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TITLE: Effect of a High Bone Turnover State Induced by Estrogen Deficiency on the
Development and Progression of Breast Cancer Bone Metastases

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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. Four-week-old and 16-week-old female Balbc/ICR Swiss athymic mice were treated with the AI letrozole (5 mg/kg/day) or control. Four-week-old female athymic mice treated with letrozole for 16 weeks had increased BMD compared to control at the total body ($p<0.0001$), mid femur ($P<0.0001$) and tibia (0.0002), but there was no difference in BMD at the spine or distal femur. Sixteen-week-old female athymic mice treated with letrozole for 26 weeks had higher BMD compared to control at the total body ($p<0.0001$), but there was no difference in BMD between the treatment groups at the spine, distal femur or tibia. Immunocompetent 4-week-old female C57B6 mice treated with letrozole for 26 weeks had decreased BMD at the total body ($p<0.0001$), spine ($p=0.0143$), femur ($p<0.0001$) and tibia ($p=0.0002$) compared to control. The AI letrozole has variable effects on BMD in young versus old athymic mice. Furthermore, there are differing effects of AI therapy on BMD in athymic versus immunocompetent mice.					
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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.

In order to perform bone metastasis experiments, our mouse model of estrogen-deficiency-induced increased bone resorption uses female BALBc/ICR Swiss athymic mice. Our previously reported data showed variable effects on bone mineral density (BMD) in this mouse model after ovariectomy (OVX) and treatment with the aromatase inhibitor (AI) letrozole. Therefore, before continuing with metastasis experiments, we needed to optimize our mouse model of estrogen deficiency-induced increased bone turnover. We performed experiments to determine if the age of the mice had an effect on treatment outcomes. 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 used 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 (1,2), letrozole may have the same effect. The immunocompetent mouse strain that we chose to use was C57B6. This strain was chosen as it has been used in our laboratory previously and has shown increased bone turnover and decreased BMD after OVX (unpublished data).

Body

Task 1: Four-week-old female BALBc/ICR Swiss athymic mice randomized to letrozole or control.

Procedures for task 1: In order to evaluate the effect of letrozole on young versus old female nude mice, twenty 4-week-old (young) BALBc/ICR Swiss athymic 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 5 mg/kg/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.

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.

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. 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.

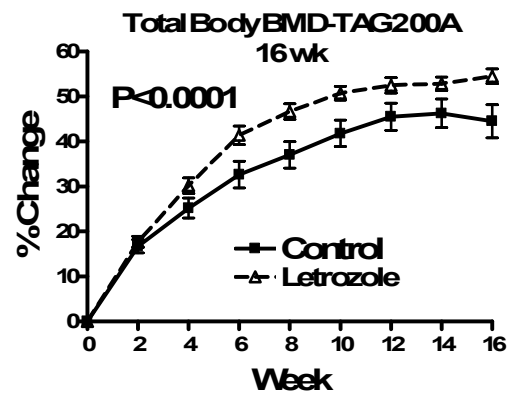
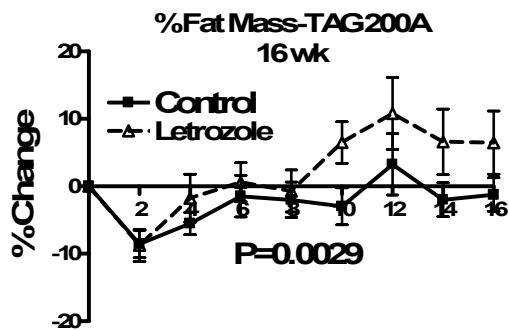
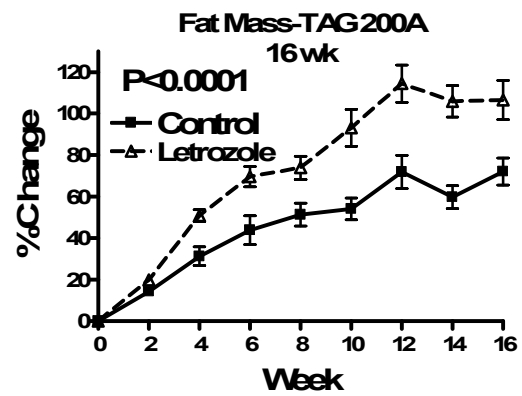
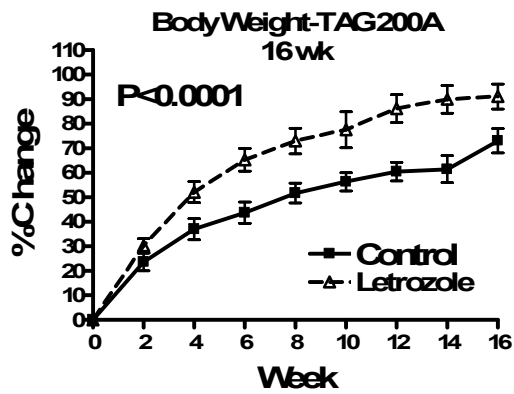


Figure 1. Body composition and BMD in 4-week-old female nude mice after 16 weeks of treatment with letrozole (5 mg/kg/day) or control. P values calculated using two-way ANOVA

