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14. ABSTRACT Aromatase inhibitors (AIs), effective treatment for breast cancer, block estrogen synthesis. Increased bone resorption and decreased bone mineral density (BMD) are predicted consequences. We hypothesized that bisphosphonates (BPs) may prevent bone loss from AI therapy. Four-week-old female nude mice were treated with letrozole (10 mcg/d), zoledronic acid (ZA) (5 mcg/kg twice weekly), letrozole + ZA or control. Mice treated with letrozole alone had lower BMD compared to control (p<0.0001; total body, spine, femur and tibia). Mice treated with ZA alone had higher BMD compared to control (p<0.0001; total body, spine, femur and tibia). MicroCT analysis of the tibia showed no difference in trabecular bone volume (BV/TV) or trabecular number, thickness or spacing in mice treated with letrozole compared to control. Treatment with ZA (+/- letrozole) resulted in a significant increase in BV/TV and trabecular number and thickness, and the structural model index indicated that the bone structure was unusually solid. ZA prevented AI-induced bone loss, but microCT and dynamic bone histomorphometry suggest reduced bone remodeling. BPs may be useful to prevent AI-induced bone loss, but further studies are needed to assess the effects of these treatments on bone quality.					
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Effect of a high bone turnover state induced by estrogen deficiency on the development and progression of breast cancer bone metastases.

Introduction

Estrogen blockade is the standard medical therapy for treatment of breast cancer and breast cancer metastases. Therapy to suppress estrogen ultimately leads to increased bone resorption and osteoporosis. Cancer treatment-induced bone loss is likely to become the most common skeletal complication of malignancy. Our hypothesis is that breast cancer bone metastases are increased when bone is in a state of high turnover resulting from estrogen deficiency, and that inhibition of increased bone resorption will reduce the development and progression of breast cancer bone metastases. We are using a mouse model to test the effects of a high bone turnover state from estrogen deficiency on breast cancer metastases to bone, and to determine if inhibition of increased bone turnover due to estrogen deficiency will reduce breast cancer metastases to bone.

Breast cancer is the second leading cause of cancer death in women. In 2007, it is predicted that 178,480 women will be diagnosed with invasive breast cancer, and that 40,460 women will die from this disease (<http://www.cancer.org/cancerinfo>). Breast cancer metastasizes to bone in greater than 80% of patients with advanced disease, causing hypercalcemia, bone pain, fractures and nerve compression (1). Treatment of breast cancer generally involves a combination of surgery, radiation therapy, chemotherapy and endocrine therapy (2).

The growth of many breast cancers is stimulated by estrogen. Therefore, treatment of breast cancer and breast cancer metastases has focused on mechanisms to block the effect of estrogen at the estrogen receptor (selective estrogen receptor modulators) and to prevent estrogen synthesis (aromatase inhibitors) (3). Aromatase is a member of the cytochrome P450 superfamily; it catalyzes the irreversible conversion of androstenedione and testosterone into estrone and estradiol respectively. Thus, AIs block the rate-limiting step in estrogen biosynthesis, reducing estrogen levels by 90-95% (4;5). Recent data suggest that AIs are more efficacious than tamoxifen. The trial comparing letrozole to placebo in early-stage breast cancer in postmenopausal women showed that letrozole, after treatment with tamoxifen for five years, significantly improved disease-free survival (6). A trial using exemestane after two to three years of tamoxifen in postmenopausal women with primary breast cancer showed a significantly improved disease-free survival as compared to five years of tamoxifen (7).

In contrast to the bone protective effects of tamoxifen, emerging data indicate detrimental effects of AIs on bone. Aromatase deficiency, due to inactivating mutations of the aromatase gene, causes severe demineralization of the skeleton despite increased testosterone levels (8;9). Demineralization was reversible with estrogen. In a model of aromatase deficiency, mice had decreased peak BMD with accelerated bone loss. These changes were reversible with estradiol (10). In the trial comparing letrozole to placebo, there was a new diagnosis of osteoporosis in 5.8% of the letrozole group versus 4.5% in the placebo group (6). The ATAC trial evaluated anastrozole, tamoxifen,

or the combination of anastrozole and tamoxifen for adjuvant treatment of postmenopausal women with early breast cancer. At 37 months, fracture incidence in the anastrozole arm was 7.1% compared to 4.4% in the tamoxifen arm (11). BMD and markers of bone turnover were measured in a subset of patients and compared to control patients with breast cancer not receiving hormone therapy. In the anastrozole arm, there was a decrease in BMD and an increase in the markers of bone turnover. In the tamoxifen arm, there was an increase in BMD and a decrease in the markers of bone turnover (12). Furthermore, studies have reported increased osteoporotic fracture risk in breast cancer survivors (13-15). The effect of AIs on BMD in healthy postmenopausal women has not been studied. These findings, however, suggest that a reduction in postmenopausal estrogen levels from AIs will result in decreased BMD.

The increased bone resorption that occurs as a result of treatment with AIs may increase bone metastases in high-risk women. Increased bone resorption releases growth factors from bone, such as transforming growth factor β (TGF β), which can then induce breast cancer cell production of osteolytic factors, such as parathyroid hormone-related protein (PTHrP) (16). Osteolytic factors promote bone destruction and tumor growth. BPs, potent inhibitors of bone resorption, reduce skeletal morbidity from bone metastases (17). By decreasing bone resorption, they may reduce the release of factors from bone that stimulate tumor growth. BPs also cause apoptosis of breast cancer cells in vitro (18;19), decrease invasion of bone matrix (20) and decrease adhesion in vitro (21;22). The BP zoledronic acid inhibits angiogenesis both in vitro and in vivo (23). It is unknown whether BPs used concomitantly with AIs will suppress bone turnover and prevent bone metastases. The answer to this question, however, will have important therapeutic implications for the skeletal health and quality of life for women with breast cancer.

Body

Hypotheses:

- 1) Bone metastases are increased when bone is in a state of high turnover caused by estrogen deficiency.**
- 2) Inhibition of increased bone turnover will prevent breast cancer bone metastases.**

Specific Aim 1: To determine the effects of estrogen deficiency, induced by ovariectomy (OVX), on the development and progression of human breast cancer metastases to bone in a mouse model (**hypothesis 1**).

Specific Aim 2: To determine the effects of estrogen deficiency, induced by treatment with the AI letrozole, on the development and progression of human breast cancer metastases to bone in a mouse model (**hypothesis 1**).

Specific Aim 3: To determine if inhibition of the increased bone resorption associated with estrogen deficiency, due to OVX or treatment with an AI, will prevent the development and progression of human breast cancer metastases to bone in a mouse model (**hypothesis 2**).

Tasks:

Task 1 (Specific Aim 1): months 01-04. Female nude mice will be randomized to OVX or sham surgery. Four weeks post surgery, intra-cardiac inoculation with the human breast cancer cell line MDA-MB-231 (MDA-MB-231) will be performed in all mice (12 mice/group).

Task 2 (Specific Aim 1): months 05-06. Analyze bone (x-ray and BMD) and tumor (x-ray, bone histology and histomorphometry) parameters from mice in Task 1.

Procedures for Tasks 1 & 2 (part 1): Forty 4-week-old female nude mice were randomized to OVX or sham surgery. At 8 weeks post surgery, 8 mice from each group were euthanized and 12 mice from each group were inoculated with MDA-MB-231 via the intra-cardiac route. The mice were followed with X-rays at baseline and then at 1-week intervals to monitor the development and progression of bone metastases. BMD was measured at baseline and then every 2 weeks for the duration of the experiment. Mice were euthanized when they developed significant bone metastases, lost more than 10% of their baseline body weight, or if they showed any signs of distress or impaired mobility.

- **OVX.** Mice were anesthetized with ketamine/xylazine and placed prone. Ovaries were excised. The mice were sutured and hydrated with 3cc of saline. The incision site was treated with an antibiotic cream and the mice were placed on a warm heating pad until they recovered from anesthesia. Control animals received sham surgeries at the same time.
- **Bone metastases model.** Tumor cells were trypsinized and resuspended in PBS to a final concentration of 10^6 cells/100 μ L immediately prior to injection. The mice were anesthetized with a ketamine/xylazine mixture and positioned ventral side up. The left cardiac ventricle was punctured through a percutaneous approach using a 26-gauge needle (16;24;25;26). For radiography, the mice were anesthetized deeply, placed prone against the detector, and exposed with an x-ray at 35 kVp for 5 seconds using a Faxitron Unit.
- **Analysis of metastases.** All radiographs from the mice were evaluated in a blinded fashion. The number and area of osteolytic bone metastases were calculated on radiographs using a computerized image analysis system (16;25;26). Quantification of lesion area and number was performed using image analysis software (Metaview/Metamorph Software). This system detects lesions as small as 0.1mm.
- **Bone & soft tissue histology & histomorphometry.** Forelimb and hindlimb long bones, spine, calvaria and soft tissues were harvested, fixed in 10% buffered formalin, decalcified in 10% EDTA and paraffin embedded. Sections for histomorphometric analysis were stained using hemotoxylin and eosin, orange G and phloxine. The following variables were measured in sections of bone to assess bone resorption (16;25;26): total bone area, total tumor area, osteoclast number per mm of tumor-bone interface and histomorphometry (using Metaview/Metamorph). In soft tissue blocks, tumor area will be measured to determine if estrogen suppression alters metastases to non-bone sites.

- **Insulin-like growth hormone-1 (IGF-1) serum levels.** Whole blood was collected by retro-orbital puncture. The collected serum was aliquoted into 20 μ l samples and stored at -80°C. IGF-1 was measured using IGF-1 RIA (American Laboratory Products Company, Windham, NH). Calculated assay sensitivity is 0.02 ng/mL; cross reactivity with IGF-2 is <0.05%. Inter-assay CV is 6%; intra-assay CV is 4%. IGF-1 was dissociated from IGF-1 binding proteins by dilution in acidic buffer, and an antibody solution containing excess IGF-2 was added to neutralize samples. IGF-1 was then measured with addition of 125 I tracer.
- **BMD measurements.** BMD was measured in anesthetized mice using a Lunar Piximus. Total body, lumbar spine, mid-femur, proximal femur and proximal tibia BMD was done at baseline and then at 2-week intervals.
- **Body composition measurements.** Body composition was measured in anesthetized mice using a Lunar Piximus. Percent fat mass and fat mass were measured at baseline and then at 2-week intervals.
- **Statistics.** Data was analyzed by ANOVA followed by Tukey-Kramer multiple comparison test for comparing > 2 groups and by the Student's t-Test for comparison of 2 treatment groups.

Results for tasks 1 & 2 – part 1 (this data was previously reported in the 2006 annual summary):

- Eight weeks after surgery there was no difference in BMD in OVX mice compared to sham mice at any site: total body ($p=0.6814$), spine ($p=0.3398$), femur ($p=0.3914$) or tibia ($p=0.3093$) (**figure 1**).
- Histomorphometry showed no difference in trabecular bone volume (TBV) in OVX mice compared to sham mice at the femur ($p=0.8634$) or tibia ($p=0.1329$) (**figure 2**).
- There was no difference in serum IGF-1 levels in the OVX mice compared to the sham mice (**figure 3**).
- In the mice inoculated with MDA-MB-231 via intra-cardiac injection, there was no difference in total body x-ray lesion area ($p=0.4728$) or tibia plus femur x-ray lesion area ($p=0.4412$) between the OVX and sham mice (**figure 4**).
- There was no difference in survival between the OVX and sham mice ($p=0.0874$) after intra-cardiac injection with MDA-MB-231 (**figure 5**).

Conclusions, potential problems and alternative strategies for tasks 1 & 2. We expected to see decreased BMD in OVX mice compared to sham mice. However, there was no difference in BMD between the 2 treatment groups. In addition, there was no difference in skeletal metastases or survival in the OVX mice compared to the sham mice after intra-cardiac injection with MDA-MB-231. A possible explanation for this is the genetic heterogeneity of our female nude mice, which are a random mix of the Balb/C and ICR Swiss breeds. This heterogeneity may explain why we have seen a different response to OVX in this experiment compared to our prior experiment. Inbred mice are known to have a variable response to OVX. For instance, Bouxsein *et al* used 5 strains of inbred mice to study the skeletal response to OVX (27). Four-month-old female mice underwent OVX or sham surgery and were euthanized 4 weeks later. The 5 strains of mice varied in terms of the site in which loss of BMD was noted, and in

whether or not they lost more trabecular versus cortical bone at each site. Li *et al* used 3 inbred mouse strains to show that genetic background influences the rate of cortical bone loss after OVX (28). Both Bouxsein *et al* and Li *et al* used older mice (16-week-old) for OVX versus sham studies. Skeletal response to OVX may differ based on age of the mice. Therefore, we elected to repeat this experiment.

It should be noted that younger mice respond more favorably to intra-cardiac injection with MDA-MB-231. Young mice will develop more bone metastases (and at a faster rate) compared to older mice, which is why the 4-week-old time point for OVX was originally chosen. This current experiment will allow us to directly compare the number and speed at which bone metastases develop in younger versus older female nude mice after intra-cardiac injection with MDA-MB-231.

Procedures for Tasks 1 & 2 (part 2a): To determine the effect of age on BMD after OVX, 40 4-week-old female nude mice were randomized to OVX or sham surgery. BMD was measured at baseline and then every 2 weeks for the duration of the experiment. Once there was a difference in BMD between the OVX and sham mice (10 weeks after surgery), 10 mice from each group were randomized to intra-cardiac injection with MDA-MB-231 or control. The mice were followed with X-rays at baseline and then at 1-week intervals to monitor the development and progression of bone metastases. Mice were euthanized when they developed significant bone metastases, lost more than 10% of their baseline body weight, or if they showed any signs of distress or impaired mobility. The 20 remaining mice (10 OVX and 10 sham) continued to undergo BMD monitoring every 2 weeks until the experiment was terminated at week 28.

- **OVX.** Please see above.
- **Bone metastases model.** Please see above.
- **Analysis of metastases.** Please see above.
- **Bone & soft tissue histology & histomorphometry.** Please see above.
- **Insulin-like growth hormone-1 (IGF-1) serum levels.** Please see above.
- **BMD measurements.** Please see above.
- **Body composition measurements.** Please see above.
- **Micro-computed tomography (micro-CT).** Micro-CT 40 (Scanco Medical, Bassersdorf, Switzerland) was used to assess skeletal changes in the right proximal tibia from each mouse. Variables measured included trabecular bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular spacing (Tb.Sp), structural model index (SMI), geometrical degree of anisotropy (DA), volumetric BMD (vBMD) and calculated connectivity density (ConnD).
- **Colony-forming units (CFU) assays.** Bone marrow cells from the femurs and tibias (3 mice/group) were used to determine the effect of OVX on fibroblast (CFU-F) and osteoblast (CFU-OB) progenitor cells. CFU-OB: Bone marrow cells

were flushed from femurs and tibias, combined, rinsed and resuspended in 15% fetal bovine serum (FBS) containing α -minimum essential medium (α MEM), 50 ug/ml ascorbic acid and 10mM β -glycerophosphate to support mineralization. Cells were plated (1×10^6 cells/well) and cultured for 28 days and then fixed with 10% Formalin and stained for 10 minutes with a 2% solution of Alizarine Red S dissolved in water with pH adjusted to 4.2. Using light microscopy, CFU-OB quantified by direct counting of all stained nodules that are Alizarin Red S-positive. *CFU-F*: Bone marrow cells were flushed from femurs and tibias, combined, rinsed and resuspended in 15% FBS containing α MEM, 50 ug/ml ascorbic acid and 10mM β -glycerophosphate. Cells were plated (2.5×10^6 cells/well) and cultured for 9 days and then fixed with 10% formalin and stained with alkaline phosphatase. Using light microscopy, a colony was defined as the presence of at least 50 alkaline phosphatase-positive cells.

- **Osteoclast formation assay.** Bone marrow cells were flushed from femurs and tibias, combined, rinsed and resuspended in 15% FBS containing α MEM and 10nM $1,25 (\text{OH})_2$ vitamin D_3 . Cells were then plated (2×10^6 cells/well) and cultured for 7 days and then stained with tartrate-resistant acid phosphatase (TRAP). Using light microscopy, osteoclasts were quantitated as the number of TRAP (+) multinucleated cells per well.
- **Statistics.** Please see above.

Results for tasks 1 & 2 – part 2a: (data not previously reported)

- At 28 weeks, OVX mice had significantly increased body weight ($p < 0.0001$), fat mass ($p < 0.0001$) and % fat mass ($p < 0.0001$) compared to the sham mice (**figure 6**). At 28 weeks, OVX mice had significantly decreased total body BMD ($p < 0.0001$) (**figure 6**), and significantly decreased BMD at the spine ($p < 0.0001$) and proximal tibia ($p < 0.0001$) compared to sham mice (**figure 7**). Interestingly, there was no difference in BMD between the OVX and sham mice at the distal femur, and the OVX mice demonstrated increased BMD at the mid femur ($p < 0.0001$) compared to the sham mice (**figure 7**).
- Histomorphometry revealed that there was no difference in TBV between the OVX and sham mice at the proximal tibia or distal femur (**figure 8**).
- Micro-CT revealed that trabecular bone in the proximal tibia did not differ between the OVX and sham mice (**figure 9**).
- Micro-CT revealed that trabecular bone in the distal femur did not differ between the OVX and sham mice (**figure 10**).
- Bone marrow cultures from OVX mice exhibited a greater number of CFU-fibroblasts ($p < 0.0001$), CFU-osteoblasts ($p < 0.0001$) and TRAP-positive osteoclasts ($p = 0.0005$) compared to sham mice (**figure 11**).
- Although baseline IGF-1 levels did not differ between the OVX and sham mice, 28 weeks after surgery the OVX mice had higher IGF-1 levels than the sham mice ($p = 0.0253$) (**figure 12**). However, pre-OVX IGF-1 levels were not significantly different from post-OVX IGF-1 levels, and pre-sham surgery IGF-1

levels were not significantly different from post-sham surgery IGF-1 levels (**figure 12**).

- Total body X-ray lesion area was greater in the sham surgery mice compared to the OVX mice ($P < 0.0001$) (**figure 13**).

Conclusions, potential problems and alternative strategies for tasks 1 & 2 (part 2a). In our initial OVX versus sham surgery experiment using 4-week-old female nude mice, there was no difference in BMD between the 2 treatment groups. In addition, there was no difference in skeletal metastases or survival in the OVX mice compared to the sham mice after intra-cardiac injection with MDA-MB-231. A possible explanation for this was the genetic heterogeneity of the female nude mouse, which is a random mix of the Balb/C and ICR Swiss breeds. However, in this repeat experiment, we saw significantly decreased BMD in the OVX mice at the total body, spine and proximal tibia. Although there was no difference in BMD at the distal femur, BMD was increased in the mid femur in the OVX mice compared to the sham mice. Interestingly, histomorphometry did not show decreased TBV at the distal femur and proximal tibia in the OVX mice compared to the sham surgery mice. Micro-CT data also did not show decreased BV/TV in the femur or tibia in OVX or sham surgery mice. Therefore, the changes in BMD that were noted using LUNAR PIXImus, which provides a two-dimensional view of bone density, may be due to changes in cortical bone, which was not analyzed by micro-CT or bone histomorphometry. In order to explore this further, the femurs and tibia are currently being reanalyzed by micro-CT in order to better assess the cortical bone.

Bone marrow cultures from OVX mice exhibited a greater number of CFU-fibroblasts, CFU-osteoblasts and TRAP-positive osteoclasts compared to sham mice. This indicates that there is increased bone turnover in the OVX mice compared to the sham mice. IGF-1 levels were also increased in the OVX mice compared to the sham mice. However, pre and post surgery IGF-1 levels did not differ for the OVX mice or the sham mice. Interestingly, the sham mice demonstrated earlier bone metastases and larger bone metastases compared to the OVX mice. The reason for this is not clear. The human breast cancer cell line MDA-MB-231 is estrogen receptor (ER)-negative. Therefore, the estrogen production in the sham mice should not have an effect on the MDA-MB-231 cell growth or metastasis. However, there are two ERs, ER-alpha and ER-beta. The MDA-MB-231 cells are ER-alpha negative, but they do have low levels of ER-beta. It may be that the estrogen production in the sham mice is enough to stimulate the low levels of ER-beta in the MDA-MB-231 cell line. Further experiments are necessary to determine the cause of increased bone metastasis in the sham versus OVX mice.

Procedures for Tasks 1 & 2 (part 2b): To determine the effect of age on BMD after OVX, 40 16-week-old female nude mice were randomized to OVX or sham surgery. BMD was measured at baseline and then every 2 weeks for the duration of the experiment. Once there was a difference in BMD between the OVX and sham mice (8 weeks after surgery), 10 mice from each group were randomized to intra-cardiac injection with MDA-MB-231 or control. The mice were followed with X-rays at baseline

and then at 1-week intervals to monitor the development and progression of bone metastases. Mice were euthanized when they developed significant bone metastases, lost more than 10% of their baseline body weight, or if they showed any signs of distress or impaired mobility. The 20 remaining mice (10 OVX and 10 sham) continued to undergo BMD monitoring every 2 weeks until the experiment was terminated at week 20.

Results for tasks 1 & 2 – part 2b: (data not previously reported)

- At 20 weeks, OVX mice had significantly increased body weight ($p < 0.0001$), fat mass ($p < 0.0001$) and % fat mass ($p < 0.0001$) compared to the sham mice (**figure 14**). At 20 weeks, OVX mice had significantly decreased total body BMD ($p = 0.0048$) (**figure 14**), and significantly decreased BMD at the spine ($p < 0.0001$) and distal femur ($p < 0.0001$) and mid femur ($p = 0.0409$) compared to sham mice (**figure 15**). There was no difference in BMD between the OVX and sham mice at the proximal tibia (**figure 15**).
- Micro-CT revealed that trabecular bone in the proximal tibia did not differ between the OVX and sham mice with the exception of “degree of anisotropy”, which was slightly increased in the OVX mice compared to the sham mice ($p = 0.0316$) (**figure 16**).
- Micro-CT revealed that trabecular bone in the distal femur did not differ between the OVX and sham mice (**figure 17**).
- Bone marrow cultures from OVX mice exhibited a greater number of CFU-fibroblasts ($p < 0.0001$) and CFU-osteoblasts ($p = 0.0001$) compared to sham mice (**figure 18**). Unfortunately, there was a technical problem with the osteoclast formation assay.
- IGF-1 levels did not differ between the OVX and sham mice at baseline or at 20 weeks after surgery (**figure 19**). In addition, pre-OVX IGF-1 levels were not significantly different from post-OVX IGF-1 levels, and pre-sham surgery IGF-1 levels were not significantly different from post-sham surgery IGF-1 levels (**figure 19**).
- Total body X-ray lesion area was greater in the sham surgery mice compared to the OVX mice ($P = 0.0043$) (**figure 20**).

Conclusions, potential problems and alternative strategies for tasks 1 & 2 (part 2b). These experiments demonstrated that 16-week-old female nude mice have decreased BMD after OVX at the total body, spine, distal femur and mid femur as compared to sham mice. There was no difference in BMD at the proximal tibia, which is in contrast to the previous experiment in which the 4-week-old female nude mice did lose bone at the proximal tibia after OVX. In addition, the 4-week-old female nude mice did not lose BMD at the distal femur after OVX, and BMD was actually increased at the mid femur after OVX. Therefore, 4-week-old (young) and 16-week-old (old) female nude mice do lose bone after OVX, but the sites of BMD loss appear to differ in young versus old female nude mice.

Micro-CT data did not show any difference in trabecular bone parameters for the femur or tibia after OVX or sham surgery in 16-week-old female nude mice. The one exception to this was “degree of anisotropy,” which was greater in the tibias of OVX mice compared to sham mice. Degree of anisotropy is a calculation that estimates the distance from the center of the bone (tibia) to each trabeculae. Given that trabecular number, thickness and separation did not differ between the OVX and sham mice, the significance of a difference in degree of anisotropy is not clear. Histomorphometry for this experiment has not yet completed but, as with the 4-week-old mice, the femurs and tibias for the 16-week-old mice are being reanalyzed by micro-CT to determine if the changes in BMD are attributable to changes in cortical bone as opposed to trabecular bone.

Bone marrow cultures from OVX mice exhibited a greater number of CFU-fibroblasts and CFU-osteoblasts compared to sham mice, indicating increased bone turnover in the OVX mice compared to the sham mice. In contrast to the 4-week-old mice, IGF-1 levels in the 16-week-old mice were not significantly different between the OVX and sham mice. Pre and post surgery IGF-1 levels also did not differ for the OVX mice or the sham mice. Interestingly, as with the 4-week-old mice, the 16-week-old sham mice demonstrated earlier bone metastases and larger bone metastases compared to the OVX mice. Again, the reason for this remains unclear.

Task 3 (Specific Aim 2): months 07-10. Female nude mice will be randomized to therapy with the AI letrozole versus control, administered via subcutaneous (sc) injection. After 4 weeks of treatment with letrozole or control, each group of mice will be randomized to intra-cardiac injection with MDA-MB-231 or control (12 mice/group).

Task 4 (Specific Aim 2): months 11-12. Analyze bone (x-ray and BMD) and tumor (x-ray, bone histology and histomorphometry) parameters from mice in Task 3.

Task 5 (Specific Aim 3A): months 13-16. Female nude mice will be randomized to OVX or sham surgery or to treatment with letrozole or control. At time of OVX/sham surgery or initiation of letrozole/control therapy, the mice in each of the 4 treatment groups will be randomized to receive twice weekly sc injections of zoledronic acid (ZA) or control. After 4 weeks, all mice will undergo intra-cardiac injection with vehicle (control for MDA-MB-231)(12 mice/group).

