

The Effect of Continuous Infusion of Human Parathyroid Hormone on Bone Architecture in Female Mice

by

Rahel E. Eisenberg

Submitted to the Department of Mechanical Engineering in Partial Fulfillment of the Requirements for the Degree of

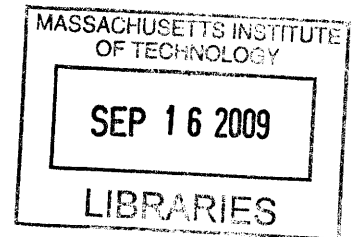
Bachelor of Science in Engineering as recommended by the Department of Mechanical Engineering at the

Massachusetts Institute of Technology

June 2009

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ABSTRACT

This research sought to create an animal model of secondary hyperparathyroidism through continuous infusion of parathyroid hormone (PTH) in adult female mice, and to subsequently study the catabolic effects of PTH. Osmotic pumps were implanted subcutaneously and infused either vehicle, 40  $\mu\text{g}/\text{kg}/\text{day}$  of PTH or 80  $\mu\text{g}/\text{kg}/\text{day}$  of PTH for either 2 week or 4 week duration. Total body bone mineral density (BMD) and bone mineral content (BMC), trabecular and cortical bone microarchitecture, and serum calcium levels were measured. There were no significant differences in BMD or BMC in either study in mice infused with either dosage, nor were there significant differences in femoral cortical bone microarchitecture. Trabecular bone in the distal femur as well as the L<sub>5</sub> vertebrae was also largely preserved. This data indicate that short term infusion of PTH in normal mice does not mimic clinical characteristics and therefore may not be an appropriate model of secondary hyperparathyroidism and the catabolic effects of PTH.

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## Introduction

Bone is a highly active tissue which is continually degrading and regenerating itself. The coupling of the bone formation and resorption processes help maintain strong bones by simultaneously removing old bone and replacing it with new bone. However, as people age their bones tend to become more fragile due to bone loss. This phenomenon, especially in women, is best known as osteoporosis.

The effects of bone loss can be devastating to patients and significantly alter their quality of life and the activities in which they can participate. As bone loss occurs, bone density decreases causing the mechanical properties of the bone, such as its Young's modulus and yield stress, to also decrease. The decrease in these mechanical properties is responsible for the higher incident rate of fractures seen in patients suffering from osteoporosis. Estrogen and calcium deficiencies play roles in bone loss. While the mechanisms through which estrogen effects bone mineral density and microarchitecture are well characterized, the mechanisms through which hyperparathyroidism induces skeletal fragility is less developed.

There are three basic ways to develop bone fragility. The first is simply failing to produce strong bones during ones growth, while the second two ways involve the bone remodeling process, in other words, bone resorption and formation. As mentioned previously, bone resorption and formation are coupled processes and therefore either excessive bone resorption and/or an inadequate bone formation response constitute the other two ways through which one can develop skeletal fragility and associated elevated risk of fracture.<sup>1</sup>

Bone remodeling can occur in both the trabecular and cortical bone. The process begins with when precursor stem cells become osteoclasts, bone resorbing cells, through interaction with cells of osteoblastic, bone building, roots. Once formed, the osteoclasts digest the bone quickly. Subsequently, formation begins, but is a much longer process during which waves of new osteoblasts continue the matrix building process while old cells are incorporated into the bone.<sup>2</sup> The difference in the time required for each cell type to do its respective job suggests that any increase in the rate of bone resorption will yield a net decrease in bone mass. Similarly, if osteoblasts fail to respond to the bone resorption process, this too can result in net bone loss. Both aforementioned means of bone loss are present in osteoporotic patients.<sup>3</sup>

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<sup>1</sup> Raisz LG 2005 Pathogenesis of osteoporosis: concepts, conflicts, and prospects. Journal of Clinical Investigation 115:3318-3325.

<sup>2</sup> Raisz LG 2005.

<sup>3</sup> Raisz LG 2005.

Both estrogen and calcium deficiencies are considered to be the primary forces resulting in osteoporosis. Studies have shown that post-menopausal women, deficient in estrogen, have higher rates of both bone resorption and bone formation.<sup>4</sup> However, the increase in resorption rate is greater and therefore bone loss is induced. Similarly, calcium deficiencies and vitamin D deficiencies can result in secondary hyperparathyroidism by decreasing serum calcium, which in turn leads to bone loss.<sup>5</sup>

As a contributor to osteoporosis, it is important to better understand the mechanism through which secondary hyperparathyroidism induces bone loss. The defining characteristic of hyperparathyroidism is a continuously elevated level of parathyroid hormone (PTH). While the continuous elevation of PTH in the blood is believed to be associated with the induced bone loss that occurs in patients with hyperparathyroidism, the association has yet to be fully understood or quantified. Therefore, this thesis research sought to develop an animal model to study the effects of hyperparathyroidism on bone to develop a better understanding of the relationship between continuous high levels of PTH and bone resorption.

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<sup>4</sup> Ebeling PR, Atley LM, Guthrie JR, Burger HG, Dennerstein L, Hopper JL, Wark JD 1996 Bone turnover markers and bone density across the menopausal transition. *Journal of Clinical Endocrinology and Metabolism* 81:3366-3371.

<sup>5</sup> Raisz LG 2005.



# Background

## ***Parathyroid Hormone***

Parathyroid hormone (PTH) regulates blood serum calcium levels. It does so by stimulating calcium re-absorption in the kidney and inducing bone resorption. For example, low serum calcium levels cause an increase in the secretion of PTH from the parathyroid. The elevated levels of PTH then induce more bone resorption as well as increase the calcium re-absorption in the kidney and intestine. The re-absorption of the calcium as well as calcium liberated by bone resorption cause the blood serum calcium concentration to increase. As the serum calcium level returns to normal, negative feedback is sent to the parathyroid gland and it secretes less PTH.<sup>6</sup> This cycle allows the parathyroid, through the secretion of PTH, to maintain a balanced level of calcium in the blood.

Although PTH causes bone resorption in order to maintain a balanced blood serum calcium level, PTH is known to have a dual effect on bone; it stimulates both bone resorption and bone formation. PTH's anabolic effect has been well studied and occurs when administered intermittently at low doses, modeling short term fluctuations.<sup>7</sup> The bone forming effect of intermittent administration has been shown to increase total body bone mineral density (BMD), improve trabecular bone microarchitecture and reduce risk of fracture<sup>8-9</sup>. This is partially attributable to the effect that PTH has on cells of osteoblast roots and the coupling of the bone formation and resorption processes, however many other cellular processes involving PTH receptors and precursor osteoblasts are also responsible for the anabolic effect.<sup>10</sup>

In contrast, PTH's catabolic effect is associated with continuously elevated levels of PTH which are seen in patients with hyperparathyroidism. When improperly regulated, PTH in turn is unable to successfully regulate blood serum calcium, and therefore continuously high levels of PTH are also associated with

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<sup>6</sup> Partridge NC, Li X, Qin L. 2006 Understanding Parathyroid Hormone Action. New York Academy of Sciences 1068: 187-193

<sup>7</sup> Partridge NC, Li X, Qin L. 2006.

<sup>8</sup> Neer RM, Arnaud CD, Zanchetta JR, Price R, Gaich GA, Reginster JY, Hodsmann AB, Eriksen EF, Ish-Shalom S, Genant HK, Wang O, Mitlak BH 2001 Effect of parathyroid hormone (1-34) on fractures and bone mineral density in post-menopausal women. New England Journal of Medicine 344:1434-1441.

<sup>9</sup> Dempster DW, Cosman F, Kurland ES, Zhou H, Nieves J, Woelfert L, Shane E, Plavetic K, Muller, Bilezikian J, Lindsay R 2001 Effects of daily treatment with parathyroid hormone on bone microarchitecture and turnover in patients with osteoporosis: A paired biopsy study. Journal of Bone and Mineral Research 16:1846-1853.

<sup>10</sup> Robert A Nissenson, "Parathyroid Hormone and Parathyroid-Related Protein," in *Osteoporosis, Two Volume Set, Volume 1-2*, ed. R Marcus, D Feldman, D Nelson, CJ Rosen (Elsevier Academic Press, 2008), 283-316.

hypercalcemia, elevated blood serum calcium, which is indicative of PTH's catabolic effect to bone in humans.<sup>11</sup> These effects are seen in patients with both types of hyperparathyroidism, primary and secondary.

### ***Primary Hyperparathyroidism***

Primary hyperparathyroidism is the result of overproduction of PTH, typically caused by an adenoma on the parathyroid gland, and is characterized by severe hypercalcemia, nephrolithiasis, and osteoporosis. It is associated with a decrease in bone mineral density, especially in post-menopausal women without estrogen supplementation, and bone loss at cortical sites. Trabecular bone microarchitecture is generally preserved, although some studies have shown a slight decrease there as well.<sup>12</sup>

In the 1970s, detection of hyperparathyroidism became easy due to the creation of the autoanalyser which was able to diagnose hypercalcemia, an indicator of hyperparathyroidism. Therefore most cases now are asymptomatic at the time of diagnosis.<sup>13</sup> Currently, diagnosis is done using PTH assays and checking the level of 25-hydroxyvitamin D. Although most cases are asymptomatic, it is still believed that parathyroidectomy is the best course of treatment. Studies have demonstrated that surgery halts bone loss, and actually increases BMD.<sup>14</sup>

Although surgery has positive effects, it is not always recommended. Patients must meet certain requirements including serum calcium levels > 1mg/dL above the upper limits of normal, reductions in bone mineral density particularly in peri- or postmenopausal women and men, or being under 50 years of age. When these requirements are not met, it has been determined that a patient's condition can be safely monitored.<sup>15</sup>

### ***Secondary Hyperparathyroidism***

Unlike primary hyperparathyroidism, secondary hyperparathyroidism is not caused by adenoma on the parathyroid gland, but is generally the result of renal failure and/or deficiency in calcium intake and vitamin D levels.<sup>16</sup> General characteristics are high fracture rate and low bone mineral density, particularly in

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<sup>11</sup> Bilezikian JP, Silverberg SJ 2004 Clinical Practice. Asymptomatic primary hyperparathyroidism. *New England Journal of Medicine* 350:1746-1751.

<sup>12</sup> Khan A, Bilezikian J 2000 Primary hyperparathyroidism: pathophysiology and impact on bone. *Canadian Medical Association Journal* 163(2):184-7.