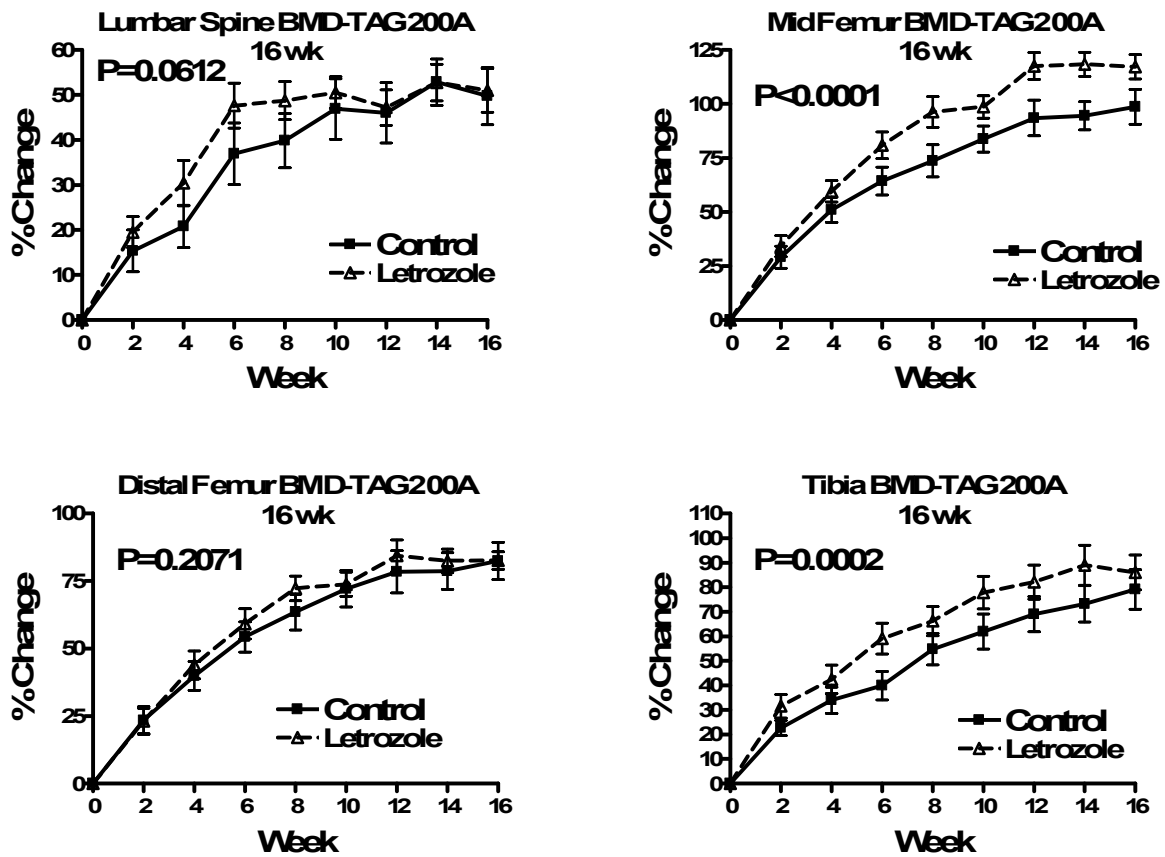


Figure 2. BMD in 4-week-old female nude mice after 16 weeks of treatment with letrozole (5 mg/kg/day) or control. P values calculated using two-way ANOVA.

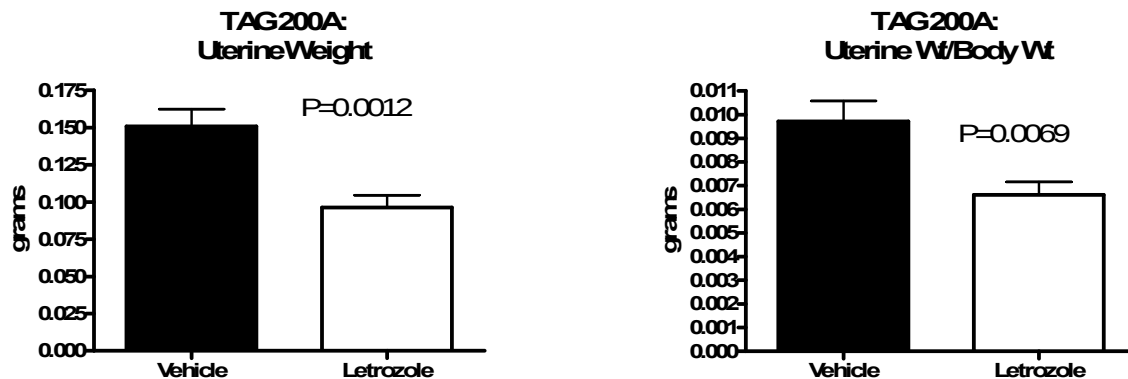


Figure 3. Uterine weight and uterine weight/body weight in 4-week-old female nude mice after 16 weeks of treatment with letrozole (5 mg/kg/day) or control. P values calculated using two-way ANOVA.



Figure 4. CFU and osteoclast formation assays using femurs and tibias (3 mice/group) from female nude mice after 16 weeks of treatment with letrozole or control. P values calculated using two-way ANOVA.

Results for task 1:

- 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 1**).
- 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. BMD did not differ between the treatment groups at the distal femur (**figure 2**).
- 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 3**).
- After 16 weeks of treatment, bone marrow cultures showed increased CFU-osteoblasts ($p = 0.0002$) and TRAP+ osteoclasts ($p = 0.0398$) in letrozole-treated mice compared to controls (**figure 4**).

Task 2: Sixteen-week-old female BALBc/ICR Swiss athymic mice randomized to letrozole or control.

Procedures for task 2: In order to evaluate the effect of letrozole on young versus old female nude mice, twenty 16-week-old (old) BALBc/ICR Swiss athymic 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 16-week-old female nude mice were euthanized after 26 weeks of treatment.