Task 6 (Specific Aim 3A): months 17-18. Analyze bone (x-ray and BMD) and tumor (x-ray, bone histology and histomorphometry) parameters from mice in Task 5.

Task 7 (Specific Aim 3B): months 19-22. Female nude mice will be randomized to OVX or sham surgery or to treatment with letrozole or control. At time of OVX/sham surgery or initiation of letrozole/control, the mice in each of the 4 treatment groups will be randomized to receive twice weekly sc injections of ZA or control. After 4 weeks, all mice will undergo intra-cardiac injection with MDA-MB-231 (12 mice/group).

Task 8 (Specific Aim 3B): months 22-23. Analyze bone (x-ray and BMD) and tumor (x-ray, bone histology and histomorphometry) parameters from mice in Task 7.

Task 9 (Specific Aims 1-3): months 24-36. Analyze data and prepare manuscripts and reports.

For tasks 3-9 (Part 1): To study the effects of AIs on bone, and whether or not the skeletal effects of AIs on bone could be prevented with concomitant treatment with the BP ZA, 40 4-week-old female nude mice were randomized to treatment with: 1) control, 2) letrozole, 3) ZA or 4) letrozole + ZA (10 mice/group). Mice were euthanized after 14 weeks of treatment.

- **Treatment of mice with an AI.** Mice were treated with letrozole 10 mcg/day/sc starting on day zero and continuing through the end of the experiment. Control mice were administered the same volume of vehicle/day/sc.
- **Treatment of mice with a BP.** Mice were treated with 5 mcg/kg/sc of ZA twice weekly, starting on the day zero and continuing through the end of the experiment. The dose of ZA was determined by previous experiments in the Guise laboratory. Control mice were injected with the same volume of vehicle sc twice weekly.
- **IGF-1 PCR.** Total RNA was extracted from homogenized mouse liver in Trizol reagent and treated with DNase prior to quantitative PCR (qPCR). cDNA was generated using an oligo dT-primed Qiagen Omniscript RT kit. qPCR was then performed using a Qiagen SYBR green PCR kit and Bio-Rad MyiQ Single Color Real-time PCR Detection System (Bio-Rad, Hercules, CA).
- **Mechanical loading.** MTS 858 Bionix materials test system (MTS Systems Corp, Eden Prairie, MN) was used to analyze the right tibia and femur from each mouse. Variables measured included peak load and stiffness.
- **Dynamic histomorphometry.** Dynamic histomorphometry was used to assess bone formation rate (BFR) & mineral apposition rate (MAR). On days 1 and 7, mice underwent intraperitoneal (IP) injection with calcein 0.02 mg/gm body weight. On day 4, the mice underwent IP injection with tetracycline 0.03 mg/gm body weight. The mice were then euthanized on day 10. Lumbar spines were embedded in methyl-methacrylate. Seven-micrometer-unstained longitudinal sections were cut and analyzed by epifluorescence microscopy. The histomorphometric examination was performed using Metamorph software and a Leica microscope. All trabecular bone measurements were made at $\times 200$ magnification.

Results for tasks 3-9 - Part 1 (this data was previously reported in the 2006 annual summary):

- After 13 weeks of treatment, mice treated with letrozole alone had lower BMD compared to control ($p < 0.0001$; total body, spine, proximal femur and tibia) (**figure 21**). In the mid-femur, however, mice treated with letrozole alone, as compared to control, did not have lower BMD after 13 weeks of treatment ($p = 0.0961$), but a difference in BMD was seen after 7 weeks of treatment ($p = 0.0435$) (**figure 22**). Mice treated with ZA alone had higher BMD compared to control ($p < 0.0001$; total body, spine, mid-femur, proximal femur and tibia). Mice treated with letrozole + ZA achieved the same BMD as mice treated with ZA alone at the spine and tibia, but had greater BMD than mice treated with ZA

alone at the mid-femur ($p<0.0001$), proximal femur ($p<0.0001$) and total body ($p<0.0023$) (**figure 21**).

- Histomorphometry demonstrated that mice treated with letrozole alone had the same TBV as mice treated with control at the proximal femur, tibia and lumbar spine (**figure 23**). Mice treated with ZA (+/- letrozole) had increased TBV compared to letrozole alone at the proximal femur and tibia. In the lumbar spine, mice treated with ZA alone had increased TBV compared to both letrozole ($p<0.05$) and letrozole + ZA ($p<0.05$).
- MicroCT analysis of the proximal tibia showed no difference in BV/TV, SMI or Tb.N, Tb.Th or Tb.Sp in mice treated with letrozole alone compared to control. Treatment with ZA (+/- letrozole) resulted in a significant increase in BV/TV, Tb.N and Tb.Th, and the SMI indicated that the bone structure was unusually solid (**figure 24**).
- Dynamic bone histomorphometry of the lumbar spine demonstrated decreased BFR and MAR in mice treated with letrozole, ZA or the combination compared to control (**figure 25**).
- Mechanical testing showed no difference in peak load or stiffness for either the femur or tibia in the letrozole-treated mice compared to the control mice (**figure 26**).
- Liver IgF-1 Real-time RT PCR demonstrated decreased IGF-1 expression in letrozole-treated mice compared to control ($p<0.01$), an effect that was reversed with the addition of ZA (**figure 27**). There was no difference in serum IGF-1 levels in mice treated with letrozole alone compared to control (**figure 27**), although there was decreased serum IGF-1 levels in the ZA alone mice compared to control ($p<0.05$).
- To assess the effect of letrozole on bone formation, calvaria obtained from 4-day-old mice were cultured for 7 days with media (BGJ) alone, positive control (insulin) or letrozole. Histomorphometry demonstrated that letrozole did not stimulate new bone formation and, when combined with insulin, did not inhibit new bone formation (**figure 28**).

Conclusions for tasks 3-9 (Part 1). Letrozole decreased BMD in female nude mice, an effect prevented by concomitant treatment with ZA. MicroCT and histomorphometry analyses indicate that the mechanism involves reduced bone remodeling with no direct effect of the treatment on bone formation. BPs may be useful to prevent AI-induced bone loss, but further studies are needed to assess the effects of these treatments on bone quality.

Procedures for tasks 3-9 (Part 2): To study the effects of AIs on bone without the confounding effects on tumor growth, we used the ER-negative human breast cancer cell line MDA-MB-231. Therefore, the AI will have no direct effects on the tumor, and any observed effect on bone metastases should be due to the expected increase in bone turnover. Twenty 4-week-old female nude mice underwent inoculation with MDA-MB-231 via intra-cardiac injection. One week later, the mice were randomized to treatment with the AI letrozole or control (10 mice per group). Mice were euthanized

when they developed significant bone metastases, lost more than 10% of their baseline body weight, or if they showed any signs of distress or impaired mobility.

Results for tasks 3-9 -Part 2 (this data was previously reported in the 2006 annual summary):

- After 3 weeks of treatment, mice treated with letrozole accrued less BMD at the proximal femur ($p=0.0161$), but achieved the same BMD as control mice at the total body, spine and tibia (**figure 29**).
- After 4 weeks of treatment, x-ray analysis demonstrated that there was no difference in total body lesion area between the letrozole-treated mice, as compared to the control mice, after intra-cardiac injection with MDA-MB-231 (**figure 30**).

Conclusions, potential problems and alternative strategies for tasks 3-9 (Part 2).

BMD and the development and progression of breast cancer bone metastases after inoculation with MDA-MB-231 did not differ between the letrozole and control groups. The mice either died or were euthanized within 4 weeks of intra-cardiac inoculation of MDA-MB-231. It may have been too early to see a significant change in bone turnover and, in turn, on the development and progression of breast cancer bone metastases. Therefore, the decision was made to start the letrozole (or control) and after 4 weeks of treatment, when changes in BMD were seen in an earlier experiment using letrozole, inoculate the mice with MDA-MB-231 via intra-cardiac injection.

Procedures for tasks 3-9 (Part 3): Twenty-six 4-week-old female nude mice were randomized to treatment with letrozole or control. After 4 weeks of treatment, all mice underwent inoculation with MDA-MB-231 via intra-cardiac injection. Mice were euthanized when they developed significant bone metastases, lost more than 10% of their baseline body weight, or if they showed any signs of distress or impaired mobility.

Results for tasks 3-9 -Part 3 (this data was previously reported in the 2006 annual summary):

- After 8 weeks of treatment, there was no difference in BMD between the letrozole and control mice at any site (**figure 31**).
- X-ray analysis demonstrated that there was no difference in total body lesion area between the letrozole-treated mice, as compared to the control mice, after intra-cardiac injection with MDA-MB-231 (**figure 32**).

Conclusions, potential problems and alternative strategies for tasks 3-9 (Part 3).

Mice treated with letrozole versus control did not differ in BMD or in the development and progression of breast cancer bone metastases after intra-cardiac inoculation with MDA-MB-231. We questioned whether or not there was a problem with the letrozole. Novartis kindly supplied us with a new supply of letrozole in order to determine the skeletal changes seen in female nude mice after treatment with letrozole versus control.

Procedures for tasks 3-9 (Part 4): To study the effects of AIs on bone, 60 4-week-old female nude mice were randomized to treatment with either letrozole or control. BMD was measured at baseline and then every 2 weeks. Three time points were chosen to euthanize the mice and harvest the bones for analysis: 4 weeks, 23 weeks and 33 weeks. Ten mice per group were euthanized at each time point. Estrogen deficiency from AI therapy is expected to decrease uterine weight. Therefore, uterine weights were measured after the mice were euthanized to ensure that letrozole was resulting in an expected and measurable effect.

Results for tasks 3-9 -Part 4 (this data was partially reported in the 2006 annual summary):

- After 4 weeks of treatment, there was no difference in BMD between the letrozole and control mice at any site (**figure 33**).
- After 4 weeks of treatment, there was no difference in TBV in the femur ($p=0.2268$) or tibia ($p=0.9691$) of letrozole-treated mice compared to the control mice (**figure 34**).
- After 4 weeks of treatment, there was no difference in uterine weight or uterine weight/body weight between the letrozole-treated mice compared to the control mice (**figure 35**).
- MicroCT analysis of the femur and tibia after 4 weeks of treatment did not show a significant difference in trabecular bone volume (BV/TV%), although a trend toward increased BV/TV% in the femurs of letrozole-treated mice was observed ($p=0.0659$) (**figure 36**). However, 4 weeks of treatment with letrozole induced marked increases in skeletal microarchitecture. Significant increases in ConnD ($p=0.0012$) and Tb.N ($p=0.0538$), Tb.Th ($p=0.0280$) and Tb.Sp ($p=0.0348$) were observed in the femurs of letrozole-treated mice, but not in the tibias (**figures 37 and 38**).
- After 23 weeks of treatment, there was no difference in body weight, body composition or total body BMD in letrozole and control mice (**figure 39**).
- After 23 weeks of treatment, letrozole-treated mice had increased BMD at the lumbar spine ($p=0.0003$) and mid-femur ($p=0.0026$) compared to control mice (**figure 40**). There was no significant difference in BMD between letrozole and control-treated mice at the distal femur or proximal tibia.
- After 23 weeks of treatment, letrozole-treated mice had significantly lower uterine weight ($p=0.0148$) and uterine weight/body weight ($p=0.0107$) compared to the control mice (**figure 41**).
- After 23 weeks of treatment, micro-CT demonstrated significantly increased BV/TV% in both the femur ($p=0.0002$) and tibia ($p<0.0001$) of letrozole-treated mice compared to control (**figure 42**).
- After 23 weeks of treatment, bone marrow cultures from the tibias and femurs of letrozole treated mice showed significantly increased CFU-fibroblasts ($p<0.0001$), CFU- osteoblasts ($p<0.0001$) and TRAP-positive osteoclasts ($p=0.0076$) compared to the bone marrow cultures from control mice (**figure 43**).

- After 29 weeks of treatment, body weight was significantly greater in the letrozole-treated mice compared to control mice ($p=0.0020$) but there were no significant differences in body composition or total body BMD (**figure 44**).
- After 29 weeks of treatment, letrozole-treated mice had increased BMD at the lumbar spine ($p<0.0001$) and mid-femur ($p<0.0001$) compared to control mice (**figure 45**). There was no significant difference in BMD between letrozole and control-treated mice at the distal femur or proximal tibia.
- After 33 weeks of treatment, letrozole-treated mice had significantly lower uterine weight/body weight ($p=0.0424$) compared to the control mice (**figure 46**). However, although there was trend towards decreased uterine weight in the letrozole-treated mice, the difference in uterine weights between the treatment groups did not meet statistical significance ($p=0.0577$) (**figure 46**).
- After 33 weeks of treatment, micro-CT did not demonstrate significant differences in %BV/TV in either the femur or tibia ($p<0.0001$) between the letrozole or control treatment groups (**figure 47**).
- After 33 weeks of treatment, bone marrow cultures from the tibias and femurs of letrozole treated mice showed significantly increased CFU-fibroblasts ($p=0.0001$), CFU- osteoblasts ($p<0.0001$) and TRAP-positive osteoclasts ($p=0.0047$) compared to the bone marrow cultures from control mice (**figure 48**).

Conclusions, potential problems and alternative strategies for tasks 3-9 (Part 4).

Unlike the results of the initial letrozole experiment (tasks 3-9; part 1), this experiment did not show differences in BMD (using Lunar Piximus) in letrozole-treated mice compared to control mice in the total body, distal femur or proximal tibia. Interestingly, in this experiment, letrozole-treated mice had increased BMD in the lumbar spine (noted by week 13) and in the mid-femur (noted by week 19) compared to the control mice. Micro-CT data did not show increased %BV/TV in the femur or tibias of letrozole treated mice at the 4 or 33 week time points, but did at the 23 week time point. Of interest, the uterine weights were not significantly different between the letrozole and control mice at the 4 or 33 week time points, but uterine weight was significantly decreased in the letrozole-treated mice at the 23 week time point.

Despite the conflicting Lunar PIXImus and micro-CT data, the bone marrow cultures did show increased CFU-osteoblasts, fibroblasts and TRAP-positive osteoclasts at all 3 time points. Therefore, there is evidence that there is increased bone turnover in the letrozole-treated mice as compared to control.

After consulting with Dr. Evan Simpson, who works with aromatase-knockout mice, it appears that the letrozole-induced suppression of uterine weight was not profound. Per communication with Dr. Simpson, 90% decrease in uterine weight is expected with complete estrogen deficiency. Therefore, the dose of letrozole may not be sufficient to induce complete estrogen deficiency in the female nude mouse. Therefore, we plan to use a higher dose of letrozole in our next experiment (5 mg/kg/day subcu), which was a dose sufficient to produce profound estrogen deficiency in a rat model (29). In addition, as in the OVX versus sham experiment, we wanted to investigate the effects of letrozole in young (4-week-old) versus old (16-week-old) female nude mice.

Letrozole likely has site-specific skeletal effects in the female nude mouse. In addition, the genetic heterogeneity of the female nude mouse may be contributing to the conflicting results from these experiments. The T cell defect in the nude mouse may also complicate the skeletal response to letrozole. Therefore, we plan to use letrozole in an immunocompetent mouse strain in order to clarify the skeletal response to letrozole in mice. Just as OVX produces a variable skeletal response in different inbred mouse strains (27,28), letrozole may have the same effect. The immunocompetent mouse strain that we have chosen is C57B6. This strain was chosen as it has been used in our laboratory previously and has shown increased bone turnover in decreased BMD after OVX.

Procedures for tasks 3-9 (Part 5): In order to evaluate the effect of letrozole on young versus old female nude mice, twenty 4-week-old (young) female nude mice were randomized to treatment with letrozole or control. In order to determine if the conflicting results of letrozole were due to inadequate dosing, a higher dose of letrozole (5 mg/kg/day) was chosen for this experiment. BMD was measured at baseline and then every 2 weeks. The 4-week-old female nude mice were euthanized after 16 weeks of treatment.

- **Treatment of mice with an AI.** Mice were treated with letrozole 125 mcg/day/sc starting on day zero and continuing through the end of the experiment. Control mice were administered the same volume of vehicle/day/sc.

Results for tasks 1 & 2 – part 2a: (data not previously reported)

- After 16 weeks of treatment, letrozole-treated mice had significantly increased body weight ($p < 0.0001$), fat mass ($p < 0.0001$), % fat mass ($p = 0.0029$) and total body BMD ($p < 0.0001$) compared to control mice (**figure 49**).
- After 16 weeks of treatment, letrozole-treated mice had significantly increased BMD at the mid femur ($p < 0.0001$) and proximal tibia ($p = 0.0002$) and there was a trend toward increased BMD at the spine ($p = 0.0612$) compared to control mice (**figure 50**). BMD did not differ between the treatment groups at the distal femur (**figure 50**).
- After 16 weeks of treatment, letrozole-treated mice had significantly decreased uterine weight ($p = 0.0012$) and uterine weight/body weight ($p = 0.0069$) compared to the control mice (**figure 51**).

Conclusions, potential problems and alternative strategies for tasks 3-9 (Part 5).

This experiment showed increased BMD at the total body, mid femur and proximal tibia in the letrozole-treated mice compared to the control mice. This data is similar to the data from the previous experiment (part 4), which used the lower dose of letrozole. In part 4, the total body BMD did not differ between treatment groups at the 23 and 33 week time points, but it was increased in the letrozole-treated mice in this experiment. In part 4, the lumbar spine BMD was increased in the letrozole-treated mice at both the 23 and 33 week time points, and there was a trend toward lumbar spine BMD in the letrozole-treated mice in this experiment. Mid femur BMD was increased in the letrozole-treated mice in part 4 at both time points, and was also increased in the letrozole-treated mice in this experiment. Distal femur BMD was not different between treatment groups in part 4 at

either time point, and was not different between treatment groups in this current experiment. Proximal tibia BMD was not different between treatment groups in part 4 at either time point, but was significantly increased in the letrozole-treated mice in this current experiment. It is difficult to determine if the increased BMD in the letrozole-treated mice was due to the increased dose of letrozole. Interestingly, although uterine weight and the ratio of uterine weight to body weight were both significantly decreased in the letrozole-treated mice in this experiment, the degree of suppression is not different from that seen in part 4 (at the 23 week time point). It is possible that the letrozole has a plateau-effect in the nude mouse, and a higher dose will not necessarily increase the degree of estrogen suppression in our model.

Bone histomorphometry, micro-CT and CFU-assay data are still pending for this experiment. In addition, 20 16-week-old female nude mice have recently begun treatment with the increased dose of letrozole to determine the effect of letrozole in young versus old female nude mice.

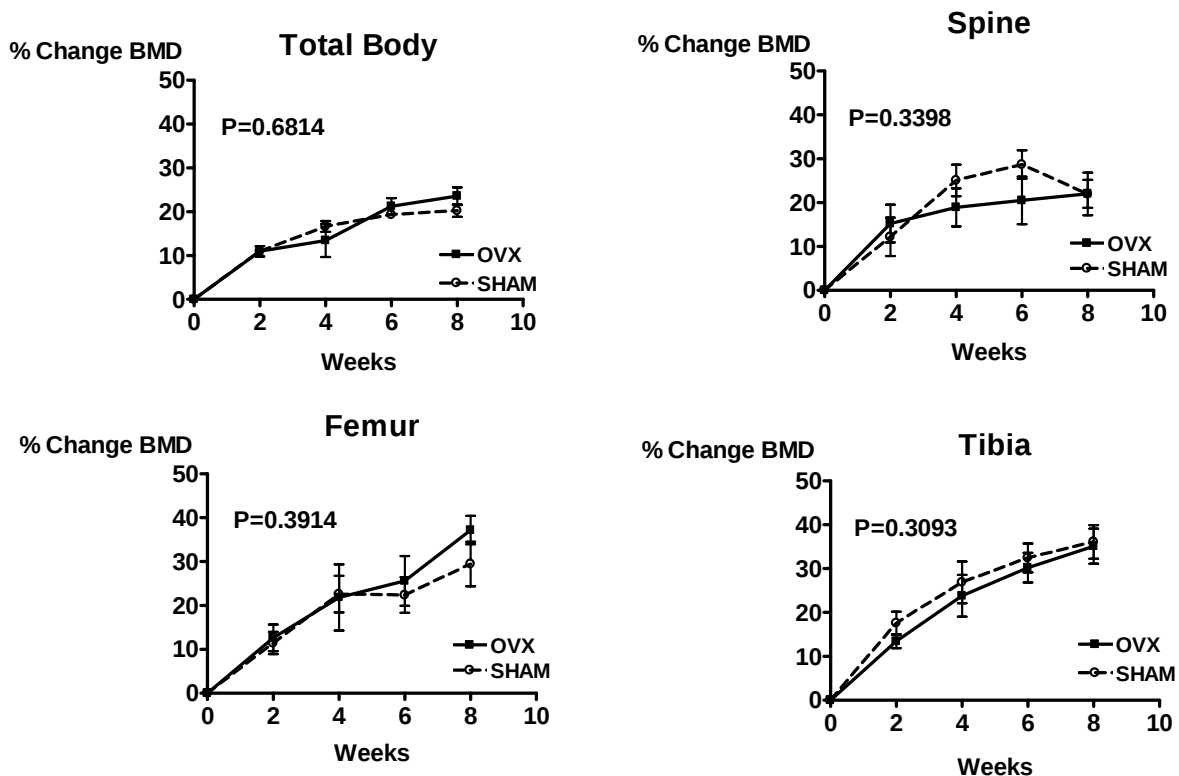


Figure 1. BMD in female nude mice after OVX versus Sham surgery. There was no difference in BMD between the OVX and Sham mice at any site: A) total body, B) spine, C) femur and D) tibia. P values determined by two-way ANOVA.

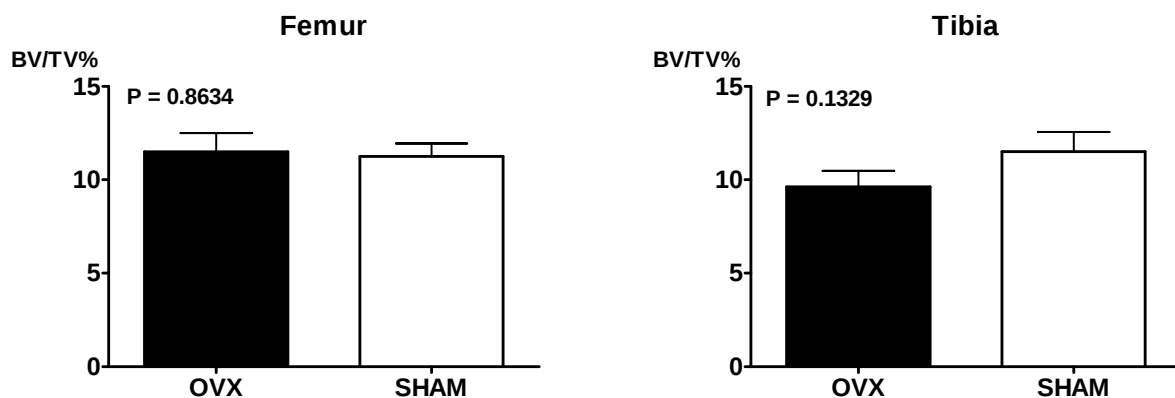


Figure 2. Trabecular bone volume (BV/TV%) in female nude mice after OVX or Sham surgery. There was no significant difference in BV/TV% between the 2 treatment groups in the proximal femur or proximal tibia. P values determined by two-way ANOVA.

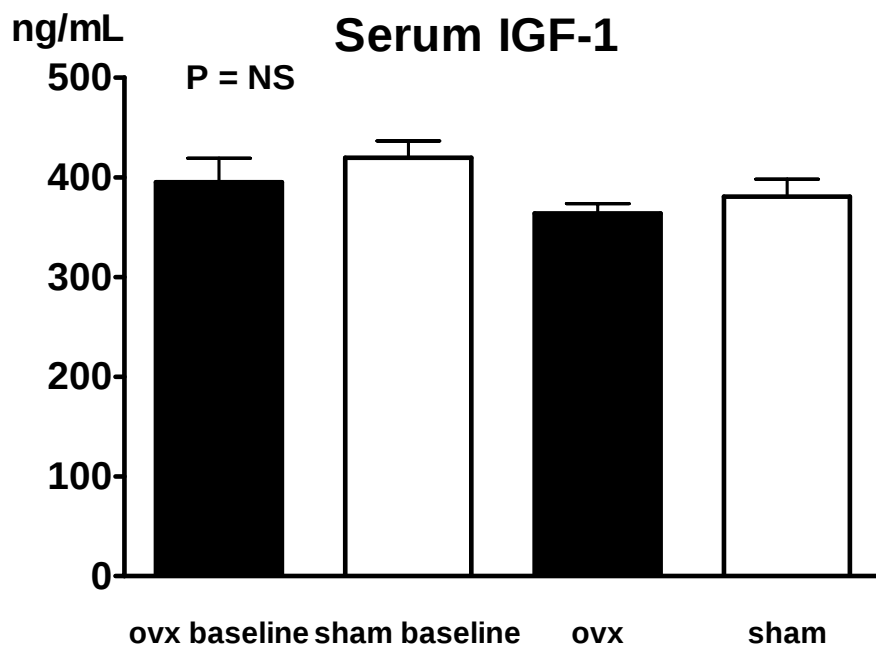


Figure 3. Serum IGF-1 levels in female nude mice pre and post OVX or Sham surgery. There was no difference in IGF-1 levels in OVX versus sham mice. P values determined by two-way ANOVA.

