Pamidronate Administration during Pregnancy and Lactation Induces Temporal Preservation of Maternal Bone Mass in a Mouse Model of Osteogenesis Imperfecta

[1,2]Diana Olvera, [1]Rachel Stolzenfeld, [1]Emily Fisher, [1]Bonnie Nolan, [1]Michelle S. Caird, [1,2]Kenneth M. Kozloff

[1]Department of Orthopaedic Surgery, University of Michigan, Ann Arbor, MI
[2]Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI

Abstract

During pregnancy and lactation, the maternal skeleton undergoes significant bone loss through increased resorption to provide the necessary calcium supply to the developing fetus and suckling neonate. This period of skeletal vulnerability has not been clearly associated with increased maternal fracture risk, but these physiological conditions can exacerbate an underlying metabolic bone condition like osteogenesis imperfecta. While bisphosphonates are commonly used in post-menopausal women, there are cases where pre-menopausal women taking bisphosphonates become pregnant. Given bisphosphonates' long half-life, there is a need to establish how bisphosphonates affect the maternal skeleton during periods of demanding metabolic bone changes that are critical for the skeletal development of their offspring. In the present study, pamidronate amplified pregnancy-induced bone mass gains and lactation-induced bone loss was prevented. This preservation of bone mass was less robust when pamidronate was administered at late stages of lactation compared to early pregnancy and first day of lactation. Pregnancy-induced osteocyte osteolysis was also observed and was unaffected with pamidronate treatment. No negative skeletal effects were observed in offspring from pamidronate-treated dams in spite of lactation-induced bone loss prevention. These findings provide important insight into a treatment window for when pamidronate is most effective in preserving maternal bone mass and provides significant insight to the maternal changes in bone metabolism that maintain calcium homeostasis crucial for fetal and neonatal bone development.

Keywords: Osteogenesis imperfecta < DISEASES AND DISORDERS OF/RELATED TO BONE, Antiresorptives < THERAPEUTICS, Preclinical Studies < ANIMAL MODELS

Introduction

Pregnancy and lactation are known as periods of significant maternal bone loss due to changes in calcium homeostasis required for fetal bone mineralization and breast milk production. Since the decrease of maternal bone has been shown to be transitory, this reproductive period has not been clearly associated with osteoporosis or increased maternal fracture risk [1]. However, it remains uncertain if these physiologic conditions define a period of vulnerability for the maternal skeleton associated with a rare underlying metabolic bone disorder like osteogenesis imperfecta (OI).

Limited information on the maternal skeletal outcome following pregnancy and lactation in women with OI is known [2-4], however fractures and other complications have been reported [5-8]. Currently, bisphosphonates (BPs) are the most widely prescribed anti-resorptive for the treatment of metabolic bone diseases associated with excessive bone resorption [9, 10]. Through their high affinity for calcium ions, these synthetic analogues bind to hydroxyapatite crystals within the bone matrix to mediate an inhibitory effect on active osteoclasts to decrease bone turnover and increase bone mineral density [11-17]. Although the majority of patients treated with BPs are postmenopausal, children and women of childbearing age are increasingly being prescribed these treatments [18-20]. Because BPs can remain sequestered within the bone matrix until they are taken up by osteoclasts, there is rising concern that fetal exposure could occur in cases where the mother has been subjected to BP treatment prior to conception. Additionally, it has been shown that other medication and drugs have the potential to pass from the mother's blood stream into the breast milk and reach the nursing infant [21-24]. Although clinical case studies have reported the benefits of BPs for the treatment of pregnancy and lactation-associated osteoporosis (PLO), glucocorticoid- induced osteoporosis, hypercalcemia and other complications during pregnancy and lactation [19, 25-28], data on the effects of BP exposure of the mother and possibly the offspring during pregnancy or lactation remains limited.

Currently BPs have been classified by the FDA as holding a category C pregnancy risk because animal reproduction studies have shown adverse effects, and no adequate and well-controlled studies in humans have been reported [29]. Our current understanding of BP effects during pregnancy rely heavily on animal studies, which have shown that these drugs are capable of crossing the placenta, thus putting the fetus at risk for exposure *in utero* [30-33]. Although these animal studies showed that BP exposure led to low birth weight, underdevelopment, and skeletal retardation, toxic levels up to 10x therapeutic dosage used clinically were administered in order to assess their safety during pregnancy and lactation. Two toxicity animal studies have also explored the effects of BPs during lactation where severe and mild hypocalcaemia was observed due to disruption in maternal calcium metabolism [34-37]. Of the case studies that are available, neither mother nor infant demonstrated serious adverse effects, however a few studies did report

low birth weight, low gestational age and transient asymptomatic hypocalcaemia in the offspring [38-46].

Recommendations regarding the use of BPs during lactation are often conflicting since it is not entirely known how much of a given drug passes the placenta or diffuses into breast milk and studies don't agree on a common conclusion. Thus, there remains a need for developing systematic studies on BP effect when treating the maternal skeleton during a period of demanding metabolic bone changes that are critical for the skeletal development of their offspring. In the present study we sought to determine a therapeutic window for when pamidronate (PAM) is most potent on the maternal skeleton and how exposure affects fetal and neonatal development. We hypothesized that PAM resorption effects on the maternal skeleton will be highly dependent on the time of administration since bone metabolism is constantly changing throughout pregnancy and lactation. Offspring are at risk of PAM exposure if BPs cross the placenta or diffuse into the breast milk even if the mother has stopped treatment prior to conception, due to BPs long-half-life and their release into the bloodstream following maternal bone resorption. Additionally, since the maternal skeleton serves as a calcium reservoir to maintain calcium homeostasis during ossification and mineralization of their offspring, BPs have the potential to interfere with maintaining this balance and indirectly affect fetal/neonatal bone development. To explore these questions, we tested BP effects using a single injection of pamidronate at different stages of pregnancy and lactation in Brtl/+ dams harboring an OI-causing defect. This knock-in mouse model reproduces the phenotype of moderately severe type IV OI [47-49] and has been used to explore therapeutic efficacy of anti-resorptive and anabolic interventions in growing and aged mice [50-55]. Additionally, we genotyped and sexed offspring from treated and untreated Brtl/+ dams to explore the sequela of events resulting from PAM exposure on the maternal skeleton. In the present study, a temporal effect on the preservation of bone mass was observed when PAM was administered at different stages of pregnancy and lactation. This protective effect was more modest when given during pregnancy (E15) and ceased to exist when PAM was given during the late stages of lactation. Despite preventing lactation-induced bone loss, no adverse effect on fetal and neonatal bone development was found, suggesting additional sources of calcium are capable of responding to the demands placed on the maternal skeletal.

Materials and Methods

Experimental design

Brtl/+ mice with a mixed background of SV129/CD-1/C57BL/6S were derived from heterozygous Brtl/+ and WT parental strains [47]. To identify temporal maternal skeletal changes during pregnancy and lactation in OI and how these skeletal changes are affected

with PAM, female Brtl/+ (n = 10/group) mice between 12 and 18 week of age were randomly assigned to receive a single tail vein injection of either pamidronate (PAM 3 mg/kg) or vehicle (PBS) during one of five time points (prior to conception (PC), gestation day 15 (E15), lactation day 1 (D1), lactation day 10 (D10) or lactation day 15 (D15)). This single PAM dose represents a standard clinical dosage that has been used during pregnancy [19]. For successful timed pregnancies, female Brtl/+ were mated with experienced male WT breeders and pregnancy was assessed by examining for a vaginal plug. Pregnant mice were caged individually and were continued on standard chow diet (5L0D, PicoLab, St. Louis, MO, USA) throughout pregnancy and lactation. Standard chow contained a calcium concentration of 6.6% and 4.6 IU/gm of vitamin D. To isolate the effects of pregnancy, mothers, along with litter, were euthanized at birth. To assess both pregnancy and lactation effects, mother and litter were euthanized at weaning (D21). As a control, age- matched virgin female Brtl/+ mice (n = 10) were assessed to establish a skeletal baseline uninfluenced by pregnancy and lactation. A summary of the experimental design for maternal and neonatal assessment of skeletal changes during pregnancy and lactation under the influence of PAM is shown in Figure 1. All protocols and procedures were approved by the University of Michigan's Committee on Use and Care of Animals.