<sup>13</sup> Khan A, Bilezikian J 2000.

<sup>14</sup> Bilezikian JP, Khan AA, Potts JT *et al.* 2009 Guidelines for the Management of Asymptomatic Primary Hyperparathyroidism: Summary Statement from the Third International Workshop. *Journal of Clinical Endocrinology and Metabolism* 94(2):335-339.

<sup>15</sup> Bilezikian JP, Khan AA, Potts JT *et al.* 2009.

<sup>16</sup> Raisz LG 2005.

the femoral neck. Regardless of the cause, bone turnover still increases in response to the elevated PTH level and is associated with cortical bone loss while preserving trabecular architecture.<sup>17</sup>

Diagnosis is typically done by radiograph or bone density measurement, since the primary effect is skeletal. However, patients also generally have slightly lower than normal serum calcium levels and elevated levels of PTH. In patients suffering from secondary hyperparathyroidism due to vitamin D deficiency, the prescribed treatment is vitamin D supplementation in doses of 30 ng/ml or higher.<sup>18</sup>

### ***Related Work***

Various studies have tried to understand the dual actions PTH has on bone. While many have been successful in creating animal models that exhibit PTH's anabolic effects when administered intermittently, few have been able to produce the catabolic effect produced by continuously high levels of PTH, an effect clinically encountered in hyperparathyroidism.

Iida-Klein *et al.* (2005) delivered continuous hPTH(1-34) of 40 µg/kg/day to 10 week old, non-Ovx, C57BL/J6 female mice via Alzet 2002 miniosmotic pumps for 14 days. Their BMD measurements by DXA showed no difference between the control and the hPTH(1-34) infused mice at any site of scanning (femur, tibia, lumbar vertebrae 4-5). However, assessment of the bone micro-architecture by µCT showed a significantly lower trabecular connectivity density in the PTH infused mice versus the controls. Though only the difference in connectivity density was statistically significant, other µCT measured parameters, such as trabecular number, and thickness also tended to be lower in the PTH infused mice. In addition to showing differences in bone micro-architecture between PTH infused mice and control mice, many measured biochemical markers, such as serum calcium and bone resorption marker (mTRAP), also supported the hypothesis that continuous administration of PTH has a catabolic effect on bone. The total serum calcium in the PTH infused mice was 35% higher than in the control mice, showing induced hypercalcemia.<sup>19</sup>

V. Shen *et al.* (2000) studied the effects of continuously elevated levels of PTH in female, estrogen deficient and estrogen satiated, 7 month old Sprague-Dawley rats. They continuously delivered a 30 µg/kg/day dose of PTH to Ovx mice either

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<sup>17</sup> Lips P 2001 Vitamin D Deficiency and Secondary Hyperparathyroidism in the Elderly: Consequences for Bone Loss and Fractures and Therapeutic Implications. *Endocrine Review* 22(4):477-501.

<sup>18</sup> Raisz LG 2005.

<sup>19</sup> Iida-Klein A, Lu SS, Kapadia R, Burkhart M, Moreno A, Dempster DW, Lindsay R 2005 Short-term continuous infusion of human parathyroid hormone 1-34 fragment is catabolic with decreased trabecular connectivity accompanied by hypercalcemia in C57BL/J6 mice. *Journal of Endocrinology* 186:549-557.

in combination with or without estrogen for 4 weeks. This study demonstrated the catabolic effect of continuously elevated PTH level in Ovx, estrogen deficient mice, and the ability of estrogen to protect the bone from this effect. Bone loss, as measured by BMD, in PTH treated rats became statistically significant compared to the control at all sites of measurement (distal, midshaft, and proximal femur) after 4 weeks of treatment only, though serum calcium levels were significantly higher by 2 weeks of treatment. Additionally, through bending tests, they showed a significant decrease in femoral neck strength of the PTH treated mice after 4 weeks of treatment, further substantiating the evidence that continuous elevation of PTH in estrogen deficient rats is deleterious to bone, such as increased cortical porosity, preserved trabecular bone microarchitecture, and increased osteoclast recruitment.<sup>20</sup>

In the same study as Shen *et al.*, H. Zhou *et al.* studied the effects of continuous PTH administration on vertebral trabecular and cortical bone (same 7 month old, Ovx, female, estrogen deficient and replete, Sprague-Dawley rats). Histomorphometry showed that PTH treatment alone partially in Ovx rats partially restored the trabecular bone in the vertebrae, however they also saw that it induced an increase in cortical porosity. In estrogen replete, PTH treated rats, they showed that trabecular bone architecture was fully restored to that of the control. Overall, they found that Ovx rats treated with 30 µg/kg/day of PTH exhibited the same bone weakening characteristics as postmenopausal women with primary hyperparathyroidism.<sup>21</sup>

Based on these studies, we designed our study to take into account both the 2 and 4 week time points and to test the continuous administration of two different PTH doses, 40 and 80 µg/kg/day. Based on Iida-Klein *et al.*'s 2 week results, many of which almost reached significant differences, as well as Shen *et al.*'s and Zhou *et al.*'s significant results at 4 weeks, we hoped that after treating mice with PTH for 4 weeks significant differences between PTH infused and control groups would be reached. Additionally, we wanted to explore the effects of higher doses of PTH in mice after only two weeks of treatment. Our study setup allowed us to see both the time and dose dependence of continuous PTH administration in mice.

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<sup>20</sup> Shen V, Birchman R, Wu DD, Lindsay R 2000 Skeletal Effects of Parathyroid Hormone Infusion in Ovariectomized Rats With or Without Estrogen Repletion. *Journal of Bone and Mineral Research* 15:740-746.

<sup>21</sup> Zhou H, Shen V, Dempster DW, Lindsay R 2001 Continuous Parathyroid Hormone and Estrogen Administration Increases Vertebral Cancellous Bone Volume and Cortical Width in the Estrogen-Deficient Rat. *Journal of Bone and Mineral Research* 16:1300-1307.

## Materials and Methods

### *Animals*

Ten to eleven week old female C57BL/J6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The animals arrived in the laboratory one week prior to the implantation of the Alzet pumps, and were handled daily prior to surgery. They were housed 4 animals per cage and were maintained on a 12:12-h light-dark cycle with free access to mouse chow (7964 Harlan Teklad F6 Rodent Diet, 1.35% Ca, Harlan Teklad, Indianapolis, IN, USA) and water. The animal protocol was approved by the ethical committee on animal care and use at Beth Israel Deaconess Medical Center, Boston, MA.

### *Materials*

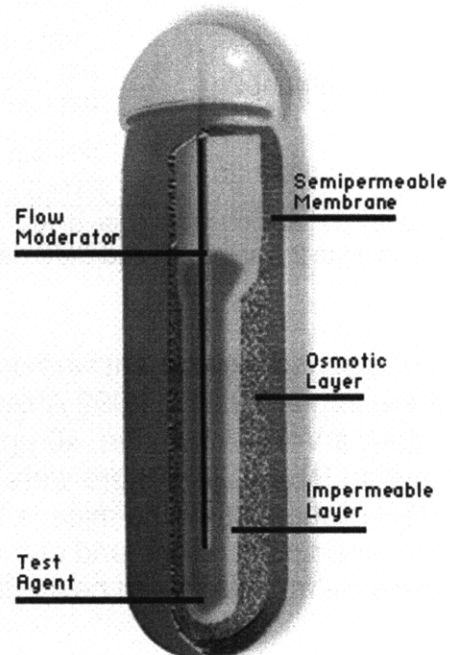
hPTH(1-34) was purchased from Bachem (Torrence, CA, USA).

Alzet miniosmotic pumps, 2002 and 2004 pump models purchased from Durect Corporation (Cupertino, CA, USA).

QuantiChrom Calcium Assay kit was purchased from BioAssay Systems (Hayward, CA, USA).

### *The Mini Osmotic Pump*

ALZET osmotic pumps allow the continuous delivery of a substance to a subject. The pump has five main components: the semipermeable membrane, the osmotic layer, the impermeable layer, the flexible agent reservoir, and the flow moderator (Figure 1). The high osmolality of the osmotic layer allows water to flow into the pump through the semipermeable membrane. As water enters the osmotic layer, also called the salt sleeve, it compresses the flexible reservoir which contains the test solution causing the solution to be released from the pump as its predetermined rate. The delivery rate is determined only by the water permeability of



**Figure 1.** ALZET mini-osmotic pump schematic.

the semipermeable membrane, and is independent of the test solution.<sup>22</sup>

The only difference between the two pump models used in these experiments, the 2002 and 2004 models, is the duration of time for which they can be used. Though the two pumps are exactly the same size and have the same size reservoir (200  $\mu$ L), the 2002 model works for only two weeks, while the 2004 model can be used for up to four weeks. This functional difference is accomplished by the different delivery rates of each pump; while the 2 week pump's delivery rate is 0.5  $\mu$ L/hr, the 4 week pump delivers at half that rate, 0.25  $\mu$ L/hr. The delivery rate is determined by the water permeability of the semipermeable membrane of each pump model.

### ***Experimental Design***

We performed two separate experiments. The first was a four week experiment in which 24 mice were implanted with 2004 Alzet miniosmotic pumps containing either a vehicle solution, 40  $\mu$ g/kg/day of human PTH(1-34), or 80  $\mu$ g/kg/day of human PTH(1-34). There were 8 mice in each experimental group, and all mice were sacrificed at the end of their 28<sup>th</sup> day of treatment.

**Table 1. Experimental Design**

Time (weeks)	0	2	4
Experiment 1	VEH (n = 8) PTH-40 (n = 8) PTH-80 (n = 8)		Sacrifice
Experiment 2a	VEH (n = 6) PTH-40 (n = 6) PTH-80 (n = 6)	Sacrifice	
Experiment 2b	PTH-40 (n = 3) PTH-80 (n = 3*)	Re-surgery	Sacrifice

\* One PTH-80 infused mouse died 3 weeks into treatment after bleeding on day 21. Her data is included in 2 week BMD and BMC data, but excluded all other data.

