CALCITONIN MODULATES SKELETAL MINERAL LOSS DURING LACTATION THROUGH INTERACTIONS IN MAMMARY TISSUE AND DIRECTLY THOUGH OSTEOCLASTS IN BONE

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A thesis submitted to the
School of Graduate Studies
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Department of Biomedical Sciences
Faculty of Medicine
Memorial University of Newfoundland

2009

St. John's Newfoundland

ABSTRACT

The physiological role of calcitonin in mammals has been in question since it discovery. Many have speculated that the *calcitonin/calcitonin gene-related peptide (ctcgrp)* gene has endured simply because of its alternate mRNA pathway to produce calcitonin gene-related peptide-α (CGRP-α) in neural tissue. Nevertheless, a few studies have persisted in suggesting that mammalian calcitonin is vital in times of increased calcium demand, such as during lactation. Unfortunately, these surgical based studies were fraught with experimental problems stemming from lack of *true* absence of calcitonin and confounding effects of thyroid ablation. This doctoral work, therefore, was carried out to test the theses that: *calcitonin is required physiologically during lactation to protect the maternal skeleton against excessive resorption of mineral* and that *lactational bone losses in the absence of calcitonin involve a complex physiological circuitry between the breast, bone and brain.* Studies were carried out using the murine *ctcgrp* null mouse model, which is completely void of calcitonin from conception onwards. *Ctcgrp* nulls were compared to the normal, wild-type (WT) siblings as controls.

We have demonstrated that although calcitonin is unnecessary during pregnancy and the post-weaning period, it is *indeed* required physiologically during lactation since in its absence over half (51. 6% ± 4.6%) of the vertebral bone mineral content (BMC) is lost versus that seen in the WT (24.4% ± 5.4%). BMC losses were also significant within the hindlimb region and at the total body level. Loss of calcitonin during lactation is associated with increased trabecular thinning and separation. Despite these significant architectural changes, however, bone strength is no different in the *ctcgrp* null versus WT, likely due to a higher baseline bone volume in the *ctcgrp* null.

Mechanisms underlying significant BMC losses in the *ctcgrp* null versus the WT include increased mammary production of PTHrP, increased circulating PTH and increased bone resorption. Pituitary contributions to bone loss appear to be less significant. Excess calcium lost during lactation does not occur as a result of compromised intestinal absorption or increased urinary excretion but is most likely lost in the milk supply.

Our studies therefore confirm an important physiological role of calcitonin in protecting the maternal skeleton against excessive resorption during lactation. Important physiological pathways regulating bone resorption at this time include the breast and bone, with potential contributions coming from the brain. Furthermore, the post-weaning *ctcgrp* null skeleton may offer an ideal context in which skeletal recovery could be examined because anabolic mechanisms are likely upregulated.

ACKNOWLEDGEMENTS

1200 mice, 4016 aerobics classes, a family emergency, 85, 000 air miles, a real job and several steep learning curves later … I would like to take a moment to thank those that provided kind support and encouragement throughout my graduate program:

Dr. Christopher Kovacs, my supervisor, for your expertise, time and patience, as well as your appreciation for my dietetics background and the ability to connect bench side efforts to the clinical reality.

Dr. Gary Paterno and Dr. Robert Gendron, my supervisory committee, for helping define the scope of my project in the early days and providing your invaluable knowledge about the research process.

Dr. Bob Gagel and Dr. Ana Hoff at the University of Texas M. D. Anderson Cancer Center, for your research efforts as well as the production and permission to use the *ctcgrp* null model.

The Canadian Institutes of Health Research, Dietitians of Canada, The American Society of Bone and Mineral Research, The Canadian Society for Endocrinology and Metabolism, and Memorial University for generously funding my research.

The past and present inhabitants of the Calcium Research Laboratory (and associates) - Neva, Beth, Charlene, Kerri, Chris S., Adam and Darryl for your help, acceptance, and for making day-to-day life interesting.

Dr. Barbara Roebothan, for encouragement, support and honest advice since the beginning of my university career. It was great to have a professional mentor that could look through both the research lens as well as that of a Registered Dietitian.

My aerobics community (Mews Centre, Nubody's, YMCA, the Works) for helping keep me motivated.

Mom and Dad, Betty and Kevin, for being on the receiving end of my debriefings and offering endless encouragement. Jennifer, for listening and not asking 'when are you going to finish?'and finally to Raymond for putting up with my absence and for not being a lemming.

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1-αOH	1-alpha Hydroxylase
3-PBF	3 Point-Bend Fixture
3-PBT	3 Point Bend Test
AM	Adrenomedullin
ANOVA	Analysis of Variance
BMC	Bone Mineral Content
BMD	Bone Mineral Density
Bp	Base Pairs
BLKSW	Black Swiss
BSALP	Bone-specific alkaline phosphatase
Ca++	Ionized Calcium
CaR	Calcium Receptor
CGRP-alpha	Calcitonin gene related peptide-alpha
CGRP-beta	Calcitonin gene related peptide-beta
CNS	Central Nervous System
CRLR	Calcitonin receptor-like receptor
CTR	Calcitonin Receptor
CTX	Carboxyterminal cross-linking telopeptide
Dpd	Deoxypyridinoline
DPX	p-xylene-bis-pyridinium bromide
DXA (DEXA)	Duel-Energy X-ray Absorptiometer

ECaC	Epithelial Calcium Channels
ELISA	Enzyme Linked ImmunoSorbent Assay
EPM	Eroded Perimeter
FC	Field Calibration
FGF	Fibroblast Growth Factor
FSH	Folicle-Stimulating Hormone
GC	Glucocorticoids
GFR	Glomerular Filtration Rate
GH	Growth Hormone
GnRH	Gonadotrophin-Releasing Hormone
HET	Heterozygous
HHM	Humoral Hypercalcemia of Malignancy
HOM	Homozygous
IGF-1	Insulin Like Growth Factor-1
IL	Interleukin
IMD	Intermedin
IRMA	Immunoradiometric Assay
K1	Klotho Gene
LH	Luteinizing Hormone
MAP	Mean Arterial Pressure
M-CSF	Macrophage Colony-Stimulating Factor
MEN2A	Multiple Endocrine Neoplasia Type 2A

mm	Millimeter
MTC	Medullary Thyroid Carcinoma
mRNA	Messenger Ribonucleic Acid
n	Number of Observations
NaCl	Sodium Chloride
NEO	Neomycin
NS	Not-Significant
ObS	Osteoblasts Surface
OC	Osteocalcin
OcS	Osteoclasts Surface
OD	Optical Density
OPG	Osteoprotegrin
Opn	Osteopontin
OS	Osteoid Surface
p	Probability
PBM	Peak Bone Mass
PICP	Procollagen type I C propeptide
PINP	Procollagen type I N propeptide
PHV	Peak Height Velocity
PIH	Pregnancy-Induced Hypertension
PKC	Protein Kinase C
PRL	Prolactin

PTH	Parathyroid Hormone
PTHrP	Parathyroid Hormone-Related Protein
PYD	Pyridinoline
QC	Quality Control
qCT	Quantitative Computed Tomography
RAMPs	Receptor-Activity-Modifying Proteins
RANK	Receptor Activator of Nuclear Factor Kappa B
RANKL	Receptor Activator of Nuclear Factor Kappa B Ligand
ROI	Region of Interest
RMPs	Receptor Modulating Proteins
RNA	Ribonucleic Acid
RPF	Renal Plasma Flow
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SE	Standard Error
sCT	Salmon Calcitonin
STC	Stanniocalcin
STD	Standard
TGFβ	Transforming Growth Factor Beta
TRAP	Tartrate-Resistant Acid Phosphatase
TRPV	Transient Receptor Potential Vanilloid
TX	Thyroidectomy
vBMD	Volumetric Bone Mineral Density

VDR	Vitamin D Receptor
WT	Wild-type

LIST OF APPENDICES

I. Introduction

A. Preamble

The following work provides important new evidence of a critical role for calcitonin during the reproductive period, as determined by the examination of mice lacking the *ctcgrp* gene. Whether calcitonin has a physiological role in mammalian mineral metabolism has been in question for the past 40 years, since its discovery (1, 2). To better appreciate the metabolic alterations during reproduction in the absence of calcitonin, this review will begin by describing how calcium metabolism is normally regulated in the non-pregnant human and rodent. Next, calcium and bone homeostasis during normal pregnancy, lactation and the post-weaning period, will be reviewed, including the prior studies that first examined whether calcitonin plays a role during reproduction, and thereby formed the basis of this doctoral research project.

B. Functions of the Adult Skeleton

The skeletal system has evolved to include an intricate framework of bones, cartilage, ligaments and tendons. Bones partner with muscles to form the musculoskeletal system which allows movement. Other evident purposes of the adult skeleton include: protecting the internal organs from damage and aiding in hematopoiesis, the formation of blood cells in the marrow (3). Much less obvious, however, is the role that the skeleton plays in mineral homeostasis (4). Bone contains 99% of the calcium that is present in the mammalian organism (3, 4). If circulating calcium levels decrease below normal, calcium is released from the skeleton, in part through the action of parathyroid hormone (PTH) stimulated bone resorption, to meet the immediate metabolic needs (4). In contrast, when

blood calcium levels increase above normal, excess calcium can be partly stored in the bone matrix (4). This dynamic function of bone is particularly challenged in the maternal skeleton during pregnancy, lactation and the post-weaning period (5 - 8).

C. Bone Mass and Density

Bone mass is the amount of bone present in the skeleton. Bone mineral content (BMC), however, is the amount of mineral in grams contained in the skeleton at a certain site. Ash weight can be used as a surrogate for true BMC in lab animals or excised bones. In contrast, bone mineral density (BMD) properly refers to bone mass per unit volume (g/cm^3). In clinical practice volumetric BMD (vBMD) is rarely measured because it requires computed tomographic scanning and high dose radiation exposure (9, 10). More commonly, a 2-dimensional measurement, areal bone density (g/cm^2), is used to approximate volumetric BMD (9, 10). Clinically, less invasive techniques such as dual-energy x-ray absorptiometry (DXA) are used to estimate *in vivo* BMC and areal BMD (9, 10). These parameters are used to assess an individuals risk for fracture, or conditions characterized by low bone mass and increased skeletal fragility, such as osteoporosis (11, 12). In humans, and in many other species, a consistent pattern is seen in bone mass with age (11-14) (**Figure 1**).

1. Skeletal Growth and Achievement of Peak Bone Mass

During infancy, childhood and adolescence bones grow significantly, not only in length but also in width (13, 14). These changes are facilitated by the sex steroids as well as other hormones and factors such as growth hormone (GH) and insulin-like growth

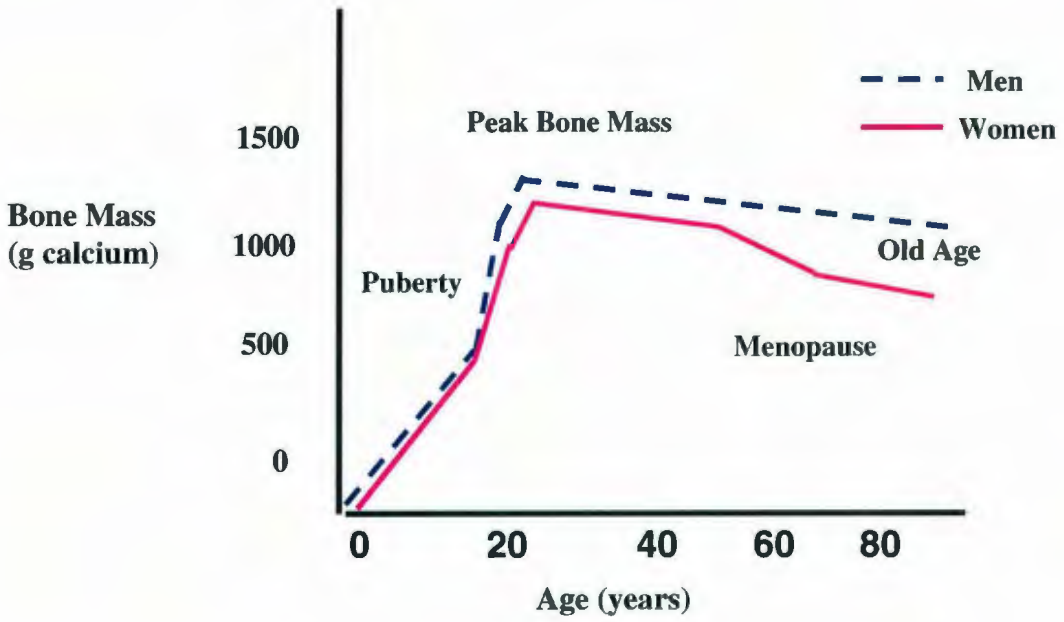


Figure 1: Pattern in Bone Mass with Age. During childhood and puberty bone mass increases significantly (13, 14). Peak bone mass (PBM) is achieved during young adulthood. Inevitably, endocrine changes cause a subsequent decrease in bone mass at menopause, as well as with old age (11-14). Typically bone loss in men occurs more gradually and at a later age (11 - 14).

factor I (IGF-I) (13, 14). At the cellular level, skeletal growth is accompanied by the processes of bone modeling and remodeling (13, 14). Modeling is the process by which new bone is developed and remodeling is the dynamic process in which mature bone mass is maintained through the coordinated activities of the bone cells (i.e. the osteoblasts, osteoclasts and osteocytes) (3). Thus, contrary to its static-like appearance, bone actually exists in a dynamic state (3). Bone accumulates microfractures from day-to-day damage and these areas of microfracture are broken down via osteoclasts and replaced with healthy new bone via the actions of osteoblasts (3). Bone modeling and remodeling are intricate processes controlled by many hormones, growth factors, transcription factors, and intracellular signaling events (3).

Peak bone mass (PBM) is achieved when the skeleton has reached its genetic and environmental capacity for a maximum bone mineral content/bone mineral density (BMC/BMD) (13 - 16). The mineral that exists at PBM is used as a storehouse for life (3, 13). The precise age at which PBM occurs can vary depending on gender and the site of the skeleton (i.e. axial versus appendicular) and the technique used to estimate it (12, 13). In general, PBM had been shown to occur anywhere from 17-35 years of age, with the mid-20's being a commonly accepted time point (**Figure 1**) (13, 14). Peak height velocity (PHV), or the point in which we attain our adult height, typically occurs within two years of puberty (13, 14).

Some of the factors that have been shown to influence the achievement of PBM and PHV include dietary intake (e.g. calcium, vitamin D, protein) (15), biomechanical loading (e.g. body weight, weight bearing physical activity) (15), timing of puberty (i.e. flux of gonadal steroids) (13, 14) and various genes (e.g. vitamin D receptor (VDR),

collagen receptor, estrogen receptor-α, transforming growth factor-β (TGFβ), α2HS-glycoprotein, collagen 1α1 and the calcium receptor (CaR)) (16). It has been estimated that 60–80% of the variance in BMD is determined by genetic factors (12-16).

Prior to the achievement of PBM, bones exist in a relatively impressionable state (17, 18). Studies in young athletes who play racquet sports (e.g. tennis and squash) have shown that the 'dominant arm' has significantly greater BMD than the 'non-dominant' arm (17, 18). This effect, however, is not nearly as striking in individuals who start playing sports as adults, after the achievement of PBM (17, 18).

After PBM has been achieved, bone mass plateaus (**Figure 1**) (14). This relatively steady state typically persists throughout adulthood and is maintained through the balanced activities of the osteoblasts and osteoclasts (i.e. equal formation and resorption rates) (14). In the normal adult skeleton this cellular balance typically cannot be altered to gain bone mass, once PBM has been achieved. Unfortunately, however, many factors can cause the adult skeleton to readily lose bone (11, 12, 15, 16).

2. Loss of Bone Mass

Some of the factors that could contribute to loss of bone mass include: nutritional deficiencies (e.g. calcium, vitamin D, protein, etc) (19), prolonged periods of skeletal unloading (e.g. extended bed-rest or lack of gravitational force on bone seen during space missions) (20), endocrine disorders (e.g. hyperthyroidism, hyperparathyroidism), bone marrow disorders (e.g. leukemia), gastrointestinal conditions (e.g. celiac disease or inflammatory bowel disease) (21), long-term use of glucocorticoids (GC) (21) and age-related endocrine changes (11).

Inevitable age-related endocrine changes are accompanied with a multitude of alterations in the bone microenvironment, many of which are not completely understood (11, 12). In women, a rapid loss of bone occurs in the first 5 years of menopause and then a more gradual loss follows thereafter (**Figure 1**) (11, 12). In men, declines in bone mass are also seen (11, 12, 15). Typically, however, bone losses in men occur more gradually and at a later age (**Figure 1**) (15). Unfortunately, bone mass that is lost as a result of age or any of the other aforementioned reasons, is largely irreversible (11, 12, 15, 20, 21).

3. Quantitative Assessment of Bone

Quantitative assessment of bone tissue may include an array of techniques, including: evaluation of bone mass, bone strength, markers of bone turnover, and histomorphometric analysis (static and dynamic) (22- 27). The techniques that are most relevant to this research will be mentioned in more detail below.

Bone Mineral Content and Bone Mineral Density

DXA is frequently used in the clinical setting to assess areal BMD and BMC (22, 23). DXA uses two low energy x-ray wavelengths that are relatively specific to being blocked by calcium (22, 23). The amount of x-ray reaching the detector is inversely proportional to the grams of calcium that lies between the x-ray source and the detector. The areal BMD is subsequently determined by dividing by the cross-sectional area of bone (22, 23). A low score may indicate that the bone is less dense than average and thus may be more prone to fracture. A limitation using areal BMD, is that the value is not corrected for height and weight, and thereby the size of the bone being measured. Thus, a

bone of normal density, but smaller width than average will give a low areal BMD reading (22, 23).

Computed tomography, in contrast, provides three-dimensional imaging and reports BMD as a volumetric density measurement (22, 23). This method can be quantitative (i.e. qCT) and is capable of isolating the region of interest (ROI) from surrounding tissues and providing information pertaining to the trabecular microarchitecture (i.e. trabecular separation distribution, trabecular thickness distribution, etc.). The qCT radiation dose is much higher than that of DXA, and that is why it is not routinely used for this purpose (22, 23). For smaller analyses, such as the assessment of rodent bone, micro CT (μCT) is also used.

Bone Strength

Biomechanical tests are often performed on bone biopsies, in animals, to quantitatively evaluate strength and elasticity (24). Bone strength is considered the capacity to resist the application of force and bone elasticity is the ability to deform under stress (24). Compromised strength and elasticity, resulting from excess demineralization, can result in increased bone fragility (24). Bone biomechanics is a complex field with established nomenclature (25). Commonly used biomechanical tests include the 3-point bend test (3PBT) and the vertebral crush test.

The 3PBT is a flexural test that provides information regarding the amount of load (i.e. force in Newton's) that can be supplied to the bone until it breaks (i.e. failure point) and the displacement of the bone from its starting position after failure (**Figure 2**)

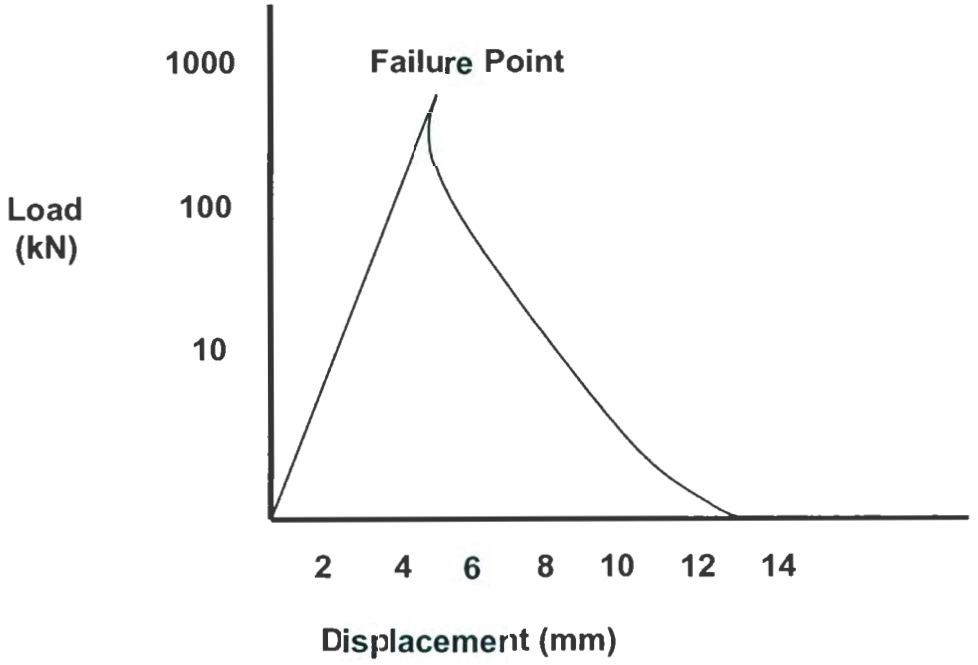


Figure 2: Standard Load/ Displacement Biomechanical Curve. During a 3-point bend flexural test (3PBT) an increasing load (kilonewtons (kN)) is applied until the bone cannot with stand the additional weight. This is noted on the curve as the failure point. The distance the bone travels from its initial position is noted as its displacement millimeters (mm).

(24). This test can also provide values for the modulus of elasticity (i.e. the degree to which the sample deforms as determined by the degree of stiffness) (24). The main advantage of a 3PBT is the simplicity of the specimen preparation and analysis (24). However, this method also has some disadvantages: the results of the testing method are sensitive to specimen and loading geometry (24).

The vertebral crush test, in contrast, is a compressive test, that supplies an evenly distributed load on the bone sample. Similar to the 3PBT, the main limitation of the vertebral crush test is the geometry of the bone. If a specimen has an irregular surface, the progressive test load may initially come into contact with a 'high-point' (i.e. a large irregularity) on the bone surface and cause erratic spikes on the load/displacement curve (**Figure 3**). These spikes may make it challenging to determine the mean failure point of each specimen.

Bone Histomorphometry

Bone biopsy samples can be assessed by looking at the microscopic organization via histomorphometry (25, 26). Samples can be examined both statically and dynamically. Static assessment includes quantification of such parameters as: number of osteoblasts, osteoclasts, osteocytes, osteoid surface, resorption surface, total surface and void surface. These measurements are typically expressed as length (mm), area (mm^2) or absolute number and are obtained using computer-assisted software programs. Dynamic histomorphometry, in contrast, uses fluorochromes, such as tetracycline, that binds to newly formed bone at the unmineralized bone interface (bone/osteoid) where it presents

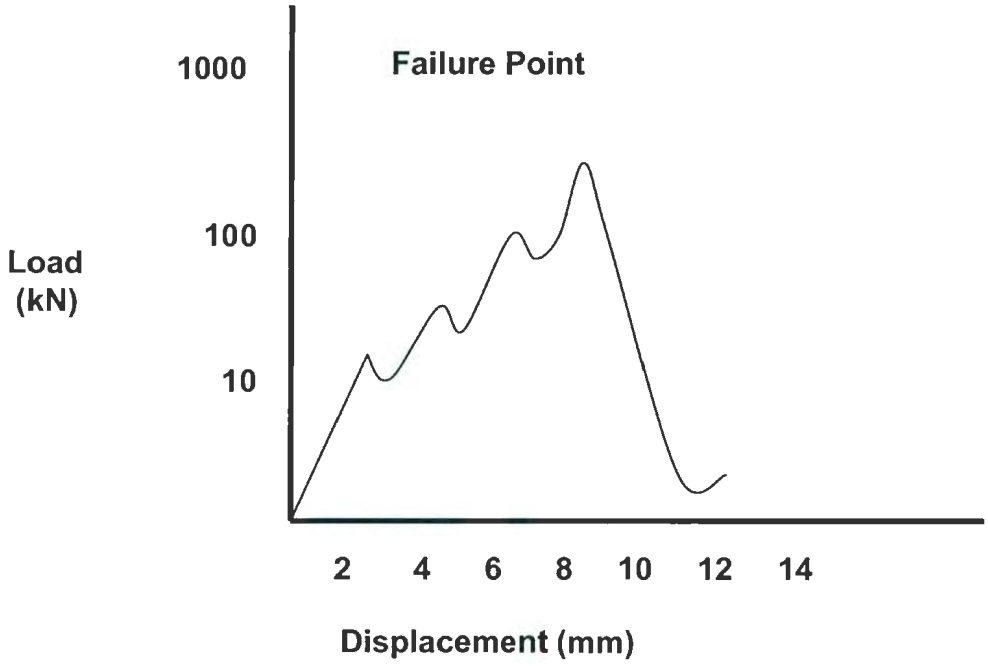


Figure 3: Vertebral Crush Test Load/ Displacement Biomechanical Curve. Erratic spikes may be seen on the load/displacement curve if a specimen has an irregular surface. These spikes make it problematic to determine the mean failure point of each specimen. The distance the bone travels from its initial position is noted as its displacement (mm).

as a linear fluorescent band under UV light microscopy. The double-labeling process involves two separate dosing periods, typically 11-14 days apart, and thus bone formation during this time interval can be quantified by measuring the distance between the two fluorescent labels. A relatively larger distance between fluorescent labels means that the rate of bone formation is higher than if the bands were closer together, or if they happened to be superimposed or absent.

Bone Turnover

Biochemical markers of bone turnover are products present in the blood or urine as a result of bone formation or resorption, which are also used to assess bone turnover (27). This non-invasive technique is often used to complement static and dynamic histomorphometric analysis or used in place of it where bone biopsy is not commonly done (e.g. in humans). Bone turnover markers provide a reliable measure of bone formation and resorption in grouped data (e.g. population trials). Values are compared to normal controls to indicate if there is a relative change (27). Bone turnover markers, however, are not as reliable when looking at an individual subject.

Osteoblasts secrete a number of proteins in the serum that are used to approximate bone formation. Commonly used bone formation markers include bone-specific alkaline phosphatase (BSALP), procollagen type I C propeptide (PICP), procollagen type I N propeptide (PINP), and osteocalcin (OC) (27).

In contrast, bone resorption markers can be measured in both the serum and urine. Resorption markers typically assess the presence of bone collagen degradation (e.g. carboxyterminal cross-linking telopeptide (CTX) of bone collagen deoxypyridinoline

(DPD), pyridinoline (PYD), and crosslinked peptide of the carboxy-terminal telopeptide of type I collagen (ICTP), or are products secreted directly by osteoclast cells (e.g. tartrate-resistant acid phosphatase (TRAP)) (27). Bone resorption can also occur as a result of osteocyte activity, or a process called osteocytic osteolysis (3).

D. Calcium Homeostasis in the Adult

Calcium is necessary in humans as well as in other species for: nerve transduction, muscle contraction, growth, as well as cellular metabolism (4, 5, 28, 29). Although unique mechanisms for calcium regulation have evolved distinctly in different species (e.g. fish mainly use their gills to exchange calcium with the environment, whereas humans use calcium from food in the gut) deviations from a normal calcium 'set-point' may result in adverse effects in all species (4, 8, 28, 29).