Non-therapeutic fluorescent bisphosphonate probe

A single injection of commercially available far-red fluorescent pamidronate (FRFP) Osteosense-680EX (Perkin Elmer, Bedford, MA, USA) was administered in Brtl/+ pregnant dams (gestation day 19) to assess if BPs crossed the placenta. The administered dosage was 2nmols of FRFP buffered in PBS in a tail vein injection volume of 150 µL. For comparison, this non-therapeutic fluorescent bisphosphonate probe is ~80nmol/kg which is significantly lower than our 3mg/kg PAM therapeutic dose which is ~10750nmol/kg. Controls received a 150 µL tail vein injection of PBS.

FRFP Imaging

To assess if BPs crossed the placenta, we looked for FRFP incorporation into the fetal skeleton. Pregnant Brtl/+ dams (gestation day 19) were euthanized within 24 hours of FRFP injection. Embryos were fresh frozen in chilled isopentane at −70°C in O.C.T compound embedding medium (Fisher Chemical Co., Santa Clara, CA, USA) and stored at -80°C. Frozen samples were sectioned to a thickness of 10μm using the Kawamoto film technique [56] and a Leica CM3050S cryostat (Leica Microsystems, Germany). Fluorescent images from tissue sections were acquired with a 20x dry objective using a Zeiss Axiovert 200M inverted microscope (Carl Zeiss, Germany).

Micro-computed tomography (μCT)

To analyze maternal and neonatal bone morphological changes after pregnancy and lactation, excised L3 vertebrae and right femora were first fixed in 10% neutral buffered formalin. Following fixation, both vertebrae and femurs were scanned in water using high-resolution μCT (Brucker Skyscan 1176, Kontich, Belgium). Image acquisition was performed at 50 kV and 800 μA with a 0.3° rotation step and a 0.25 mm aluminum filter for filtration of beam hardening artifacts. Manufacturer-provided hydroxyapatite phantoms were scanned with the same parameters to calibrate and compute volumetric BMD. Individual two-dimensional cross-sectional images were reconstructed into three-dimensional volumes with 9 μm isotropic voxel size using NRecon software (version 1.6.5.8, Bruker).

Two volumes of interest were established to analyze the effect of PAM during pregnancy and lactation on the femur. A 2 mm (maternal) and 1 mm (neonatal) VOI was centered midway between the lateral third trochanter and the distal femoral growth plate for cortical analysis and segmented using a user-defined global threshold derived from calculating the average thresholds over a range of samples. For trabecular analysis, a VOI spanning 15% of total femoral length for maternal bone and 7% for neonatal bone was placed at the metaphysis just proximal to the distal growth plate. Manual contours were used to isolate the trabecular compartment and further segmentation was performed using an automatic adaptive thresholding algorithm.

For vertebral analysis, a VOI was placed between the cranial and caudal endplates of maternal and neonatal L3 vertebrae. Vertebral cortical and trabecular bone were separated through manual contouring to denote the outer and inner boundaries of the cortex and segmented with automatic adaptive thresholding. The areas of interest were analyzed with CTAn software (version 1.15.4.0, Bruker). Bone architecture parameters analyzed include: (trabecular number – TbN, trabecular thickness – TbTh, bone volume fraction – BV/TV, cortical thickness – CTh, and bone mineral density – BMD). Representative femoral and vertebral isosurfaces were obtained using commercially available software (MicroView Advanced Bone Analysis Application, GE Healthcare Pre-Clinical Imaging, London, ON, Canada).

Backscatter scanning electron microscopy

Previous studies have shown that osteocyte osteolysis plays an important role during lactation [57-60]. To assess physiologic osteocytic remodeling in our study, lacunar area was measured on maternal cortical bone of right femora. Following microCT imaging, maternal right femora were embedded without decalcification in methyl methacrylate and sectioned transversely below the lateral third trochanter using an Isomet low-speed diamond saw (Buehler Ltd., Lake Bluff, NY). Two hundred-micron sections were then polished and mounted on aluminum stubs using an alcoholic colloidal graphite solution. Images of osteocyte lacunae on the sectioned bone surface were acquired with a scanning electron microscope equipped with a backscattered electron detector (BSEM, Tescan MIRA3 FEG- SEM, Czech) at 30 kV accelerating voltage, 300 pA current, and 10 mm working distance. Acquired high contrast images were then converted into binary mask using ImageJ (NIH, Bethesda, MD) and areas were measured for all lacunae in the anterior, posterior, lateral and medial 0.33 mm x 0.33 mm quadrants of the femoral cortex. No regional differences were observed so all quadrants were combined to represent an overall average.

Biomechanical testing

To assess the mechanical effects during pregnancy and lactation and the influence of PAM during these events, maternal L5 vertebra and left femora were loaded to failure in compression and four-point bending, respectively, using an MTS 858 Mini-Bionix servo-hydraulic testing system (MTS Systems Corp., Eden Prairie, MN, USA). All specimens were kept hydrated in lactated ringer's solution (LRS) prior to mechanical testing. The vertebral body was vertically aligned along its loading axis with an alignment pin (attached to lower platen and extending through the spinal column) and compressed to failure at a displacement rate of 0.05 mm/second. For four-point bending, the posterior surface of the femur was oriented in tension and the mid-diaphysis was loaded to failure at a displacement rate of 0.5 mm/second. Force and vertical displacements were continuously recorded throughout each test by a 50 lb load cell (Sensotec, Columbus, OH, USA) and an external linear variable differential transducer (LVDT; Lucas Schavitts, Hampton, VA, USA), respectively. A custom- developed LABVIEW program was used to calculate the mechanical properties for both tissues.

Tartrate-resistant acid phosphatase staining

Qualitatively, histochemical staining of the biochemical marker tartrate-resistant acidic phosphatase (TRAP) has been used to identify osteoclasts. To assess TRAP in maternal and neonatal trabecular bone, right proximal tibiae were fixed (4% neutral buffered formalin), decalcified (10% EDTA for 21 days) and embedded in paraffin. Ribbons of serial sections (6μm) were cut with an automated Leica RM2255 (Leica, Wetzlar, Germany) rotary microtome and disposable low profile stainless steel blades (Accu-Edge, 4689). Collected sections were mounted on Superfrost/Plus glass slides (Fisher,

Pittsburgh, PA., USA) and left to dry overnight on a 40°C slide warmer. Staining for TRAP was carried out with an Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma-Aldrich, St. Louis, MO., USA) according to the manufacturer's instructions. Images were acquired using an upright microscope (Nikon Eclipse Ni-U) associated with a DS-Fi2 digital camera, NIS BR software (Nikon France, Champigny-sur-Marne, France) and a 20x dry objective. Histomorphometric analysis of maternal osteoclasts was acquired from two ROIs; the first was placed proximal to the growth plate at a span of 5 mm, while the second spanned 12.5 mm placed 5 mm distal to the growth plate. For neonates, due to high mineralized bone concentrated below the growth plate, a single 17.5 mm ROI was placed 12.5 mm distal the growth plate. In addition, neonatal osteoclasts from mothers exposed to PAM at timepoints PC, E15 and D1 were analyzed to assess osteoclast number and surface on offspring skeletal development when exposed to PAM. To assess osteoclast number per bone area and osteoclast surface percentage, the number and surface of TRAP-positive multinucleated cells containing three or more nuclei was quantified for both mothers and neonates as well as the bone surface within the ROI using Bioquant software (Bioquant Image Analysis Corporation, Nashville, TN., USA).

Statistics

Maternal bone architecture data are reported through box plots where treated (grey) groups are superimposed onto untreated (white) groups. Maternal variations in bone architecture, biomechanics, osteoclasts osteolysis, and osteoclasts between untreated/treated groups and virgin controls were determined using a one-way ANOVA followed by Holm–Sidak test for multiple comparison. To determine differences between untreated and treated groups a two-way ANOVA was applied to analyze both maternal and neonatal parameters. Differences with p-values <0.05 were considered significant. All statistical analyses were carried out using Graph Pad Prism (version 7.04, San Diego, CA, USA). Data are presented as mean+SD, or by individual values. Box plots, when presented, display mean (horizontal line), 25^{th} and 75^{th} percentiles (box) and minima and maxima (bars).

