021$). The interaction can also be interpreted as the simple effect of

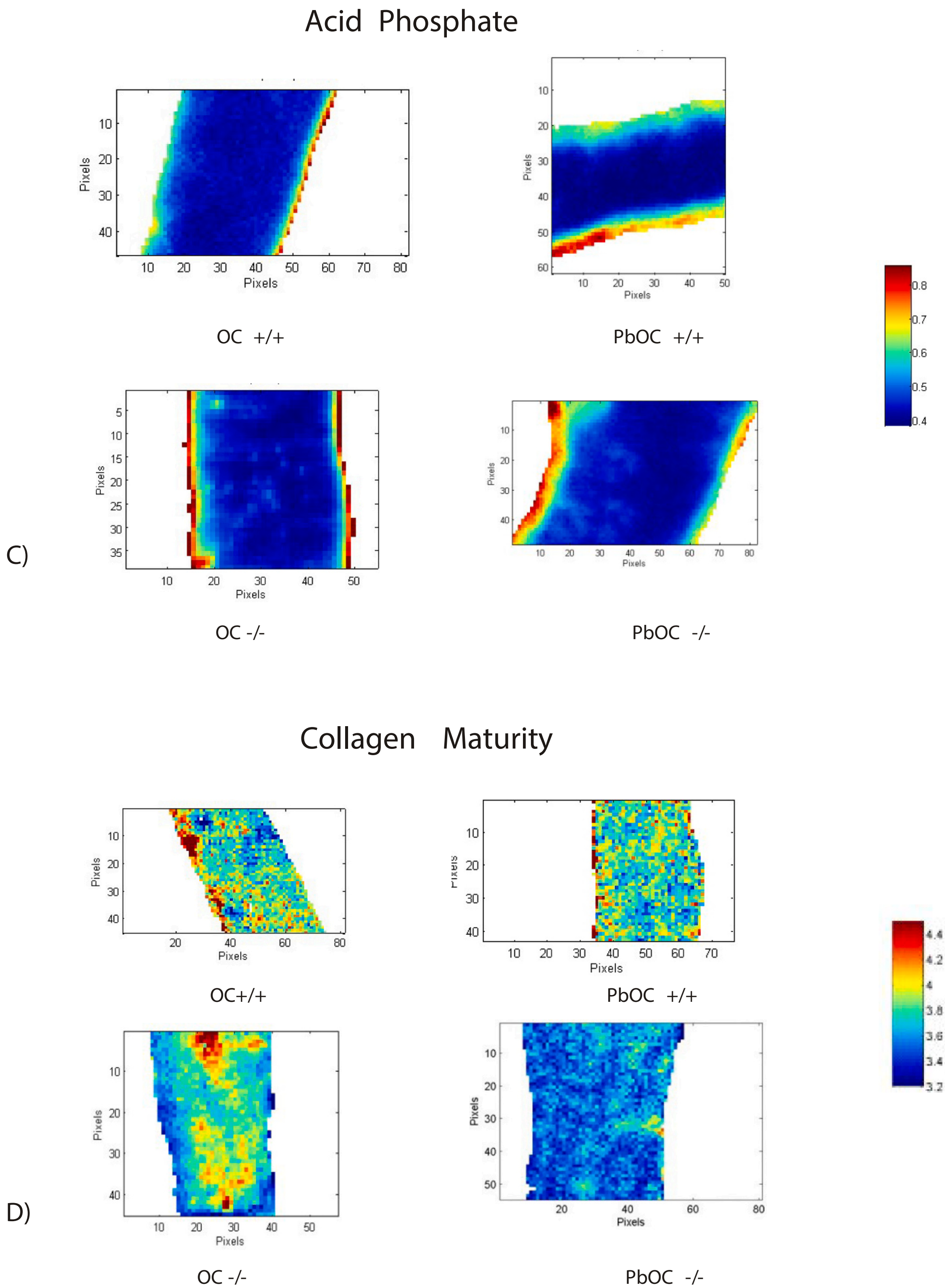


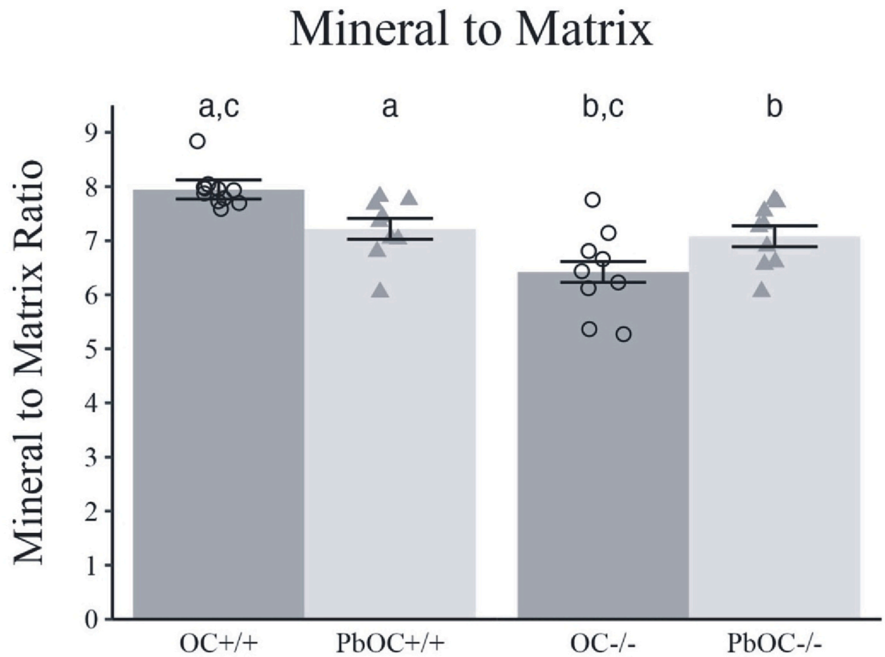
Fig. 1. (*continued*).

osteocalcin on collagen maturity in the absence of lead is not significant but in the presence of lead it is significant. Collagen maturity is significantly reduced ($p < 0.021$) in PbOC−/− bones as compared to PbOC+/+.

3.4. Serum biomarkers and matrix osteocalcin

The bone formation marker, P1NP, had a significant dependence on both lead and osteocalcin while the bone resorption marker, CTX, showed a significant dependence only on lead (Fig. 3). Bone formation, as indicated by serum P1NP concentration, was increased in the absence of osteocalcin (OC−/− and PbOC−/−) (Berezovska et al., 2019) as compared to mineral containing osteocalcin (OC+/+ and PbOC+/+). Serum P1NP levels were also increased with lead treatment (PbOC+/+ and PbOC−/−) versus the control (OC+/+ and OC−/−) mineral.