In general, calcium homeostasis refers to the series of physiological events that maintain the concentration of serum calcium such that normal body function is sustained and health is not compromised (4, 28, 29). Physiological compartments involved in the exchange of calcium include: dietary intake, intestinal absorption, fecal excretion, renal excretion and reabsorption, and bone formation and resorption (**Figure 4**). In 'positive calcium balance' dietary intake (i.e. mainly through dairy products and/or supplementation) and retention are greater than the corresponding losses in the urine, feces and intestines (28). In negative calcium balance, however, intestinal, urine and fecal losses exceed calcium intake and retention (28). A growing adolescent could be considered in a state of positive calcium balance (28). In contrast, an individual sustaining prolonged bed rest, in a postmenopausal state, or receiving glucocorticoid treatment could

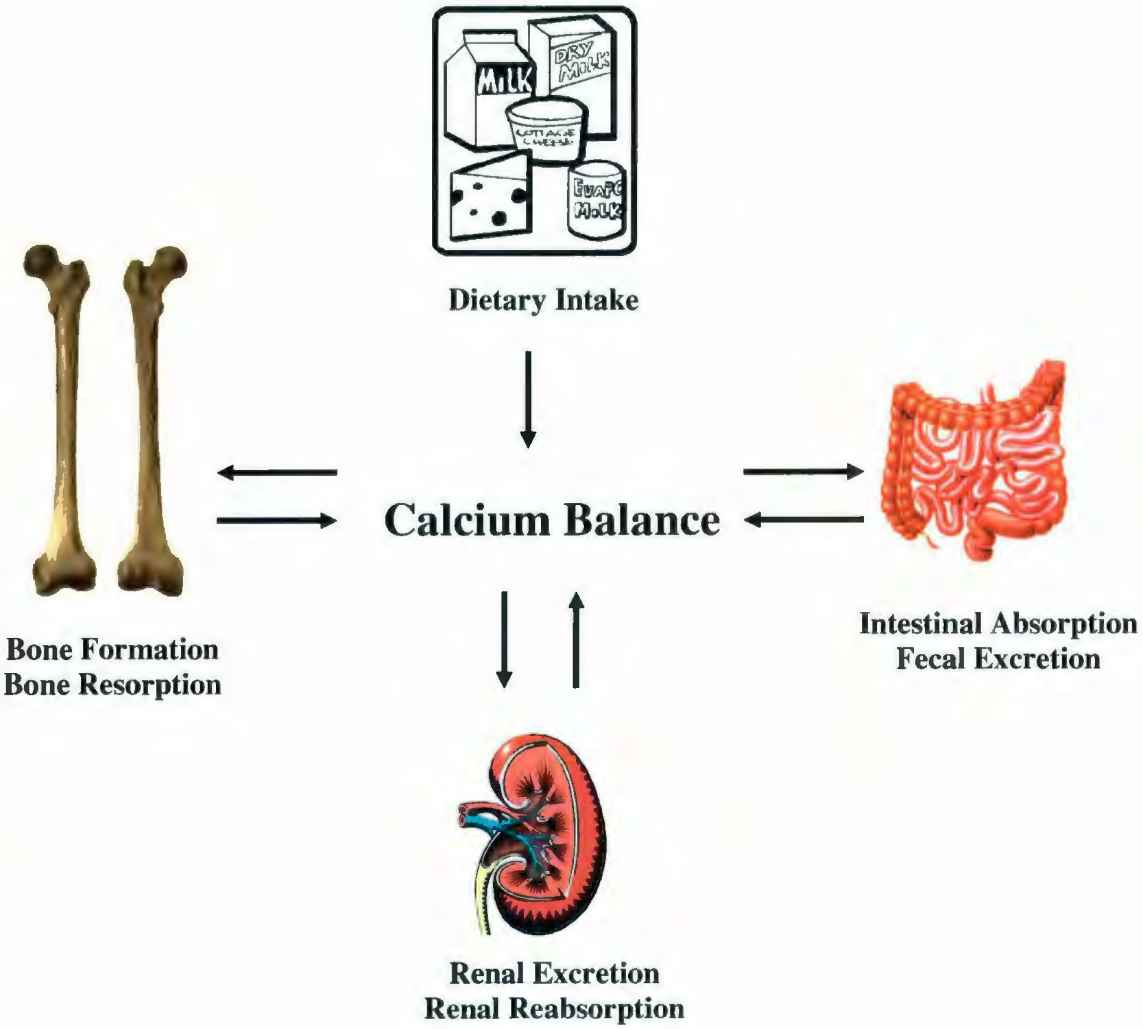


Figure 4: Calcium Balance. Physiological compartments involved in calcium exchange include: dietary intake, intestinal absorption, fecal excretion, renal excretion and reabsorption, and bone formation and resorption.

likely be considered in negative mineral balance. In zero balance, there is no net loss or gain of calcium (28).

1. Forms of Calcium

Calcium is found in the body in three main forms: bone calcium, extra- cellular or blood calcium and intracellular calcium (4, 29). About 99% of the total calcium in the body is made up of bone calcium in the form of hydroxyapatite, a crystalline structure caught in the mineral phase (4, 29). The remaining 1% exists in a pool that can rapidly be exchanged with the extracellular calcium (4, 29). The serum contains calcium in three different forms: ionized or free, protein bound (i.e. bound to albumin or globulins), or complexed to different anions (i.e. phosphate, citrate or bicarbonate) (4). Intracellular calcium is mainly found within the mitochondria and microsomes, bound to phosphates in blood and muscle (4).

All forms of calcium are tightly regulated (28, 29). Total blood calcium is often used for the clinical evaluation of calcium status because of the ease of use of automated spectrophotometric techniques (30, 31). Free or ionized calcium, although affected by pH (32), may be the more precise indicator of clinical calcium status. Through the use of calcium sensitive electrodes free calcium is now being used more frequently (30). If severe enough, both low blood calcium (i.e. hypocalcemia) and high blood calcium (i.e. hypercalcemia) can result in cardiovascular, neuromuscular, renal, gastrointestinal and skeletal complications (33, 34).

2. The Intestines and Calcium Homeostasis

The intestines are highly involved in the regulation of calcium, especially during: childhood, adolescence, early adulthood, pregnancy and lactation (28). The normal daily intestinal absorption of calcium can range from 200 – 1000 mg, depending mainly upon dietary calcium intake (28). Two main mechanisms are used to absorb calcium in the small intestines of mammals: passive or diffusional, paracellular absorption and active, saturable transcellular absorption (28). Both processes are largely dependent upon 1, 25-dihydroxyviatmin D (28).

Paracellular absorption is driven by an electrochemical gradient and varies per segment of the intestine (28). If dietary calcium intake is low absorption efficiency increases to enhance what calcium is available (28). In contrast, if calcium intake is beyond its saturation point (i.e.≈ 1000 mg) absorption will plateau (28). Active, transcellular absorption, begins with calcium being imported into the intestinal cells via a calcium channel created by the calcium transport protein 1 (i.e. transient receptor potential vanilloid type 6 (TRPV6)). This process is induced by 1, 25 dihydroxyvitamin D and estradiol (28). Subsequently calcium is stored into vesicles containing calcium binding protein calbindin 9kDa and is shipped to the basolateral surface of the cell where it is then exported into the extracellular fluid and blood via either a sodium calcium exchanger or a calcium-magnesium-dependent ATPase (28). A recent study showed that calbindin D9k null mice were fully able to absorb calcium from the intestine in response to 1,25-dihydroxyvitamin D3 (35). These authors concluded that calbindin D9k is not required for vitamin D-induced intestinal calcium absorption in the small intestine (35).

3. The Kidneys and Calcium Homeostasis

The kidneys also play an important role in delivering adequate calcium to the growing fetus and neonate (4-7, 28, 36, 37). Approximately 10 grams of calcium are filtered by the kidneys each day in humans (4-7, 28, 36, 37). The mechanisms responsible for calcium reabsorption vary depending upon the segment of kidney involved (28, 36 - 38). The vast majority of calcium is reabsorbed passively via a sodium dependent paracellular pathway (28, 36 - 38). Active calcium transport, in contrast, is controlled by a transcellular pathway that involves three processes: entry of calcium across the luminal membrane via the transient receptor potential (TRP) super family channels (i.e.TRPV5 and TRPV6), transfer through the cytoplasm where the calcium is buffered by calcium-binding proteins (i.e. calbindin-D_{28K} and calbindin D_{9K}), and active transport of calcium across the basolateral membrane by the Na^+/Ca^{2+}-exchanger and Ca^{2+}-ATPase (28, 36-38). TRPV5 and TRPV6 have been found to be controlled by 1, 25 dihydroxyvitamin D, dietary calcium intake and estrogen (38). Renal calcium handling is also controlled by PTH, calcitonin, parathyroid hormone-related protein (PTHrP), fibroblast growth factor-23 (FGF-23) and Klotho, a recently discovered gene that is prominently expressed in the kidneys (28, 38-42).

Ninety-eight percent of the calcium that enters the kidneys is reabsorbed from the tubular system back into blood, which maintains blood calcium levels within a narrow physiological range (28, 36, 37). Hypercalcemia results in an increase in renal calcium filtration whereas hypocalcemia results in a decrease in renal calcium filtration (28, 33, 34). Tubular reabsorption may be stimulated by PTH, PTHrP, phosphate, thiazide

diuretics, alkalosis, etc (28, 36, 37). Reabsorption may decrease as a result of hypercalcemia, phosphate deficiency, acidosis, etc (28, 36, 37).

4. Bone and Calcium Homeostasis

Two morphologically distinct types of bone exist in the skeleton, compact (i.e. cortical bone) and spongy (i.e. cancellous or trabecular bone) (3). Both types of bone assist in maintaining calcium homeostasis through functioning as a vast pool of mineral that can be drawn upon in times of need (3, 5). These types of bone will be referred to as cortical and trabecular bone for consistency within this research paper. Cortical bone consists of tightly packed haversian systems containing blood vessels (3). Trabecular bone, in contrast, consists of plates (trabeculae) adjacent to irregular cavities that contain bone marrow (3). Trabecular bone has a higher degree of porosity relative to cortical bone (3). This porosity provides a vast amount of free surfaces and subsequently more cellular components. As a result, trabecular bone is considered more metabolically responsive to stimuli than cortical bone (3).

5. Hormones and Calcium Homeostasis

Through the kidneys, intestines and bone, the following hormonal players help maintain normal calcium homeostasis in the adult: PTH, 1, 25-dihydroxyvitamin D3, PTHrP, the sex steroids and calcitonin.

Parathyroid Hormone

One of the predominant hormonal players involved in the regulation of

mammalian calcium homeostasis is PTH (43). PTH, in mammals, is secreted by the chief cells of the parathyroid glands (43). Secretion of PTH is regulated by the extracellular calcium concentration (Ca^{2+}) and the calcium receptor (CaR), a G protein-coupled receptor located on the chief cells of the parathyroid glands (44). Activation of the CaR on the parathyroid cells, by small increases in blood ionized calcium, cause an immediate inhibition of PTH secretion followed by decreases in PTH gene expression and parathyroid cellular proliferation (43, 44). In contrast, low calcium levels result in reduced binding of Ca^{2+} to the CaR (i.e. a negative feedback-loop), and thus PTH is then released from the parathyroids (43, 44). These actions help sustain extracellular calcium levels within an acceptable physiological range (43, 44). The physiological significance of PTH can be illustrated by considering studies on human hypoparathyroidism (45), as well as mice null for the *PTH* gene (46 - 50). Hypocalcemia and hyperphosphatemia have been found in both PTH null mice and individuals with hypoparathyroidism, both which require supplementation with calcium to restore normocalcemia (48-50).

Within the kidneys PTH: stimulates the reabsorption of calcium within the proximal tubules, inhibits the reabsorption of phosphorus in the distal tubules and directly stimulates the 1-α-hydroxylase (1-α-OH) enzyme in the formation of 1, 25-dihydroxyvitamin D (47). All of these actions help increase the normal physiological range of calcium in the serum.

Within bone, the pharmacological actions of PTH are multifaceted and complex, resulting in both anabolic (i.e. bone building) and catabolic (i.e. bone breaking down) effects (47, 51-56). Most recently the anabolic affects of PTH have been noted and confirmed using intermittent administration of exogenous PTH (51 - 53). Daily

administration of PTH has been found to increase bone mass and reduce fracture risk (53, 54). PTH has been confirmed to stimulate of preosteoblast proliferation, reversal of quiescent lining cells to active osteoblasts, increased osteoblast activity, and increased osteoblast life span by the prevention of apoptosis (53). This anabolic effect of pulsatile PTH has validated its use in the clinical treatment of post-menopausal osteoporosis. More research is needed to explore the molecular and cellular mechanisms underlying the anabolic effects of PTH, and whether it may be involved in physiological processes such as the rapid remineralization of the maternal skeleton post-weaning.

Within bone, the catabolic effects of PTH are well documented (55, 56). PTH binds to the PTH1 receptor on the bone forming osteoblast cells and the marrow stromal cell precursors (55, 56). Upon activation of these cells, PTH causes an increase in the expression of receptor activator of nuclear factor kappaB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) (55, 56). Both RANKL and M-CSF are expressed in preosteoblastic cells (56). RANKL is a member of the tumor necrosis factor (TNF) ligand super family. In the presence of M-CSF, RANKL binds to RANK receptors on the osteoclast precursor cells and promotes the differentiation, survival of mature, multinucleated bone digesting osteoclasts cells (55, 56). RANKL has also been shown to increase the resorptive capacity of the osteooclast cells, thus aiding in the release of calcium into the blood (55, 56). At the same time chronic administration of PTH also causes osteoblasts to decrease the expression of osteoprotegerin (OPG) a soluble "decoy" receptor for RANK (55, 56). Decreased OPG results in less competition for binding to the RANK receptor and thus RANKL can bind more often (55, 56). In summary, more RANK/RANKL binding equals more calcium released into the blood. This work will

focus more on the physiological and not pharmacological effects of PTH through the assessment of circulating PTH levels throughout reproduction. Given that PTH can have catabolic (i.e. bone-resorbing effects), it is reasonable to consider that PTH over activity might contribute to the lactational losses of bone mass that support milk production and neonatal growth.

1, 25-dihydroxyvitamin D3

1, 25-dihydroxyvitamin D3 is the biologically active form of vitamin D (i.e. calcitriol) formed in the kidneys after hydroxylation by the 1-αOH enzyme (57, 58). 1, 25-dihydroxyvitamin D3 binds to its intracellular hormone receptor, the vitamin D3 receptor (VDR) where it interacts with DNA on a multitude of target cells to produce an array of biological effects (57).

1, 25-dihydroxyvitamin D3 aids in the maintenance of blood calcium primarily through its actions in the intestine and bone (57). Within the small intestine, 1, 25-dihydroxyvitamin D3 enhances calcium entry into the epithelial calcium channels (ECaC). Once in the intestinal cells, 1, 25 dihydroxyvitamin D3 activates several calcium transport proteins (e.g. calbindin 9K, calmodulin) which help to shuffle calcium across the cytoplasm. 1, 25 dihydroxyvitamin D3 then activates Ca^{2+}-ATPase which actively pumps calcium across the basal lateral surface of the intestine into the circulation. In addition, 1, 25-dihydroxyvitamin D3 also aids in the release of calcium into the blood by stimulating the maturation of osteoclasts, and thus subsequent resorption of mineral (57).

Vitamin D deficient patients may present with normal to low blood ionized calcium, elevated PTH and possibly low phosphorus levels (57). Vitamin D deficiency

may occur as a result of lack of a functional VDR, dietary deficiency or lack of adequate sunlight (57). Several metabolic bone diseases may result from vitamin D deficiency, such as rickets and osteomalacia (57). Mice null for the VDR experience hypocalcemia, rickets and alopecia (58). Provision, however, of a high-calcium, high-phosphorus, lactose-supplemented diet by the third week of life in these mice prevents abnormalities in mineral ion homeostasis indicating that absence of the VDR does not have to have adverse physiological effects, if the diet is modified accordingly (58).

Parathyroid Hormone-Related Protein

PTHrP is also involved in calcium and bone homeostasis in both humans and rodents (59). Both PTH and PTHrP share amino terminal regional homology as well as a common receptor, the PTH1R (59). PTHrP is present in an array of cells including: bone, mesenchymal, smooth muscle, mammary, parathyroid, intestinal, pancreas and placenta (60- 67). PTHrP has shown to be important in: endochondral bone formation, mammary gland development, placental calcium transfer, tooth development, smooth muscle functioning as well as being responsible for most cases of humoral hypercalcemia of malignancy (HHM) (60- 67). PTHrP is having an increasingly appreciated role in calcium homeostasis during reproduction, as will be explained in Section I and M.

Sex Steroids

The gonadal steroids, including the estrogens, androgens and progesterone, play an important role in calcium and bone homeostasis in animals and humans (68, 69). Removal of the gonads in both men and women is associated with bone loss due to

increased bone resorption, and a decrease in bone formation. Studies in mice with knock out of the estrogen receptor or androgen receptor have provided supporting data regarding the *in vivo* actions of sex steroids on bone (69). Sex steroid effects on bone are likely multifaceted and complex, including possible prolongation of the osteoblast lifespan and control of osteoclastogenesis (69). Low estrogen is linked with an increase in the secretion of various cytokines (e.g. interleukin (IL) -1, IL-6, M-CSF and RANKL (69). Increased cytokine activity causes the recruitment and activation of more bone resorbing osteoclast cells, and a decrease in the antiresorbing agent, OPG (69). Minute-to-minute regulation of blood calcium in humans and rodents is unlikely to be affected by the gonadal steroids (69).

E. Calcitonin in the Adult

Another hormonal player involved in calcium homeostasis is calcitonin, a 32 amino acid peptide discovered in 1962 as a hormone postulated to regulate the 'normal level' or '*tone*' of calcium in the circulation (70-73). Initial investigators perfused blood containing high levels of calcium into the thyroid and parathyroid glands of dogs and sheep and discovered the subsequent release of a substance that lowered the blood calcium (71-75). At first it was believed that this hypocalcemic agent was produced by the parathyroid glands (71- 73). Shortly thereafter, however, it was demonstrated that calcitonin was produced specifically within the parafollicular cells ('C' cells) of the thyroid gland giving rise to the name 'thyrocalcitonin' (74, 75). More recent studies have shown calcitonin is also produced by the brain, breast, placenta and other neuroendocrine cells (76 – 80) (**Figure 5**).

Calcitonin has been postulated to help regulate normal calcium homeostasis mainly through its biological actions on the bone and kidneys (70, 76, 77). Calcitonin binds to the calcitonin receptors (CTR) on the osteoclast cells to directly inhibit both the number and resorptive capacity of these bone resorbing units, thus decreasing the amount calcium released into the circulation (77, 81). This property of calcitonin has resulted in its use in the treatment of conditions characterized by intensified bone resorption, such as osteoporosis, Paget's disease, and hypercalcemia of malignancy (70). Calcitonin also inhibits tubular reabsorption of calcium by the kidneys and thus increases the amount of calcium lost in the urine (70, 76). Although calcitonin is often referred to as being part of the triad of important calcium regulating hormones, along with the aforementioned 1, 25-dihydroxyvitain D3 and PTH, most of the effects of calcitonin are seen only at pharmacological doses and thus it has been speculated by many that calcitonin may be physiologically unimportant in mammals (1). Supporting this theory is the fact that there is no significant deficiency syndrome linked solely to the absence of the thyroid, a major source of calcitonin (1, 82 - 84). This lack of an effect is illustrated in those who have had their thyroid glands surgically removed, thus eliminating a significant source of calcitonin. These individuals appear to have non-significant alterations in calcium metabolism (83, 84). Furthermore, individuals with medullary thyroid carcinoma (MTC), and thus increased circulating levels of calcitonin (i.e. > 100 pg/ml), do not appear to exhibit any alterations in calcium regulation (85, 86).

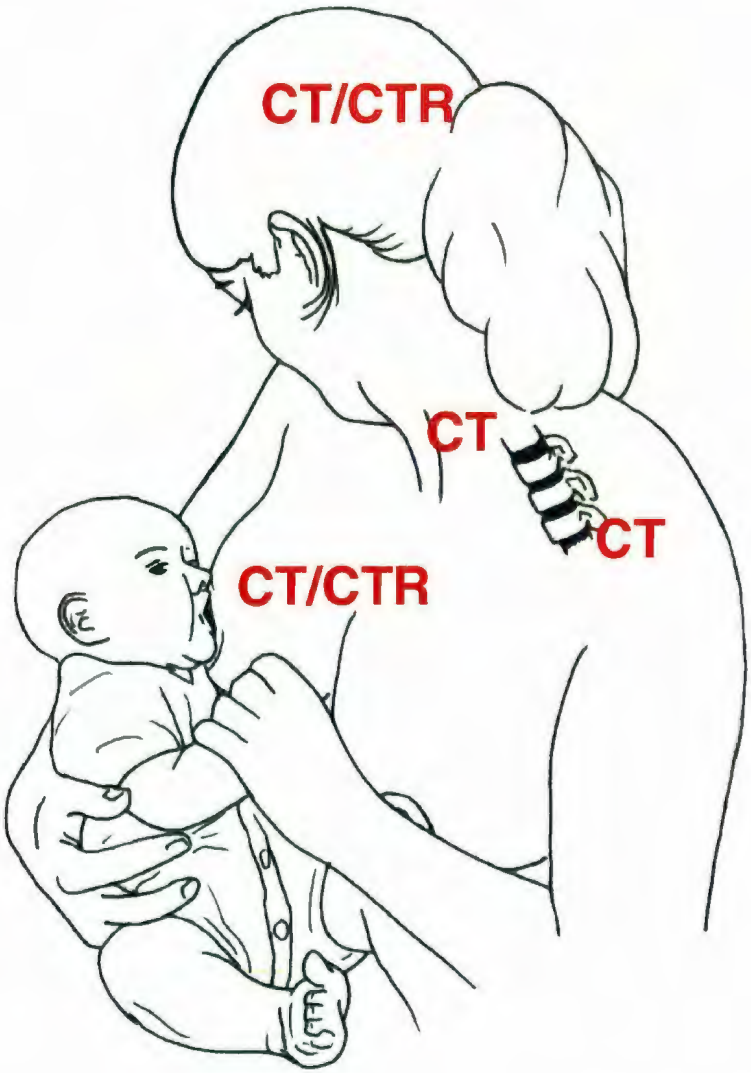


Figure 5: Location of Calcitonin and the Calcitonin Receptor within Human Physiology. Calcitonin is released in the systemic circulation after being produced by the C-cells of the thyroid gland. Calcitonin is also produced in the breast during reproduction, as well as in the pituitary lactotrophs of the brain (78 - 80). Calcitonin receptors (CTR) are located in the brain, breast, and on the osteoclasts in bone (78 - 81). *Image by C.S. Kovacs, 2005.*

There are, however, several studies that suggest calcitonin is important within mammalian physiology (70, 76, 85-90). Some of these suggested functions include: acting as an antiresorption agent for the management of bone resorping conditions (86 - 90), a gastrointestinal regulator/satiety controller (87, 88), a hypocalcemic agent/calciuric hormone (76), a tumor marker (85, 86), a regulator of acid-base balance to prevent dehydration (70), and finally, a regulator of the maternal skeleton during reproduction (1, 89, 90), just to name a few. The latter potential function, being the focus of interest, will be discussed in detail in Section *J* (Pregnancy), Section *N* (Lactation) and Section *R* (the Post-Weaning Period). First, however, an introduction to the calcitonin gene family, calcitonin receptors, and an overview of what is known about the physiological role of calcitonin based on genetic ablation studies in mice.

1. The Calcitonin Gene Family

The calcitonin gene super-family includes calcitonin, calcitonin gene-related peptide (CGRP- α), amylin, adrenomedullin (AM), as well as the recently described adrenomedullin 2 (AM2) (also referred to as intermedin (IM)) and calcitonin receptor-stimulating peptide (91, 92). For the purposes of this review emphasis will be placed on calcitonin and CGRP-α only.

Calcitonin and CGRP-α are derived from the *Calca* or *CALCA* gene. The older nomenclature '*ctcgrp*' will be used to denote the gene throughout this dissertation **(Figure 6)**, which is encoded on Chromosome 7 and 11 in mice and in humans respectively (70, 91 - 94). Alternative processing of the initial 6.5 kb gene transcript

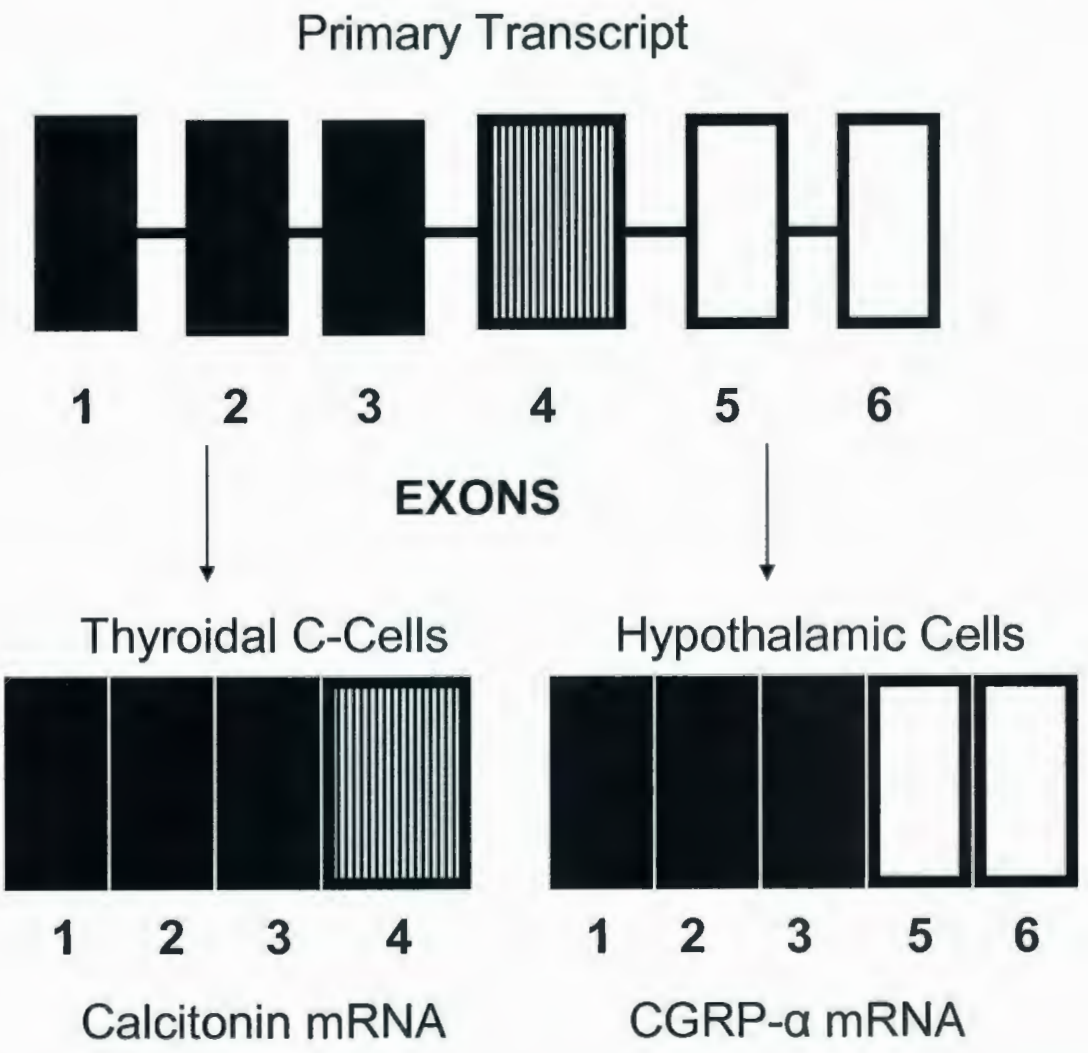


Figure 6: The *Calcitonin* Gene. Calcitonin and CGRP-α are derived from the *Calcitonin* gene. Alternative processing of the initial gene transcript results in the production of a 141-residue calcitonin-specific precursor and a 128-residue CGRP-α specific precursor. Post-translational modification of these pro-hormones results in the thyroidal C cells producing primarily calcitonin transcript and the hypothalamus producing mainly the transcript for CGRP-α. Of the 6 exons spanning the *ctcgrp* gene, 1-3 are present in both calcitonin and CGRP-α mRNA, exon 4 contains the calcitonin-coding sequence and exon 5 encodes the CGRP-α form sequence.

results in the production of a 141-residue calcitonin-specific precursor and a 128-residue CGRP-α specific precursor (70, 91 - 94). Post-translational modification of these pro-hormones result in the thyroidal C cells producing mainly calcitonin transcript and the hypothalamus producing the transcript for CGRP-α **(Figure 6)** (70, 91 - 94). CGRP and its receptors in the nervous system and in peripheral tissues are mainly involved in maintaining vascular tone, ingestive behavior, modulation of the autonomic and endocrine systems and possibly in the etiology of migraine (95- 97). Of the 6 exons spanning the *ctcgrp* gene, 1-3 are present in both calcitonin and CGRP-α mRNA (although exon 1 is not translated), exon 4 contains the calcitonin-coding sequence and exon 5 encodes the CGRP-α form sequence (70). There also exists a CGRP-β gene, however there is no calcitonin mRNA produced from the β gene (70, 91, 92).

