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### Authors

Conley, Travis  
Richardson, Cardius  
Pacheco, Juan  
et al.

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## Bone manganese is a sensitive biomarker of ongoing elevated manganese exposure, but does not accumulate across the lifespan

Travis E. Conley<sup>a</sup>, Cardius Richardson<sup>a</sup>, Juan Pacheco<sup>b</sup>, Neil Dave<sup>c</sup>, Thomas Jursa<sup>a</sup>, Stefano Guazzetti<sup>d</sup>, Roberto G. Lucchini<sup>d,e</sup>, Scott Fendorf<sup>b</sup>, Robert O. Ritchie<sup>c</sup>, Donald R. Smith<sup>a,\*</sup>

<sup>a</sup>Department of Microbiology and Environmental Toxicology, University of California, Santa Cruz, CA, 95064, USA

<sup>b</sup>Department of Earth System Science, Stanford University, Stanford, CA, 94305, USA

<sup>c</sup>Department of Materials Science & Engineering, University of California, Berkeley, CA, 94720, USA

<sup>d</sup>Department of Occupational and Environmental Medicine, University of Brescia, Spedali Civili 1, 25125, Brescia, Italy

<sup>e</sup>Department of Environmental Health, Florida International University, Miami, FL, 33139, USA

### Abstract

Studies have established associations between environmental and occupational manganese (Mn) exposure and executive and motor function deficits in children, adolescents, and adults. These health risks from elevated Mn exposure underscore the need for effective exposure biomarkers to improve exposure classification and help detect/diagnose Mn-related impairments. Here, neonate rats were orally exposed to 0, 25, or 50 mg Mn/kg/day during early life (PND 1–21) or lifelong through ~ PND 500 to determine the relationship between oral Mn exposure and blood, brain, and bone Mn levels over the lifespan, whether Mn accumulates in bone, and whether elevated

\*Corresponding author. University of California, Santa Cruz, Department of Microbiology and Environmental Toxicology, 1156 High Street, CA, 95064, USA. drsmith@ucsc.edu (D.R. Smith).

#### Credit author statement

Travis E. Conley: Formal analysis, Writing – original draft preparation, Writing – review & editing, Visualization. Cardius Richardson: Methodology, Formal analysis, Investigation. Juan Pacheco: Methodology, Formal analysis, Investigation. Neil Dave: Formal analysis, Investigation. Thomas Jursa: Formal analysis, Investigation. Stefano Guazzetti: Formal analysis. Roberto G. Lucchini: Conceptualization, Methodology, Investigation, Writing – review & editing. Scott Fendorf: Methodology, Supervision. Robert O. Ritchie: Methodology, Supervision. Donald R. Smith: Conceptualization, Methodology, Formal analysis, Writing – original draft preparation, Writing – review & editing, Visualization, Supervision.

#### Approval of human and animal studies

All human and animal studies were approved by the relevant Institutional Review Boards and Institutional Animal Use and Care Committee and adhered to institutional guidelines. Further information on this approval is described in Sections 2.1 and 2.3, as well as the Italian ethics committee approval documents for human studies included with this submission.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envres.2021.112355>.

bone Mn altered the local atomic and mineral structure of bone, or its biomechanical properties. Additionally, we assessed levels of bone Mn compared to bone lead (Pb) in aged humans (age 41–91) living in regions impacted by historic industrial ferromanganese activity. The animal studies show that blood, brain, and bone Mn levels naturally decrease across the lifespan without elevated Mn exposure. With elevated exposure, bone Mn levels were strongly associated with blood Mn levels, bone Mn was more sensitive to elevated exposures than blood or brain Mn, and Mn did not accumulate with lifelong elevated exposure. Elevated early life Mn exposure caused some changes in bone mineral properties, including altered local atomic structure of hydroxyapatite, along with some biomechanical changes in bone stiffness in weanlings or young adult animals. In aged humans, blood Mn ranged from 5.4 to 23.5 ng/mL; bone Mn was universally low, and decreased with age, but did not vary based on sex or female parity history. Unlike Pb, bone Mn showed no evidence of accumulation over the lifespan, and may not be a biomarker of cumulative long-term exposure. Thus, bone may be a useful biomarker of recent ongoing Mn exposure in humans, and may be a relatively minor target of elevated exposure.

## Keywords

Manganese; Developmental exposure; Biomarker; Bone; Accumulation

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## 1. Introduction

Environmental manganese (Mn) exposure is a growing public health concern in the U.S. and other countries due to expanding evidence that children may be exposed to harmful levels of Mn exposure from multiple sources, including drinking water (Bouchard et al., 2007, 2011; Wasserman et al., 2006), soil and dust (Gunier et al., 2014a, 2014b; Lucchini et al., 2012a), and their diet (Crinella, 2012). Recent epidemiological studies have shown that elevated Mn exposure is associated with reductions in Full Scale IQ and verbal comprehension, along with impaired attention, impulse control, and fine motor function in children and adolescents (Bouchard et al., 2011; Ericson et al., 2007; Mora et al., 2018; Oulhote et al., 2014). Occupational exposures from mining, dry-cell battery production, and ferromanganese alloy plants have also been associated with adverse health outcomes in adults, including Mn-induced parkinsonism and other neurodegenerative conditions (Kwakye et al., 2015; Long et al., 2014; Lucchini et al., 1999; Smith et al., 2007).

The improved understanding of the health risks from elevated Mn exposure have led to an increased need for effective exposure biomarkers to help detect and diagnose Mn-related impairments, especially given that children are more vulnerable to elevated exposures and the neurotoxic effects of Mn than adults, and exposure risk changes over the lifespan (Erikson et al., 2007; Kern et al., 2010; Ljung and Vahter, 2007; Mora et al., 2015a). Reported biomarkers for Mn exposure have included blood, hair, saliva, urine, nails, and teeth (Arora et al., 2012a; Butler et al., 2019; Claus Henn et al., 2010; Gil et al., 2011; Haynes et al., 2015; Laohaudomchok et al., 2011; Lucchini et al., 2012a; Mora et al., 2015a, 2018; Oulhote et al., 2014; Ward et al., 2018). Blood and urine Mn levels appear to reflect only recent exposures over the span of several days to weeks (Cowan et al., 2009; Järvisalo et al., 1992; Smith et al., 2007), while hair and nail Mn levels have been reported

to reflect exposures on the scale of ~3–12 months (Eastman et al., 2013; Jursa et al., 2018; Laothaudomchok et al., 2011; Reiss et al., 2015; Ward et al., 2018). Teeth or skeletal Mn levels may reflect exposures over longer periods of months to years, based on recent studies (Arora et al., 2012b; Austin et al., 2017; Claus Henn et al., 2018; Horton et al., 2018; Rolle-McFarland et al., 2018).

The extent that candidate Mn exposure biomarkers are associated with adverse health outcomes, such as cognitive and behavioral deficits, is mixed (Haynes et al., 2015; Lucchini et al., 1999, 2012a; Mora et al., 2015a; Smith et al., 2007). For example, some studies have reported associations between Mn levels in hair, blood, and teeth with cognitive or behavioral impairments (Haynes et al., 2015; Menezes-Filho et al., 2011; Mora et al., 2015a), while others have reported no association between blood and urinary Mn with health outcomes (Lucchini et al., 1999, 2012b; Smith et al., 2007). In the case of tooth Mn levels, recent studies suggest that higher prenatal dentine Mn levels are associated with improved visual spatial abilities, impulse control, and attentional function, whereas higher postnatal dentine Mn levels are associated with no, or adverse neurobehavioral effects, in children depending on age, sex, and outcome (Bauer et al., 2017; Claus Henn et al., 2018; Horton et al., 2018; Mora et al., 2015a). These differences across studies regarding the extent that the Mn exposure biomarker(s) are associated with adverse health effects may result in part from exposure misclassification, further underscoring the need for an improved understanding of Mn exposure and exposure biomarkers over the lifespan.

Bone Mn represents a potential biomarker of long-term Mn exposure, as Mn levels have been shown to increase in bone during developmental periods, and Mn in bone has been estimated to account for roughly 40% of body Mn (Andersen et al., 1999; Aschner and Aschner, 2005; O'Neal et al., 2014). The basis for Mn incorporation into bone mineral may be due in part to Mn<sup>2+</sup> serving somewhat as a biologic analog to Ca<sup>2+</sup> (Frausto da Silva and Williams, 2001). This Mn<sup>2+</sup> - Ca<sup>2+</sup> relationship in mineralized tissues may be similar to the well-established relationship between Pb<sup>2+</sup> and Ca<sup>2+</sup>, which leads to the accumulation of lead (Pb) in mineralized tissues and the utility of bone and tooth Pb levels as biomarkers of cumulative Pb exposure (Hu, 1998; Smith et al. 1996; Téllez-Rojo et al. 2004; Specht et al. 2016; Arora et al. 2012b). Moreover, recent technological advances have led to the development of portable neutron activation systems for *in vivo* assessment of bone Mn levels in humans (Liu et al., 2013; Pejovi -Mili et al., 2009; Rolle-McFarland et al., 2018), suggesting the emerging feasibility of assessing bone Mn levels as an exposure biomarker to complement other tissue measures currently in use. Additionally, the fact that Mn is an essential nutrient that plays a role in skeletal development and maintenance, while Pb serves no essential biological function, may inform differences in bone-Pb vs. bone-Mn interactions (Andersen et al., 1999; Aschner and Aschner, 2005; O'Neal et al., 2014). If Mn accumulates in bone with elevated exposure, similar to Pb, bone Mn may prove to be an informative biomarker to assess Mn body burden over the lifespan.

Here, we investigated the relationship between oral Mn exposure and tissue Mn levels over the lifespan in a rodent model of early postnatal vs. lifelong Mn exposure. Specifically, we determined whether Mn accumulates in bone over the lifespan, the extent that bone Mn levels were associated with elevated Mn levels in the brain, and whether elevated

bone Mn altered the mineral structure or physical properties of bone. We also assessed the levels of skeletal Mn in aged humans living in regions impacted by historic ferromanganese alloy plant activity to determine typical bone Mn levels in environmentally-exposed adults. Finally, regarding accumulation of Mn into bone over the lifespan and the utility of bone Mn as an integrative biomarker of Mn exposure, we define the term “accumulation” as a net increase in bone Mn levels over time with steady-state exposure. Collectively, these findings further elucidate the impact of Mn exposure on bone tissue across the lifespan and establish the potential benefits of using bone as a biomarker of Mn exposure.