Results

Maternal and neonatal body mass are not affected with pamidronate treatment

Maternal body mass increased during pregnancy (through E15), decreased immediately following birth (D1) and increased again through weaning (D15) (Figure 2A) in both PAM and PBS treated groups. By the end of lactation (D21), maternal body mass reached a common value regardless of PAM treatment status or time of treatment (Figure 2B). At 21 days of age, the body mass of genotyped female pups from PBS-treated dams did not differ from the body mass of genotyped female pups from PAM-treated dams (Figure 2C). The same observations were seen in genotyped male pups. Furthermore, consistent with the reduced body size in OI, the body mass of HET pups was significantly lower than the body mass of WT pups in both females and males. This suggests that neonatal

and maternal body mass was not affected by maternal PAM exposure during bone formation. The number of sudden deaths of pups in the litters was evenly distributed between PAM-treated (n = 9) and PBS-treated groups (n = 11), consistent with prior reports of spontaneous deaths in Brtl/+ pups ([47]). In addition, one PAM and two PBS treated dams were euthanized due to morbidity prior to the end of the experiment.

Pamidronate effects on pregnancy and lactation-induced bone changes are highly dependent on time of treatment

In Brtl/+ dams, pregnancy alone significantly increased vertebral Tb.N by 37% but significantly decreased Tb.Th and TB.Sp, resulting in an overall increase in maternal bone volume fraction of 28% (Figure 3A, Supplemental Figure 1; Treatment Timepoint PC; End Timepoint Birth). The femur was less susceptible to these effects, as pregnancy had no effect on Tb.N or Tb.Sp but significantly decreased Tb.Th (7%), resulting in a 15% net bone loss (Figure 3B, Supplemental Figure 1; Treatment Timepoint PC; End Timepoint Birth). Conversely, lactation induced a loss in Tb.N in both vertebra (7%) and femur (39%) as seen in Figure 3A and Figure 3B (Treatment Timepoint PC-D15; End Timepoint Weaning). Together, these changes led to a 13% loss of vertebral bone volume fraction and an even greater femoral loss of 43% compared to virgin dams. When Brtl/+ dams were treated prior to conception with PAM, greater gains in vertebral Tb.N were obtained following pregnancy while no significant further gains were observed in the femur when compared to PBS- treated dams. Strikingly, when dams were carried out to the end of lactation (weaning of their pups at day 21), PAM induced a treatment-induced preservation of bone volume fraction through retained Tb.N that was directly related to timing of injection in both vertebra and femur. A modest preservation of bone mass was observed in the vertebra when PAM was administered prior to conception while significant gains of 58% were observed in the femur. PAM had no effect on Tb.Th regardless of time of treatment. In the cortical structure, pregnancy alone did not induce changes in vertebral or femoral C.Th, however, by weaned date, C.Th was reduced 16% and 10% in the vertebra and femur, respectively. Despite preservation of trabecular bone mass during the early stages of pregnancy and lactation (PC, E15 and D1) with PAM intervention, preservation of C.Th was only observed when PAM was administered prior to conception in both the vertebra (Figure 3C; Treatment Timepoint PC; End Timepoint Weaning) and the femur (Figure 3D; Treatment Timepoint PC; End Timepoint Weaning).

To qualitatively analyze the effects of PAM when administered at different stages of pregnancy and lactation, representative microCT images were extracted from virgin females and from dams at D21 post-weaning to visualize changes in maternal trabecular and cortical bone morphology. Lactation- induced bone loss was observed in both the vertebra and femur of PBS- treated dams (Figure 3E). When PAM was administered prior to conception, pregnancy and lactation-induced bone loss was completely prevented in Brtl/+ dams (Figure 3F). When PAM was administered at E15 during pregnancy and D1 of lactation, a temporal preservation of bone mass was observed. No further preservation

of trabecular structure was observed when dams were treated with PAM at D10 and D15 of lactation, suggesting trabecular bone loss had already occurred prior to this time point.

PAM-induced preservation of trabecular and cortical bone increases stiffness but not strength during pregnancy and lactation

Like the response seen in trabecular bone, a temporal effect was also observed in stiffness for both the vertebra and femur of PAM-treated dams. With pregnancy alone, a 57% increase in vertebral stiffness was observed in PBS-treated dams (Figure 4A), consistent with pregnancy-induced gains observed in Tb.N. No stiffness changes were observed in the femur following pregnancy alone, but a 10% femoral stiffness loss was observed by the end of lactation (Figure 4B). When PAM was administered prior to conception, vertebral stiffness in dams increased by 151% following pregnancy alone while a 50% increase was observed when assessed following lactation. Femoral stiffness assessed at birth and treated prior to conception showed an increase of 15% and an increase of 21% when assessed following lactation compared to virgin dams. Treatment with PAM induced a temporal effect on both vertebral and femoral stiffness when treated with PAM at different timepoints during pregnancy and lactation similar to the protective effect observed in their trabecular structure. Unlike stiffness, ultimate load in the vertebra did not show a temporal effect with PAM treatment. Instead, when dams were treated with PAM prior to conception and assessed at birth, a sustained effect was observed in vertebral max load, but an overall 26% was noted by weaned date regardless of PAM timepoint administration. Unlike the vertebra, a temporal effect with PAM treatment was observed in femoral ultimate load to failure when assessed at weaning. When dams were treated with PAM prior to conception a 6% increase in ultimate load to failure was observed at birth, however, when assessed at weaning ultimate load decreased by 10% compared to virgin dams. This protective effect on femoral max load ceased to exist when PAM was administered at late stages of lactation. Biomechanical results are summarized in Figure 4 and Supplemental Table 1.

BPs cross the placenta

To assess if BPs cross the placenta, a fluorescent bisphosphonate imaging agent (Osteosense 680EX) was administered when Brtl/+ were in late stages of gestation (E19). As seen in Figure 5, fluorescent images of the fetal vertebral body showed localization of BPs during late stages of gestation, confirming that BPs can cross the placenta.

Neonates at birth and weaning did not show skeletal adverse effects from maternal pamidronate exposure

Pregnancy and lactation represent a challenging period for the maternal skeleton as high demands of calcium are required for the healthy development of the neonatal skeleton. Since pregnancy and lactation-induced bone loss was prevented in PAM-treated dams, assessing neonatal skeletal development was imperative. At birth, following maternal

PAM exposure during pregnancy alone, neonates (1 day of age) from dams treated with PAM prior to conception showed no effects from BP exposure during their early stages of skeletal formation as no differences in BMD were observed in either the vertebra or the femur (Figure 6A). Following maternal PAM exposure during pregnancy and lactation, at 21 days of age, sexed Brtl/+ and WT pups from PAM-treated dams continued to show no effect from PAM exposure on trabecular and cortical structures (Figure 6B). To further assess that PAM exposure during pregnancy and lactation did not induce any adverse effects in the neonate's skeletal formation, Safranin O-stained sections reveal no morphological differences between pups from PBS and PAM treated dams (Supplementary Figure 2). Thus, despite preventing pregnancy and lactation-induced bone loss, no effects were observed in pups from PAM-treated dams compared to pups from PBS-treated dams.

Pamidronate increases maternal osteoclast number and surface but has no effect on neonates exposed to PAM during early stage of skeletal formation and lactation

To examine the presence of osteoclasts following maternal PAM exposure during pregnancy and lactation, mature osteoclasts positive for TRAP staining were identified in maternal tibia for all groups carried out through the end of lactation. TRAP+ mature osteoclasts (Figure 7A; N.Oc/BS) were present in both PAM and PBS-treated dams, albeit statistically more in PAM treated Brtl/+ dams. However, no differences were observed in osteoclast- surface coverage (Figure 7A; Oc.S/BS) between PAM and PBS-treated dams. To further assess the effect of the potential exposure to PAM during skeletal development, osteoclast number and surface in day 21 neonates from PBS and PAM treated dams were also analyzed following maternal intervention at prior to conception, E15 and D1. No differences in osteoclast number and surface coverage (Figure 7B) between neonates from PAM and PBS-treated dams were observed suggesting that either our PAM dosage provides a low exposure risk during crucial periods of skeletal development or that PAM has not yet taken an effect on neonatal bone.