2. Calcitonin Receptors

The peptides within the calcitonin family interact with two main seven transmembrane G protein-coupled receptors - the CTR and the calcitonin receptor- like receptor (CRLR) (70, 92, 98- 102). Calcitonin receptors are located within osteoclasts, mammary tissue during lactation as well as the nervous system (70, 92, 99 - 103). Partial homologous members of the CTR family including: CGRP, adrenomedullin and amylin, show some cross-reactivity to the CTR (100, 101). Receptor modulating proteins (RMPs) include a family of proteins that heterodimerize with the CTR or the CRLR to generate different receptor phenotypes (98, 102). Known RMPs include CGRP-receptor

component protein and a family of receptor-activity-modifying proteins (RAMPs) (98, 102).

3. Genetic Ablation of Genes within the Calcitonin Family

To better understand the physiological role of the genes within the calcitonin family, several mouse models have been genetically engineered in which the coding sequences for the following alleles: *ctcgrp-α, CGRP-α, CTR, amylin, AM* and *CRLR* have been deleted via homologous recombination (91 - 93). Each genetically engineered colony produces WT, Heterozygous (HET) and Homozygous (HOM) mice. In the *ctcgrp-α* mouse model, for example, genetically manipulated colonies include WT mice that have two normal *ctcgrp* alleles (i.e. these animals are genetically normal), HET mice that have one *ctcgrp* allele plus one mutant allele (i.e. neomycin cassette) and HOM mice (referred to as the 'null') that have 2 mutant *ctcgrp* alleles. It is important to note that null mice are completely void of the resulting peptide from conception onward (i.e. calcitonin, CGRP, CTR, etc) (91 - 93, 103- 108). In some genetic knockout mouse colonies, the absence of a particular gene results in null embryonic lethality (e.g. the CTR null). A summary of the findings on these models is summarized in **Table 1**.

In the original published report on *ctcgrp* nulls, mice were described as having significantly greater trabecular bone volume and increased bone formation at 1 and 3 months of age, versus WT mice (103). *Ctcgrp* nulls were found to be more responsive to exogenous human PTH, responding with greater increases in serum calcium and bone resorption markers, an effect that was completely reversible with calcitonin supplementation (103). In addition, it was found that *ctcgrp* null mice maintained BMC

Table 1: Summary of Calcitonin Gene Ablation Study Findings

Mouse Model	Reference No.	Summary of Findings (versus that seen in the normal, WT sibling)
Calcitonin/calcitonin gene related peptide-α (ctcgrp) *ctcgrp* null	103	***Initial phenotype:*** Unique inbred homozygous colony created in a high bone mass mouse background (C57BL/6). F3 generation were compared to unrelated WT mice and were found to: - have greater trabecular bone volume and increased bone formation at 1 and 3 months of age - be more responsiveness to exogenous human PTH - maintain BMC post ovariectomy
	104, 105	***Revised phenotype:*** F3 generation knockout model (103) backcrossed into a pure C57BL/6 mouse background (F10 generations). C57BL/6 *ctcgrp* null when compared to WT littermates were found to have: - no increase in bone mass with age - high bone turnover with age
	106	increased trabecular thinning and cortical remodeling at 3 & 6 months of age as well as 12 and 18 months of age in females
Calcitonin gene related peptide-α (CGRP-α) null	106	mice suffer from osteopenia at all ages
Calcitonin Receptor (CTR) null	107	no CTR null embryos present after 10.5 days post coitum CTR HET mice have increased bone mass due to increased bone formation
Amylin null	108	enhanced bone resorption resulting in bone loss
Calcitonin Receptor-Like Receptor (CRLR) null	109	CRLR null embryos die between 13.5 and 14.5 days of gestation. CRLR null embryos have cardiovascular abnormalities
Adrenomedullin (AM) null	110	AM nulls experience vascular abnormalities and elevated blood pressure

post-ovariectomy, versus WT mice, which lost 36% of their trabecular bone volume after two months (103). The authors concluded from this initial study that the *ctcgrp* gene reduces bone resorption in hypercalcemic states and assists in the regulation of bone formation (103).

It is important to note, however, that the aforementioned study on *ctcgrp* null mice (103) was fraught with significant confounders that limit the interpretation of the results. More specifically, mice were created in a high bone mass background strain (i.e.C57BL/6), inbred to each other, and then compared to unrelated WT mice from background strains with inherently lower bone mass (104). Also, considering that the mouse strain was created by homozygous matings, results simply may have been due to spontaneous mutations created through generations of inbreeding.

After these initial studies, it was noted, that *ctcgrp* nulls, when compared to *related* WT littermates did not exhibit the same increase in bone mass with age (104). The *ctcgrp* knockout mice (F3 generation) (103) were subsequently backcrossed into a pure C57BL/6 mouse background (F10 generation) (105). These mice demonstrated a striking phenotype characterized by increased trabecular remodeling, trabecular and cortical thinning, and cortical porosity (105). The cortical thinning lead to morphophological changes of vertebral bodies characterized by a 5% reduction of the height/width ratio in 12 and 18 month-old animals (105). The earliest indication of increased remodeling was presence in 3 and 6 month-old animals. By 12 months of age a striking phenotype had emerged characterized by trabecular and cortical thinning with evidence of cortical tunneling and collections of osteoclasts. The authors concluded that, contrary to initial findings (103) *ctcgrp* nulls had a profound and progressive phenotype characterized by

increased trabecular thinning and cortical remodeling versus that of the WT. These mice, however, did retain the greater calcemic response to PTH (105) as described earlier (103).

Consistent with R.F. Gagel's findings (105) Huebner and colleagues (106) also found that *ctcgrp* null mice experienced a high bone turnover with age. Histomorphometric analysis performed at the age of 12 months revealed significant increases in not only bone formation, but also bone resorption (106). Huebner also studied a mouse model lacking only *CGRP*-α (106). In the *CGRP*-α null mouse model exon 5 of the calcitonin gene, which encodes the sequence of *CGRP*-α, was replaced with a neomycin resistance gene (106). Mice missing only *CGRP*-α showed an osteopenic phenotype at all ages studied (106). The authors concluded that in addition to the previously described increase of bone formation in the *ctcgrp*-α null mice, there is also increased bone resorption with age (106). This suggests that calcitonin and/or CGRP may have a dual action as an inhibitor of bone remodeling, which may explain why alterations of serum calcitonin levels in humans do not result in major changes in BMD (106).

To study the consequences of the complete ablation of the murine CTR gene, exons E6 and E7 of the mCTR gene were deleted (107). Deletion of those exons effectively disabled all CTR isoforms. CTR null mice were found to be embryonic lethal (107). CTR HET mice, however, were shown to have increased bone mass due to increased bone formation (107). This again could possibly be the result of backcrossing with mice with a naturally high BMC or possibly an effect subsequent to lack of calcitonin and/or CGRP-α because there is no dominant functional receptor for the ligand to act upon.

Both calcitonin and amylin are ligands for the CTR. Mice with deletion of the amylin gene, exhibit bone loss due to enhanced bone resorption (108). Also a gene-targeted knockout model of the CRLR gene has also been generated and studied (109). Mice HET for the targeted CRLR allele appear normal, survive to adulthood, and reproduce normally (109). However, HET matings fail to produce viable CRLR null pups. CRLR null embryos die between embryonic day 13.5 and 14.5 of gestation (109). These embryos have multiple cardiovascular abnormalities, including thin vascular smooth muscle walls and small hearts also previously shown to be exhibited by AM null mice (110).

Thus, to summarize from the above studies, absence of calcitonin (and other genes from the calcitonin family) has been linked with increased bone turnover (104 – 110). Some of these results, however, remain unpublished and evidently more work needs to be completed to confirm inconsistencies and to factor in the effects of animal background on resulting bone mass. Understanding of the role of the calcitonin family of peptides is certainly not complete, manipulation of the mouse genome and production of knockout models has certainly contributed information about the physiological roles of peptides from the calcitonin family. More information, however, is required to fully extrapolate the role of calcitonin in bone biology. To date the role of calcitonin or CGRP-α during the reproductive period has not been examined. This will be the particular focus of this research.

F. Rodent Calcium Homeostasis versus Human Calcium Homeostasis

Due to the highly invasive nature of many experiments (e.g. biomechanical

testing, tissue biopsy, etc) involved in exploring calcium homeostasis and the pathogenesis of bone disease, animal models are often used instead of humans. Rodents, in particular, are a promising model because of; their relative cheap cost and availability, our awareness of their biological properties and ability to manipulate their genome, their fast generation time, and the relative ease in which we can experimentally control their environment and behavior (111). Unfortunately, however, there are also possible limitations with these models in studying calcium and bone metabolism, including the difficulty of accurately assessing the microstructures of the skeleton (i.e. due to the small size of the bones), obtaining adequate sample sizes of tissue and blood for analysis, anatomical differences in the skeletal structure, number of offspring, etc.

Rodents, however, do experience similar physiological alterations in calcium and bone metabolism during reproduction (e.g. loss of BMC during lactation, and subsequent restoration of mineral post-weaning) and share significant gene homology to humans (6-8). Thus, rodents provide a sound starting point for molecular biological experimentation.

G. Unique Periods of Bone Mineral Loss and Accretion

Studies in both animals and humans have revealed several unique physiological periods in which substantial bone mass can be lost at a rapid rate, yet completely regained afterwards. These unique periods include: antler mineralization (112), egg shell formation (113), as well as lactation and post-weaning (5-8, 114-124).

During these periods mineral is temporarily borrowed from the adult skeleton to provide for the immediate needs (e.g. mineralization of antlers, formation of egg shell, formation of milk, etc). After the requirements have been met, the skeleton completely

and rapidly rebuilds itself, returning to its former state (5-8, 114-124). The mechanisms underlying these processes have been largely unexplored. For the purpose of this review, bone alterations during pregnancy, lactation and the post-weaning period will be the focus.

1. Pregnancy, Lactation and the Post-Weaning Period

Pregnancy, lactation and the post-weaning period are accompanied with significant alterations in bone mass (5-8, 114-117, 119, 122-125). To accommodate the rapidly growing fetus, the production of milk for the neonate, and the reconstruction of the maternal skeleton post-weaning, respectively, maternal calcium and bone homeostasis must undergo major physiological adjustments **(Figure 7)** (5-8, 114-125). Perhaps the most remarkable of these alterations is the ability of the maternal skeleton to rapidly and fully remineralize itself after such a significant bone loss during lactation (5-8, 29-40, 114-125). Changes during reproduction occur at the cellular level as well as systemically (5-8, 29-40, 114-125). The metabolic alterations required for the attainment/utilization of mineral during these unique periods varies, and is different from the non-pregnant state (5-8, 29- 40, 114-131). More detail regarding the specific systemic alterations that accompany pregnancy, lactation as well as the post-weaning period will be discussed in subsequent sections regarding each of these time periods.

H. Maternal Calcium and Bone Metabolism during Pregnancy

Pregnancy is a time of incredible demand on maternal calcium and bone metabolism, as mineral must be actively transported across the placenta to support the

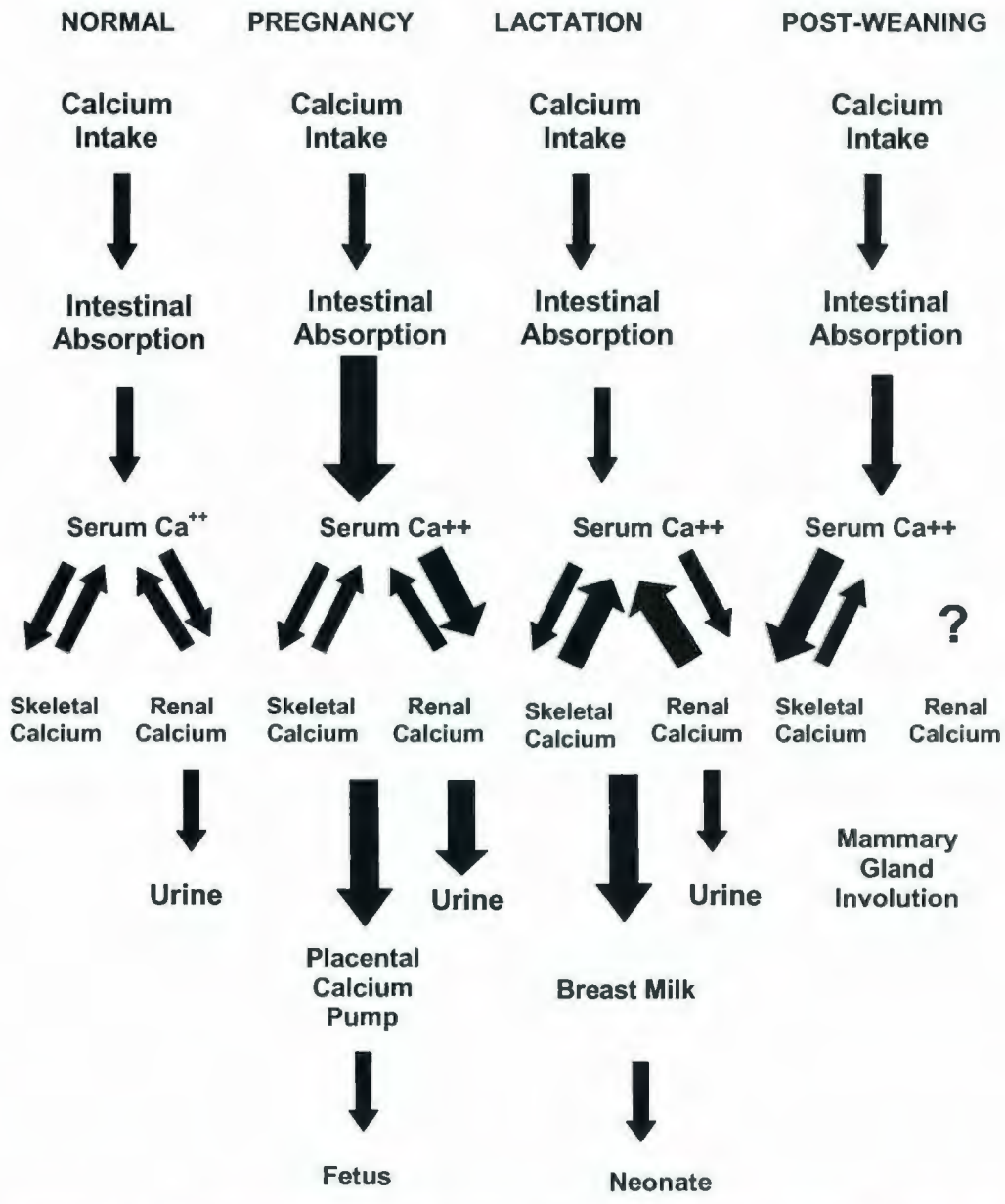


Figure 7: Calcium homeostasis during human pregnancy, lactation and the post-weaning period versus normal. Arrow thickness indicates relative increase or decrease versus normal. Image adapted from: Kovacs CS and Kronenberg HM, Maternal-Fetal Calcium and Bone Metabolism during Pregnancy, Puerperium, and Lactation, Endocrine Reviews, 1997 Dec; 18(6):832-72. *Copyright, The Endocrine Society. Permission for use can be seen in Appendix A.*

growing fetus (6-8, 29-36). The specific biological strategies that the maternal skeleton has adapted to meet the mineral needs of the growing fetus, are only partly understood (6-8). At birth the average human fetus contains 20 – 30 grams of calcium, the vast majority of which is accreted during the last trimester (121- 125). In fact, the daily calcium requirement of the growing human fetus increases from approximately 50 mg/day during the 22nd week of gestation up to 330 mg/day at 35 weeks of gestation (121- 125). To help meet these rising demands for mineral the fetus has adapted its own mechanisms for calcium regulation through unique adaptations within the placenta, kidneys, bone, and intestine. Calcium is not only required for fetal skeleton mineralization but also cellular growth, signal transduction and neurotransmitter release (6-8). The dominant biological mechanism in place to meet the increased calcium demands of pregnancy appears to be a doubling in maternal intestinal calcium absorption, facilitated at least in part by the actions of 1, 25-dihydroxyvitamin D **(Figure 7)** (126-129). Alterations in the hormonal regulators of pregnancy will be reviewed first.

I. Alterations in Hormonal Regulators of Calcium Homeostasis during Pregnancy

The hormonal milieu of pregnancy is different than that seen in the non-pregnant state (6-8, 122-131). Normal pregnancy induced alterations in: PTH, 1, 25-dihydroxyvitamin D, PTHrP, the gonodal steroids and calcitonin are discussed below.

1. Parathyroid Hormone

In human studies using intact immunochemical assays, serum PTH levels have been shown to decrease during early pregnancy and then return to mid-normal range by

the end of gestation (6-8, 130, 131). Older studies suggested pregnancy was a time of hyperparathyroidism (132, 133). These studies, however, were flawed by the use of assays that measured non-biologically active fragments of PTH in the circulation (132, 133). Women who suffer from hypoparathyroidism during pregnancy may have fewer hypocalcemic symptoms, and require less calcium supplementation (6-8). This implies lack of a role for PTH during pregnancy and suggests the normal increase in intestinal calcium absorption and circulating 1, 25-dihydroxyvitamin D at this time.

In contrast to humans, a few reports in rats suggest that pregnancy is a time of hyperparathyroidism (134, 135). Preliminary studies show that mice lacking the *PTH* gene are generally hypocalcemic and hyperphosphatemic at baseline line when compared to their WT littermates (48-50). PTH null mice have a low fertility rate and appear to be more susceptible to spontaneous death from anesthetic (an effect that was reduced when the diet was supplemented with additional calcium). During pregnancy PTH null mice receiving a 1% standard calcium chow experienced a slightly lower blood ionized calcium levels when compared to PTH null mice receiving a 2% fortified calcium chow and to the WT siblings (48-50). Despite these mineral alterations at baseline no apparent difference was observed in the cycling of BMC during pregnancy versus that experienced that of the WT littermates (48-50).

2. 1, 25-dihydroxyvitamin D

In humans 1, 25-dihydroxyvitamin D levels double beginning at early pregnancy and this level is maintained until term (126, 127, 134, 136-138). Increases in 1, 25-dihydroxyvitamin D may be attributed primarily to upregulation of the renal 1α-OH

enzyme (126). Renal 1α-OH stimulates formation of 1, 25 dihydroxyvitamin D. PTH typically stimulates 1α-OH, completing the last hydroxylation in the formation of active vitamin D (i.e. 1, 25-dihydroxyvitamin D3) in the kidneys (126). During pregnancy in humans, however, it is unlikely that PTH is the cause of the doubling in 1, 25-dihydroxyvitamin D because the rise in 1, 25-dihydroxyvitamin D begins while the PTH levels have fallen to the low-normal range (126). The maternal kidneys are speculated to be the dominant source as shown by an anephric woman who had no increase in 1, 25-dihydroxyvitamin D during pregnancy and the evidence that renal 1α-OH is upregulated (6-8). Also contributing to the rise in 1, 25-dihydroxyvitamin D3 are the placenta and fetal kidneys (6-8).

Preliminary experiments in mice null for the VDR showed that pregnancy rescued low bone mass and normalized intestinal calcium absorption (139). *Vdr* null mice entered pregnancy with a lower total body BMC (0.381 ± 0.003 grams versus 0.519 ± 0.004 grams in WT, $p<0.02$) (139). *Vdr* null mice, however, experienced a relatively greater increase in BMC during pregnancy versus that seen in the WT mice when compared to the respective baseline BMC value (i.e. *Vdr* nulls increased to 158% of the baseline BMC versus WT mice that increased to 122% of their baseline BMC value ($p<0.02$)). Findings indicated that duodenal 45 calcium was 78.7%± 3.8% in non-pregnant WT mice and $94.1\% \pm 8.8\%$ in *Vdr* null mice during pregnancy (139). Thus, based on these findings it appears that 1, 25 dihydroxyvitamin D and the associated VDR are not the only mechanisms responsible for increasing calcium absorption, because in the absence of the VDR calcium absorption is still increased during pregnancy. It appears that the increase

in calcium absorption seen during pregnancy also occurs via pathways independent of the VDR and/or 1, 25 dihydroxyvitamin D.

3. Parathyroid Hormone-Related Protein

Studies in humans and rodents have shown that serum PTHrP levels begin to increase towards late pregnancy (6-8, 121-124, 140 -144). PTHrP is produced by a vast number of both maternal and fetal tissues (including the mammary glands, placenta, fetal parathyroids, osteoblasts in bone, amnion and umbilical cord) and thus the exact source of PTHrP during pregnancy is hard to determine (6-8, 121-124, 140-144). In rats and in humans PTHrP has been suggested to regulate myometrial tone, blood flow and the relaxation of smooth muscle during pregnancy and delivery (121-126, 140-144). The amino-terminal portion of PTHrP has been shown to stimulate the renal 1α-OH enzyme and skeletal calcium resorption (59, 60). The mid-molecular portion of PTHrP specifically in the fetus has been shown to stimulate placental calcium transfer (145), and the carboxy-terminal portion, also known as 'osteostatin', has been shown to inhibit osteoclastic bone resorption and may protect the maternal skeleton from excessive resorption of mineral (146).

Thus, from the above studies it can be concluded that there are several theoretical mechanisms by which PTHrP could serve a functional role during pregnancy. Despite these findings, however, low circulating maternal PTHrP levels throughout most of gestation suggest that, if PTHrP does play a role during pregnancy it is most likely local and not systemic.

4. Sex Steroids and Other Hormones of Pregnancy

Substantial increases in circulating prolactin (PRL) and placental lactogen (PL) (also called chorionic somatomammotrophin) are seen during pregnancy in both humans and rodents (5-7, 147). Prolactin (PRL) stimulates the glandular production of colostrum and milk proteins (147). The secretion of GnRH and the gonadotropins (i.e. follicle stimulating hormone (FSH), leutinizing hormone (LH)) are inhibited during pregnancy, thus eliminating menstrual cycles at this time (5-7, 147). The involvement of these hormones in the regulation of calcium metabolism during pregnancy is largely unknown (4-7, 147). Some studies suggest that PRL and placental lactogen may stimulate intestinal calcium absorption and the synthesis of PTHrP at this time (5-7).

J. Calcitonin during Pregnancy

Serum calcitonin levels are increased during pregnancy in rodents and humans versus that of non-pregnancy (5-7, 124, 138, 148, 149). Contributions to this rise in circulating calcitonin could include the C-cells of the thyroid, the placenta and breast. Elevations in calcitonin during pregnancy provided the early rationale behind the scientific speculation that calcitonin may protect the maternal skeleton during reproduction, and perhaps during other times of greater physiologically need (5-7, 124, 138, 148-154).

Thyroidectomy (TX) was the method of choice to investigate the possible physiological role of calcitonin in early studies (82-84, 150-154). Considering the fact that the thyroid is a major source of calcitonin production, it was thought at the time that removal of the thyroid would result in production of a 'calcitonin deficient state' (150 -

152). Investigators used sheep, rats and goats that had undergone TX with subsequent thyroid replacement to examine bone mass changes throughout reproduction (150-152). Some of the studies found that these animals had lower BMC/BMD values after periods of pregnancy and lactation versus their sham operated counterparts whereas other investigators found no effect of TX (150-152). These results were inconclusive as to whether or not calcitonin may be involved in protection of the maternal skeleton from excessive losses of mineral during pregnancy, and more significantly during lactation. Unfortunately, however, extrathyroidal calcitonin sources, found in the breast, brain placenta and throughout the vast neuroendocrine system were not appreciated at this time (78-80), and thus these early studies were flawed by lack of a truly calcitonin deficient model. Whether or not calcitonin plays a critical role in calcium metabolism during pregnancy was unanswered by these particular animal studies.

K. Systemic Adaptations to Pregnancy

The large demands for mineral throughout pregnancy are met primarily through the following systemic alterations in the maternal intestines, kidneys and skeleton **(Figure 7).**

1. Intestinal Adaptations during Pregnancy

Mineral balance studies, as well as kinetic studies using calcium isotopes (^{48}Ca, ^{44}Ca, ^{42}Ca) have shown an increase in intestinal calcium absorption by as much as 50-80% compared to non-pregnant controls during human and rodent pregnancy **(Figure 7)** (5-8, 118, 123 -127, 129, 149, 153 -158). This increase in intestinal calcium absorption

has been shown to begin during early pregnancy and to continue until the end of gestation, providing an important mechanism by which the maternal skeleton adapts to meet its own mineral needs, as well as the needs of the growing fetus.

Up-regulation of intestinal calcium absorption during pregnancy may be in part due to increases in: calcium ATPase, calcium binding protein mRNA levels or TRPV6 mRNA expression in the intestine (126, 156, 157). These increases could lead to more calcium pumped out of the intestinal cell into the circulation. This rise in intestinal calcium absorption during pregnancy may allow the maternal skeleton to store calcium in advance of the fetal demands later in pregnancy. The increased intestinal calcium absorption appears to be a major maternal adaptation to meet the fetal need for calcium (126, 156, 157).

2. Renal Adaptations during Pregnancy

Urinary calcium excretion is increased during pregnancy (i.e. absorptive hypercalciuria caused by increased gastrointestinal absorption of calcium) **(Figure 7)** (158, 159). Glomerular filtration rate (GFR) and renal plasma flow (RPF) have been found to be upregulated by 40-65% and 50-85%, respectively in normal women with uncomplicated pregnancy (158, 159). Hypercalciuria occurs throughout all trimesters with the exception, in some circumstances, such as under fasting conditions (73, 158, 159).

3. Skeletal Adaptations during Pregnancy

Pregnancy is characterized by alterations in maternal calcium metabolism at the

level of the skeleton (5-7, 117, 119, 124, 160-169). Studies in humans mainly focus on markers of bone turnover with scant data considering BMC/BMD measurements during the pre and post partum periods (155, 160, 164, 165). Studies in rodents, in contrast have considered BMC/BMD alterations throughout the full reproductive period (162, 168). Data is also available for bone markers as well as alterations in bone volume (161, 162). On rare occasions fragility fractures may occur during pregnancy (165). Specific details pertaining to skeletal adaptations during pregnancy are noted below.

Markers of Bone Turnover during Pregnancy

In healthy pregnant women serum markers of bone formation, including: OC, BSAP and PICP showed a biphasic pattern with decreases in bone formation in early pregnancy, followed by a significant increase in the third trimester and post partum (122, 124, 125, 141). It is important to note, however, that these variables are often difficult to interpret due to the increased GFR of pregnancy, the effect of hemodilution during pregnancy, fetal and placental contributions to the markers in the urine and absence of pre-pregnancy (baseline) values for comparison (5-7). Thus, one may carefully conclude that bone turnover is increased during pregnancy, but more data is needed in this area.

Flux in Bone Mass during Pregnancy

Due to fetal exposure to x-rays DXA is typically avoided during human pregnancy (5-8). In contrast, animal studies commonly use DXA as a rapid and precise method to assess bone status throughout reproduction (5-8, 161, 162 -173). It is important to note, however, that DXA measurements may be confounded by drastic alterations in maternal

weight, soft tissue composition, and fluid shifts during pregnancy (166). These physiological changes could result in artifactual alterations in BMD (166). Despite the lack of sequential BMD measurements throughout full gestation periods in humans, there are a few studies that have considered pre-pregnancy and/or the post-partum period (122, 155, 164, 167).

Findings using DXA analysis pre and post pregnancy in humans have shown variable results from losses in total body and vetebral BMD during pregnancy (122), to no significant change (164). Numbers in these studies were generally low and baseline BMD measurements were often obtained at inconsistent time periods. Problems may also arise when baseline BMC measurements are taken before the individual has reached their peak bone mass, and thus post-pregnancy bone mass assessment may falsely represent general growth and not bone mass accretion due to reproduction. One human study suggested that pregnancy effects the maternal skeleton by producing a fluctuation in cancellous bone volume in which early temporary bone loss through trabecular thinning is restored in its entirety through the addition of trabeculae that produce a modestly more complex system of thinner more numerous bars by the end of gestation (174). Increases in bone volume may also be adaptive mechanisms to accommodate the weighted fetus and neonate (174).