## 2. Materials and methods

### 2.1. Rodent subjects

All subjects were born in-house from nulliparous timed-pregnant Long Evans rats (obtained from Charles River on gestational age 18 d). Twelve to 24 h after parturition (designated PND 1, birth = PND 0), litters were sexed, weighed, and culled to eight pups per litter such that each litter was composed of five to six males and the remainder females. Only one male per litter was assigned to a particular Mn treatment condition. Animals (dams and weaned pups) were fed Harlan Teklad rodent chow #2018 (reported by the manufacturer to contain 118 mg Mn/kg) and housed in polycarbonate cages at a constant temperature of  $21 \pm 2$  °C. At PND 22, all pups were weaned and pair-housed (two rats per cage) with an animal of the same Mn treatment group and maintained on a reversed 10:14 h light/dark cycle. Animals reported in the present study were littermates of animals that underwent behavioral testing for attentional, impulse control, and fine motor functions over ~PND 30–120 (animals sacrificed on PND 24 and 66), or were the behaviorally tested animals that were sacrificed following microdialysis measurement of brain neurotransmitter levels prior to sacrifice (median PND 490, range PND 292–889); findings from the behavioral and microdialysis studies are reported elsewhere (Beaudin et al., 2015, 2013; Beaudin et al., 2017a,b; Beaudin et al., 2017a,b; Lasley et al., 2020). Males were exclusively used because studies have suggested that males may be more sensitive than females to developmental Mn neurotoxicity (Kern et al., 2010; Lucchini et al., 2012a; Takser et al., 2003), and attentional dysfunction is two to three times more prevalent in boys than girls (Feldman and Reiff, 2014; Willcutt, 2012). All animal procedures were approved by the institutional IACUC (protocols Smitd0912 and 234193) and adhered to National Institutes of Health guidelines set forth in the *Guide for the Care and Use of Laboratory Animals*. Criteria for exclusion of animals from the study were based on overt signs of poor animal health, including loss of body weight, absence of grooming, impaired function, and death; no animals were excluded from the study based on these criteria.

### 2.2. Manganese exposure protocol

Neonatal rats were orally exposed to Mn doses of 0, 25, or 50 mg Mn/kg/d starting on PND 1 through weaning on PND 21 (early postnatal Mn exposure), or throughout life until the end of the study. For dosing over PND 1–21, Mn was delivered once daily directly into the mouth of each pup (~20 µL/dose) via a micropipette fitted with a flexible polyethylene pipet tip (Fisher Scientific, Santa Clara, CA, USA). Control animals received the vehicle solution. For the Mn dosing solution, a 225 mg Mn/mL stock solution of MnCl<sub>2</sub> was prepared by

dissolving  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  with Milli-Q™ water; aliquots of the stock solution were diluted with a 2.5% (w/v) solution of the natural sweetener stevia to facilitate oral dosing of the pups. Oral Mn exposure post-weaning (PND 22 – end of study) occurred via the animals' drinking water. For this, a 42 mg Mn/mL stock Mn solution was prepared as above and diluted with tap water to a final concentration of 420  $\mu\text{g}$  Mn/mL in a polycarbonate carboy. The stock solutions were made fresh weekly, and water bottles were refilled with fresh water two to three-times per week. Water bottle weights were recorded at refilling to determine water intake per cage, and daily Mn intake per kg body weight was estimated based on daily measured body weights of the two rats housed per cage. Drinking water Mn concentrations were adjusted weekly as needed to maintain target daily oral Mn intake levels of 25 or 50 mg/kg/d based on measured water intake rates. This Mn exposure regimen is relevant to children exposed to elevated Mn via drinking water, diet, or both; pre-weaning exposure to 50 mg Mn/kg/d produces a relative increase in Mn intake that approximates the increase reported in infants and young children exposed to Mn-contaminated water or soy-based formulas (or both) (Beaudin et al., 2015, 2013; Beaudin et al., 2017a,b; Kern et al., 2010; Kern and Smith, 2011). Chronic oral exposure to the same daily Mn dose was maintained after weaning via drinking water to model the situation where children may continue to suffer chronic elevated Mn exposures from a variety of environmental sources (e.g., contaminated well water, dust, etc.) (Bouchard et al., 2011; Lucas et al., 2015; Oulhote et al., 2014). Our prior studies have shown that this Mn exposure regimen did not produce any overt signs of Mn toxicity, or changes in body iron status, based on blood hematocrit levels at PND 24 and 66 and blood, plasma, or brain tissue iron levels (Beaudin et al., 2013, 2017a; Kern et al., 2010; Kern and Smith, 2011).

### 2.3. Human subjects

Forty-nine subjects (30 female, 19 male) scheduled to undergo hip joint replacement due to hip osteoarthritis or femur head fracture (International Classification of Disease codes M16 and S72, respectively) consented to provide a bone and blood sample for metal analysis. The subjects resided in one of three geographically distinct sites within the province of Brescia, Italy: Vallecamonica, an area with historical ferromanganese alloy production for over a century that ended in 2001; Bagnolo Mella, an area with currently active ferromanganese alloy industrial activity since 1974; or Garda Lake, a tourist region with no history of ferromanganese alloy activity (Lucchini et al., 2007). The mean age of participants was  $82.1 \pm 9$  years for females and  $74.0 \pm 10$  years for males. Intact femoral head bone samples were collected from all 49 subjects, while 44 blood samples were collected several days before surgery (26 female, 18 male). In addition, parity history was obtained from all female subjects via questionnaire. The study was approved by the ethical committee of the responsible local health authority or hospital ("ASL Vallecamonica – Sebino," "Comitato Etico dell'Azienda Ospedaliera di Desenzano del Garda" and "Comitato Etico dell'Azienda Ospedaliera Spedali Civili di Brescia") and informed consent was obtained from each subject prior to sample collection.

### 2.4. Sample collection

Rat blood, brain, and bone tissues for Mn analyses were collected from PND 24, PND 66, and ~PND 490 rats ( $n = 10$ –16/treatment group and time point), as reported in Beaudin

et al. (2013, 2015, 2017a). Briefly, animals were euthanized via sodium pentobarbital overdose (75 mg/kg intraperitoneal injection) and exsanguination, and whole blood (2–3 mL) was collected from the left ventricle of the surgically-exposed heart and stored in EDTA Vacutainers at  $-20^{\circ}\text{C}$  for analyses. Whole brain was immediately removed, bisected into hemispheres, and the hind-brain regions of each hemisphere collected and stored at  $-80^{\circ}\text{C}$  for Mn concentration determinations (forebrain was dedicated to other outcome measures). The right and left femur was dissected free of the hindlimb and adherent soft tissue and periosteum removed with a stainless-steel scalpel.

Human bone samples of the intact femoral head removed during hip arthroplasty were stored at  $-20^{\circ}\text{C}$  in sterile polyethylene containers until processing for analyses. There was no visible sign of bone degeneration in any of the bone samples. Whole blood samples were collected with butterfly catheters into trace metal free Vacutainers. Within a HEPA filtered-air laboratory, bone-core samples containing subchondral and trabecular regions were obtained by drilling through the center of the femur head (anterior-posterior axis) using a custom-fabricated, hollow titanium alloy drill bit (5.4 mm internal diameter), as reported elsewhere (Smith et al., 1996).

## 2.5. Blood, brain, and bone tissue analyses for metal concentrations

Within a trace metal clean HEPA filtered-air laboratory, aliquots of rat or human whole blood were digested overnight at room temperature with 16 N  $\text{HNO}_3$  (Optima grade, Fisher Scientific), followed by addition of  $\text{H}_2\text{O}_2$  and Milli-Q water. Digestates were centrifuged ( $13,000\times g$  for 15 min) and the supernatant collected for Mn analysis. For rat brain, aliquots of homogenized hind-brain tissue ( $\sim 200$  mg wet weight) were dried to a constant weight at  $65^{\circ}\text{C}$  then digested with hot 16 N  $\text{HNO}_3$ , evaporated and redissolved in 1 N  $\text{HNO}_3$  for analyses. For rat bone, the right femur was bisected and any blood/bone marrow within the femur shaft was removed and the bone rinsed with ultrapure Milli-Q water. For human bone samples,  $\sim 1$ – $2$  mm thick sections of the femoral head bone cores were dissected from the cores using a stainless-steel scalpel to obtain analytical samples from within the bone core. Both rat and human bone samples were rinsed repeatedly with 1% quartz-distilled  $\text{HNO}_3$  and ultrapure water, dried to a constant weight at  $65^{\circ}\text{C}$ , then digested with hot 16 N  $\text{HNO}_3$ , evaporated and redissolved in 1 N  $\text{HNO}_3$  for analyses. For Mn and Pb (human samples only) analyses, rhodium and thallium (human samples only) were added to sample aliquots as internal standards, and Mn and Pb levels determined using a Thermo Element XR inductively coupled plasma – mass spectrometer in low (Pb) or medium (Mn) resolution, measuring masses  $^{55}\text{Mn}$ ,  $^{208}\text{Pb}$ ,  $^{103}\text{Rh}$ , and  $^{205}\text{Tl}$  (the latter two for internal standardization). External standardization for Mn and Pb used certified SPEX standards (Spex Industries, Inc., Edison, NJ). National Institutes of Standards and Technology SRM 1577b (bovine liver) and 1486 (bone meal) were used to evaluate procedural accuracy. The analytical detection limit for Mn in blood, brain, and bone was 0.018, 0.015, and 0.003 ng/mL, respectively, while the analytical detection limit for Pb in human bone was 0.005 ng/mL.

## 2.6. Synchrotron-based analyses using XRD, XANES, and EXAFS

Bone samples from rat femurs were analyzed at the Synchrotron Radiation Laboratory, Stanford University. X-ray diffraction (XRD) measurements were performed at an energy of

17,600 eV on Beamline 7–2. Samples were pulverized and set into a 0.3 mm quartz capillary tube. XRD patterns were collected in  $Q(A^{-1})$  space up to  $Q = 8 A^{-1}$ . X-ray absorption spectroscopy (XAS) experiments, including X-ray absorption near edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) were performed at beamline 11–2 in fluorescence mode with a 100-element Ge detector. Whole sections of the femur specimens were placed between two layers of Kapton tape and inside an aluminum holder. Samples were placed inside an environmental chamber with a continuous He gas flow to avoid scattering and absorption by air. Raw data treatment and quantitative analyses were performed using the Demeter platform (Ravel and Newville, 2005).