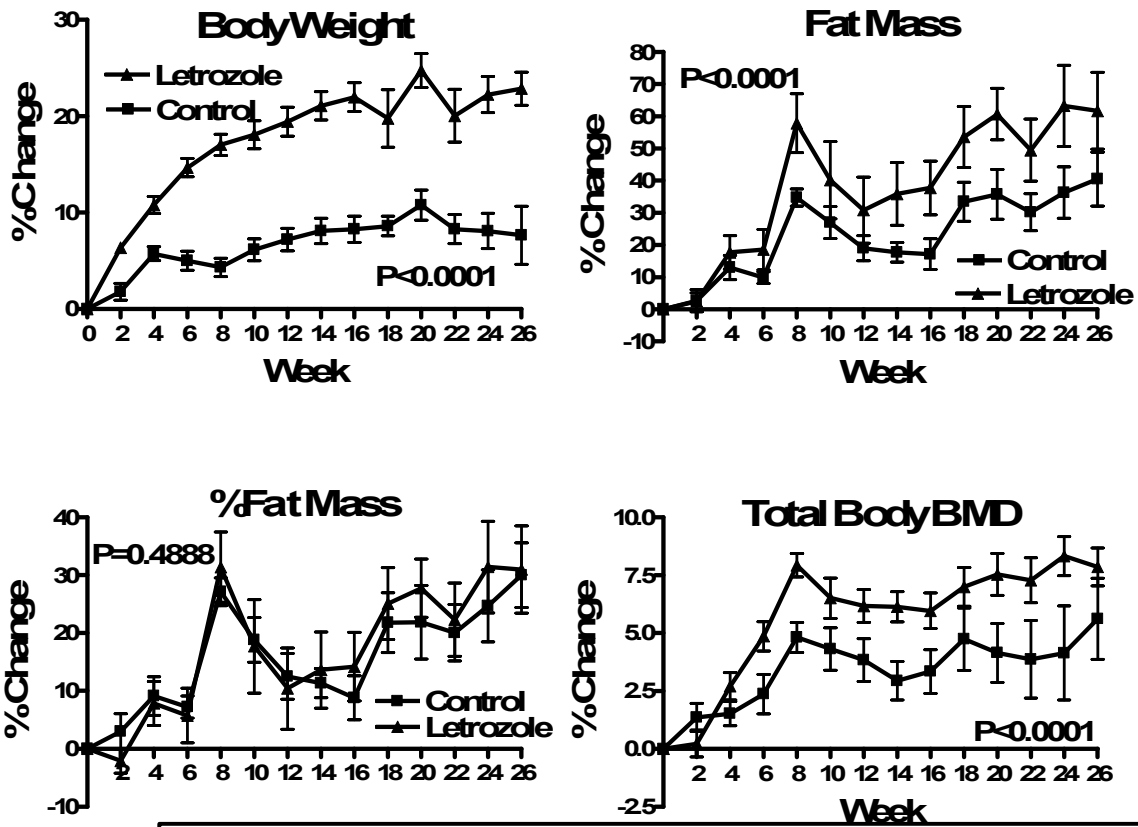


Figure 5. Body composition and BMD in 16-week-old female nude mice after 26 weeks of treatment with letrozole (5 mg/kg/day) or control. P values calculated using two-way ANOVA.