Pamidronate did not further increase osteocyte lacunar area during pregnancy

Backscattering imagining was used to analyze PAM effects on maternal osteocyte osteolysis during pregnancy and lactation at standard locations of the femoral cortical bone (Figure 8A). Consistent with other studies, a significant increase in osteocyte osteolysis was observed following pregnancy in PBS- treated dams (Figure 8B). A similar increase was observed when dams were treated with PAM, suggesting that PAM intervention had no effect on lacunar size. By the end of lactation, when calcium demand is low, osteocyte osteolysis decreased and PAM continued to show no effect. Representative images of the differences in lacunar area under these conditions are shown in Figure 8C.

Discussion

The present study shows that pregnancy led to maternal gains in vertebral trabecular bone mass observed at parturition, while lactation induced maternal cortical and trabecular bone loss in both vertebra and femur observed at weaning in Brtl/+ mice harboring an OI-inducing Gly->Cys mutation in *col1a1*. PAM intervention led to a temporal retention of maternal cortical and trabecular bone in both vertebra and femur. When PAM was administered prior to conception (PC), bone mass gains due to pregnancy were amplified and lactation-induced bone loss was prevented. This protective effect was more modest when given during pregnancy (E15) and ceased to exist in the late stages of lactation (D10 and D15). Furthermore, pregnancy induced osteocyte osteolysis which was unaffected with PAM treatment. Despite preventing lactation-induced maternal bone loss, no negative skeletal effects from PAM exposure on offspring were observed. Since the use of BPs is increasing in premenopausal women with diseases of high bone remodeling rates like OI, these findings provide important clinical insight on a window for when PAM is most effective in preserving maternal bone mass and how maternal exposure may affect embryonic and neonatal skeletal development.

Current knowledge on the effect of BPs during pregnancy remains limited and is based almost entirely on animal toxicity studies that show transplacental effects of BPs [30-33]. Many of the adverse effects noted in these studies are likely dose related, however fewer studies exist that examine sub-toxic doses. No adverse maternal or offspring effects were observed in a rat and rabbit study using low dosages of incadronate, however when dosage was increased, dam death, retarded fetal ossification and abnormal tooth growth were noted [33]. Human data on BP effects during pregnancy is also limited. Scattered clinical cases reported uneventful pregnancies under BP treatment [19, 44, 61], but a few case studies did show low birth mass corresponding to lower gestational age as well as an increased incidence of spontaneous abortions [62]. However, due to the rarity of these outcomes these effects may have arisen from underlying conditions affecting the mother rather than BP itself.

To date, two animal toxicity studies have explored the effects of BPs during lactation. Both bovine and rat studies showed that EHDP and Cl2MBP, respectively induce hypocalcaemia in response to the inhibition of lactation-induced bone loss [34, 35]. Several other animal studies have shown evidence of post-lactation recovery of maternal bone loss [65-67] which may occur independent of preceding resorption events. When zoledronate was administered to mice at the beginning of lactation, bone formation persisted after forced weaning despite prevention of lactation-induced bone loss [68]. With regards to human data, there is a dearth of case studies reporting the effects of BPs when administered prior to, or during lactation. Of the human case studies that are available, both mother and infant did not demonstrate serious adverse effects [39, 41, 62, 69], however one report cited an OI mother with a year history of alendronate and pamidronate gave birth to a asymptomatic hypocalcaemic newborn [38].

The present study differs from prior animal studies by using a single clinical dose compared to the high doses previously used to evaluate BP safety during pregnancy and lactation. We observed that pregnancy triggered a significant increase in trabecular bone volume fraction of Brtl/+ dams, similar to results observed in other animal studies [70, 71]. However, this increase in bone mass was only observed in the vertebral body as no significant differences were noted in the distal femur. During pregnancy, studies have shown that there is an accumulation of calcium in the maternal skeleton to serve as a calcium reservoir during lactation [72]. Other animal studies have also reported that following conception, there is a rapid gain of skeletal mass and its utilization begins around late pregnancy when the fetal skeleton starts to form and develop [73, 74]. This process is then followed by a decline in maternal bone mass during lactation, as the neonatal skeleton begins to mineralize [75, 76]. Similar findings were observed in the present study, as trabecular bone mass significantly decreased in both the vertebra and distal femur during lactation. While other studies report greater changes in the vertebra [77], our results showed greater lactation-induced bone loss in the distal femur. This may be due to the timing of neonatal weaning, as other studies report lactation results following forced weaning (12 days of lactation). In our study, mice were weaned naturally on day 21 which may have led to some percentage of bone to be recovered by day 21 since the lactation demand is reduced as neonates transition to chow. However, these lumbar results corroborate with other studies that show a significant decrease in trabecular thickness and not number in the vertebra following lactation [68, 78]. Although pregnancy did not induce any response on cortical bone, significant thinning of the cortical bone was observed following lactation, consistent with results reported in other studies [72, 75, 79].

Since we confirmed through FRFP that BPs cross the placenta and that PAM intervention prevented lactation-induced bone loss in maternal bone, it was vital to assess if fetal and neonatal bone development was affected as well. Surprisingly, vertebral and femoral BMD of newborn WT and Brtl/+ mice (1 day of age) was unchanged when BP was administered to mothers prior to conception or during the embryonic stage. We propose four possible pathways of neonatal exposure that occurs when dams are treated with PAM during different stages of pregnancy and lactation as shown in Figure 9. 1) Treating with PAM prior to conception (PC) or BP recycling resulting from bone resorption during high periods of high calcium demand might expose offspring during both the embryonic stage (placental crossing) or neonatal stage (lactation and breast milk) prior to weaning. 2) Treating with PAM during gestation, may lead BP in the bloodstream to potentially cross the placenta, exposing the embryo to PAM (E15). 3) PAM treatment during lactation may lead to diffusion of BPs into the breast milk, exposing neonates to PAM during a time they highly depend on their mothers for survival (D1). 4) Lastly, PAM can indirectly interfere with fetal and neonatal development by inhibiting maternal bone resorption. This in return can disrupt calcium homeostasis and prevent offspring from receiving the necessary calcium supply for healthy bone development. Embryonic bone ossification has been reported to start near gestation day 14 [80]. Therefore,

administration of PAM to mothers during early stages of fetal bone ossification may lead to BP exposure to the offsping through placental crossing and localization on mineralized fetal bone during the embryonic stage. During lactation, it has also been well established that the demand for calcium is greatest when the offspring is solely dependent on the mother for nourishment and decreases as offspring are introduced to outside food sources [65, 81]. As a result, when the maternal skeleton was treated with PAM at either D1, D10 or D15 of lactation, risk of exposure through diffusion into the breast milk was likely greatest at the beginning of lactation. Because of the long half-life of BPs, treating dams prior to conception may expose fetal and neonatal bone to PAM through bisphosphonate recycling secondary to maternal bone resorption. Alternatively, PAM can indirectly interfere with fetal and neonatal skeletal development secondary to inhibition of maternal bone resorption. Direct disruption of maternal calcium homeostasis through anti-resorptive effects may prevent offspring from receiving the necessary calcium supply for healthy bone development. Despite these potential exposures to PAM, Brtl/+ and WT offspring (21 days of age) from treated and untreated mothers showed no differences in trabecular bone mass or cortical thickness. These findings suggest that the single PAM exposure had no effect on skeletal development whether potentially transferred through the placenta, breast milk or BP recycling resulting from maternal bone resorption, or through altered maternal calcium homeostasis.

We observed no difference in osteoclast surface or number in maternal tibiae between virgin controls and PBS-treated dams following lactation, despite the trabecular bone loss observed during this time. It is possible that we missed the window of high osteoclast number/surface since assessment was performed at time of weaning when bone turnover rate has slowed down (as seen from our D10 and D15 treated dams), however, no osteoclast assessment was performed following parturition. To support these findings, there is accumulating evidence that the conclusion of lactation is associated with maternal metabolic changes to reverse bone loss and increase bone formation in order to recover the maternal skeleton and rebuild its mineral storage in preparation for the next reproductive period [84]. In fact, female mice are in fertile estrus about 10 and 24 hours after giving birth, suggesting that this window of recovery is particularly short in mice. Furthermore, osteoclasts become apoptotic immediately with the cessation of lactation [85]. Similarly, we did not observe an effect of PAM on osteoclast surface coverage, however greater osteoclast numbers were observed with treatment. The literature on the effect BPs have on osteoclast number remains mixed. In rats treated with a high dose of zoledronate, the number of osteoclasts increased significantly, and a number of other studies have shown that the number of osteoclasts could be unaffected or even become elevated during BP treatment [86-88]. Alternatively, another study showed that treatment with zoledronic acid reduced osteoclast surface when administered for both short and long durations [89].