The second experiment was primarily a two week experiment in which 24 mice were implanted with 2002 Alzet miniosmotic pumps. Again, the pumps contained either a Vehicle solution, 40  $\mu$ g/kg/day of human PTH(1-34), or 80  $\mu$ g/kg/day of human PTH(1-34). There were 6 mice in the vehicle group, and 9 in each of the PTH groups. 18 of the mice, all 6 controls and 6 mice from each PTH group, were sacrificed at the end of their 14<sup>th</sup> day of treatment. The 6 remaining mice were re-implanted with new 2002 Alzet pumps, containing their respective PTH

<sup>22</sup> Alzet Osmotic Pumps "How does it work" 4 May 2009, <<http://www.alzet.com/products/howdoesitwork.php>>.

solutions, on the 15<sup>th</sup> day of the experiment. These mice continued treatment for another 13 days at which point they were sacrificed, with total treatment duration of 28 days.

## ***Procedure***

### ***Group Assignment***

For both experiments, one day prior to pump implantation, baseline DXA scans were performed (PIXImus2; GE Lunar Madison, WI, USA). To ensure that no experimental group started the treatment period with significantly different baseline bone parameters, the mice were assigned to their experimental group only after the baseline scans were analyzed. Total body bone mineral density (BMD, g/cm<sup>2</sup>) was used as the determining parameter.

### ***PTH Preparation***

The procedures for pump filling, and implantation were the same for both of the experiments.

Two days prior to implantation, the mice were weighed, and their average weight was used to determine the PTH concentrations of the administered solutions:

$$concentration = weight * dose * \frac{1}{r} \quad \text{eq. (1)}$$

where *weight* represents the average weight of all the animals in grams, *dose* is the desired dosage of PTH in µg/kg/day, and *r* represents the dispensation rate of the pump in µL/day.

Using the determined concentration, the volume of solution required to produce the desired dosages of PTH was determined.

$$V = \frac{hPTH(1-34)}{concentration} \quad \text{eq. (2)}$$

where *V* is the total volume in mL, *hPTH(1-34)* is the amount of human PTH(1-34) in mg, and *concentration* is the previously determined concentration needed to produce the desired dosage in mg/mL. The composition of the solution was 1% HCl, 2% mouse serum, and 97% saline solution.

The 80 µg/kg/day dosage solution was mixed first. The PTH was dissolved in 90% of the saline required for the solution, and then the required amounts of mouse serum and HCl were added. The pH of the solution was read by a pH

meter (Corning Model 430 pH meter) and slowly decreased to a pH of 4.0 by adding HCl in 2-5  $\mu\text{L}$  increments. Once the pH was brought to 4.0, the remaining 10% of saline was added. Finally, the solution was sterilized through a filter and deposited into a sterile vial. Pumps were filled according to the manufacturer's guidelines with the 80  $\mu\text{g}/\text{kg}/\text{day}$  dosage solution and submerged in a vial containing sterile saline.

The amount of remaining 80  $\mu\text{g}/\text{kg}/\text{day}$  solution was then calculated and diluted to make the 40  $\mu\text{g}/\text{kg}/\text{day}$  solution. Going from an 80  $\mu\text{g}/\text{kg}/\text{day}$  solution to a 40  $\mu\text{g}/\text{kg}/\text{day}$  solution, requires a doubling of the total amount of solution and the addition of no more PTH. Therefore, the amount of HCl, mouse serum, and saline added to the solution was 1%, 2%, and 97% of the remaining solution respectively. The pumps were filled according to the manufacturer's guidelines with the 40  $\mu\text{g}/\text{kg}/\text{day}$  solution and placed in a vial containing sterile saline.

Finally, the pumps for the control animals were filled with the vehicle solution and placed in a vial containing sterile saline.

The 4 week pumps were filled 48 hours prior to pump implantation, while the 2 week pumps were filled only 24 hours prior to pump implantation, per the manufacturer's guidelines. Once filled, they were left to prime in a water bath at 37°C until implantation.

### ***Pump implantation***

All surgical equipment was sterilized, and a sterile surgical field was created. Partial aseptic techniques were used during surgery.

Animals were anesthetized using Isoflurane, and Buprenex (0.1 mg/kg) was administered. The dorsal region of the mouse was shaved to remove fur and hair. The area was cleaned with 70% isopropyl alcohol followed by iodine and then again cleansed with 70% isopropyl alcohol. While pulling the skin away from the body with forceps, a single 5-7 mm transverse incision was made above the scapula. Using homeostats and blunt dissection techniques, a pocket was created between the subcutaneous skin and outer muscle fascia. The pump was implanted into the created pocket delivery end first. The incision was then approximated and closed with wound skin clips.

Once surgery was complete, the animals were placed on gauze under a heating lamp where they were allowed to recover. Once consciousness was regained, they were placed back in their cages (4 animals per cage).

There were 6 mice in the second experiment, with two week duration pumps that were treated for a total of 28 days. For these mice, on the 15<sup>th</sup> day of treatment the pump was removed, a DXA scan was performed, and subsequently a new pump with the same dose of PTH was implanted. The surgical procedure for the



re-surgery was essentially the same as before, the only difference being the removal of the original pump. To do this, an incision was made approximately where the original incision had been made. The pumps were pushed out through this incision and then, after being scanned, the new pumps were inserted through this same incision.

### ***Post-Surgery***

Post surgical observations were made twice in the first 24 hours. Buprenex (0.1 mg/kg) was administered twice more, approximately 8 hours and 20 hours after surgery. Post surgical observations continued to be made at least once daily for 5 days post surgery, monitoring the mice for any signs of stress, pain, or abnormal behavior. Their wound clips were removed after 10 days, and the mice were monitored for the duration of the experiment.



**Figure 2.** A mouse 7 days after pump implantation. See the shaved area and the wound clips as well as the outline of the pump down the mouse's left side.

Most animals showed no signs of pain or discomfort, continuing to move around the cage and eat normally. However, two animals showed signs of distress, one in the 4 week experiment and one in the two week experiment. They both excessively scratched the area around the incision and wound clips. One developed bald patches around both the incision and on her right hind leg. After consulting the in-house veterinarian, the bald patches were treated daily with Betadine and a topical antibiotic to keep the area from becoming infected.

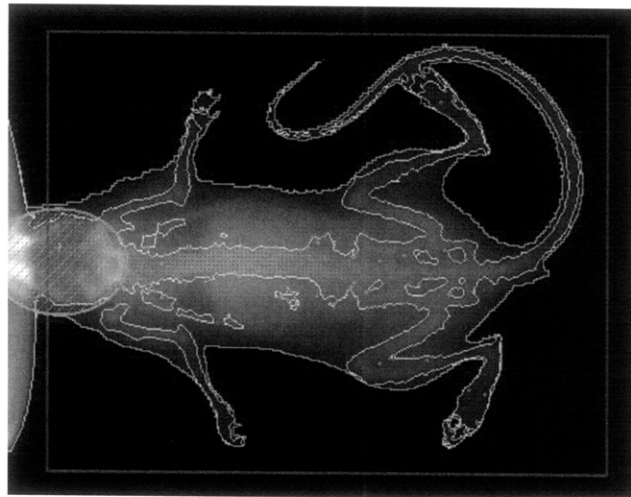
The other distressed mouse tore through her skin around the incision, and her pump became partially exposed. The pump was re-inserted, and the area was closed with wound clips. Both mice showed signs of improvement and lasted the full duration of the experiment.

### ***Blood collection by submandibular bleeding***

Blood samples of 100-200  $\mu\text{L}$  were collected from all mice at baseline (one day before pump implantation), and after 4, 7, and 14 days. For mice that went to 28 days, blood was also collected at 21 and 28 days. All but terminal blood samples were collected by submandibular bleeding techniques. The mice were anesthetized using Isoflurane and then bled. At the 4 and 7 day time points, the mice were administered subcutaneous injections of saline (20  $\mu\text{l}$ ). Terminal blood samples were collected by cardiac puncture. The serum was separated from whole blood and stored at  $-80^{\circ}\text{C}$  for the use in biochemical assays.

### ***Total body BMD and BMC by DXA***

Total body bone mineral density (BMD,  $\text{g}/\text{cm}^2$ ) and bone mineral content (BMC, g) were measured using dual-energy x-ray absorptiometry (DXA) at baseline and end of the study (PIXImus2, GE Lunar Cop., Madison, WI, USA).<sup>23-24</sup> Terminal scans were performed after sacrifice and pump removal. Additionally, as previously mentioned, the 6 mice that had new pumps put in at 2 weeks also had a DXA scan performed between the removal of the old pump and the insertion of the new pump. In order to perform the scans, the mice were briefly anesthetized using Isoflurane and placed on the platform of the PIXImus2. Due to the metal in the implanted pump, obtaining midpoint DXA scans for the other animals was impossible. The machine was calibrated with the manufacturer's phantom prior to scanning the mice.



**Figure 3.** Image of PIXImus2 evaluation. Can see the outline of the skeleton. Skeleton inside the rectangle and outside the oval is included in the evaluation of BMD and BMC.

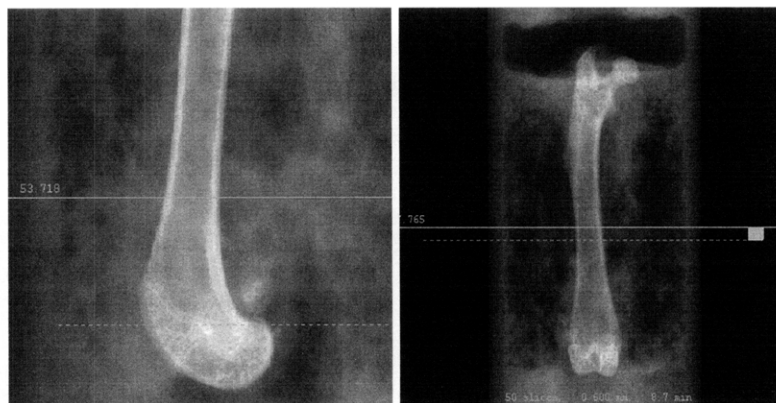
<sup>23</sup> Nagy TR, Clair AL 2000 Precision and accuracy of dual-energy X-ray absorptiometry for determining in vivo body composition of mice. *Obesity Research* 8:392-398.

<sup>24</sup> Iida-Klein A, Lu SS, Yokayama K, Dempster DW, Nieves J, Lindsay R 2003 Precision, accuracy, and reproducibility of dual X-ray absorptiometry measurements in mice in vivo. *Journal of Clinical Densitometry* 6:25-33.

The scans from the PIXImus2 were used to measure bone mineral density (BMD,  $\text{g}/\text{cm}^2$ ) and bone mineral content (BMC, g). They were analyzed using the GE Lunar PIXImus2 software. The whole body, except for the head, was included in the manually defined area of interest (Figure 3).