The histomorphometry measurements showed the same significant



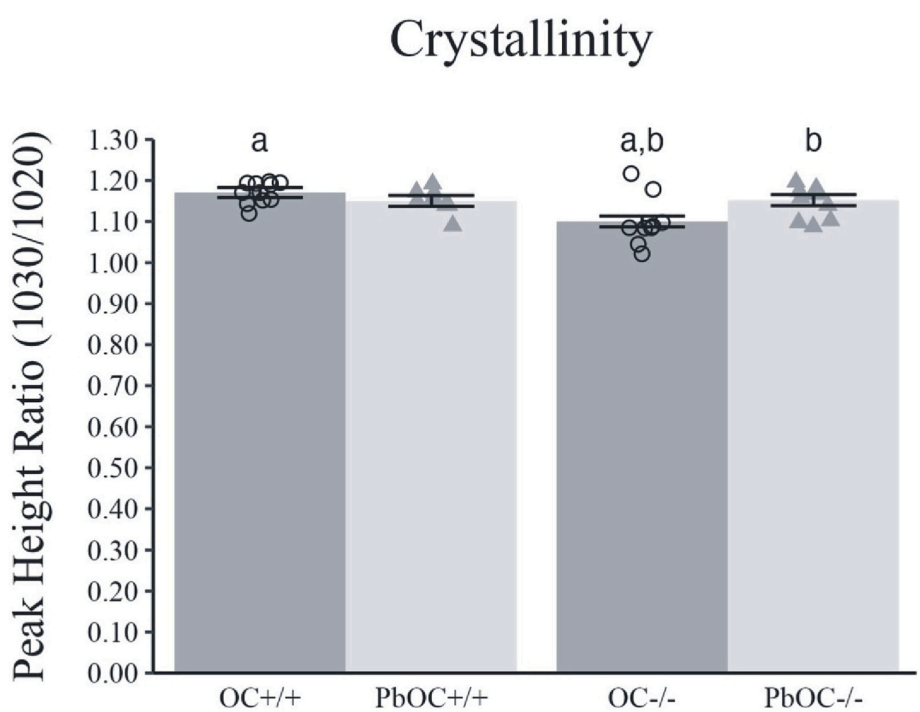


Fig. 2. a: The MANOVA gave a significant main effect for osteocalcin (Pillai's trace $p < 0.005$) and a significant interaction effect for Pb^{2+} and osteocalcin ($p < 0.001$) for the 4 FTIR imaging parameters. Univariate tests showed significant interaction effects for the Mineral/Matrix and the Crystallinity parameters ($p < 0.009$) shown. Interaction effects were analyzed by a simple effects analysis described in the Results section. Plots of the model estimated means for mineral/matrix and crystallinity obtained from FTIR images for each bone group are shown. Groups labeled with the same letter are significantly different (a: $p < 0.009$, b: $p < 0.02$, c: $p < 0.001$).
b: The MANOVA gave a significant main effect for osteocalcin (Pillai's trace <0.005) and a significant interaction effect for Pb^{2+} and osteocalcin ($p < 0.001$) for the 4 FTIR imaging parameters. Univariate tests showed significant interaction effects for the Acid Phosphate and the Collagen Maturity parameters ($p < 0.027$) shown. Interaction effects were analyzed by a simple effects analysis described in the Results section. Plots of the model estimated means for acid phosphate and collagen maturity obtained from FTIR images for each bone group are shown. Groups labeled with the same letter are significantly different (a: $p < 0.001$, b: $p < 0.021$). (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