Data in Black Swiss (BLKSW) mice suggest gains in bone mass during gestation up to 10-15% above baseline **(Figure 8)** (168). With larger litter sizes, bone mass gains in rodents during pregnancy could possibly be an adaptive mechanism to protect the maternal skeleton and prepare for the mineral requirements of the developing fetus and later for milk production for 12 or more suckling pups (168). Gains in bone mass and

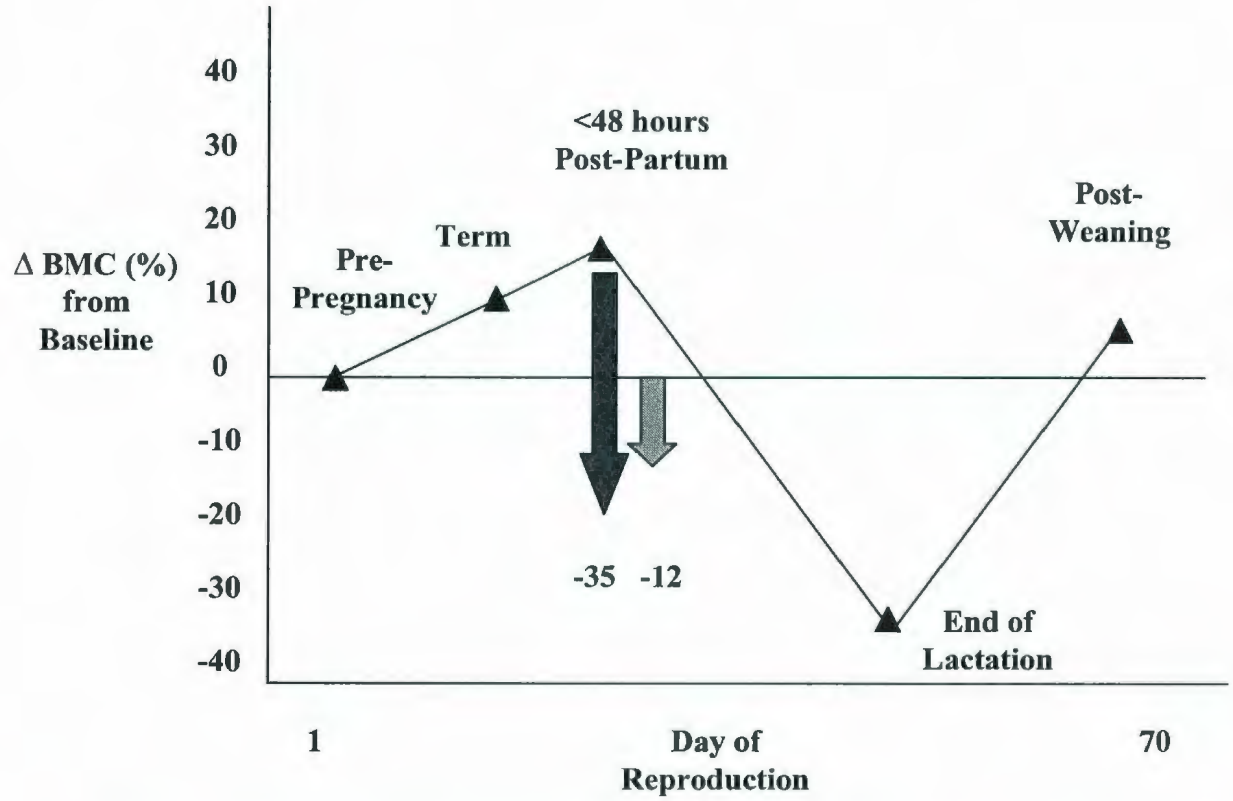


Figure 8: Bone Mineral Content (BMC) pattern throughout reproduction in normal, *Blackswiss* mice. Normal Black Swiss (BLKSW) mice gain BMC during pregnancy, peaking at < 48 hours post-partum (168). These gains have not been noted in human studies (155, 156). During lactation in BLKSW mice BMC levels drop -12% from baseline, and as much as -35% from peak BMC levels at < 48 hours post-partum (168). Remarkably, there is full recovery, and in fact a temporary overshoot, of BMC post weaning (168). Serial BMC measurements in humans have shown losses of -5 to -10% in BMC during lactation from immediate postpartum also a complete regain after weaning (5-8). Losses may be more significant in mice due to significant litter sizes. BLKSW data obtained from Sharpe and Kovacs *IBMS/JSBMR* 2003.

volume during pregnancy have also been seen in rats (161). In one study in rats, at the end of pregnancy there was an increase in cortical periosteal bone formation and an increase in cortical volume (161). Up to a 40% increase in cancellous bone volume has been observed in the lumbar spine of rats during pregnancy compared with age-matched virgin controls (163). More research is needed in this area to fully understand the effect of pregnancy on bone mass. Reviews of epidemiologic studies in both humans and animals have shown that the alterations in bone mass during pregnancy do not have negative effects on long-term fracture risk, likely because of the reversibility of these alterations (122, 155, 163, 164). On rare occasion, a woman may suffer from fragility fractures during or immediately after pregnancy (165). Alterations in mineral metabolism during pregnancy (i.e. increased bone resorption) may exasperate complications related to a pre-existing low bone mass (165). Baseline (i.e. pre-pregnant) bone mass measurements are often unavailable, and thus problems associated with a pre-existing low bone mass may be unforeseen. This 'pregnancy-associated osteoporosis' may be due to other systemic causes such as viral infections, ischemia or trauma.

L. Maternal Calcium and Bone Metabolism during Lactation

Following parturition the mammalian system promptly shifts its metabolic priority from providing the growing fetus with its mineral needs to supplying copious amounts of calcium for transfer across mammary epithelial cells into the milk (5-7). The calcium content of human breast milk has been shown to range anywhere from 25 -36 mg/100ml (169-171). This translates into a typical maternal loss of 210–400 mg of calcium into the breast milk each day, although losses of up to 1000 mg a day have been observed (172).

These lactational demands impose a challenge to the maternal calcium regulatory system of both humans and animals, which is even greater than the demands of pregnancy.

Unlike pregnancy, however, the main maternal adaptation to meet the demands of lactation is not a doubling of intestinal calcium absorption, but rather an increase in skeletal resorption of calcium from the maternal skeleton (5-8, 155, 168, 173-175). Proof of increased bone resorption comes from histomorphometric analysis of bone in animals (5-8, 156, 168). In humans, serum and urine markers of bone formation and resorption have been used to assess bone turnover during lactation (because of the invasive nature of bone biopsy). Markers of bone resorption (TRAP, Dpd/creatinine) are increased 2-3 fold during lactation (5-8, 141, 116, 119). Thus, despite the proportional metabolic requirements during pregnancy and lactation, the mechanism by which mineral is obtained varies quite significantly (5-8). It has been noted that PTHrP, in the context of low estrogen, may be the primary mediator of skeletal calcium mobilization during lactation (**Figure 9**) (175).

M. Alterations in Hormonal Regulators of Calcium Homeostasis during Lactation

The hormonal milieu of lactation varies from that of pregnancy, as well as from the non-pregnant state (5-8, 149, 154, 155, 175 - 183). Lactation induced alterations in: PTH, 1, 25-dihydroxyvitamin D, calcitonin, PTHrP and the gonadal steroids are discussed below.

1. Parathyroid Hormone

Older studies in humans, which used assays that also measured biologically

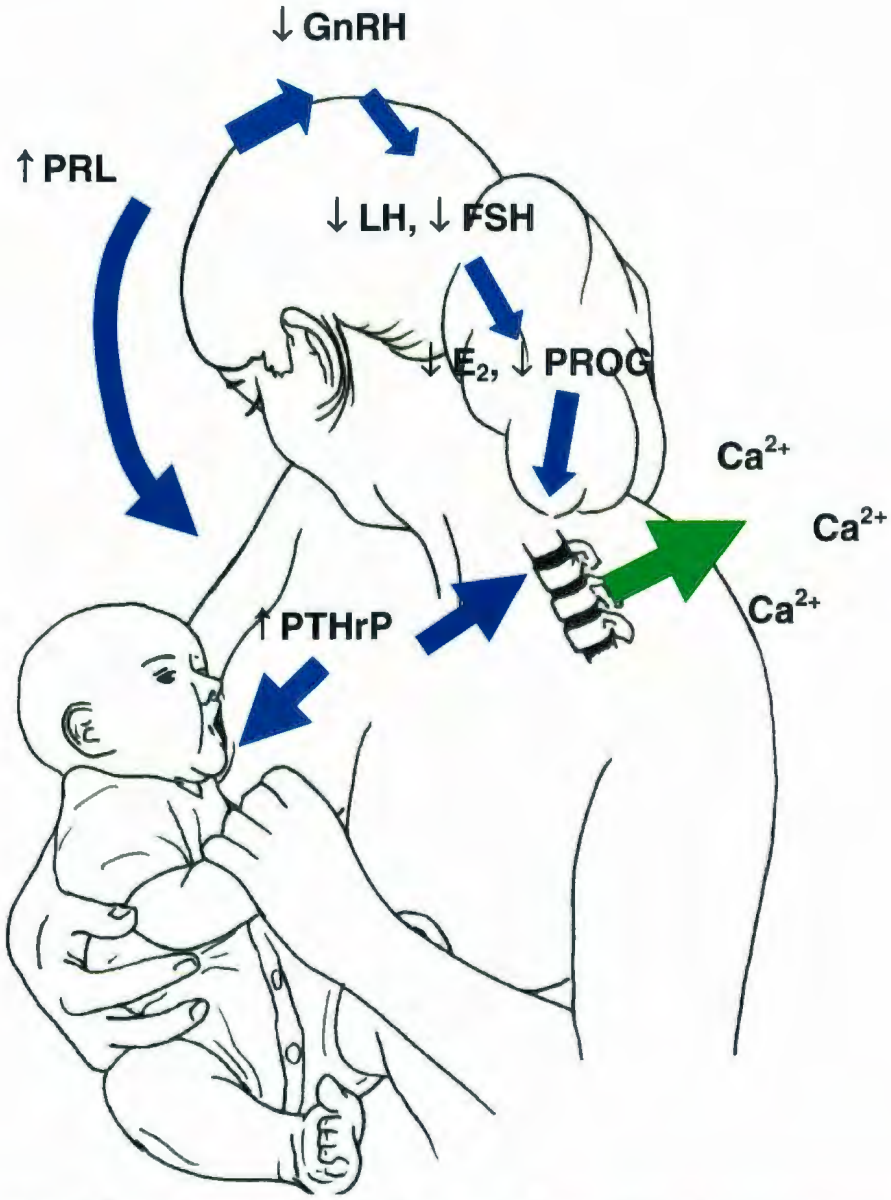


Figure 9: Maternal Calcium and Bone Adaptations during Lactation. High circulating prolactin levels during lactation and suckling suppress the gonadotropin-releasing hormone (GnRH) pulse centre in the hypothalamus, resulting in suppression of the pituitary gonadotropins (i.e. luteinizing hormone (LH) and folicle-stimulating hormone (FSH) (175). This cascade of events causes a suppression of the ovaries, ovulation and production of estradiol. Hypoestrogenemia continues throughout lactation. PTHrP, produced by the breast during lactation, along with low circulating estrogen, may be the primary cause of skeletal calcium mobilization during lactation. *Image by C.S. Kovacs, 2005.*

inactive fragments of PTH, have shown increases in PTH during lactation (176). In contrast, however, newer intact PTH assays, clearly show a decrease in serum PTH levels by as much as 50% during lactation (5-8, 149, 155, 164, 177, 178). Consistent with this finding is the fact that lactating hypoparathyroid women have been shown to have normalized blood calcium and phosphorus levels and thus do not require additional calcium and calcitriol supplementation (179). As well, hypoparathyroid women have shown to lactate successfully (179). This suggestion of lack of a role of PTH during lactation directed many researchers towards the potential role of PTHrP at this time. PTH levels in rats and mice also decrease during lactation. Preliminary experiments in mice lacking the PTH gene have shown that BMC losses during lactation in PTH null mice are no different than in the WT littermates (48-50).

2. 1, 25-Dihydroxyvitamin D

In humans 1, 25-dihydroxyvitamin D levels are typically unaltered by lactation (5-7, 124, 127). Levels of 1,25-dihydroxyvitamin D decrease from that of pregnancy lactation to that of non-pregnancy during lactation (5-7, 124, 127). In one study, however, women nursing twins were found to have higher 1, 25-dihydroxyvitamin D levels than women nursing singletons (184).

Studies in lactating rats, in contrast, have shown elevated levels of 1, 25-dihydroxyvitamin D (180). These elevations in circulating 1, 25-dihydroxyvitamin D have been prevented by supplementing the diet with additional calcium (180). Furthermore, the specific role of 1, 25 dihydroxyvitamin D in regulating the cycling of BMC during lactation has been tested using a genetically engineered mouse model

lacking the VDR (139). During these studies *Vdr* null mice were switched to a 2% calcium chow at 10 weeks of age to increase fertility (139). The *Vdr* null BMC dropped by 0.137 grams (36%) from pregnancy to late lactation and the WT mice BMC dropped by 0.188 grams (36.2%) during the same period. Although these values did not vary significantly from each other (i.e. the bone mass lost during lactation was of a similar magnitude), the *Vdr* null still had a BMC of 0.465 grams (122% of the respective baseline BMC versus 85% percent in the WT siblings) compared to the respective pre-pregnancy baseline BMC values (138). Due to the fact that VDR deficient mice have an increase in BMC during pregnancy suggests that during lactation calcium absorption may occur independently of the VDR and 1,25 dihydroxyvitamin D (139).

3. Parathyroid Hormone-Related Protein

Despite the decline in circulating PTH during lactation in both rodents and humans, increased urinary cAMP excretion, as seen hypoparathyroidism, provided indirect evidence of another PTH-like hormone functioning at this time (184, 185). The peptide is thought to be PTHrP. In humans, PTHrP mRNA expression is increased in mammary tissue during lactation (185 – 189) **(Figure 9)**. Furthermore, an abundant amount of PTHrP is secreted into the milk (191, 192). It is currently unknown if PTHrP, present in the milk, plays an important role in the developing neonate (191, 192).

Studies in mice have suggested that PTHrP is part of a lactational mammary-bone feedback loop **(Figure 9)** (186 - 193). Proof of a role of PTHrP is illustrated in studies using mice that have been genetically engineered to lack mammary gland PTHrP mRNA and milk PTHrP (193) (i.e. mice carrying a *β-lactoglobulin* promoter-driven *Cre*

transgene, one null *PTHrP* allele, and one *floxed PTHrP* allele). Mammary epithelial expression of Cre during late pregnancy and lactation resulted in efficient deletion of the *PTHrP* gene. Findings illustrated that mice with PTHrP absent from the mammary tissue during lactation had significantly lower circulating PTHrP levels versus their WT littermates (193). These mice were found to lose less BMC as assessed by DXA by day 11 of lactation versus their WT litter- mates (i.e. control mice had a BMC of 0.044 ± 0.001 g/cm^2 versus *BLG-Cre*/PTHrP$^{lox/-}$ mice had a BMC of 0.048 ±0.002 g/cm^2, $p<0.01$) (193). Thus, these finding suggest that the mammary tissue is a major contributor to circulating PTHrP levels and that mammary PTHrP levels positively correlate with bone resorption rates at this time (193). Thus, mammary tissue PTHrP is a contributor to lactational bone loss, but not all bone loss is contributed to PTHrP, as *BLG-Cre*/PTHrP$^{lox/-}$ mice still experienced lactational BMC losses (193).

Studies in mice have also demonstrated that the CaR, which becomes expressed on mammary epithelial cells at the transition from pregnancy to lactation, regulates PTHrP production and calcium transport (188,189). Increasing concentrations of calcium and neomycin suppressed PTHrP secretion by mammary epithelial cells in vitro (188). In vivo, however, it was shown that systemic hypocalcemia increased PTHrP production. (189). Authors of this work concluded that, in mice, PTHrP is required to stimulate the osteoclastic bone resorption needed to induce the normal loss of BMC during lactation (189).

4. Sex Steroids and Other Hormones of Lactation

In both humans and rodents prolactin stimulates lactogenesis, or milk production,

by acting together with cortisol and insulin to stimulate the transcription of the genes that encode milk proteins (194- 196). As lactation is being established prolactin levels are consistently elevated **(Figure 8)**. After this period, however, prolactin levels increase in response to the suckling reflex stimulus (194-196). Elevated circulating prolactin levels during lactation suppress the gonadotropin-releasing hormone (GnRH) pulse centre in the hypothalamus, resulting in suppression of the pituitary gonadotropins (i.e. luteinizing hormone (LH) and folicle-stimulating hormone (FSH) (194). This cascade of events causes a suppression of the ovaries, and thus ovulation, ultimately decreasing production of estrogen (194-196).

Hypoestrogenemia has been widely assumed as an important trigger leading to increased bone resorption during lactation. Studies in mice, however, have shown that low estradiol explains only part of lactational bone loss (175). Mice treated with estrogen during lactation experience a 50-60% reduction in the amount of bone lost by DXA when compared with untreated mice (175). It is important to note, however, that treatment in this study provided estrogen doses that were 4-fold higher than in normal circulation, thus providing results for pharmacological overtreatment, and not simply physiological replacement. Even with pharmacological doses of estrogen mice still continued to lose 5–7% of their bone mass between days 4 and 12 of lactation (175). Thus, the lactational bone phenotype is likely due to other factors in addition to low estrogen levels.

In rats, high circulating prolactin levels of lactation have been shown to stimulate PTHrP production *in vivo* thus reinforcing the brain, as a physiological contributor to the mammary-bone feedback loop, and ultimately skeletal bone loss **(Figure 8)** (140). Reinforcing this link is the fact that PTHrP has been shown to be elevated in states of

hyperprolactinemia other than lactation (197). Both prolactin and PTHrP are induced by suckling and prolactin has been shown to upregulate its own receptors (140, 197). As a consequence, increased prolactin may possibly perpetuate PTHrP's bone resorbing effects on the maternal skeleton **(Figure 8)** (140, 187, 197). One study in rats also suggested that PTHrP production from the lactating mammary gland is suppressed by progesterone (185). Progesterone dramatically falls after delivery, thus possibly disinhibiting PTHrP production by lactating mammary glands and resulting in considerable amounts of PTHrP secreted into the milk (189, 190). Furthermore, skeletal losses during lactation occur irrespective of ovariectomy, adrenalectomy, or subsequent pregnancy (5-8).

N. Calcitonin during Lactation

Studies in lactating humans and animals have shown elevated calcitonin levels in the serum during lactation, even in the absence of the thyroid (150, 152, 153, 184). High circulating calcitonin levels during lactation, like that of pregnancy, have been speculated to have a role in the protection of the maternal skeleton at this time (150, 152, 153, 184).

Several lines of evidence exist to suggest a possible role for calcitonin during lactation in addition to the aforementioned studies on post-thyroidectomized sheep, rats and goats (refer to section J. *Calcitonin during Pregnancy*). Levels of calcitonin in the breast milk of humans have been found at concentrations significantly higher then that in the maternal serum (153), as well mothers nursing twins have been found to have slightly higher calcitonin levels verses their singleton nursing counterparts (122). In rats calcitonin has been found to be expressed in the gonadotropes of the anterior pituitary gland (78) and when administered intravenously it has also shown to inhibit prolactin release from

rat anterior pituitary cells (198- 200) which may directly affect milk production. As well, targeted overexpression of calcitonin in gonadotrophs of transgenic mice leads to chronic hypoprolactinemia (201). In rodents, CTR mRNA expression is induced in the mammary epithelium during pregnancy and lactation, suggesting that calcitonin may have paracrine regulatory activity in this tissue at that time (79, 202). As well, calcitonin and the CTR are also expressed in T47D and MCF7 breast cancer cell lines.

Whether or not calcitonin or the CTR has an essential role in calcium metabolism during lactation was largely unanswered by the aforementioned studies in sheep, rats and goats (150, 152, 153, 184). This research was carried out before targeted strategies to manipulate the mammalian genome were created, and many before extrathyroidal sources of calcitonin were appreciated. Therefore, a true model of calcitonin deficiency during lactation has not been examined until now. Studies of calcium metabolism in the complete absence of calcitonin could reveal possible physiological roles of calcitonin during lactation.

O. Systemic Adaptations to Lactation

The large demands for mineral throughout pregnancy are met by several systemic alterations in the maternal intestines, kidneys and skeleton **(Figure 6)**.

1. Intestinal Adaptations during Lactation

In humans intestinal calcium absorption falls to a level that is no different than that of non-pregnancy during lactation **(Figure 6)** (5-8, 117, 119, 121 – 123, 136, 149, 156). This translates to approximately 30% of dietary calcium intake. This decline in

calcium absorption from pregnancy to that of lactation is thought to occur mainly as a consequence of the respective decrease to normal in 1, 25-dihydroxyvitamin D levels at this time (5-8, 117, 119, 121 – 123, 136, 149, 156). This decrease in intestinal absorption may suggest that calcium needs are being met primarily through other homeostatic mechanisms (i.e. increased skeletal reabsorption) (5-8, 117, 119, 121 – 123, 136, 149, 156). In addition, clinical studies of calcium supplementation show evidence that suggests that optimal lactation and maternal bone health does not depend on an increase in calcium intake by the breastfeeding mother (6-8).

Unlike humans, intestinal calcium absorption in rats, however, appears to remain elevated 2-fold above non-pregnancy levels **(Figure 6)** (5-7). This elevated intestinal absorption may be maintained to support a large litter size, or to accommodate a short and intense lactation period.

2. Renal Adaptations during Lactation

In humans, urinary calcium excretion decreases during lactation versus that seen during pregnancy **(Figure 6)** (5-8, 117-123, 136, 149, 156). This occurs as a result of a rise in renal tubular reabsorption of calcium (5-8, 118-123, 136, 149, 156). Low urinary calcium in the setting of high calcium in the blood suggests that tubular reabsorption of calcium might be increased to account for the reduction in calcium excretion and this is likely due to the increased physiological demand for calcium at this time. Renal calcium excretion has been shown to be similarly reduced in the lactating rat (5-7, 118 -123).

3. Skeletal adaptations during Lactation

Perhaps the most profound of all systemic changes are those that have been seen in the skeleton during lactation **(Figure 6)** (5-8, 117-123, 149, 156). The metabolic alterations are in place during lactation to ensure that adequate mineral is available for the production of breast milk and the survival of the growing neonate. On rare occasion the mineral demands of lactation may be significant enough to cause a woman to suffer from fragility fractures (203 - 206).

Markers of Bone Turnover during Lactation

In humans, histomorphometric data is unavailable during lactation due to the invasive nature of bone biopsy. Serum and urine markers of bone resorption and serum makers of bone formation are therefore used at this time to evaluate bone turnover. Markers of bone resorption (e.g. TRAP, Dpd/creatinine) are clearly elevated 2- to 3-fold during lactation and especially above values of pregnancy (5-7, 114, 115, 122, 123, 207, 208). Bone formation (e.g. OC, BSAP and PICP) markers have also been found to be generally elevated during lactation above that of pregnancy (5-7, 114, 115, 122, 123, 207, 208) but not as markedly increased as the resorption markers, thus there is net uncoupling of turnover.

Bone turnover is also elevated in rodents during lactation. Histomorphometric analysis has revealed an increase in osteoclast number during lactation as well as losses of up to 30% of bone mineral after a full lactation cycle (154, 168). Bone markers have shown a greater increase in bone resorption than bone formation (152, 168). In addition, studies in rats have shown that the large demands for mineral during successive lactation

periods is associated with a decrease in cortical and trabecular bone strength as well as a decrease in cancellous bone volume (163).

In summary, bone turnover is increased during lactation in both humans and rodents. The net effect, however, appears to be that of bone resorption.

Flux in Bone Mass during Lactation

BMC losses of 3- 10% have been found at trabecular sites (e.g. lumbar spine and hip) with smaller losses are seen at cortical sites, after 2-6 months of exclusive lactation in humans (5-7). This translates to a BMC loss of 1-3% per month while breastfeeding (5-7). BMC losses associated with post-menopausal osteoporosis are considered rapid at >1% per year in humans. Thus, losses of 1-3% per month seen during lactation are quantitatively more profound (5-7).

Rats and mice, in contrast, have shown BMC losses amounting to 25-30% of BMC via DXA at trabecular rich sites after 3 weeks of exclusive lactation **(Figure 7)** (6-8, 168) with smaller losses at cortical sites. These losses are likely more exaggerated because of the significant demands of 8-12 suckling pups (6-8, 168). These tissue density decreases in rodents have also been confirmed using μCT (6-8). Cortical periosteal bone formation and bone volume has also been shown to increase from early gestation to the end of pregnancy in rats (161, 162). It has been suggested that this early increase in bone formation, as well as the increase in bone volume may be a mechanism for storing mineral for the subsequent decrease in bone volume and BMC that results from milk production during lactation (161, 162).

Lactational BMC losses in both human and rodents are obligatory and occur irrespective of dietary calcium intake (6-8, 156, 183, 209, 210). In several randomized, placebo-controlled trials, women that received calcium supplementation daily throughout lactation lost amounts of mineral that were no different than their placebo receiving counterparts (156, 183, 209, 210). Authors concluded that calcium supplementation does not prevent bone loss during lactation (156, 183, 209, 210). Even in a study of lactating Gambian woman where calcium intake is relatively low (i.e. 283 mg/day), randomized supplementation with almost triple the amount of calcium had no effect on breast-milk calcium concentration or changes in maternal BMC (209, 210). This study suggested that, in women with low calcium intakes, there was no direct benefit from increasing calcium intake during lactation, and that physiological mechanisms operate regardless of dietary intake to supply calcium for breast-milk production. Opposing results, however, have been noted. One particular study showed that increased dietary calcium intake was associated with improved calcium balance in women with habitual intakes of <500 mg calcium per day (116).

The mechanisms underlying the bone loss that occurs during lactation are not completely understood (5-7). Hypoestrogenemia is well established as the primary contributor to the bone loss associated with post-menopausal osteoporosis (11, 12, 17, 203-208, 211). During lactation, however, low estrogen is thought to be only part of the hormonal milieu resulting in bone loss **(Figure 8)** (5-8). Treatment of lactating mice with pharmacological levels of estrogen has shown to decrease histomorphometric parameters and urinary markers of bone resorption, reducing the amount of bone loss by 50–60%, but not completely preventing it (175). It is important to note that this study may not show the

contributions of physiological levels of estrogen to bone loss, but perhaps an exaggerated model of estrogen effects based on a pharmacological dosage. Additional proof, regarding the role of estrogen in preventing bone loss lies in studies of women who have been rendered estrogen deficient through the use of GnRH agonist therapy, for the treatment of medical conditions such as endometriosis and uterine leiomyomata (212, 213). After six months of GnRH agonist therapy, women lost about 1 - 4% of their BMC at trabecular-rich sites (212, 213). Lactation, in contrast, is accompanied with only moderate estrogen deficiency, yet BMC losses amount to 5-10% at trabecular-rich sites after six months (5-8, 161, 163, 164, 167, 183).

Studies in mice have illustrated that BMC loss during lactation is attributed to both high levels of mammary-derived PTHrP and low levels of circulating estrogen (175, 193) **(Figure 8)**. More specifically, the relative contributions of estrogen and PTHrP can be highlighted through studies that show that: 1) circulating estrogen levels have been found to be low during mid-lactation and correlated negatively with bone resorption markers (175), 2) treatment of lactating mice with estrogen lowers urinary CTx levels and histomorphometric parameters of bone resorption, and reduced the amount of bone lost significantly but not completely (175) and 3) mice lacking mammary derived PTHrP lose significantly less bone mass during lactation versus the normal siblings (193). Thus, it is likely that mammary derived PTHrP during lactation acts synergistically with hypoestrogenemia to trigger accelerated bone resorption during lactation.