## 2.7. Biomechanical testing of bone strength properties

Biomechanical testing was performed on PND 66 rat femurs, which were hydrated by soaking in 37 °C Hanks' Balanced Salt Solution, HBSS; Sigma-Aldrich) for 12 h prior to testing. Each specimen was subjected to a three-point bending test; the bone was loaded such that the posterior surface was under tension and the anterior surface was under compression, using an MTS 831 electro-servo-hydraulic test machine (MTS Corp., Eden Prairie, MN). Each femur was loaded to failure at a displacement rate of 0.01 mm/s, and the load and displacement measured, the former using a calibrated 225 N load cell. After testing, a two-point average of the diameter and a six-point average of the cortical shell thickness were measured at the fracture site of each tibia using digital calipers with a 0.01 mm readout. The peak load (N) was recorded from the maximum load in each test. The corresponding yield and ultimate strengths of the central femurs ( $\sigma$ ) were calculated, in units of Pa, from the standard equation for a beam in three-point bending:

$$\sigma = \frac{PLy}{4I}$$

where respectively,  $P$  is the load at yielding (i.e., at the onset of inelastic deformation) or the maximum load reached during the bending test;  $L$  is the major span between the loading support pins;  $y$  is the distance from the center of mass; and  $I$  is the moment of inertia of the cross-section. The stiffness was measured in terms of the initial elastic slope of the load-displacement curve. In addition, the toughness (work to failure,  $W_f$ ) was calculated from the load-displacement curve as the work to fracture (energy absorption); specifically,  $W_f$  was defined (in units of  $\text{kJ/m}^2$ ) as the area under the load-displacement curve divided by twice the projected area of the fracture surface. All tests were done blinded to experimental treatment condition.

## 2.8. Statistical analysis and experimental design

Blood and brain Mn level data were analyzed using a one-way analysis of variance (ANOVA) and Tukey's *post hoc* test for pairwise comparisons. Data were log10 transformed before analysis if necessary to achieve normal distribution and variance homogeneity. In all cases, the significance level was set at  $p < 0.05$ . Tissue biomarker correlations were evaluated by generating Pearson's correlations between blood, brain, and bone across all ages and treatment groups. The slope from each Pearson's correlation output was used as the measure for comparison. For the human bone Mn and Pb data, linear regression models



were used to assess the relationship between bone metal levels and subject age, gender, and parity history. All analyses were performed with JMP Pro 15 Statistical Discovery software (SAS Institute) or R version 4.0.5 (R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>).

### 3. Results

#### 3.1. Tissue Mn decreases across the lifespan without elevated Mn exposure

In order to determine how natural changes in tissue Mn levels across the lifespan, in the absence of elevated Mn exposure, might affect tissue Mn levels in the presence of elevated exposure, we first assessed the concentrations of blood, brain, and bone Mn at PND 24, 66, and ~500 in control rats. Overall, Mn levels in all measured tissues decreased from early post-weaning life through adulthood, with reductions in tissue Mn most pronounced between PND 24 post-weaning and PND 66 young adulthood (Fig. 1a). This was evidenced by a main effect of age on blood Mn [ $F(2, 37) = 430, p < 0.0001$ ], reflecting significant differences between PND 24, 66, and ~500 groups ( $p$ 's  $< 0.0001$ ). The mean blood Mn concentration naturally decreased ~60% from PND 24 ( $24.2 \pm 0.79$  ng/mL) to PND 66 ( $9.76 \pm 0.28$  ng/mL), and decreased an additional ~40% from PND 66 to PND ~500 ( $5.76 \pm 0.28$  ng/mL), for an overall reduction of 76% from PND 24 to ~500 (Fig. 1a).

For brain Mn levels there was also a significant main effect of age [ $F(2, 34) = 145, p < 0.0001$ ], reflecting a significant reduction of ~40% between PND 24 ( $3.61 \pm 0.12$   $\mu\text{g/g}$ ) and PND 66 ( $2.13 \pm 0.031$   $\mu\text{g/g}$ ) ( $p < 0.0001$ ). Thereafter, there was a small non-significant decrease of ~8% from PND 66 to PND 500 ( $1.95 \pm 0.063$   $\mu\text{g/g}$ ) ( $p = 0.17$ ). Overall, mean brain Mn levels decreased by 46% from PND 24 to ~500 ( $p < 0.0001$ ) (Fig. 1b). Natural age-related changes in bone Mn levels showed a similar pattern as brain, with a significant main effect of age [ $F(2, 44) = 63.2, p < 0.0001$ ] that was driven by a significant reduction of ~75% from PND 24 ( $2.66 \pm 0.30$   $\mu\text{g/g}$ ) to PND 66 ( $0.65 \pm 0.062$   $\mu\text{g/g}$ ) ( $p < 0.0001$ ), but no further reduction from PND 66 to ~500 ( $0.59 \pm 0.064$   $\mu\text{g/g}$ ) ( $p = 0.95$ ). Overall, bone Mn levels significantly declined by ~78% from PND 24 to PND ~500 ( $p < 0.0001$ ) (Fig. 1c).

#### 3.2. Tissue Mn levels do not accumulate across the lifespan in the presence of elevated Mn exposure

To determine how tissue Mn levels change, and in particular whether Mn accumulates across the lifespan in the presence of continued elevated oral exposure, we measured Mn concentrations in blood, brain, and bone in weanling (PND 24), young adults (PND 66), and aged adults (~PND 500) exposed to different levels of Mn (0, 25, or 50 mg Mn/kg/day) for different exposure durations (early life from PND 1–21, or lifelong from PND 1 through PND 66 or ~PND 500).

**3.2.1. Comparison between Mn exposure groups within age groups**—First, to determine how Mn exposure restricted to the pre-weaning period or continuous throughout life affects tissue Mn levels at different life stages, we performed statistical analyses on tissue Mn levels within the post-weaning, young adult, and aged adult age groups. There

was a significant main effect of oral Mn exposure to increase blood, brain, and bone Mn levels at each of the three lifestages (ANOVA results, Supplemental Table 1). Specifically, at PND 24, blood, brain, and bone Mn levels in the early life 25 and 50 groups were significantly higher than controls ( $p$ 's < 0.0001, Fig. 2, lowercase superscripts). However, when comparing the differences between the 25 and 50 Mn dose groups at PND 24, only blood Mn levels in the early 50 group were significantly higher than the early 25 ( $p = 0.044$ ), while brain and bone levels in these two groups trended towards being significantly different ( $p = 0.066$  and  $p = 0.065$ , respectively). In the PND 66 young adult animals, all four Mn exposure groups were significantly elevated vs. controls for blood ( $p$ 's < 0.048) and bone ( $p$ 's < 0.001), while for brain only the early life 50 and lifelong 25 and 50 groups were higher than controls ( $p$ 's < 0.024; early life 25 vs controls,  $p = 0.86$ ). At the ~PND 500 aged adult life stage, differences between the lifelong and early life exposure groups became apparent, relative to controls, with the lifelong 25 and 50 groups being significantly elevated over both controls and their early life exposure group counterparts for blood ( $p$ 's < 0.012) and bone ( $p$ 's < 0.047). For brain, only the lifelong 25 and 50 groups were significantly different than controls ( $p$ 's < 0.0001).

### **3.2.2. Tissue Mn levels decline from weaning to young adulthood, even in the presence of ongoing elevated Mn exposure**

**3.2.2.1. Comparison within tissues across ages.:** Next, we determined how exposure impacts tissue Mn levels in each tissue (blood, brain, and bone) *across* the lifespan. Initially, we performed ANOVA to assess how early life Mn exposure over PND 1–21 impacts tissue Mn levels in PND 24 weanling versus PND 66 young adult animals. There was a significant main effect of age on tissue Mn levels for blood [ $F(5, 81) = 279, p < 0.0001$ ], brain [ $F(5, 80) = 91.5, p < 0.0001$ ], and bone [ $F(5, 81) = 154, p < 0.0001$ ], largely reflecting the much higher tissue Mn levels in PND 24 weanling animals compared to their PND 66 counterparts (Fig. 2, bars with \*). The interaction of age x Mn exposure group was also significant for all three tissues ( $p$ 's < 0.0001), reflecting that differences in tissue Mn levels between Mn exposure groups were significant for the PND 24 versus PND 66 animals exposed over PND 1–21. For example, blood Mn levels in PND 66 animals exposed to 25 or 50 mg Mn/kg/day over early life (PND 1–21) were significantly lower than their PND 24 counterparts ( $p$ 's < 0.0001). This same pattern was observed for brain and bone, which similarly showed significant reductions in tissue Mn concentrations in PND 66 versus PND 24 animals following early life Mn exposure ( $p$ 's < 0.0001).

Subsequently, we performed ANOVA to determine how continued lifelong exposure affected tissue Mn levels in PND 66 young adults compared to their PND 24 counterparts. For this analysis, only the lifelong Mn exposure groups at PND 24 and 66 were included. There was again a significant main effect of age on tissue Mn levels for blood [ $F(5, 81) = 250, p < 0.0001$ ], brain [ $F(5, 81) = 87.4, p < 0.0001$ ], and bone [ $F(5, 81) = 145, p < 0.0001$ ], as well as a significant age x Mn exposure group interaction ( $p$ 's < 0.0001 for all three tissues). These results reflect that there were significant reductions in tissue Mn levels from PND 24 to PND 66, despite the continuous oral Mn exposure over this time ( $p$ 's < 0.0001) (Fig. 2, bars with \*).

### 3.2.3. Tissue Mn levels decline from young to aged adulthood, even in the presence of ongoing elevated oral Mn exposure

**3.2.3.1. Comparison within tissue across ages.:** In light of the substantially greater impact of oral Mn exposure to increase tissue Mn levels in PND 24 weanlings versus their PND 66 counterparts, and specifically to determine whether prolonged oral Mn exposure resulted in the accumulation of higher tissue Mn levels in aged versus young adults, we performed subsequent analyses specifically comparing tissue Mn concentrations in the PND 66 young adult and ~PND 500 aged adult animals exposed to oral Mn throughout their lifespan. The main effects of age and Mn exposure were again significant for blood [ $F(9, 157) = 13.8, p < 0.0001$ ], brain [ $F(9, 143) = 12.7, p < 0.0001$ ], and bone [ $F(9, 176) = 17.3, p < 0.0001$ ]. However, the age x Mn exposure interaction was only significant for brain ( $p = 0.034$ ) and bone ( $p = 0.0054$ ), but not blood ( $p = 0.14$ ). The significant age x Mn interaction for brain and bone tissues reflect the fact that Mn levels in these tissues of one or both of the early life Mn exposure groups in the aged ~ PND 500 animals were significantly lower than their PND 66 counterparts, while tissue Mn levels in the PND 66 and ~PND 500 continuous lifelong exposure groups were not measurably different. In contrast, the non-significant age x Mn exposure interaction for blood reflects that blood Mn levels measurably declined from PND 66 to ~PND 500 in both the early life and lifelong Mn treatment groups, even though the latter aged adult group continued to receive daily oral Mn exposure in their drinking water (Fig. 2, # indicating significantly different from PND 66 counterpart). In fact, when comparing tissue Mn concentrations in the continuous lifelong 25 and 50 Mn exposure groups at PND 66 versus ~500, tissue Mn levels in PND ~500 aged adult animals were either lower than (blood,  $p$ 's  $< 0.014$ ) or not statistically different from (brain, bone,  $p$ 's  $> 0.72$ ) their PND 66 counterparts (Fig. 2, bars with # reflect PND 500 groups that are significantly lower than their PND 66 counterpart of the identical Mn exposure group). Collectively, these findings show that continuous lifelong oral Mn exposure does not result in Mn accumulation in these tissues.