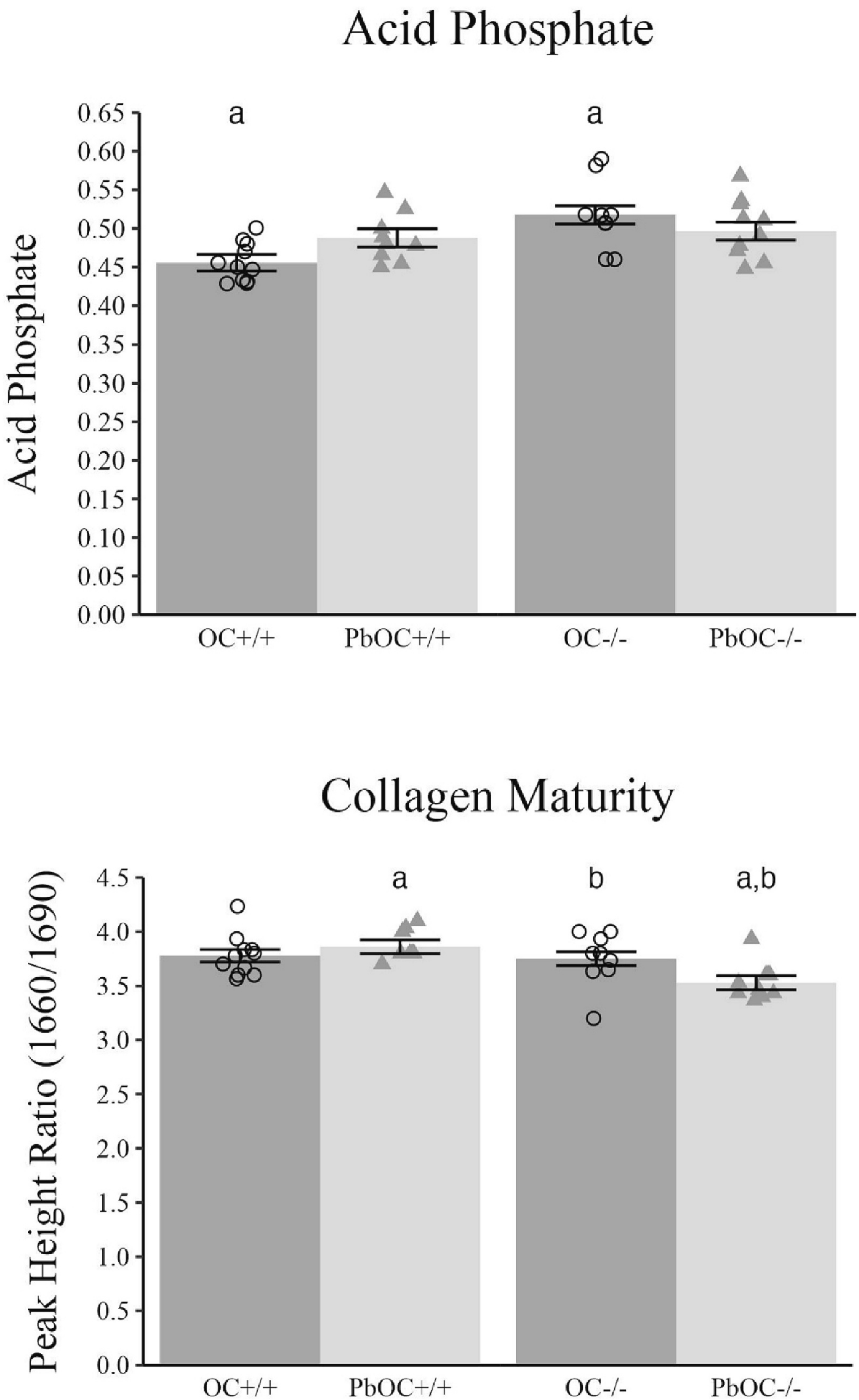


Fig. 2. (*continued*).

main effect of both lead and osteocalcin on the number of osteoblasts/bone perimeter (Table 3). The number of osteoblasts/bone perimeter was significantly increased without osteocalcin (OC−/− and PbOC−/−) vs. in the presence of osteocalcin (OC+/+ and PbOC+/+). The number of osteoblasts was also significantly increased in the presence of lead (PbOC+/+ and PbOC−/−) vs. in the absence (OC+/+ and OC−/−).