Although rare, osteoporosis may present itself during lactation (201-204). The occurrence is quite rare, and may arise from an unrelated condition or an exacerbation of a pre-existing low bone mass (203 - 206). These women may present clinically with

vertebral crush fractures during or after breastfeeding (203 - 206). At this time, PTH and calcitriol are typically normal or slightly reduced (203 - 206). In one known case of lactational osteoporosis, PTHrP levels were found to be elevated for months after weaning (205). The extent, however, to which PTHrP contributes to the reduction of bone density during lactation is only now being appreciated (5-7, 201-204).

In the long-term, for the vast majority, the consequence of lactation-induced bone loss does not appear of clinical concern (5-7). Most epidemiological studies of pre- and postmenopausal women have found that a history of lactation has no adverse effects on bone health (6-8, 183, 203, 208, 214). In fact, some studies suggest that breastfeeding may reduce the risk of spinal and hip fracture after menopause (173, 214).

4. Mammary Gland Adaptations during Lactation

Lactation is the primary physiological function of the mammary gland. Many of the unique adaptations underlying lactation still remain an enigma (8). Lactating mammary tissue has been shown to express: calcitonin, CTR, CaR and PTHrP mRNA (5-8, 79, 80, 174, 186 -191). During lactation the breast becomes a calcium-sensing organ (188). PTHrP acts through the CaR in response to extracelullar calcium levels to control the calcium, and possibly water content of milk **(Figure 8)** (187). PTHrP appears to mimic the normal bone resorbing actions of PTH, by acting through the PTH1 receptor (5-8, 189). In addition, suckling, as well as prolactin, has been shown to stimulate PTHrP production during lactation **(Figure 8)** (197). PTHrP levels are found to be extremely high in the milk of humans and animals (191, 192). This research suggests several

important pathways in which the breast, brain and bone are linked during lactation **(Figure 8)**.

P. Maternal Calcium and Bone Metabolism during the Post-Weaning Period

Despite losses of up to 5-10% of total body BMC after 6 months of exclusive lactation in humans, and losses of up to 35% of total body BMC after 3 weeks lactation in rodents, mineral is completely and rapidly restored after weaning. Within 3-6 months in humans and 3 weeks in mice BMC values via DXA have shown to be no different than that of pre-pregnancy (38-40, 215- 217).

It is notable that the adult skeleton is normally able to regain little of the mineral that it has lost (e.g. after periods of weightlessness, prolonged corticosteroid therapy, estrogen withdrawal, etc) (11, 12, 19-21) and thus, the post-lactation period is a unique interval in which the skeleton can accrete substantial mineral during adulthood (5-7). A clearer understanding of the mechanisms underlying this rapid post-weaning skeletal remineralization may help to isolate molecular pathways and specific factors involved in bone mineral accretion. These factors may be important in the establishment of novel pharmacological therapies for the treatment of metabolic conditions characterized by excessive bone loss (e.g. osteoporosis).

Weaning is typically defined as the gradual process of replacing breast milk feeding with an increasing amount of ingested nutrients. In animal studies, weaning is often the direct removal of suckling offspring from the presence of the mother (38-40, 119, 208, 215-217). In spite of the potential information that the post-weaning skeleton may reveal about the molecular biology of bone mineral accretion, limited data is

available that considers the actual mechanisms underlying the anabolic actions of the maternal skeleton at this time (215- 217). Below is a summary of what is currently known about calcium metabolism during the post-weaning period.

Q. Alterations in Hormonal Regulators of Calcium Homeostasis during the Post-Weaning Period

Weaning has also been shown to affect some of the hormonal players involved in calcium homeostasis (119, 208, 215-217). Studies considering PTH, PTHrP, VDR, the sex steroids and calcitonin in both humans and rodents are noted below.

A few studies in humans have shown elevations in PTH during the post weaning period (up to 6 months) versus that of pregnancy and lactation (119, 183). It is important to note, however, that these studies were based on single measurements at only one selected time point during the post-weaning period. A study in mice found contrasting data, in that, PTH levels returned to the non-pregnant level 7 days post-weaning (217). Furthermore, preliminary studies in mice null for the *PTH* gene suggest that PTH is not required for BMC remineralization, as the skeleton return to its pre-pregnant state post-weaning, despite lacking PTH (48-50).

PTHrP may be involved in the remineralization of the maternal skeleton post-weaning through its actions on bone or mammary tissue (215-217). Within bone PTHrP is produced by cells of the osteoblast lineage and acts through the PTH1R on mature osteoblasts (60). Six week old mice with osteoblast-specific targeted disruption of PTHrP were found to have an increase in marrow space, reduced BMD and bone volume, scattered thin spicules of trabecular bone and decreased trabecular number, when

compared to age matched control mice (219). It is possible that PTHrP produced by osteoblasts may stimulate the recovery after weaning in the absence of the hormonal milieu of lactation (i.e. high prolactin and low estradiol) (218, 219). This possibility is currently being explored in our lab. Mammary derived PTHrP could also be considered a possible candidate in aiding in skeletal recovery post-weaning. The breast is powerful source of PTHrP during lactation and might remain so during recovery. Rapid mammary tissue involution after lactation, may however over-ride this explanation. Furthermore, circulating PTHrP levels have been shown to correlate with levels of prolactin during lactation and the post weaning period (187, 217).

Preliminary studies in genetically engineered mouse models have shown that the VDR may not be required for skeletal remineralization during the post-weaning period (138). More specifically, mice lacking the VDR gene completely regain BMC levels post weaning, as per normal WT mice (139).

During weaning, or possibly earlier, ovarian function resumes causing estrogen levels to return to normal (215). This return of estrogen to the pre-pregnancy level may assist in the remineralization of the maternal skeleton (215). Physiologically, estrogen acts by inhibiting the bone resorbing osteoclasts cells, and thus it seems possible that post-weaning rise in skeletal bone mass may in part be contributed to this effect (222-224). Additional support for the anabolic effects of estrogen on bone comes from the post-menopausal period (11, 12). Estrogen receptors are present on the bone forming osteoblast cells (11, 12). At the cellular level estrogen has been shown to have an anti-apoptotic effect on osteoblasts (68, 222). It has also been demonstrated that osteoclast formation from mononuclear hematopoietic stem cells and bone resorption activity of

mature osteoclasts is inhibited by estrogen, which leads to reduced depth of resorption lacunae (223). Most estrogen effects are thought to be mediated via nuclear estrogen receptors alpha (ERα) and beta (ERβ) (222).

There are, however, a multitude of additional calcium regulating hormones and factors that have not been explored in detail during weaning. The ratio of RANKL to OPG in controlling osteoclastogenesis, and thus bone loss should be considered during weaning, as a decrease in this ratio could possibly contribute to the bone formation post-weaning. Supporting this theory is a recent finding in mice that suggested that RANKL expression is decreased during the post-weaning period (217).

Recovery of the maternal skeleton to its pre-pregnant state post weaning likely involves a number of complex metabolic pathways (215-218). It is also important to note that many rodents undergo post-partum estrus, and thus can conceive the day immediately following delivery. Thus, rodent calcium metabolism is particularly challenged, as many of these animals are concurrently pregnant and lactating. Despite the simultaneous demands for mineral, the rodent skeleton can completely remineralize after weaning.

R. Calcitonin during the Post-Weaning Period

Despite the lack of evidence that elevated or depressed calcitonin levels may have any major physiological effect in humans, a recent study in rats has implicated calcitonin in the synchronization of mineral prior to, during and post lactation (216). In this particular study, rats were shown to have a five-fold increase in maternal calcitonin levels at 24 hours post-weaning (216). These changes were concurrent with a finding of rapid inactivation and apoptosis of osteoclasts (216). The authors concluded that these changes

were similar to the reversal phase of a bone remodeling cycle, where bone formation begins and resorption slows down (216). More research is needed to investigate if calcitonin is involved during the weaning process.

It is also relevant to note that pituitary lactotrophs express the CTR (75) and pituitary calcitonin has been shown to inhibit PRL gene transcription, and lactotroph cell proliferation (78). Studies in mice have also shown that targeted overexpression of calcitonin in the pituitary gonadotrophs leads to chronic hypoprolactemia (201). Due to the fact that PRL is a key player in milk production and possibly bone turnover during lactation, a sharp increase in calcitonin post-weaning could possibly provide the necessary signaling required, initiating the anabolic cascade of events at the time.

S. Systemic Adaptations to Weaning

Much less is known about the systemic alterations in the maternal intestines, kidneys and skeleton during weaning **(Figure 6)**. The few finding that exist, are noted below.

1. Renal and Intestinal Adaptations during the Post-Weaning Period

In humans, the renal and intestinal handling of calcium has not been explored in detail during the post-weaning period. Fractional calcium absorption was found to be higher in lactating women after weaning (0.37+/-0.02) compared with non-lactating postpartum control subjects (0.31+/-0.02) (120). The authors concluded that lactation stimulates an increase in fractional calcium absorption, but the response is only apparent after weaning or the resumption of menses (120). It is also important to note that this

small difference in calcium absorption may not prove to be clinically significant. No rodent data is available reading the renal or intestinal handling of calcium during the post-weaning period.

2. Skeletal Adaptations during Post-Weaning

Limited data on bone turnover markers or histomorphometry is available post-weaning in humans. A study considering changes in bone metabolism in 10 women from pre-pregnancy through post-weaning found no significant difference in osteocalcin versus that of pre-pregnancy (226). A slight increase in BSAP was found post-weaning compared to pre-pregnancy. Another group found increases in serum osteocalcin post-weaning (227).

In comparison, a study in rats, immediately following weaning found there was a profound increase in the osteoblast population in maternal cancellous bone (216). In contrast, a more recent study in mice concluded that serum markers of bone formation stayed the same as that seen during lactation (i.e. elevated above pregnant and non-pregnant levels) (217). In this study, although no change was seen in osteoblast number or bone formation rates, there was a significant increase in osteocalcin and numbers of osteoblast colonies found in culture (217). This suggested that osteoblast signaling may indeed vary during the post-weaning period, despite no significant alteration in osteoblast number. Another study in rats showed that after lactation all indices of bone formation rates were significantly greater versus those observed in the controls, which is indicative of higher bone turnover rates (161). At 8 weeks after weaning, none of the indices of bone formation and turnover were significantly different from the controls (161).

Few studies have also considered bone resorption changes that occur in maternal osteoclasts during the transition from lactation to post-lactation. A study in rats assessed skeletal samples during and after lactation. Findings showed a rapid change in the osteoclast population after weaning, resulting in less resorptive surfaces (216). Osteoclasts were found to detach from their bone surfaces, losing their ruffled borders, and becoming fragmented, showing apoptosis within 24 hr after lactation (216). A more recent finding in mice suggested that RANKL expression is decreased during the post-weaning period (i.e. RANKL being involved in osteoclast differentiation and function) (217). More research is needed to explore the post weaning period.

Studies in humans have shown a rapid increase in femur, spine and total body BMC/BMD during the weaning period (208, 225). This has been found to be equal to a gain of 0.5-2% in BMD per month after lactation. A study in humans showed that during the weaning period women gained significantly more bone in the lumbar spine than did non-lactating women (5.5% versus 1.8%) (208). Authors concluded that earlier resumption of menses was associated with a smaller loss of bone during lactation and a greater increase of bone after weaning (208). A study in mice showed that after a 28-day period, BMD had increased by 37% within the spine, 27% within the femur and 25% for the total body (215). In another study at 8 weeks after weaning, the amount of cancellous bone volume had increased and there was no significant difference from controls (161).

It can be concluded based on limited findings, that the post-weaning period is accompanied with a significant anabolic phase in the skeleton versus that seen at other time points during reproduction or adult life. Recovery of the maternal skeleton to its pre-pregnant state appears to occur independent of PTH and 1, 25- dihydroxyvitamin D.

Possible hormonal changes in the microenvironment that may assist in skeletal anabolism include increases in estrogen and calcitonin. It is likely that the post-weaning period is accompanied by a number of unknown metabolic pathways. More studies are needed to determine the mechanisms that trigger these rapid BMC gains.

In summary, weaning as well as the previously mentioned periods of pregnancy and lactation, are accompanied with unique systemic and skeletal adaptations (5-8, 114-117, 119, 122-125). Of key interest is the role of the calcitonin gene and its peptides during these time points. This will be the focus of the work in this thesis.

II. Project Purpose, Hypothesis and Description

Research over the past 30 years has largely concluded that mammalian calcitonin at physiological doses is not required for survival (1, 2, 70, 6-8). Proof, of this theory, lies in physiological studies that have illustrated no harmful effects of calcitonin excess or deficiency (82-85). Thus, it has been speculated that calcitonin may only remain physiologically important in lower vertebrates that dwell in the calcium rich sea (1, 2, 6-8, 94).

A few studies, nevertheless, have persisted in suggesting that mammalian calcitonin is required during times of increased calcium demand, such as that seen during pregnancy and lactation (89, 90). It is important to note, however, that these older animal studies were based on surgical models of calcitonin deficiency and were fraught with confounding variables, such as side-effects of thyroid ablation and the lack of awareness of the extra-thyroidal sources of calcitonin. In light of this work and the possibility that calcitonin may have a physiological role in mammals, this doctoral research was carried out to re-test the hypothesis that calcitonin is required to protect the maternal skeleton from excessive resorption during pregnancy and lactation. Assuming this hypothesis to be true, our secondary hypothesis was that calcitonin acts at the level of the pituitary, mammary tissue and osteoclasts to regulate skeletal metabolism during lactation.

These hypotheses were approached by using a genetically engineered mouse model in which the *ctcgrp* gene has been ablated. In these genetically manipulated colonies, WT mice have both calcitonin alleles (i.e. these animals are genetically normal), HET mice have one calcitonin allele plus one mutant allele (i.e. neomycin resistant copy) and *ctcgrp* null mice have 2 mutant alleles. *Ctcgrp* null females, versus their WT sisters

were studied during pre-pregnancy, pregnancy, lactation, and during the post-weaning period. Studies examined BMC, bone microarchitecture, biomechanics, as well as the levels of various hormones and minerals in the serum, urine, milk and breast tissue. Differences seen in the null mice could then be attributed to a direct or indirect consequence of calcitonin and or CGRP-α ablation.

These studies were anticipated to provide definitive evidence about the role of calcitonin during reproduction in mice within the breast, brain and bone **(Figure 10)**, which in turn could lead to confirmatory studies in humans that lack physiological levels of calcitonin. A clearer understanding of the mechanisms underlying the rapid demineralization of the maternal skeleton during lactation and the subsequent restoration of mineral during the post-weaning period could lend invaluable information regarding the etiology of bone diseases and the creation of anabolic based therapies for the future.

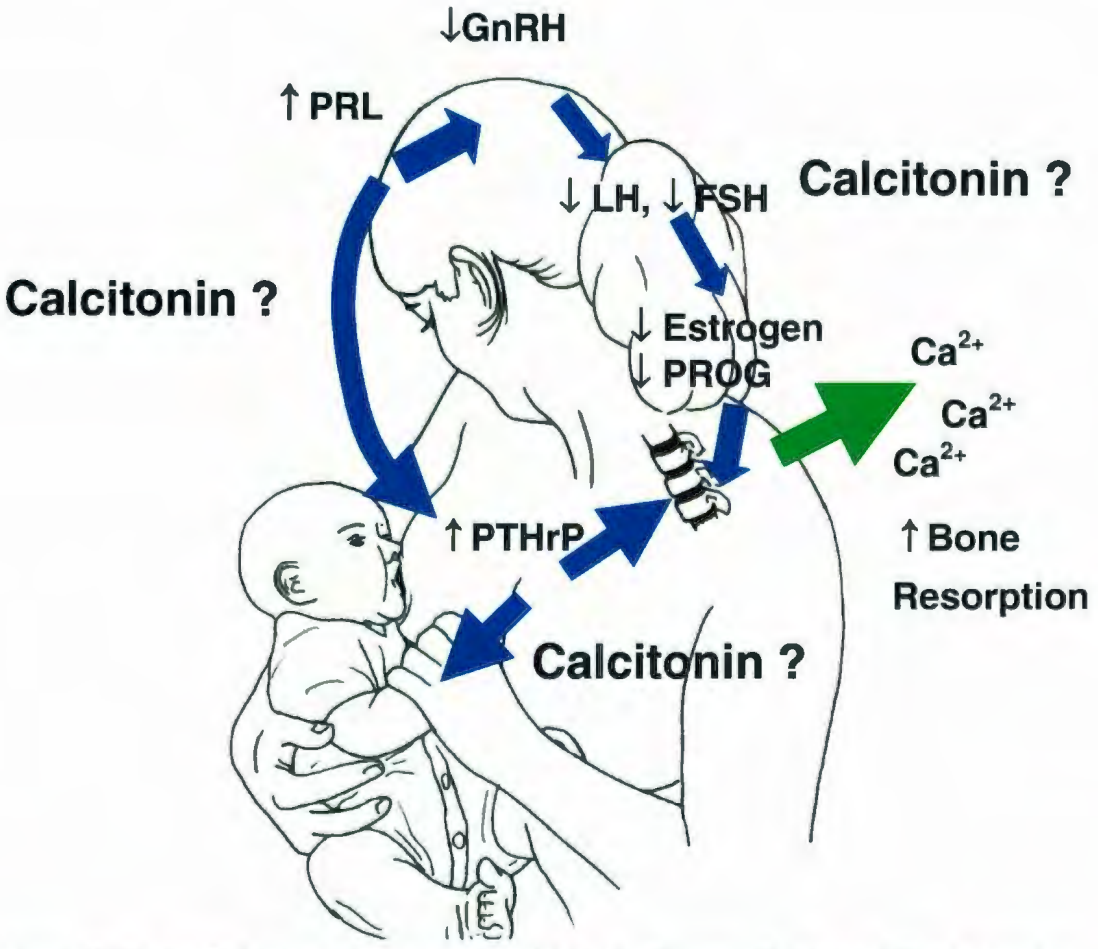


Figure 10: Does Calcitonin have a role within the Breast, Bone and Brain during Reproduction? Elevated prolactin (PRL) levels during lactation stimulate PTHrP production by mammary tissue, and suppress the GnRH pulse center in the hypothalamus, in turn suppressing the pituitary gonadotropins (LH and FSH) and then the ovaries [ovulation, estradiol, and progesterone (PROG) production]. Low estradiol and high mammary-derived PTHrP act together to enhance osteoclast-mediated bone resorption. The presence of calcitonin and its receptors (CTR) within these key areas of calcium regulation (i.e. osteoclasts, mammary tissue, and the pituitary lactotrophs) during reproduction suggests at least three pathways by which calcitonin could modulate BMC cycling during reproduction.

III. Materials and Methods

A. Animal Husbandry

1. Animals

Ctcgrp-null mice were obtained from Dr. Robert F. Gagel's group at the University of Texas, M. D. Anderson Cancer Centre. These mice were created with targeted ablation of exons 3-5 of the murine calcitonin gene and genotyped by PCR, as previously described (103). Chimeras were originally crossed with C57BL/6 mice to produce germline transmission of the targeted allele. The original strain was subsequently back-crossed into BLKWS mice (Taconic, Germantown, NY) (this is an outbred stock (NIH Swiss) with the exception of homozygosity for the non-agouti allele that gives black fur) for at least six generations, and the colony was maintained by breeding heterozygous-deleted mice together.

2. Scheduled Mating and Litter Sizes

Virgin first degree relative pairs of WT and *ctcgrp* null females were selected for study after 10 weeks of age (i.e. after peak bone mass was achieved). Experimental female mice were placed with heterozygous-deleted breeder males at approximately 17:00 hours. Mice mated overnight and then were manually checked for the presence of a vaginal mucus plug at approximately 9:00 hours (i.e. the morning after mating). The presence of a mucus plug marked gestational day 0.5. Both pregnant and non-pregnant experimental mice were removed from the presence of the breeder male. Pregnant mice were subsequently placed in separate labeled cages. Non-pregnant mice were mated again the next evening. Normal gestation for these mice was 19 days. Weaning normally

occurred by 21 days of lactation, and was ensured by removing the pups from the mother on this day. At this stage the pups typically had started eating solid food and drinking water. All mice were given a standard chow (1% calcium, 0.75% phosphorus) and water ad libitum.

Litters (i.e. number of pups from one mouse pregnancy) were counted at weaning (i.e. day 21 of lactation).

3. Housing

Mice were housed with regular light /dark cycles (9:00 am – 9:00 pm light). Non-pregnant mice were housed 4 per cage. Nestlets (Ancare) were used for nesting in addition to standard bedding. All studies were performed with the prior approval of the Institutional Animal Care Committee of Memorial University.

B. Reproductive Cycles

Full reproductive cycles lasted approximately 70 days. Pre-pregnancy was defined as the 5-10 days prior to being placed with a breeder male and after the female mice had reached 12 weeks of age and during which time the BMC was stable. Pregnancy spanned 19 days and for experimental purposes was further subdivided into 3 time points (early pregnancy: 7 days gestation, mid-pregnancy: 12-14 days gestation, and late pregnancy: 17 - 19 days gestation). Lactation spanned 21 days and day 1 was noted as soon as the pups latched on the mother for suckling. Like pregnancy, lactation was also further subdivided into 3 time periods (i.e. early lactation: 7 days, mid-lactation: 12-14 days, late-lactation:

19-21 days). Weaning was defined as the day in which the suckling pups were manually removed from the mother (day 21 of lactation), and post-weaning (also referred to as 'recovery') spanned 21 days even if BMC had recovered prior to that date. Weaning was subdivided into 3 time periods (early weaning: 7 days, mid-weaning: 12-14 days, late-weaning: 19-21 days). An additional time point for data collection included 24-48 hrs of lactation (see **Figure 11** for a Schematic of this reproductive time line).

C. Genotyping

1. Mice Identification

At weaning (i.e. 21 days of age) pups were separated into cages (4 mice per cage) based on gender. After brief anesthesia with isoflurane ™ (CDMV) mice ears were pierced with individual identification tags (i.e. the calcitonin colony was denoted with the letter 'S').

2. Tail Collection

Using sterile razor blades (Smith Brand®), mouse's tail samples (approximately 1 cm) were clipped and collected at the time of weaning. Samples were then placed in microcentrifuge tubes (Fisher Scientific) containing 500µL of lysis buffer (100mM Tris HCL, pH 8.0 / 500 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0 / 0.2% sodium dodecyl sulfate (SDS)/ 200 mM sodium chloride (NaCl)) containing 100 µg/ml proteinase K (Invitrogen). Tubes were subsequently incubated in an isotherm oven (Fisher Scientific) at 55° C overnight to digest.

Week 1 – **Pre-pregnancy** – baseline data was collected in virgin mice starting at 12 weeks of age

Week 2 – **Pregnancy** – female mice were placed with male mice until a vaginal plug was observed indcating day one of gestation

Early Pregnancy was denoted as the first week of gestation

Late Pregnancy was denoted as 17-19 days gestation, as referred to as the 'peak of pregnancy'

Week 4 – **Partuition** – after 18.5 days of gestation mice delivered pups

24-48 post-partum

Week 5 – **Early Lactation** was denoted as the 7th day of lactation

Week 6 – **Mid-Lactation** was denoted as day 12-14 of lactation

Week 7 – **Late Lactation** was denoted as 19-21 days of lactation

Weaning (day 1) – the day the pups were removed from the mother

Week 8 – **Early Weaning** – denoted as day 7 post-weaning

Week 9 – **Mid-Weaning** – denoted as day 12-14 post-weaning

Week 10 – **Late Weaning** – denoted as day 21 of post-weaning

Figure 11: Schematic Time Line of Data Collection through Full Reproductive Cycles in Mice. Samples of tissue and serum as well as DEXA scans were collected at key time points throughout reproduction.

3. DNA Extraction

After overnight incubation tail tissue was shaken by hand for 3 minutes and spun on a microcentrifuge (Thermoelectron/Fisher) for 10 minutes at a maximum speed (i.e. 13,000 rpm). The supernatant was then decanted into fresh microcentrifuge tubes (Fisher Scientific) (Fisher Scientific) containing 0.5 ml isopropanol and inverted to precipitate the mouse tail DNA. DNA was then collected using small pipette tips, placed into microcentrifuge tubes (Fisher Scientific) containing 0.5 mL water and shaken for 5 minutes to dissolve the DNA. Next 0.5 mL of phenol/ chloroform/ isoamyl alcohol (100:100:1) was then added and shaken vigorously for 60 seconds. The resulting turbid solution was then centrifuged for 2 minutes at 13,000 rpm. Then a pipette was used to remove the supernatant and it was placed into fresh eppendorf tubes. 1 ml of 0.12 M sodium acetate (NaOAc) in ethanol (ETOH) was added. The tubes were then inverted several times until the DNA was clearly seen in the solution. Samples were subsequently centrifuged for another 10 minutes (13,000 rpm) to precipitate out the DNA, supernatant was discarded and 1 ml of 70% ETOH was added. Finally the samples were spun for a final 10 minutes, ETOH discarded, and pellets were allowed to air dry. DNA pellets were resuspended in 50 – 200 μL of Tris EDTA buffer (TE) and stored at 4 ° C.

4. Polymerase Chain Reaction (PCR)

PCR was performed on mouse tail DNA samples to establish the presence of the *ctcgrp* gene. WT, HET and *ctcgrp* null animals were identified by PCR analysis using a 3-primer system specific to the *ctcgrp* gene sequence and the neomycin cassette. More specifically, the CT1 forward primer (sequence: CAG GAT CAA GAG TCA CCG CT)

was used to detect the retained portion of the WT allele, the CT3 primer (sequence: GGA GCC TGC GCT CCA GCG AA) was used to detect the deleted portion of the WT allele, and the CTN primer (sequence: GGT GGA TGT GGA ATG TGT GC) was used to detect the neomycin cassette **(Figure 12)**.

PCR cocktail was made using 10X PCR buffer ([Invitrogen] 20mM Tris-HCl, pH 8.4, 50 mM KCl), dNTPs ([Invitrogen] 0.2 mM of each dGTP, dTTP dATP, dCTP,), primers (CT1, CT3 and CTN), 50 mM $MgCl_2$ (Invitrogen), Taq DNA polymerase ([Invitrogen] 0.02 U/μL) and deionized water. Cocktail was aliquoted into PCR tubes (Fisher) (49 μL) and DNA (1.5 μL or 500 ng) was carefully added to this mix. Each tube was labeled with the mouse identification code and placed into the Peltier Thermal Cycler-Dual Alpha Blocks (PT-200 DNA Engine Thermal Cycler).

The following PCR program was used: Step 1: 94 °C for 4 minutes, Step 2: 94 °C for 1 minute, Step 3: 63° C for 1 minute (-0.5° C per cycle), Step 4: 72° C for 1 minute, Step 5: back to Step 2 repeat 10X, Step 6: 94 °C for 1 minute, Step 7: 62 ° C for 1 minute, Step 8: 72 ° C for 1 minute, Step 9: back to Step 6 repeat 30X, Step 10: 94 ° C for 1 minute, Step 11: 62 ° C for 1 minute, Step 12: 72 ° C for 5 minute, Step 13: 4 ° C forever.