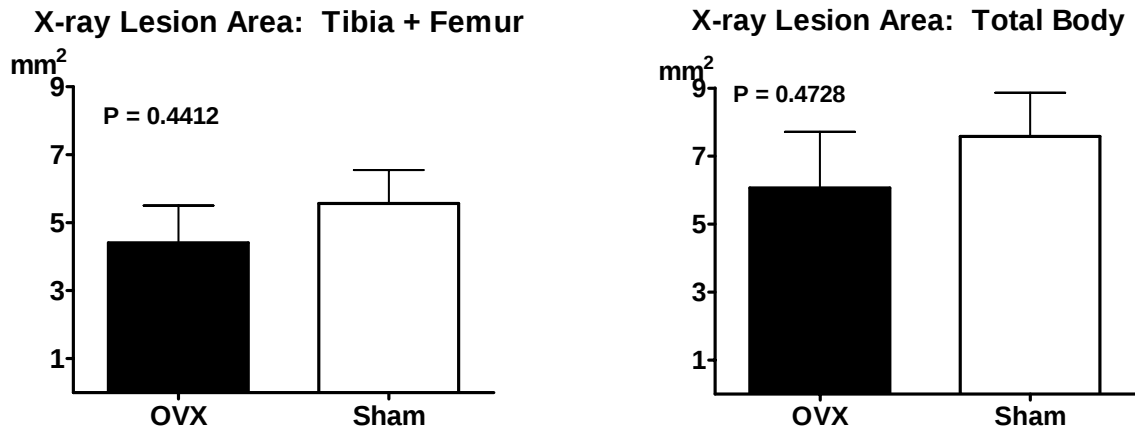


Figure 4. X-ray lesion area of bone metastases. Female nude mice were inoculated with MDA-MB-231 via intra-cardiac injection 8 weeks after OVX or sham surgery. Lesion area of bone metastases did not differ in the tibia+femur or total body between the OVX or sham treatment groups. P values determined by two-way ANOVA.

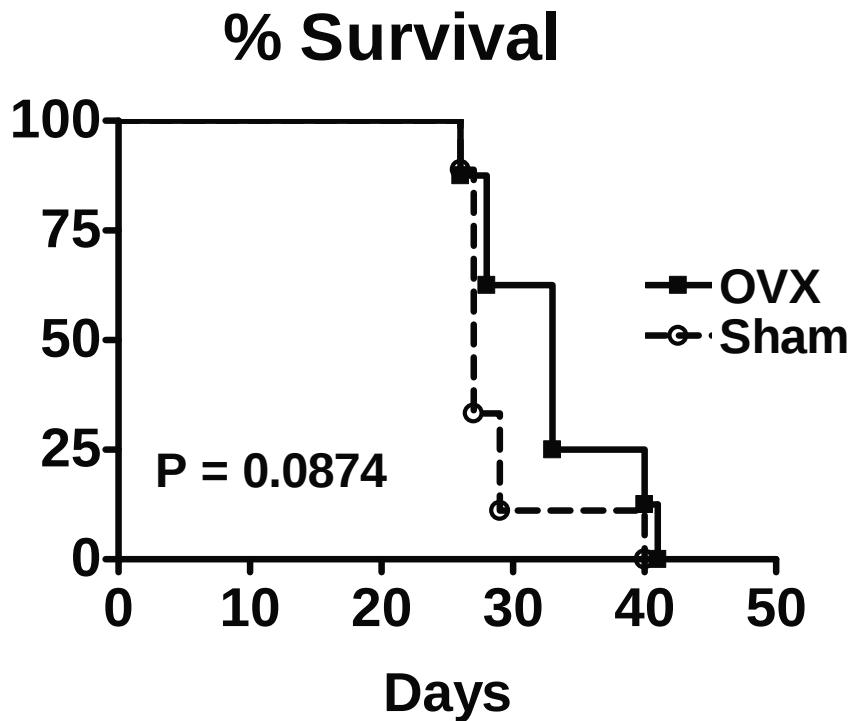


Figure 5. Survival curve for female nude mice inoculated with MDA-MB-231 via intra-cardiac injection 8 weeks after OVX or sham surgery. There was no difference in survival between the OVX or sham treatment groups.

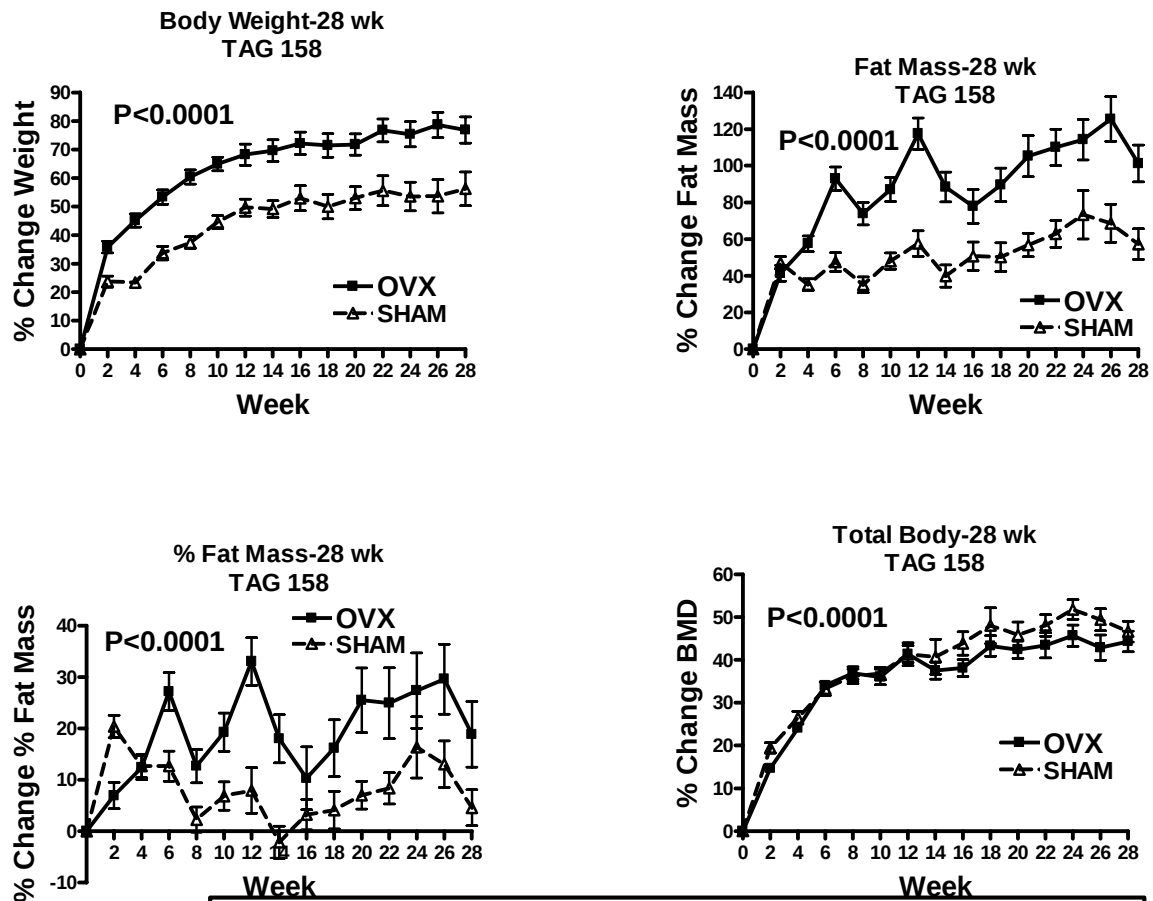


Figure 6. Body composition and BMD in female nude mice 28 weeks after OVX or sham surgery. Mice were 4-weeks-old at time of surgery. P values calculated using two-way ANOVA).

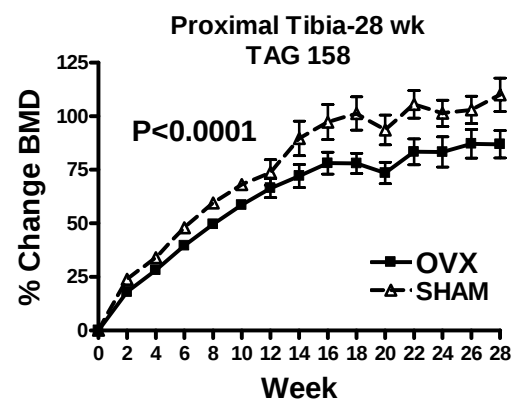
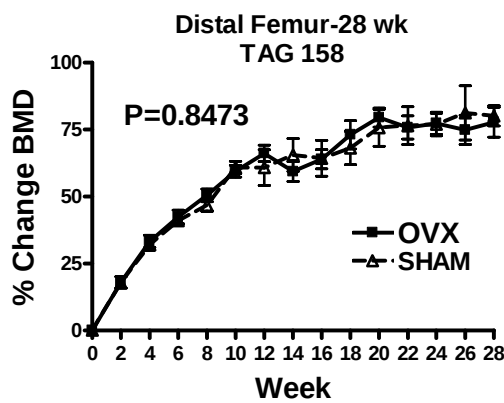
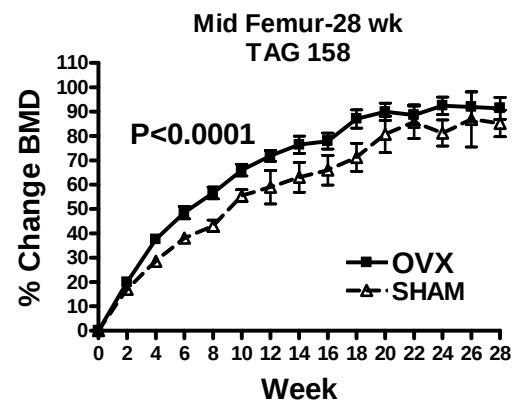
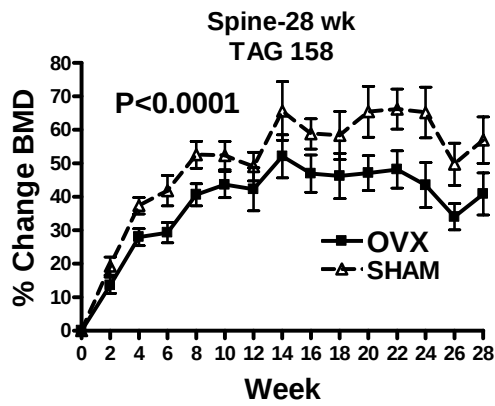


Figure 7. BMD in female nude mice 28 weeks after OVX or sham surgery. Mice were 4-weeks-old at time of surgery. P values calculated using two-way ANOVA).

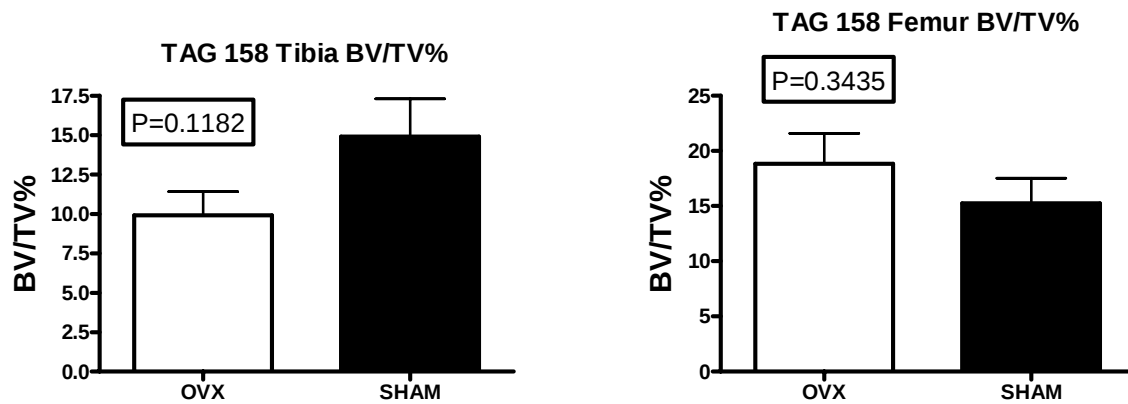


Figure 8. Trabecular bone volume in female nude mice 28 weeks after OVX or sham surgery. Mice were 4-weeks-old at the time of surgery. P values calculated using Student's t-test.

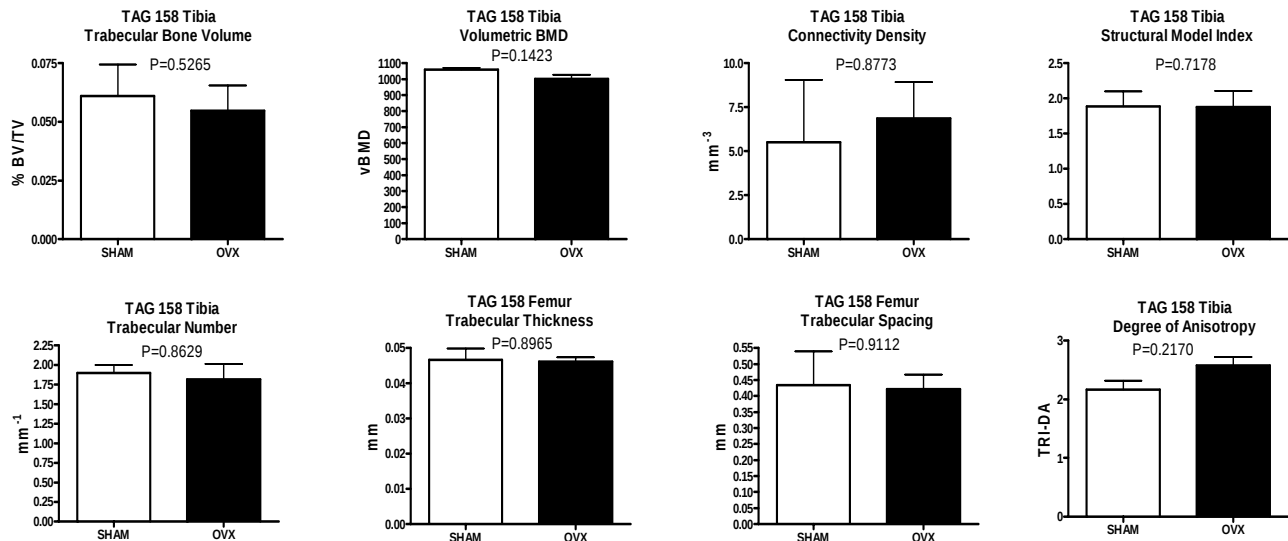


Figure 9. Micro-CT data from the right tibias of female nude mice 28 weeks after OVX or sham surgery. Mice were 4-weeks-old at time of surgery. P values calculated using Student's t-test.

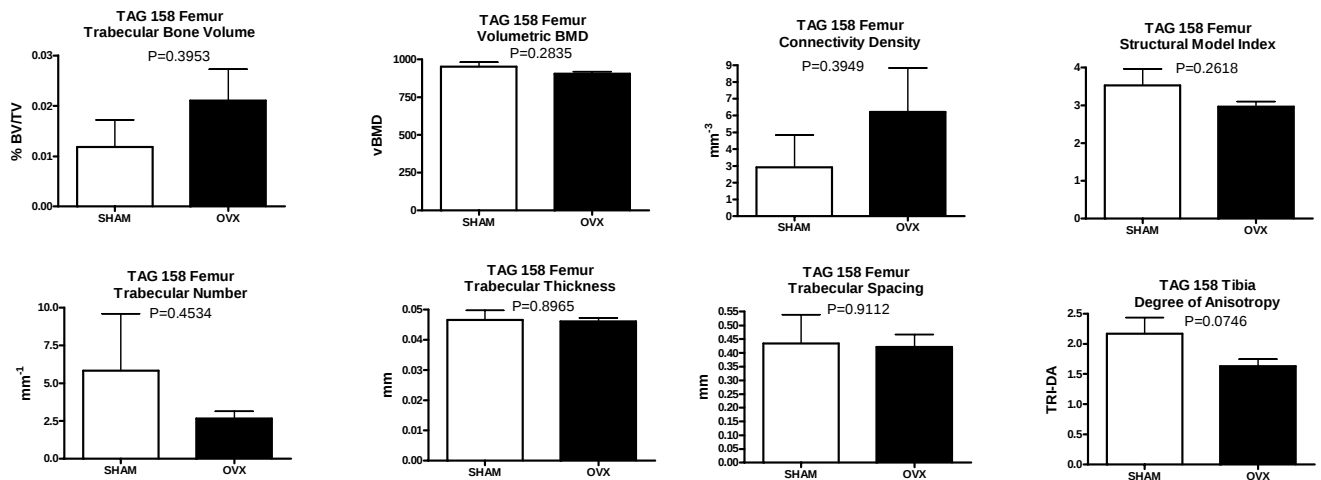


Figure 10. Micro-CT data from the right femurs of female nude mice 28 weeks after OVX or sham surgery. Mice were 4-weeks-old at time of surgery. P values calculated using Student's t-test.

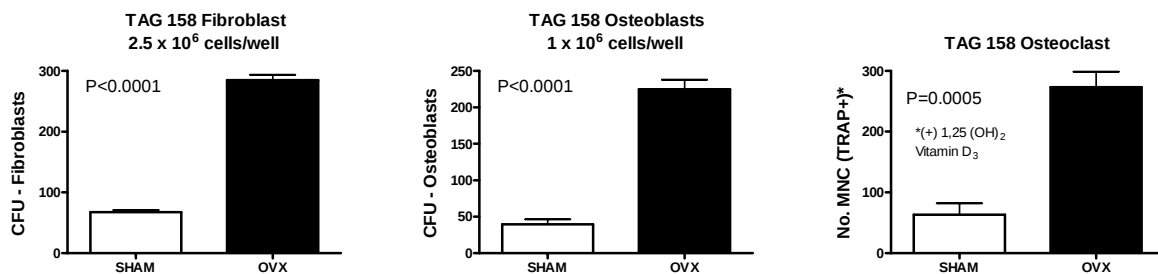


Figure 11. CFU and osteoclast formation assays using the femurs and tibias (3 mice/group) from female nude mice 28 weeks after OVX or sham surgery. Mice were 4-weeks-old at the time of surgery. P values calculated using Student's t-test.

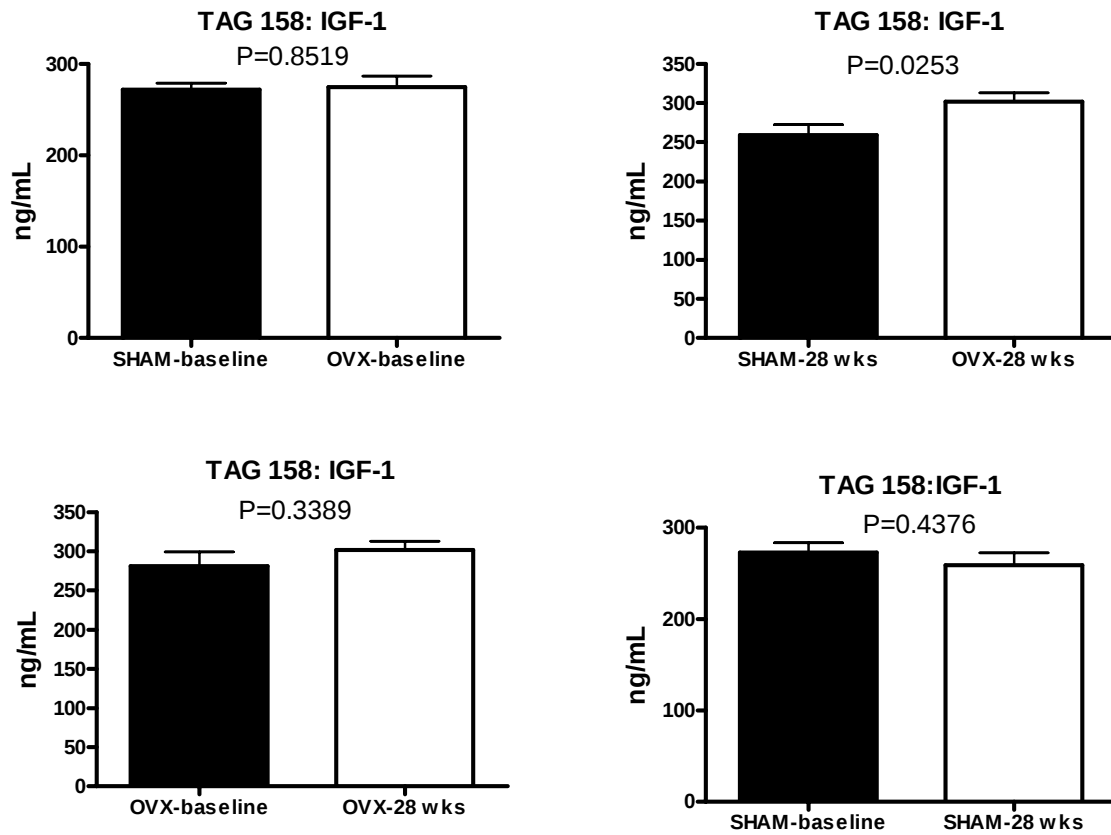


Figure 12. Serum IGF-1 levels from female nude mice 28 weeks after OVX or sham surgery. Mice were 4-weeks-old at time of surgery. P values calculated using Student's t-test.

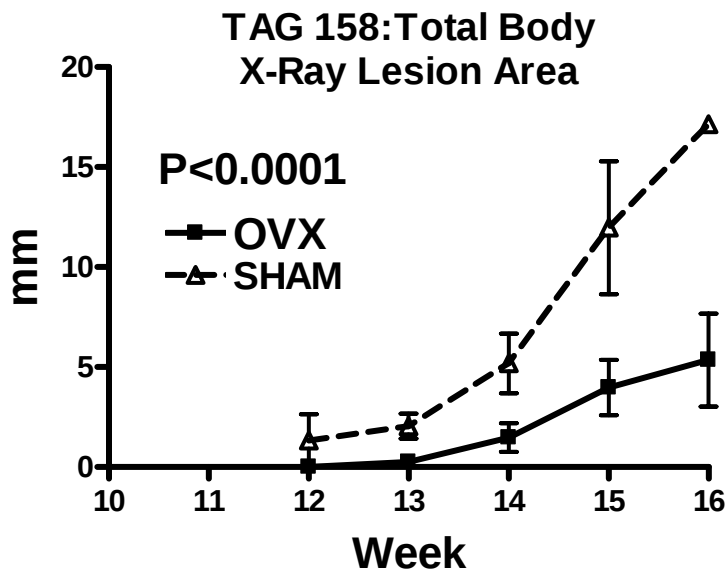


Figure 13. X-ray lesion area in female nude mice after OVX or sham surgery. Mice were 4-weeks-old at the time of surgery. Mice were inoculated with MDA-MB-231 10 weeks after surgery. P values calculated using two-way ANOVA

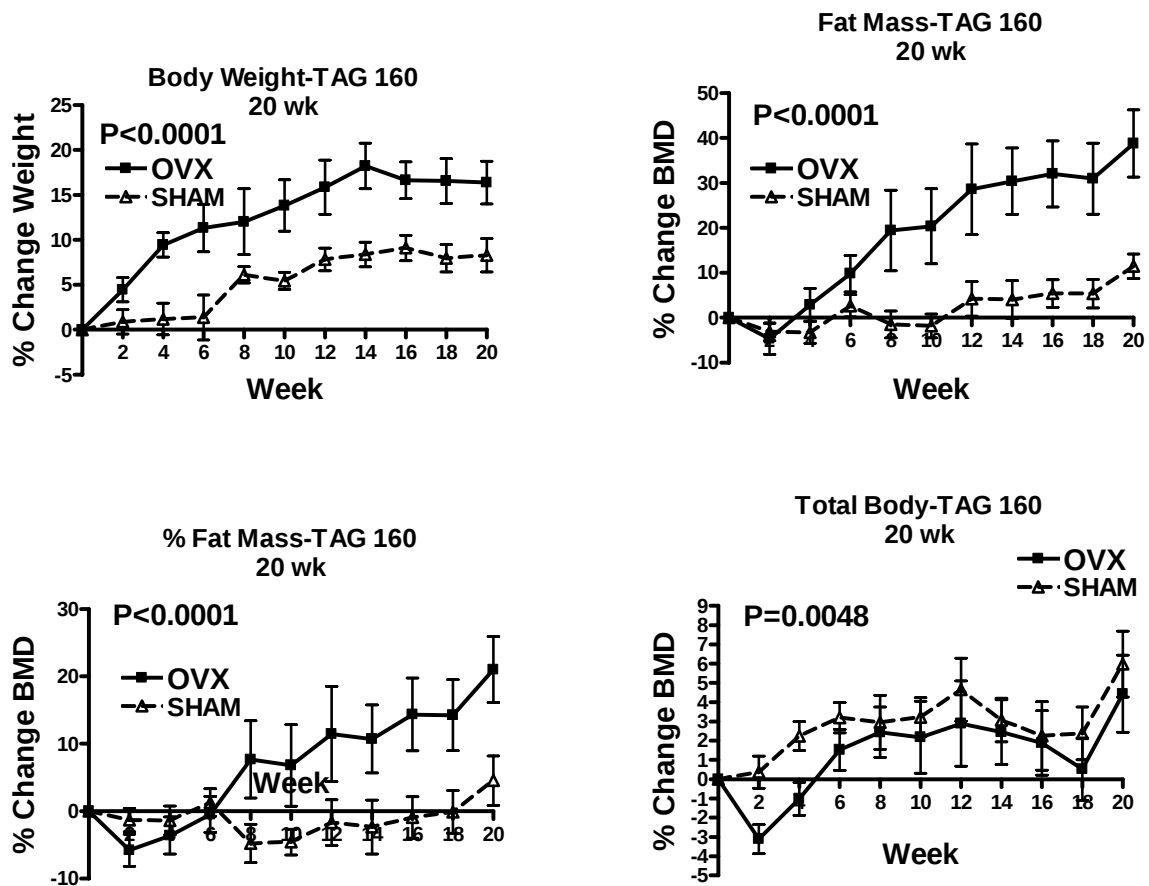


Figure 14. Body composition and BMD in female nude mice after OVX or sham surgery. Mice were 16-weeks-old at the time of surgery. P values calculated using two-way ANOVA.