We assessed osteoclast number and surface in the neonates at wean date since it would allow us to evaluate the potential risk of PAM exposure on the role of osteoclasts during

the ossification and mineralization stages of bone development. We explored timepoints PC, E15 and D1 since not only was maternal bone loss inhibited with PAM at these time points, but these time points dictate three possible routes of fetal and neonatal BP exposure. Considering that no differences on osteoclast surface and number were observed, we can conclude that in this model, BP exposure through recycling (PC), placental crossing (E15) and diffusion into the breast milk (D1) have no effect on embryonic and neonatal development. These limited effects might be due to two possibilities; First, embryonic and neonatal bone formation rate may exceed bone resorption, so osteoclasts don't play a significant role during these early stages of bone development; or second, a single PAM dosage to the mother was not enough to induce a significant biological effect in the offpsring.

Although osteoclasts have been assumed to be primarily responsible for the removal of bone during calcium homeostasis, osteocytes have also been found to play a role during periods of increased calcium demand. While some studies have shown osteocytes play a role in calcium homeostasis during pregnancy and lactation [59, 90] others have not [91]. In our study, following pregnancy, Brtl/+ dams showed an increase in lacunar area which returned to baseline by end of lactation. While these changes were modest (16%) it is possible that lacunar area was higher during the earlier stages of lactation. Prior studies have shown osteocytic perilacunar and canalicular remodeling on the twelfth [58] and fourteenth day of lactation [92] while our observations were limited to parturition and weaning only. Most importantly, although maternal bone loss was prevented with PAM intervention, PAM showed no effect on osteocyte lacunar remodeling during this crucial period of calcium demand. Conversely, others have shown inhibition of osteocyte osteolysis during lactation through calcitonin [93] and parathyroid hormone [94], which may reflect a direct action on the osteocytes through related signaling pathways.

This study has several limitations. To assess the effect of a history of BP therapy, a single PAM dosage was administered prior to conception, which does not reflect continuous BP accumulation in the maternal skeleton arising from extensive treatment prior to conception in humans. Exploring a wider range of dosages will be important to assess the full effects of bone interventions during pregnancy and lactation. It is well established that BPs have a long half-life and remain embedded in the bone matrix until liberated during osteoclastic resorption. Thus, fetal and neonatal BP effects might not be observed until later stages of rapid growth or adulthood when BPs are recycled through bone remodeling. Therefore, although no effects were observed on weaned pups, BP exposure risk should not be disregarded, and post-lactation assessment of neonatal growth should be considered. Despite inhibiting lactation-induced decrease in maternal bone mass, no effects were observed in fetal and neonatal bone development. However, we did not analyze calcium levels in mothers or their offspring to verify that PAM did not indirectly interfere with fetal and neonatal development secondary to inhibition of maternal bone resorption. Additionally, we did not biochemically analyze maternal milk to assess neonatal PAM exposure during lactation. Though studies have established that pregnancy

up-regulates intestinal calcium absorption [95], we did not monitor whether an increase in intestinal calcium absorption is observed when lactation induced bone loss is prevented with BP treatment. The present study assessed the effects of pregnancy and lactation-induced bone loss and the effects following PAM intervention using only Brtl/+ mice. Since no WT controls were analyzed, we cannot conclusively determine whether the skeletal findings in this study are dependent on OI or specific to the background strain of the mice. While other studies have shown an increase in osteoclastic bone resorption during lactation, our lack of similar observations in this study is likely due to our post-weaning timing when bone mass is already recovering.

Aside from studying the effects of bone therapeutics in animal models for their translation into human clinical trials, studying any therapeutic effect during pregnancy and lactation raises several challenges particularly since mice have a shorter gestation period (18 to 21 days) than humans (280 days). In this study, PAM was administered in mice on gestation day 15 which has been shown to approximate week 8 of pregnancy in humans [96]. Although this timepoint represents the commencement of ossification in both timelines [96, 97], the rapid skeletal development in mice significantly reduces their risk of possible transplacental exposures. As a result, PAM intervention in humans might lead to greater exposure risks in utero because 80% of their gestation period remains as opposed to 29% in mice. Once born, there is a wide variation in the developmental phases between mice and humans and correlating their relative ages can be determined through several factors [98]. For example, mice are weaned at day 21 post-birth while on average the weaning age for humans is about 6 months (180 days) [99]. Thus, in this study, neonatal mice were exposed to PAM on days 1, 10 and 15 of lactation which approximate 9, 86 and 129 days of age in humans, respectively. However, this correlation through weaned date may not accurately represent the metabolic bone state between both timelines, suggesting that further studies are necessary.

During pregnancy and lactation, significant demands are placed on the maternal skeleton to provide the necessary calcium supply required for fetal and neonatal bone formation. The changes in calcium homeostasis during these reproductive periods can influence a time-dependent BP effect on bone metabolism that is not captured in toxicology studies. The results in this study capture the temporal effects on bone metabolism when PAM was administered at different stages of pregnancy and lactation. Specifically, when the maternal skeleton was treated with PAM prior to conception, lactation-induced bone loss was prevented. Despite inhibiting lactation-induced bone loss with PAM treatment, no adverse effects were observed on neonatal bone development. Additionally, our results of osteocyte remodeling provided further insight to their contribution to the regulation of calcium homeostasis during periods of high calcium demand from the maternal skeleton. In this study, calcium release from trabecular thinning and osteocyte osteolysis during pregnancy and potentially intestinal absorption (though not tested here) may have contributed to the healthy neonatal bone development observed. These results offer a treatment window during pregnancy and lactation for when PAM is most effective and

represent a further significant insight of the maternal changes in bone metabolism due to calcium homeostasis during this reproductive period.

Acknowledgements

The authors thank Carol Whitinger for her contributions. Research funding provided by NIH (RO1AR062522, P30AR069620, S10 OD017979). Study designed and conducted by DO, RS, BN and KMK. Data collected by DO, RS, EF. Data analyzed and interpreted by DO, RS, EF, MSC, and KMK. Manuscript was written by DO and KMK and revised and approved by all authors. KMK takes responsibility for the integrity of the data analysis.

Figures

Figure 1: Assessing PAM effects at different stages of pregnancy and lactation in the maternal skeleton. Single injection of PAM treatment or PBS was administered during pregnancy and lactation in Brtl/+ dams. Virgin controls were used to establish a skeletal baseline uninfluenced by these reproductive periods.