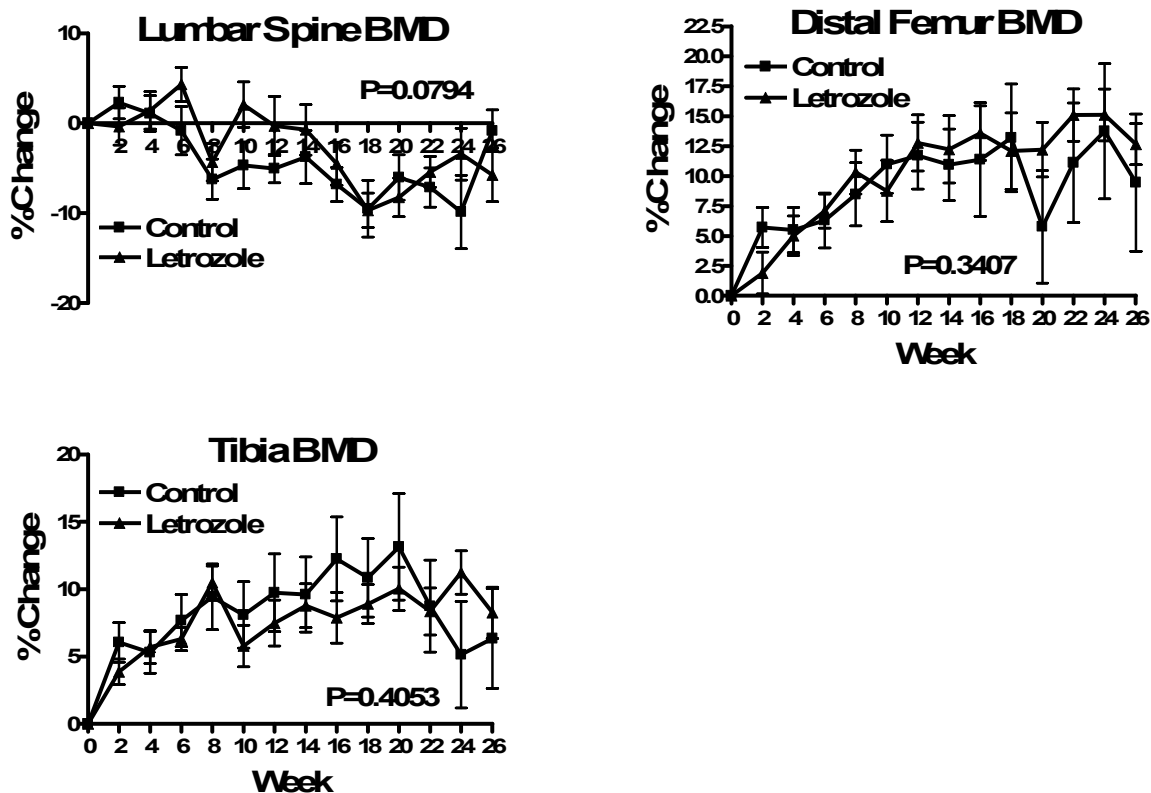


Figure 6. BMD in 16-week-old female nude mice after 26 weeks of treatment with letrozole (5 mg/kg/day) or control. P values calculated using two-way ANOVA

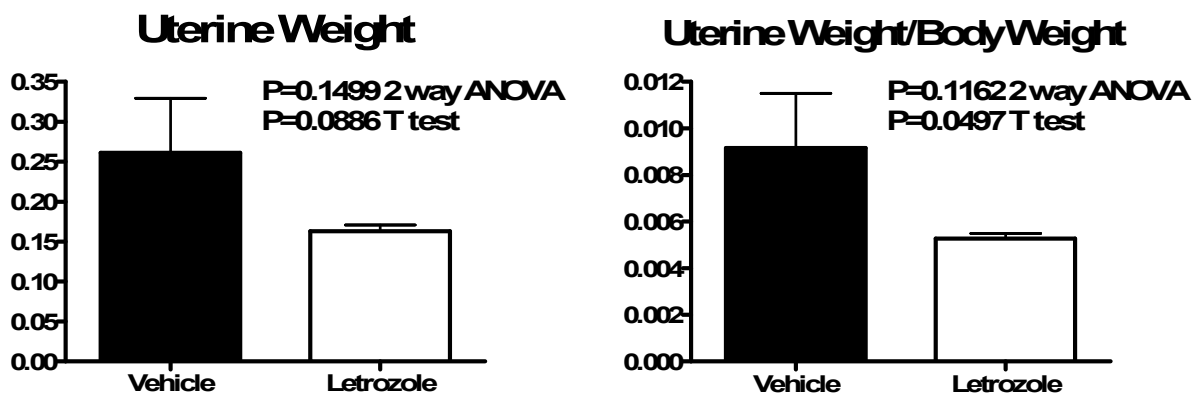


Figure 7. Uterine weight and uterine weight/body weight in 16-week-old female nude mice after 26 weeks of treatment with letrozole (5 mg/kg/day) or control. P values calculated using two-way ANOVA and Student's T-test.

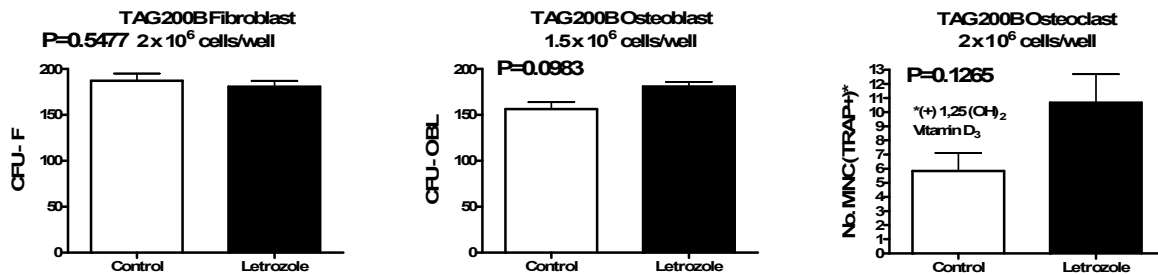


Figure 8. CFU and osteoclast formation assays using femurs and tibias (3 mice/group) from female nude mice after 26 weeks of treatment with letrozole or control. P values calculated using two-way ANOVA.

Results for task 2:

- After 26 weeks of treatment, letrozole-treated mice had significantly increased body weight ($p < 0.0001$), fat mass ($p < 0.0001$) and total body BMD ($p < 0.0001$) compared to control mice (**figure 5**).
- After 26 weeks of treatment, there was no significant difference in BMD between letrozole-treated and control mice at the spine, femur or tibia (**figure 6**).
- After 26 weeks of treatment, there was no significant difference in uterine weight or uterine weight/body weight between letrozole-treated and control mice (**figure 7**).
- After 26 weeks of treatment, bone marrow cultures showed no difference in CFU-osteoblasts, CFU-fibroblasts or TRAP+ osteoclasts between letrozole-treated and control mice (**figure 8**).

Task 3: Four-week-old female C57B6 immunocompetent mice randomized to OVX, sham surgery, letrozole or control.

Procedures for task 3: Previous experiments in the Guise laboratory showed that C57B6 mice lost BMD after OVX (unpublished data). In order to evaluate the effect of letrozole on BMD in immunocompetent mice, 64 4-week-old C57B6 mice were randomized to OVX, sham surgery, treatment with letrozole (5 mg/kg/day) or treatment with control. BMD was measured at baseline and then every 2 weeks. The 4-week-old female C57B6 mice were euthanized 26 weeks after surgery, or 26 weeks after initiation of treatment.

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.

