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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. Previous data showed variable BMD response to AI therapy in the female BALBc/ICR swiss athymic mouse. Therefore, to further define the BMD response to AI therapy in a mouse model, four-week-old female BALBc/ICR Swiss immunocompetent mice were treated with ovariectomy (OVX), sham surgery, the AI letrozole (5 mg/kg/day) or control. Ten weeks after surgery or initiation of AI/control, there was increased body weight ($p<0.0001$) and fat mass ($p<0.0001$) in OVX and letrozole-treated mice as compared to their respective controls. OVX mice had decreased BMD at the total body ($p=0.0089$), spine ($p<0.0001$), femur ($p=0.0573$) and tibia ($p=0.0045$) compared to sham mice. Letrozole-treated mice did not differ from control mice at the total body or tibia, but had decreased BMD at the spine ($p=0.0018$) and increased BMD at the femur ($p=0.0003$) compared to control. There was a significant decline in uterine weight after OVX compared to sham surgery ($p<0.0001$), but there was no difference in uterine weight between the letrozole and control mice. Therefore, the anticipated decline in estrogen levels with AI therapy was not seen in the BALBc/ICR Swiss immunocompetent mouse strain.

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Aromatase inhibitors, letrozole, breast cancer, ovariectomy, metastasis, bisphosphonates, mouse model

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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 T cell defect in the athymic mouse may also complicate the skeletal response to letrozole. Therefore, we used letrozole in two immunocompetent mouse strains, C57B6 and BALBc/ICR Swiss (the parent strain of our athymic mouse) in order to clarify the skeletal response to letrozole in mice. We also needed to determine whether there was an additional BMD response to OVX plus letrozole versus OVX alone.

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.

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.

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.

Procedures for all tasks:

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.

Treatment of mice with an AI. Mice were treated with letrozole (initially 10 mcg/day/sc then 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.

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.

Bone metastases model. Tumor cells were trypsinized and resuspended in PBS to a final concentration of 10^6 cells/100μL 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 (1-4). 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 (1,3-4). 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 (1,3-4), 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.

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.

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 (αMEM), 50 ug/ml ascorbic acid and 10mM ß-glycerophosphate to support mineralization. Cells were plated (1 x 10^6cells/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 αMEM, 50 ug/ml ascorbic acid and 10mM ß-glycerophosphate. Cells were plated (2.5 x 10^6cells/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 αMEM and 10nM 1,25 $(OH)_2$ vitamin D_3. Cells were then plated (2 x 10^6cells/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.

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 ×200 magnification.

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.

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.

Procedure for Tasks 1 & 2: 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.

Results for tasks 1 & 2 – part 1 (data and figures are in the 2006 report):

- Eight weeks after surgery there was no difference in BMD in OVX mice compared to sham mice at any site.
- Histomorphometry showed no difference in trabecular bone volume (TBV) in OVX mice compared to sham mice at the femur or tibia.
- In the mice inoculated with MDA-MB-231 via intra-cardiac injection, there was no difference in total body x-ray lesion area or tibia plus femur x-ray lesion area between the OVX and sham mice.
- There was no difference in survival between the OVX and sham mice after intra-cardiac injection with MDA-MB-231.

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 BALBc 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 (5). 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 (6). 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. The next experiment was 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.

Results for tasks 1 & 2 – part 2a (data and figures are in the 2007 report):

- At 28 weeks, OVX mice had significantly increased body weight, fat mass and % fat mass and significantly decreased total body, spine and tibia BMD compared to the sham mice. There was no difference in BMD between the OVX and sham mice at the distal femur.
- Histomorphometry revealed that there was no difference in TBV between the OVX and sham mice at the proximal tibia or distal femur.
- Micro-CT revealed that trabecular bone in the proximal tibia and distal femur did not differ between the OVX and sham mice.
- Bone marrow cultures from OVX mice exhibited a greater number of CFU-fibroblasts, CFU-osteoblasts and TRAP-positive osteoclasts compared to sham mice.
- Total body X-ray lesion area was greater in the sham surgery mice compared to the OVX mice ($P<0.0001$).

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 BALBc 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, but no difference at the distal femur. Interestingly, histomorphometry and micro-CT data did not show decreased TBV at the distal femur and proximal tibia in the OVX mice compared to the sham surgery mice. 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.

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 and figures are in the 2007 report):

- At 20 weeks, OVX mice had significantly increased body weight, fat mass and % fat mass and significantly decreased total body, spine and femur BMD compared to sham mice. There was no difference in BMD between the OVX and sham mice at the proximal tibia.
- Micro-CT revealed that trabecular bone in the proximal tibia and distal femur did not differ between the OVX and sham mice.
- Histomorphometry revealed that there was no difference in TBV between the OVX and sham mice at the proximal tibia or distal femur.
- Bone marrow cultures from OVX mice exhibited a greater number of CFU-fibroblasts and CFU-osteoblasts compared to sham mice. TRAP+ osteoclasts could not be assessed due a technical problem with the assay.
- Total body X-ray lesion area was greater in the sham surgery mice compared to the OVX mice.

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 and distal femur 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, yet the 16-week-old mice did lose BMD at this site. 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 and histomorphometry 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. 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. 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.

Procedures for tasks 3-6 (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.

Results for tasks 3-6 - Part 1 (data and figures in 2006 report):

- After 13 weeks of treatment, mice treated with letrozole alone had decreased BMD at the total body, spine, proximal femur and tibia compared to control.
- Mice treated with ZA alone had higher BMD compared to control at the 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 proximal femur and total body.
- Histomorphometry demonstrated that 1) mice treated with letrozole alone had the same TBV as mice treated with control at the femur, tibia and lumbar spine; 2) mice treated with ZA (+/- letrozole) had increased TBV compared to letrozole alone at the proximal femur and tibia and 3) mice treated with ZA alone had increased TBV in the lumbar spine compared to both letrozole and letrozole + ZA.

- 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.
- Dynamic bone histomorphometry of the lumbar spine demonstrated decreased BFR and MAR in mice treated with letrozole, ZA or the combination compared to control.
- 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.
- 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.

Conclusions, potential problems and alternative strategies for tasks 3-6 (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.

Procedures for tasks 3-6 (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 3-6 -Part 2 (data and figures are in the 2006 report):

- Mice treated with letrozole accrued less BMD at the proximal femur but achieved the same BMD as control mice at the total body, spine and tibia.
- 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.

Conclusions, potential problems and alternative strategies for tasks 3-6 (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 3-6 (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 3-6 -Part 3 (data and figures are in the 2006 report):

- After 8 weeks of treatment, there was no difference in BMD between the letrozole and control mice at any site.
- 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.