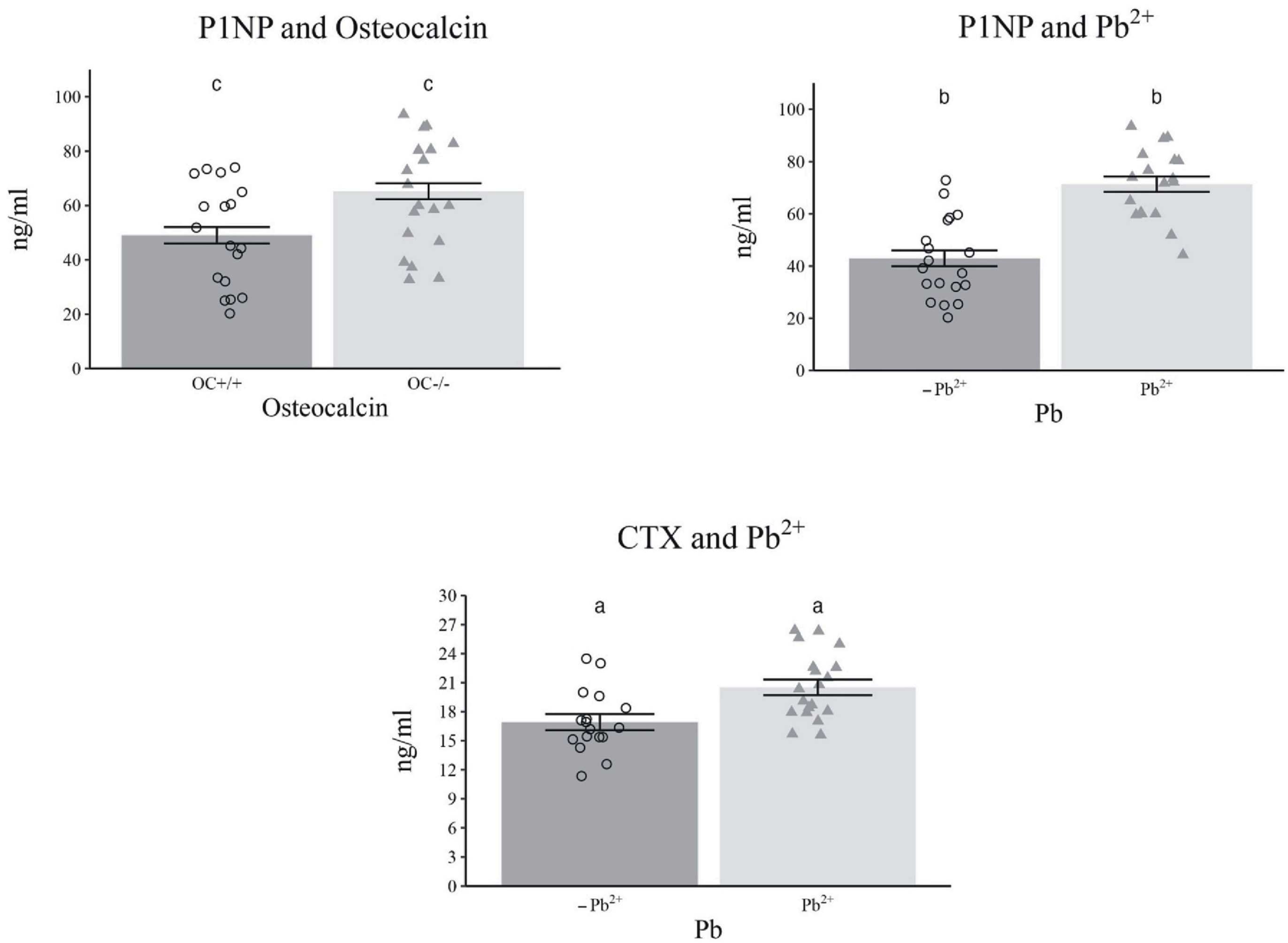


Fig. 3. The Manova gave a significant main effect for Pb^{2+} (Pillai's trace <0.001) and a significant main effect for osteocalcin (Pillai's trace = 0.001) for serum P1NP and CTX concentrations. Univariate tests showed both osteocalcin and Pb^{2+} had a significant main effect on P1NP. Only Pb^{2+} had a significant main effect on CTX. Model estimated means for these parameters were derived from the MANOVA and reported with the significance level. (OC+/+ refers to OC+/+ and PbOC+/+, OC−/− refers to OC−/− and PbOC−/−.) Groups labeled with the same letter are significantly different (a: $p = 0.004$, b: $p < 0.001$, c: $p < 0.001$).

Table 3
The MANOVA analysis reported a significant main effect of Pb^{2+} (Pillai's trace $p < 0.001$) and a significant main effect of osteocalcin (Pillai's trace $p < 0.001$) for the number of osteoblasts and osteoclasts. Univariate tests showed a significant main effect of Pb^{2+} for the number of osteoblasts/bone perimeter (no. OB/Bpm) and for the number of osteoclasts/bone perimeter (No. OC/Bpm) and a significant main effect of osteocalcin on the number of No. OB/bone perimeter as shown. The model estimated means and significance levels from the univariate analyses are presented. The estimated marginal means for bones with $+Pb^{2+}$ (PbOC+/+, PbOC−/−) or without $-Pb^{2+}$ (Ost+/+, Ost−/−) and the estimated marginal means for bones with (OC+/+, PbOC+/+) or bones without (OC−/−, PbOC−/−) osteocalcin. Groups with the same letter are significantly different (a: $p < 0.001$, b: $p < 0.001$, c: $p < 0.001$).