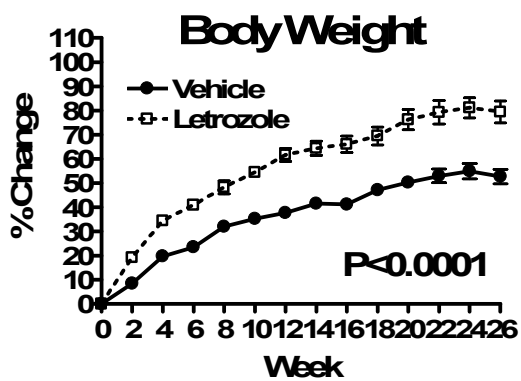
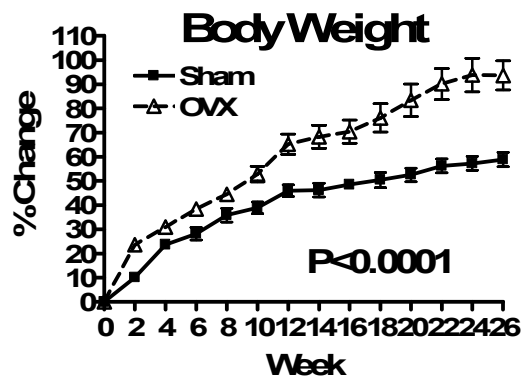
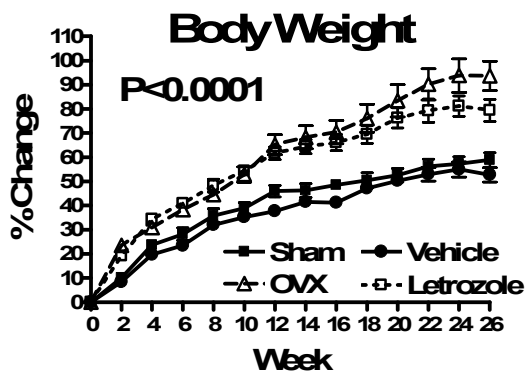


Figure 9. Body weight in 4-wk-old C57B6 mice after OVX, sham surgery, treatment with letrozole or control. P-values calculated using two-way ANOVA

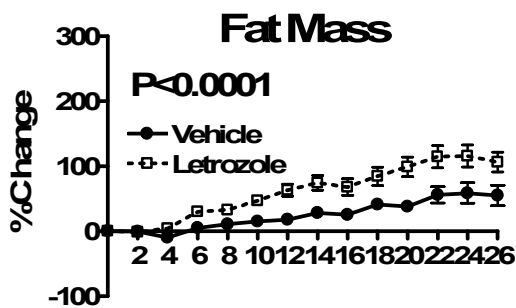
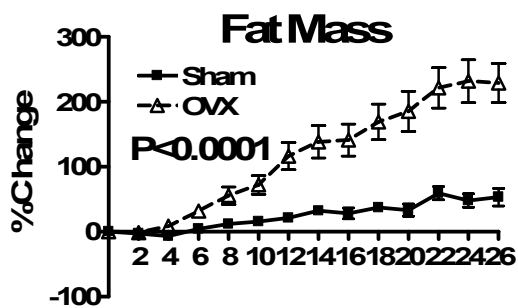
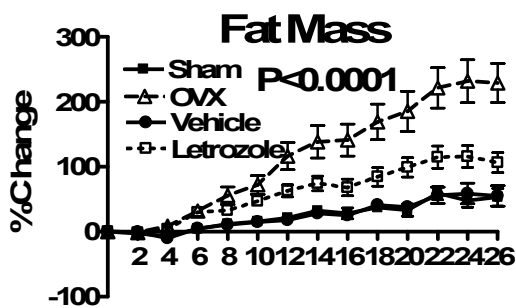


Figure 10. Fat mass in 4-wk-old C57B6 mice after OVX, sham surgery, treatment with letrozole or control. P-values calculated using two-way ANOVA

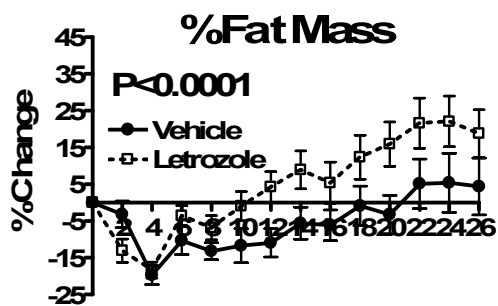
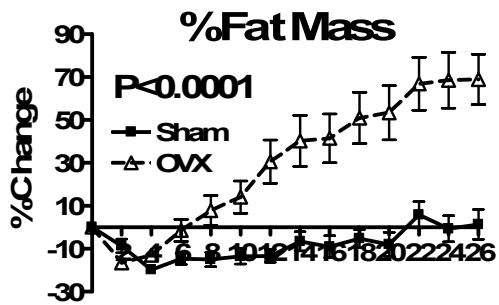
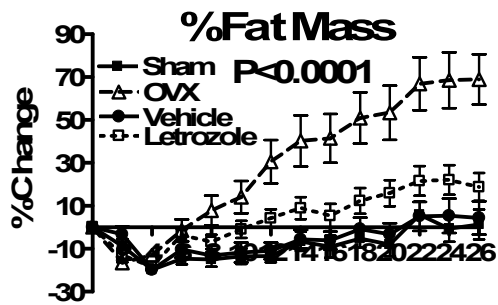


Figure 11. % Fat mass in 4-wk-old C57B6 mice after OVX, sham surgery, treatment with letrozole or control. P-values calculated using two-way ANOVA