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 3-6 (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 3-6 -Part 4 (data and figures are in the 2007 report):

- PIXImus Data:
  - After 4 weeks of treatment, there was no difference in BMD between the letrozole and control mice at any site.
  - After 23 weeks of treatment, there was no difference in total body, femur or tibia BMD between letrozole and control mice, but letrozole-treated mice had increased BMD at the lumbar spine compared to control mice.
  - After 33 weeks of treatment, there were no significant differences in total body, femur or tibia BMD between letrozole-treated and control mice, but letrozole-treated mice had increased BMD at the lumbar spine compared to control mice.
- Micro-CT Data:

- After 4 weeks of treatment, no difference in TBV in the femur or tibia. However, letrozole induced marked increases in skeletal microarchitecture of the femurs, including ConnD, Tb.N, Tb.Th and Tb.Sp.
- After 23 weeks of treatment, significant increase in TBV in both the femur and tibia of letrozole-treated mice compared to control.
- After 33 weeks of treatment, no significant difference in TBV in either the femur or tibia between the letrozole or control treatment groups.

➢ Uterine Weight:

- After 4 weeks of treatment, no difference in uterine weight or uterine weight/body weight between the letrozole-treated and control mice.
- After 23 weeks of treatment, letrozole-treated mice had significantly lower uterine weight and uterine weight/body weight compared to control mice.
- After 33 weeks of treatment, letrozole-treated mice had significantly lower uterine weight/body weight compared to the control mice.

➢ Bone Marrow Cultures:

- After 23 weeks of treatment, bone marrow cultures from tibias and femurs of letrozole-treated mice showed significantly increased CFU-fibroblasts, CFU-osteoblasts and TRAP-positive osteoclasts compared to control.
- After 33 weeks of treatment, bone marrow cultures from tibias and femurs of letrozole-treated mice showed significantly increased CFU-fibroblasts, CFU-osteoblasts and TRAP-positive osteoclasts compared to control.

Conclusions, potential problems and alternative strategies for tasks 3-6 (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, femur or tibia, but did show increased BMD in the lumbar spine in letrozole-treated mice compared to the control mice. Micro-CT data did not show increased TBV in the femurs 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. 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.

To determine if the dose of letrozole was not sufficient to induce complete estrogen deficiency in the female nude mouse, we planned 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).

Procedures for tasks 3-6 (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.

Results for tasks 3-6 – part 5 (data and figures are in the 2008 report):

- After 16 weeks of treatment, letrozole-treated mice had significantly increased body weight, fat mass, % fat mass and total body BMD compared to control mice.
- After 16 weeks of treatment, letrozole-treated mice had significantly increased BMD at the proximal tibia and there was a trend toward increased BMD at the spine compared to control mice. BMD did not differ between the treatment groups at the distal femur.
- After 16 weeks of treatment, letrozole-treated mice had significantly decreased uterine weight and uterine weight/body weight compared to the control mice.
- After 16 weeks of treatment, bone marrow cultures showed increased CFU-osteoblasts and TRAP+ osteoclasts in letrozole-treated mice compared to controls.

Conclusions, potential problems and alternative strategies for tasks 3-6 (Part 5). This experiment showed increased BMD at the total body and proximal tibia in the letrozole-treated mice compared to the control mice. This data is similar to the data from the previous experiment which used the lower dose of letrozole. Using the lower dose of letrozole, 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 the previous experiment, 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. Distal femur BMD was not different between treatment groups in the previous experiment at any time point, and was not different between treatment groups in this current experiment. Proximal tibia BMD was not different between treatment groups in the previous experiment at any 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 uterine weight/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 the previous experiment 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.

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.

Procedures for tasks 3-6 (Part 6): 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.

Results for tasks 3-6 (Part 6) (data and figures are in the 2008 report):

- After 26 weeks of treatment, letrozole-treated mice had significantly increased body weight, fat mass and total body BMD compared to control mice.
- After 26 weeks of treatment, there was no significant difference in BMD between letrozole-treated and control mice at the spine, femur or tibia.
- After 26 weeks of treatment, there was no significant difference in uterine weight or uterine weight/body weight between letrozole-treated and control mice.
- 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.

Conclusions, potential problems and alternative strategies for tasks 3-6 (Part 6). As with the 4-week-old mice, the 16-week-old nude mice had increased body weight, fat mass and total body BMD. Unlike the 4-week-old mice, there was no difference in BMD at any other site, and there was no evidence of increased bone turnover with the bone marrow culture assays. There was also no decrease in uterine weight or uterine weight/body weight. Histomorphometry and micro-CT data are still pending for this experiment.

The genetic heterogeneity of the BALBc/ICR Swiss 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 needed to assess the BMD response to letrozole in an immunocompetent mouse strain. 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 chose was C57B6 because it has been previously used in the Guise laboratory and had shown increased bone turnover and decreased BMD after OVX.

Procedures for tasks 3-6 (Part 7): 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 initiation of treatment.

Results for tasks 3-6 – part 7 (data and figures are in the 2008 report):

- Increased body weight, fat mass and % fat mass in OVX and letrozole-treated mice as compared to their respective controls.
- Decreased total body BMD, spine BMD, femur BMD and tibia BMD in OVX and letrozole-treated mice as compared to their respective controls.

- Decreased uterine weight and uterine weight/body weight in OVX and letrozole-treated mice as compared to their respective controls. There was also a significant difference in uterine weight and uterine weight/body weight in OVX mice as compared to letrozole-treated mice.
- Increased TRAP+ osteoclasts in OVX and letrozole-treated mice as compared to their respective controls.

Conclusions, potential problems and alternative strategies for tasks 3-6 (Part 7).
In this immunocompetent mouse model, we did see the expected decline in BMD and increase in bone turnover with letrozole treatment. Although the changes in BMD and in bone turnover were similar between the OVX and letrozole mice, the reduction in uterine weight was more profound with OVX than with letrozole.

We established that the effect of letrozole on BMD and bone turnover in a mouse model may be dependent on T-cells as we observed the expected decline in BMD and increase in bone resorption that we anticipated by using the immunocompetent C57B6 mouse. We needed to ascertain if there was mouse strain factor as well. Therefore, we needed to repeat this experiment using the immunocompetent BALBc/ICR Swiss parent strain of our nude mouse model.

Procedures for tasks 3-6 – part 8: In order to evaluate the effect of estrogen deficiency on BMD and bone turnover in BALBc/ICR Swiss immunocompetent mice, which is the parent strain of the athymic mice we have been using in our mouse model of breast cancer bone metastasis, 64 4-week-old BALBc/ICR Swiss 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 mice were euthanized 28 weeks after surgery, or 28 weeks after initiation of treatment.

Results for tasks 3-6 – part 8:

- Ten weeks after surgery or initiation of AI/control, there was increased body weight **(figure 1)** and fat mass **(figure 2)** in OVX and letrozole-treated mice as compared to their respective controls. Letrozole-treated mice also had increased % fat mass **(figure 3)** compared to control, but % fat mass in OVX mice did not significantly differ from that in sham mice.
- Ten weeks after surgery or initiation of AI/control, there was decreased total body BMD **(figure 4)** in OVX mice compared to sham mice, but no significant difference in total body BMD between letrozole-treated and control mice.
- Ten weeks after surgery or initiation of AI/control, there was decreased spine BMD **(figure 5)** in OVX and letrozole-treated mice compared to their respective controls.
- Ten weeks after surgery or initiation of AI/control, there was decreased femur BMD **(figure 6)** in OVX mice compared to sham mice, but there was an increase in femur BMD in letrozole-treated mice compared to control.