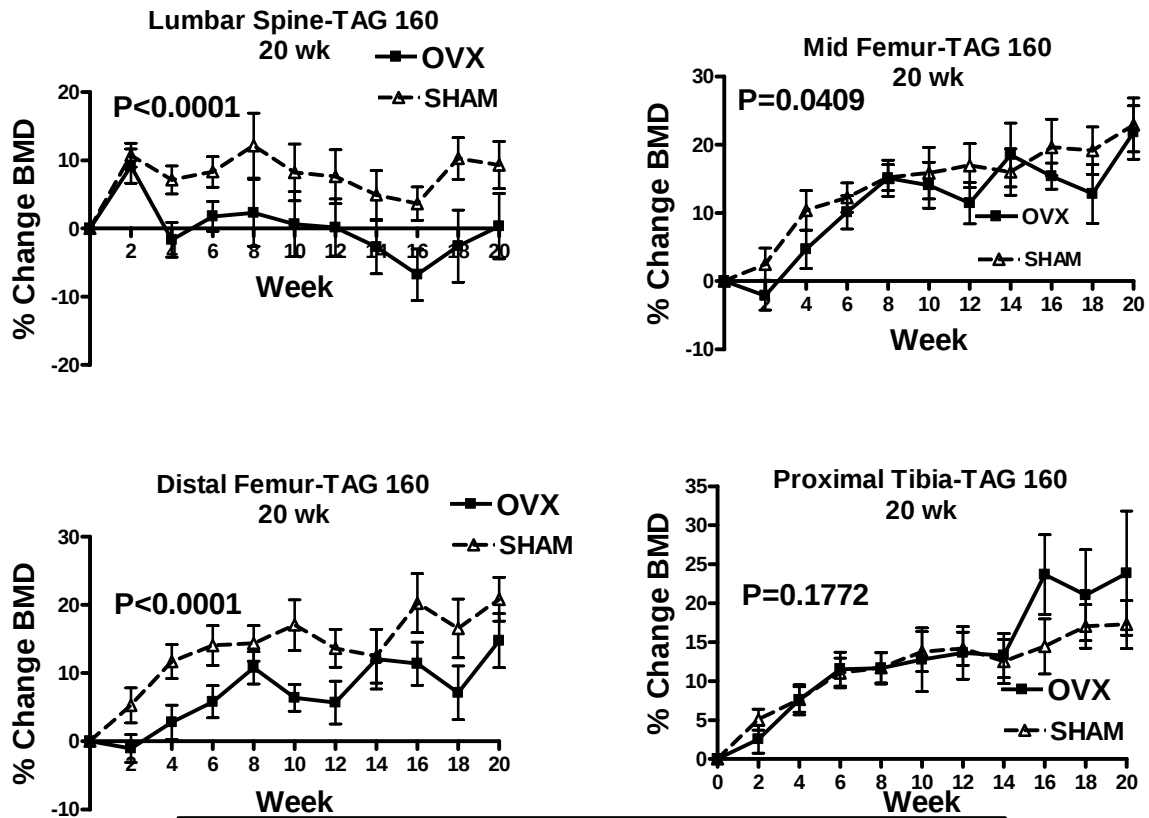


Figure 15. BMD in female nude mice after OVX or sham surgery. Mice were 16-weeks-old at the time of surgery. P values calculated using two-way ANOVA.

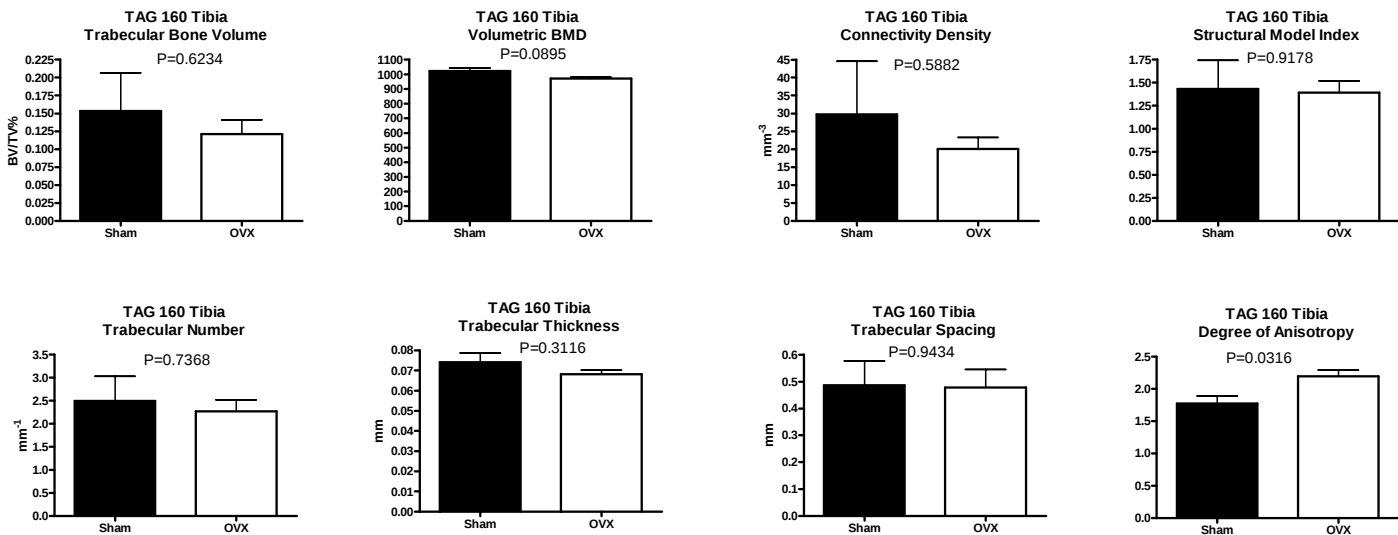


Figure 16. Micro-CT data from the right tibias of female nude mice 20 weeks after OVX or sham surgery. Mice were 16-weeks-old at time of surgery. P values calculated using Student's t-test.

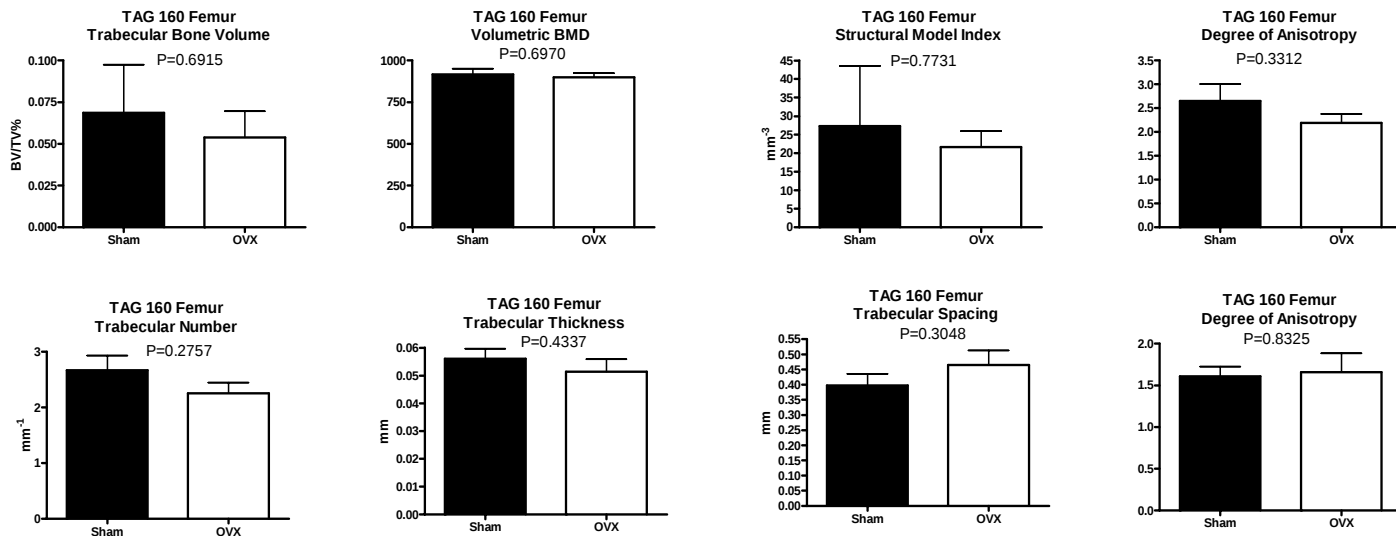


Figure 17. Micro-CT data from the right femurs of female nude mice 20 weeks after OVX or sham surgery. Mice were 16-weeks-old at time of surgery. P values calculated using Student's t-test.

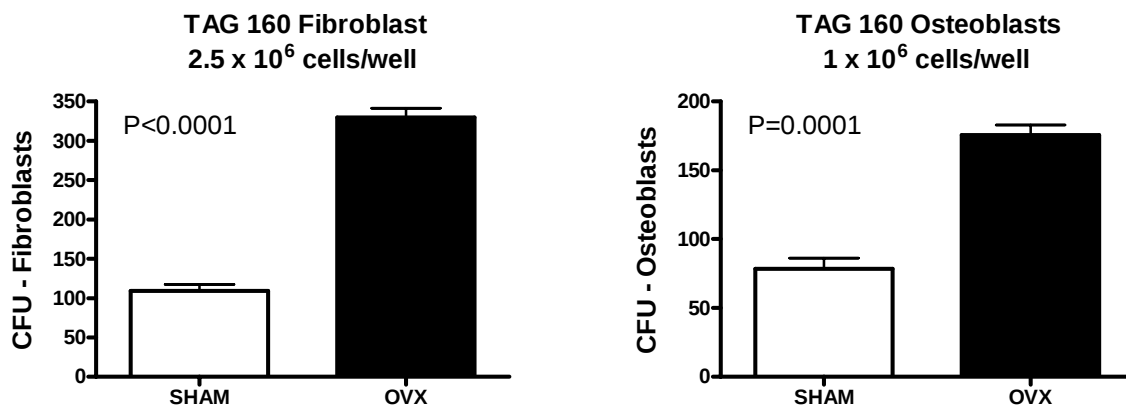


Figure 18. CFU assays using bone marrow from the femurs and tibias (3 mice/group) of female nude mice 20 weeks after OVX or sham surgery. Mice were 16-weeks-old at time of surgery. P values calculated using Student's t-test.

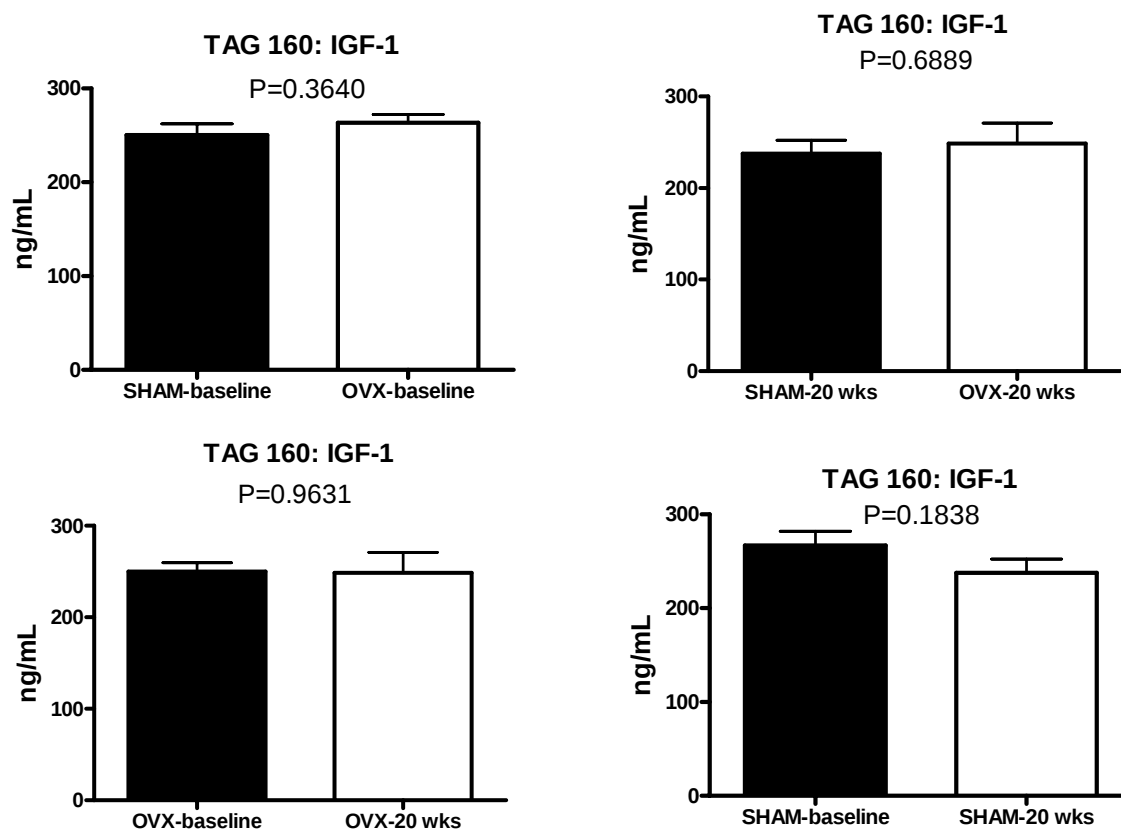


Figure 19. Serum IGF-1 levels from female nude mice pre and 20 weeks post OVX or sham surgery. Mice were 16-weeks-old at time of surgery. P values calculated using Student's t-test.

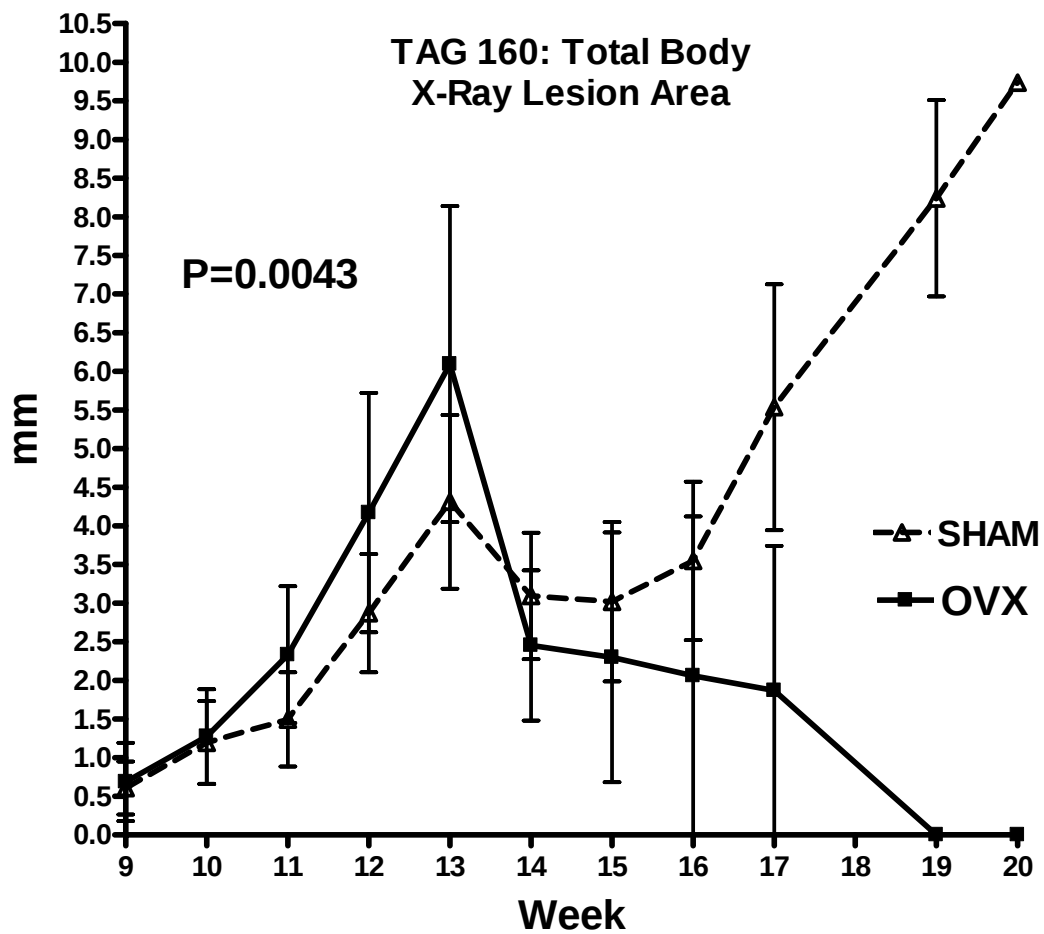


Figure 20. X-ray lesion area in female nude mice after OVX or sham surgery. Mice were 16-weeks-old at time of surgery. Mice were inoculated with MDA-MB-231 8 weeks after surgery. P values calculated using two-way ANOVA.

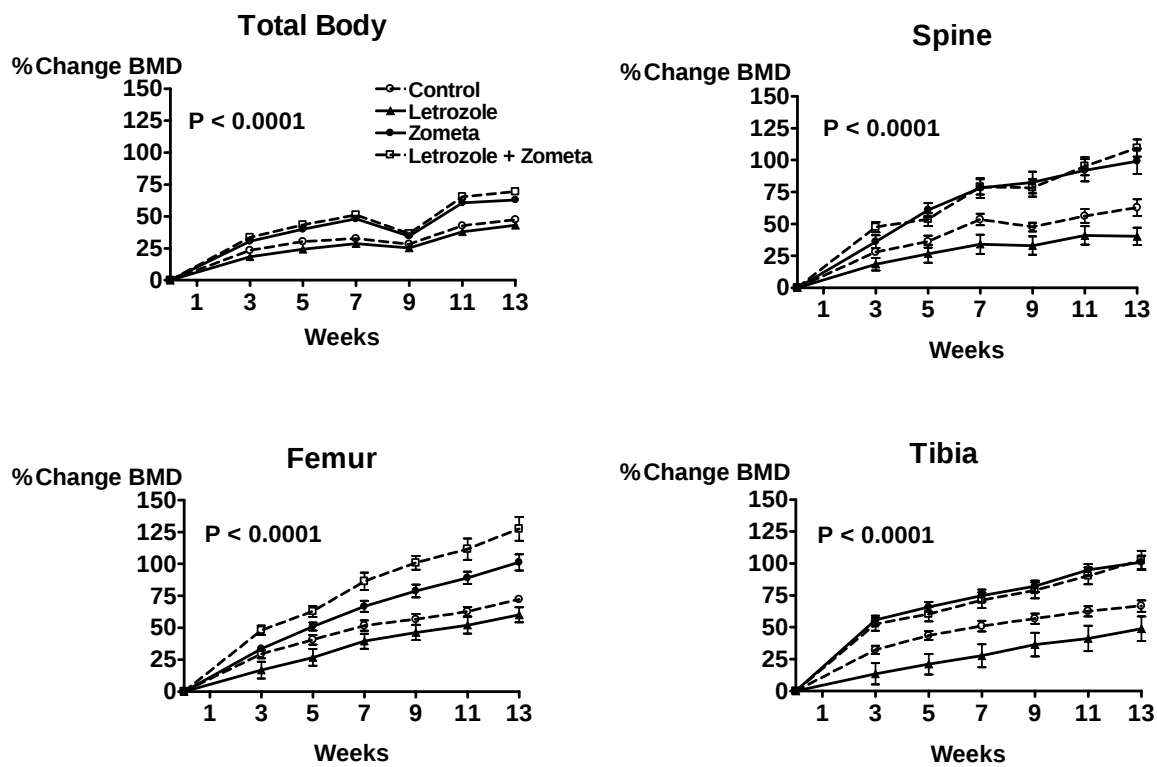


Figure 21. BMD in female nude mice after 13 weeks of treatment with control, letrozole, zometa or letrozole + zometa: total body, spine, femur and tibia.

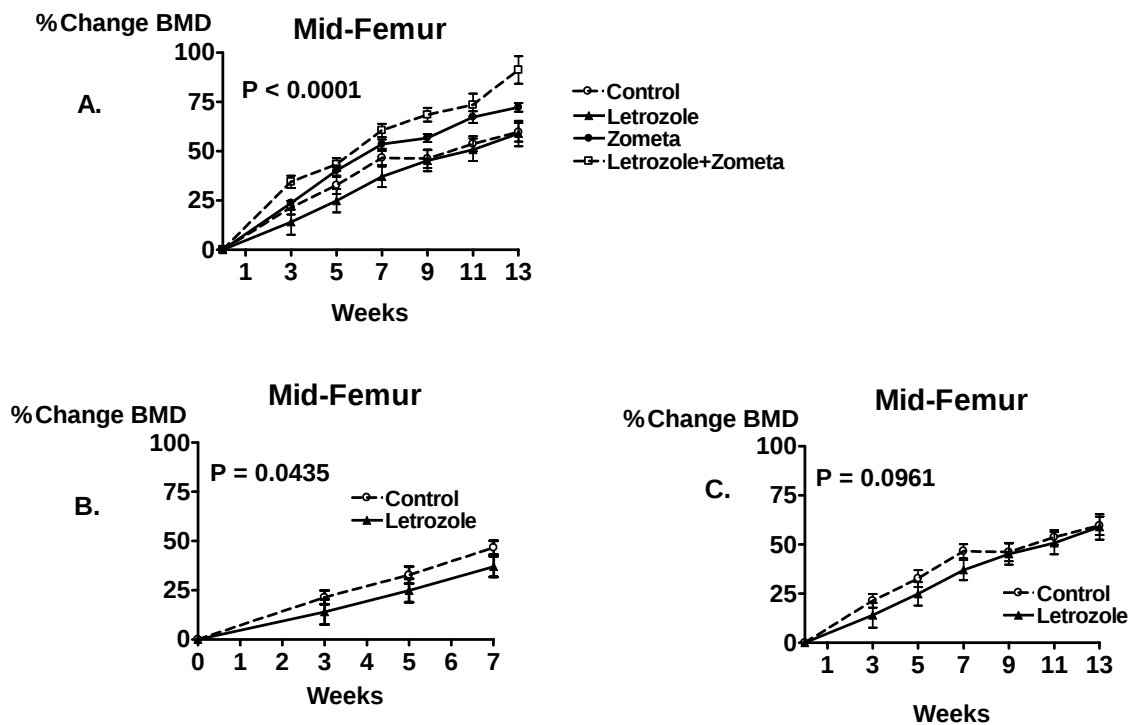


Figure 22. Mid-femur BMD in female nude mice after A) 13 weeks of treatment with control, letrozole, zometa or letrozole + zometa, B) 7 weeks of treatment with control or letrozole, and C) 13 weeks of treatment with control or letrozole. P values calculated using two-way ANOVA.

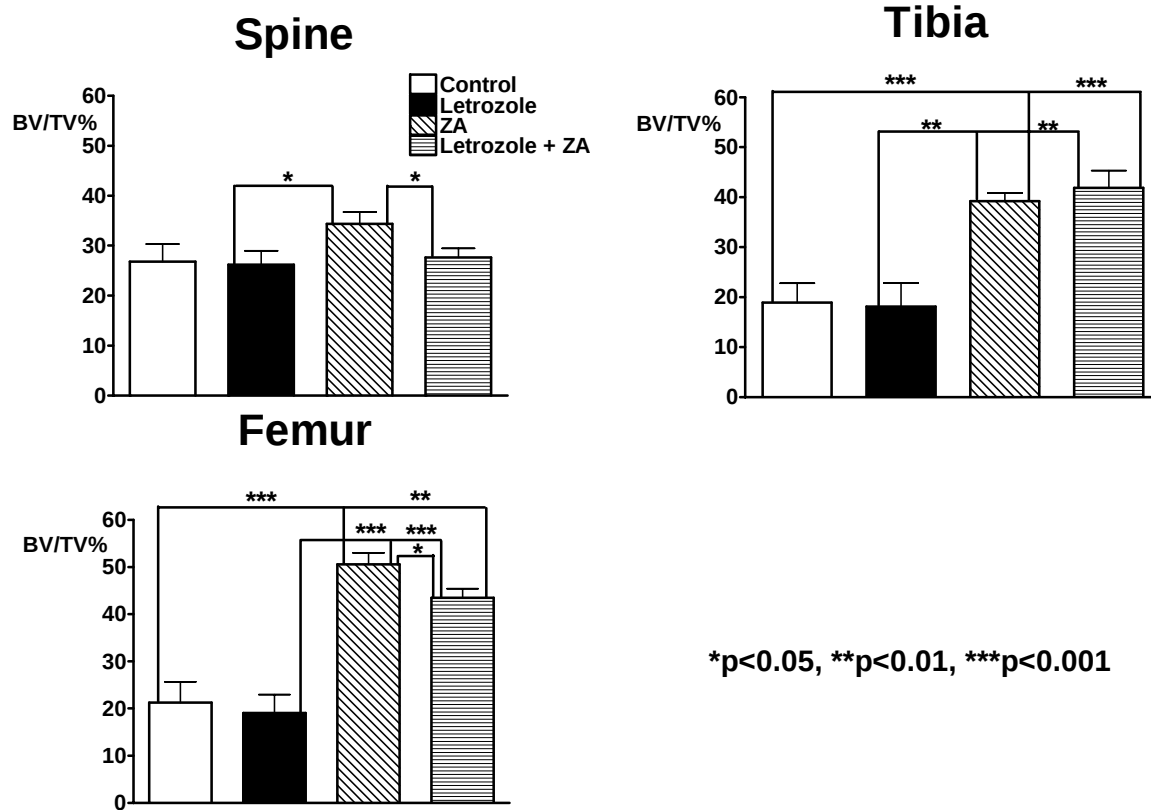


Figure 23. Trabecular Bone Volume (BV/TV%) in female nude mice after 13 weeks of treatment with control, letrozole, zometa or letrozole + zometa: femur, tibia and spine. There was no difference in BV/TV% between the control versus letrozole group at any site. P values calculated using Student's t-test.