Parameter	OC+/+	OC−/−	$-Pb^{2+}$	$+Pb^{2+}$
No. OB/Bpm			39.0 ± 1.99^{a}	58 ± 1.99^{a}
No. OB/Bpm	41.7 ± 2.0^{b}	55.26 ± 2.0^{b}		
No. OC/Bpm			0.43 ± 0.091^{c}	1.55 ± 0.091^{c}
Sample size (N)	12	12	12	12

Bone resorption, as indicated by serum CTX concentration, was affected only by the presence of Pb^{2+}. Bones containing lead had significantly higher bone resorption (PbOC+/+ and PbOC−/−) than bones without lead (OC+/+ and OC-). There was also a significant main effect of number of osteoclasts per bone perimeter with lead (Table 3). The number of osteoclast/bone perimeter was significantly higher in the presence of lead (PbOC+/+ and PbOC−/−) vs. the absence (OC+/+ and OC−/−).

The results indicate a difference in bone turnover (formation and resorption) with lead depending on the presence or absence of osteocalcin. The increase in bone turnover (formation and resorption) with lead (PbOC+/+ and PbOC−/−) and the further increase in just formation without osteocalcin (OC−/− and PbOC−/−) suggests an imbalance in bone turnover in the PbOC−/− vs. PbOC+/+ bones.

We had previously reported that Pb^{2+} increased mineral bound osteocalcin on hydroxyapatite crystals in-vitro (Dowd et al., 2001). In this study we measured mineral bound osteocalcin from lead treated and control wild-type bones in-vivo and found mineral bound osteocalcin was not significantly altered with lead treatment (1.06 ± 0.28 vs. 1.06 ± 0.16 for control and lead treated respectively).

4. Discussion

This study is the first to investigate the role of osteocalcin in both mineral and biomechanical properties in female background matched mice in normal and abnormal bone physiology (lead toxicity). This study is also the first to investigate the role of osteocalcin in the presence of an agent that increases both bone formation and resorption (lead) (Monir et al., 2010). The bone remodeling parameters can help explain the results obtained when comparing the different groups of mice. The discussion of the results between the control OC+/+ and OC−/− were previously reported (Berezovska et al., 2019).

4.1. Wild-type control vs. lead treated wild-type

In this study we exposed female mice corresponding to young adult ages and showed that elevated lead had a detrimental effect on wild-type cortical bone. The bone remodeling parameters we measured can explain our results. As we observed previously in wild-type adult female mice (Monir et al., 2010), lead significantly increased bone formation and bone resorption leading to increased bone turnover. The increased marrow area/total area, decreased bone volume/total volume are consistent with an increase in bone resorption with lead vs. OC+/+ mineral. The increased bone formation may indicate newer matrix which didn't have time to mineralize or an increase in the amount of matrix deposited leading to a lower mineral to matrix ratio in the lead treated wild-type cortical mineral.

We had previously reported a 12 % decrease in trabecular bone density and a 2.7 % decrease in cortical bone mineral density with blood lead levels reflecting a moderate level of toxicity (blood lead 33 μg/dL) in female wild-type C57/BL6 mice at 6 months of age relative to controls (Monir et al., 2010). In the current study the blood lead levels are lower (18.2 ± 2.7 μg/dL and 13.2 ± 7.2 μg/dL for lead treated wild-type and knock-out respectively) and bone lead levels are lower. Data analysis at these lead levels showed bone mineral density depended on the presence of osteocalcin with no difference in cortical nor trabecular wild-type (OC+/+) bone mineral density with lead. We also previously reported a trend toward a decreased Maximum Load (bone strength) ($p = 0.07$) with lead due to significantly impaired bone material properties at moderate lead levels in wild-type bones (Monir et al., 2010). Although the current lower lead levels also produced a high bone turnover leading to detrimental effects (lower mineral/matrix ratio and BV/TV) we found that all bone strength parameters depended on the presence of osteocalcin alone and lead had no effect. This suggests possibly a longer exposure time at these lower blood lead levels may be needed to detect differences in the biomechanical parameters between OC+/+ and PbOC+/+ bone samples.