- Ten weeks after surgery or initiation of AI/control, there was decreased tibia BMD **(figure 7)** in OVX mice compared to sham mice, but no significant difference in tibia BMD between letrozole-treated and control mice.
- Twenty-eight weeks after surgery or initiation of AI/control, uterine weight and uterine weight/body weight **(figure 8)** was significantly decreased in OVX mice compared to sham surgery. However, there was no significant difference in uterine weight or uterine weight/body weight in letrozole-treated mice compared to control.

Conclusions, potential problems and alternative strategies for tasks 3-6 (Part 8).
The 10-week data for BMD response to estrogen deprivation has been submitted for this report. The remainder of the BMD data is pending, as is the histomorphometry, micro-CT and bone marrow culture data. At this point, it does appear that the immunocompetent BALBc/ICR Swiss athymic mouse loses BMD at all sites after OVX, but has a more variable response to letrozole. After 10 weeks of treatment, letrozole has resulted in an increase in BMD at the femur, a decrease in BMD at the spine, and no change in BMD at the total body or tibia. These variable site specific effects of letrozole have been seen with the BALBc/ICR Swiss nude mouse as well. Unfortunately, there was no decline in uterine weight with letrozole treatment. Therefore, this mouse strain does not have a uniform or predictable response to AI therapy.

As in previous experiments, we needed to ascertain if mouse age at the time of estrogen deprivation has an effect on BMD and bone turnover. Therefore, in order to publish our data on the effect of OVX in the athymic mouse, we needed to determine the effect of OVX on BMD and bone turnover in the 16-week-old (old) female BALBc/ICR Swiss immunocompetent mouse.

Procedures for tasks 3-6 – part 9: In order to evaluate the effect of OVX on young versus old female BALBc/ICR Swiss immunocompetent mice, 32 16-week-old (old) BALBc/ICR Swiss mice were randomized OVX or sham surgery. The 16-week-old mice were euthanized 20 weeks after surgery.

Results for tasks 3-6 – part 9:

- Two weeks after surgery, there is no significant decrease in body weight or fat mass between OVX and sham mice, but % fat mass is significantly different between the OVX and sham mice **(figure 9)**.
- Two weeks after surgery, there is a significant decrease in total body and spine BMD in OVX mice compared to sham mice, but there is no significant difference in femur or tibia BMD **(figure 10)**.
- Twenty weeks after surgery, there was a significant difference in uterine weight and uterine weight/body weight between OVX and sham mice **(figure 11)**.

Conclusions, potential problems and alternative strategies for tasks 3-6 (Part 9).
The 2-week data for BMD response to estrogen deprivation has been submitted for this report. The remainder of the BMD data is pending, as is the histomorphometry, micro-CT and bone marrow culture data. There was a significant decline in uterine weight after OVX.

It was thought that there would be no added benefit combining OVX with letrozole treatment. However, we thought it prudent to do a small experiment looking at the effect of OVX plus letrozole.

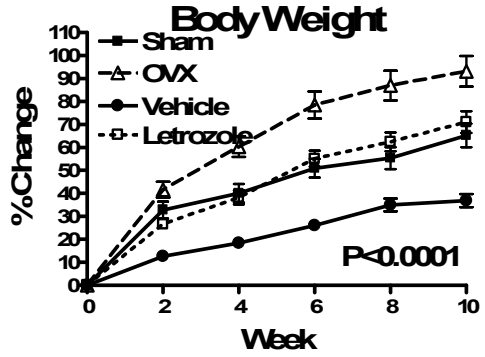
Procedures for tasks 3-6 (Part 10): In order to evaluate the effect of OVX plus letrozole, versus OVX alone, on BMD and bone turnover in BALBc/ICR Swiss immunocompetent mice, 32 4-week-old female BALBc/ICR Swiss mice were randomized OVX plus letrozole or OVX plus vehicle. The 4-week-old female mice were euthanized 28 weeks after surgery.

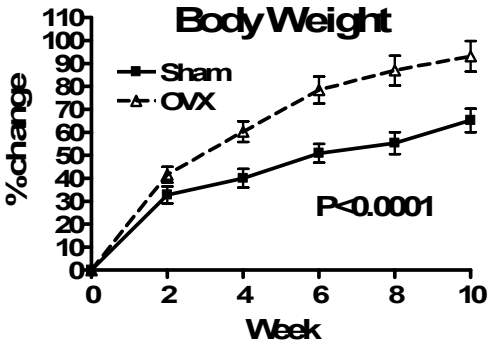
Results for tasks 3-6 (Part 10):

- Six weeks after initiation of treatment, there was a significant decrease in fat mass and % fat mass in the mice treated with OVX + letrozole compared to the mice treated with OVX + control **(figure 12)**, but there was no difference in body weight between the treatment groups.
- Six weeks after initiation of treatment, there was a significant decrease in total body and tibia BMD in the mice treated with OVX + letrozole compared to the mice treated with OVX + control **(figure 13)**, but there was no difference in spine or femur BMD between treatment groups.
- Twenty-eight weeks after surgery, there was a significant difference in uterine weight and uterine weight/body weight between OVX + letrozole mice compared to OVX + control mice **(figure 14)**.

Conclusions, potential problems and alternative strategies for tasks 3-6 (Part 10).
The 6-week data for BMD response to estrogen deprivation has been submitted for this report. The remainder of the BMD data is pending, as is the histomorphometry, micro-CT and bone marrow culture data. However, there was a significant decline in uterine weight and uterine weight/body weight in OVX + letrozole mice compared to OVX + control mice, suggesting that there is a further decline in estrogen levels when the AI letrozole is combined with OVX in this mouse model.

Figures:





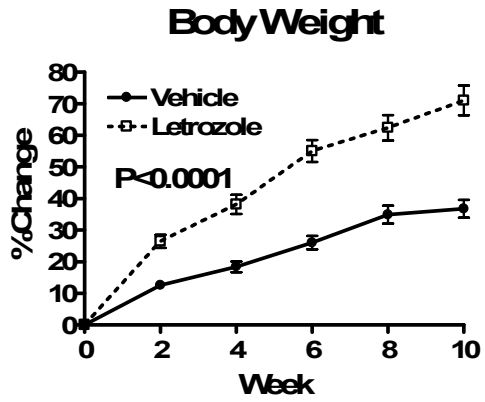
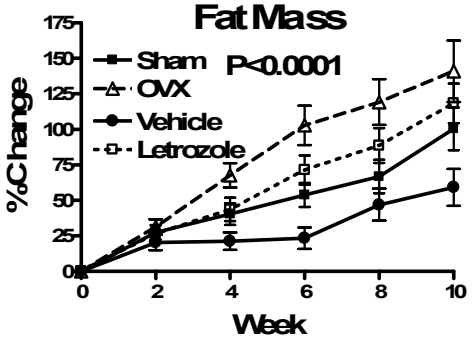
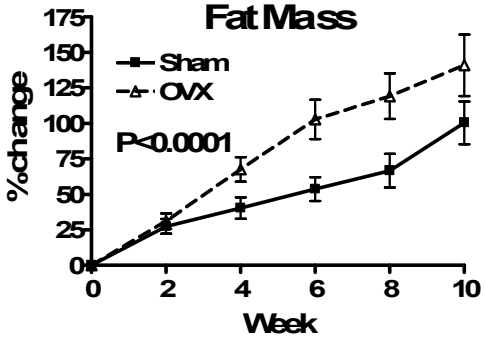