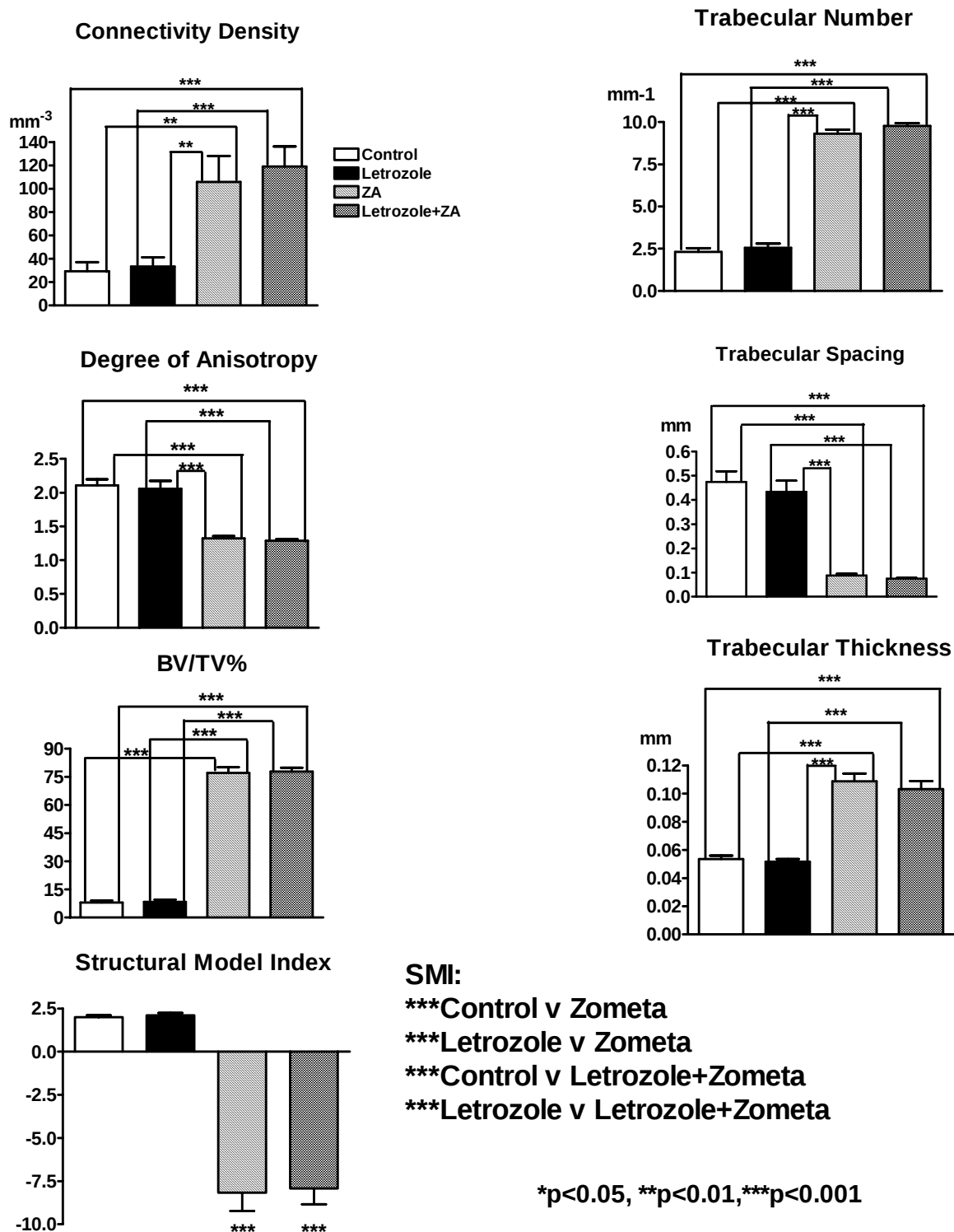


Figure 24. Micro-CT data from the right tibias of female nude mice after 13 weeks of treatment with control, letrozole, zometa or letrozole + zometa. P values calculated using Student's t-test.

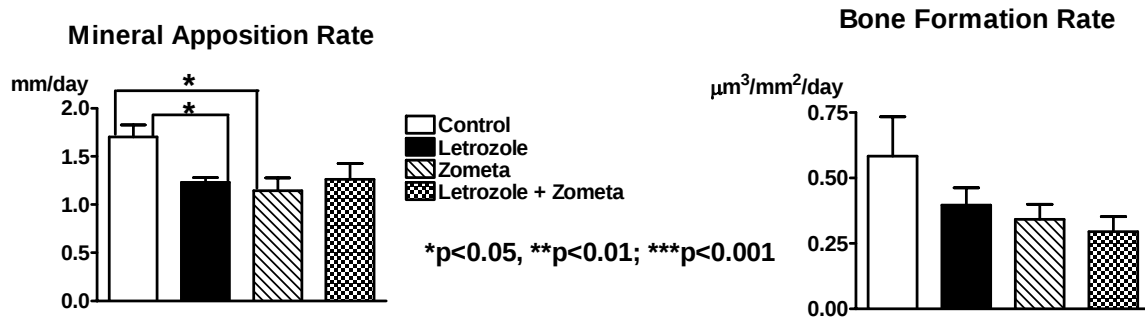


Figure 25. Mineral Apposition Rate and Bone Formation Rate in female nude mice after 13 weeks of treatment with control, letrozole, zometa or letrozole + zometa. P values calculated using Student's t-test.

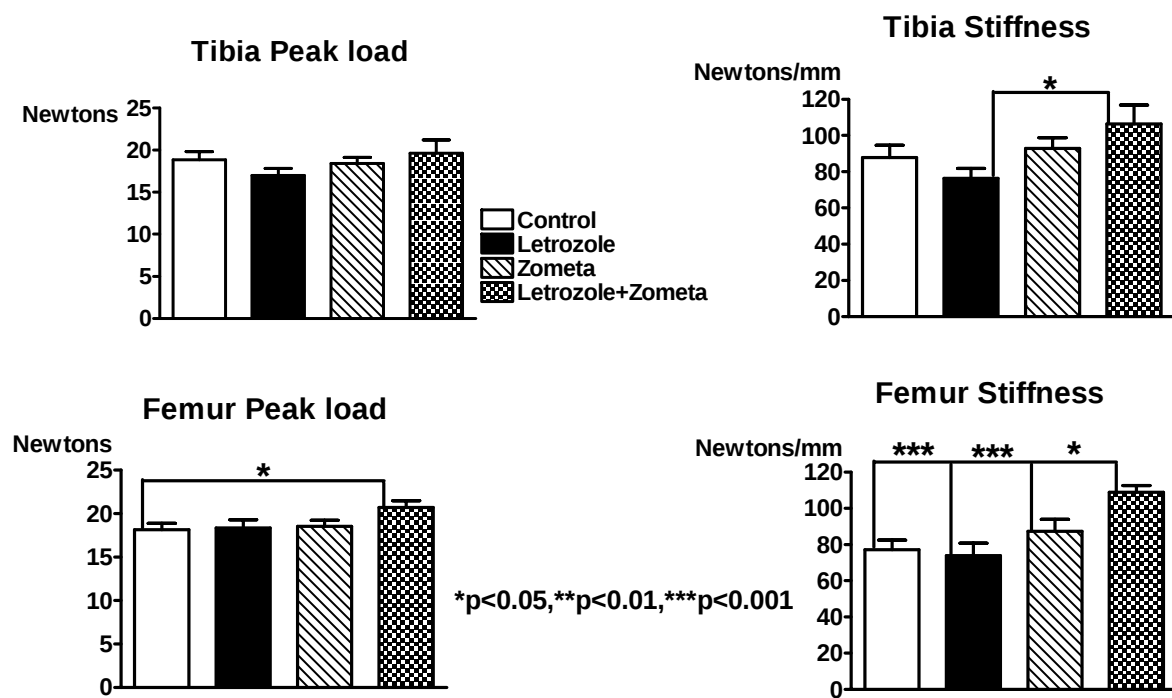


Figure 26. Mechanical loading data from the right tibias and femurs of female nude mice after 13 weeks of treatment with either control, letrozole, zometa or letrozole + zometa. P values calculated using Student's t-test.

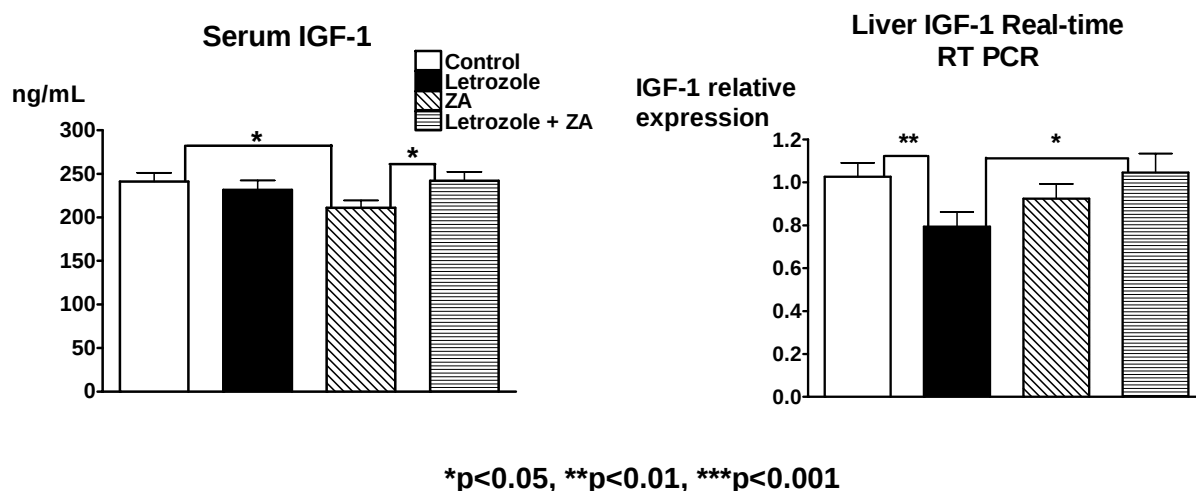


Figure 27. IGF-1 Real-time RT PCR and serum IGF-1 levels from female nude mice after 13 weeks of treatment with control, letrozole, zometa or letrozole + zometa. P values calculated using Student's t-test.

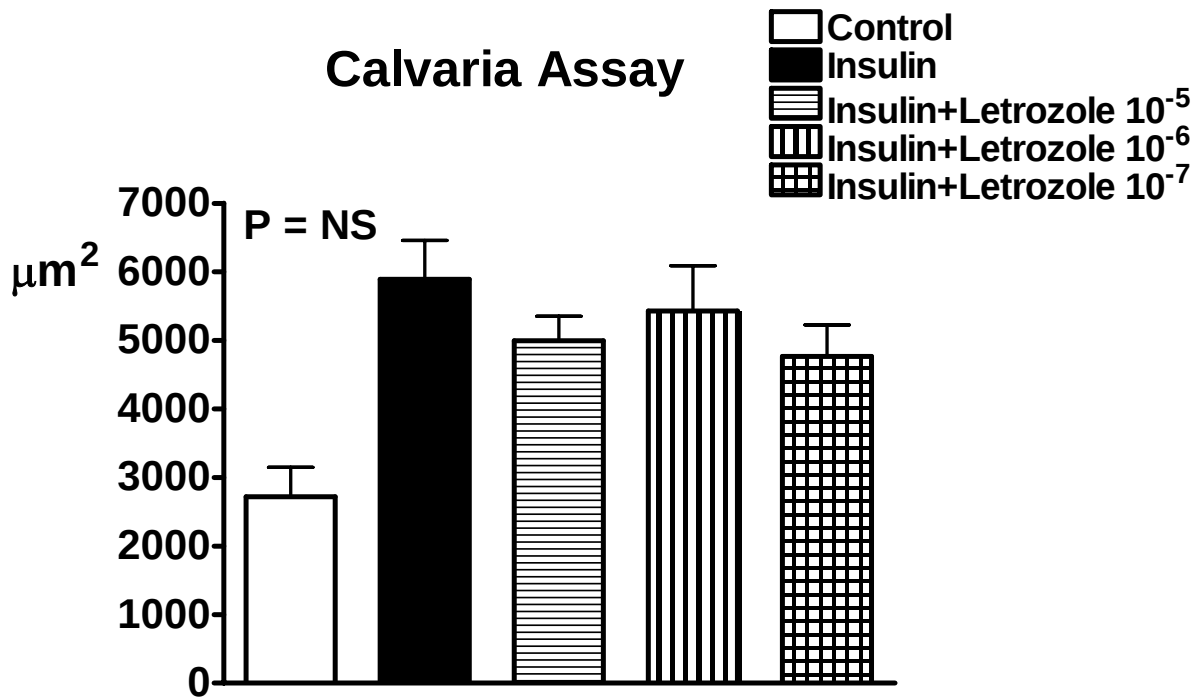


Figure 28. To assess the effect of letrozole on bone formation, calvaria obtained from 4-day-old mice were cultured for 7 days with media (BGJ) alone, positive control (insulin) or letrozole. Histomorphometry demonstrated that letrozole did not stimulate new bone formation and, when combined with insulin, did not inhibit new bone formation. P values calculated using Student's t-test.

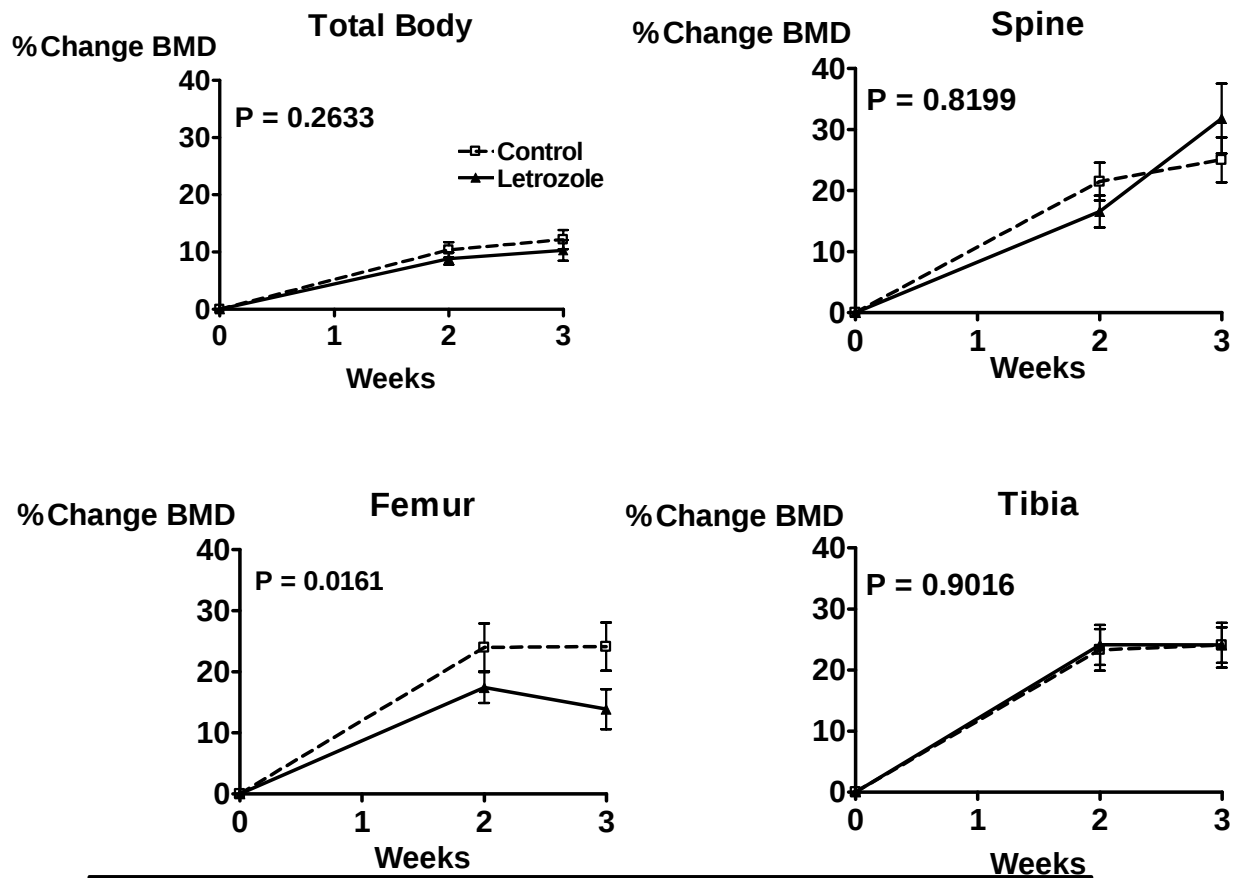


Figure 29. BMD in female nude mice after 3 weeks of treatment with letrozole or control. P values calculated using two-way ANOVA.

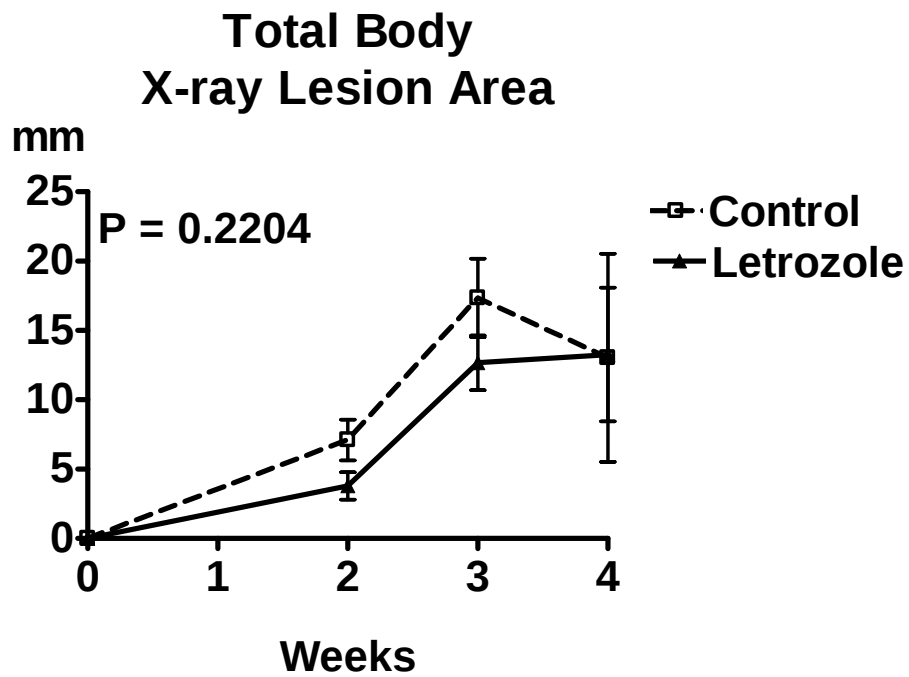


Figure 30. Total body x-ray lesion area in female nude mice after intra-cardiac injection with MDA-MB-231 and then 4 weeks of treatment with letrozole or control. Lesion area did not differ between treatment groups. P values calculated using two-way ANOVA.

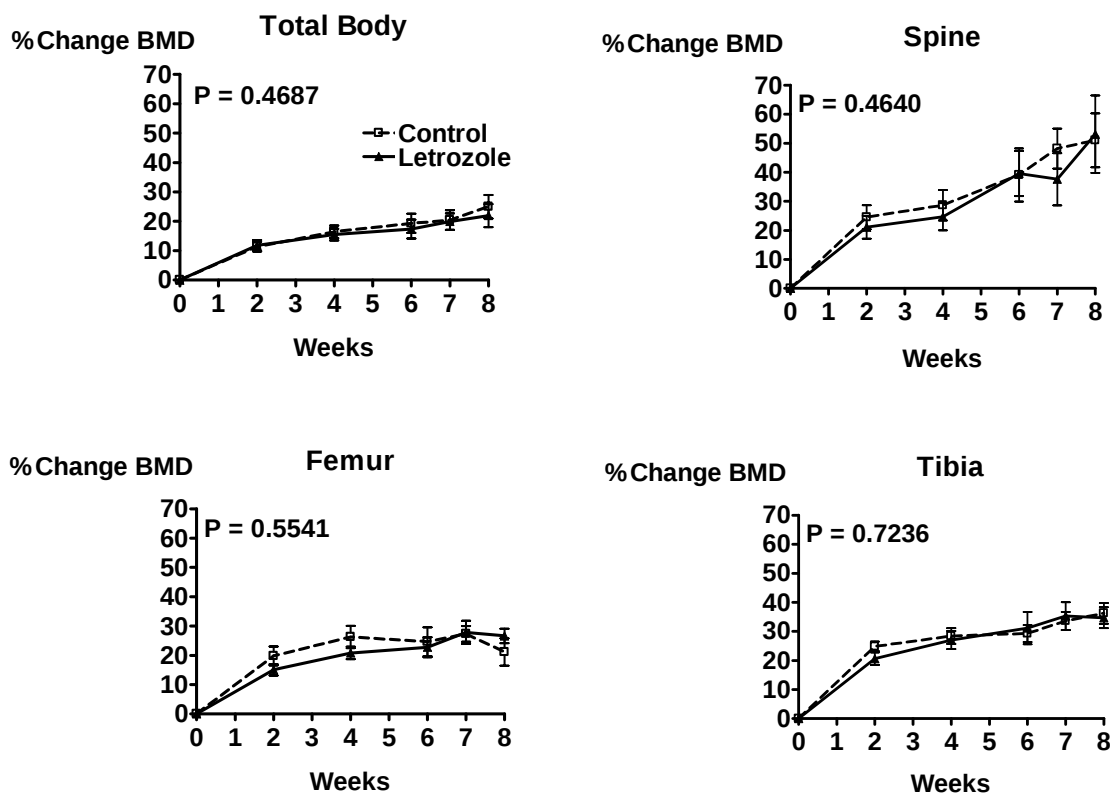


Figure 31. BMD in female nude mice after 8 weeks of treatment with control or letrozole. Mice treated with letrozole achieved the same BMD as mice treated with control at all sites: total body, spine, femur and tibia. P values calculated using two-way ANOVA.

Total Body X-ray Lesion Area

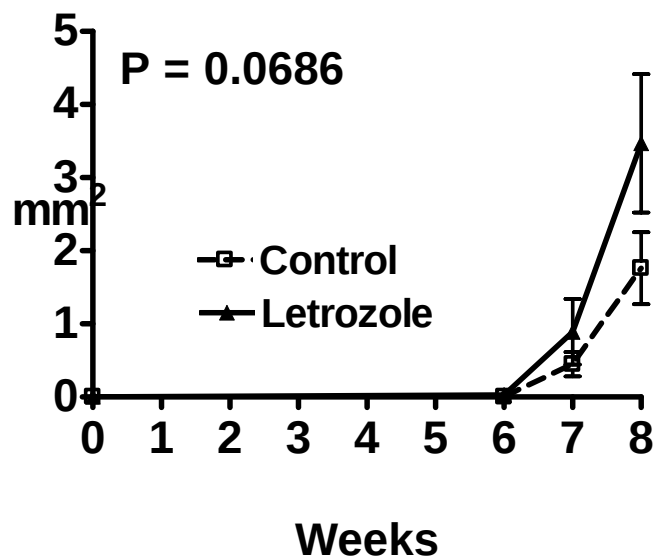


Figure 32. Total body x-ray lesion area in female nude mice treated for 5 weeks with either letrozole or control, and then inoculated with MDA-MB-231 via intracardiac injection. Lesion area did not differ between treatment groups. P value calculated using two-way ANOVA.