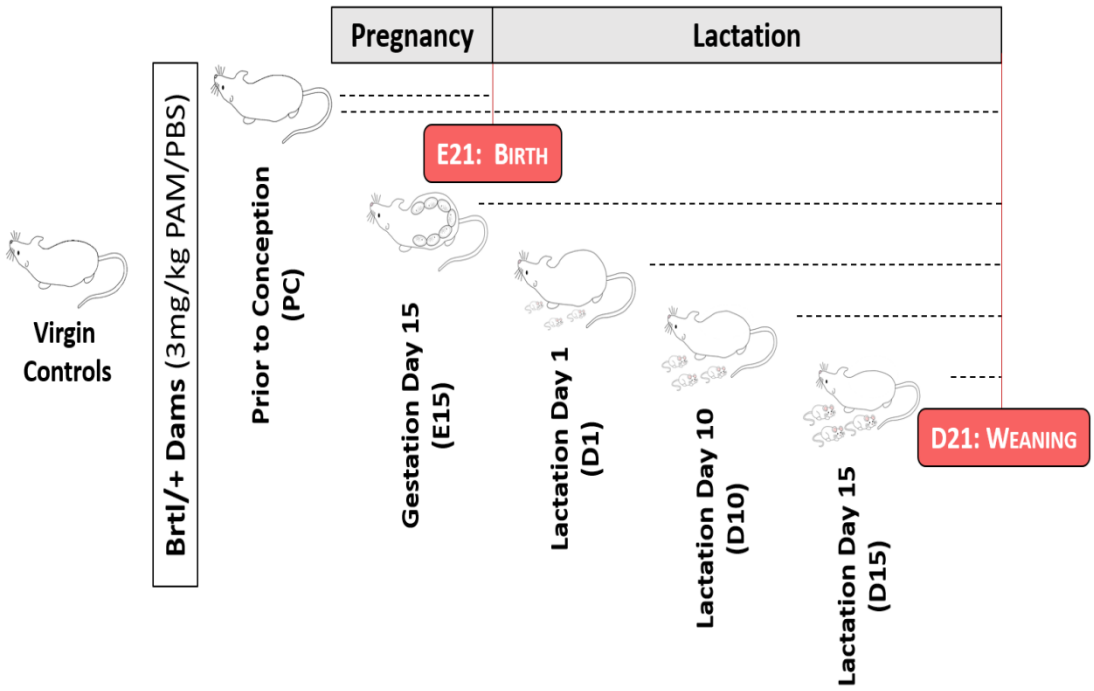


Figure 2: Maternal and neonatal body mass are not affected with pamidronate treatment. A) Maternal body mass increased due to pregnancy and lactation and no body mass differences between PBS and PAM treated groups were observed. B) Greater gains in maternal body mass were not induced with PAM treatment. Data are represented for maternal outcomes assessed at birth or weaning for dams treated with PBS (open box) and PAM (gray box).C) No differences in body mass of pups from PAM-treated dams and PBS-treated dams were observed. Results of Two-Way ANOVA factors: * $p<0.05$.

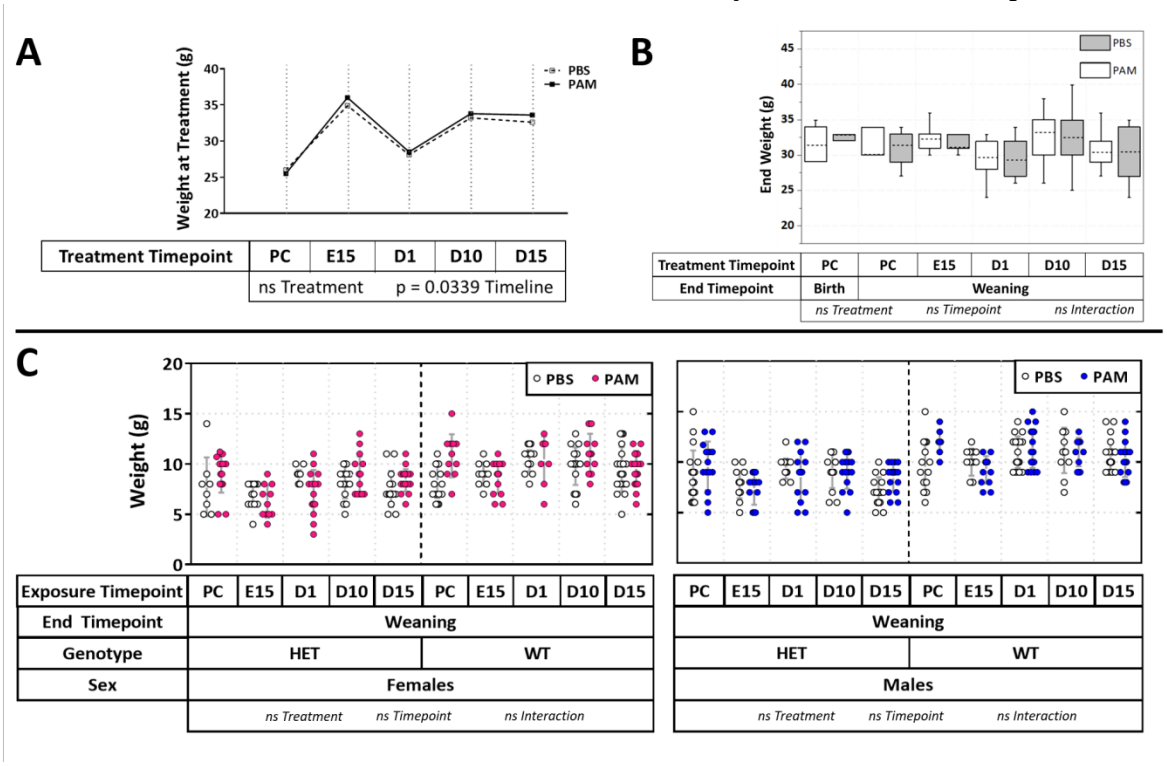


Figure 3: Pamidronate effects on pregnancy and lactation-induced bone changes are highly dependent on time of treatment. MicroCT data are represented for virgin controls at baseline (black), followed by maternal outcomes assessed at birth or weaning for dams treated with PBS (open box) and PAM (gray box). Bone microstructural analysis of the maternal Brtl/+ skeleton showed a temporal response to PAM intervention during pregnancy and lactation in both vertebra (A) and femur (B). A less robust temporal effect was observed in maternal cortical bone of both vertebra (C) and femur (D). Representative microCT isosurfaces of the distal femur metaphysis and the vertebral body reflect maternal bone structure in response to pregnancy and lactation in both untreated (E) and treated dams (F). Results of One-Way ANOVA factors: PAM and (PBS) compared to virgin controls: ++++ p<0.00005, +++ p<0.0005, ++ p<0.005, and + p<0.05. Results of Two-Way ANOVA factors: **** p<0.00005, *** p<0.0005, ** p<0.005, and * p<0.05.