Figure 1. Body weight in 4-week-old BALBc/ICR swiss mice 10 wks after surgery or treatment with letrozole (5 mg/kg/day) or control. P values calculated using two-way ANOVA





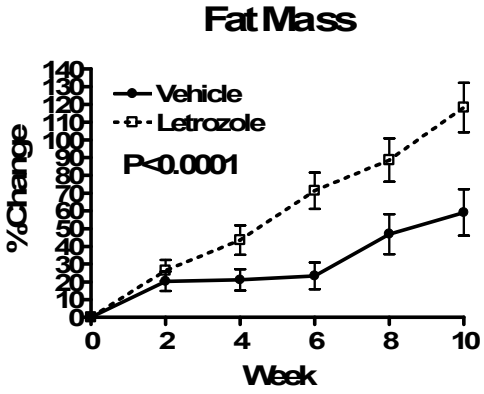
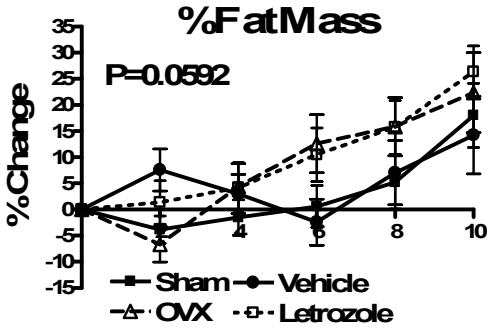
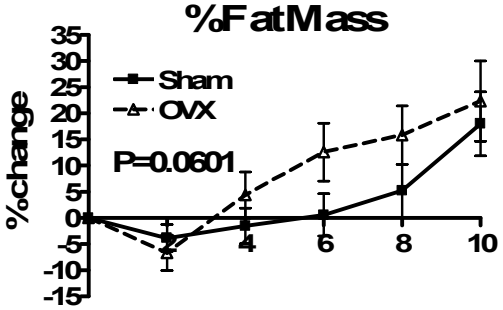


Figure 2. Fat mass in 4-week-old BALBc/ICR swiss mice 10 wks after surgery or treatment with letrozole (5 mg/kg/day) or control. P values calculated using two-way ANOVA





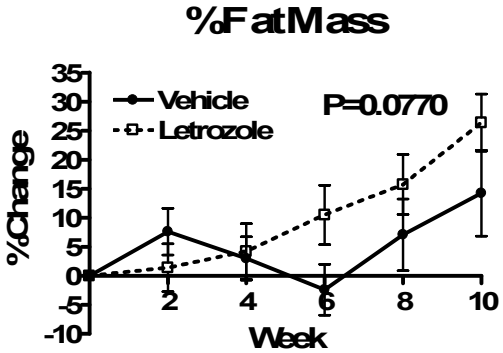
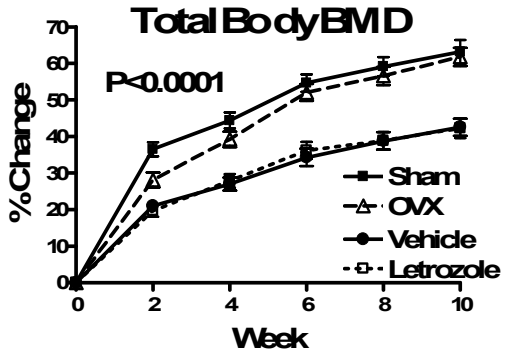
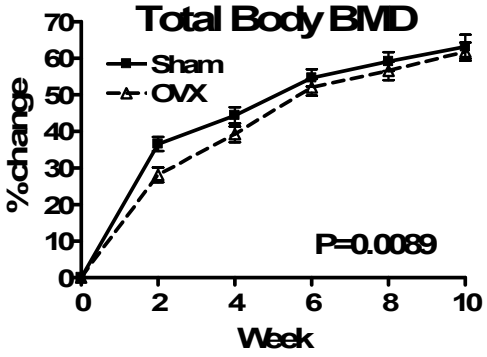


Figure 3. % Fat mass in 4-week-old BALBc/ICR swiss mice 10 wks after surgery or treatment with letrozole (5 mg/kg/day) or control. P values calculated using two-way ANOVA





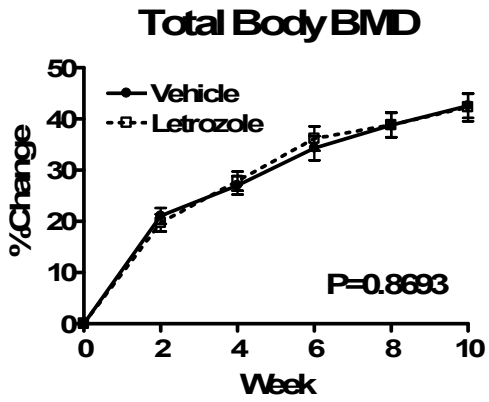
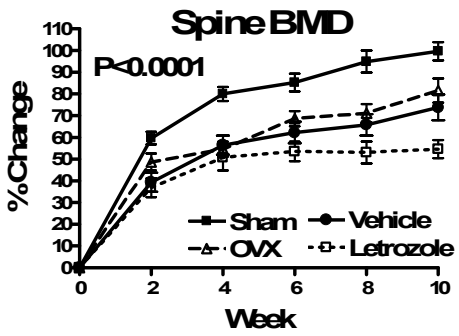
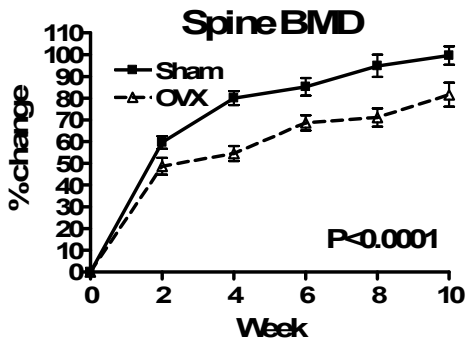


Figure 4. Total Body BMD in 4-week-old BALBc/ICR swiss mice 10 wks after surgery or treatment with letrozole (5 mg/kg/day) or control. P values calculated using two-way ANOVA