Notably, modest reductions in tissue Mn are evident between PND 66 and ~500 in the early life Mn groups that were exposed over PND 1–21. For example, blood Mn levels in the PND ~500 control, early life 25, and early life 50 groups were significantly lower than their PND 66 counterparts ( $p = 0.041$  for controls,  $p$ 's  $< 0.009$  for the Mn groups). In slight contrast, brain Mn levels in the early life 50 ~PND 500 aged adults were measurably lower than their PND 66 counterparts ( $p = 0.016$ ), while there was no measurable reduction between the young and aged adults in the control and early 25 Mn groups ( $p$ 's  $> 0.44$ ). For bone, Mn levels in the ~PND 500 early life 25 and 50 groups were measurably lower than in their PND 66 counterparts ( $p$ 's  $< 0.023$ ), but there was no difference between controls ( $p = 0.99$ ).

### 3.2.4. There is no relationship between bone Mn levels and age in aged adult animals exposed to lifelong elevated oral Mn—

In order to further address whether chronic oral Mn exposure results in the accumulation of Mn in bone tissue, we performed linear regression analyses between animal age and bone Mn level in the aged adult lifelong 25 and 50 Mn-exposed animals according to their age at sacrifice. The age range for the PND ~500 aged adult lifelong 25 (PND 388–617, median 490) and 50 (PND 273–624, median 486) Mn-exposed animals varied because animals were also used in microdialysis

studies of brain neurotransmitter release that spanned these prolonged intervals, as noted above (Lasley et al., 2020). Results show that there is no relationship between age and bone Mn level in either the continuous lifelong 25 ( $R = 0.091$ ,  $p = 0.24$ ) or lifelong 50 ( $R = 0.029$ ,  $p = 0.46$ ) Mn exposure groups, and neither regression slope is significantly different from zero ( $p$ 's  $> 0.23$ ), further substantiating that there is no evidence of Mn accumulation in bone tissue with prolonged elevated oral Mn exposure (Supplemental Fig. S1).

Overall, early pre-weaning life showed the greatest susceptibility to elevated oral Mn exposure, based on the highest tissue Mn levels at PND 24 for all tissues. Notably, the very elevated tissue Mn levels in PND 24 weanling animals decline significantly with age, especially between early post-weaning and young adulthood, even in the presence of continuous oral Mn exposure. However, there remains a significant relationship between elevated ongoing oral Mn dose and tissue Mn levels within the young adult and aged adult age classes, indicating that tissues Mn levels, and most notably bone Mn in adult animals, are measurably affected by ongoing oral Mn exposure. Finally, there is clear evidence that Mn does not accumulate in brain or bone tissue with prolonged lifelong exposure from young adulthood into aged adulthood.

### 3.3. Sensitivity of tissue Mn increases to oral Mn exposure

Based on the dose-response relationship between continuous lifelong oral Mn exposure and tissue Mn levels across the lifespan, we explored the association between tissue Mn levels in order to determine which of the three tissues is the most sensitive biomarker of ongoing oral Mn exposure. For this, we used Pearson's correlations of best-fit regressions between bone and blood, brain and blood, and bone and brain Mn levels, and the resultant regression fit (Pearson's  $R$  and associated  $p$ -value) and the regression sensitivity (slope) for specific comparisons. In order to compare regression parameters across tissues with inherently different Mn concentration units (i.e., ng/mL for blood vs.  $\mu\text{g/g}$  for brain and bone), values within a tissue and age group for each individual animal were normalized to their respective average control group value (i.e., %control), and these normalized values were then used in the regression analyses.

**3.3.1. Bone Mn is strongly associated with blood Mn levels—**Among all pairwise correlations made for each pair of tissues and treatment groups across ages, all of the correlations were statistically significant ( $p$ 's  $< 0.05$ ), except the relationship between brain and bone Mn for the lifelong exposure groups at PND 500 ( $p = 0.38$ ) (Fig. 3, bar with #). When comparing regression slopes of brain versus blood, bone versus blood, and brain versus bone for all ages and treatment groups, a pattern was observed in which the slopes of the bone versus blood linear regressions were notably steeper than the brain versus blood and brain versus bone regressions. For example, while both the bone versus blood and brain versus blood correlations are statistically highly significant ( $p$ 's  $< 0.0001$ ), the slopes of the bone versus blood regressions (e.g., 3.18 for PND 66) are substantially steeper compared to the brain vs. blood regressions slope (e.g., 0.1 for PND 66) (Fig. 3; Supplemental Figs. S2a and b, respectively), indicating a much greater relative increase in bone Mn for a given increase in blood Mn. These findings suggest that bone Mn levels may be a more sensitive biomarker of ongoing elevated oral Mn exposure than either blood or brain Mn.

### 3.4. Mn exposure and bone properties

**3.4.1. Elevated bone Mn does not alter the gross crystalline structure of bone mineral**—In light of the proportionally greater increase in bone Mn with elevated exposure compared to blood or brain Mn levels (i.e., in PND 24 animals a ~30-fold increase in bone Mn between the control and 50 mg Mn/kg/d exposure groups, whereas blood and brain Mn levels increased by ~10-fold and ~3.6-fold, respectively), and the very high levels of bone Mn attained in young weanling animals (i.e., ~80 µg/g or higher in animals exposed to 50 mg Mn/kg/d over PND 1–21), the possibility exists that elevated Mn exposure may affect the properties of bone mineral during skeletal growth, as has been shown to occur with elevated Pb exposures (Álvarez-Lloret et al., 2017; Beier et al., 2016; Monir et al., 2010). To explore this, we used X-ray diffraction (XRD) analysis to identify the gross crystalline structure properties of femur bone mineral in PND 24 rats. For analysis, a single femur sample was selected from each of the control, 25 and 50 mg Mn/kg/day groups with measured bone Mn levels in the alternate femur from the same animal of 2.4, 58, 166 µg/g, respectively. All three samples yielded XRD spectra comparable to the hydroxyapatite standard, indicating no significant alteration in mineral particle size and gross crystalline structure of the bone mineral in the Mn-exposed animals (Supplemental Fig. S3).

**3.4.2. At elevated levels, Mn in bone exists as Mn<sup>2+</sup>**—While Mn is known to exist in the Mn<sup>2+</sup> and Mn<sup>3+</sup> valence states within vertebrate organisms, the vast majority of cellular Mn is present as Mn<sup>2+</sup> (Gunter et al., 2006; Reaney et al., 2002, 2006). Mn<sup>2+</sup> is also known to serve somewhat as a biologic analog to Ca<sup>2+</sup> (Frausto da Silva and Williams, 2001). Thus, bone Mn may be expected to exist largely in the Mn<sup>2+</sup> valence state, though it is not known whether elevated Mn exposure may lead to incorporation of other valence states of Mn in bone, with possible implications for altered bone mineral structure. To address this, we used X-ray absorption near edge structure (XANES) analysis to determine whether Mn exposure altered the valence state of Mn in bone mineral in the same 25 and 50 mg Mn/kg/d bone mineral samples used for XRD analyses above. Results show that in control (2.4 µg Mn/g) and elevated bone Mn samples, Mn exists in the Mn<sup>2+</sup> valence state, with no detectable Mn<sup>3+</sup> or Mn<sup>4+</sup> (Supplemental Fig. S4).

**3.4.3. Elevated bone Mn alters the local atomic structure of the hydroxyapatite matrix**—Mn plays a role in many cellular processes throughout the body, and has the ability to form complexes with a variety of local atomic structures in multiple cell types (Aschner and Aschner, 2005; Geszvain et al., 2012). Given this, we explored whether elevated Mn in bone tissue may alter the local atomic structure of hydroxyapatite bone mineral, using extended X-ray absorption fine structure (EXAFS) analysis for the same elevated Mn bone sample (166 µg/g) used for the XRD and XANES analyses reported above. While the bulk X-ray diffraction analyses indicate no co-precipitation of Mn phases into the bone structure, analysis of the local structure through EXAFS indicates that Mn is substituting for Ca<sup>2+</sup>, as evidence by the Mn<sup>2+</sup>-Ca<sup>2+</sup> coordination into the hydroxyapatite structure (Table 1). The contraction of the distance between Ca<sup>2+</sup> and hydroxyapatite is due to the smaller atomic size of Mn<sup>2+</sup> relative to Ca<sup>2+</sup> (Supplemental Fig. S5).

**3.4.4. Elevated bone Mn may alter the physical properties of bone**—Based on the X-ray analyses above, it is possible that due to the incorporation of Mn into the bone matrix, elevated Mn exposure may alter the mechanical properties of bone resulting from potential effects on other aspects of bone metabolism, such as bone cell metabolism, organic matrix synthesis, etc. To address this, we conducted biomechanical analyses using an electro-servo-hydraulic test instrument to measure the mechanical properties of femurs in PND 66 rats from all five Mn exposure groups ( $n = 13\text{--}16/\text{group}$ ) (femurs from PND 24 rats were not fully mineralized and were unsuitable for biomechanical analysis). These analyses generated outcomes of stiffness, yield strength, ultimate strength, and work of fracture (Beier et al., 2016; Conti et al., 2012). Overall, there was a significant main effect of postnatal Mn exposure on femur stiffness [ $F(4, 69) = 4.91, p = 0.0016$ ]. Specifically, there was a significant increase in femur stiffness in the early life 25 and 50 Mn groups, compared to controls (2283 and 2199 MPa vs. 1489 MPa, respectively;  $p$ 's  $< 0.0035$ ). In contrast, lifelong Mn exposure caused a trending, but statistically non-significant increase in femur stiffness in the lifelong 50 group (1988 MPa), relative to controls ( $p = 0.093$ ), while there was a smaller non-significant increase in the lifelong 25 group (1975 MPa,  $p = 0.107$ ). There were no measurable differences in femur stiffness between the early and lifelong 25 and 50 Mn exposure groups in the PND 66 animals ( $p$ 's  $> 0.52$ ) (Fig. 4).

In addition to femur stiffness, we also evaluated other physical properties of bone, including yield strength, ultimate strength, and work of fracture, to determine whether they were altered by elevated bone Mn levels. There was no significant main effect of Mn exposure on yield strength [ $F(4, 67) = 0.43, p = 0.78$ ], ultimate strength [ $F(4, 66) = 1.84, p = 0.13$ ], or work of fracture [ $F(4, 67) = 0.78, p = 0.54$ ] (data not shown).