Previous animal studies have reported alterations in bone remodeling parameters or bone mineral densities with lead. Chronic lifetime low lead exposure in rats resulted in decreased bone mass and bone formation (Beier et al., 2013) which involved depression of Wnt signaling partly through elevation of sclerostin (Beier et al., 2013; Beier et al., 2015). Chronic exposure to lead at mid to high levels from birth to adolescence produced an increased bone density due to an increased bone formation rate and a decrease in bone resorption in female adolescent mice (Beier et al., 2016). We have shown increased bone remodeling (resorption and formation) at low to mid-level lead exposure from late adolescence to middle adult age female mice in this study as well as a previous study (Monir et al., 2010). Higher lead exposure in female rats from adult to older age produced an increase in bone resorption resulting in osteopenia (Brito et al., 2014). These studies show that the effect of lead depends on species, age of exposure and duration of exposure as well as lead level. Possibly lead effects bone resorption more when exposed at older ages as seen in the animal studies as well as in older humans (Potula et al., 2005). None the less, all reports show that lead has some detrimental effect on wild-type bone, regardless of age and duration of exposure, further emphasizing its toxicity.

4.2. Osteocalcin knock-out control vs. lead treated osteocalcin knock-out

Bone formation and bone resorption were significantly increased in lead exposed mineral (PbOC+/+ and PbOC−/−) versus the controls. The increased turnover is responsible for the increase in total volume, total area, and I_{min} by increased bone formation vs. control OC−/− bones. The increase in marrow area/total area with lead, due to increased bone resorption, would also play a role in the increased size and decrease in bone volume to total volume in cortical bone vs. OC−/−. The increased turnover with lead also contributed to the increase in trabecular total volume as well as a decreased trabecular bone volume to total volume. Since connectivity density is normalized to total volume, the connectivity density would be decreased in the PbOC−/− compared to the OC−/− bones as well.

Lead also significantly increased bone resorption over that of the control OC−/− mineral. This was corroborated by a significant lead induced increase in marrow area/total area which correlates with bone resorption. It is also consistent with the increased number of osteoclasts with lead. The increased remodeling leads to an improvement in some cortical bone mineral properties such as significant increases in bone mineral density, mineral to matrix ratio and crystallinity in the PbOC−/− cortical bone vs. the OC−/− bone. Since there was no significant difference in the mineral between the two groups the increase in mineral to matrix ratio must be due to a decrease in the amount of matrix in the PbOC−/− bone as a result of an increase in bone resorption. The increase in crystallinity in the PbOC−/− bone compared to the OC−/− could also be due to the increased bone resorption. Smaller crystals are dissolved first and are resorbed before larger crystals (Gourion-Arsiquaud et al., 2009).

All bone strength parameters: stiffness, size-adjusted stiffness, maximum moment and size adjusted maximum moment were found to be dependent on the presence of osteocalcin alone. Despite improving some bone mineral properties lead had no effect on bone strength at the lower lead concentrations used in this study. Therefore there is no significant difference in strength and stiffness between OC−/− and PbOC−/− bone. The lack of a difference in size-adjusted stiffness and maximum moment indicates the material properties were not significantly different between.

OC−/− and PbOC−/− mineral. The lower mineral to matrix ratio in OC−/− bones and reduced collagen maturity in PbOC−/− bones may contribute similarly to the bone strength. This is further support for a role of osteocalcin with bone strength even in the presence of low levels of a toxic agent.

4.3. Lead treated wild-type vs. lead treated osteocalcin knock-out

Most cortical bone mineral properties showed no difference between PbOC+/+ and PbOC−/− bones. Since lead had a significant main effect on total volume, BV/TV, total area, marrow area/total area there was no significant difference in these parameters whether osteocalcin was present or not. There was no significant difference in mineral to matrix ratio, crystallinity nor acid phosphate mineral content between the PbOC−/− and PbOC+/+ cortical bones.