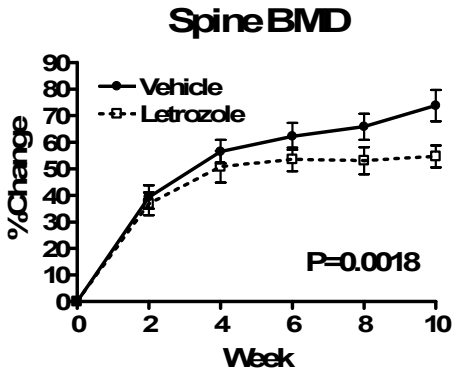
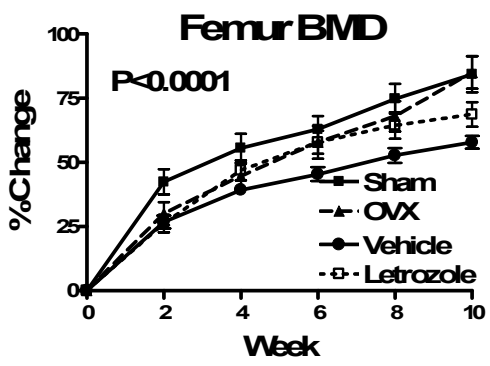
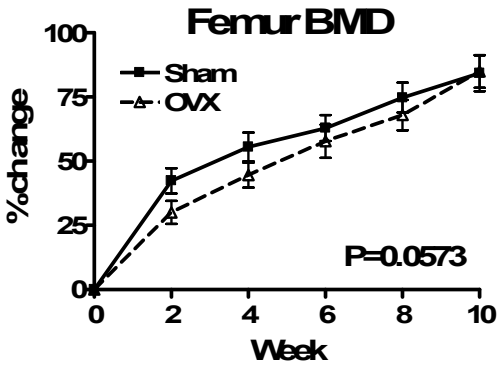


Figure 5. Spine BMD in 4-week-old BALBc/ICR swiss mice 10 wks after surgery or treatment with letrozole (5 mg/kg/day) or control. P values calculated using two-way ANOVA.





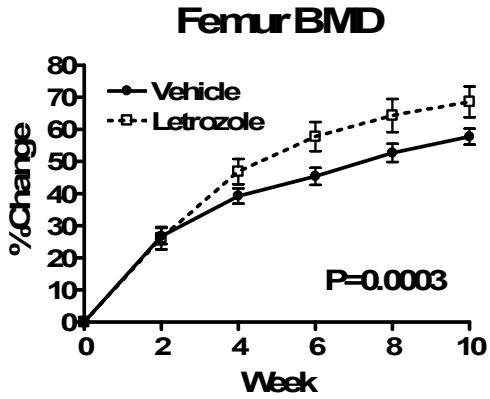
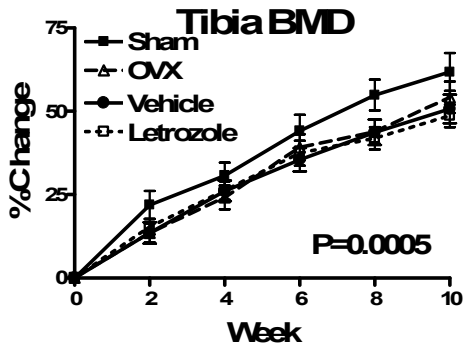
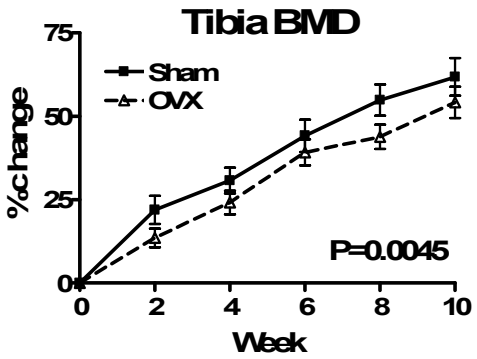


Figure 6. Femur BMD in 4-week-old BALBc/ICR swiss mice 10 wks after surgery or treatment with letrozole (5 mg/kg/day) or control. P values calculated using two-way ANOVA.





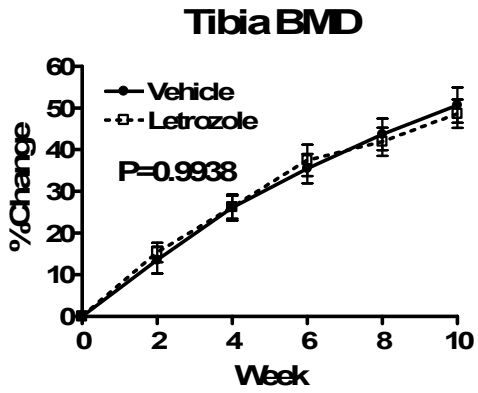
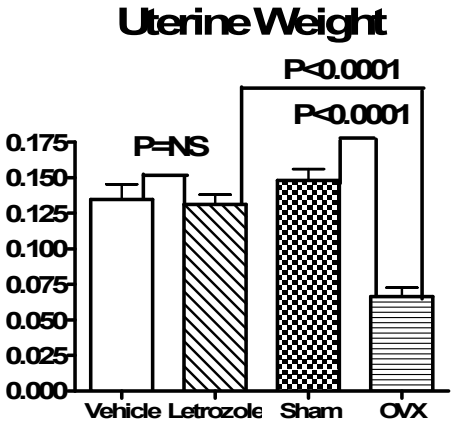


Figure 7. Tibia BMD in 4-week-old BALBc/ICR swiss mice 10 wks after surgery or treatment with letrozole (5 mg/kg/day) or control. P values calculated using two-way ANOVA



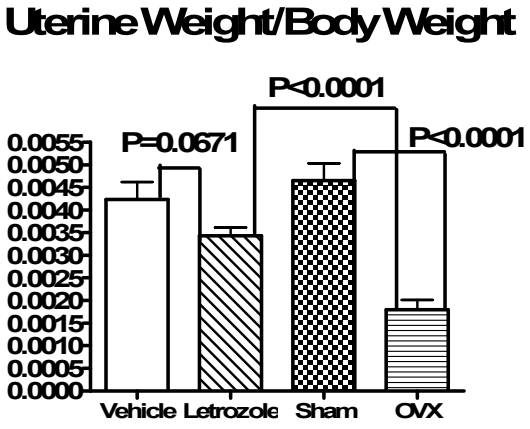
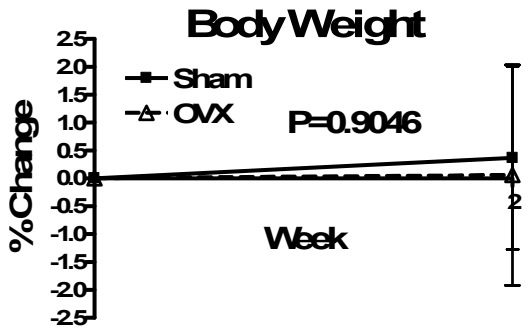


Figure 8. Uterine weight and uterine weight/body weight in 4-week-old BALBc/ICR Swiss mice 28 weeks after surgery or treatment with letrozole (5 mg/kg/day) or control. P values calculated using Students t-Test.



