INTRODUCTION:
During osteoporosis, excessive bone resorption results in increased risks for fracture in postmenopausal women. The current FDA approved anti-resorptive drugs essentially inhibit osteoclast formation and/or activity but also impair fracture healing by blocking callus remodeling. New generations of drugs have been developed to inhibit bone resorption without totally abrogating osteoclast function. Thus, cathepsin-K inhibitors (CatK-I) have been developed as novel therapeutic agents to prevent and/or treat osteoporosis. While the CatK-I, Ódanacatib, has been shown to protect against bone loss in human osteoporotic patients as well as in preclinical studies, the effects of CatK-I on fracture healing remain unknown. Therefore, we compared the effects of the bisphosphonate, alendronate (ALN), to the CatK-I (L235) on bone repair using a mouse model of femoral fracture.

METHODS:
Animals: Wild type C57BL/6J mice were purchased from Jax Laboratories (Bar Harbor, ME) and housed at the University of Connecticut Health Center animal facilities according to state and federal law requirements. Mice were divided into groups of 8 which were daily given either the 0.75% carboxymethyl cellulose vehicle control or the CatK-I via gavage at 300 mg/kg body weight (bw) from day 0 post-fracture to the time of necropsy (days 14, 21 and 28). For the ALN group, mice subcutaneously received 0.15 mg/kg bw of ALN twice a week and euthanized at the day 7 and 21 time points.

Fracture procedure: The left limbs of mice from each group were fractured using the Einhorn device. The status of fracture and ongoing callus formation was monitored via X-ray at days 7, 10, 14, 21 and 28 post-fracture using a Faxitron (Wheeling, IL), and via µCT imaging at days 14, 21 and 28 post-fracture using a scanner device (Scanco Medical AG, Brütisellen, Switzerland).

Histological and histomorphometric analyses: Five-micron thick serial sections of fractured limbs were stained with Safranin O/Fast Green (SO/FG). Histomorphometric analysis was performed using NIS-Elements BR 3.0 (Nikon, Melville, NY).

TRAP staining: Deparaffinized sections were stained using the Kamiya TRAP staining kit (Kamiya biomedical company, Seattle, WA)

Serum CTX and PINP assays: Serum collected from mice at day 21 post-fracture was used to measure the level of C-terminal telopeptides of type I collagen (CTX; RatLap® EIA) and N-terminal propeptide of type I procollagen (PINP; Immunodiagnostic System Inc. AZ) as biomarkers for bone resorption and bone formation respectively.

RESULTS:
CatK-I treatment shows less mineralized callus compared to Alendronate: Radiologic assessments of fracture calluses indicated that, similar to the CON group, fracture calluses from either CatK-I or ALN treated mice were not mineralized at day 7 postfracture. By day 14 all calluses were mineralized and their sizes were not significantly different. However, the CatK-I and ALN groups maintained high mineralization density at day 21 compared to the CON group. These dense calluses were maintained in the CatK-I treated fractures at day 28 compared to CON, although they were smaller than at day 21.

We complemented our X-ray analyses with microCT imaging to quantitatively measure the degree of mineralization and remodeling of fracture calluses between day 14 and day 28 post-fracture. The fraction of mineralized callus over total callus was significantly increased by 20%, 31%, and 63% at days 14, 21, and 28 post-fracture, respectively in the CatK-I treated mice compared to the CON groups. At day 21 post-fracture, ALN significantly increased mineralized callus fraction by 96% compared to the CON and by 50% compared to the CatK-I.

CatK-I stage-specifically affects bone fracture repair: We performed histological analyses by staining SO/FG in the fractured limbs harvested from all groups at days 14, 21, and 28 post-operation. At 14 days post-fracture CatK-I treated mice exhibited slightly smaller calluses without visible changes in bony tissue compared to the CON groups. By day 21 post-fracture, calluses from both CatK-I and ALN groups are slightly bigger with more mineralized bony tissue and residual cartilaginous tissue compared to the CON groups. Callus remodeling was delayed in the CatK-I group as evidenced by a bigger callus size with residual mineralized bony tissue compared to CON group at day 28 post-fracture. These changes in callus size as well as callus content in cartilage and bone were quantified via histomorphometric analyses.

Increased number of osteoclasts following CatK-I treatment: We performed TRAP staining in fracture calluses of all groups at day 14, 21, and 28 post-fracture. We found that both CON and CatK-I treated mice showed positive cells in their bony calluses at day 14 post-fracture. At day 21 post fracture calluses from the CatK-I treated groups displayed very strong TRAP staining indicative of increased number of osteoclasts compared to the CON and ALN treated groups. This abundance of TRAP positive cells in the calluses of CatK-I treated groups persisted at day 28 post-fracture.

Inhibited osteoclast function by CatK-I during fracture repair: Serum CTX isolated from CatK-I mice at day 21 post-fracture was measured by ELISA and found to be significantly elevated by 64% compared to the CON groups. As expected, the ALN groups showed reduced serum CTX by 53% compared to the CON group. However, when levels of serum CTX were normalized to the number of osteoclasts per surface area within the fracture calluses, relative levels of serum CTX in both CatK-I and ALN groups were significantly decreased compared to that of the CON groups. When we examined bone formation rate via assessment of serum PINP in these experimental groups, we found that CatK-I did not show any difference on bone formation compared to CON. However, as expected, ALN groups showed decreased osteoblast activity compared to the CON groups.

Figure 1. The effects of CatK-I on fracture repair

(A) Micro-CT rendering of the mineralized fracture calluses (top panel) and their transverse section (bottom panel) shows increased mineralization in CatK-I treated mice and to a higher extent in ALN treated mice at day 21 post-fracture. (B) The fraction of mineralized calluses over total calluses was quantified in all experimental groups. (C) Histological sections stained with SO/FG showing residual unmineralized cartilaginous calluses (Red color) and increased mineralized bony tissue (green) in CatK-I and ALN specimens compared to CON at day 21 post-fracture. (D) Areas of bone and cartilaginous calluses were quantified using histomorphometrical analysis. *p<0.05 vs. CON, #p<0.05 vs. CatK-I

DISCUSSION:
The data indicate that even though treatment with CatK-I delayed fracture callus remodeling, it did not prevent fractures from healing. The degree of mineralization of unresorbed calluses was significantly higher in fracture calluses of mice treated with ALN than those treated with the CatK-I. The fact that the number of TRAP positive cells was much higher in the calluses of CatK-I treated mice indicates that these osteoclasts are not functional and further demonstrates the efficacy of CatK-I to inhibit resorption rather than abrogating it. The CTX and PINP data support the concept that, opposite to the traditional anti-resorptive drugs such as ALN, CatK-I uncouples bone resorption and bone formation. We hypothesize that CatK-I may enhance bone formation. Dynamic histomorphometric analyses are currently being performed to determine the rate of bone formation in response to CatK-I treatment.