### ***Bone architecture by $\mu\text{CT}$***

After terminal DXA scans were performed, the fifth lumbar vertebral body ( $L_5$ ), right femur, and left femur were dissected and cleaned of soft tissue. The right femurs were placed in 70% ethanol to preserve them for histology, while both the  $L_5$  vertebrae and left femur were wrapped in saline soaked gauze and frozen in order to preserve them for  $\mu\text{CT}$  scanning and future mechanical testing.



**Figure 4.** 2D  $\mu\text{CT}$  images showing the regions of the femur that were scanned and subsequently evaluated. Distal femur shown on the left, femoral midshaft shown on the right.

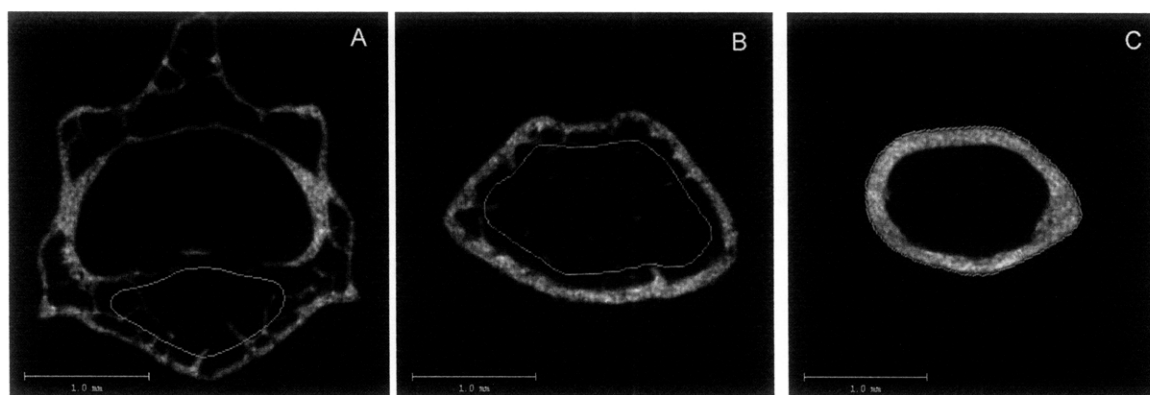
Trabecular and cortical bone architecture was assessed using a high resolution desktop tomographic imaging system ( $\mu\text{CT}40$ , Scanco Medical AG, Basserdorf Switzerland). Trabecular bone architecture was assessed for the  $L_5$  vertebrae as well as the distal femur, and the cortical bone morphology was evaluated at the femoral midshaft. CT images were reconstructed in  $1024 \times 1024$  -pixel matrices and stored in 3-D arrays. The resulting grayscale images were segmented using a constrained Gaussian filter ( $\sigma = 0.8$ , support = 1) to remove noise, and an image specific threshold determined by an adaptive-iterative algorithm,<sup>25,26,27</sup> was used to extract the structure of the mineralized tissue.

<sup>25</sup> Ridler T, Calard S 1978 Picture thresholding using an iterative selection method. IEEE Transactions on System, Man and Cybernetics. SMC-8:630-632.

<sup>26</sup> Meinel L, Fajardo R, Hofmann S, Langer R, Chen J, Snyder B, Vunjak-Novakovic G, Kaplan D 2005 Silk implants for the healing of critical size bone defects. Bone 37:688-698.

For the vertebrae, transverse CT slices (12  $\mu\text{m}$  isotropic voxel size) were acquired for the entire vertebral body. The trabecular bone region within the vertebral body, excluding the superior and inferior endplates, was manually identified (Figure 5A). For the distal femur, 204 transverse CT slices were acquired starting at the tip of the growth plate and extending 2500  $\mu\text{m}$  proximally (Figure 4). The region of evaluated slices began at the tip of the growth plate and extended to  $\sim 1800$   $\mu\text{m}$  above the growth plate. The region of interest, trabecular bone excluding the primary spongiosa, was manually identified and outlined on each CT slice (Figure 5B). Parameters including bone volume fraction (BV/TV, %), trabecular thickness (Tb.Th,  $\mu\text{m}$ ), trabecular number (Tb.N,  $\text{mm}^{-1}$ ), trabecular separation (Tb.Sp,  $\mu\text{m}$ ), structural model index (SMI), and connectivity density (ConnDens,  $\text{mm}^{-3}$ ) were computed.

The cortical bone architecture was determined from the femoral midshaft, at which 50 transverse CT slices were obtained (Figure 4). Again, the region of interest was manually identified on each CT slice (Figure 5C). Measured parameters included total cross-sectional area (TA,  $\text{mm}^2$ ), cortical bone area (BA,  $\text{mm}^2$ ), medullary area (MA,  $\text{mm}^2$ ), cortical thickness (CortTh,  $\mu\text{m}$ ), and bone area fraction (BA/TA, %).



**Figure 5.** 2D  $\mu\text{CT}$  images showing the regions of trabecular bone evaluation in the  $L_5$  vertebrae (A) and the distal femur (B), and the region of cortical bone evaluation in the femoral midshaft (C).

### ***Blood serum calcium measurement***

Total serum calcium was determined in duplicate by the QuantiChrom<sup>TM</sup> Calcium Assay Kit (DICA-500). The kit uses a phenolsulphonephthalein dye which turns a very stable blue color when binds specifically with free calcium. When read at 612 nm, the optical density is directly proportional to the calcium concentration of the sample.

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<sup>27</sup> Rajagopalan S, Lu L, Yaszemski MJ, Robb RA 2005 Optimal segmentation of microcomputed tomographic images of porous tissue-engineering scaffolds. *Journal of Biomedical Materials Research Part A* 75:877-887.

Mice were chosen at random from each of the 6 groups (VEH, PTH-40, and PTH-80 from both the 2 week and 4 week studies). Serum from days 4, 7, 14 and 28 (n = 2, 4, 4, 6 from each group respectively) were included in the evaluation.

The optical densities of the samples were read at 610 nm (VMax Kinetic Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). Calculation of the serum calcium levels was done according to manufacturer instructions. After determining that there was no statistically significant difference between the serum calcium levels at any single time point between mice with the same PTH dosage from the 2 and 4 week studies, the groups of the same time point and dosage were combined.

### ***Data analysis***

Standard descriptive statistics were computed. ANOVA was used to assess the effect of PTH dose on skeletal composition. Posthoc testing was performed using Fisher's protected least squares difference (PLSD). All tests were two-tailed, with differences considered significant at  $p < 0.05$ . Data are presented as mean  $\pm$  SE, unless otherwise noted.

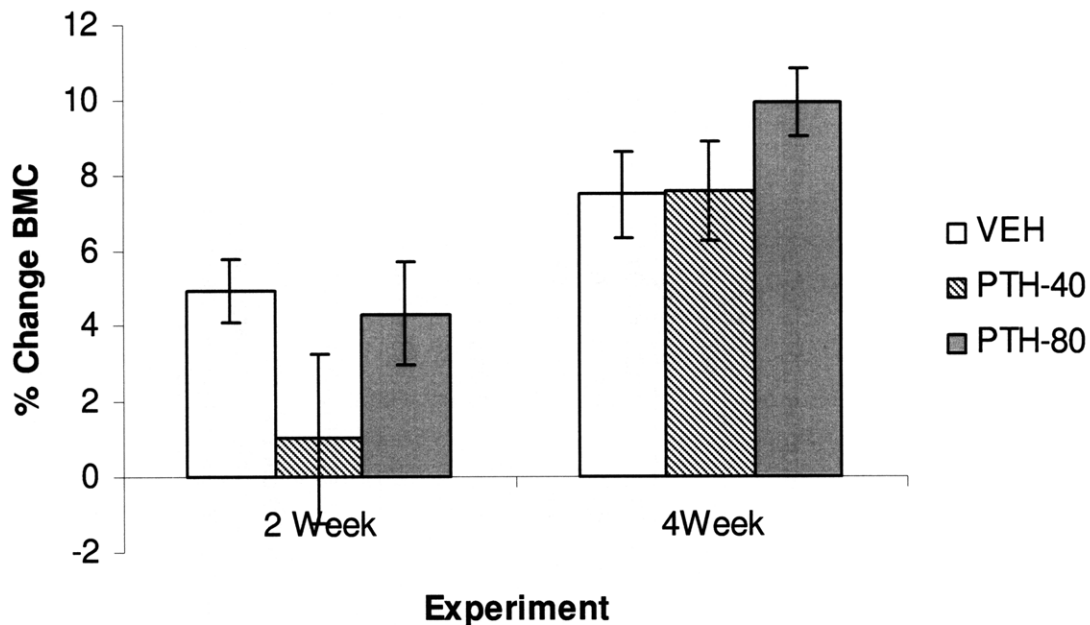


## Results

### **Total body BMD and BMC by DXA**

At baseline (10 or 11 weeks of age) there were no significant differences in either total body BMD or BMC between PTH infused groups and the control groups in the two or four week duration studies. After two weeks of infusion, BMC tended to increase less in PTH-40 mice than in VEH and PTH-80 mice, although this difference did not become significant ( $p = 0.15$  versus control, Table 2, Figure 6).

After four weeks of PTH infusion, BMC tended to increase more in PTH-80 mice than in either VEH or PTH-40 mice, but also did not reach significance ( $p = 0.14$  versus control, Table 2, Figure 6). Changes in BMD after two and four weeks exposure to continuous PTH were also similar in all groups, any apparent increases occurring due to growth of the animals and their normal corresponding gain of cortical bone (Table 2).



**Figure 6.** Percent change in total body BMC after 2 and 4 weeks of exposure to continuous PTH. Data reported as mean  $\pm$  SE.