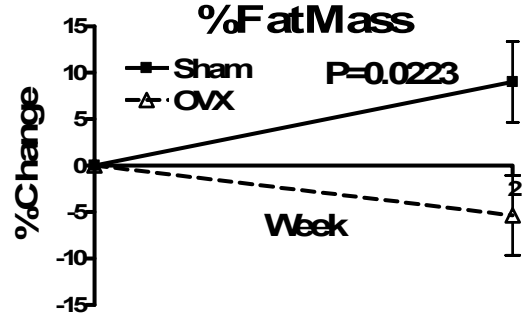
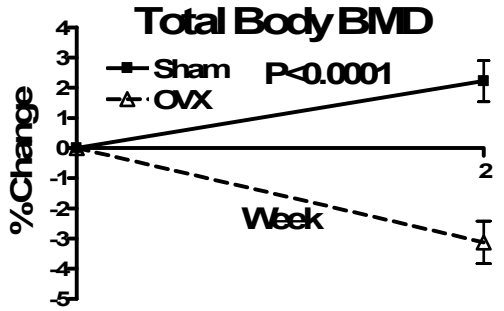
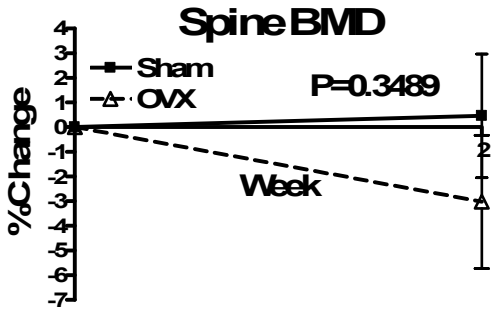
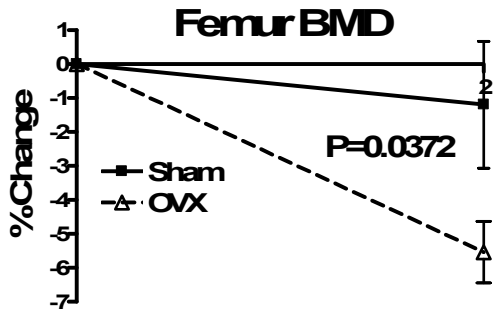


Figure 9. Body composition in 16-week-old BALBc/ICR swiss mice 2 wks after OVX or sham surgery. P values calculated using two-way ANOVA







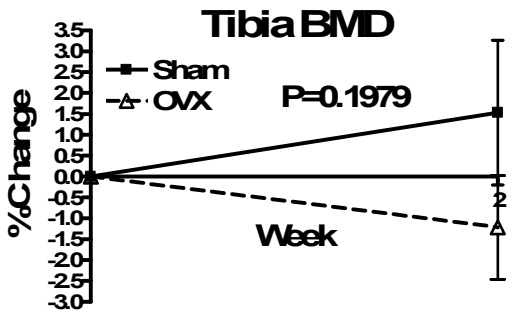
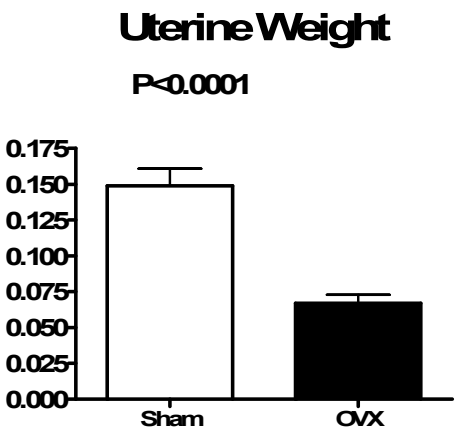


Figure 10. BMD in 16-week-old BALBc/ICR swiss mice 2 wks after OVX or sham surgery. P values calculated using two-way ANOVA



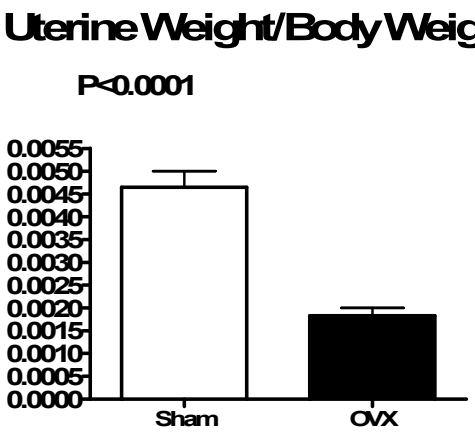
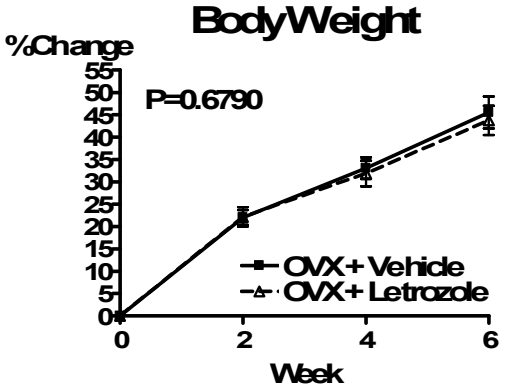
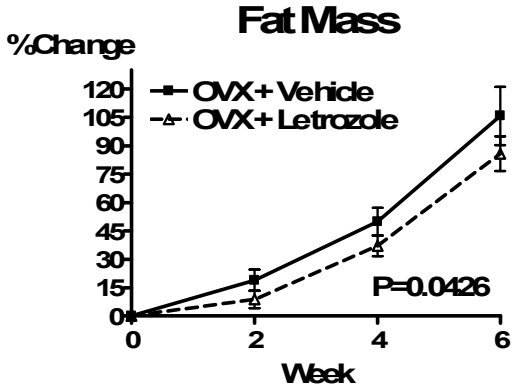


Figure 11. Uterine weight and uterine weight/body weight in 16-week-old BALBc/ICR Swiss mice 20 weeks after OVX or sham surgery. P values calculated using Students t-Test.





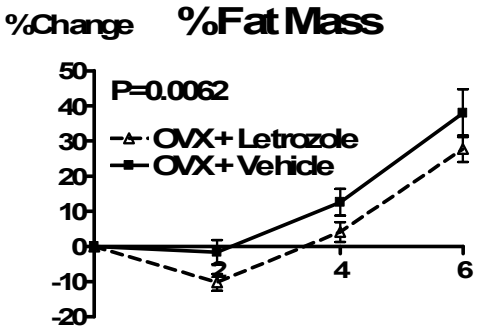


Figure 12. Body composition in 4-week-old BALBc/ICR swiss mice 6 wks after OVX +/- treatment with letrozole. P values calculated using two-way ANOVA.