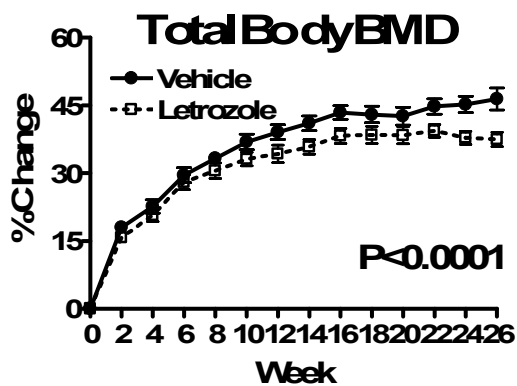
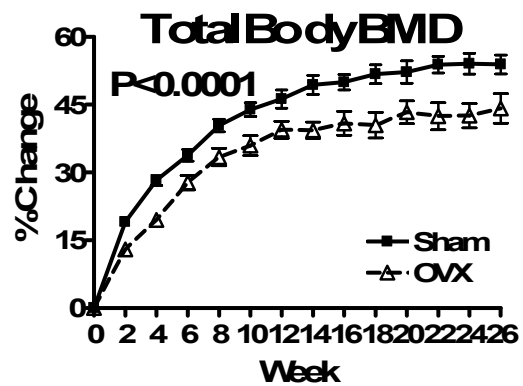
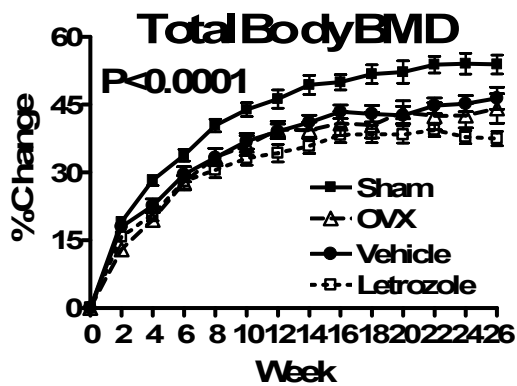


Figure 12. Total Body BMD in 4-wk-old C57/B6 mice after OVX, sham surgery, treatment with letrozole or control. P-values calculated using two-way ANOVA.

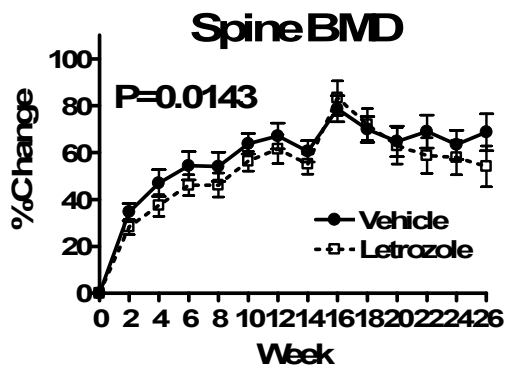
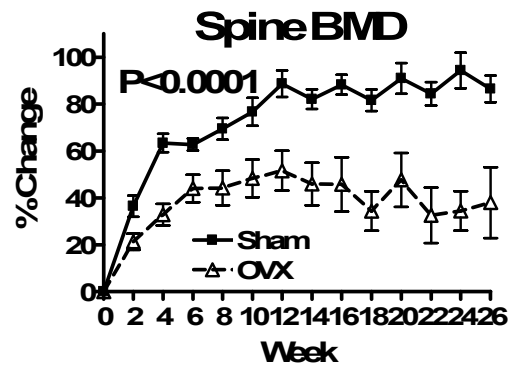
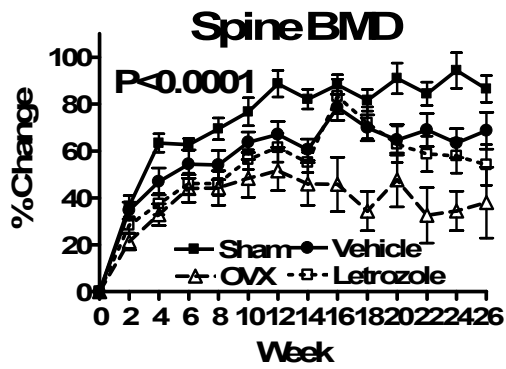


Figure 13. Spine BMD in 4-wk-old C57B6 mice after OVX, sham surgery, treatment with letrozole or control. P-values calculated using two-way ANOVA.

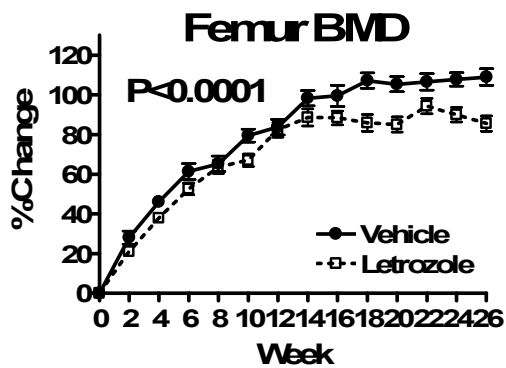
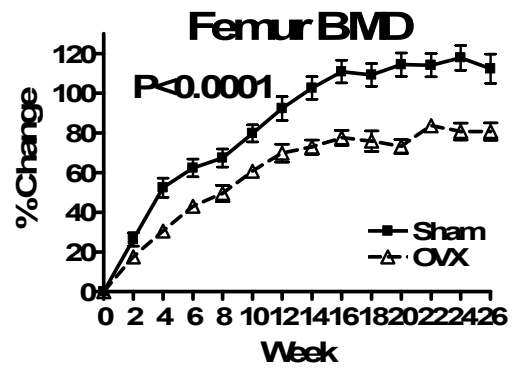
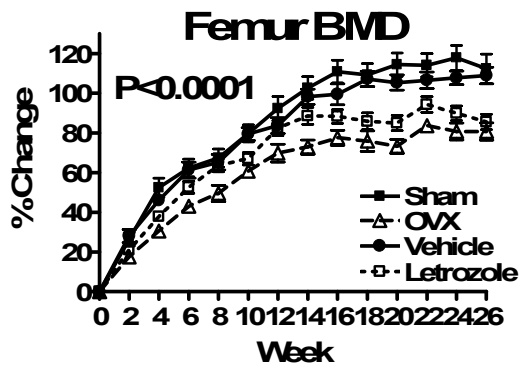