### 3.5. Bone Mn and Pb levels in aged humans

**3.5.1. Bone Mn levels do not differ by sex, but tend to decrease with age**—We measured bone and blood Mn and Pb levels in environmentally-exposed aged adults, since this life stage is under-represented in environmental exposure studies, and because they may further inform whether Mn accumulates in the human skeleton and hence the potential utility of bone as a biomarker of cumulative Mn exposure. Bone samples were obtained from 30 female and 19 male subjects, age 41–95, undergoing hip arthroplasty surgery; blood samples were collected from 44 of these subjects (26 female, 18 male). Subjects were lifelong residents of the Province of Brescia, Italy, which contains varying amounts of ferromanganese industrial activity that started in the early 1900s, therefore inducing early life and lifelong exposure for all participants. Blood Mn levels were  $9.76 \pm 3.88$  ng/mL in females (mean  $\pm$  SD, range 6.03–23.5 ng/mL), and  $9.49 \pm 3.12$  ng/mL in males (range 5.42–15.3 ng/mL). Bone Mn levels in these aged subjects were universally low, ranging from 0.014 to 0.17  $\mu\text{g/g}$  (mean  $0.038 \mu\text{g/g} \pm 0.024$  SD,  $n = 49$ ), and there was no relationship between blood and bone Mn levels. Bone Mn levels in males (mean  $0.046 \mu\text{g/g} \pm 0.035$  SD, median  $0.036 \mu\text{g/g}$ , range 0.017–0.17  $\mu\text{g/g}$ ,  $n = 19$ ) trended towards being slightly higher compared to females (mean  $0.034 \mu\text{g/g} \pm 0.013$  SD, median  $0.033 \mu\text{g/g}$ , range 0.014–0.069  $\mu\text{g/g}$ ,  $n = 30$ ) [ $F(1, 48) = 3.231, p = 0.079$ ]. Notably, one male subject who had a history of occupational metal exposure had a relatively high bone Mn concentration of 0.17  $\mu\text{g/g}$ . When this individual was excluded from the analysis, the statistically trending effect of

higher bone Mn in males was no longer present [ $F(1, 47) = 1.628, p = 0.208$ ]. While there was no effect of sex on bone Mn, there was a trending, but statistically non-significant effect of age ( $p = 0.073$ ), suggesting that bone Mn levels decrease by ~1% per year (sexes combined) over the age range of our study group (Fig. 5a).

Blood Pb levels in these subjects were  $38.2 \pm 23.2$  ng/mL in females (mean  $\pm$  SD, range 9.17–107 ng/mL), and  $81.6 \pm 57.8$  ng/mL in males (range 25.1–241 ng/mL). Using an ANCOVA-like linear model, we also assessed the effect of sex and age on bone Pb levels. Sex was a significant predictor of bone Pb levels in humans ( $\beta = 1.49, p < 0.0001$ ), with males (mean  $5.96 \mu\text{g/g} \pm 4.23$  SD, median  $5.04 \mu\text{g/g}$ , range 1.16–17.5  $\mu\text{g/g}$ ,  $n = 19$ ) having higher Pb concentrations than females (mean  $1.93 \mu\text{g/g} \pm 1.74$  SD, median  $1.33 \mu\text{g/g}$ , range 0.28–7.15  $\mu\text{g/g}$ ,  $n = 30$ ). The male with the highest bone Mn (0.17  $\mu\text{g/g}$ ) had a bone Pb level (5.76  $\mu\text{g/g}$ ) that was near the mean for males. Age was also a significant predictor of bone Pb ( $\beta = 0.031, p = 0.027$ ). Also, as with Mn, there was no significant relationship between blood and bone Pb levels. The overall model fit was  $R^2 = 0.45$  [ $F(1, 45) = 18.57, p < 0.0001$ ], showing that bone Pb levels in males are ~3.5-times higher than in females, and that overall bone Pb levels increase ~3.3% per year (Fig. 5b).

### 3.5.2. Parity history is not associated with altered bone Mn or bone Pb levels

—Prior studies have established a relationship between bone Pb levels and pregnancy/parity history in women (Gulson et al., 1997; Hernandez-Avila et al., 2000). Furthermore, maternal skeletal Pb is known to be remobilized during pregnancy and lactation (Franklin et al., 1997; Gulson et al., 1997, 2004; Manton et al., 2003). In light of this, we explored the relationship between parity history and bone Mn levels in aged females with zero ( $n = 3$ ), one ( $n = 5$ ), two ( $n = 6$ ), three ( $n = 7$ ), or more than three ( $n = 4$ ) children (parity information was unavailable for five female subjects) using linear regression analysis. Parity history had a non-significant, but statistically trending main effect on bone Mn levels ( $\beta = 0.092, p = 0.054$ ), that was largely influenced by a single female with seven children. Removing this individual resulted in a clearly non-significant regression coefficient ( $\beta = 0.098, p = 0.15$ ). Similarly, there was no relationship between parity history and bone Pb levels in females when analyzed assuming log-linearity, similar to Mn above ( $\beta = -0.11, p = 0.94$ ).

## 4. Discussion

Our animal model findings demonstrate that Mn levels in blood, brain, and bone decline naturally with age in the absence of elevated exposure, and do not accumulate in the presence of prolonged elevated oral Mn exposure. Among these tissues, bone Mn is the most responsive biomarker of ongoing oral Mn exposure. While X-ray-based analyses of bone samples show that with elevated exposure  $\text{Mn}^{2+}$  can replace  $\text{Ca}^{2+}$  in the hydroxyapatite mineral, the gross physical structure of hydroxyapatite bone mineral is not measurably altered. However, elevated Mn exposure does alter bone stiffness, suggesting that elevated exposure may cause alterations in the physical properties of bone not captured in the X-ray-based analyses. Data from our animal model are complemented by bone Mn analyses in aged humans, showing that bone Mn decreases with age, with no effect of sex, or parity history in females. Collectively, these findings indicate that bone may be a useful biomarker

of recent ongoing oral Mn exposure in humans, and that bone may be a relatively minor target of elevated Mn exposure, based on the limited functional alterations reported here.

#### 4.1. Tissue Mn naturally declines with age in the absence of elevated Mn exposure

Results in our rodent model show that, in the absence of elevated exposure, blood, brain, and bone Mn levels naturally decline with age across the lifespan (Fig. 1). The reduction in tissue Mn levels from early life to late adulthood likely reflects a decline in the absorption/retention of Mn with age, presumably reflecting a lower biological need for Mn in adulthood relative to early developmental life. The primary evidence for this comes from studies in animal models and adult humans. For example, Davidsson et al. (1989) reported Mn absorption rates in adult humans of 8.2%, 2.4%, and 0.7% for human milk, cow's milk, and soy formula, respectively. These human findings are corroborated by rodent studies showing that adult rats absorb <5% of ingested Mn (Ballatori et al., 1987; Mena, 1974). In contrast, young rats <15 days of age absorb over 40% of ingested Mn (Keen et al., 1986; Miller et al., 1975; Pappas et al., 1997). Our data show that in control animals, blood, brain, and bone Mn levels decrease substantially from weaning (PND 24) to young adulthood (PND 66), and blood Mn levels (but not brain or bone Mn) decline further from young adulthood to aged adulthood (PND ~500) (Fig. 1). This pattern is consistent with the greater control of intestinal and hepatic regulation of oral Mn intake on blood Mn levels in adults, and may also reflect the relatively shorter residence time of Mn in blood compared to brain and bone (Cowan et al., 2009; Crossgrove and Zheng, 2004; Jarvisalo et al., 1992; O'Neal et al., 2014; O'Neal and Zheng, 2015; Smith et al., 2007).

#### 4.2. Tissue Mn levels do not accumulate across the lifespan in the presence of elevated Mn exposure, but instead reflect ongoing recent exposure

The question of whether Mn accumulates in tissues with elevated exposures over the lifespan is of tremendous interest in identifying exposure biomarkers that may reflect temporally integrated or cumulative exposures, and for identifying potential target tissues of toxicological effects. Here, we define "accumulation" of Mn as a net increase in tissue Mn levels over time with steady-state elevated exposure. Based on this definition, our results show that continuous oral Mn exposure in rodents throughout life did not lead to the accumulation of Mn in blood, brain, or bone (Fig. 2). In fact, tissue Mn levels decline over the lifespan from post-weaning to young adulthood for all tissues, and blood Mn continues to decline from young adulthood into aged adulthood, even in the presence of ongoing elevated oral Mn exposure (Fig. 2a, lifelong 25 and 50 groups at PND ~500). The lack of Mn accumulation in bone with prolonged ongoing oral exposure is further illustrated when considering only the aged adult animals continuously exposed to oral 25 or 50 mg Mn/kg/d since birth; these animals were sacrificed at ages spanning PND 292–889 (median PND 490) due to experimental constraints in brain microdialysis measurements (Lasley et al., 2020). Regression analyses of bone Mn level versus age yields non-significant regression fits ( $p$ 's = 0.24), with slopes that do not measurably differ from zero (Fig. 3 and Supplemental Fig. S1). Together, these data underscore that age-related regulation of oral Mn uptake and elimination plays a significant role in modulating susceptibility to elevated oral Mn exposure, in that the early developmental life stage where oral Mn exposure produces the highest tissue Mn levels is also the life stage of greatest susceptibility to Mn neurotoxicity



(Beaudin et al., 2013; Beaudin et al., 2017a,b; Beaudin et al., 2017a,b; Claus Henn et al., 2018; Conley et al., 2020; Mora et al., 2015a; Oulhote et al., 2014).

It is noteworthy that blood Mn levels measurably declined from young adulthood (PND 66) to aged adulthood (~PND 500) in the presence of ongoing elevated oral Mn exposure (Fig. 2a, see lifelong 25 and 50 groups), while brain and bone Mn levels did not (Fig. 2 b, c). This may suggest that for tissues with longer Mn residence times compared to blood (i.e., brain and bone), continuous elevated oral Mn exposure may offset the relative influence of natural age-based reductions in tissue Mn levels. This suggestion is supported by the fact that the ~PND 500 aged animals continuously exposed to elevated oral Mn exposure had significantly higher blood, brain, and bone Mn levels compared to controls, whereas the early life Mn exposed groups did not differ from controls at this age (Fig. 2).