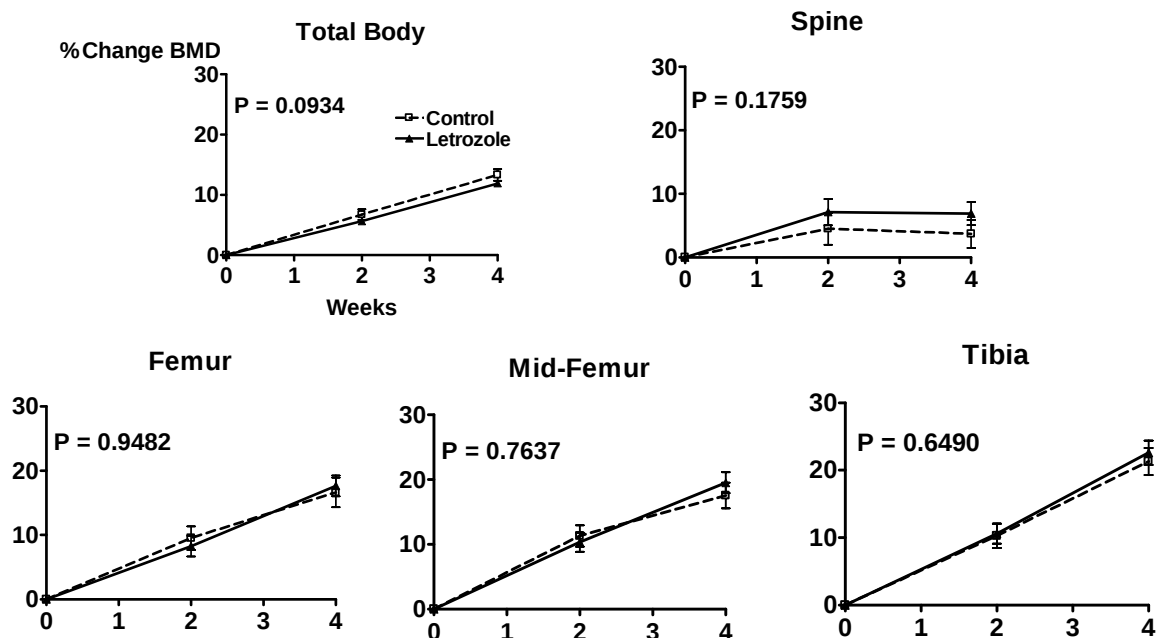


Figure 33. BMD in female nude mice after 4 weeks of treatment with letrozole or control. There was no difference in BMD at any site between the 2 treatment groups. P values calculated using two-way ANOVA.

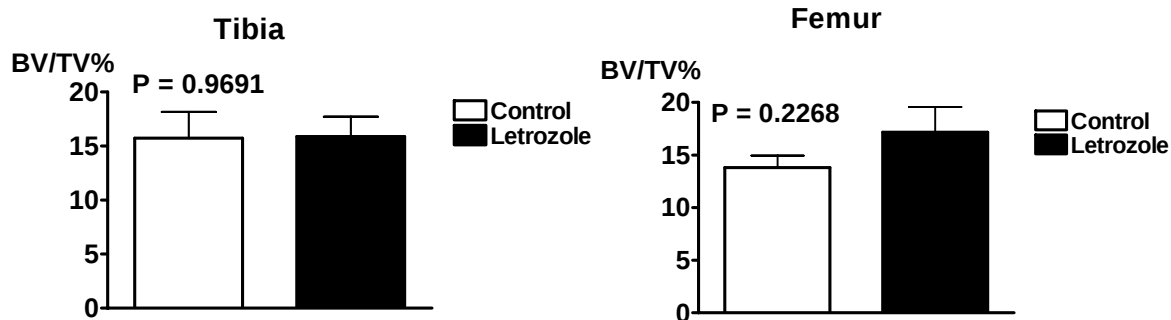


Figure 34. Trabecular bone volume (BV/TV%) in female nude mice after 4 weeks of treatment with letrozole or control. There was no difference in BV/TV% in the tibia or femur between the 2 treatment groups. P values calculated using Student's t-test.

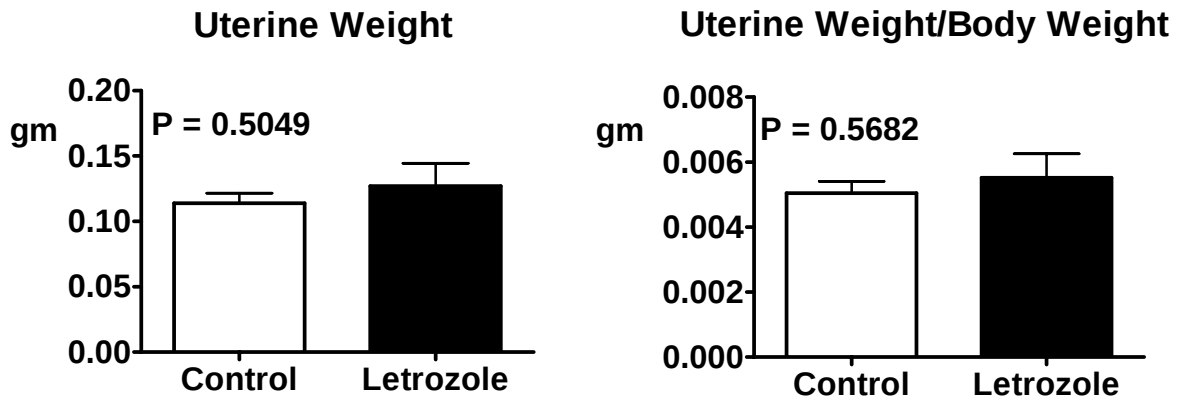


Figure 35. Uterine weight and uterine weight/body weight in female nude mice after 4 weeks of treatment with letrozole or control. There was no difference in either uterine weight or uterine weight/body weight between treatment groups. P values calculated using Student's t-test.

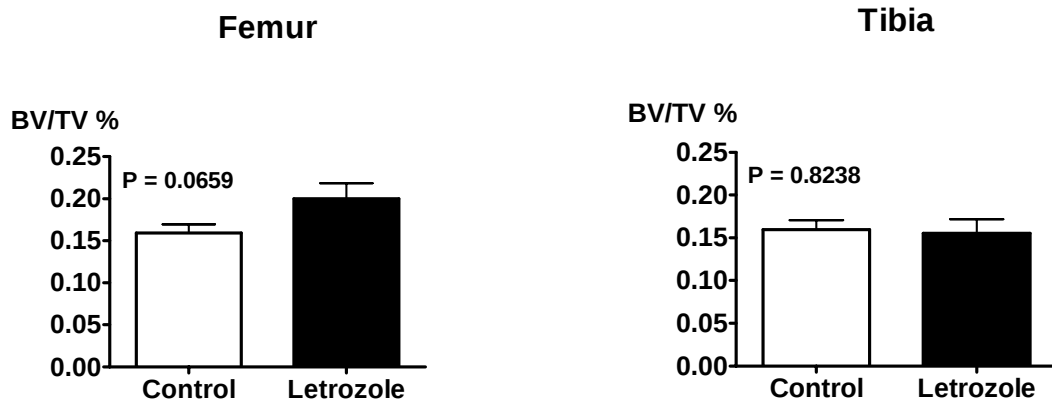


Figure 36. MicroCT analysis of trabecular bone volume (BV/TV%) of right femur and tibia from female nude mice after 4 weeks of treatment with letrozole or control. A significant difference in BV/TV% was not noted in the femur or tibia, although a trend toward increased BV/TV% in the femurs of letrozole-treated mice was observed (p=0.0659). P values calculated using Student's t-test.

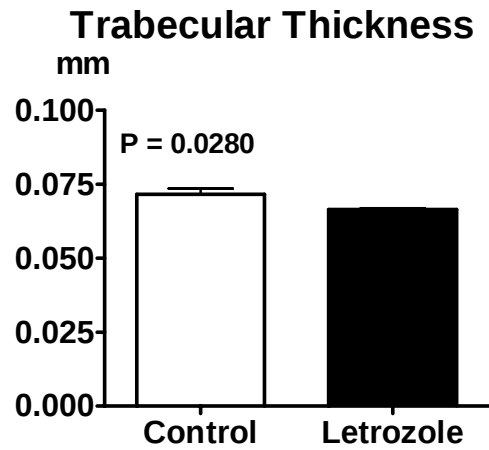
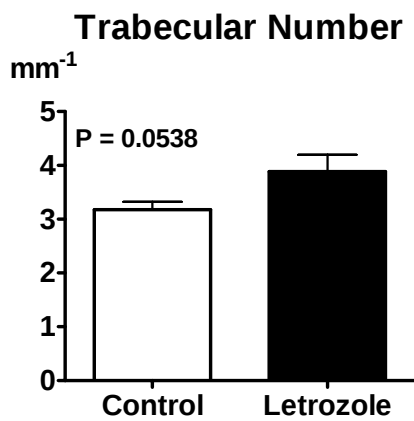
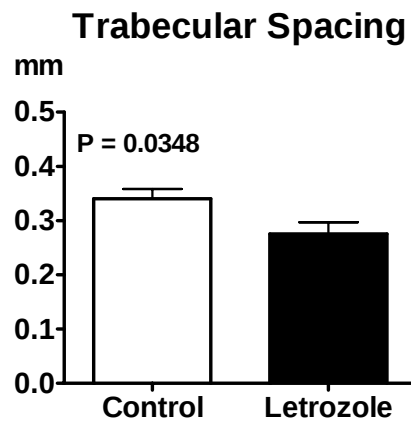
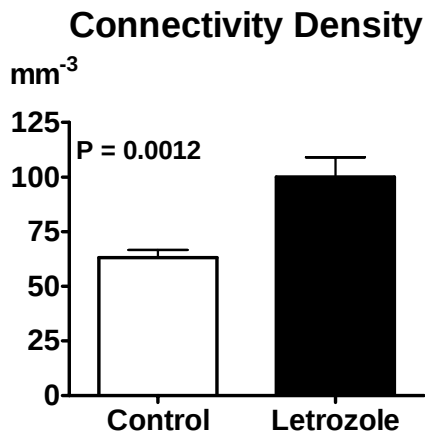


Figure 37. MicroCT analysis of the right femur from female nude mice after 4 weeks of treatment with letrozole or control. P values calculated using Student's t-test.

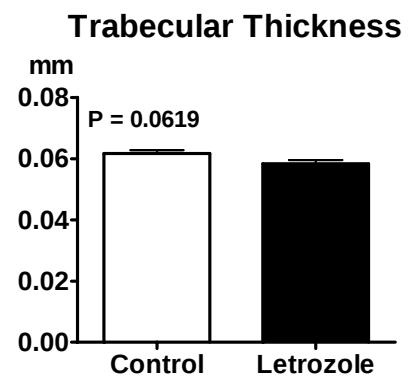
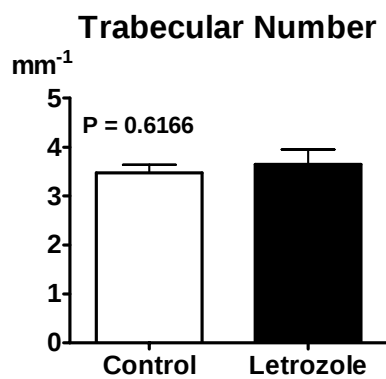
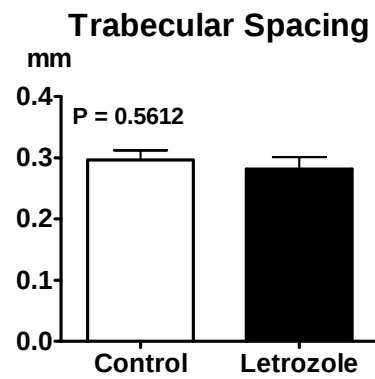
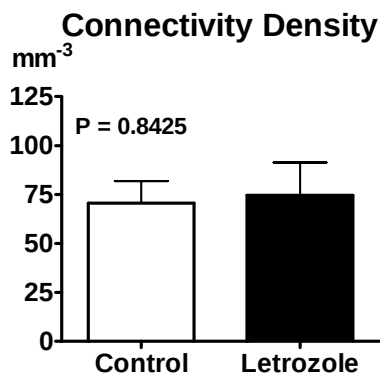


Figure 38. MicroCT analysis of the right tibia from female nude mice after 4 weeks of treatment with letrozole or control. P values calculated using Student's t-test.

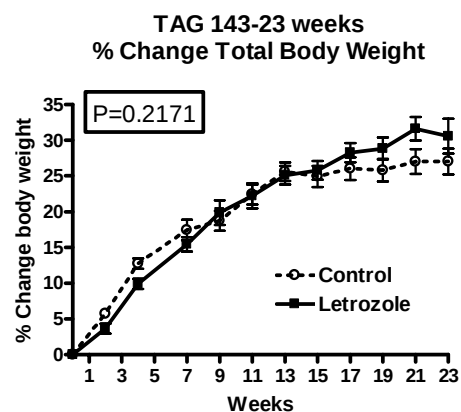
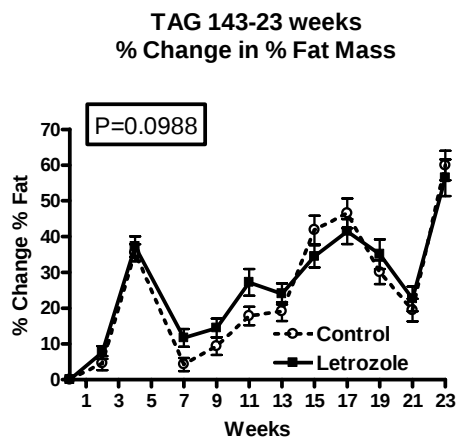
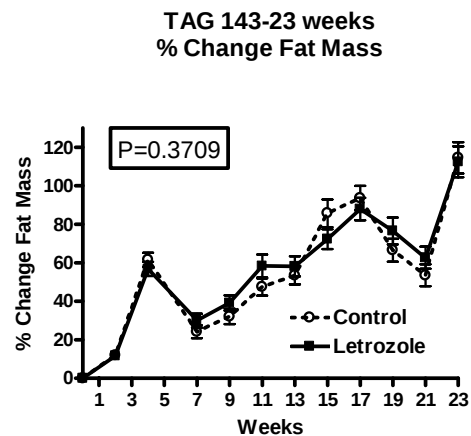
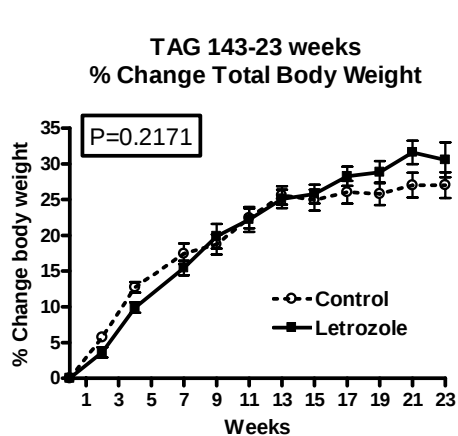


Figure 39. Body composition and total body BMD in 4-week-old female nude mice treated with letrozole or control. P values calculated using two-way ANOVA.

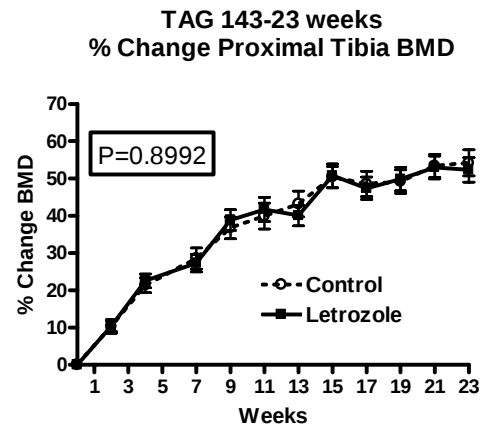
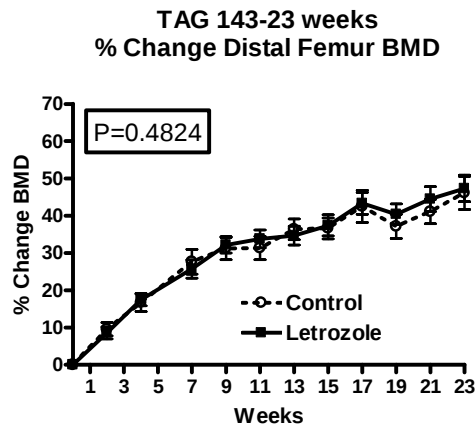
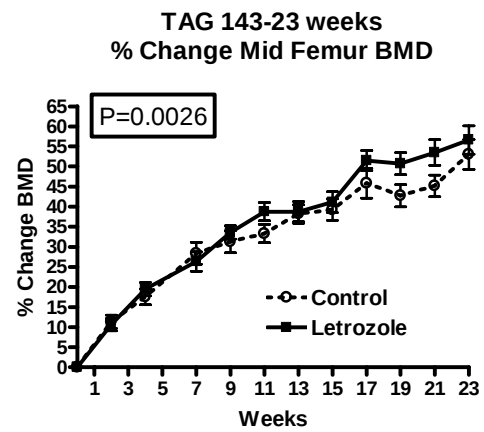
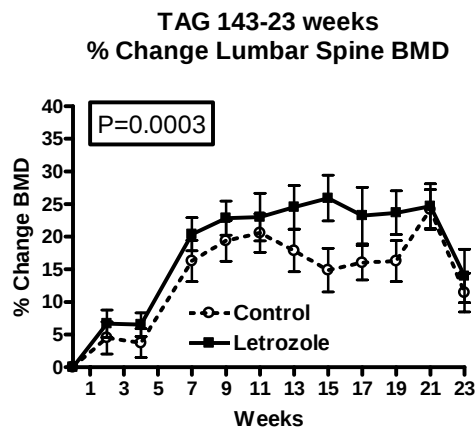


Figure 40. BMD in 4-week-old female nude mice treated with letrozole or control. P values calculated using two-way ANOVA.

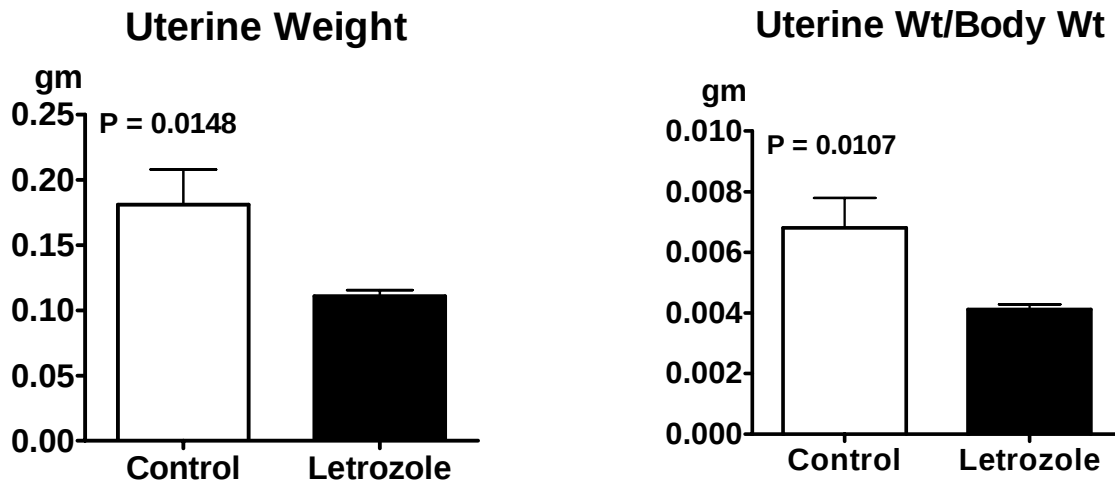


Figure 41. Uterine weight and uterine weight/body weight in female nude mice after 23 weeks of treatment with letrozole or control. Uterine weight and uterine weight/body weight was significantly lower in the letrozole-treated mice compared to the control mice. P values calculated using the student's t-test.



Figure 42. MicroCT analysis of the femur and tibia from 4-week-old female nude mice after 23 weeks of treatment with letrozole or control. P values calculated using student's t-test.

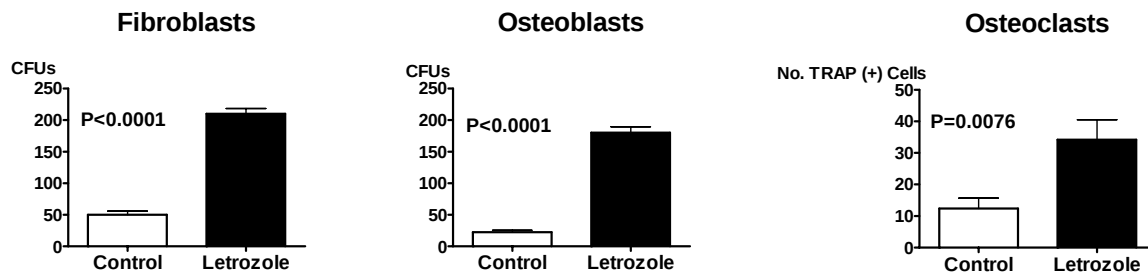


Figure 43. CFU and osteoclast formation assays using femurs and tibias (3 mice/group) from female nude mice after 23 weeks of treatment with letrozole or control. P values calculated using Student's t-test.

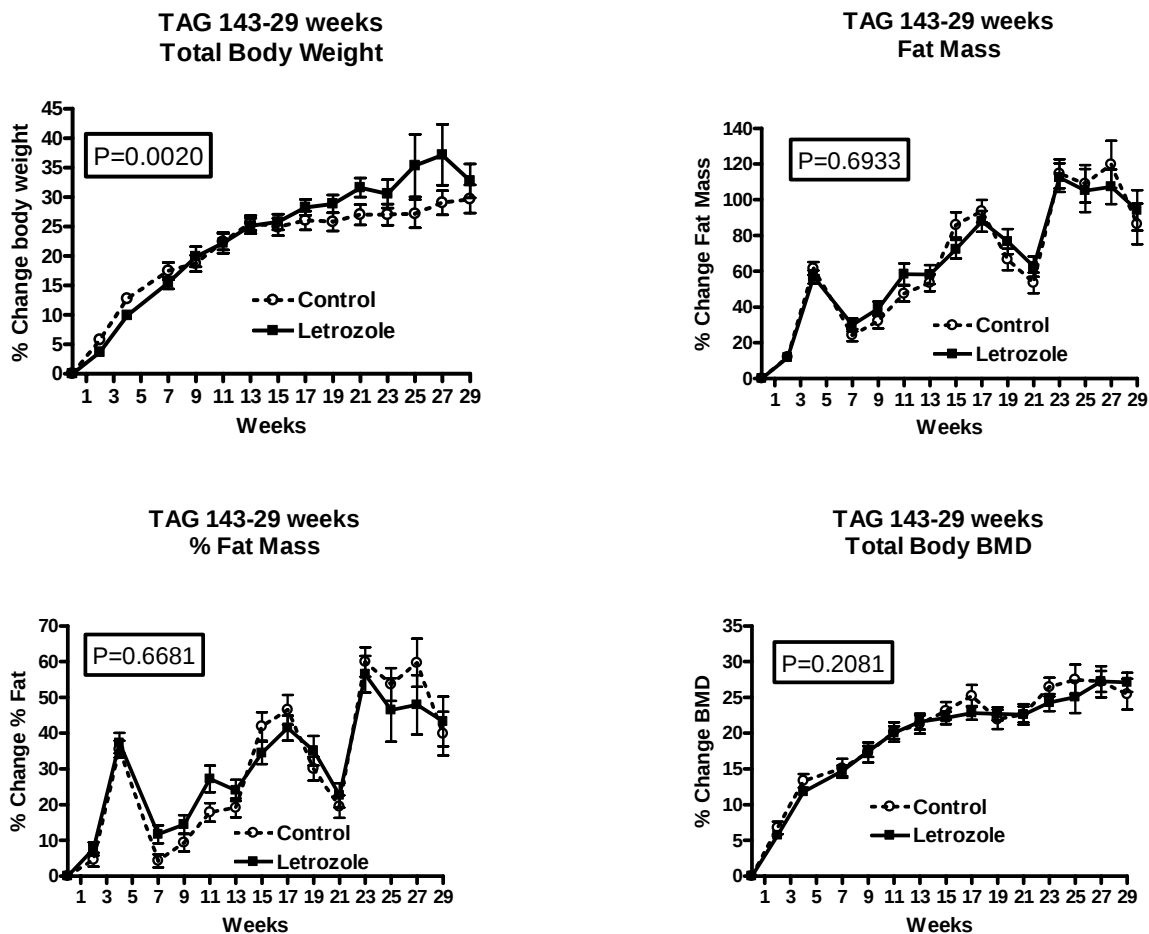


Figure 44. Body composition and total body BMD in female nude mice treated with letrozole or control for 29 weeks. P values calculated using two-way ANOVA.

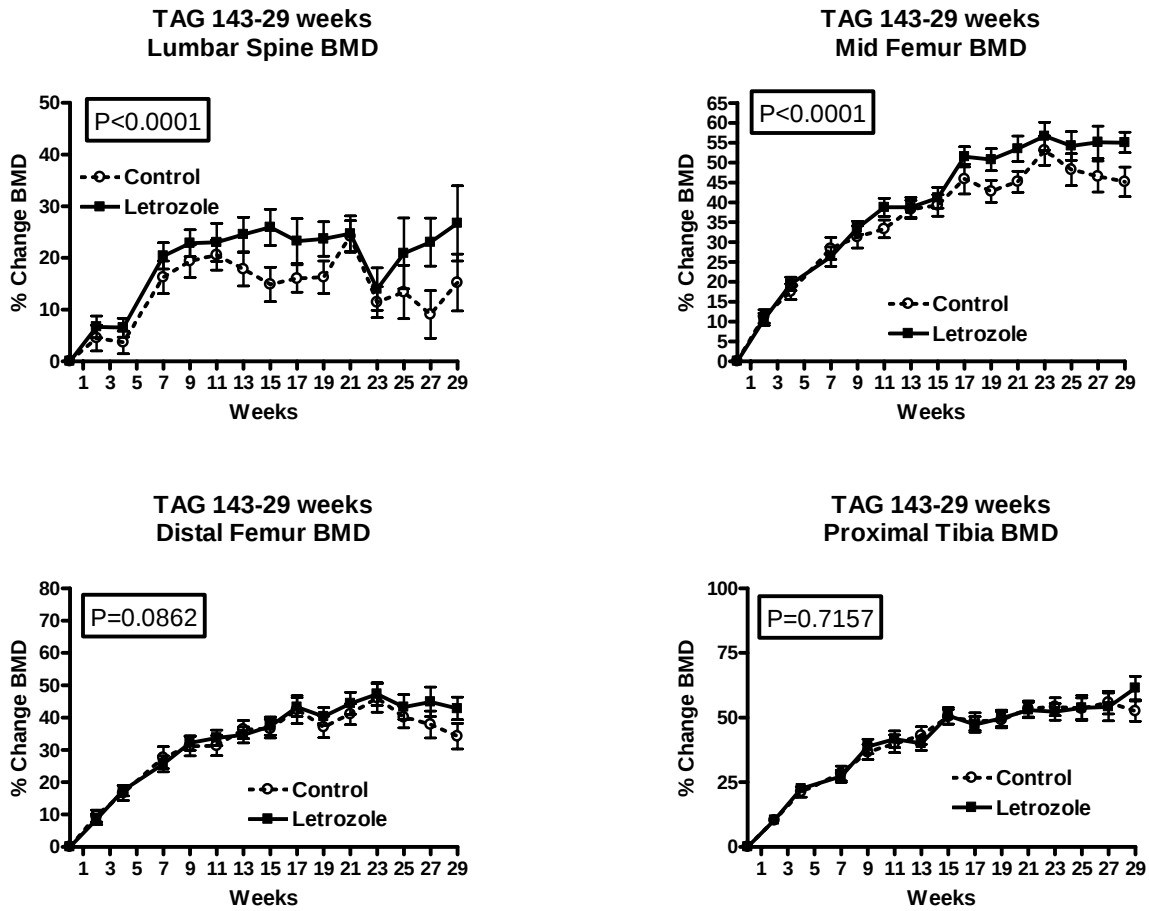


Figure 45. BMD in female nude mice treated with letrozole or control for 29 weeks. P values calculated using two-way ANOVA.