**Table 2. Effects of continuous infusions of hPTH(1-34) on Total Body BMC and BMD assessed by DXA**

		2 Week*			4 Week*		
		VEH (n = 6)	PTH-40 (n = 9)	PTH-80 (n = 9)	VEH (n = 8)	PTH-40 (n = 8)	PTH-80 (n = 8)
BMC (g)	Baseline	0.339 ± 0.005	0.336 ± 0.011	0.332 ± 0.009	0.336 ± 0.010	0.336 ± 0.009	0.335 ± 0.008
	Terminal	0.356 ± 0.006 <sup>a</sup>	0.339 ± 0.012	0.346 ± 0.010 <sup>a</sup>	0.362 ± 0.011 <sup>a</sup>	0.361 ± 0.008 <sup>a</sup>	0.368 ± 0.008 <sup>a</sup>
	%Change**	4.931 ± 0.834	1.045 ± 2.256	4.330 ± 1.355	7.472 ± 1.159	7.560 ± 1.303	9.914 ± 0.878
BMD (g/cm <sup>2</sup> )	Baseline	0.045 ± 0.001	0.045 ± 0.001	0.045 ± 0.001	0.045 ± 0.001	0.044 ± 0.0005	0.045 ± 0.001
	Terminal	0.0470 ± 0.001 <sup>a</sup>	0.046 ± 0.001	0.047 ± 0.001	0.046 ± 0.001 <sup>a</sup>	0.046 ± 0.001 <sup>a</sup>	0.045 ± .0005 <sup>a</sup>
	%Change**	3.504 ± 1.239	1.345 ± 1.456	2.602 ± 1.501	2.169 ± 0.633	2.623 ± 0.978	3.402 ± 1.107

Data are reported as mean ± SE.

\* 2 week data is from the 2 week time point for all animals in the 2002 pump, 2 week study. 4 week data is from the 2004 pump, 4 week study, only.

\*\* %Change = (Terminal – Baseline)/Baseline x 100.

<sup>a</sup>  $p < 0.05$  between baseline and terminal value of a particular group as determined by an unpaired t-test.

No significant differences between experimental groups as determined by ANOVA. Differences considered significant at  $p < 0.05$ .

BMC, bone mineral content; BMD, bone mineral density.



**Table 3. Effects of continuous infusions of hPTH(1-34) on Total Body BMC and BMD assessed by DXA for re-surgery mice**

		2wk VEH* (n = 6)	4wk VEH* (n = 8)	PTH-40** (n = 3)	PTH-80** (n = 2)
BMC (g)	Baseline (0 weeks)	0.339 ± 0.005	0.336 ± 0.010	0.332 ± 0.017	0.311 ± 0.014
	2 weeks	0.356 ± 0.006 <sup>ε</sup>	--	0.310 ± 0.012 <sup>a, β</sup>	0.314 ± 0.014 <sup>a</sup>
	4 weeks	--	0.362 ± 0.011 <sup>ε</sup>	0.365 ± 0.004	0.355 ± 0.012 <sup>ε</sup>
	%Change (0-2wks) <sup>#</sup>	4.931 ± 0.834	--	-6.271 ± 1.610 <sup>a, b</sup>	0.907 ± 0.234 <sup>a</sup>
	%Change (0-4wks) <sup>#</sup>	--	7.472 ± 1.159	10.554 ± 5.104	14.133 ± 1.032
	%Change (2-4wks) <sup>##</sup>	--	--	18.005 ± 4.041	13.111 ± 1.285
BMD (g/cm <sup>2</sup> )	Baseline (0 weeks)	0.045 ± 0.001	0.045 ± 0.001	0.045 ± 0.001	0.043 ± 0.001
	2 weeks	0.047 ± 0.001 <sup>ε</sup>	--	0.045 ± 0.001 <sup>β</sup>	0.044 ± 0.001
	4 weeks	--	0.046 ± 0.001 <sup>ε</sup>	0.047 ± 1.7e-4	0.047 ± 3.5e-4 <sup>ε</sup>
	%Change (0-2wks) <sup>#</sup>	3.504 ± 1.239	--	0.498 ± 2.632	2.775 ± 0.199
	%Change (0-4wks) <sup>#</sup>	--	2.169 ± 0.633	6.065 ± 3.022	7.760 ± 0.437 <sup>b</sup>
	%Change (2-4wks) <sup>##</sup>	--	--	5.543 ± 1.290	4.851 ± 0.629

Data are reported as mean ± SE.

\* VEH data at the 2 week time point is taken from the 2 week, 2002 pump, experiment; n = 6. VEH data at the 4 week time point is taken from the 4 week, 2004 pump, experiment.

\*\* Mice in these groups were had a second surgery after two weeks of treatment to implant a new 2002 model pump.

<sup>#</sup> %Change = (Terminal – Baseline)/Baseline x 100.

<sup>##</sup> %Change = (4 weeks – 2 weeks)/(2 weeks) x 100.

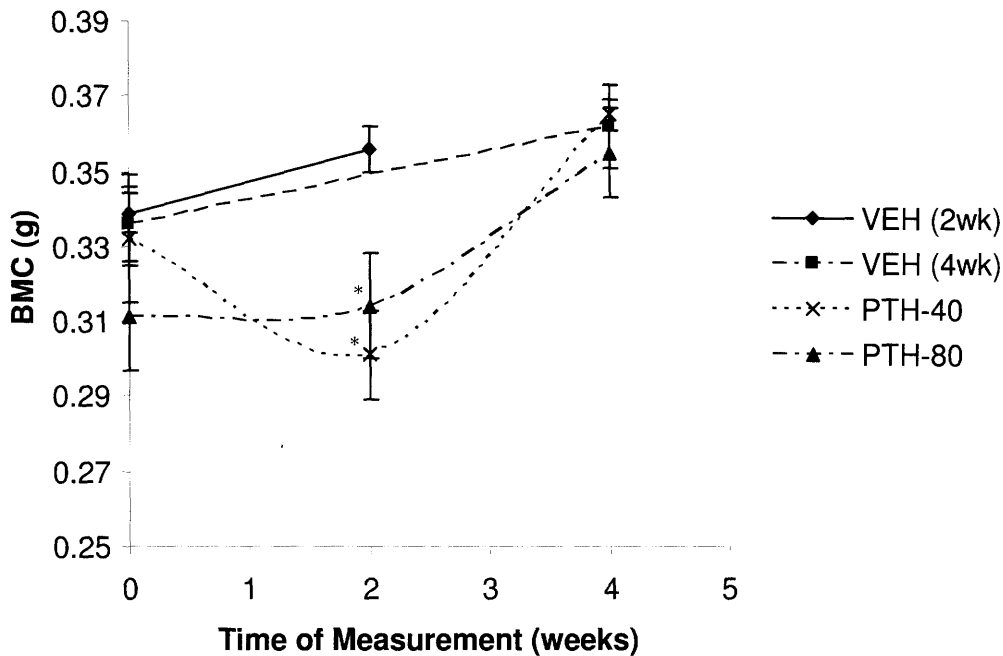
<sup>a</sup>  $p < 0.05$  for PTH vs. VEH, determined by ANOVA

<sup>b</sup>  $p < 0.05$  for PTH-40 vs. PTH-80, determined by ANOVA.

<sup>ε</sup>  $p < 0.05$  between baseline and time-point values of a particular group as determined by an unpaired t-test.

<sup>β</sup>  $p < 0.05$  between 2wk and 4wk values of a particular group as determined by an unpaired t-test.

BMC, bone mineral content; BMD, bone mineral density.



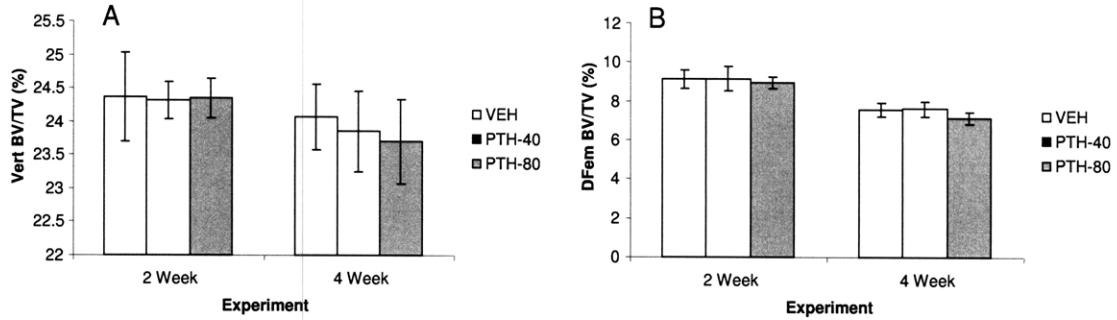
**Figure 7.** BMC (g) of the re-surgery group at the 0, 2, and 4 week time points. VEH (2wk) data from the 2 week, 2002 pump, VEH group. VEH (4wk) data from the 4 week, 2004 pump, group. \*  $p < 0.05$  as compared to VEH, determined from ANOVA.

At two weeks, the re-surgery PTH-40 and PTH-80 mice had lower than usual BMCs as compared with the rest of their groups at two weeks, and thus their BMCs were significantly lower than the VEH-treated group at this time point ( $p = .005$  and  $.02$  respectively as compared to VEH, Table 3, Figure 7). Not only did the PTH-40 group's BMC increase less than that of the VEH mice, but their BMC actually decreased after the first two weeks of treatment (Figure 7). After four weeks of PTH infusion, both PTH groups' BMC had recovered to that of the VEH, the PTH-40 and PTH-80 mice increasing their BMC by  $18.055 \pm 4.041 \%$  and  $13.111 \pm 1.285 \%$  respectively (Table 3, Figure 7).

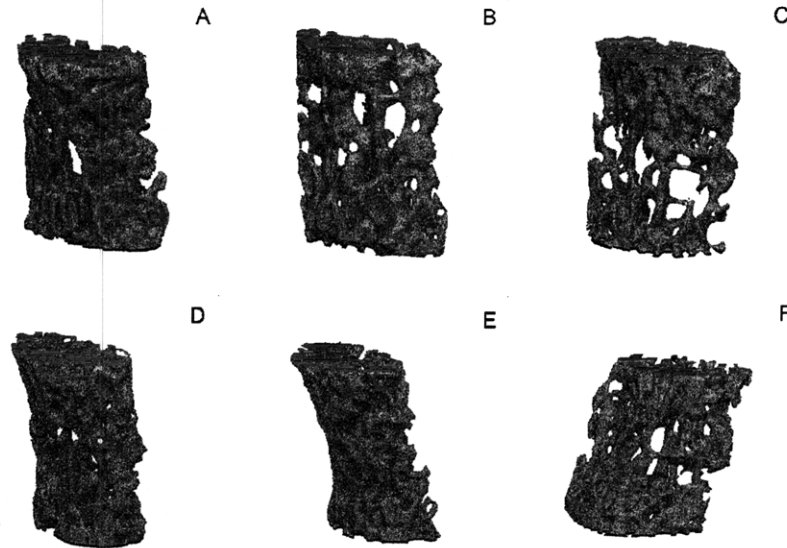
As with their BMC measurements, the BMD response in the PTH re-surgery groups was also very different after two and four weeks of treatment. Most of the PTH groups' BMD change occurred over the second two weeks of treatment; PTH-40 mice BMD increased approximately 10 times as much during the second two weeks of treatment while the PTH-80 mice gained 2 times as much during the latter part of the experiment (Table 3). BMD values never reached significant differences as compared to the VEH-treated mice.