Our findings in rodents showing that bone Mn levels reflect ongoing oral exposures, but do not accumulate Mn with elevated exposures spanning birth through aged adulthood, have important implications for studies of environmentally and occupationally exposed humans. In particular, these results suggest that bone Mn levels may not be a good biomarker of cumulative oral Mn exposure integrated over prolonged durations, as has been shown for bone Pb levels (Aufderheide et al., 1981; Hu, 1998; Hu et al., 1998). This conclusion contrasts somewhat the findings of Rolle-McFarland et al. (2018), who reported that Mn accumulated in bone tissue in a population of 60 Chinese industrial workers. That study used neutron activation to measure bone Mn *in vivo*, and characterized occupational Mn exposure using a Cumulative Exposure Index (CEI) that relied partly on worker questionnaire responses to classify occupational exposure into qualitative exposure rankings of high, medium, or low. While the CEI approach is commonly used in occupational studies where significant challenges exist in performing comprehensive exposure assessments spanning months to years, it may not have been able to accurately distinguish whether higher bone Mn levels were reflecting Mn accumulation from prolonged exposure (e.g., years to decades) from the influence of more recent higher exposures over shorter durations proximal to the bone Mn measurements. It is also noteworthy that the exposure pathway in that study was inhalation, whereas in the present study it was oral, and there is some evidence that Mn exposures may follow different toxicokinetic profiles following oral versus inhalation exposures (Roels et al., 1997).

#### **4.3. Bone Mn levels appear to be a more sensitive biomarker of ongoing oral Mn exposure than either blood or brain Mn**

Although we found no evidence of Mn accumulation in bone in aged adult rats with continuous oral exposure, our biomarker sensitivity analyses show that bone is a more sensitive biomarker to ongoing oral Mn exposure than blood or brain (Fig. 3). This interpretation is supported by comparison of tissue Mn levels among controls and the high dose Mn group (50 mg Mn/kg/day) across tissues at PND 24, the age group where the continuous oral Mn exposures produced the highest tissue Mn levels. For example, blood Mn levels increased ~10-fold (from 24 ng/mL in controls to 247 ng/mL in animals exposed to 50 mg Mn/kg/d over PND 1–21), and brain Mn increased ~3.6-fold (from 3.6 to 12.8 µg/g), whereas bone Mn increased ~29-fold (from 2.7 to 77 µg/g). The greater relative

increase of bone Mn levels with ongoing elevated exposure, compared to relative increase in blood or brain Mn, is also evidenced by the slopes of the bivariate Pearson's regressions of normalized tissue Mn levels (Fig. 3). The bone vs blood (Y vs. X) regression slopes in the PND 24, 66, and ~500 lifelong Mn exposure groups range from 1.86 to 3.28 (mean  $2.72 \pm 0.76$  SD), showing there is a several-fold greater relative increase in bone Mn for a given increase in blood Mn. Moreover, the bone vs. blood regression slopes are >10-fold steeper than the slopes of the brain vs. blood regressions (0.12–0.27 for the three age groups), showing that a given increase in blood Mn produces a much greater increase in bone Mn than brain Mn levels. Collectively, these findings suggest that bone Mn levels may be a more sensitive biomarker of ongoing oral Mn exposure than levels of Mn in other tissues. While Mn does not accumulate in bone per se, bone Mn levels do appear to reflect an intermediate exposure duration of weeks to months, i.e., longer than the duration for blood, consistent with the estimated 143 days (~4.7 months) half-life of Mn in bone reported by O'Neal et al. (2014).

#### **4.4. Elevated Mn exposure caused some changes in bone mineral properties, accompanied by changes in the physical properties of bone**

The oral Mn exposure conditions that produced elevated bone Mn levels in adult animals did not result in changes to the gross mineral structure of hydroxyapatite in bone (Supplemental Fig. S3). However, EXAFS analysis revealed that elevated Mn exposure did lead to changes in the local atomic structure of hydroxyapatite mineral due to the substitution of  $\text{Mn}^{2+}$  for  $\text{Ca}^{2+}$  in the coordination of the hydroxyapatite mineral structure (Supplemental Fig. S5). Not unexpectedly, the substituted Mn was in the  $\text{Mn}^{2+}$  valence state, consistent with prior evidence showing that the predominant Mn valence state in eukaryotic cells is  $\text{Mn}^{2+}$  (Aschner and Aschner, 2005; Reaney et al., 2002; Reaney and Smith, 2005).

The finding that elevated bone Mn altered the local atomic structure, but not the gross crystalline structure of hydroxyapatite mineral is noteworthy, in light of the changes in the physical properties of bone determined through our biomechanical testing. We evaluated a number of physical properties of femurs from PND 66 rats, including stiffness, yield strength, ultimate strength, and work, but only femur stiffness was measurably increased with Mn exposure, and only in the early life 25 and 50 Mn exposure groups compared to controls (bone stiffness trended higher in the lifelong 25 and 50 Mn groups, but did not reach significance) (Fig. 4). This finding of minimal biomechanical alterations to bone containing elevated Mn levels suggests that bone may be a target organ for Mn effects, though likely not as sensitive and impacted as other organ systems such as the brain, particularly when considering the elevated bone Mn levels observed here versus levels reported in environmentally or occupationally exposed humans. For example, bone Mn levels in the PND 66 young adult animals exposed to the highest Mn dose (i.e., 50 mg Mn/kg/d over PND 1–21 or lifelong) averaged ~2.4–3.2  $\mu\text{g/g}$ , though levels were much higher in the younger PND 24 weanlings exposed over PND 1–21 (mean 77  $\mu\text{g/g}$ , Fig. 2). By comparison, bone Mn levels of 0.89–2.6  $\mu\text{g/g}$  have been reported in occupationally exposed adults using neutron activation analysis (Rolle-McFarland et al., 2018; Wells et al., 2018).

Other toxic metals such as Pb, which is also known to substitute for  $\text{Ca}^{2+}$  in the hydroxyapatite mineral matrix, and to some extent cadmium, have been shown to significantly reduce bone mineral density and strength in mice (Monir et al., 2010), along with decreasing trabecular bone surface area and increasing risk of fracture in rats (Álvarez-Lloret et al., 2017). Alterations to bone metabolism following Pb exposure have been reported in epidemiological studies, including a recent study from Ravibabu and colleagues, in which long-term occupational Pb exposure in a group of 176 male Pb-battery workers was positively associated with significantly higher levels of biomarkers of bone formation (i.e., serum bone-specific alkaline phosphatase) and bone resorption (i.e., serum pyridinoline, tartarate-resistant acid phosphatase-5b, urinary hydroxyproline), along with a negative association with serum osteocalcin (Ravibabu et al., 2020). Similarly, Akbal et al. found a negative association between occupational Pb exposure and vertebral bone density in middle-aged male Pb-battery workers in Turkey (Akbal et al., 2014). Long-term exposures to other metals, such as cadmium, are also associated with harmful effects to the human skeleton, including reduced bone mineral density, higher incidence of fractures, and higher risk of osteoporosis (Engström et al., 2012; Wallin et al., 2016).

#### **4.5. Bone Mn levels in aged humans decrease with age, and do not vary with sex or parity history**

In order to determine bone Mn and Pb levels in environmentally exposed aged humans (mean age of 82 and 74 years for females and males, respectively), and whether bone Mn levels varied with age, sex and parity, we obtained intact femoral head bone samples from female ( $n = 30$ ) and male human ( $n = 19$ ) subjects from the province of Brescia, Italy, undergoing hip joint replacement due to osteoarthritis. Our findings show that bone Mn levels are generally very low in these aged adults (mean  $0.038 \mu\text{g/g}$ ), and that there was a trending  $\sim 1\%$  per year reduction in bone Mn levels with age (Fig. 5). We did not find a significant relationship between bone Mn and sex, which may reflect a similar rate of osteoarthritis and osteoporosis in these male and female subjects who underwent joint arthroplasty. It is noteworthy that there is limited information on bone Mn levels in aged humans from other studies; Budis et al. (2014) reported mean levels of  $0.18 \mu\text{g/g}$  (dry weight) in the cartilage and adjacent compact bone of femoral heads in a Polish population, aged 32–82 years, while Chang et al. (2018) reported mean concentrations of  $0.704 \mu\text{g/g}$  (dry weight) in the greater trochanter of older adults, aged 63 years  $\pm 10$  SD. Others, such as Kuo and colleagues, found mean bone Mn levels of  $0.59 \mu\text{g/g} \pm 0.74$  SD (dry weight) in the femoral heads of Taiwanese males and females, aged 61–80 years (Kuo et al., 2000). Prior research has shown that osteoarthritis and osteoporosis are more prevalent in women, and that both conditions lead to loss of bone mineral over time (Alswat, 2017; Maleki-Fischbach and Jordan, 2010; Zhang and Jordan, 2010). We also found that there was no relationship between bone Mn levels and parity history in females. We may have expected a decrease in bone Mn levels with parity history, given that a number of studies have reported that maternal blood Mn levels increase from  $\sim 7 \text{ ng/mL}$  to  $\sim 17\text{--}25 \text{ ng/mL}$  over the course of pregnancy and into the postnatal lactation period (Mora et al., 2015b; Yamamoto et al., 2019), suggesting that there may be increased mobilization of maternal bone Mn with mobilization of bone mineral over pregnancy and lactation, as has been shown with bone Pb (Gulson et al., 1997, 2004; Manton et al., 2003; Téllez-Rojo et al., 2004).

Bone Pb levels represent a valuable point of comparison relative to Mn since both metals are able to substitute with  $\text{Ca}^{2+}$  in hydroxyapatite mineral, and elevated Pb exposures are similarly associated with industrial activity (Fleming et al., 1997; Grimsley and Adams-Mount, 1994; Hernberg, 2000; Kaufman et al., 1994; Rudolph et al., 1990), and there is a substantial body of evidence on Pb in the skeleton (Marcus, 1985; O'Flaherty, 1993; Rabinowitz et al., 1973; R dulescu and Lundgren, 2019). For example, studies have shown that Pb accumulates in the skeleton with age, that the skeleton can contribute 40–70% of circulating blood Pb in environmentally exposed adults (Gulson et al., 1997; Smith et al., 1996), and that there is significant remobilization of bone Pb with bone mineral mobilization over pregnancy and lactation (Gulson et al., 1997, 2004; Manton et al., 2003; Téllez-Rojo et al., 2004). Here we show that bone Pb levels in these aged adults (mean 3.49  $\mu\text{g/g}$ , median 2.20  $\mu\text{g/g}$ ) were ~100-fold higher than bone Mn levels, and that bone Pb levels were associated with both age and sex. Specifically, bone Pb levels increased with age by approximately 3.3% per year over the age range of these subjects (41–95 years), and males have ~3.5-times higher bone Pb levels compared to females (Fig. 5). These findings are consistent with multiple studies that show males tend to have higher tissue Pb levels relative to females, in part due to presumably higher likelihood of elevated environmental and/or occupational exposure (Hu, 1998; Hu et al., 1991; Smith et al., 2002). Additionally, the lack of a relationship between bone Pb and parity history in females was somewhat unexpected as Pb has been reported to remobilize during pregnancy and lactation as bone mineral turnover increases (Gulson et al., 1997, 2004; Manton et al., 2003; Téllez-Rojo et al., 2004).