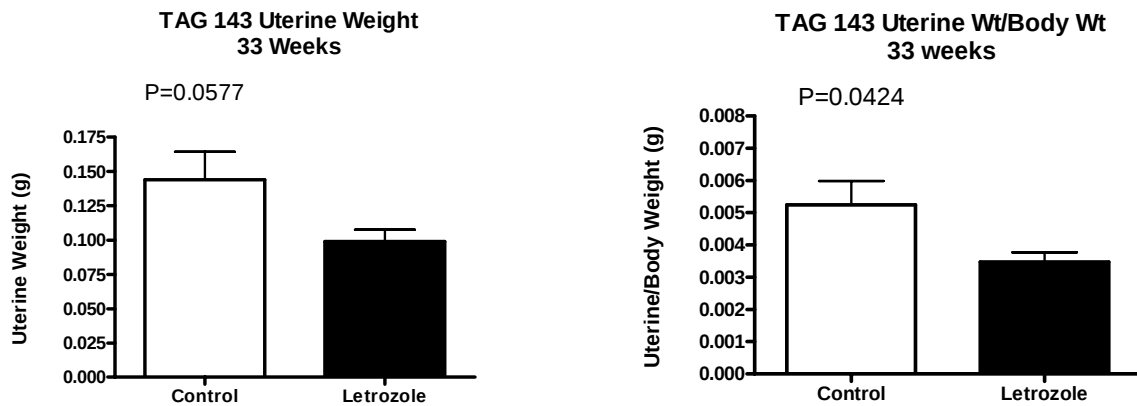


Figure 46. Uterine weight and uterine weight/body weight in female nude mice after 33 weeks of treatment with letrozole or control. P values calculated using the student's t-test.

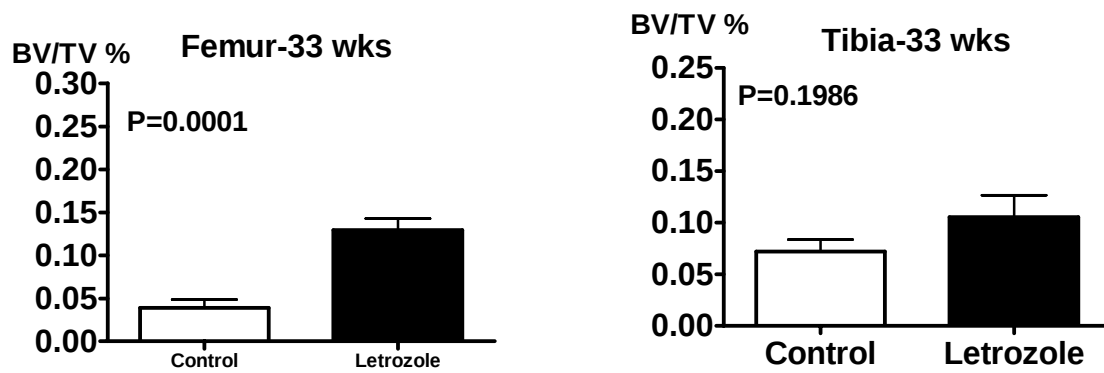


Figure 47. MicroCT analysis of the femur and tibia from female nude mice after 33 weeks of treatment with letrozole or control. P values calculated using student's t-test.

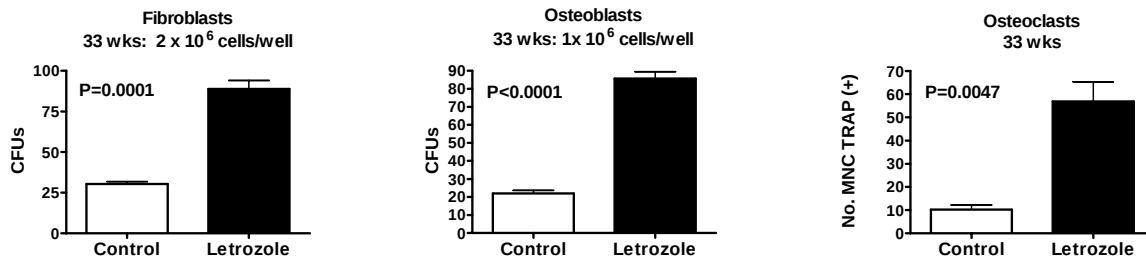


Figure 48. CFU and osteoclast formation assays using femurs and tibias (3 mice/group) from female nude mice after 33 weeks of treatment with letrozole or control. P values calculated using Student's t-test.

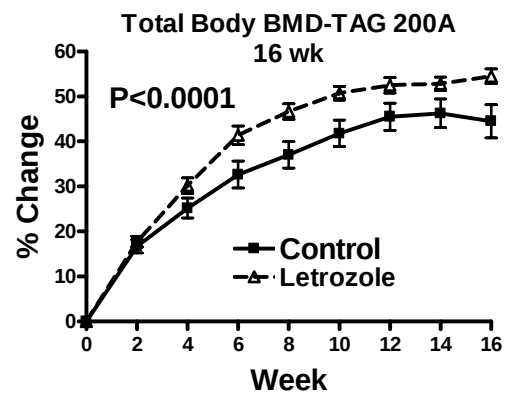
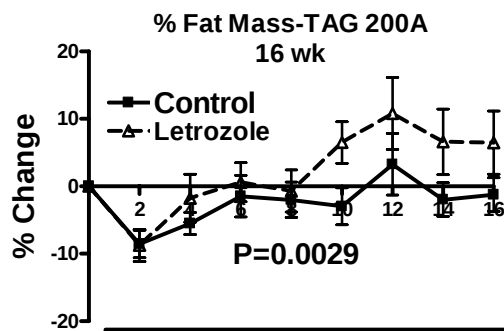
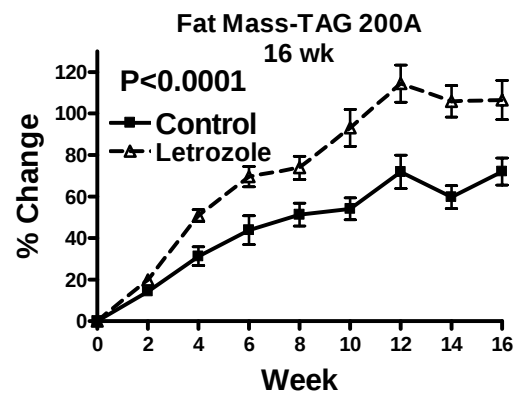
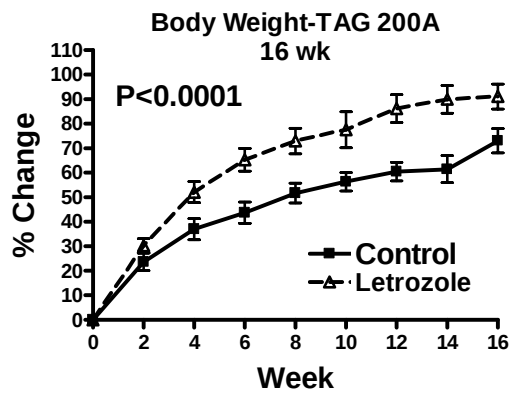


Figure 49. Body composition and BMD in 4-week-old female nude mice after 16 weeks of treatment with letrozole (5 mg/kg/day) or control. P values calculated using two-way ANOVA.

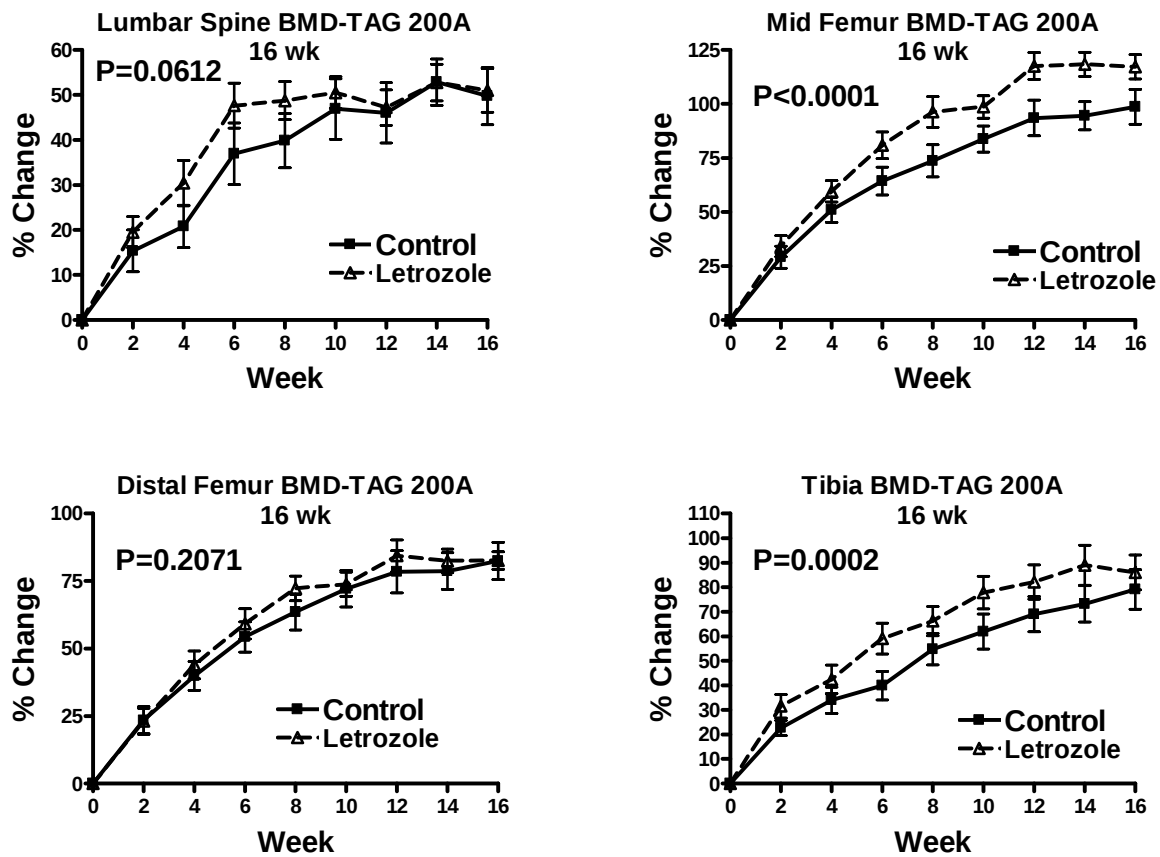


Figure 50. BMD in 4-week-old female nude mice after 16 weeks of treatment with letrozole (5 mg/kg/day) or control. P values calculated using two-way ANOVA.

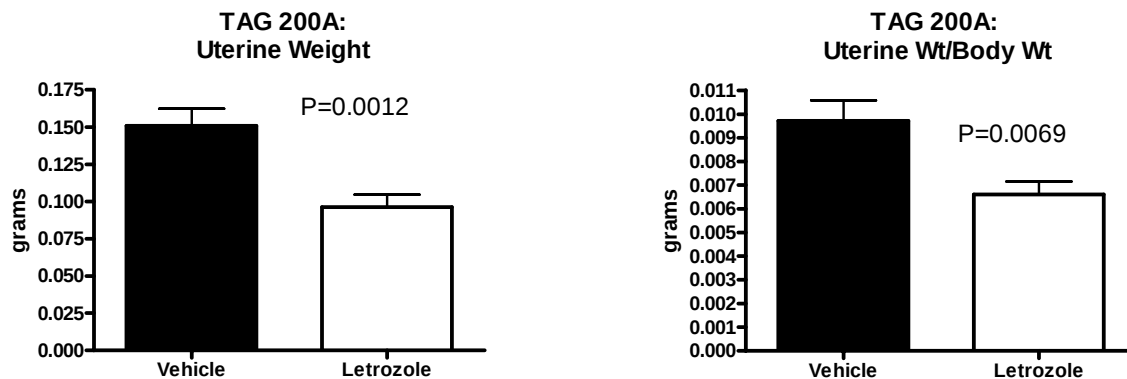


Figure 51. Uterine weight and uterine weight/body weight in 4-week-old female nude mice after 16 weeks of treatment with letrozole (5 mg/kg/day) or control. P values calculated using two-way ANOVA.

Key Research Accomplishments

Results for tasks 1 & 2 – part 1 (this data was previously reported in the 2006 annual summary):

- Eight weeks after surgery there was no difference in BMD in OVX mice compared to sham mice at any site: total body ($p=0.6814$), spine ($p=0.3398$), femur ($p=0.3914$) or tibia ($p=0.3093$) (**figure 1**).
- Histomorphometry showed no difference in trabecular bone volume (TBV) in OVX mice compared to sham mice at the femur ($p=0.8634$) or tibia ($p=0.1329$) (**figure 2**).
- There was no difference in serum IGF-1 levels in the OVX mice compared to the sham mice (**figure 3**).
- In the mice inoculated with MDA-MB-231 via intra-cardiac injection, there was no difference in total body x-ray lesion area ($p=0.4728$) or tibia plus femur x-ray lesion area ($p=0.4412$) between the OVX and sham mice (**figure 4**).
- There was no difference in survival between the OVX and sham mice ($p=0.0874$) after intra-cardiac injection with MDA-MB-231 (**figure 5**).

Results for tasks 1 & 2 – part 2a: (data not previously reported)

- At 28 weeks, OVX mice had significantly increased body weight ($p<0.0001$), fat mass ($p<0.0001$) and % fat mass ($p<0.0001$) compared to the sham mice (**figure 6**). At 28 weeks, OVX mice had significantly decreased total body BMD ($p<0.0001$) (**figure 6**), and significantly decreased BMD at the spine ($p<0.0001$) and proximal tibia ($p<0.0001$) compared to sham mice (**figure 7**). Interestingly, there was no difference in BMD between the OVX and sham mice at the distal femur, and the OVX mice demonstrated increased BMD at the mid femur ($p<0.0001$) compared to the sham mice (**figure 7**).
- Histomorphometry revealed that there was no difference in TBV between the OVX and sham mice at the proximal tibia or distal femur (**figure 8**).
- Micro-CT revealed that trabecular bone in the proximal tibia did not differ between the OVX and sham mice (**figure 9**).
- Micro-CT revealed that trabecular bone in the distal femur did not differ between the OVX and sham mice (**figure 10**).
- Bone marrow cultures from OVX mice exhibited a greater number of CFU-fibroblasts ($p<0.0001$), CFU-osteoblasts ($p<0.0001$) and TRAP-positive osteoclasts ($p=0.0005$) compared to sham mice (**figure 11**).
- Although baseline IGF-1 levels did not differ between the OVX and sham mice, 28 weeks after surgery the OVX mice had higher IGF-1 levels than the sham mice ($p=0.0253$) (**figure 12**). However, pre-OVX IGF-1 levels were not significantly different from post-OVX IGF-1 levels, and pre-sham surgery IGF-1 levels were not significantly different from post-sham surgery IGF-1 levels (**figure 12**).
- Total body X-ray lesion area was greater in the sham surgery mice compared to the OVX mice ($P<0.0001$) (**figure 13**).

Results for tasks 1 & 2 – part 2b: (data not previously reported)

- At 20 weeks, OVX mice had significantly increased body weight ($p<0.0001$), fat mass ($p<0.0001$) and % fat mass ($p<0.0001$) compared to the sham mice (**figure 14**). At 20 weeks, OVX mice had significantly decreased total body BMD ($p=0.0048$) (**figure 14**), and significantly decreased BMD at the spine ($p<0.0001$) and distal femur ($p<0.0001$) and mid femur ($p=0.0409$) compared to sham mice (**figure 15**). There was no difference in BMD between the OVX and sham mice at the proximal tibia (**figure 15**).
- Micro-CT revealed that trabecular bone in the proximal tibia did not differ between the OVX and sham mice with the exception of “degree of anisotropy”, which was slightly increased in the OVX mice compared to the sham mice ($p=0.0316$) (**figure 16**).
- Micro-CT revealed that trabecular bone in the distal femur did not differ between the OVX and sham mice (**figure 17**).
- Bone marrow cultures from OVX mice exhibited a greater number of CFU-fibroblasts ($p<0.0001$) and CFU-osteoblasts ($p=0.0001$) compared to sham mice (**figure 18**). Unfortunately, there was a technical problem with the osteoclast formation assay.
- IGF-1 levels did not differ between the OVX and sham mice at baseline or at 20 weeks after surgery (**figure 19**). In addition, pre-OVX IGF-1 levels were not significantly different from post-OVX IGF-1 levels, and pre-sham surgery IGF-1 levels were not significantly different from post-sham surgery IGF-1 levels (**figure 19**).
- Total body X-ray lesion area was greater in the sham surgery mice compared to the OVX mice ($P=0.0043$) (**figure 20**).

Results for tasks 3-9 - Part 1 (this data was previously reported in the 2006 annual summary):

- After 13 weeks of treatment, mice treated with letrozole alone had lower BMD compared to control ($p<0.0001$; total body, spine, proximal femur and tibia) (**figure 21**). In the mid-femur, however, mice treated with letrozole alone, as compared to control, did not have lower BMD after 13 weeks of treatment ($p=0.0961$), but a difference in BMD was seen after 7 weeks of treatment ($p=0.0435$) (**figure 22**). Mice treated with ZA alone had higher BMD compared to control ($p<0.0001$; total body, spine, mid-femur, proximal femur and tibia). Mice treated with letrozole + ZA achieved the same BMD as mice treated with ZA alone at the spine and tibia, but had greater BMD than mice treated with ZA alone at the mid-femur ($p<0.0001$), proximal femur ($p<0.0001$) and total body ($p<0.0023$) (**figure 21**).
- Histomorphometry demonstrated that mice treated with letrozole alone had the same TBV as mice treated with control at the proximal femur, tibia and lumbar spine (**figure 23**). Mice treated with ZA (+/- letrozole) had increased TBV compared to letrozole alone at the proximal femur and tibia. In the lumbar spine, mice treated with ZA alone had increased TBV compared to both letrozole ($p<0.05$) and letrozole + ZA ($p<0.05$).
- MicroCT analysis of the proximal tibia showed no difference in BV/TV, SMI or Tb.N, Tb.Th or Tb.Sp in mice treated with letrozole alone compared to control. Treatment with ZA (+/- letrozole) resulted in a significant increase in BV/TV, Tb.N and Tb.Th, and the SMI indicated that the bone structure was unusually solid (**figure 24**).
- Dynamic bone histomorphometry of the lumbar spine demonstrated decreased BFR and MAR in mice treated with letrozole, ZA or the combination compared to control (**figure 25**).

- Mechanical testing showed no difference in peak load or stiffness for either the femur or tibia in the letrozole-treated mice compared to the control mice (**figure 26**).
- Liver IgF-1 Real-time RT PCR demonstrated decreased IGF-1 expression in letrozole-treated mice compared to control ($p<0.01$), an effect that was reversed with the addition of ZA (**figure 27**). There was no difference in serum IGF-1 levels in mice treated with letrozole alone compared to control (**figure 27**), although there was decreased serum IGF-1 levels in the ZA alone mice compared to control ($p<0.05$).
- To assess the effect of letrozole on bone formation, calvaria obtained from 4-day-old mice were cultured for 7 days with media (BGJ) alone, positive control (insulin) or letrozole. Histomorphometry demonstrated that letrozole did not stimulate new bone formation and, when combined with insulin, did not inhibit new bone formation (**figure 28**).

Results for tasks 3-9 -Part 2 (this data was previously reported in the 2006 annual summary):

- After 3 weeks of treatment, mice treated with letrozole accrued less BMD at the proximal femur ($p=0.0161$), but achieved the same BMD as control mice at the total body, spine and tibia (**figure 29**).
- After 4 weeks of treatment, x-ray analysis demonstrated that there was no difference in total body lesion area between the letrozole-treated mice, as compared to the control mice, after intra-cardiac injection with MDA-MB-231 (**figure 30**).

Results for tasks 3-9 -Part 3 (this data was previously reported in the 2006 annual summary):

- After 8 weeks of treatment, there was no difference in BMD between the letrozole and control mice at any site (**figure 31**).
- X-ray analysis demonstrated that there was no difference in total body lesion area between the letrozole-treated mice, as compared to the control mice, after intra-cardiac injection with MDA-MB-231 (**figure 32**).

Results for tasks 3-9 -Part 4 (this data was only partially reported in the 2006 annual summary):

- After 4 weeks of treatment, there was no difference in BMD between the letrozole and control mice at any site (**figure 33**).
- After 4 weeks of treatment, there was no difference in TBV in the femur ($p=0.2268$) or tibia ($p=0.9691$) of letrozole-treated mice compared to the control mice (**figure 34**).
- After 4 weeks of treatment, there was no difference in uterine weight or uterine weight/body weight between the letrozole-treated mice compared to the control mice (**figure 35**).
- MicroCT analysis of the femur and tibia after 4 weeks of treatment did not show a significant difference in trabecular bone volume (BV/TV%), although a trend toward increased BV/TV% in the femurs of letrozole-treated mice was observed ($p=0.0659$).

(figure 36). However, 4 weeks of treatment with letrozole induced marked increases in skeletal microarchitecture. Significant increases in ConnD ($p=0.0012$) and Tb.N ($p=0.0538$), Tb.Th ($p=0.0280$) and Tb.Sp ($p=0.0348$) were observed in the femurs of letrozole-treated mice, but not in the tibias **(figures 37 and 38).**

- After 23 weeks of treatment, there was no difference in body weight, body composition or total body BMD in letrozole and control mice **(figure 39).**
- After 23 weeks of treatment, letrozole-treated mice had increased BMD at the lumbar spine ($p=0.0003$) and mid-femur ($p=0.0026$) compared to control mice **(figure 40).** There was no significant difference in BMD between letrozole and control-treated mice at the distal femur or proximal tibia.
- After 23 weeks of treatment, letrozole-treated mice had significantly lower uterine weight ($p=0.0148$) and uterine weight/body weight ($p=0.0107$) compared to the control mice **(figure 41).**
- After 23 weeks of treatment, micro-CT demonstrated significantly increased BV/TV% in both the femur ($p=0.0002$) and tibia ($p<0.0001$) of letrozole-treated mice compared to control **(figure 42).**
- After 23 weeks of treatment, bone marrow cultures from the tibias and femurs of letrozole treated mice showed significantly increased CFU-fibroblasts ($p<0.0001$), CFU- osteoblasts ($p<0.0001$) and TRAP-positive osteoclasts ($p=0.0076$) compared to the bone marrow cultures from control mice **(figure 43).**
- After 29 weeks of treatment, body weight was significantly greater in the letrozole-treated mice compared to control mice ($p=0.0020$) but there were no significant differences in body composition or total body BMD **(figure 44).**
- After 29 weeks of treatment, letrozole-treated mice had increased BMD at the lumbar spine ($p<0.0001$) and mid-femur ($p<0.0001$) compared to control mice **(figure 45).** There was no significant difference in BMD between letrozole and control-treated mice at the distal femur or proximal tibia.
- After 33 weeks of treatment, letrozole-treated mice had significantly lower uterine weight/body weight ($p=0.0424$) compared to the control mice **(figure 46).** However, although there was trend towards decreased uterine weight in the letrozole-treated mice, the difference in uterine weights between the treatment groups did not meet statistical significance ($p=0.0577$) **(figure 46).**
- After 33 weeks of treatment, micro-CT did not demonstrate significant differences in %BV/TV in either the femur or tibia ($p<0.0001$) between the letrozole or control treatment groups **(figure 47).**
- After 33 weeks of treatment, bone marrow cultures from the tibias and femurs of letrozole treated mice showed significantly increased CFU-fibroblasts ($p=0.0001$), CFU- osteoblasts ($p<0.0001$) and TRAP-positive osteoclasts ($p=0.0047$) compared to the bone marrow cultures from control mice **(figure 48).**

Results for tasks 1 & 2 – part 2a: (data not previously reported)

- After 16 weeks of treatment, letrozole-treated mice had significantly increased body weight ($p<0.0001$), fat mass ($p<0.0001$), % fat mass ($p=0.0029$) and total body BMD ($p<0.0001$) compared to control mice (**figure 49**).
- After 16 weeks of treatment, letrozole-treated mice had significantly increased BMD at the mid femur ($p<0.0001$) and proximal tibia ($p=0.0002$) and there was a trend toward increased BMD at the spine ($p=0.0612$) compared to control mice (**figure 50**). BMD did not differ between the treatment groups at the distal femur (**figure 50**).
- After 16 weeks of treatment, letrozole-treated mice had significantly decreased uterine weight ($p=0.0012$) and uterine weight/body weight ($p=0.0069$) compared to the control mice (**figure 51**).