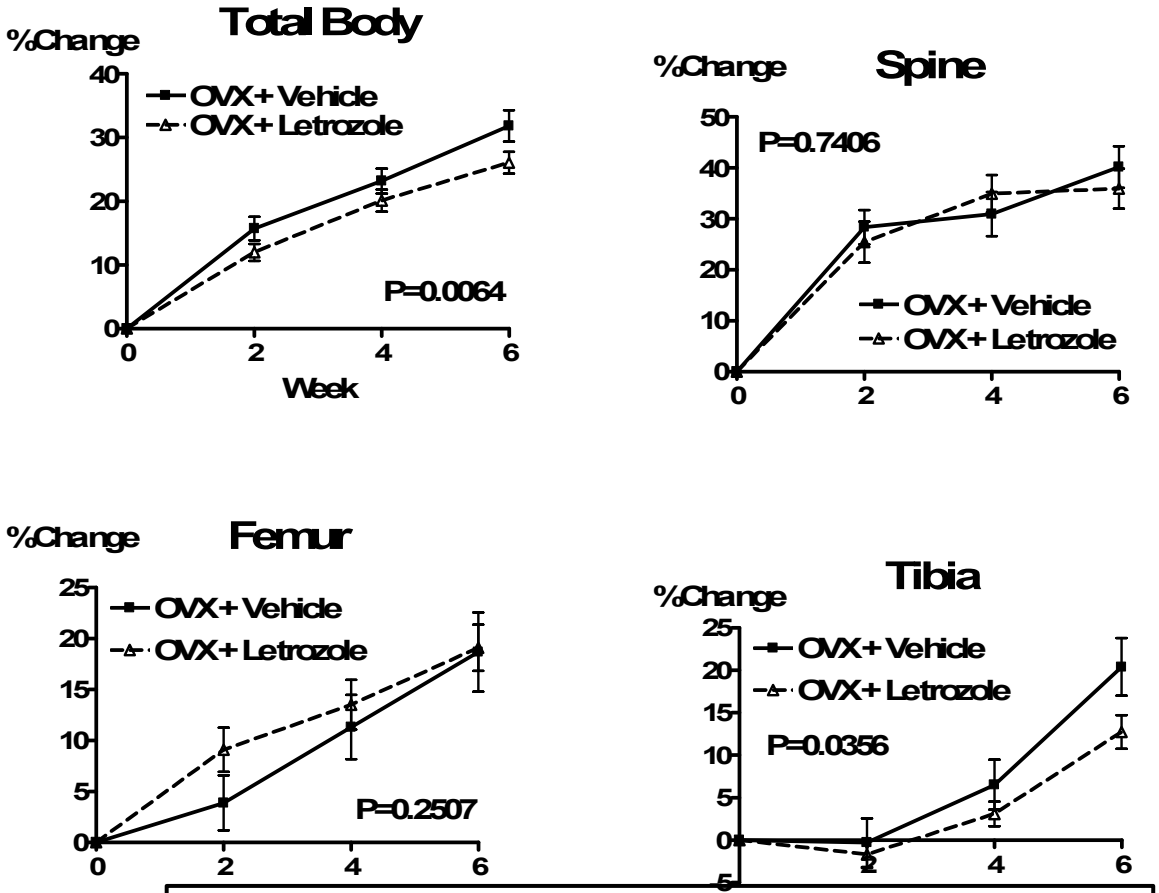


Figure 13. Total body BMD in 4-week-old BALBc/ICR swiss mice 6 wks after OVX +/- treatment with letrozole. P values calculated using two-way ANOVA.

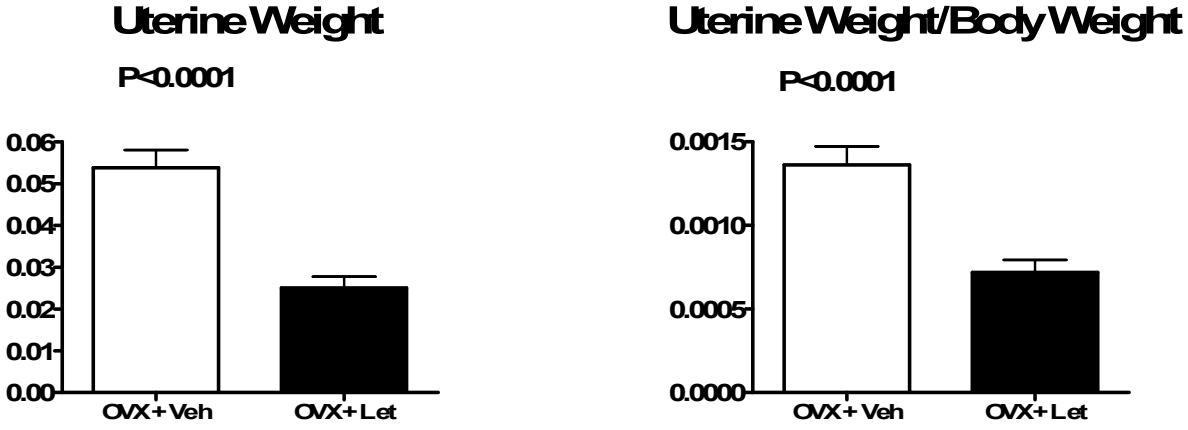


Figure 14. Uterine weight and uterine weight/body weight in 4-week-old BALBc/ICR Swiss mice 28 weeks after OVX +/- treatment with letrozole. P values calculated using Students t-Test.

Key Research Accomplishments

- Significantly decreased BMD (using Lunar PIXImus) in 4-week-old BALBc/ICR Swiss athymic mice after OVX, compared to sham surgery, at the total body, spine and proximal tibia, but there was no difference in BMD at the distal femur. Histomorphometry and micro-CT data did not show decreased TBV at the distal femur and proximal tibia in the OVX mice compared to the sham surgery mice. Bone marrow cultures from OVX mice exhibited a greater number of CFU-fibroblasts, CFU-osteoblasts and TRAP-positive osteoclasts compared to sham mice, thus indicating increased bone turnover after OVX compared to sham surgery.
- Significantly decreased BMD (using Lunar PIXImus) in 16-week-old BALBc/ICR Swiss athymic mice after OVX, compared to sham surgery, at the total body, spine and distal femur. There was no difference in BMD at the proximal tibia. 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 and histomorphometry data did not show a difference in trabecular bone parameters for the femur or tibia after OVX or sham surgery. 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.
- No difference in skeletal metastases or survival in BALBc/ICR Swiss athymic mice (4-week-old) after OVX as compared to sham surgery after intra-cardiac injection with MDA-MB-231. In the 16-week-old mice, sham surgery resulted in earlier and larger skeletal metastases after intra-cardiac injection with MDA-MB-231, the reasons for which remain unclear.
- An initial experiment showed that letrozole decreased BMD at all sites in female BALBc/ICR Swiss athymic mice, an effect prevented by concomitant treatment with ZA. However, repeat experiments showed that BMD in BALBc/ICR Swiss athymic mice treated with letrozole (either 4-week or 16-week-old) either did not differ, or there were site specific increases in BMD, as compared to control. Despite this, bone marrow cultures showed increased bone turnover in the athymic mice treated with letrozole versus control.
- There was no difference in the development and progression of breast cancer bone metastases after intra-cardiac inoculation with MDA-MB-231 in 4-week-old BALBc/ICR Swiss athymic mice treated with letrozole or control.
- Letrozole and OVX induced the anticipated decline in BMD and increases in bone turnover in the immunocompetent C57B6 mouse model.
- The immunocompetent BALBc/ICR Swiss mouse loses BMD at all sites after OVX, but has a more variable BMD response to letrozole. There was no decline in uterine weight after letrozole treatment. Therefore, this particular mouse strain does not have a uniform or predictable response to AI therapy.
- In the BALBc/ICR Swiss mouse, there does appear to be a further decline in estrogen levels when the AI letrozole is combined with OVX, as evidenced by a

more profound reduction in uterine weight after OVX plus letrozole versus OVX alone.

Key Training Accomplishments

As a result of this award, I was able to stay on as faculty at the University of Virginia (UVA) in the Division of Endocrinology and Metabolism after I finished my fellowship in clinical endocrinology. Although I was initially 80% research and 20% clinical, at the end of the award (March 2008), I changed to a 100% clinical position and took on the additional role of Director of the Metabolic Bone Disease clinic at UVA. In my clinical role, I see patients with breast cancer and bone complications from their breast cancer therapy. Dr. Guise was very influential in my obtaining a faculty position at UVA upon finishing my fellowship.