#### 4.6. Study strengths and limitations

There are several strengths and limitations of this study that may provide context for interpreting our findings. Study strengths include the use of a prolonged animal exposure design to evaluate both early life and lifelong Mn exposure paradigms into aged adulthood, along with a controlled and well-characterized Mn exposure regimen that is relevant to human environmental exposures (see Beaudin et al., 2017a for a detailed exposure rationale). Additional strengths are the comprehensive use of advanced analytical methods (i.e., XRD, XANES, EXAFS) to assess whether Mn accumulates in bone tissue, and whether Mn exposure affected the mineral and atomic structure of bone relative to the physical biomechanical properties. Finally, the evaluation in aged humans to determine the relationship between age, sex, and parity history on bone Mn relative to bone Pb levels is an additional strength of this study. There are also several noteworthy limitations of this study, including using only femurs, which are predominantly cortical bone, to assess bone Mn levels in the animal studies. Others may find different results using bones that are primarily trabecular in composition. Further, inhalation is an important route of exposure for occupationally exposed adults, while our rodent model was limited to oral exposures. Regarding our human subjects, the diagnosis of osteoarthritis and/or osteoporosis in this group may have influenced the levels of bone Mn measured in our samples, and may not necessarily be generalized to a healthier population.

## 5. Conclusions

Collectively, our study provides a framework for comparing the sensitivity of Mn exposure biomarkers using a variety of tissue types. These findings indicate that Mn does not accumulate in bone, brain, or blood across the lifespan with continued elevated exposure, though bone may be a useful biomarker of recent ongoing oral Mn exposure in humans. Additionally, bone may be a relatively minor target of elevated Mn exposure, based on the limited functional alterations reported here.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements and conflict of interest disclosure

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## Abbreviations:

<b>CEI</b>	Cumulative Exposure Index
<b>EDTA</b>	ethylenediaminetetraacetic acid
<b>EXAFS</b>	extended X-ray absorption fine structure
<b>Mn</b>	manganese
<b>Pb</b>	lead
<b>PND</b>	postnatal day
<b>XANES</b>	X-ray absorption near edge structure
<b>XAS</b>	X-ray absorption spectroscopy
<b>XRD</b>	X-ray diffraction

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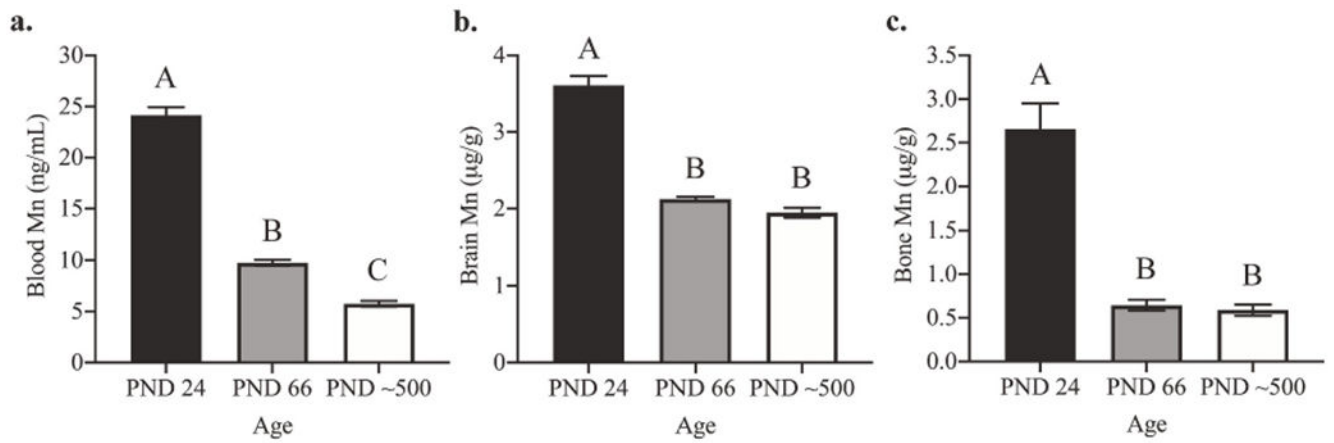
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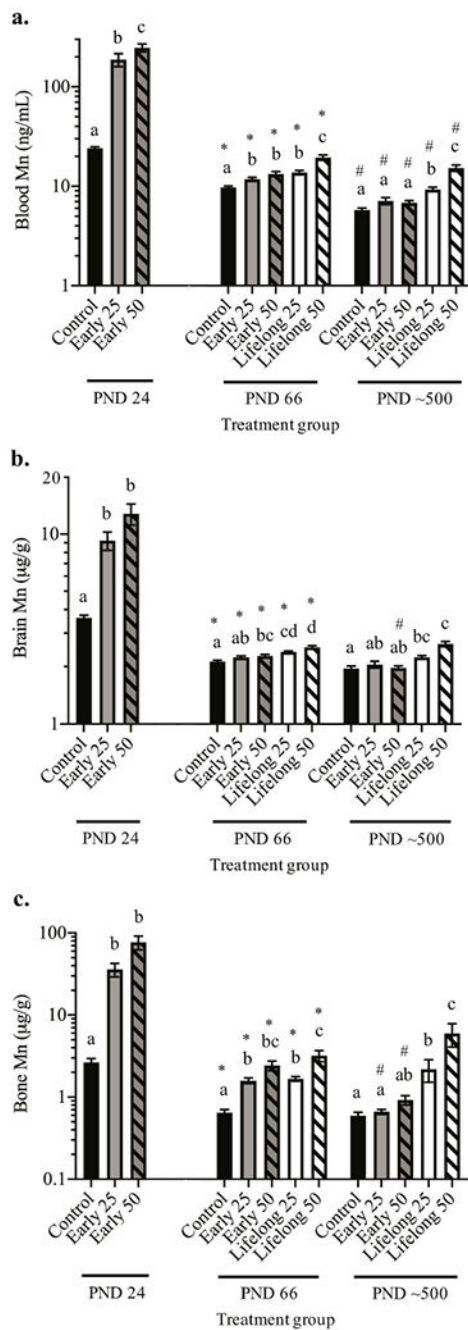
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**Fig. 1. Tissue Mn naturally decreases across the lifespan without elevated Mn exposure.** Bar chart shows tissue Mn levels for (a) blood (ng/mL), (b) brain ( $\mu\text{g/g}$  dry weight), and (c) bone ( $\mu\text{g/g}$  dry weight) in control animals that received no elevated Mn exposure ( $n = 10\text{--}16$  animals/treatment group). Data are least squares means  $\pm$  SEM values generated from one-way ANOVA. Bars with different superscripts are statistically different ( $p < 0.05$ ), based on Tukey's multiple comparisons test.



**Fig. 2. Tissue Mn does not accumulate in blood, brain, or bone across the lifespan in the presence of elevated oral Mn exposure.**

Bar charts show tissue Mn levels for (a) blood (ng/mL), (b) brain (µg/g dry weight), and (c) bone µg/g on a log10 scale, grouped by treatment group and age (n = 10–16 animals/treatment and age group). Data are least squares means ± SEM values generated from one-way ANOVA. Bars with different superscripts are statistically different ( $p < 0.05$ ), based on Tukey’s multiple comparisons test. Lowercase superscripts are comparisons within an age across treatment groups. \* represents comparison between age groups for PND 66

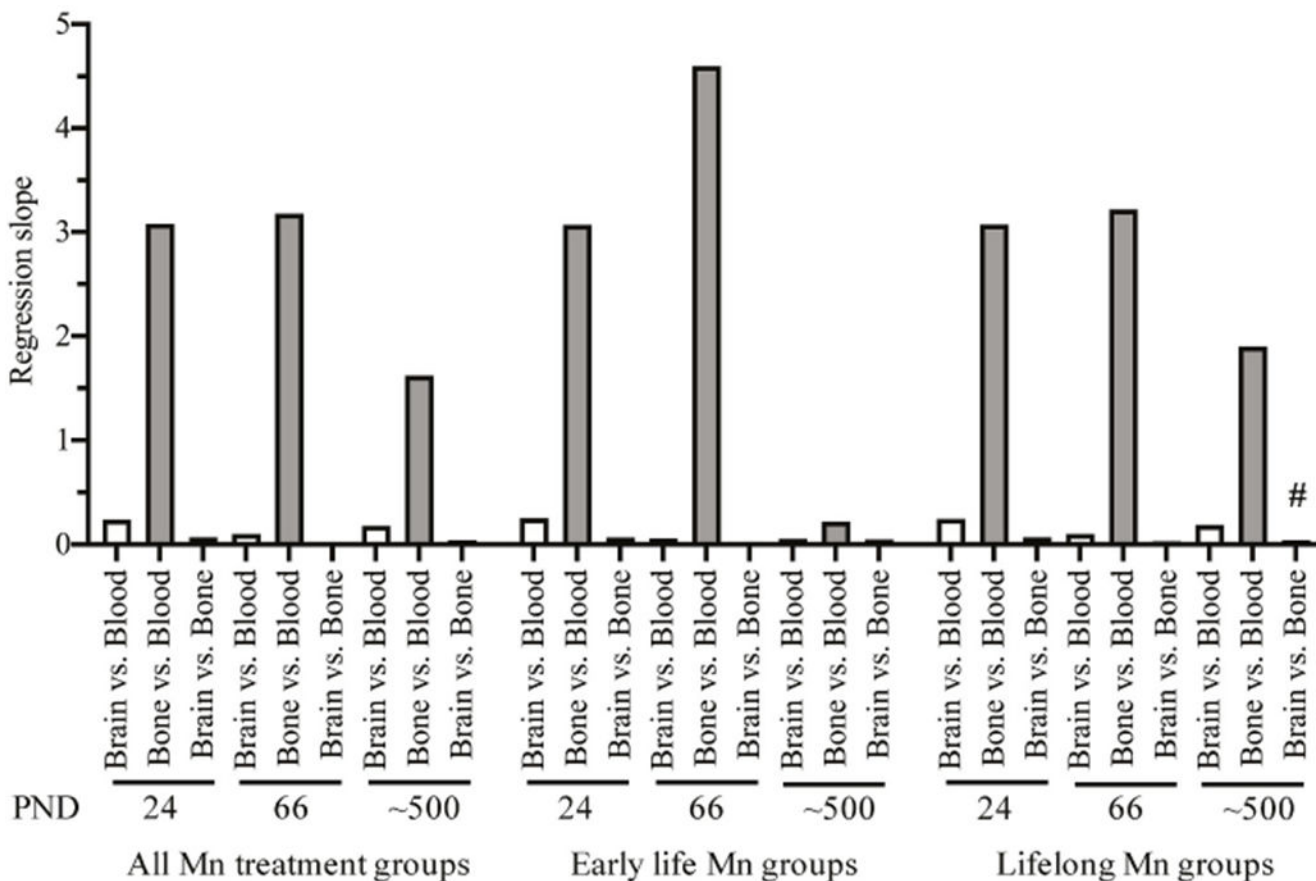
that are statistically different from their PND 24 counterpart of the same exposure dose. # represents comparison between ages for PND ~500 that are statistically different from their PND 66 counterpart for the same exposure group.