5. Gel Electrophoresis

Electrophoresis was performed on PCR products using a 1.2% agarose gel (2 g agarose [Invitrogen], 10 mL of 10X TAE [pH 8.0, 0.12M EDTA, 0.40 M Tris, 11.5% Glacial Acetic Acid] and 90 mL of deionized water, 0.06% ethidium bromide [Invitrogen] or 0.20% SYBR ® Safe). The gel solution was microwaved for 1 minute, briefly mixed and microwaved again for 1.3 minutes. Next, the solution was poured into a gel mold

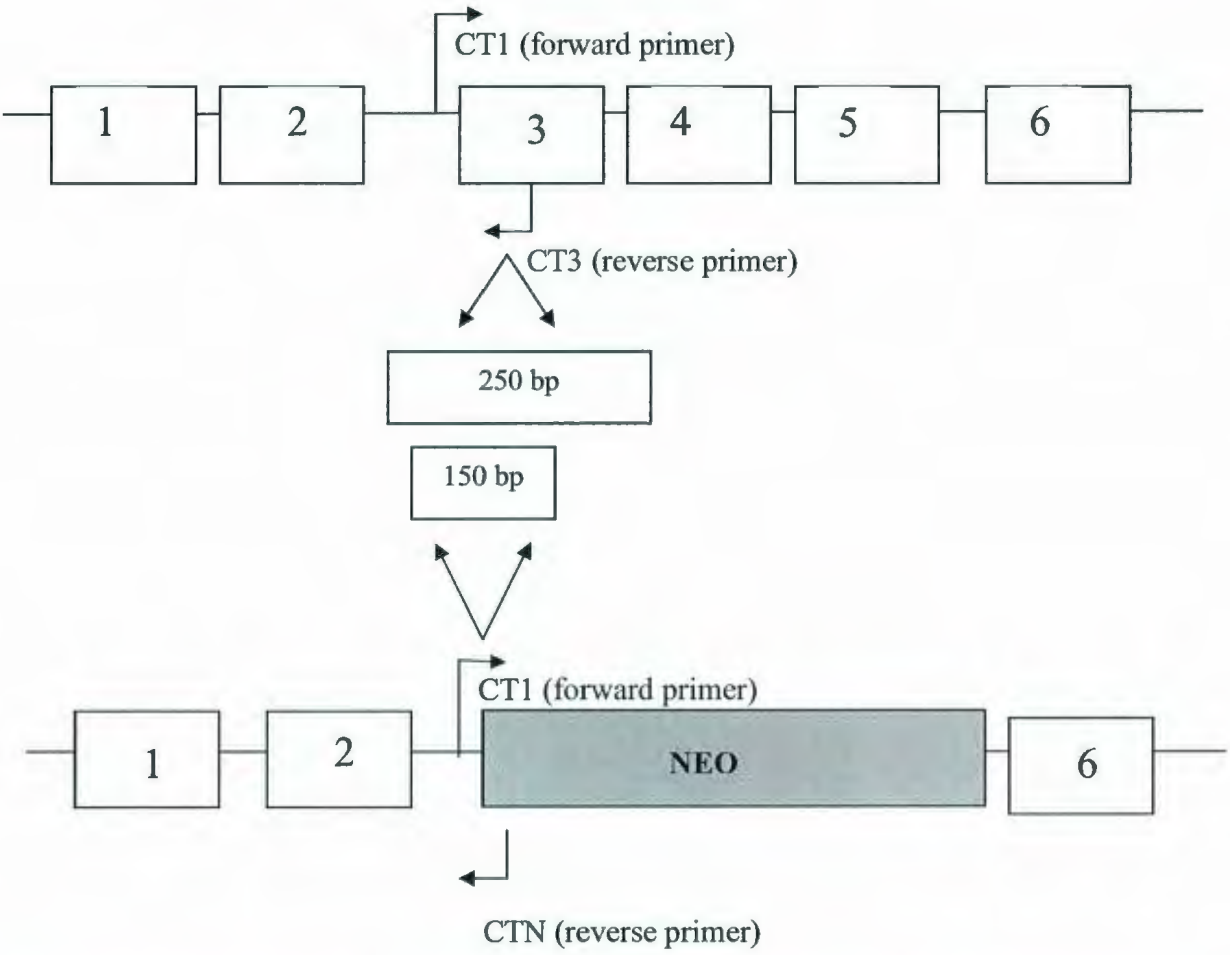


Figure 12. Schematic of the Alignment of Primers used to distinguish the Retained and Deleted Portion of the ***ctcgrp* gene.** WT, HET and *ctcgrp* null animals were identified by PCR analysis using a 3-primer system specific to the *ctcgrp* gene sequence and the neomycin cassette. The CT1 forward primer was used to detect the retained portion of the WT allele, the CT3 primer was used to detect the deleted portion of the WT allele, and the CTN primer was used to detect the neomycin cassette.

with well inserts and cooled for 10 minutes. Gel buffer (630 mL deionized water and 70 mL 10X TAE) was added and gel inserts were carefully removed. Two microliters of Orange G (10 mL 10x TAE, 50 mL Glycerol, 500 mg Orange G powder) was then added to each of the tubes of DNA and mixed. Ten microliters of each DNA/dye solution was then loaded into each of the wells. The power supply was then connected (200 volts) and the gel timed to run for approximately 30 minutes. After this time the gel was then exposed to UV light and an image was captured using Chemi-Imager Software and printed on Mitsubish Thermal paper (Perkin Elymer).

The presence of a single 250 base pair (bp) band indicated that the mouse was WT, a band at 150 bp indicated that the mouse was a NULL, and finally a mouse with both the 250 bp and 150 bp bands was a HET **(Figure 13)**.

D. Data Collection and Storage

1. Litter Sizes

At the time of weaning (21 days) litter numbers were counted for WT and *ctcgrp* null mice. Due to normal losses of pups after birth, this number is normally lower than the number of pups that would have been observed at 24-48 hours after birth.

2. Serum

Serial blood samples were taken by either bleeding the tail vein or by cardiac puncture. Tail vein blood was collected by cutting the tip of the mouse tail with a sterile razor blade and manually 'milking' blood down the tail vein into 1.5 mL micro-centrifuge tubes (Fisher Scientific). On several occasions multiple bleeds were required

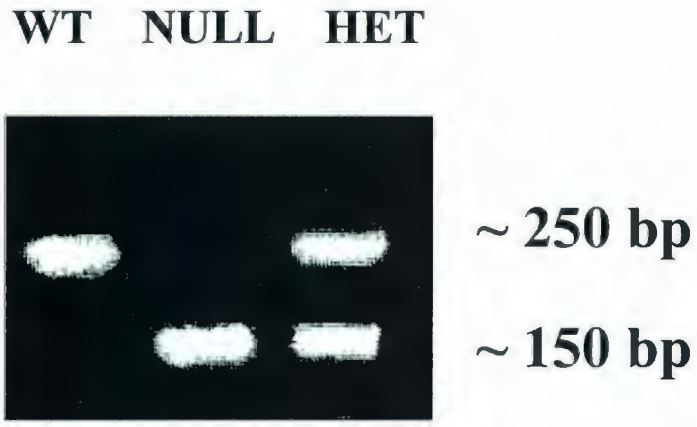


Figure 13: Genotyping by Polymerase Chain Reaction (PCR) and Gel Electrophoresis. The presence of a single 250 base pair (bp) band indicated that the mouse was WT, a band at 150 bp indicated that the mouse was NULL for the *ctcgrp* gene (i.e. *ctcgrp* null*)*, and finally a mouse with both a 250 bp and 150 bp band was HET for the *ctcgrp* gene. Wild-type = WT, Heterozygous = HET and Homozygous = HOM.

to obtain adequate volumes of tail blood for analysis. Cardiac puncture was performed, prior to euthanization, to obtain larger blood volumes. For this procedure, mice were anesthetized with a single intraperitoneal (i.p.) injection of a combination of ketamine hydrochloride (Wyeth®) and Xylazine (Bayer). Mice were then held with one hand firmly holding the scapular region together, to prevent limb or head movement. Sterile 5 cc syringes (LuerLOK®) were attached to 22 gauge needles (PrecisionGlide®) and skin was pierced beneath the mouse ribcage (approximately 2 centimeters under the xiphoid process) at a slight angle until the heart was penetrated. Blood from the heart was subsequently drawn into the syringe, emptied into 1.5 mL microcentrifuge tubes (Fisher Scientific) (after needles were removed from the syringes to prevent red blood cell damage) and spun on a microcentrifuge (Thermoelectron) for approximately 5 minutes at a maximum speed (i.e. 13,000 rpm) to separate out serum (i.e. the clear liquid separated from clotted blood). Serum was then pipetted into clean 1.5 mL microcetrifuge tubes (Fisher Scientific), labeled and placed at -20° C.

3. Plasma

Aprotinin solution (0.0133 g Aprotinin [Sigma] in 1 mL 0.5 EDTA) was used to rinse syringes, needles, and microcentrifuge tubes (Fisher Scientific) prior to collection of plasma. Whole blood was drawn via cardiac puncture (as per above method) into syringes containing 10 µL of aprotinin solution and transferred to microcentrifuge tubes (Fisher Scientific) containing 3 µL of aprotinin. The samples were immediately spun on a microcentrifuge (Thermoelectron) for approximately 5 minutes at a maximum speed (i.e. 13,000 rpm) to obtain plasma (i.e. the liquid component of blood with suspended blood

cells). Plasma was then pipetted into clean 1.5 mL microcetrifuge tubes (Fisher Scientific), labeled and immediately placed at -70° C.

4. Urine

Urine was collected by allowing the mice to void into autoclaved, empty cages. Clean capillary tubes (Fisherbrand) were then used to gather the pools of urine. A capillary tube bulb (Drummond Scientific Co.) was then used to void the urine out of the capillary tubes into clean microcentrifuge tubes (Fisher Scientific) for storage at -20° C.

5. Milk

Pups were separated from their mothers 1 hour before milking. Mothers were provided with a subcutaneous injection of 1 IU of Oxytocin (in 100 µL of solution) (Hospira) to stimulate milk let down. A milking apparatus was assembled using a laboratory vacuum attached to rubber tubing **(Figure 14)**. A disposable pipette tip (Fisher Scientific) was inserted into the end of the rubber tubing (pointed side inward). Mouse fur was dampened with water and alveolar area was cleared for the ease of pumping milk. The round open end of the pipette tip was then inserted over the alveolar area of each mammary gland and light suction was applied by turning on the vacuum. A pumping-like action was simulated with the pipette to promote the ejection of milk. Sterile capillary tubes were used to collect the milk droplets from the inside of the disposable pipette tips. A capillary bulb was used to empty the milk from the capillary tubes into microcentrifuge tubes (Fisher Scientific). Tubes were labeled and milk was stored at -20 ° C.

Milk was corrected for variations in protein content using a Bovine Serum Albumin Standard (BSA) protein assay kit 23227 (Pierce). Samples were diluted (1 μL milk in 100 μL deionized water) for the protein concentration of the milk to fall within the range of the standard curve (i.e. 25 μL - 2000 μL). Assay was performed according to the kit instructions.

6. Mammary Glands

Mammary glands were harvested from WT and *ctcgrp* null mice after a rapid cervical dislocation during mid-lactation, and late lactation (prior to this time they were not clearly visible). A lower abdominal incision was made with small surgical scissors and a clean cut was continued directly up through the mid-line of the mouse. Additional incisions were made traveling up each hind-limb to allow for full visualization and access to the mouse mammary glands. Embedded lymph nodes were removed with scissors and forceps. 5 sets of yellow/beige coloured mammary glands were clearly visible: one set under each upper-limb extending around the neck, 1 set adjacent to the thoracic region of the spine extending around the back, and 2 more sets in the abdominal and groin region **(Figure 15)**. Samples were quickly collected using autoclaved forceps and scissors rinsed with DEPC treated water. Mammary samples were then either snap-frozen in liquid nitrogen and placed at -70°C (for RNA extraction and analysis), or placed immediately in 10% formalin (37% formaldehyde, 10 x PBS pH 7.3, distilled water) to be embedded and sectioned for histomorphometry.

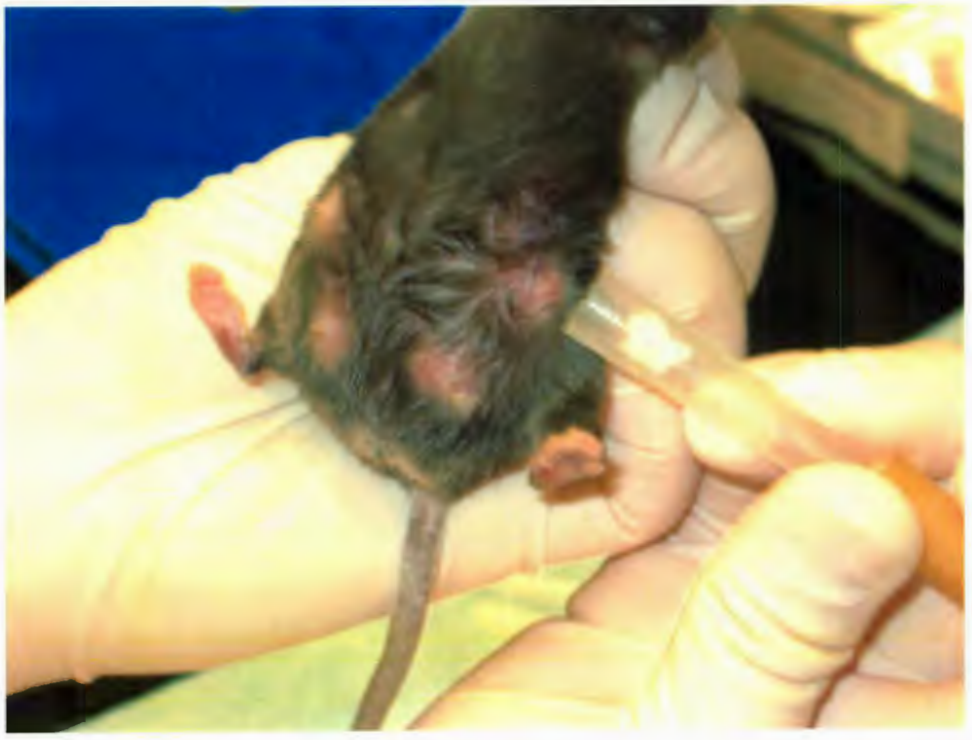


Figure 14: Apparatus used to Collect Milk from Mice during Lactation

A pipette tip was inserted into the end of the rubber tubing. The round open end of the pipette tip was then inserted over the alveolar area of each mammary gland and light suction was applied by turning on the vacuum. A pumping-like action was simulated with the pipette until milk ejected. *Photo taken by Danielle Ings.*

7. Pituitary Glands

Pituitary glands were harvested from WT and *ctcgrp* null mice after a rapid cervical dislocation during mid-lactation. To harvest pituitary samples incisions were made in the inferior portion of the mandible using small surgical scissors. Then, several cuts were made through the midline of the mandible exposing the skull. Using the same tools the skull was carefully removed and the brain was shifted to expose the underlying pituitary gland (i.e. small white coloured gland in back of brain) **(Figure 16).** Using forceps rinsed with DEPC treated water, pituitary samples were removed, snap-frozen in liquid nitrogen and placed at -70°C (for RNA extraction and analysis).

8. Bone

At various time points (i.e. non-pregnancy, late pregnancy, mid-lactation and late-lactation) mouse femur, tibia, fibula and spine regions were harvested. After euthanisation via cervical dislocation, hindlimb bones from WT and *ctcgrp* null mice were quickly harvested. Initially hair and flesh was removed using forceps and scissors. The heads of the femurs were then carefully separated from the ileum and pubis using circular motions **(Figure 17).** Spine regions were subsequently harvested by making three complete incisions with a disposable razor blade (Smith Brand®), one just below the cervical line (i.e. where dislocation occurs), the second just slightly above the lumbar region, and a third at the centre of this sample (i.e. creating 2 samples). Left hind limb and upper spinal regions were stored at -20°C in small labeled vials for biomechanical analysis **(Figure 17).** Right hind limbs and lower spinal regions were placed immediately in 10% formalin

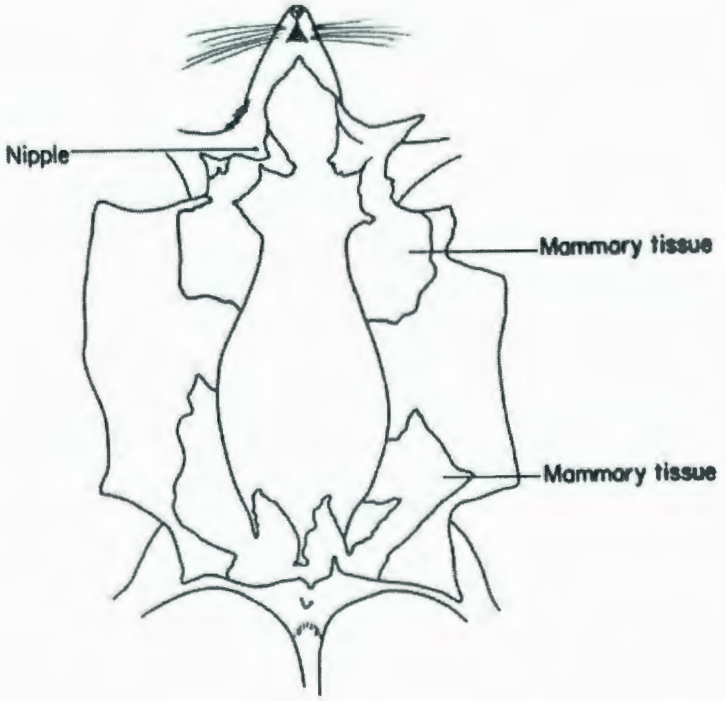


Figure 15: Location of Mouse Mammary Tissue. Reprinted from *The Anatomy of the Laboratory Mouse* by Margaret J. Cook. Copyright © 1965, with permission from Elsevier (**Appendix B**). Adapted for the Web by: Mouse Genome Informatics The Jackson Laboratory Bar Harbor, Maine, May 2005.

(37% formaldehyde, 10 x PBS pH 7.3, distilled water) to be embedded in plastic and sectioned for future histomorphometric analysis.

E. Bone Mineral Content

BMC was measured using the PIXImus 2 duel-energy x-ray absorptiometer (DXA) (GE Lunar). The corresponding software was PIXImus version 2.1 with Microsoft Windows 2000. A standard phantom (Fat 11.9% and BMC 0.063 grams) was used to perform quality control calibrations (QC) with the PIXImus daily. If QC failed, a field calibration was performed. If field calibration did not pass the PIXImus software was reinstalled, and global parameters reentered.

Brief anesthesia was induced using isoflurane (Minrad Inc.). Individual mice were placed in a small enclosed chamber (i.e. glass jar with sealed lid, containing a paper towel soaked with a small volume of anesthesia). After this time, anesthesia was maintained anywhere from 15 minutes to several hours with a single intraperitoneal (i.p.) injection of either thiopental sodium (Abbott Laboratories) or a combination of ketamine hydrochloride (Wyeth®) and Xylazine (Bayer).

Mice were subsequently placed on holding trays. Orientation of the mouse on the PIXImus tray was critical to maintain BMC reproducibility with <1% precision error. Spine regions were manually straightened, and the head and ear tags were excluded from active scanning regions of the DXA because of inconsistency of BMC in this area (**Figure 18**). In addition, as pregnancy progressed the expanding size of the mouse's body surpassed the capacity of the holding tray, and thus only the body and limbs could be included.

Figure 16: Location of mouse pituitary gland in the brain. Image obtained from *Mouse Atlas of Gene Expression* (www.mouseatlas.org). This work is licensed under a Creative Commons Attribution 2.5 License (www. creativecommons.org/licenses /by/2.5/).

Total body (minus head) and regional (spine, hind limb) BMC (grams) measurements were obtained for each mouse. Absolute values were normalized to the non-pregnant baseline BMC measurements (e.g. *ctcgrp* null BMC measurements for the peak of pregnancy were expressed as a ratio of the respective BMC measurement for each *ctcgrp* null mouse at baseline) **(Figure 18)**.

Initially, to determine the pattern of BMC, mice were scanned every day throughout full reproductive cycles. During this time anesthesia overdose caused deaths in a few cases. Later it was decided that mice would be scanned every other day, and eventually only at representative time points (i.e. baseline, late pregnancy, late lactation, post weaning) to ensure that the expected BMC changes were occurring **(Figure 18)**. Due to the x-ray exposure dams were euthanized at the end of the post- weaning, recovery phase as well as all pups from these pregnancies after they were weaned at 21 days of age.

Previous quality control studies were conducted in the Calcium Research Lab to determine fetal contributions to total body BMC. Mice on day 18.5 of pregnancy were scanned immediately before and after a C-section in which the pups were removed; this determined that the fetal skeletons contributed 1% or less to the apparent maternal BMC and was, therefore, negligible (data not shown).

F. Micro Computed Tomography

Both WT and *ctcgrp* null mice were euthanized at baseline and late lactation via cervical dislocation. Right femurs were immediately harvested and adjacent flesh was carefully taken off using forceps. Femur samples were then fixed in paraformaldehyde for

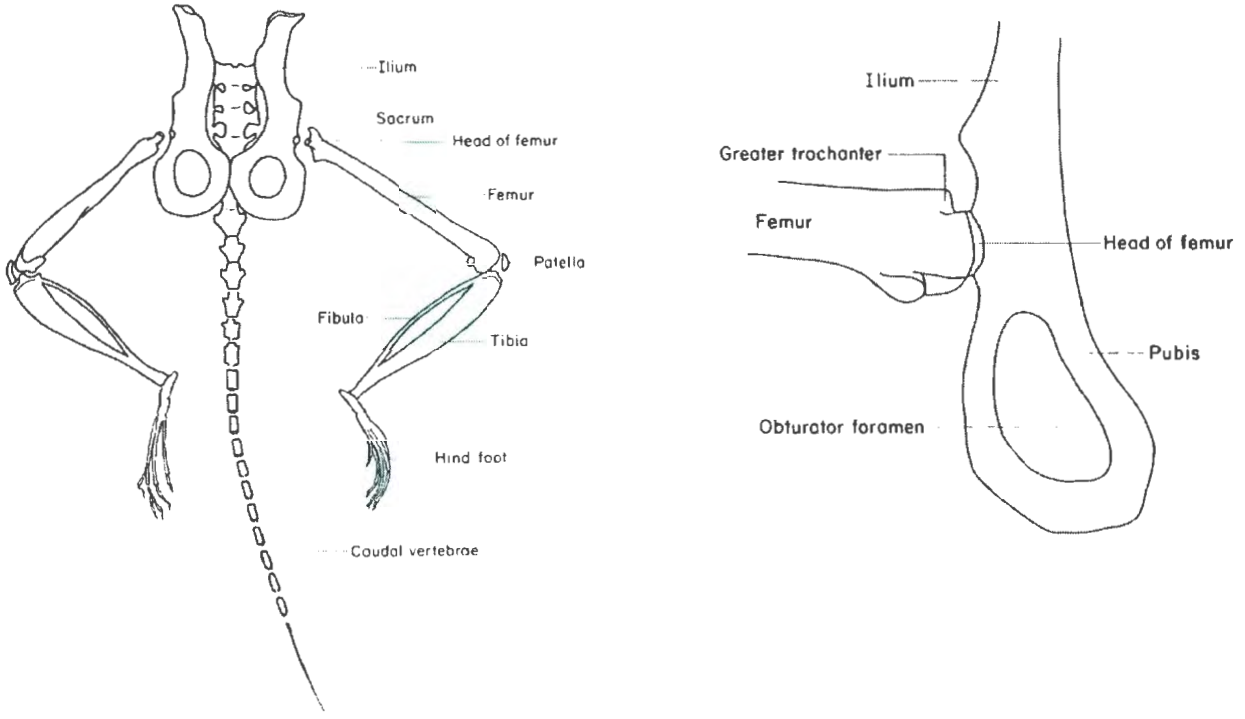


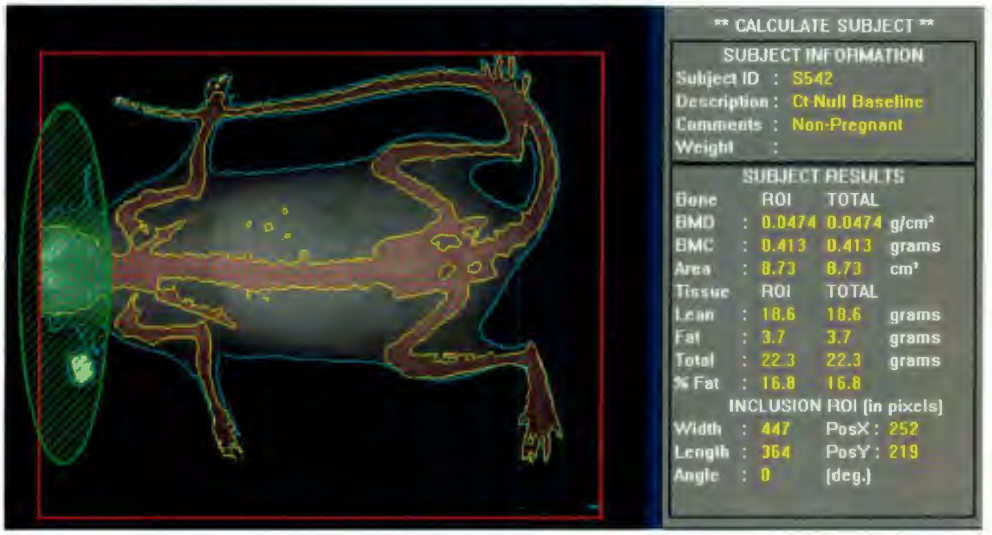
Figure 17: Collection of mouse hind limbs. The heads of the femurs were carefully separated from the ileum and pubis using a circular motion. Reprinted from *The Anatomy of the Laboratory Mouse* by Margaret J. Cook. Copyright © 1965, with permission from Elsevier **(Appendix B)**. Adapted for the Web by: Mouse Genome Informatics The Jackson Laboratory Bar Harbor, Maine, May 2005.

18 hours. After this time bones were rinsed, stored in 10 x phosphate-buffered saline at room temperature, and labeled until analysis.

The quantitative and qualitative μCT analysis for each femur was completed at the Centre for Bone and Periodontal Research at McGill University using the SkyScan 1072 (SkyScan,Vluchtenburgstraat 3, B-2630 Aartselaar, Belgium). This instrument has a 20-100 keV/0-250 μA sealed, air-cooled, microfocus x-ray source with a polychromatic beam derived from a tungsten target with a spot size of less than 5 μm at 4 W. For these analyses, the x-ray source was operated at 100 kV and at 98 μA (maximum power). Images were captured using a 12-bit, cooled CCD camera (1024 x 1024 pixels) coupled by a fiber optics taper to the scintillator.

Samples were scanned at a magnification resulting in a pixel size of 10.94 μm. Using a rotation step of 0.9 degrees and an exposition time of 2240 ms for each step, a total of 206 images was generated giving a scanning time of 30 min. The cross-sections along the specimen axis were reconstructed using Cone-Beam Reconstruction Software (SkyScan), with a distance between each cross-section of 27.371 μm. Each cross-section was reduced in half size to facilitate the analysis, giving of a voxel of 27.371 x 27.371 x 27.371 μm^3. CT-analyzer and 3D Creator software (both from SkyScan) were used to analyze and to do 3D rendering respectively. Data produced included mean trabecular thickness, mean trabecular spacing, total volume, anisotropy, volume fraction (%), volume (cubic mm), surface (square mm), surface/ volume (%) and structure model index (SMI) (**Figure 17**). See **Appendix D** for detailed protocol.

(a)



(b)

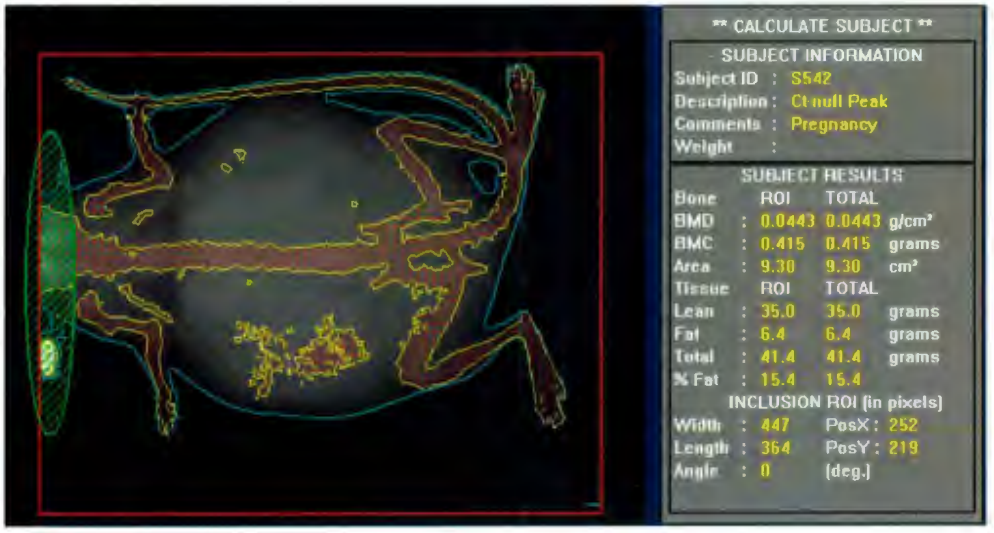


Figure 18: Bone Mineral Content (BMC) Assessment via PIXImus Bone Densitometry. (a) Sample PIXImus image at baseline (non-pregnancy), (b) Sample PIXImus scan at late pregnancy (17.5 days gestation).