Reportable Outcomes

Publications:

Scientific Journals:

1. Perkins DL, Listman JA, Marshak-Rothstein A, **Kozlow W**, Kelley VR, Finn PW, Rimm IJ. 1996 Restriction of the TCR repertoire inhibits the development of memory cells and prevents autoimmunity in lpr mice. *Journal of Immunology*. 156(12):4961-8.
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2. Meeting Report from Skeletal Complications of Malignancy IV; April 28-30, 2005 in Bethesda, Maryland, USA. This symposium was jointly sponsored by The Paget Foundation for Paget's Disease of Bone and Related Disorders, the National Cancer Institute and the University of Virginia School of Medicine. Authors: Robert L. Vessella, Theresa A. Guise, Edward S. Susman, Larry J. Suva, Gregory A. Clines, Scott L. Kominsky, Kristy L. Weber, John M. Chirgwin, Laurie K. McCauley and **Wende Kozlow**. Published on BoneKEy: www.bonekey-ibms.org.

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Abstracts:

1.**Kozlow W**, Mohammad K, McKenna R, Niewolna M, Suva LJ, Rosen C and Guise TA. 2005 Aromatase inhibition causes lower bone density than ovariectomy in mice, an effect prevented by bisphosphonates. *Journal of Bone and Mineral Research*. 20(Suppl 1):S313.

2.**Kozlow W**, Mohammad K, McKenna R, Niewolna M and Guise TA. 2005 Aromatase inhibition results in lower bone density than ovariectomy in mice, an effect prevented by bisphosphonates. *Journal of Bone and Mineral Research*. 20(Suppl 2):S34.

3.**Kozlow W**, Mohammad K, McKenna R, Walton H, Niewolna M, Dilley JD, Suva LJ and Guise TA. 2006 Aromatase inhibition results in gain of bone density in the spine and femur in female nude mice. *Journal of Bone and Mineral Research*. 21(Suppl 1):S83.

Presentations:

1. Blackard Symposium, Richmond, VA, 2005

2. Breast Cancer Research Program (BCRP) Leading Innovative Networking and Knowledge Sharing (LINKS) Meeting 2006 Baltimore, MD, 2006

3. American Society for Bone and Mineral Research (ASMBR) annual meeting, Honolulu, Hawaii 2007 (*upcoming event- submitted abstract below*)

▪ **Completed Courses:**

- Spring 2005 School of Continuing & Professional Studies
*BIMS 710 RESEARCH ETHICS S 1.0
- Fall 2005 School of Continuing & Professional Studies
*HES 700 INTRO TO BIOSTATISTICS A 3.0

▪ **Certifications:**

- Endocrine University: March 11-16, 2006 (certified by American College of Endocrinology (AACE))
- AACE Thyroid Ultrasound and FNA Biopsy Accreditation Course®: March 11-12, 2006 (certified by AACE)
- Certified Clinical Densitometrist (CCD): March 15, 2006 (certified by International Society for Clinical Densitometry)
- Certified, Endocrinology, Diabetes and Metabolism 2006

▪ **Awards:**

- Women in Endocrinology 2006 Abstract Award in recognition of the abstract submitted to Endocrine Society 2006.
- Award for Translational Cancer Research from the V Foundation – American Association for Cancer Research; awarded 04/19/05.

▪ **Abstracts (provided in the appendix):**

1. Skeletal Complications of Malignancy IV Meeting; April 2005; Bethesda, MD
2. University of Virginia Medicine Research Day; May 2005; Charlottesville, VA
3. American Society Bone & Mineral Research (ASBMR) annual meeting; September 2005; Nashville, TN
4. Endocrine Society annual meeting; June 2006; Boston, MA
5. ASBMR annual meeting; September 2006; Philadelphia, PA

6. Cancer and Bone Society's IV International Meeting on Cancer Induced Bone Disease; December 2006; San Antonio, TX
7. AMBMR annual meeting; September 2007; Honolulu, HI

▪ **Employment:**

- March 1, 2006: Director of the endocrinology elective for UVA medical students and medicine residents
- July 1, 2006: Assistant Professor of Research, Division of Endocrinology and Metabolism, Department of Medicine, University of Virginia (80% research and 20% clinical)

Conclusion

Tamoxifen therapy is bone-sparing, but its use in breast cancer is rapidly superseded by AIs. Unlike tamoxifen, AI therapy for breast cancer results in high bone turnover. This leads to osteoporosis and fractures. It may increase breast cancer bone metastases. Women treated with AIs can expect to remain on therapy for a prolonged period of time. Therefore, it is important to assess the long-term consequences of AI therapy, including its effects on skeletal health. If AIs result in increased bone turnover and decreased BMD, concomitant treatment with antiresorptive agents must be considered. Our mouse model will 1) define the effect of a high bone turnover state induced by breast cancer therapy on the development and progression of breast cancer bone metastases and 2) test effective therapy to prevent increased bone turnover and breast cancer bone metastases. Preventing metastases to bone blocks the progression to Stage IV cancer, which is generally incurable. The findings from this multidisciplinary research protocol may reduce the morbidity from osteoporosis and bone metastases experienced by breast cancer patients.

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Appendices

1. Abstract from the Skeletal Complications of Malignancy IV Meeting; April 2005; Bethesda, MD:

Aromatase Inhibition Results in Lower Bone Density Than Ovariectomy in Mice an Effect Prevented by Bisphosphonates. W.M. Kozlow, K. Mohammad, R. McKenna, M. Niewolna and T.A. Guise. Department of Internal Medicine, Division of Endocrinology and Metabolism, University of Virginia, USA.

Aromatase inhibitors have emerged as superior to tamoxifen to treat breast cancer. These drugs block estrogen synthesis by inhibiting the rate-limiting step in the conversion of testosterone and androstenedione to estradiol and estrone, respectively. This reduction in estrogen synthesis can be anticipated to increase bone resorption, thereby decreasing bone density. Clinical trials confirm that aromatase inhibitors reduce bone density in breast cancer patients. Bisphosphonates, inhibitors of bone resorption, may be useful to prevent bone loss due to aromatase inhibitor therapy. To study the effect of estrogen deficiency on bone mineral density (BMD) in mice, we performed 2 experiments. In the first study, 4-week-old female nude mice were randomized to bilateral ovariectomy or sham surgery (n = 12/group). BMD was measured at baseline and then every 2 weeks with GE Lunar PIXImus. At 8 weeks post-surgery, there was no difference in BMD in the ovariectomized mice compared to the sham control mice at any site: total body (p = 0.6814), spine (p = 0.3398), femur (p = 0.3914) and tibia (p = 0.3093). Next, we studied the effect of aromatase inhibitors +/- bisphosphonates on BMD. Forty 4-week-old female nude mice were randomized to 4 treatment groups: control (0.3% hydroxypropyl cellulose in PBS), letrozole (10 mcg SQ QD), zoledronic acid (5 mcg/kg SQ twice weekly) or letrozole (10 mcg SQ QD) plus zoledronic acid (5 mcg/kg SQ twice weekly). BMD was measured at baseline and then every 2 weeks for 13 weeks. Mice treated with letrozole alone had significantly lower BMD compared to mice treated with control at all sites: total body (p < 0.0001), spine (p = 0.0002), femur (p = 0.0005) and tibia (p < 0.0001). Mice treated with zoledronic acid had higher BMD compared to mice treated with control at all sites: total body (p < 0.0001), spine (p < 0.0001), femur (p < 0.0001) and tibia (p < 0.0001). Mice treated with letrozole plus zoledronic acid achieved the same bone density as mice treated with zoledronic acid alone at the spine (p = 0.8546) and tibia (p = 0.2169), but had greater bone density than mice treated with zoledronic acid alone at the femur (p < 0.0001) and total body (p < 0.0023). Thus, the aromatase inhibitor, letrozole, caused a reduction in bone density in female nude mice that was greater than that observed with ovariectomy alone. This bone loss was prevented by concomitant treatment with zoledronic acid.

These results indicate that medical castration with aromatase inhibitors causes more profound bone loss than with ovariectomy. This may be due to the fact that aromatase inhibitors result in complete blockade of estrogen production compared to ovariectomy, where adrenal androgens may still be converted to estrogens by peripheral aromatase activity. Nonetheless, the significant bone loss induced by aromatase inhibition can be prevented with bisphosphonate therapy. Bisphosphonates may potentially be used for primary prevention against bone loss when therapy with an aromatase inhibitor is indicated.

2. Abstract from the University of Virginia Medicine Research Day; May 2005; Charlottesville, VA:

AROMATASE INHIBITION RESULTS IN LOWER BONE DENSITY THAN OVARECTOMY IN MICE, AN EFFECT PREVENTED BY BISPHOSPHONATES. Wende Kozlow, Khalid Mohammad, Ryan McKenna, Maryla Niewolna and Theresa A. Guise.

Aromatase inhibitors (AIs) have emerged as superior to tamoxifen to treat breast cancer. These drugs block estrogen synthesis by inhibiting the rate-limiting step in the conversion of testosterone and androstenedione to estradiol and estrone, respectively. Reduction in estrogen synthesis can be anticipated to increase bone resorption, thereby decreasing bone mineral density (BMD). Bisphosphonates (BPs), inhibitors of bone resorption, may prevent bone loss due to AI therapy. To study the effect of estrogen deficiency on BMD in mice, we performed 2 experiments. In the first study, 4-week-old female nude mice were randomized to bilateral ovariectomy (ovx) or sham surgery (n=12/group). BMD was measured at baseline and then every 2 weeks with GE Lunar PIXImus. At 8 weeks post-surgery, there was no difference in BMD in the ovx mice compared to the sham mice at any site: total body (p=0.6814), spine (p=0.3398), femur (p=0.3914) and tibia (p=0.3093). Next, we studied the effect of AIs +/- BPs on BMD. Forty 4-week-old female nude mice were randomized to 4 treatment groups: control (0.3% hydroxypropyl cellulose in PBS), letrozole (10 mcg SQ QD), zoledronic acid (ZA) (5 mcg/kg SQ twice weekly) or letrozole (10 mcg SQ QD) plus ZA (5 mcg/kg SQ twice weekly). BMD was measured at baseline and then every 2 weeks for 13 weeks. Mice treated with letrozole alone had significantly lower BMD compared to mice treated with control at all sites: total body (p<0.0001), spine (p=0.0002), femur (p=0.0005) and tibia (p<0.0001). Mice treated with ZA had higher BMD compared to mice treated with control at all sites: total body (p<0.0001), spine (p<0.0001), femur (p<0.0001) and tibia (p<0.0001). Mice treated with letrozole plus ZA achieved the same bone density as mice treated with ZA alone at the spine and tibia, but had greater bone density than mice treated with ZA alone at the femur (p<0.0001) and total body (p<0.0023). The AI caused a reduction in BMD in female nude mice that was greater than that observed with ovx alone. This bone loss was prevented by concomitant treatment with ZA. These results indicate that medical castration with AIs causes more profound bone loss than with ovx. BPs may potentially be used for primary prevention against bone loss when therapy with an AI is indicated.

3. Abstract from the American Society Bone & Mineral Research (ASBMR) annual meeting; September 2005; Nashville, TN:

Aromatase Inhibition Causes Lower Bone Density Than Ovariectomy in Mice, an Effect Prevented by Bisphosphonates. W. Kozlow¹, K. Mohammad¹, R. McKenna¹, M. Niewolna¹, L. Suva², C. Rosen³, T.A. Guise¹. ¹Internal Medicine, University of Virginia, Charlottesville, VA, USA, ²Orthopaedic Surgery, University of Arkansas, Little Rock, AR, USA, ³Jackson Laboratory, Bar Harbor, ME, USA.

Aromatase inhibitors (AIs), effective treatment for breast cancer, block estrogen synthesis by inhibiting the conversion of testosterone and androstenedione to estradiol and estrone. Increased bone resorption and decreased bone mineral density (BMD) are predicted consequences. We hypothesized that bisphosphonates (BPs) may prevent bone loss from AI therapy. We studied the effect of estrogen deficiency on bone remodeling in 4-week-old female nude mice that underwent ovariectomy (ovx) or sham surgery. Ovx and sham mice did not differ in BMD (assessed by DXA) or in histomorphometric assessment of trabecular bone volume. Next, to study the effect of AIs +/- BPs on bone remodeling, 4-week-old female nude mice were treated with letrozole (10 mcg/d), zoledronic acid (ZA) (5 mcg/kg twice weekly), letrozole (10 mcg/d) + ZA (5 mcg/kg twice weekly) or control. Mice treated with letrozole alone had lower BMD compared to control mice ($p < 0.0001$; total body, spine, femur and tibia). Mice treated with ZA alone had higher BMD compared to control mice ($p < 0.0001$; total body, spine, femur and tibia). Mice treated with letrozole plus ZA achieved the same BMD as mice treated with ZA alone at the spine and tibia, but had greater BMD than mice treated with ZA alone at the femur ($p < 0.0001$) and total body ($p < 0.0023$). MicroCT analysis of the proximal tibia showed no difference in bone volume (BV/TV), structural model index, or trabecular number, thickness or spacing in mice treated with letrozole alone compared to control. Treatment with ZA (+/- letrozole) resulted in a significant increase in BV/TV and trabecular number and thickness, and the structural model index indicated that the bone structure was unusually solid. Dynamic bone histomorphometry of the lumbar spine demonstrated decreased bone formation and mineral apposition rates in mice treated with letrozole, ZA or the combination compared to control. Serum testosterone concentrations were increased in mice treated with letrozole compared to control. Serum IGF-1 concentrations were similar in all groups. These data indicate that aromatase inhibition with letrozole caused lower BMD in female nude mice than that observed with ovx. The greater effect of AIs compared to ovx may be due to reduced adrenal androgen conversion to estrogen. ZA prevented AI-induced bone loss, but microCT and dynamic bone histomorphometry suggest reduced bone remodeling. BPs may be useful to prevent AI-induced bone loss, but further studies are needed to assess the effects of these treatments on bone quality.

4. Abstract for the Endocrine Society annual meeting; June 2006; Boston, MA:

Aromatase Inhibition Results in Loss of Bone Mineral Density, an Effect Prevented by Bisphosphonates. Wende M Kozlow¹, Khalid Mohammad¹, Ryan McKenna¹, Maryla Niewolna¹, Larry J Suva² and Theresa A Guise¹. ¹Department of Endocrinology and

Metabolism, University of Virginia, Charlottesville, Virginia, United States, 22908 and
²Department of Orthopaedic Surgery, University of Arkansas for Medical Sciences, Little Rock, Arkansas, United States, 72205.

Aromatase inhibitors (AIs), effective treatment for breast cancer, block the conversion of androstenedione and testosterone into estrone and estradiol. Anti-cancer therapies that suppress estrogen lead to increased bone resorption and the loss of bone mineral density (BMD). Cancer treatment-induced bone loss will likely become one of the most common skeletal complications of malignancy. We hypothesized that bisphosphonate (BP) treatment may prevent increased bone resorption from AI therapy, and impact bone formation.

To study the effect of AIs +/- BPs on bone remodeling, 4-week-old female nude mice were treated with letrozole (10 mcg/d), zoledronic acid (ZA) (5 mcg/kg twice weekly), letrozole + ZA, or control. BMD was assessed by DXA. Mice treated with letrozole alone had lower BMD compared to control ($p < 0.0001$; total body, spine, femur and tibia). Mice treated with ZA alone had higher BMD compared to control ($p < 0.0001$; total body, spine, femur and tibia). Mice treated with letrozole + ZA achieved the same BMD as mice treated with ZA alone at the spine and tibia, but had greater BMD than mice treated with ZA alone at the femur ($p < 0.0001$) and total body ($p < 0.0023$). MicroCT analysis of the proximal tibia showed no difference in bone volume (BV/TV), structural model index (SMI), or trabecular number (Tb.N), thickness (Tb.Th) or separation in mice treated with letrozole alone compared to control. Treatment with ZA (+/- letrozole) resulted in a significant increase in BV/TV, Tb.N and Tb.Th, and the SMI indicated that the bone structure was unusually solid. Dynamic bone histomorphometry of the lumbar spine demonstrated decreased bone formation and mineral apposition rates in mice treated with letrozole, ZA or the combination compared to control.

To assess the effect of letrozole on bone formation, calvaria obtained from 4-day-old mice were cultured for 7 days with media (BGJ) alone, positive control (insulin) or letrozole. Histomorphometry demonstrated that letrozole did not stimulate new bone formation and, when combined with insulin, did not inhibit new bone formation.

Letrozole decreased BMD in female nude mice, an effect prevented by concomitant treatment with ZA. MicroCT and histomorphometry indicate that the mechanism involves reduced bone remodeling with no direct effect of the treatment on bone formation. BPs may be useful to prevent AI-induced bone loss, but further studies are needed to assess the effects of these treatments on bone quality.

5. Abstract for the ASBMR annual meeting; September 2006; Philadelphia, PA:

Aromatase Inhibition Results in Gain of Bone Mineral Density in the Spine and Femur in Female Nude Mice. W. Kozlow¹, K. Mohammad¹, C. R. McKenna^{*1}, H. Walton^{*1}, M. Niewolna^{*1}, J. D. Dilley^{*2}, L. J. Suva², T. A. Guise¹. ¹Internal Medicine, University of Virginia, Charlottesville, VA, USA, ²Orthopaedic Surgery, University of Arkansas for Medical Sciences, Little Rock, AR, USA.

Aromatase inhibitors (AIs), effective treatment for breast cancer, block the conversion of androstenedione and testosterone into estrone and estradiol. Suppression of estrogen leads to increased bone resorption and the loss of bone mineral density (BMD). Therefore, cancer treatment-induced bone loss will likely become one of the most common skeletal complications of malignancy. We hypothesized that the AI letrozole would result in loss of BMD in female nude mice.

Four-week-old female nude mice were treated with letrozole (10 mcg/d) or control. BMD was assessed at baseline and every 2 weeks thereafter. Surprisingly, mice treated with letrozole had increased BMD compared to control at the mid femur ($p=0.0030$) and spine ($p=0.0002$). There was no difference in BMD between control and letrozole-treated mice at the total body, proximal femur or proximal tibia. MicroCT analysis of the femur after 4 weeks of treatment did not show a significant difference in trabecular bone volume (BV/TV), although a trend toward increased BV/TV in the letrozole-treated mice was observed ($p=0.0659$). However, 4 weeks of treatment with letrozole induced marked increases in skeletal microarchitecture. Significant increases in connectivity density ($p=0.0012$) and trabecular number ($p=0.0538$), thickness ($p=0.0280$) and separation ($p=0.0348$) were observed in the femurs of letrozole-treated mice, but not in the tibias. Interestingly, these data differ from published data using immunocompetent aromatase null mice, suggesting that differences in T-cell populations in nude mice may account for these distinct effects on bone density and architecture.

In a separate experiment, 4-week-old female nude mice were treated with the bisphosphonate zoledronic acid (ZA) (5 mcg/kg) twice weekly +/- letrozole (10 mcg/d) for 14 weeks. Mice treated with letrozole + ZA had increased BMD at the proximal femur ($p<0.0001$) and total body ($p=0.0003$) compared to ZA alone but, by histomorphometric analysis, bone formation rates were not increased. Similarly, letrozole did not stimulate or inhibit osteoblast number or bone formation in ex-vivo cultures of neonatal mouse calvariae.

In conclusion, letrozole increased BMD at the spine and mid femur and increased trabecular architecture in the femur. This effect, pronounced in the presence of bisphosphonate treatment, was not due to a direct effect of letrozole on bone formation. Unlike in intact immunocompetent mice, letrozole appears to have site-specific effects on the skeletons of nude mice.

6. Abstract for the Cancer and Bone Society's IV International Meeting on Cancer Induced Bone Disease; December 2006; San Antonio, TX

The aromatase inhibitor letrozole has site-specific effects on the skeletons of female nude mice. W Kozlow, K Mohammad, M. Niewolna, C.R. McKenna, H. Walton and T.A. Guise. Internal Medicine, University of Virginia, Charlottesville, VA, USA.

Aromatase inhibitors (AIs) block conversion of androstenedione and testosterone to estrone and estradiol and have emerged as superior to tamoxifen to treat breast cancer. Suppression of estrogen causes loss of bone mineral density (BMD). To study the effect of estrogen deficiency on BMD in mice, we performed 2 experiments. Four-week-

old female nude mice were randomized to ovariectomy (ovx) or sham surgery. BMD was measured at baseline and then every 2 weeks with Lunar PIXImus. At 10 weeks post-surgery, ovx mice had lower BMD than sham mice at the spine ($p<0.0001$) and proximal tibia ($p<0.0001$), and greater BMD than sham mice at the mid femur ($p<0.0001$). There was no difference in BMD at the total body ($p=0.1385$) or distal femur ($p=0.4306$). Next, 4-week-old female nude mice were treated with the AI letrozole (10 mcg/d) or control. BMD was assessed at baseline and every 2 weeks. Uterine weight/body weight was decreased in letrozole-treated mice compared to control ($p=0.0265$). Surprisingly, letrozole-treated mice had increased BMD compared to control at the mid femur ($p=0.0025$) and spine ($p<0.0001$). There was no difference in BMD at the total body, distal femur or proximal tibia. In bone marrow cultures, letrozole-treated mice exhibited a greater number of colony forming unit (CFU)-fibroblasts ($p<0.0001$), CFU-osteoblasts ($p<0.0001$) and TRAP-positive osteoclasts ($p=0.0076$) compared to control mice.

Estrogen depletion by different modalities had different effects on bone. Ovx decreased BMD at the spine and proximal tibia but increased BMD at the mid femur, where cortical bone predominates. In contrast, letrozole increased BMD at the spine and mid femur but had no effect on BMD at the other sites. Letrozole increased bone marrow fibroblast, osteoblast and osteoclast progenitor cells. In conclusion, letrozole has site-specific effects on the skeletons of nude mice as well as effects on multiple bone marrow progenitor cells.

7. AMBMR annual meeting; September 2007; Honolulu, HI

Ovariectomy Decreases Bone Mass in Young and Old Female Athymic Mice. W. Kozlow, K. Mohammad, C. R. McKenna, H. Walton, M. Niewolna, T. A. Guise Internal Medicine, University of Virginia, Charlottesville, VA, USA.

Ovariectomy (OVX) has been reported to have no effect on trabecular bone mass in female athymic (nude) mice because these mice lack T cells ⁽¹⁾. However, recent data has demonstrated trabecular, but not cortical, bone loss 4 weeks after OVX in 6-week-old female nude mice ⁽²⁾. The effect of OVX on bone mass in female nude mice may be related to mouse age at the time of surgery.

To determine the effect of mouse age (at the time of OVX) on bone mass, 4-week-old (young) and 16-week-old (old) female BALB-c nude mice were randomized to OVX or sham surgery (sham). Bone mineral density (BMD), as assessed by Lunar PIXImus, was assessed at baseline and every 2 weeks thereafter. At 20 weeks, the young OVX mice had decreased BMD at the total body ($p=0.0056$), spine ($p<0.0001$) and proximal tibia ($p<0.0001$) compared to the sham mice. Decreased BMD was noted as early as 2 weeks after OVX in the total body and proximal tibia, and by 4 weeks after OVX in the lumbar spine. Although there was no difference in BMD at the distal femur, BMD was surprisingly increased at the mid femur ($p<0.0001$) in the OVX mice compared to the sham mice. However, histomorphometry demonstrated no difference in trabecular bone volume at the distal femur or proximal tibia between the OVX mice and sham mice.

Twenty weeks after surgery, the old OVX mice had decreased BMD at the total body ($p=0.0048$), spine ($p<0.0001$), mid femur ($p=0.0409$) and distal femur ($p<0.0001$) as compared to the sham mice. Decreased BMD was noted as early as 2 weeks after OVX in the total body and distal femur, and by 4 weeks after OVX in the lumbar spine and mid femur. There was no difference in BMD at the proximal tibia.

At 20 weeks, differences between the OVX and sham mice were greater in the young mice versus the old mice: 3.4% versus 1.5% total body; 18.3% versus 9% spine; 9.2% versus 1.1% mid femur; 3.8% versus 6.1% distal femur; 20.1% versus 6.6% proximal tibia.

Bone marrow cultures from OVX mice exhibited a greater number of colony forming unit (CFU)-fibroblasts ($p<0.0001$ for young and old), CFU-osteoblasts ($p<0.0001$ young, $p=0.0001$ old) and TRAP-positive osteoclasts ($p=0.0005$ young) compared to sham mice.

These experiments show that OVX does have an effect on bone mass at multiple sites in female nude mice. OVX-induced decreases in bone mass were seen in both young and old female nude mice, but the differences between the OVX and sham mice were more profound in the younger mice as compared to the older mice. Bone marrow cultures revealed that the lower bone mass was associated with increased bone turnover.

⁽¹⁾ Cenci S et al. *J Clin Invest.* 2000;106: 1229-37.

⁽²⁾ Lee S-K et al. *J Bone Miner Res.* 2006; 21:1704-12.