Figure 14. Femur BMD in 4-wk-old C57B6 mice after OVX, sham surgery, treatment with letrozole or control. P-values calculated using two-way ANOVA

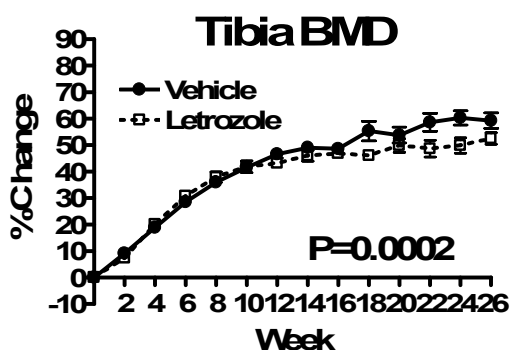
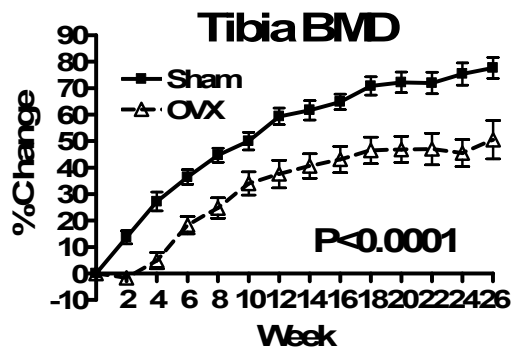
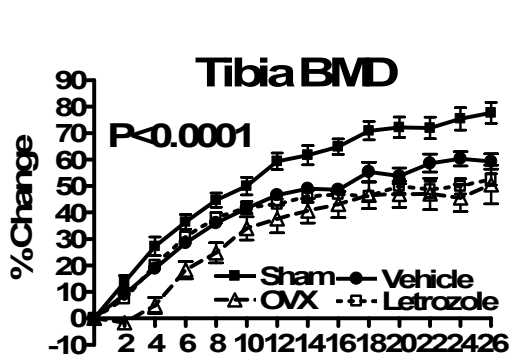


Figure 15. Tibia BMD in 4-wk-old C57B6 mice after OVX, sham surgery, treatment with letrozole or control. P-values calculated using two-way ANOVA

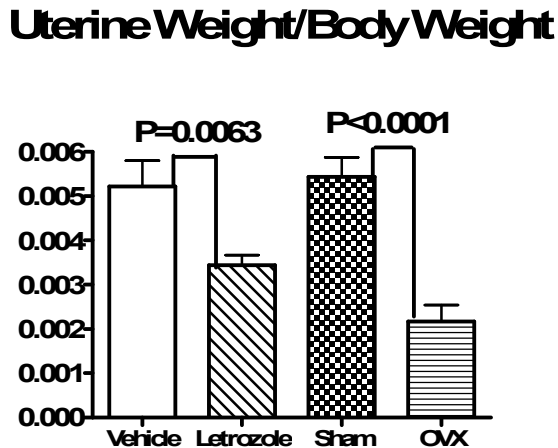
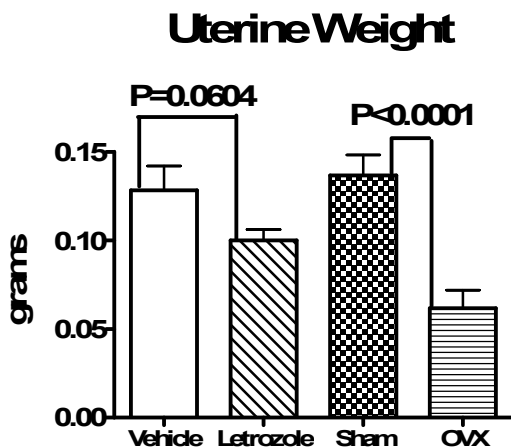


Figure 16. Uterine weight and uterine weight/body weight in 4-week-old C57B6 mice after OVX, sham surgery, letrozole or control. P values calculated using two-way ANOVA

Osteoclasts 2x10⁶ cells/well

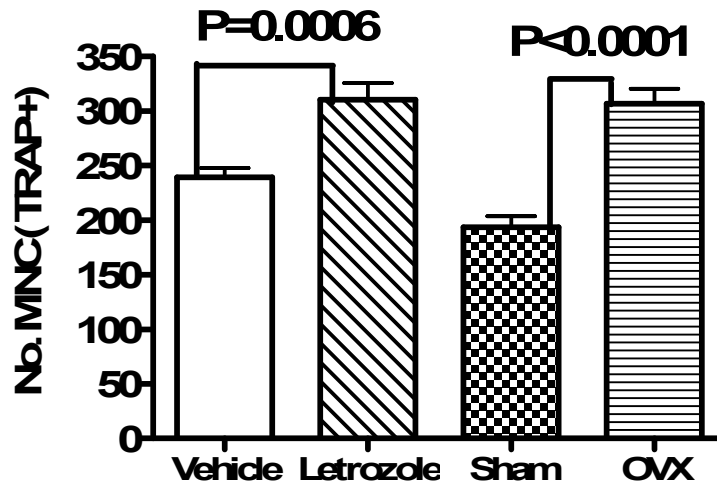


Figure 17. Osteoclast formation assay using femurs and tibias (3 mice/group) from C57B6 mice after OVX, sham surgery, letrozole or control. P values calculated using Student's T-test.