G. Biomechanical Testing

Specimens were kept frozen until a few hours before testing and were then thawed to room temperature prior to biomechanical analysis (Instron Series 3340 electromechanical single column test instrument). Several biomechanical fixtures were subsequently attached to assess the strength of WT and *ctcgrp* null mouse bones. Each fixture is described below.

1. Three-Point Bend Test

Cortical bone strength was assayed using a 3 point-bend fixture (3PBF) attached to an Instron Series 3340 electromechanical single column test instrument (Instron) **(Figure 20a)**. Instron Series IX/s software package was used for the data acquisition, control and analysis. A compression test was selected from a list of standardized methods. Tibias were carefully separated from fibulas and femurs at room temperature. Each tibial sample was carefully and evenly placed across the lower part of the 3PBF (i.e. the 2 point end). Sample specifications (e.g. thickness, identification number and the corresponding reproductive time point (e.g. baseline, lactation) was entered into the software. The length) were measured with the millimeter (mm) side of a ruler. Values were subsequently entered into the software, along with the sample identification machine was balanced (i.e. zero force, zero distance) and the cross-head was lowered so that the10kN load cell with the anterior component of the 3PBF was approximately 2-3 cm above the mid-shaft of the tibia. The crosshead speed was set to 10 mm/ minute and the start button was manually activated. The cross-head proceeded to descend onto the specimen until it could no longer tolerate the force applied to it. This break point (i.e. point of failure, or

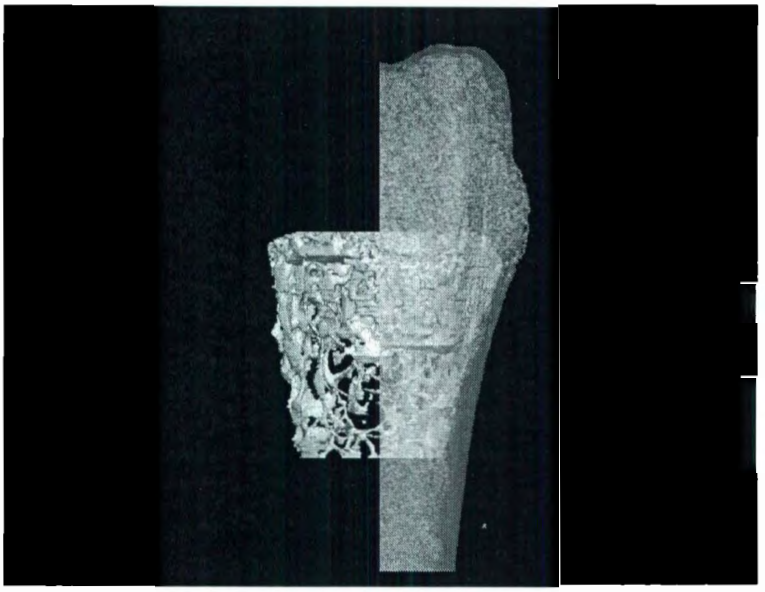


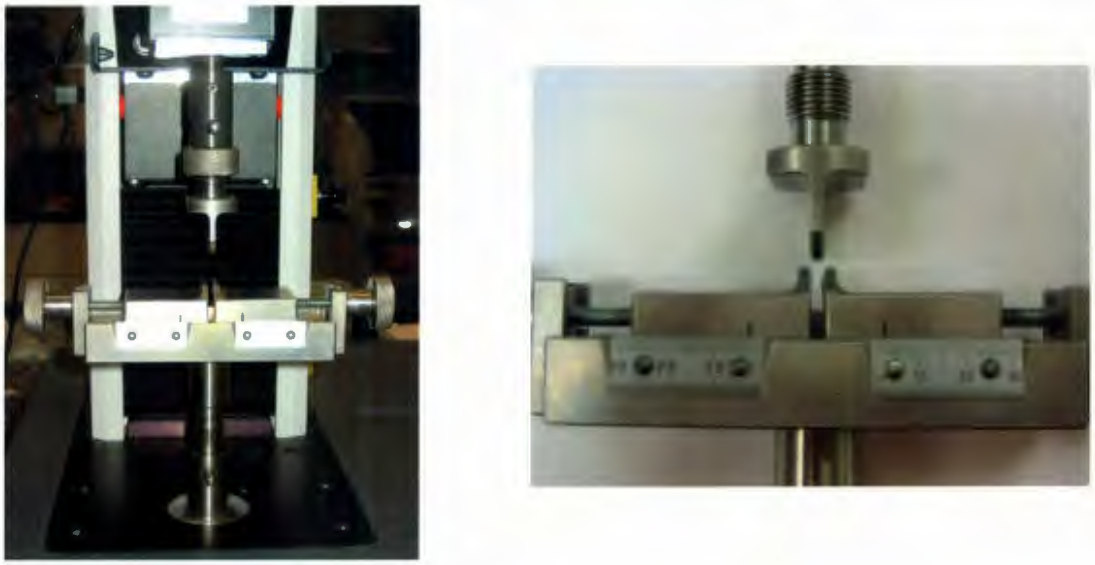
Figure 19: MicroComputed Tomographic (μCT) Image of a Mouse Femur. Sample SkyScan 1072 image of mouse femur showing trabecular thickness distribution and trabecular separation distribution (McGill University, Centre for Bone and Periodontal Research).

spike on the curve) was recorded, as well as the load-displacement curve from which other biomechanical parameters were derived. The bone fragments were then discarded. Values for load (kN), displacement (cm) and slope were then placed in a spreadsheet for analysis.

2. Crush Test

To provide a more extensive analysis of both cortical and trabelcular bone, a 'crush fixture' was conceptualized and designed at Memorial University. Two round and flat pieces of metal (2.5 cm x 2.5 cm x 1 cm) were attached to a fixture stem and scaled to fit into the Instron Series 3340 electromechanical testing device **(Figure 20b)**. Single thoracic vertebral bodies were cut from intact thoracic regions and used for analysis. The superior and inferior endplates were removed from the cortical rims to even the surface. Samples were lodged at the centre of the lower fixture plate. As per the 3PBT, specifications were measured and values were entered into the software, along with the sample identification number and corresponding reproductive time point. The machine was balanced and the cross-head was lowered approximately 2-3 cm above the vertebral sample. The crosshead speed was set to10 mm/ minute and the start button was manually activated. The cross-head proceeded to descend onto the specimen until it could no longer tolerate the amount of force applied to it, or a maximum force was reached (in this case the cross-head would retract at 10kN). This break point (i.e. point of failure, or spike on the curve) was recorded from the load-displacement curve from which other biomechanical parameters were derived. The compressed vertebral bodies were then discarded.

(a)



(b)

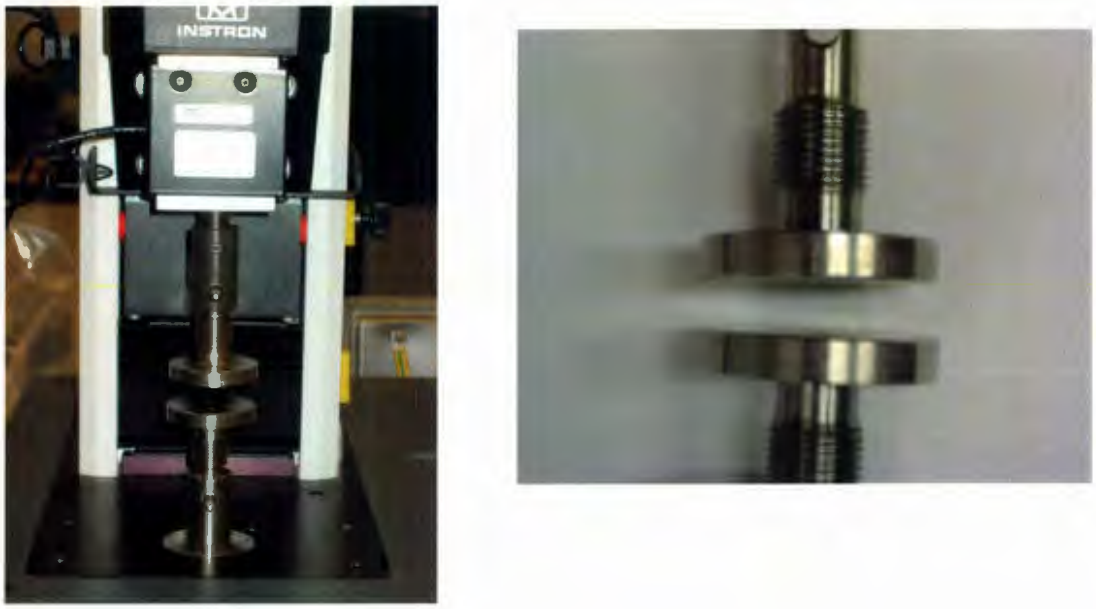


Figure 20: Biomechanical Fixtures used to assess bone strength (a) Three-point bend fixture used to assess the strength of long bones, and (b) crush fixture used to assess the strength of irregular and flat bones.

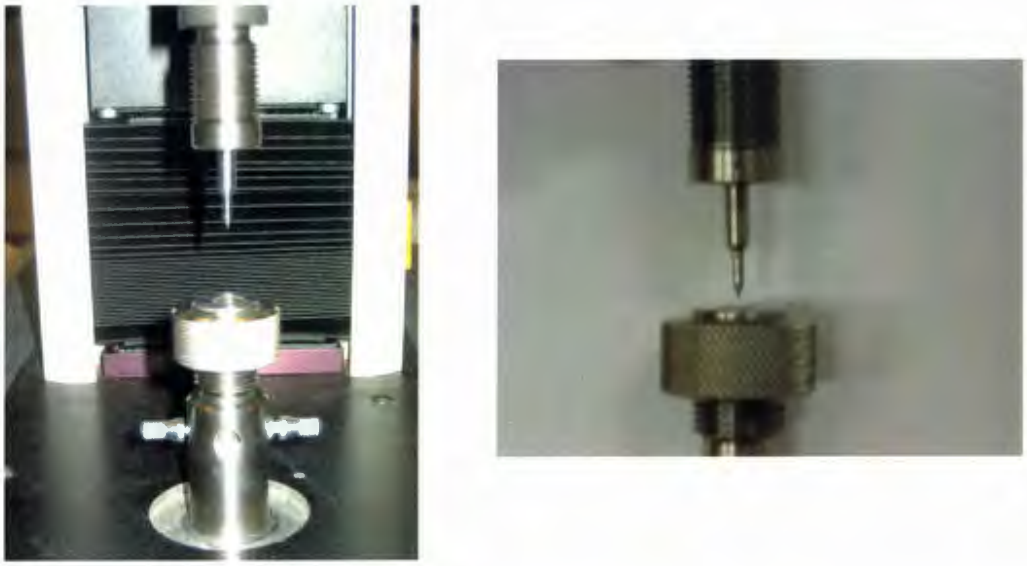
3. Narrow Pointed Fixture Compression Test

A 3rd fixture was designed to assess primarily trabecular bone strength **(Figure 21a)**. The conceptualization of this fixture came from Dr. Molgaard from the Faculty of Engineering, Memorial University of Newfoundland. The design was executed by the Machine shop at the Health Sciences Centre, Memorial University. The descending component of the fixture included a long, narrow metal component with a cone shaped pointed tip (2.5 cm x 3 mm) **(Figure 21a)**. The bottom of the fixture (i.e. the stationary component) included a small round incision drilled into it, accommodating the ascending portion of the fixture upon descent **(Figure 21a)**. The fixture was intended to penetrate through the vertebral cross section, without disturbing the cortical rim. The vertebral cross section was lodged directly on top of the insert in the bottom fixture. By readjusting the crosshead, the ascending fixture was placed 2-3 cm above the vertebral cross section. The machine was then balanced (i.e. zero force, zero distance) and the test was initiated. The crosshead speed was set to 10 mm/ minute and the start button was manually activated. The cross-head proceeded to descend onto the specimen until it could no longer tolerate the amount of force applied to it, or a maximum force was reached. Point of failure was recorded (i.e. first spike on graph), as well as the load-displacement curve from which other biomechanical parameters could be derived.

4. Narrow Flat Fixture Compression Test

Furthermore, a 4th biomechanical fixture was designed with slight modifications from the 3rd fixture **(Figure 21b)**. This fixture was also designed to assess

(a)



(b)

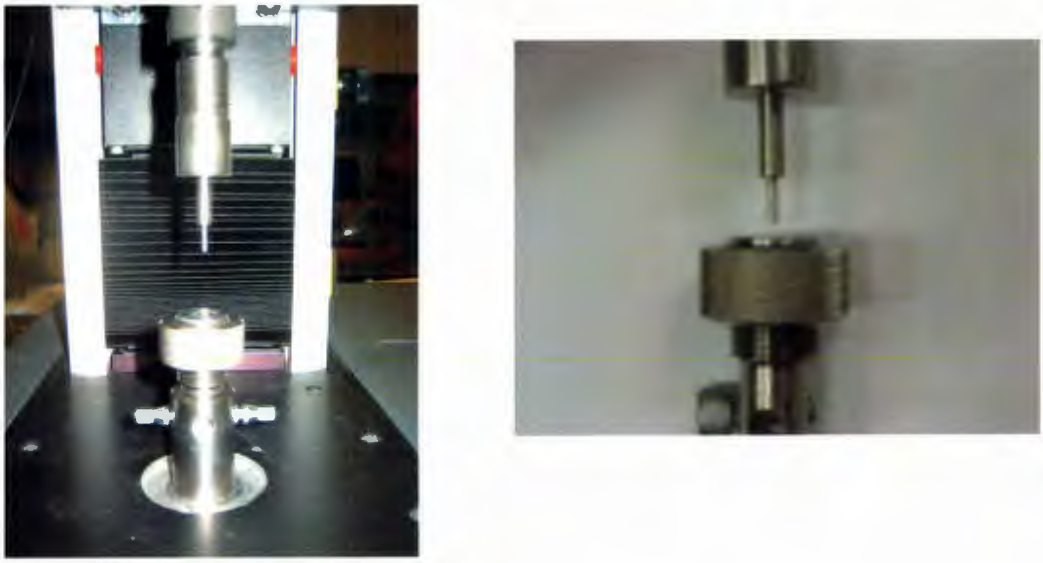


Figure 21: Biomechanical Fixtures used to assess bone strength (a) Narrow pointed fixture and (b) Narrow flat fixture. Both fixtures were custom designed for the assessment of trabecular (cancellous) contributions to bone strength.

trabecular strength. This design was executed by the Machine shop at the Health Sciences Centre, Memorial University. The descending component of the fixture included a long, narrow piece of metal with a flat round tip (2.5 cm x 3 mm). The bottom of the fixture (i.e. the stationary component) was the same as the lower portion of the 3rd fixture. This fixture was intended to penetrate through the vertebral cross section, and evenly penetrate the trabecular framework within the cortical shell of the vertebral bodies. The vertebral cross section was lodged directly on top of the insert in the bottom fixture. By readjusting the crosshead, the ascending fixture was placed 2-3 cm above the vertebral cross section. The machine was then balanced (i.e. zero force, zero distance) and the test was started. Point of failure was recorded (i.e. first spike on graph), as well as the load-displacement curve from which other biomechanical parameters could be derived.

H. Mineral Assessment

1. Whole Blood Ionized Calcium

Whole blood ionized calcium was measured on fresh mouse tail blood using a Chiron Diagnostics 634 Ca++/pH Analyzer (Chiron Diagnostics) with high, medium and low test controls (Bayer HealthCare). Samples were collected directly from mouse tail veins at room temperature using heparinized capillary tubes (Bayer). Time points assessed included: baseline, late pregnancy, 24-48 hours post-partum, early, mid and late lactation, as well as post partum.

2. Flame Atomic Absorption Spectroscopy and Milk Calcium

Maternal milk calcium content was analyzed via flame atomic absorption

spectroscopy (Perkin-Elmer 2380 Atomic Absorption Spectrometer). High, medium and low calcium standards were made using nitric acid and increasing concentrations of a calcium reference solution (Fisher Scientific) (i.e. 0.25 µg/ml, 0.75 µg/ml, 1.5µg/ml). Whole milk samples were diluted (5 µl milk: 1000 µl nitric acid) and assessed in duplicate on the atomic absorption spectrometer (Perkin-Elmer 2380 Atomic Absorption Spectrometer). Milk was subsequently corrected for variations in protein content using a protein assay with Bicinchoninic acid (BSA™ Protein Assay Kit).

3. Urine Calcium and Creatinine Correction

Calcium content of maternal urine samples was assessed using a colorimetric assay (Sigma-Aldrich) as per kit instructions. Urine was subsequently normalized to creatinine concentrations. Creatinine was measured using an alkaline picrate colormetric assay (Diagnostic Chemicals Limited, Ref N. 221-30).

I. Hormone Physiology

1. Parathyroid Hormone (PTH)

The PTH content of maternal serum was measured at baseline (pre-pregnancy), late pregnancy, early lactation, late lactation, and post-weaning (21 days post - lactation) in both WT and *ctcgrp*-null mice using a rat 96 well PTH intact enzyme-linked immunosorbent assay (ELISA) kit (Immutopics) that is also reactive against mouse PTH. Two different goal polyclonal antibodies to rat intact PTH were used in the kit: an antibody specific to the mid-region/C-terminal portion (39-84) of PTH biotinylated (for capture), and another antibody specific to the PTH N-terminal region (1-34) conjugated

with the enzyme horseradish peroxidase (for detection). The sensitivity of the kit was 1.6 pg/mL of PTH. Assay was performed according to the kit instructions, with each sample being assayed in duplicate when possible. The absorbance of each sample was taken on a spectrophotometric kinetic microtiter plate reader (Molecular Devices) capable of reading absorbance at 400 nm and 750 nm.

2. Estradiol (17- beta Estradiol)

Serum Estradiol was assessed using an enzyme immunoassay (EIA) (Cayman Chemical Catalog No.582251). The detection limit of the assay was 8 pg/mL. The assay was based on the competitive binding of either free estradiol or an estradiol tracer (estradiol linked to an acetylcholinesterase (AChE) molecule) for a limited amount of estradiol-specific rabbit antiserum binding sites. The amount of estradiol tracer able to bind to the rabbit antiserum was inversely proportional to the concentration of free estradiol in each well. The assay was carried out according to kit instructions with each sample being assayed in duplicate when possible. The absorbance of each sample was then taken on a spectrophotometric kinetic microtiter plate reader (Molecular Devices) capable of reading absorbance from 400 nm to 750 nm.

3. Prolactin

Serum Prolactin was assessed using a RIA by Dr. A.F. Parlow (National Hormone and Peptide Program, Harbor, UCLA Med Center, CA, USA). The following immuno-reactants were used in the RIA: antiserum to mouse prolactin (rabbit), highly purified mouse prolactin antigen for iodination and mouse prolactin reference preparation (1

ampoule containing 5 micrograms, lyophilized in 1 ml 1% bovine serum albumin in PBS). All reactants were added to the RIA tubes at a single sitting, at refrigerator temperature, in the sequence of a) buffer, b) standard or unknown, c) radiolabelled antigen, and d) antiserum at a final, tube dilution of 1:400,000. The reactants were then incubated at room temperature for 24 hours, prior to the addition of the second antibody. Standard curves were then created for mouse prolactin concentrations and unknown values were subsequently calculated.

4. Parathyroid Hormone Related Protein (PTHrP)

Mouse plasma PTHrP was measured using an in-house PTHrP radioimmunoassay (RIA) specific to the N-terminal of PTHrP designed by Dr. T.J. Martin (St Vincent's Institute of Medical Research, Melbourne, Australia) **(Appendix C)**. In brief, on day 1: standards, plasma samples and assay buffer were incubated, tracer was added and incubated overnight at room temperature. On day 2: anti-goat Sac-Cel was added and incubated for 30 minutes at room temperature, 1 ml of distilled water was added to each tube, samples were then centrifuged and supernatant was aspirated. Finally pellets were counted using a gamma counter (Beckman Coulter $_{TM}$ LS 6500). Normal human plasma (stored at -80° C) was used in lieu of the serum from haemochromatosis patients as per the protocol **(Appendix C)**.

J. Markers of Bone Turnover

1. Deoxypyridinoline (Dpd) crosslinks

Dpd crosslinks, a common bone resorption marker, was quantified in the urine of WT verses *ctcgrp* null mice at baseline, late pregnancy, late lactation and the post-weaning period using a METRA DPD EIA (Quidel). The limit of the kit was 1.1 nM/L. The assay was performed according to the kit instructions, with each urine sample being assayed in duplicate. The absorbance of each sample was then taken on a spectrophotometric kinetic microtiter plate reader (Molecular Devices) capable of reading absorbance at 400 nm and 750 nm. Dpd was then expressed relative to creatinine in order to correct for variations in urine concentration. Creatinine was measured using an alkaline picrate colormetric assay (Diagnostic Chemicals Limited, Ref N. 221-30). The assay was based on the reaction between the sample and sodium picrate, where creatinine combines with the reagent to form a red-orange complex. Final calculations involved dividing the Dpd value (nmol/L) by the creatinine value (mmol/l) of each sample (creatinine mg/dl x 0.088 = mmol/l). The assay was performed according to kit instructions with final results were expressed as nmol Dpd/mmol creatinine

2. Osteocalcin

Serum osteocalcin, a common bone formation marker, was quantified in the serum of *ctcgrp* null mice verses WT at: baseline, late pregnancy, early lactation, late lactation and during the post weaning period (21 days post-lactation). A two- site IRMA (Immutopics) was used for this analysis. The IRMA utilized two different antibodies specific to mouse osteocalcin; a polyclonal goat antibody recognizing the mid-region C-

terminal region (immobilized onto a plastic bead for capture) and a polyclonal goat antibody recognizing the amino terminal region (radiolabeled for detection). The assay was performed according to the kit instructions with each sample being assayed in duplicate. The assay sensitivity was 0.1 ng/mL. Each tube was counted for 1 minute on a1480 WALLARD Wizard^{TM} automatic gamma counter (PerkinElmer) and counts were subsequently recorded.

K. Immunohistochemistry

Immunohistochemistry was completed to immunolocalize PTHrP in the mammary epithelium during lactation in the WT versus *ctcgrp* null mice. A peroxidase antioperoxidase protocol was used (**Appendix E**). In brief paraffin embedded mammary tissue were dewaxed with xylene, rehydrated in ethanol, submerged in methanol with 1% hydrogen peroxide to block endogenous peroxidase activity of the sections. These sections were then incubated with a primary antiserum of rabbit anti PTHrP. This antiserum was denoted 'r87' and was obtained from Dr. Jack Martin's lab at the University of Melbourne. The 'r87' antiserum was specific to the amino terminal region of PTHrP. For each sample duplicate a separate negative control slide containing non-immune rabbit serum at the same dilution was also used. All sections were then rinsed and incubated with a secondary antibody (i.e.goat anti rabbit immunoglobulin) for 30 minutes. Sections were again rinsed and incubated in diaminobenzidine, tris and water, tris buffer, rinsed and stain with Mayer's haematoxylin for 30-60 seconds and dehydrated through graded absolute ethanol ethanol 75% , 90%, 100% x 2. Finally samples were rinsed in xylene and mounted in p-xylene-bis-pyridinium bromide (DPX).

L. RNA Extraction

Total RNA was extracted from whole mammary glands using an RNeasy Midi Kit (Quiagen). Approximately 100 g of mouse mammary gland yielded 0.6-1.3 μg of RNA per μl of solution.

M. cDNA Synthesis

cDNA synthesis was performed on mouse mammary gland RNA samples using the Superscript III First-Strand Synthesis System (Invitrogen).

N. Real- Time Quantitative RT-PCR

Quantitative real-time RT-PCR data and analysis were obtained using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). For the calcitonin receptor (CTR), the calcium receptor (CaR), and the Vitamin D receptor (VDR) Taqman® Gene Expression Assays were used that included pre-designed primers and probes for optimal amplification (Applied Biosystems) along with Taqman® Universal PCR Master Mix (Applied Biosystems). All gene expression assays have a FAM reporter dye at the 5' end of the TaqMan MGB probe and a non-fluorescent quencher at the 3' end of the probe. Other gene specific primers used for this study were as follows; for parathyroid hormone related peptide (PTHrP) (forward) 5'-TCCACACAGCCGAAATCAGAGCTA -3'; (reverse) 5'-TTCTCCTGTTCTCTGCGTTTCCCA-3', for1-alpha hydroxylase, (forward) 5', CCAGAGCGCTGTAGTTTCTCATCA-3', (reverse)

5'ATGAAGGTTTCTGTGTCAGGAGGG-3', for calcium transport protein subtype 1, (forward) 5' ATCGATGGCCCTGCGAACT 3', (reverse) 5' CAGAGTAGAGGCCATCTTGTTGCTG 3'(20), for epithelial calcium channel, (forward) ATTGACGGACCTGCCAATTACAGAG 3', (reverse) 5' GTGTTCAACCCGTAAGAACCAACGGTC 3' (20). These primers were used along with SYBR® Green PCR Mastermix. The real-time PCR thermal cycler protocol included 1 cycle at 50 °C for 2 minutes, 1 cycle at 95 °C for 10 minutes, and 40 cycles of 95 °C for 15 seconds and 1 minute at 60.0 °C. For primers using SYBR® Green PCR Mastermix the real-time PCR program was the same, but without the first cycle at 50 °C for 2 minutes. All samples were performed in triplicate and expression ratios were calculated from differences in threshold cycles (C_T), which represents the PCR cycle at which the an increase in reporter fluorescence above the baseline signal can first be detected. GAPDH was used as a control gene.

O. Duodenal Calcium Absorption

Duodenal calcium absorption was measured in non-pregnant versus mid-lactating WT and *ctcgrp* null mice as previously described (228). In brief, fasted mice were anesthetized with an i.p. injection of a combination of ketamine hydrochloride (Wyeth®) and Xylazine. After animals were anesthethized a closed duodenal loop of approximately 1.5 cm in length was created using surgical suture material and closed tied loops at each end of the segmented area. Next 16uCi/mL of $Calcium^{45}$ (in 40 µl of buffer containing NaCl 150mM, CaCl2 2mM (the calcium carrier) and Tris-HCl 30 mM) was injected into this loop. After 10 minutes the loop was excised and mice were subsequently euthanized.

The duodenal sections were immediately solubilized in glass vials containing Scintigest and stored at 65°C overnight. The next day radioactivity was counted on a liquid scintillation counter to determine the amount of calcium absorbed. Non-viable loops were detected and discarded.

P. Calcitonin and CGRP-α Dose Finding and Rescue Experiment

Dose finding experiments were conducted to determine the amount of salmon calcitonin (SCT) (Rhone-Poulenc Rorer) was required to prevent losses of BMC during lactation. A mean intramuscular (i.m.) dose of 0.067 μmoles or 10 IU of SCT (Rhone-Poulenc Rorer) was provided daily in 60μl of saline (vehicle) beginning on the day of delivery and throughout 21 days of lactation to representative WT and *ctcgrp* null mice. In addition other WT and *ctcgrp* null mice received equimolar doses (0.067 μmoles or 255 μg in 60 μl of saline) of rat CGRP-α (American Peptide Co Lot: S02011A1). Control mice received once daily i.m. injections of either 100 μl of 0.9% saline.