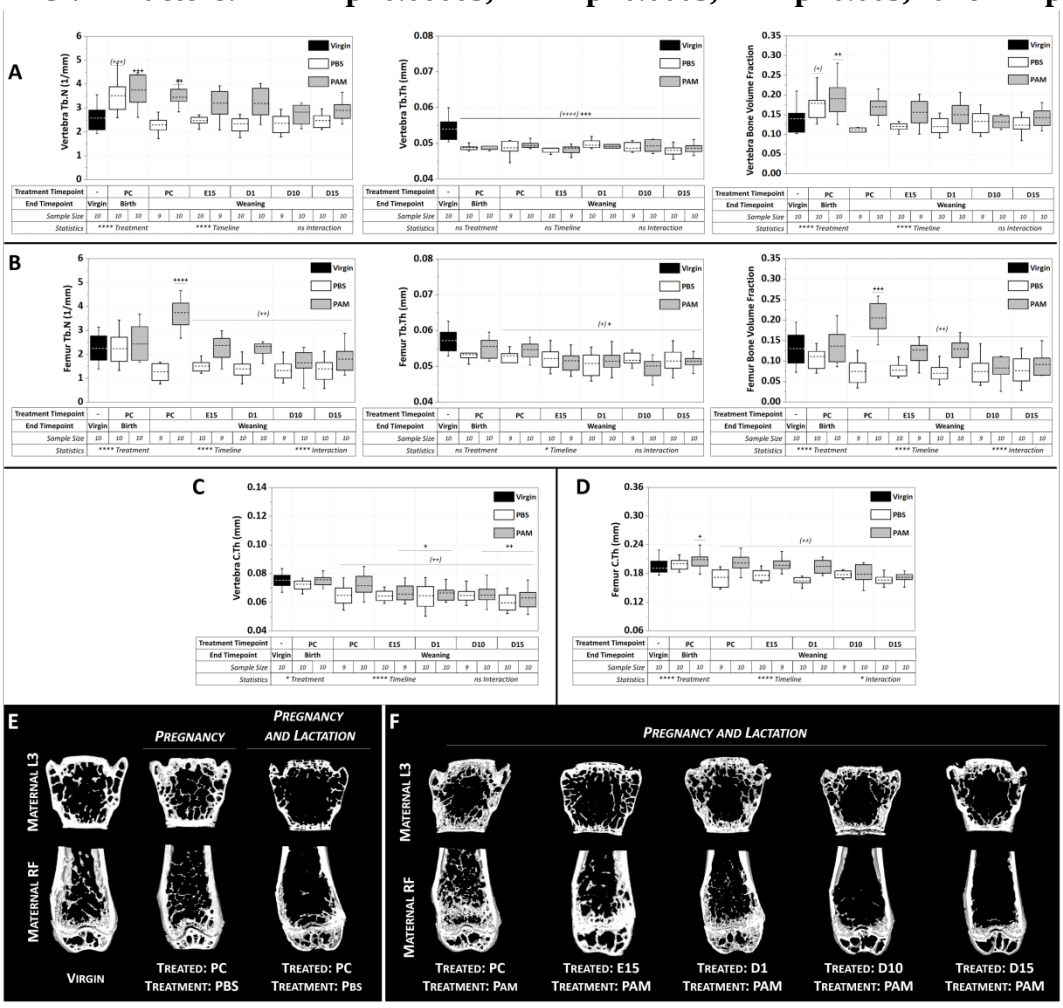
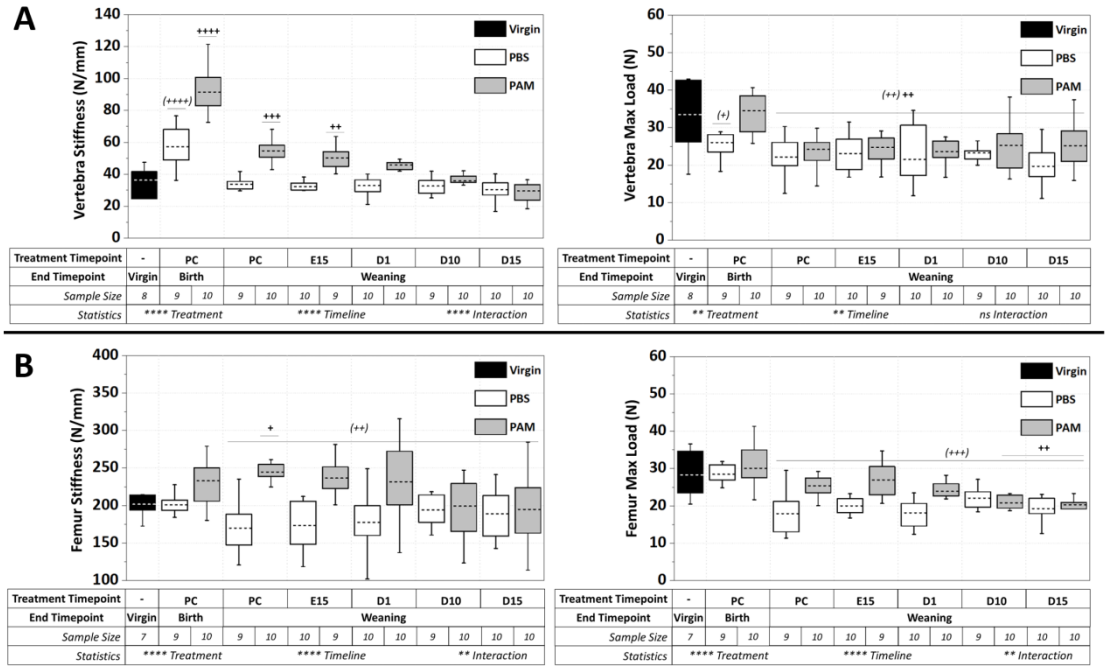


Figure 4: PAM-induced preservation of trabecular and cortical bone increases stiffness but not strength during pregnancy and lactation. Data for biomechanical properties are represented for virgin controls at baseline (black), followed by maternal outcomes assessed at birth or weaning for dams treated with PBS (open box) and PAM (gray box). A) With PAM treatment PC, significant gains in vertebral stiffness were observed following pregnancy and lactation but no preservation in maximum load to fracture was observed. Similarly, in the femur, PAM showed a sustained effect on stiffness and also a loss in maximum load to fracture (B). Results of One-Way ANOVA factors: PAM and (PBS) compared to virgin controls: ++++ p<0.00005, +++ p<0.0005, ++ p<0.005, and + p<0.05. Results of Two-Way ANOVA factors: **** p<0.00005, *** p<0.0005, ** p<0.005, and * p<0.05.



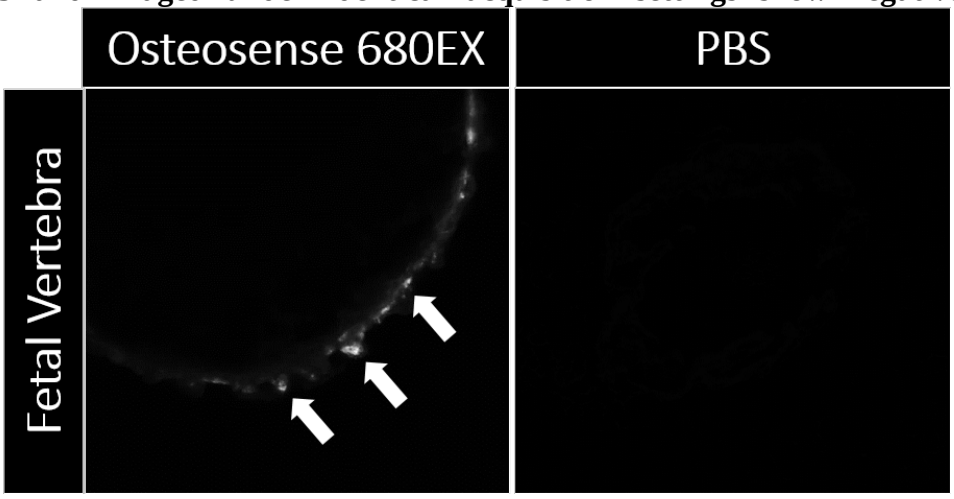


Figure 5: BPs cross the placenta. Fluorescent non-therapeutic BPs localized in fetal vertebral body during late stages of gestation. Control tissues from animals receiving PBS and imaged under identical acquisition settings show negative fluorescence.

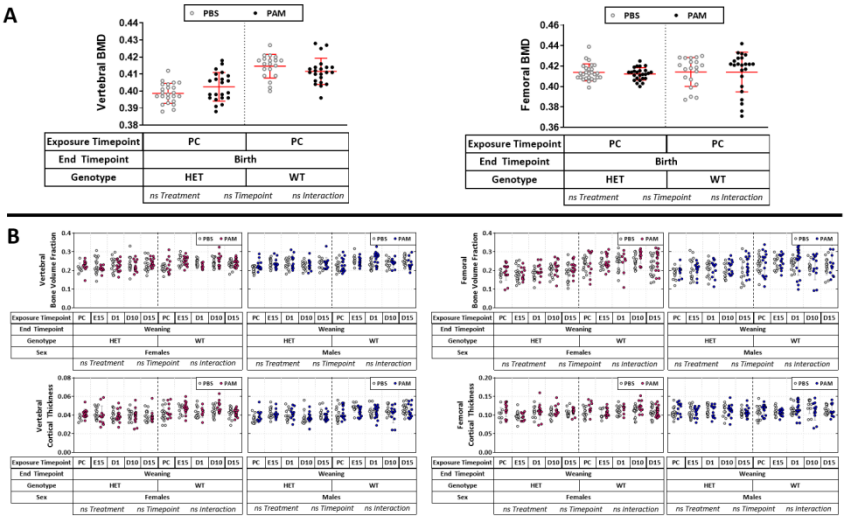


Figure 6: Neonates at birth and weaning did not show skeletal adverse effects from maternal pamidronate exposure. A) No changes in vertebral and femoral bone mineral density were observed at birth in sexed Brtl/+ and WT offspring following PAM exposure during the embryonic stage. B) At 21 days of age, sexed Brtl/+ and WT neonates from PBS and PAM treated dams showed no change in bone mass or cortical thickness when exposed to PAM at different stages of their skeletal development. Results of Two-Way ANOVA factors: **** p<0.00005, *** p<0.0005, ** p<0.005, and * p<0.05.

Figure 7: Pamidronate increases maternal osteoclast number and surface but has no effect on neonates exposed to PAM during early stage of skeletal formation and lactation. Maternal osteoclast number/surface data are represented for virgin

controls at baseline (black), followed by maternal outcomes assessed at birth or weaning for dams treated with PBS (open box) and PAM (gray box).A) Histomorphometric quantification of TRAP-stained tibial bone sections showed that PAM treatment increased maternal osteoclast number and surface coverage compared to PBS-treated dams. No temporal effect was observed with PAM treatment. B) Osteoclasts number and surface in pups from PAM- treated dams was not significantly altered with PAM exposure at PC, E15 and D1 compared to pups from PBS- treated dams. Results of One-Way ANOVA factors: PAM and (PBS) compared to virgin controls: ++++ p<0.00005, +++ p<0.0005, ++ p<0.005, and + p<0.05. Results of Two-Way ANOVA factors: **** p<0.00005, *** p<0.0005, ** p<0.005, and * p<0.05.