Results for task 3:

- Increased body weight (**figure 9**), fat mass (**figure 10**) and % fat mass (**figure 11**) in OVX and letrozole-treated mice as compared to their respective controls.
- Decreased total body BMD (**figure 12**), spine BMD (**figure 13**), femur BMD (**figure 14**) and tibia BMD (**figure 15**) in OVX and letrozole-treated mice as compared to their respective controls.
- Decreased uterine weight and uterine weight/body weight (**figure 16**) in OVX and letrozole-treated mice as compared to their respective controls. There was a further decline in uterine weight ($p=0.0033$) and uterine weight/body weight ($p=0.0061$) in OVX mice as compared to letrozole-treated mice.
- Increased TRAP+ osteoclasts (**figure 17**) in OVX and letrozole-treated mice as compared to their respective controls.

Key Research Accomplishments

- AI therapy in 4-week-old athymic mice results in increased bone turnover and site-specific (total body, mid femur, proximal tibia) increases in BMD.

- AI therapy in 16-week-old athymic mice does not result in increased bone turnover, and increases in BMD was limited to the total body.
- AI therapy in an immunocompetent mouse model results in increased bone turnover and decreased BMD at all sites (total body, spine, femur and tibia).
- Loss of BMD and estrogen suppression appear to be more profound in OVX mice, as compared to AI-treated mice, in an immunocompetent mouse model.

Reportable Outcomes

Oral Presentation, 29th Annual Meeting of the American Society for Bone and Mineral Research, Honolulu, Hawaii, 2007.

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.

Conclusion

Tamoxifen therapy is bone-sparing, but its use in breast cancer is being rapidly superseded by AIs. Unlike tamoxifen, AI therapy for breast cancer results in high bone turnover. This leads to osteoporosis and fractures. Interestingly, the use of AIs in our athymic mouse model has yielded results that differed from what we expected. In 4-week-old (young) athymic mice, AI therapy resulted in increased BMD at the total body, mid femur and proximal tibia compared to the control mice. This data is similar to the data from our previously reported experiments. Despite these increases in BMD, bone turnover was increased in the 4-week-old letrozole-treated mice as evidenced by the increase in CFU-osteoblasts and TRAP+ osteoclasts. Furthermore, reduction in uterine weight and uterine weight/body weight in the 4-week-old letrozole-treated mice indicates that there was suppression of estrogen. 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.

In 16-week-old (old) athymic mice, we did see an increase in body weight, fat mass and total body BMD in the letrozole-treated mice as compared to control. However, there was no difference in BMD in letrozole-treated mice, compared to control, at the spine, femur or tibia. Furthermore, there was no difference in uterine weight or uterine weight/body weight in the 16-week-old letrozole-treated mice compared to the control mice. CFU-fibroblasts, CFU-osteoblasts and TRAP+ osteoclasts also did not differ between the letrozole-treated and control mice. Therefore, the timing of the initiation of AI therapy does have a profound effect on treatment outcomes in this athymic mouse model, where the younger mice experience a reduction in uterine weight, an increase in bone turnover and an increase in BMD at multiple sites, but the older mice do not.

Given the results of these experiments, it was important to establish the effect of AI therapy on BMD in an immunocompetent mouse model. We chose to use C57B6 mice, as these mice had previously demonstrated a decrease in BMD after OVX in the Guise laboratory. We directly compared the standard method of inducing estrogen deficiency, OVX, with AI therapy in this mouse model. Interestingly, we did see a profound reduction in BMD at all sites when we used letrozole in this immunocompetent mouse model. There was also an increase in TRAP+ osteoclasts in the letrozole-treated mice. We also appreciated an increase in body weight and fat mass, and a decline in uterine weight and uterine weight/body weight in the letrozole-treated mice. Interestingly, mice treated with

OVX had more profound suppression of uterine weight and uterine weight/body weight than the letrozole-treated mice. Furthermore, although all differences were statistically significant, it did appear that there were more profound differences in BMD between the OVX and sham C57B6 mice as compared to the letrozole-treated and control-treated C57B6 mice. Therefore, T cells may play a role in responsiveness to AI therapy.

There is also the question of whether or not there would be a further increase in bone turnover, and a further loss of BMD, in mice treated with both OVX *and* letrozole as opposed to OVX or letrozole alone. Future experiments to further elucidate the effects of estrogen deficiency on BMD and bone turnover in a mouse model will evaluate the effect of OVX *plus* letrozole-treatment on BMD and bone turnover in both an immunocompetent and an athymic mouse model. We also plan to evaluate the effects of AI therapy in the BALBc/ICR Swiss immunocompetent mouse (parent strain of the athymic mice we have been using). As previously shown with OVX (1), AI-induced bone loss may vary among inbred strains of mice.

References

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 2. Li CY, Schaffler MB, Wolde-Semait HT, Hernandez CJ, Jepsen KJ. Genetic background influences cortical bone response to ovariectomy. *Journal of Bone & Mineral Research* 2005; 20(12):2150-8.
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