I have been mentored by both Dr. Theresa Guise and Dr. Richard Santen for the duration of the award. Through their mentoring and their assistance with interpretation of my data, I have been able to do this research, and have made the appropriate changes to the experiments in order to try and solve the issues that have arose, and in order to better define this mouse model of estrogen deficiency.

Reportable Outcomes

Publications:

1.Guise TA, Kozlow WM, Heras-Herzig A, Padalecki SS, Yin JJ, Chirgwin JM. 2005 Molecular mechanisms of breast cancer metastases to bone. *Clinical Breast Cancer*. 5 suppl (2):S46-53.

2.Kozlow W and Guise TA. 2005 Breast Cancer Metastasis to Bone: Mechanisms of Osteolysis and Implications for Therapy. *Journal of Mammary Gland Biology and Neoplasia*. 10(2):169-80.

3.Vessella RL, Guise TA, Susman ES, Suva LJ, Clines GA, Kominsky SL, Weber KL, Chirgwin JM, McCauley LK and Kozlow W. 2006 Meeting Report from Skeletal Complications of Malignancy IV. *BoneKEy-Osteovision*. 3(3):15-42.

Abstracts:

1.Abstract (poster) 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 (poster) University of Virginia Medicine Research Day; May 2005; Charlottesville, VA.

AROMATASE INHIBITION RESULTS IN LOWER BONE DENISTY THAN OVARIECTOMY 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. 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.

*presented as a poster at the 27th ASBMR annual meeting in Nashville, TN

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 (poster) 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 Kozlow[1], Khalid Mohammad[1], Ryan McKenna[1], Maryla Niewolna[1], Larry J Suva[2] and Theresa A Guise[1]. [1]Department of Endocrinology and Metabolism, University of Virginia, Charlottesville, Virginia, United States, 22908 and [2]Department 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.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.

*presented as a poster at the ASBMR annual meeting; September 2006; Philadelphia, PA

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 (poster) 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.W. Kozlow, K. Mohammad, C. R. McKenna, H. Walton, M. Niewolna, T. A. Guise. Ovariectomy Decreases Bone Mass in Young and Old Female Athymic Mice. 2007 *Journal of Bone and Mineral Research.22* (Suppl 1):S40.

* oral presentation at the 29th ASBMR annual meeting; September 2007; Honolulu, HI

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.

8. Abstract (poster) for the Era of Hope Meeting 2008.

W. Kozlow, K. Mohammad, C. R. McKenna, H. Walton, M. Niewolna, T. A. Guise. High Bone Turnover May Increase Breast Cancer Metastasis to Bone. Internal Medicine, University of Virginia, Charlottesville, VA, USA.

Tamoxifen is bone-sparing, but its use in breast cancer is being rapidly superseded by aromatase inhibitors (AIs). Unlike Tamoxifen, AI therapy for breast cancer results in high bone turnover which can lead to 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 from estrogen deficiency. We developed a mouse model of high bone turnover from estrogen deficiency to study its effect on breast cancer metastasis to bone.

To induce high bone turnover, 4-wk-old female athymic mice were randomized to ovariectomy (OVX), sham surgery (sham), treatment with the AI Letrozole (125 mcg/sc/d) or treatment with vehicle. Bone mineral density (BMD), as assessed by Lunar PIXImus, was done at baseline and then every 2 weeks. Mice were sacrificed 28 weeks after surgery and 16 weeks after treatment with Letrozole or vehicle. OVX mice had significantly decreased uterine weight ($P<0.0001$) compared to sham mice, thus confirming estrogen depletion. OVX mice had increased body weight ($P<0.0001$) and fat mass ($P<0.0001$) and decreased BMD at the total body ($P=0.0257$), spine ($P<0.0001$) and tibia ($P<0.0001$) compared to sham mice. BMD did not significantly differ between OVX and sham mice at the femur. Letrozole mice also had decreased uterine weight ($P=0.0012$) compared to vehicle. As with OVX, Letrozole mice had increased body weight ($P<0.0001$) and fat mass ($P<0.0001$) compared to vehicle. However, in contrast to OVX, Letrozole mice had *increased* BMD at the total body ($P<0.0001$) and tibia ($P=0.0002$) compared to vehicle. There was no difference in BMD at the spine or femur between Letrozole and vehicle mice.

Histomorphometry showed no difference in trabecular bone volume at the femur or tibia when comparing OVX to sham surgery or Letrozole to vehicle. However, bone marrow cultures from OVX mice exhibited a greater number of colony forming unit (CFU)-fibroblasts ($P<0.0001$), CFU-osteoblasts ($P<0.0001$) and TRAP-positive osteoclasts ($P=0.0002$) as compared to sham mice. Bone marrow cultures from Letrozole mice also exhibited a greater number of CFU-fibroblasts ($P=0.0001$), CFU-osteoblasts ($P<0.0001$) and TRAP-positive osteoclasts ($P=0.0047$) compared to vehicle. These data indicate a high bone turnover state in both OVX and Letrozole mice as compared to the control mice.

In another experiment, 4-wk-old female nude mice were randomized to OVX, sham surgery or to daily treatment with Letrozole or vehicle, and then subsequently underwent inoculation with the estrogen-receptor negative human breast cancer cell line MDA-MB-231 via intra-cardiac injection. Mice were x-rayed at baseline and then at 1-week intervals to monitor for development and progression of bone metastases. Interestingly, total body x-ray lesion area was greater in sham mice compared to OVX ($P<0.0001$), but there was no difference in total body lesion area between the Letrozole and vehicle mice.

A high bone turnover state is induced by estrogen deficiency from breast cancer therapy. However, OVX and Letrozole had different effects on bone mass. Further

studies are needed to characterize the effects of OVX and AI therapy on bone. OVX mice were protected from breast cancer metastasis to bone. The reason for this finding is unclear, but will be the topic of further investigation.

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.

Positions:

2006 – Present	Assistant Professor of Medicine, Department of Medicine, Division of Endocrinology and Metabolism, University of Virginia
2006 – Present	Director, Endocrinology & Metabolism Elective for Medical Students & Residents, Division of Endocrinology and Metabolism, University of Virginia
2008 – Present	Director, Metabolic Bone Disease Clinic, University of Virginia

Salary support from this award:

Wende Kozlow
Christopher Ryan McKenna
Maria Niewolna
Mark Conaway

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. We wanted to develop a mouse model to define the effect of a high bone turnover state induced by breast cancer therapy on the development and progression of breast cancer bone metastasis, and to test effective therapy to prevent increased bone turnover and breast cancer bone metastasis. The method of inducing estrogen deficiency, OVX or AI therapy, has resulted in variable effects on BMD in our

mouse model that do not reflect what is seen in the clinical setting. More work will need to be done to define the effect of AI therapy on BMD and bone turnover in a mouse model before it can be used to study the effect of breast cancer bone metastasis in the setting of estrogen deficiency and high bone turnover.

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Appendices

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