### Trabecular bone microarchitecture by $\mu$ CT

After two weeks of treatment, the trabecular bone microarchitecture in both the L<sub>5</sub> vertebrae and the distal femur of the PTH groups did not differ from the vehicle group (Table 4, Figure 8A-B). Similarly, there were no significant differences between the PTH-40 and PTH-80 groups after two weeks, nor were there any trends towards significance in any of the other measured parameters (Table 4).



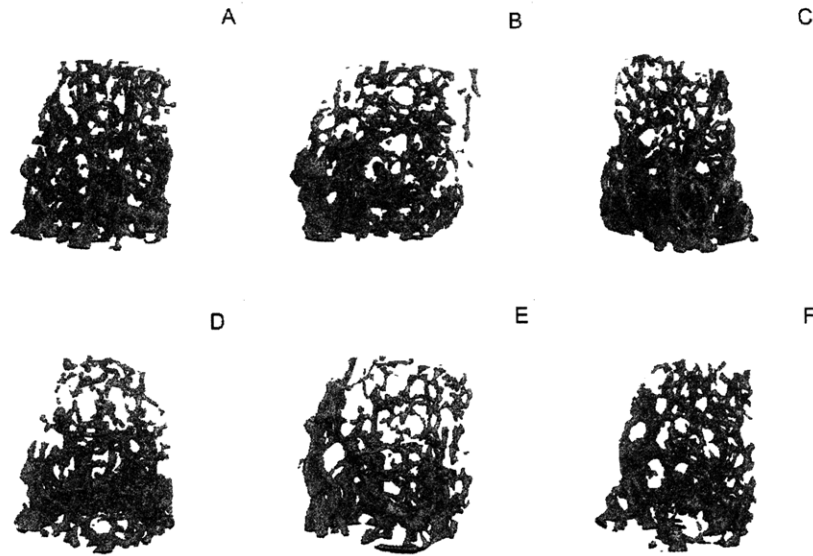
**Figure 8.** Trabecular BV/TV (%) for L<sub>5</sub> (A) and distal femur (B) in VEH and PTH treated mice for durations of 2 and 4 weeks (mean  $\pm$  SE).



**Figure 9.** 3D images showing the trabecular microarchitecture of the L<sub>5</sub> vertebrae by  $\mu$ CT analysis. A, B and C are VEH, PTH-40, and PTH-80 vertebrae, respectively, from the 2 week study. D, E, and F are VEH, PTH-40, and PTH-80 vertebrae, respectively, from the 4 week duration study.

After four weeks of treatment, trabecular number was about 7% lower and trabecular separation was about 8% higher in the L<sub>5</sub> vertebrae of the PTH-80

infused mice versus both VEH and PTH-40 treated ( $p = .03$  and  $0.03$  respectively versus VEH,  $p = 0.02$  and  $0.02$  versus PTH-40, Table 5, Figure 9D-F). In the distal femur trabecular BV/TV did not differ among the groups. However, trabecular thickness tended to be higher in both PTH infused groups as compared to VEH treated mice, although this did not reach significance ( +4.3%,  $p = 0.087$  and  $0.064$  for PTH-40 and PTH-80 versus VEH respectively, Figure 10E-F). As after two weeks of treatment, the BV/TVs of the three groups were relatively the same (Table 4, Figure 8A-B).



**Figure 10.** 3D images showing the trabecular microarchitecture of the distal femur by  $\mu$ CT analysis. A, B and C are VEH, PTH-40, and PTH-80 femurs, respectively, from the 2 week study. D, E, and F are VEH, PTH-40, and PTH-80 femurs, respectively, from the 4 week duration study.

In the pilot, re-surgery experiment (measurements taken after four weeks) trabecular bone volume and architecture tended to be worse in PTH-40 infused mice than in either PTH-80 or vehicle infused mice. In the L<sub>5</sub> vertebrae, the BV/TV of the PTH-40 group was significantly lower than the PTH-80 group but only tended to be lower than the control group ( $p = 0.031$  and  $0.15$  respectively, Table 5). Conversely, the BV/TV in the L<sub>5</sub> of the PTH-80 mice tended to be higher than that of the control mice ( $p = 0.15$ , Table 5). Additionally, the SMI of the PTH-40 mice was significantly higher than those of the control and PTH-80 groups, indicating a more rod-like structure of the trabeculi (+26.4%,  $p = 0.023$  and +37.2%,  $p = 0.019$  respectively, Table 5) Trabecular number and connectivity density in the L<sub>5</sub> also tended to be lower for PTH-40 compared to both vehicle and PTH-80 (Table 5).

The trabecular microarchitecture differences were less apparent in the distal femur of the re-surgery group. There tended to be fewer trabeculi and consequently a

higher trabecular separation in the PTH-40 mice compared with the control ( $p = 0.14$  and  $0.15$  respectively, Table 5). SMI and BV/TV did not differ among groups.

**Table 4. Bone microarchitecture at the L<sub>5</sub> lumbar spine, distal femur, and midfemur assessed by  $\mu$ CT**

	2 Week*			4 Week*		
	VEH (n = 6)	PTH-40 (n = 6)	PTH-80 (n = 6)	VEH (n = 8)	PTH-40 (n = 8)	PTH-80 (n = 8)
<b>L<sub>5</sub> vertebrae: trabecular</b>						
BV/TV (%)	24.36 ± 0.66	24.31 ± 0.28	24.35 ± 0.30	24.07 ± 0.49	23.85 ± 0.60	23.70 ± 0.63
Tb.N (mm <sup>-1</sup> )	4.562 ± 0.09	4.519 ± 0.09	4.519 ± 0.035	4.515 ± 0.08	4.532 ± 0.09 <sup>#</sup>	4.223 ± 0.09 **
Tb.Th (μm)	55.10 ± 0.7	54.97 ± 0.4	55.02 ± 0.3	55.45 ± 0.7	55.04 ± 0.3	56.00 ± 0.3
Tb.Sp (μm)	215.68 ± 5.2	215.73 ± 3.5	214.48 ± 1.7	216.80 ± 4.6	215.34 ± 5.9 <sup>#</sup>	235.11 ± 6.3**
ConnD (1/mm <sup>3</sup> )	146.59 ± 4.6	146.53 ± 7.4	150.492 ± 4.5	141.89 ± 5.1	145.94 ± 4.7	147.75 ± 8.5
SMI	0.839 ± 0.08	0.911 ± 0.05	0.881 ± 0.05	0.869 ± 0.07	0.914 ± 0.08	0.887 ± 0.05
<b>Distal femur: trabecular</b>						
BV/TV (%)	9.097 ± 0.48	9.145 ± 0.63	8.923 ± 0.31	7.550 ± 0.36	7.603 ± 0.40	7.128 ± 0.29
Tb.N (mm <sup>-1</sup> )	3.759 ± 0.10	3.840 ± 0.10	3.805 ± 0.03	3.435 ± 0.08	3.347 ± 0.11	3.312 ± 0.09
Tb.Th (μm)	52.93 ± 1.4	51.28 ± 1.3	51.52 ± 0.6	51.64 ± 0.7	53.37 ± 0.69	53.52 ± 0.6
Tb.Sp (μm)	266.10 ± 8.5	259.18 ± 6.9	259.90 ± 2.2	292.07 ± 7.3	301.68 ± 10.9	303.16 ± 8.5
ConnD (1/mm <sup>3</sup> )	48.55 ± 5.2	53.12 ± 5.9	44.23 ± 1.8	32.31 ± 3.8	34.52 ± 1.8	32.42 ± 3.5
SMI	3.100 ± 0.07	3.065 ± 0.09	3.135 ± 0.06	3.290 ± 0.07	3.253 ± 0.06	3.299 ± 0.07
<b>Midfemoral: cortical</b>						
TA(mm <sup>2</sup> )	1.615 ± 0.03	1.527 ± 0.07	1.612 ± 0.01	1.570 ± 0.03	1.591 ± 0.03	1.611 ± 0.03
BA (mm <sup>2</sup> )	0.638 ± 0.01	0.591 ± 0.03	0.635 ± 0.01	0.628 ± 0.01	0.632 ± 0.02	0.638 ± 0.01
MA (mm <sup>2</sup> )	0.997 ± 0.03	0.936 ± 0.04	0.977 ± 0.01	0.943 ± 0.02	0.960 ± 0.03	0.973 ± 0.02
BA/TA (%)	39.56 ± 0.50	38.66 ± 0.79	39.39 ± 0.55	40.01 ± 0.65	39.74 ± 0.84	39.63 ± 0.43
Cort.Th (μm)	155.00 ± 1.1	148.67 ± 4.5	155.50 ± 2.2	156.37 ± 2.9	156.25 ± 3.3	156.50 ± 1.3

Data are reported as mean ± SE.

\*Showing data from the 2002 pump, 2 week, group and the 2004 pump, 4 week group; no re-surgery group data is reported here.

\*\* $p < 0.05$  for PTH vs. VEH within experiment (2 or 4 week), determined by ANOVA.

<sup>#</sup> $p < 0.05$  for PTH-40 vs. PTH-80 within experiment (2 or 4 week), determined by ANOVA.

BV/TV, bone volume/tissue volume; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; ConnD, connectivity density; SMI, structure model index; TA, total area; BA, bone area; MA, medullary area; Cort.Th, cortical thickness.