Lead produced more detrimental effects in the knock-out cortical matrix and bone turnover as compared to PbOC+/+. The bone formation (P1NP) and resorption (CTX) were significantly increased by lead and the lack of osteocalcin additionally increased the bone formation rate in the PbOC−/− versus PbOC+/+ mineral. This is supported by an increase in I_{min} in PbOC−/− vs. PbOC+/+ bones as well as increases in osteoblast number without osteocalcin and in the presence of lead. Lead significantly reduced the collagen maturity (ratio of mature/immature collagen crosslinks) in the PbOC−/− bones compared to the PbOC+/+ bones. The first step in the formation of collagen crosslinks is the lysyl oxidase catalyzed conversion of lysyl or hydroxylysyl residues within collagen telopeptides to aldehydes. Condensation of the telopeptide aldehyde with a neighboring lysine or hydroxylysine residue produces a bivalent immature crosslink. In time the immature crosslinks are converted into trivalent mature crosslinks by further reaction with telopeptide aldehydes (Knott and Bailey, 1998). Since the PbOC−/− bones have a significantly higher bone formation rate compared to PbOC+/+ there would be more newly formed osteoid deposited with immature collagen crosslinks. Indeed a study using Raman spectroscopy revealed newly deposited bone had a lower collagen crosslink ratio indicating reduced collagen maturity (McNerny et al., 2015). Similarly, a reduction in immature collagen crosslinks was reported in osteoporotic bone where resorption exceeds formation (Oxlund et al., 1996).

Both stiffness and strength were significantly affected by the presence of osteocalcin and reduced in its absence (both OC−/− and PbOC−/−). Lead, at this lower concentration did not play a major role in strength reduction in PbOC−/− bones. However the reduction in collagen maturity and increased I_{min} in PbOC−/− vs. OC−/− or PbOC+/+ indicates the PbOC−/− bones are not proportionally stronger for their increased size due to poor material quality. Indeed reduced collagen maturity (McNerny et al., 2015) along with higher cross sectional area was shown to result in lower bone material quality (Bivi et al., 2012) and reduced strength (Oxlund et al., 1995). This negative effect on bone material qualities may explain why lead didn't reverse the effect of low osteocalcin on bone strength.

This study further supports the role of osteocalcin as a coupler of bone formation and resorption. We had previously confirmed the initial report indicating increase in bone formation with no effect on bone resorption in the OC−/− mineral (Ducy et al., 1996; Berezovska et al., 2019), suggesting osteocalcin plays a role in coupling. However a later report disagreed with this and found no difference in bone formation in the OC−/− vs. OC+/+ bones (Moriishi et al., 2020). This current study also shows an imbalance in bone turnover without osteocalcin, with greater formation, even in the presence of Pb^{2+}, a toxic agent which increases bone turnover. Osteocalcin may couple the two processes by inhibiting bone formation so it is more closely matched to resorption. An osteocalcin receptor found on human osteoblast cells was proposed to be involved in the inhibition of bone formation (Bodine and Komm, 1999).

Our previous study showed moderate levels of lead, in the presence of osteocalcin (PbOC+/+), produced a trend toward reduced strength and significantly reduced bone material properties (Monir et al., 2010). The current study showed lower lead levels had no significant effect on bone strength in bones containing osteocalcin (PbOC+/+). In the absence of osteocalcin (PbOC−/−) lower lead levels significantly reduced bone stiffness, strength and material properties due mainly to the reduction of osteocalcin in these bones. However, since our data shows PbOC−/− bones had impaired collagen maturity and reduced trabecular BV/TV compared to OC−/− it is quite possible that years of exposure to low lead or moderate levels of lead may have additional negative effects on bone strength and fractures at low osteocalcin levels seen in the elderly or in diabetics.

CRediT authorship contribution statement

Gozde Yildirim – sample collection, data collection, data analysis, assisted in manuscript preparation
William Budell - sample collection, data collection, data analysis
Olga Berezovska - data collection, data analysis
Sarah Yagerman - data collection, data analysis
Syeda Maliath – data analysis
Paul Mastrokostas - data analysis
S.M. Tommasini – data analysis, manuscript critical review
Terry L. Dowd – conception, data analysis, manuscript writing.

Declaration of competing interest

All authors declare they have no conflict of interest.

Data availability

After publication authors will share data upon request.

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Appendix A. Supplementary data

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