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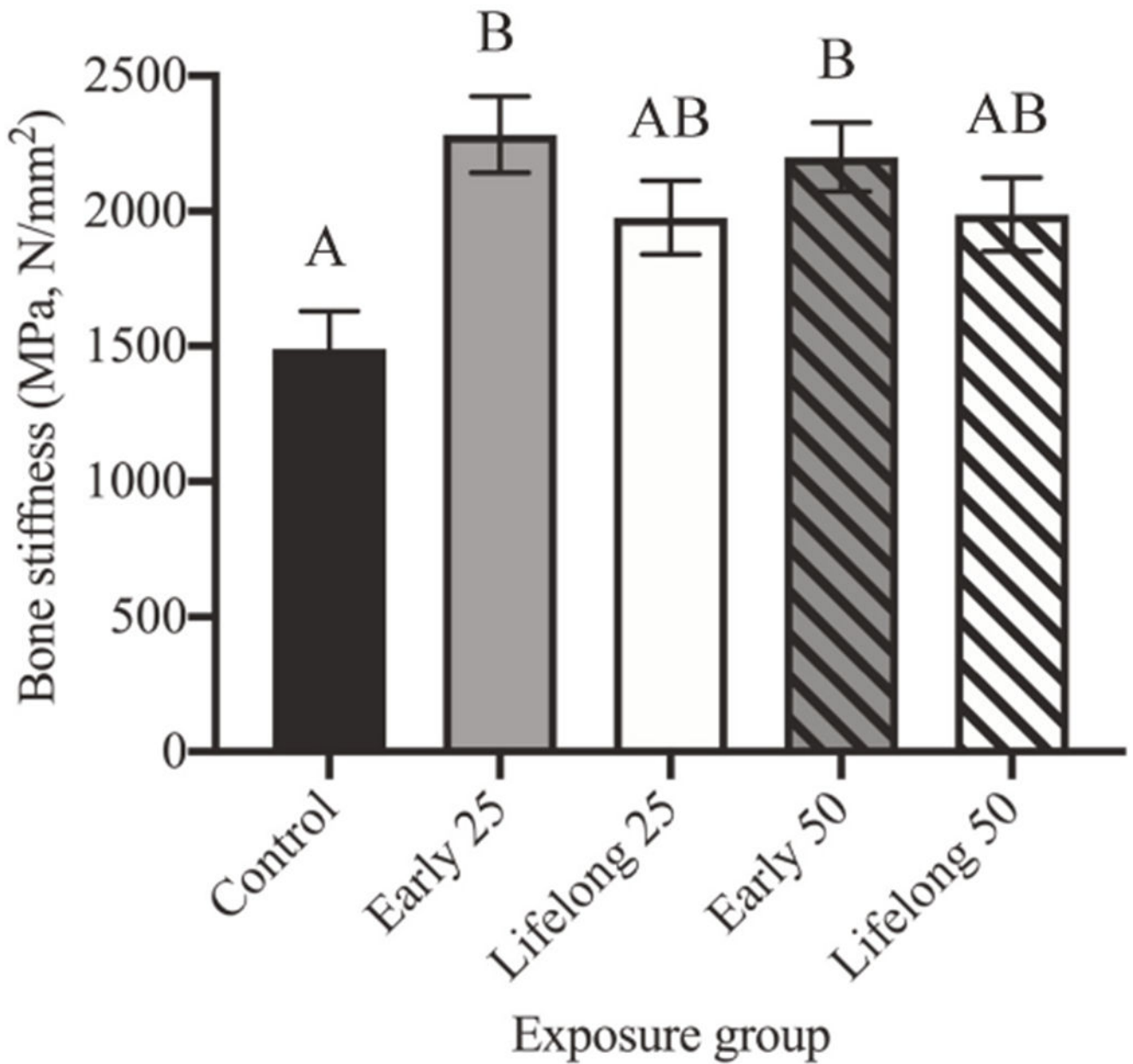
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**Fig. 3. Bone Mn levels are highly sensitive to increases in blood Mn levels.**

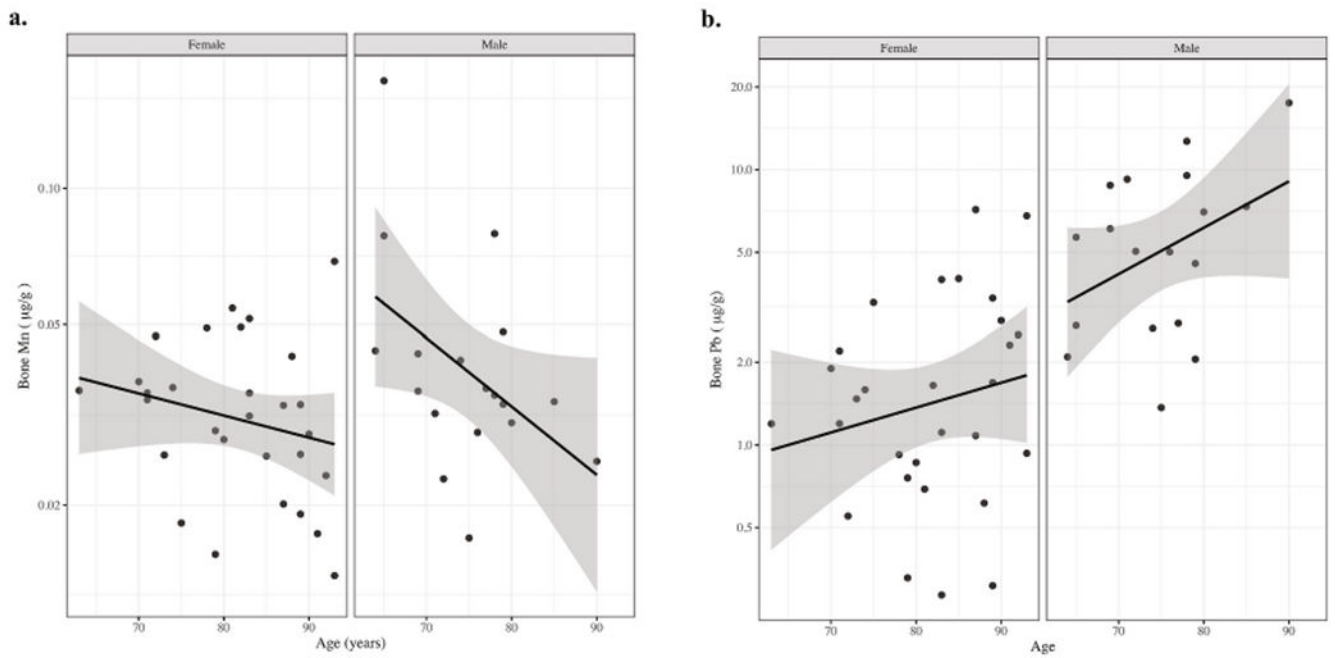
Data are regression slopes (Y vs. X) from Pearson’s correlation coefficient generated from tissue Mn values normalized to percentage of age matched controls separately for blood, brain, and bone tissues. All Pearson’s correlations were significant (Pearson’s R from 0.0101 to 0.508, with associated p-values,  $p < 0.05$ ), with the exception of one indicated with # ( $p = 0.38$ ).





**Fig. 4. Postnatal Mn exposure increases bone stiffness.**

Bar chart shows femur stiffness in PND 66 rats (MPa, N/mm<sup>2</sup>, n = 13–16 animals/treatment group). Data are least squares means  $\pm$  SEM values generated from the ANOVA statistical model with all five treatment groups. Bars with different superscripts are statistically different ( $p < 0.05$ ), based on Tukey's multiple comparisons test.



**Fig. 5. In aged humans, bone Mn trends towards a decrease with age, while bone Pb levels increase with age.**

Data are individual human bone concentrations of (a) Mn and (b) Pb in  $\mu\text{g/g}$ , as a function of age for females ( $n = 26$ ) and males ( $n = 18$ ). Trendlines with 95% confidence intervals reflect analyses described in section 3.5.1. Bone Mn (a) showed a trending decrease with age ( $p = 0.073$ ), but no relationship with sex ( $p = 0.208$ ). Bone Pb (b) increased significantly with age ( $p = 0.027$ ) and with sex, showing greater levels in males than females ( $p < 0.0001$ ).

**Table 1**  
**EXAFS results and comparison with the interatomic distances between hydroxyapatite and a femur bone sample from a PND 24 rat exposed to Mn 50 mg/kg/d over PND 1–21.**

N is the number of atoms of oxygen (O), phosphorus (P), or calcium (Ca) in the central atom Mn coordination. D is the interatomic distance in angstroms ( $\text{\AA}$ ), and  $\sigma^2$  is the Debye-Waller Factor in angstroms squared ( $\text{\AA}^2$ ), which reflects the degree of X-ray scattering due to thermal motion; both of these values were unremarkable in this dataset.

Central Atom Mn Coordination	Interatomic Distance and Debye-Waller Factor	Mn 50 mg/kg/d	Hydroxyapatite
Mn–O1 (N = 6)	$D(\text{\AA})$	2.15 (0.01)	2.40
	$\sigma^2(\text{\AA}^2)$	0.0075 (0.0001)	
Mn–O2 (N = 3)	$D(\text{\AA})$	2.41 (0.01)	2.80
	$\sigma^2(\text{\AA}^2)$	0.0075 (0.0001)	
Mn–P1 (N = 3)	$D(\text{\AA})$	2.91 (0.02)	3.21
	$\sigma^2(\text{\AA}^2)$	0.0097 (0.0002)	
Mn–P2 (N = 3)	$D(\text{\AA})$	3.13 (0.02)	3.60
	$\sigma^2(\text{\AA}^2)$	0.0054 (0.0020)	
Mn–P3 (N = 2)	$D(\text{\AA})$	3.42 (0.02)	3.55
	$\sigma^2(\text{\AA}^2)$	0.0034 (0.0001)	
Mn–O3 (N = 9)	$D(\text{\AA})$	3.93 (0.03)	3.72
	$\sigma^2(\text{\AA}^2)$	0.0095 (0.0003)	
Mn–Ca1 (N = 6)	$D(\text{\AA})$	3.94 (0.05)	4.19
	$\sigma^2(\text{\AA}^2)$	0.0286 (0.0006)	