**Table 5. Bone microarchitecture at the L<sub>5</sub> lumbar spine, distal femur, and midfemur assessed by  $\mu$ CT for mice who had pump replaced at 2 weeks.**

	4 Week *		4 Week, Re-surgery*	
	VEH (n = 8)		PTH-40 (n = 3)	PTH-80 (n = 2)
<b>L<sub>5</sub> vertebrae: trabecular</b>				
BV/TV (%)	24.07 ± 0.49		22.43 ± 0.71 <sup>#</sup>	25.95 ± 1.91
Tb.N (mm <sup>-1</sup> )	4.515 ± 0.08		4.248 ± 0.183	4.662 ± 0.317
Tb.Th (μm)	55.45 ± 0.7		55.93 ± 0.90	56.10 ± 0.10
Tb.Sp (μm)	216.80 ± 4.6		229.87 ± 13.43	210.80 ± 13.80
ConnD (1/mm <sup>3</sup> )	141.89 ± 5.1		131.50 ± 7.38	159.93 ± 32.01
SMI	0.869 ± 0.07		1.181 ± 0.035 <sup>**#</sup>	0.742 ± 0.096
<b>Distal femur: trabecular</b>				
BV/TV (%)	7.550 ± 0.36		6.370 ± 0.011	7.100 ± 0.010
Tb.N (mm <sup>-1</sup> )	3.435 ± 0.08		3.2000 ± 0.094	3.411 ± 0.140
Tb.Th (μm)	51.64 ± 0.7		52.57 ± 1.42	51.30 ± 1.70
Tb.Sp (μm)	292.07 ± 7.3		312.87 ± 9.12	295.20 ± 12.00
ConnD (1/mm <sup>3</sup> )	32.31 ± 3.8		26.02 ± 3.58	33.88 ± 1.21
SMI	3.290 ± 0.07		3.409 ± 0.075	3.329 ± 0.113
<b>Midfemoral: cortical</b>				
TA(mm <sup>2</sup> )	1.570 ± 0.03		1.566 ± 0.019	1.564 ± 0.053
BA (mm <sup>2</sup> )	0.628 ± 0.01		0.627 ± 0.003	0.654 ± 0.030
MA (mm <sup>2</sup> )	0.943 ± 0.02		0.939 ± 0.021	0.910 ± 0.022
BA/TA (%)	40.01 ± 0.65		40.05 ± 0.62	41.80 ± 0.53
Cort.Th (μm)	156.37 ± 2.9		156.67 ± 1.9	163.50 ± 3.5

Data are reported as mean ± SE.

\* VEH data from the 2004 pump, 4 week group. PTH data from the 2002 pump, 4 week, re-surgery group.

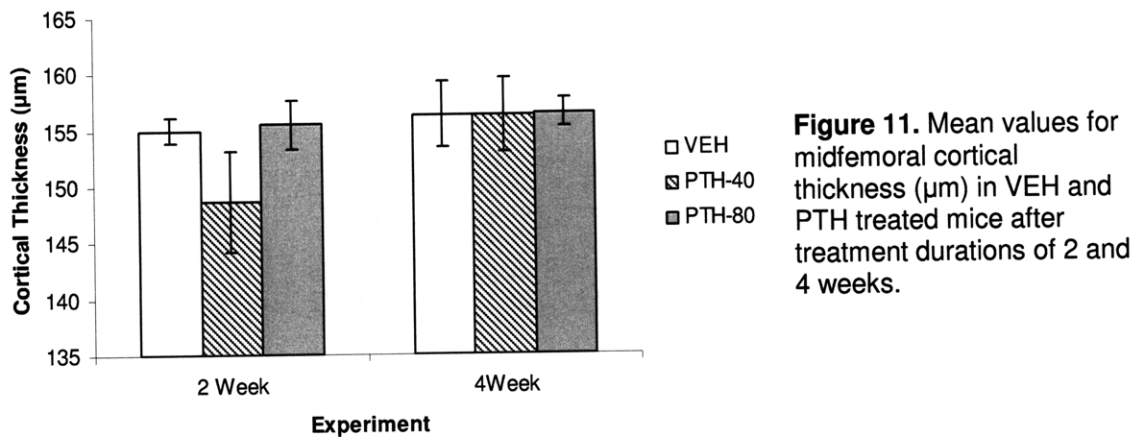
\*\* $p < 0.05$  for PTH vs. VEH, determined by ANOVA.

<sup>#</sup> $p < 0.05$  for PTH-40 vs. PTH-80, determined by ANOVA.

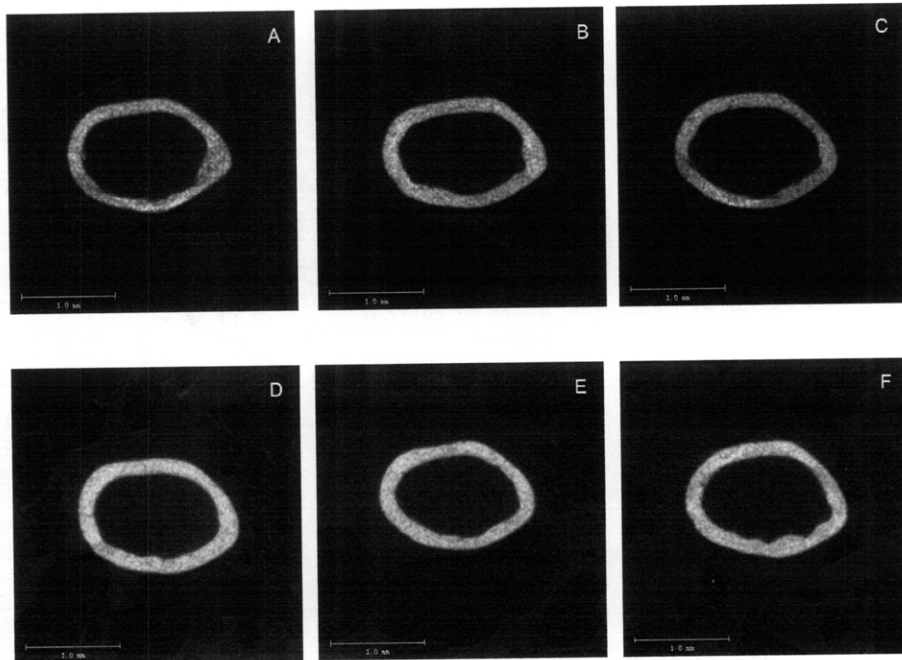
BV/TV, bone volume/tissue volume; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; ConnD, connectivity density; SMI, structure model index; TA, total area; BA, bone area; MA, medullary area; Cort.Th, cortical thickness.

## Femoral cortical bone microarchitecture by $\mu$ CT

In the two week experiment, cortical bone affects were observed only in the PTH-80 group. Although parameters did not reach significant differences, mid-femoral total cross sectional-area ( $p = 0.14$ ,  $p = 0.15$  versus VEH and PTH-80 respectively), cortical bone area ( $p = 0.06$ ,  $p = 0.08$  versus VEH and PTH-80 respectively) tended to be smaller in the PTH-40 infused mice as compared to both the vehicle and PTH-80 groups (Table 4). Additionally, the cortical thickness generally lower than vehicle and PTH-80 mice ( $p = 0.13$ ,  $p = 0.10$  versus VEH and PTH-80 respectively, Table 4, Figure 11A-C, Figure 12).



**Figure 11.** Mean values for midfemoral cortical thickness ( $\mu\text{m}$ ) in VEH and PTH treated mice after treatment durations of 2 and 4 weeks.



**Figure 12.** 2D images showing the midfemoral cortical bone microarchitecture by  $\mu$ CT analysis. A, B and C are VEH, PTH-40, and PTH-80 femurs respectively from the 2 week study. D, E, and F are VEH, PTH-40, and PTH-80 femurs respectively from the 4 week duration study.



In contrast, by four weeks of treatment, any trends that had been seen at two weeks were no longer apparent. All measured cortical bone parameters were similar across all experimental groups (Table 4).

As with the previous four week experiment, the re-surgery, 4 week experiment also showed no dose dependent PTH response in the cortical bone. Likewise, all measured parameters were similar across the experimental groups (Table 5).

## Serum calcium

At baseline, there were no significant differences among group in serum Ca. Serum Ca remained relatively constant within each experimental group over the duration of the experiment (Table 6, Figure 13). After 2 weeks of infusion, PTH-40 mice had significantly lower serum calcium levels as compared with PTH-80 (-14%,  $p = 0.038$ ). However, there were no other significant changes over time (Table 6, Figure 13).

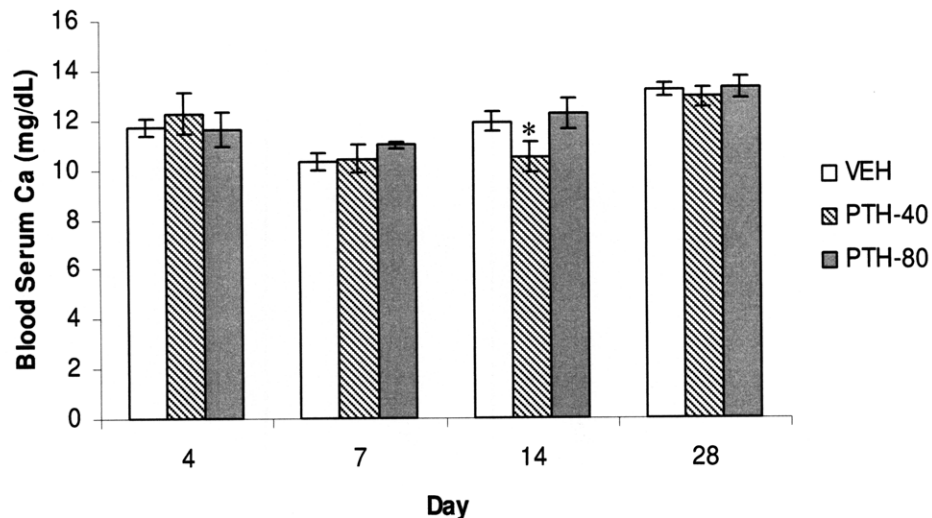
**Table 6. Blood serum calcium measurements (mg/dL)**

Day Measured	VEH	PTH-40	PTH-80
4 (n = 4)	11.727 ± 0.350	12.270 ± 0.833	11.651 ± 0.711
7 (n = 8)	10.331 ± 0.336	10.442 ± 0.534	10.999 ± 0.132
14 (n = 8)	11.918 ± 0.414	10.504 ± 0.622*	12.230 ± 0.594
28 (n = 6)	13.229 ± 0.251	12.904 ± 0.397	13.297 ± 0.403

Data is reported as mean ± SE.

n = 4, 8, 8, 6 for days 4, 7, 14, and 28 respectively. Mice were randomly chosen from each of the 2 experiments (2 weeks and 4 weeks). After determining that there was no significant difference between the blood at the same time point from the different experiments, the mice from each time point were combined into a single group yielding these n values.

\* $p < 0.05$  for PTH-40 vs. PTH-80, determined by ANOVA.