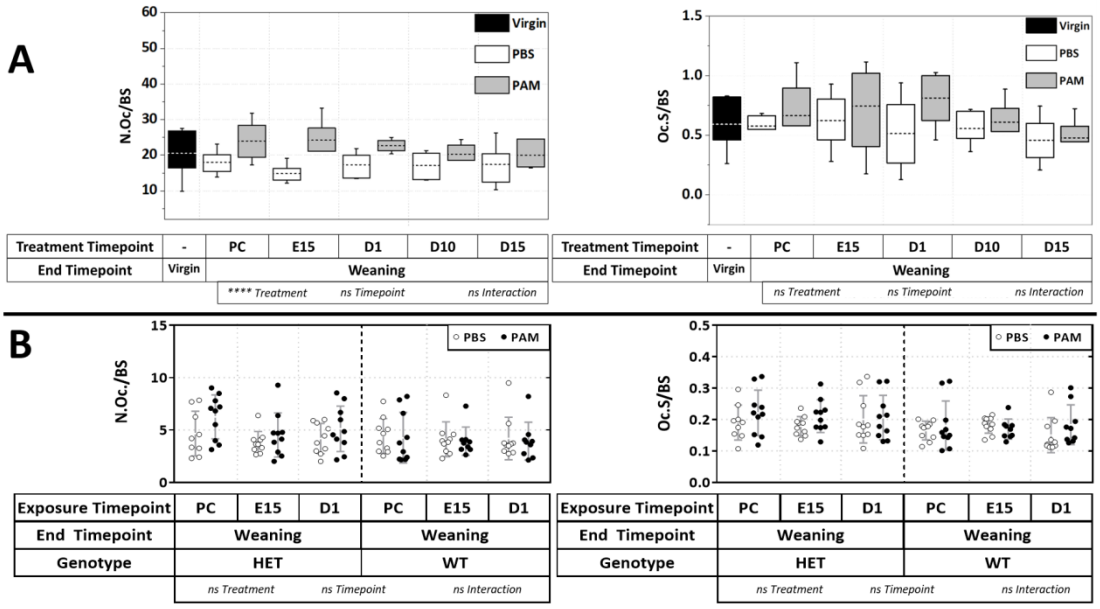


Figure 8: Pamidronate did not further increase osteocyte lacunar area during pregnancy. A) Panels show the location of measurements and representative images of lacunae from PBS and PAM treated dams. B) Pregnancy induced osteocytic lacunar enlargement in the femur as measured by backscatter electron microscopy. By the end of lactation, osteocyte lacunar area remodeled back to baseline. Lacunar data are represented for virgin controls at baseline (black), followed by maternal outcomes assessed at birth or weaning for dams treated with PBS (open box) and PAM (gray box). C) Representative SEM backscatter panels of cortical bone for virgin dams, and dams after pregnancy or lactation. Results of One-Way ANOVA factors: PAM and (PBS) compared to virgin controls: ++++ p<0.00005, +++ p<0.0005, ++ p<0.005, and + p<0.05. Results of Two-Way ANOVA factors: **** p<0.00005, *** p<0.0005, ** p<0.005, and * p<0.05.

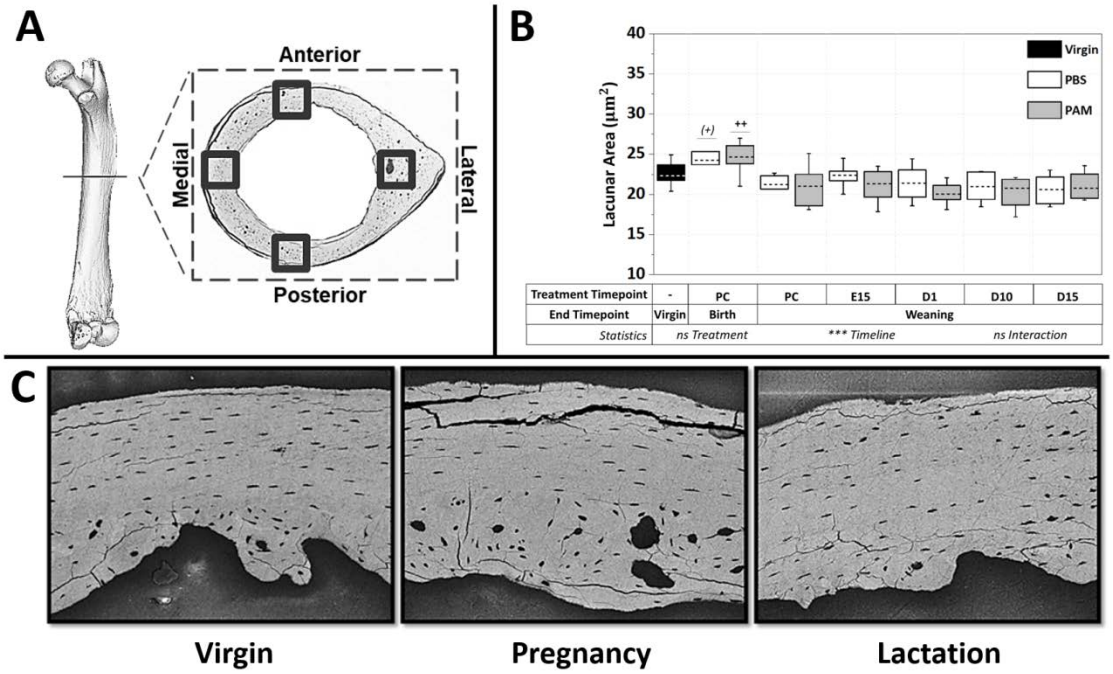
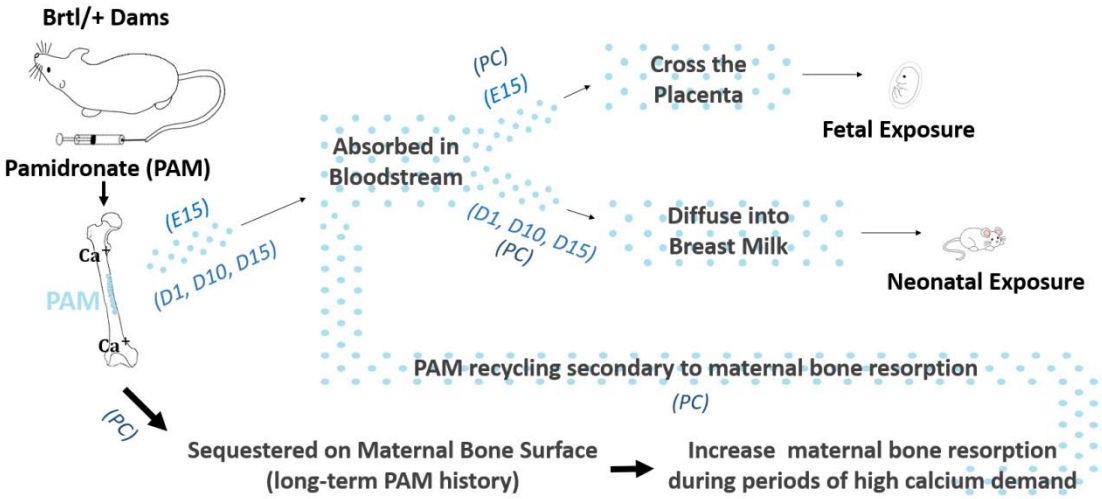


Figure 9: *Assessing PAM effects on offspring bone on offspring bone development from PAM exposure*. Proposed pathways of PAM exposure on fetal and skeleton bone development when the maternal skeleton is treated with BPs during the different stages of pregnancy and lactation. Not shown, potential indirect effect on calcium homeostasis from osteoclastic inhibition of maternal bone resorption during fetal and neonatal bone development.



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