**Figure 13.** Blood serum calcium levels at 4 time points. Mice were randomly selected from each of the 2 (re-surgery mice included) and 4 week experiments. After showing no significant differences between experimental groups across the different experiments at a given time point, data from all experiments were combined to yield this data (n = 4, 8, 8, 6 for day 4, 7, 14 and 28 respectively) \*  $p < 0.05$  versus PTH-80. Represented as mean ± SE.

## Discussion

This experiment sought to develop a model of secondary hyperparathyroidism in mice and subsequently examine the skeletal consequences of continuous, high dose PTH. In PTH-treated mice we expected to see a catabolic effect in the cortical bone, relatively preserved trabecular bone microarchitecture, and an overall decrease in total body BMC and BMD. However, whereas we observed relative preservation of trabecular bone, there were no deleterious effects in cortical bone at either 2 or 4 weeks of treatment. Similarly, there seemed to be little dose dependency, as neither PTH group, 40 µg/kg/day nor 80 µg/kg/day, induced the expected catabolic response to PTH in cortical bone.

Both BMC and BMD showed no significant response to PTH infusion of either dose after 2 or 4 weeks of treatment (Table 2). Although BMC did tend to increase less during two weeks of treatment in the PTH-40 infused mice as compared with both VEH and PTH-80, it was clearly very variable across the entire group with a standard error twice that of the average change (PTH-40 %Change BMC =  $1.045 \pm 2.256$  % at 2 weeks). Additionally, one would expect that after four weeks of treatment, any catabolic response apparent after two weeks of treatment would have increased. However, after four weeks of treatment, and trend supporting a catabolic response in PTH infused mice had disappeared.

The lack of PTH effect on cortical bone is further supported by the evaluation of its microarchitecture. Although PTH-40 treated mice showed smaller midfemoral cross-sectional area and bone area, as well as thinner cortical thickness as compared to both the VEH and PTH-80 treated mice at two weeks, the differences were not significant, nor did they reach significance after four weeks of treatment (Table 3). Rather, by four weeks of treatment, these parameters were indicating that there was little cortical bone degradation as a product of PTH infusion.

Longitudinal BMC and BMD data were only available for the re-surgery PTH infused groups. While the data seems to support the notion that there is a difference in response during the first two weeks of treatment as compared to the second two weeks of treatment, it must be considered that the sample sizes for each group were very small ( $n = 3$  and  $2$  for PTH-40 and PTH-80 respectively). Additionally, although the re-surgery mice's data happened to be significant at two weeks, there were no significant differences in the BMC, BMD and their respective %changes of the PTH-groups in the two week experiment as a whole, in which the re-surgery mice were included. Therefore, although BMC measurements of the re-surgery group suggest that a two week duration PTH-40 infusion better supports a model of secondary hyperparathyroidism than a 4 week duration infusion, one cannot conclude anything due to the sample size. However, it might be interesting to further investigate the difference between two

and four weeks of infusion by conducting a re-surgery experiment with larger sample sizes.

Unlike cortical bone parameters which tended towards significance after two weeks, trabecular bone microarchitecture exhibited trends towards significance after four weeks of treatment. Additionally, as opposed to seeing effects in the PTH-40 infused group, the effect was more clearly established in the PTH-80 infused group, with a significant decrease in trabecular number and corresponding increase in trabecular separation in the vertebrae as compared to both VEH and PTH-40 treated mice. As expected, trabecular microarchitecture in the distal femur was preserved.

Not only do patients suffering from secondary hyperparathyroidism experience cortical bone loss, as indicated by their lower than normal BMC and BMD, which we did not observe in this study, they also typically experience high levels of blood serum calcium. Due to the fact that PTH is secreted in response to a low serum calcium level in order to restore it to normal, we expected that continuous elevated PTH would cause a corresponding elevated serum calcium level. Therefore, high serum calcium would be an indication that PTH levels were successfully increased in the mice. However, blood serum is generally well regulated, and there are reported cases of hyperparathyroidism in which calcium levels remain normal.<sup>28</sup> In order to better ascertain that the mice did in fact exhibit elevated levels of PTH, serum phosphorus measurements should be taken. A decrease in serum phosphorus would be a good biochemical indication that the mice were responding to an elevated PTH level. (These assays are currently being conducted).

Although Iida-Klein *et al.* also showed no effect of PTH infusion on cortical bone, our results differ from theirs in that they demonstrated a catabolic effect on trabecular architecture in mouse bone after infusion of a 40 µg/kg/day PTH dosage for two weeks through a significant decrease in trabecular connectivity density. Additionally, they shared that PTH infusion induced hypercalcemia after two weeks with a 35% increase in serum calcium, whereas this study saw no effect on serum calcium at any measured time-point. Typical cause for concern in serum calcium levels is designated at 1 mg/dL above the upper limits of normal,<sup>29</sup> about 10.3 mg/dL,<sup>30</sup> constituting only a 10% increase. An increase of 35% in serum calcium would put it at a non-physiologic level. This difference could be accounted for in the fact that they measured serum total calcium, whereas we measured free (ionized) calcium. It is believed that ionized calcium measurements are more meaningful than total calcium measurements because

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<sup>28</sup> Bilezikian JP, Khan AA, Potts JT *et al.* 2009.

<sup>29</sup> Bilezikian JP, Khan AA, Potts JT *et al.* 2009.

<sup>30</sup> Ladenson JH, Bowers GN Jr 1973 Free Calcium in Serum. II. Rigor of Homeostatic Control, Correlations with Total Serum Calcium, and Review of Data on Patients with Disturbed Calcium Metabolism. *Clinical Chemistry* 19(6):575-582.

the ionized calcium is the portion of serum calcium free to interact with its surroundings.<sup>31</sup>

One explanation as to why no clear catabolic effect was seen in PTH infused mice is because the skeletal response to PTH infusion is sensitive to estrogen levels in both rats and mice.<sup>32-33</sup> These studies strongly suggest that when delivered to estrogen replete animals, continuous infusion of PTH was reported to be anabolic to bone, as evidenced by restoration of both BMD and femoral neck loading strength. Since our mice were not ovariectomized, it is possible that their endogenous estrogen levels shielded the bone from PTH's catabolic effects. It would be interesting to repeat the experiment with ovariectomized, thus estrogen deficient, mice to see if the results then show a viable model in mice for secondary hyperparathyroidism. Similarly, vitamin D deficiency is also known to be a cause of secondary hyperparathyroidism and induce bone loss.<sup>34</sup> Therefore, the relationship that vitamin D plays should also be further investigated to determine if this might have limited the catabolic action of continuous PTH infusion.

Another reason as to why no catabolic effect was seen in the bone of the PTH infused mice could be that the catabolic action was not occurring at the distal and midfemoral portions of the bone. Rather, it was occurring in regions of the bone that we were unable to measure by  $\mu$ CT or DXA. Effects in other sections of the bone could be identified through histomorphometry.

Clearly, there is more work to be done to develop an animal model for secondary hyperparathyroidism. Although we showed a preservation of trabecular bone microarchitecture, there was no evidence of the expected catabolic effect on cortical bone. The data presented supports the notion that catabolic effects seen clinically are not caused only by elevated levels of PTH, but by many other endogenous factors as well such as estrogen and vitamin D. In the future these factors and their relationship to PTH and bone loss will need to be further assessed. Additionally, other models of secondary hyperparathyroidism that may be more reproducible include low calcium diet, which has been shown to increase PTH and induce both trabecular and cortical bone loss,<sup>35</sup> and parathyroid targeted overexpression of the cyclin D1 oncogene, which has been

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<sup>31</sup> Forman DT, Lorenzo L 1991 Ionized calcium: its significance and clinical usefulness. *Annals of Clinical and Laboratory Science* 21(5): 297-304.

<sup>32</sup> Shen V, Birchman R, Wu DD, Lindsay R 2000.

<sup>33</sup> Masiukiewicz US, Mitnick M, Grey AB, Insogna KL 2000 Estrogen modulates parathyroid hormone-induced interleukin-6 production *in vivo* and *in vitro*. *Endocrinology* 141(7):2526-2531.

<sup>34</sup> Raisz LG 2005.

<sup>35</sup> Pierroz DD, Rufo A, Bianchi EN, Glatt V, Capulli M, Rucci N, Cavat F, Rizzoli R, Teti A, Bouxsein ML, Ferrari SL 2009 Beta-Arrestin2 regulates RANKL and ephrins gene expression in response to bone remodeling in mice. *Journal of Bone and Mineral Research*. 24(5):775-784.

shown to exhibit biochemical hyperparathyroidism and bone abnormalities,<sup>36</sup> should also be further investigated.

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<sup>36</sup> Imanishi Y, Hosokawa Y, Yoshimoto K, Schipani E, Mallya S, Papanikolaou A, Kifor O, Tokura T, Sablosky M, Ledgard F, Gronowicz G, Wang TC, Schmidt EV, Hall C, Brown EM, Bronson R, Arnold A 2001 Primary hyperparathyroidism caused by parathyroid-targeted overexpression of cyclin D1 in transgenic mice. *Journal of Clinical Investigation*. 107(9):1093-1102.

## Conclusion

This thesis research sought to develop an animal model of secondary hyperparathyroidism in mice and further quantify the relationship between continuous PTH infusion and bone loss. Two different doses of hPTH(1-34) were infused into mice for either two or four weeks, after which their trabecular and cortical bone microarchitecture was evaluated as were their bone mineral content and bone mineral density. Analysis of the different bone parameters showed that PTH infusion of either dose, for either duration of time produced little catabolic effect in bone. The lack of response to PTH could be due in part to the mice not being estrogen deficient and/or vitamin D deficient, both of which could protect the bone from the catabolic effect of PTH; these effects should be further investigated in the future. Additionally, our analysis of serum calcium levels showed no significant increase in either PTH infused group after either experiment duration, and therefore phosphorus serum levels should be measured as an indicator of successfully raising PTH levels during infusion.

In conclusion, continuously infusing 40 µg/kg/day or 80 µg/kg/day of PTH into normal mice for either two or four weeks was unsuccessful in developing an animal model of secondary hyperparathyroidism. Factors influencing continuous PTH's catabolic and anabolic effects on bone remain to be elucidated. Further investigation is needed in order to better understand PTH's role in secondary hyperparathyroidism as well as how it interacts with estrogen and vitamin D during the bone remodeling process.





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