Mice were briefly anesthetized with isoflurane (as per method previously described) in order to receive the injection. A small area on the hindlimb was sterilized with 70% ethanol prior to i.m. injection. Bone densitometry was used every second day throughout the experiment to precisely determine the excursion of BMC. Mice and pups were euthanized post rescue experiment.

Q. Statistical Analysis

Data was analyzed using SYSTAT 5.2.1 for Macintosh (SYSTAT Inc, Evanston,

IL). ANOVA was used for the initial analysis; Tukey's test was used to determine which pairs of means differed significantly from each other. Real-time quantitative RT-PCR results were analyzed by the $2^{-\Delta\Delta C}T$ method where the target and reference are amplified in separate wells (21). Two-tailed probabilities are reported, and all data are presented as mean ± SE. Where statistical significance between pairs of means exists, this is indicated on the respective figure or table. If no pairs of means have a notation to suggest that they were statistically significant from each other, this means that they were not.

Furthermore, planned group sizes were based on several power calculations. Power is the probability of finding a statistically significant difference at a given "P" value with a specific number of subjects in each group. Calculations revealed that for DEXA experiments, to obtain a power of 95% ($p < 0.10$) with an anticipated difference between means of 20%, 5 mice were required in each group. The actual group size of mice in a few cases was more than 5 due to the fact that initially we thought we needed more mice for these experiments. Unfortunately, mice were not always readily available and some died during or after anesthesia and some ate their pups and therefore could not be studied during lactation. In fact, the observed BMC difference was 100% between the two groups (the doubling of BMC loss) so that the group size of 5 for a predicted 20% difference was more than adequate. In contrast, for the biomechanical testing, we later learned that due to the variability of expected results with this technique that colleagues often use a sample size of 15-20 per group, and thus we had underestimated the numbers required for that particular procedure. Also,

biomechanical testing was a new procedure for our lab and we didn't know all of its inherent problems.

IV Results

A. Litter Sizes in WT, HET and *Ctcgrp* Null Mice

Initially, the number of live pups present at the time of weaning (i.e. day 21 of lactation) was counted to determine if absence of calcitonin would affect postnatal litter size. Results indicated that there was no difference in litter size between the WT, *ctcgrp* HET and *ctcgrp* null female mice at the time of weaning **(Figure 22)**. Mean litter sizes were as follows: WT 8.8 pups ± 0.6 (n=9), HET 7.9 pups ± 0.6 (n=11) and *ctcgrp* null 7.6 pups ± 0.5 (n=18) (p=0.33). Only WT and *ctcgrp* nulls were used for subsequent experiments.

Previous findings, in contrast, showed that the number of viable fetuses in utero of *ctcgrp* null females was significantly lower (7.9 pups ± 0.4) than the number of *ctcgrp* HET females (9.1 pups ± 0.3) ($p<0.02$) when assessed on embryonic day 17.5 or 18.5 post cessarian section (233). Variations in litter sizes between genotypes before birth versus that seen at weaning may result from unfavorable changes in calcium metabolism in the *ctcgrp* null in utereo. Possible reasons for lack of a significant difference in litter size between genotypes at weaning could be: potential survival advantages after birth for the *ctcgrp* null or selected culling of the WT pups after birth. It is also possible that this result is simply a chance occurrence or the difference persisted but couldn't be detected with smaller numbers counted post-natally. A larger sample size might have been needed to detect a difference in litter size between genotypes.

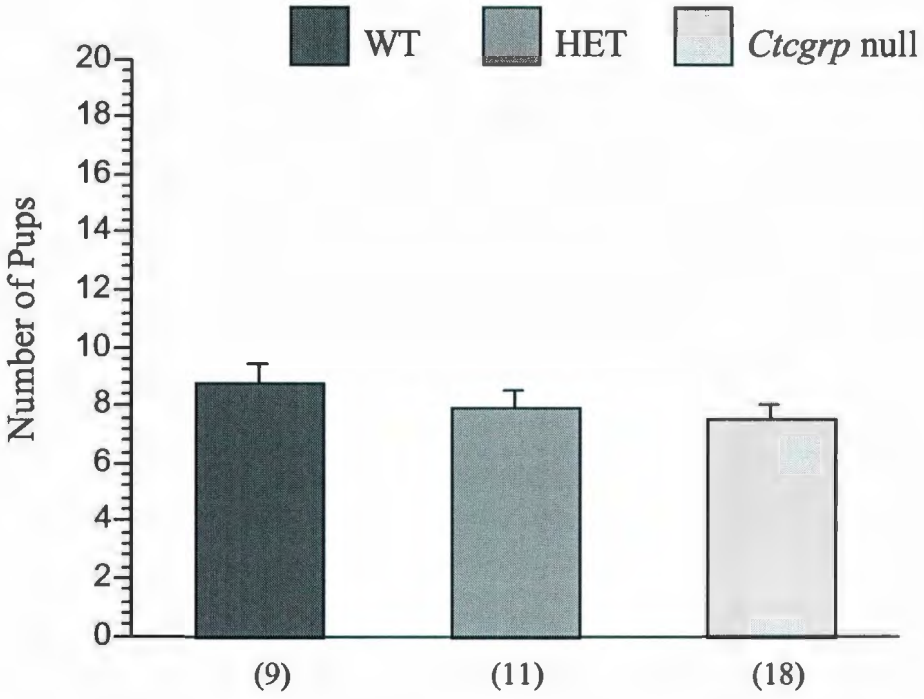


Figure 22: Litter Sizes in WT, HET and *Ctcgrp* null mice. At weaning the litter sizes (mean ± SE) were not significantly different between *ctcgrp* null, HET and WT female mice. Numbers in parentheses represent the number of mice studied.

B. Bone Mineral Content

Mice were taken, and serially scanned by DXA every second day throughout reproduction to determine if loss of calcitonin would affect the normal cycling of BMC during reproduction compared to the normal siblings. Calcitonin normally suppresses bone resorption by directly inhibiting the number and function of the bone digesting osteoclast cells (77). Therefore lack of calcitonin, in the *ctcgrp* nulls, was hypothesized to cause uninhibited bone resorption and a subsequent decrease in BMC during reproduction, a time of increased calcium demand.

Findings indicated that at the baseline age of 10-12 weeks, *ctcgrp* null mice had a higher mean BMC (0.397 g ± 0.016 g) (n=15) by DXA versus that seen in the WT siblings (0.383 g ± 0.021 g) (n=8) **(Figure 23)**. Baseline BMC values, however, were not statistically significant between genotypes. Previous research using contact x-rays of vertebral bodies and tibias also showed that baseline bone density trended higher in 1- and 3-month-old female *ctcgrp* null mice versus WT mice of the same age (103). Within this particular study by Hoff and colleagues (103), *ctcgrp* null mice were also found to have a higher trabecular bone volume in vertebral bodies at 1 and 3 months and in the proximal tibia at 3 months (103).

After 18.5 days of pregnancy whole body BMC in WT mice increased by 15.9% ± 3.4% above baseline (i.e. an increase of 0.061g) (n=5). In *ctcgrp* null mice, gains in total body BMC during pregnancy increased by 9.9% ± 2.6% above the respective pre-pregnancy baseline values (i.e. an increase of 0.039 g) (n=7). Thus, mean gains in mineral during pregnancy were no different in *ctcgrp* nulls versus that seen in the normal WT siblings (p=0.365) **(Figure 22)**.

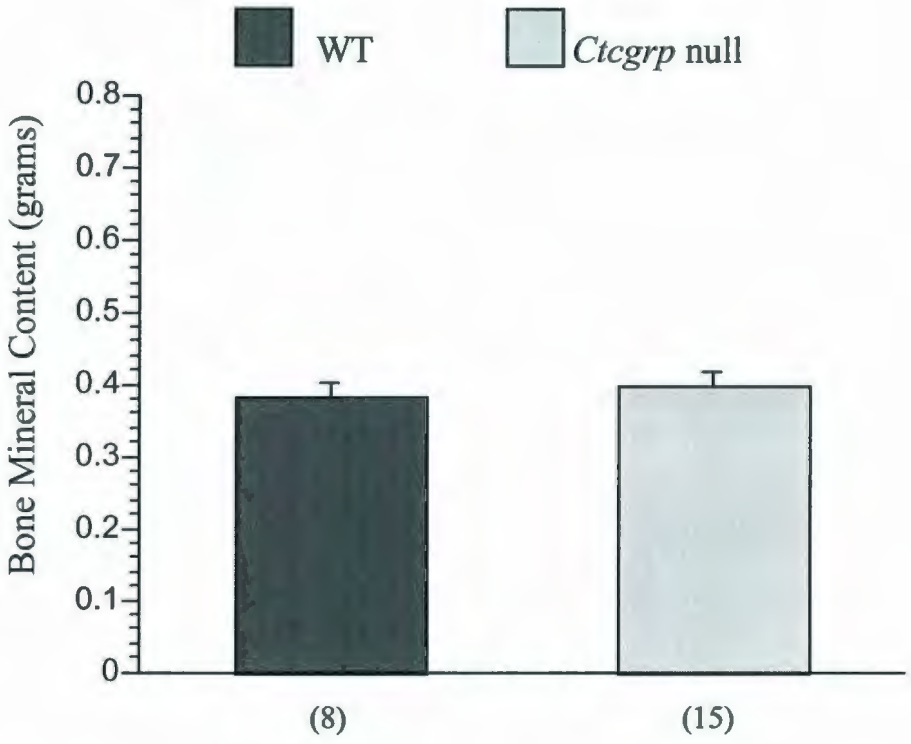


Figure 23: Baseline BMC (grams) in WT and *Ctcgrp* null mice. At the baseline age of 10-12 weeks *ctcgrp* null mice had a BMC (mean ± SE) that was not significantly different than in the WT siblings ($p = 0.605$) Numbers in parentheses represent the number of mice studied.

After 7 days of lactation, however, *ctcgrp* null mice were found to have lost significantly more total body BMC via DXA (-10.1 % ± 3.1%, i.e. a decrease of 0.0401 grams) (n=7) when compared to the loss experienced by the WT sisters (-4.5% ± 3.7%, i.e. a loss of 0.017 g) (n=5) (p=0.000) and the respective pre-pregnancy baseline BMC values. After 14 days of lactation this significant result was seen again, with *Ctcgrp* null mice having lost significantly more BMC (-29.6% ± 3.1%, i.e. a loss of 0.118 g) (n=7) versus WT mice (-15.7% ± 3.7%, i.e. 0.060 g) (n=5) (p=0.00). Most noticeable, however, were BMC values at 21 days of lactation (i.e. at the end or 'trough' of lactation). At this time, *ctcgrp* null mice were found to have lost almost double the amount of total body BMC (-32.3% ± 2.3%, i.e. a loss of 0.128 g) (n=7) by DXA versus that seen in the WT siblings at this time (-19.9% ± 3.0%, i.e. a loss of 0.076 g) (n=5) with respect to the respective pre-pregnancy baseline BMC values (p<0.020) **(Figure 24).**

Regional BMC measurements (i.e. hind limb and cervical spine) were also assessed by DXA at baseline and throughout reproduction in WT and *ctcgrp* null mice. Baseline hindlimb BMC values were as follows: WT 0.075 g ± 0.003 g (n=5) and *ctcgrp* null 0.068 g ± 0.002 g (n=7). During pregnancy hindlimb BMC increased by 0.05% ± 3.4% (i.e. an increase of 0.004 g) (n=5) and 7.2% ± 2.9% (i.e. an increase of 0.005 g) (n=7) in the WT and *ctcgrp* null mice respectively, when compared to the baseline BMC values. These values were not significantly different (p=0.530) **(Figure 25).** After 21 days of lactation, however, losses in the hind limb of *ctcgrp* null mice (i.e. -28.4 % ± 2.9 %) (n=7) were significantly greater than that in the hind limb of the WT siblings (-12.9% ± 3.4 %) (n=5) (p=0.00) when compared to the respective pre-pregnancy

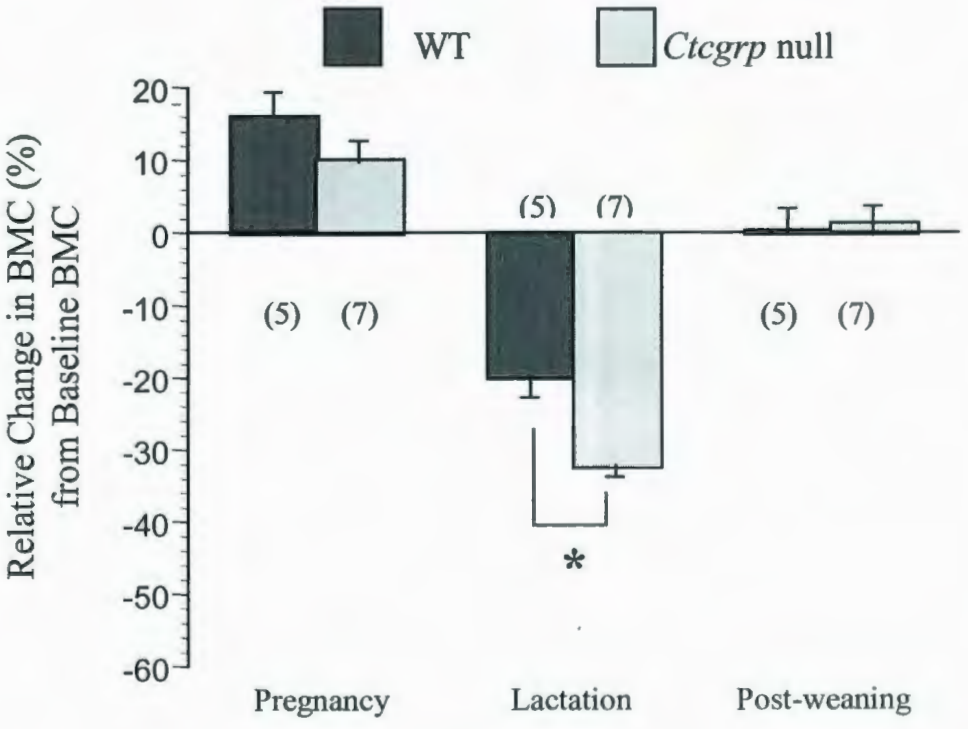


Figure 24: Total Body BMC changes in WT and *Ctcgrp* null mice throughout reproduction relative to Baseline BMC. Gains in total body BMC (mean ± SE) during pregnancy were not significantly different between WT and *ctcgrp*-null mice. During lactation, however, *ctcgrp* null mice lost significantly more total body BMC compared to the WT siblings (*p=0.002). Despite these losses during lactation, both WT and *ctcgrp*-null mice completely regained BMC levels post-weaning. Numbers in parentheses represent the number of mice studied.

baseline BMC values (i.e. lactation BMC losses: WT 0.010 g, *ctcgrp* null 0.019 g) **(Figure 25)**.

Baseline spine BMC values were as follows: WT 0.075g ± 0.003 g, *ctcgrp* null 0.076 g ± 0.003 g. During pregnancy spine BMC levels decreased by -4.0%± 5.4% (i.e. a loss of 0.003 g) (n=5) and -12.6 % ± 4.6 % (i.e. a loss of 0.010 g) (n=7) in the WT and *ctcgrp* null mice, respectively, when compared to baseline BMC values. These values were not significantly different. Again, during lactation, however, losses in the lower spine in the *ctcgrp* nulls (-51.6% ± 4.6%) (n=7) were over double that seen in the WT (-24.4% ± 5.4%) (n=5) (p=0.00) at that time (i.e. lactation BMC losses: WT 0.018 g, *Ctcgrp* null 0.039g) **(Figure 26)**.

Despite the significant losses of BMC seen during lactation in the WT and more significantly in the *ctcgrp* nulls, both genotypes restored their BMC to their respective baseline values after weaning **(Figure 24)**. Full recovery, however, took several additional days in the *ctcgrp* null mice (18.1 ± 0.8 days) (n= 7) compared to the WT siblings (13.1± 1.1 days) (n=4) (p=0.0065). This finding was not surprising considering the degree of BMC loss in the *ctcgrp* null.

It is important to note that for most of these experiments there were notable discrepancies in the group sizes of mice. Sample numbers did not turn out as consistent as expected due to unanticipated deaths during experiments, as well as challenges with timed pregnancies and therefore mice being available at the same time for comparison.

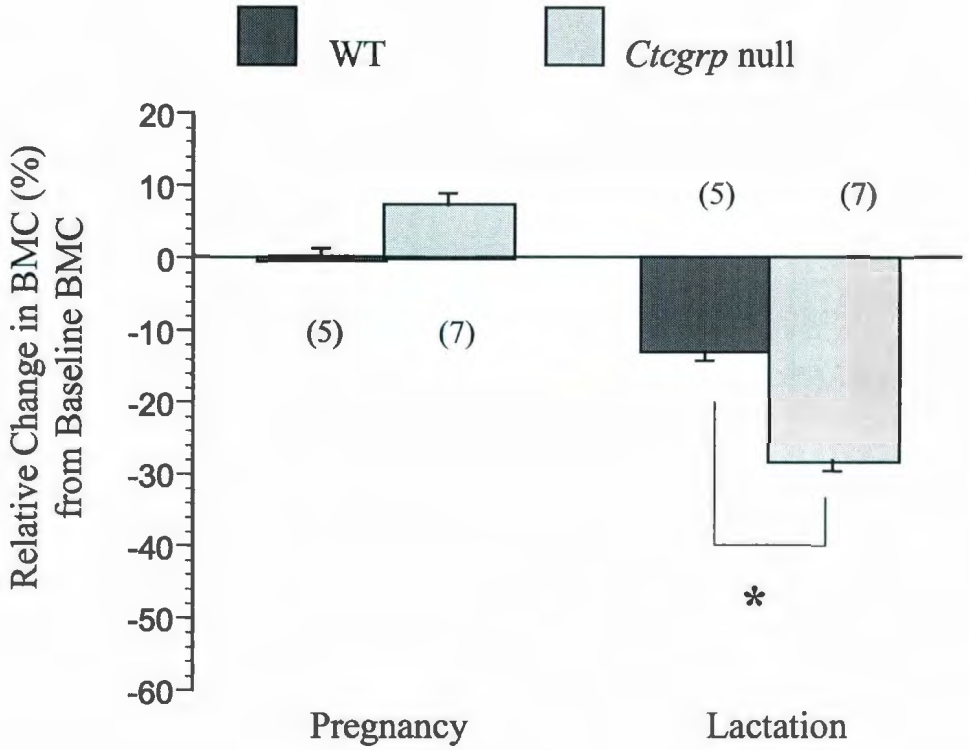


Figure 25: Hindlimb BMC changes in WT and *Ctcgrp* null mice during Pregnancy and Lactation relative to Baseline BMC. Gains in hindlimb BMC (mean ± SE) during pregnancy were not significantly different between WT and *ctcgrp* null mice. During lactation, however, *ctcgrp* null mice lost significantly more hindlimb BMC compared to the WT siblings (*p=0.005). Numbers in parentheses represent the number of mice studied.

C. Calcitonin and *CGRP*-α Supplementation during Lactation

In an effort to prove that lack of calcitonin, and not lack of CGRP- α, was the cause of the significant BMC losses during lactation, a peptide rescue experiment was performed. Initially a series of dose-finding experiments were completed to determine if provision of salmon calcitonin (sCT) could prevent excessive losses of BMC in the *ctcgrp* null (i.e. 'rescue' the BMC loss to a level that was no different than that seen in the WT siblings) **(Figure 27)**. Initially, i.m. injections of 50 IU of sCT (in 250µl of solution) was provided daily as a 'maximum dosage' based on a calculation of 50% of the concentration that has been found in previous studies to suppress lactation (i.e. approximately 100 IU) (234-236). It was found that this dosage resulted in no lactational loss in BMC, and in fact a slight BMC gain was observed in the *ctcgrp* null when compared to the peak of pregnancy BMC (i.e. 103.0% ± 4.2%) **(Figure 27)**. In contrast, a mean daily i.m. dose of both 10 IU and 1 IU (in 60µl and 35µl solution respectively) was found to prevent excessive losses of BMC during lactation in *ctcgrp* null mice to a level that was no different than that seen in the WT siblings.

Based on these findings, 10 IU of sCT was selected as the concentration of sCT that could reduce the lactational BMC loss in the *ctcgrp* null mice to a level that was no different than that seen in the WT. Equimolar doses of rat CGRP-α were calculated and

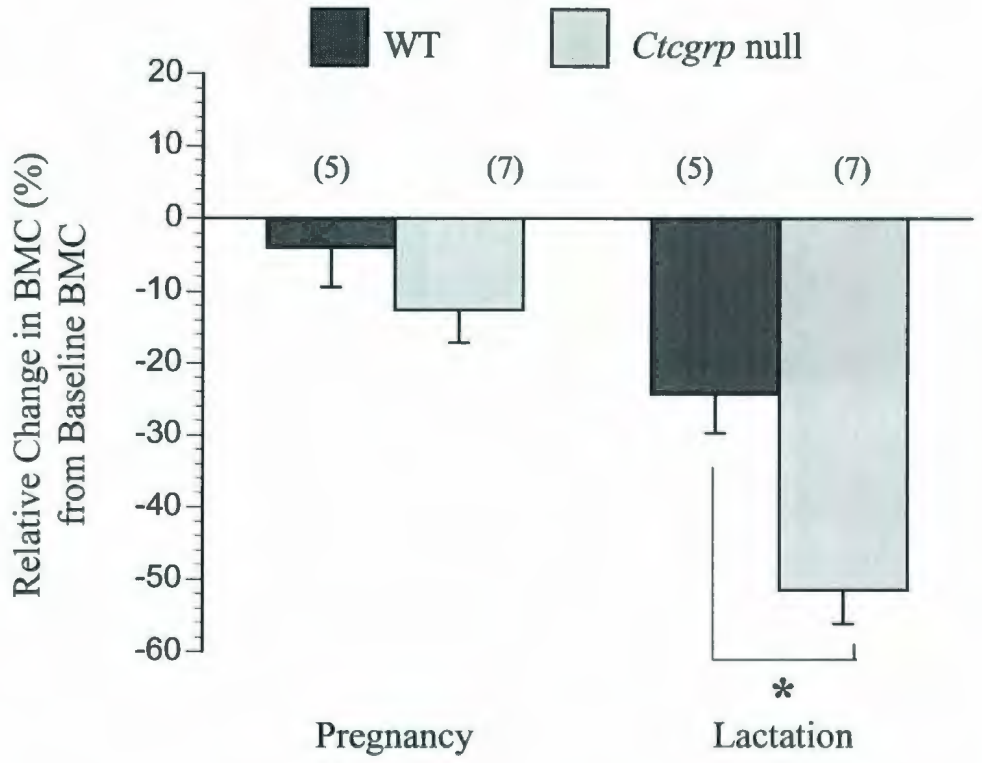


Figure 26: Spine BMC changes in WT and *Ctcgrp* null mice throughout Pregnancy and Lactation relative to Baseline BMC. Spine BMC (mean ± SE) losses during pregnancy were not significantly different between WT and *ctcgrp* null mice. During lactation, however, *ctcgrp* null mice lost significantly more spine BMC compared to the WT siblings (*p=0.003). Numbers in parentheses represent the number of mice studied.

subsequently a rescue experiment was carried out to determine which peptide (or both) would rescue the *ctcgrp* null BMC phenotype during lactation.

In the rescue experiment it was found that daily injections of 10 IU of sCT prevented the excess loss of BMC in *ctcgrp* null mice to levels that were not significantly different than that seen in the WT siblings after 21 days (-24.0% ± 2.7% in *ctcgrp* null vs. -25.0% ± 3.5% in the WT) **(Figure 28)**. All experimental BMC values were based on respective peak BMC values during pregnancy. If once-daily injections of peptide hadn't worked, twice-daily injections would have been provided in experiments. If this did not suffice, subcutaneous pumps could have been used. We wanted to avoid the use of surgically implanted subcutaneous pumps, however, as they could have potentially interfered with lactation as well as the behavior of the mice.

Next to determine if absence of CGRP-α was also contributing to the exaggerated loss of BMC during lactation in the *ctcgrp* null versus WT equimolar dosages of rat CGRP-α were provided in the same manner throughout lactation. Finding indicated that rat CGRP-α did not blunt the loss of mineral seen during this time period in the *ctcgrp* null as did the injections of sCT. *Ctcgrp* null mice that received the equimolar dose of CGRP-α, lost the same amount as saline treated animals, which was significantly more BMC than the *ctcgrp* null mice receiving sCT treatment (-54.3% ± 2.7%) (n=3) **(Figure 28)**.

The lower number of experimental animals in the *CGRP-α* treated group should be noted. This was due to several factors: the high cost of the custom *CGRP-α* peptide meant that only a few mice could be afforded this treatment, as well one mouse died,.

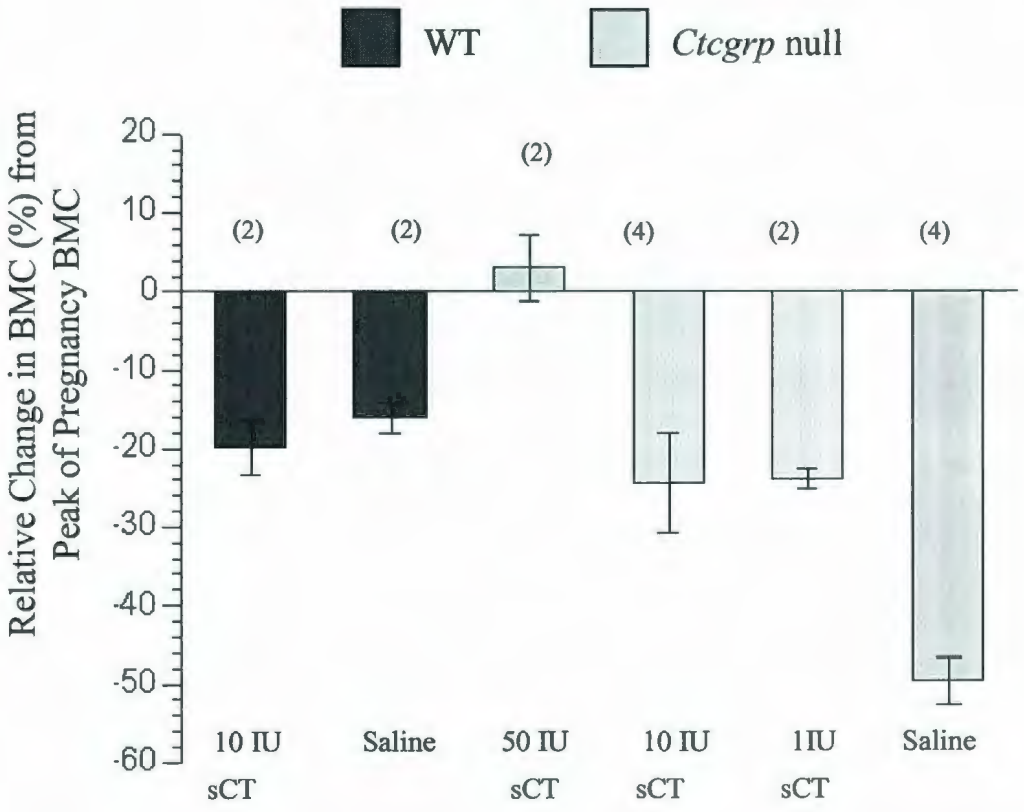


Figure 27: BMC Changes during Lactation in WT and *Ctcgrp* null Mice upon Administration of Varying Doses of Salmon Calcitonin (sCT) and Saline (controls). 60µl (10IU) of sCT administered i.m. once daily throughout lactation resulted in a loss of BMC (mean ± SE) in the *ctcgrp* null that was no different than that seen in the WT siblings receiving saline. In contrast, *ctcgrp* null mice that received 250µl (50 IU) of sCT were found to gain BMC during lactation. Numbers in parentheses represent the number of mice used in this dose finding experiment.

bringing the number from 4 to 3. Also, a dose finding experiment was not completed for CGRP-α because an equimolar dosage of calcitonin was considered 'equal'.

A limitation of this experimentation was the fact that there was no positive control for CGRP. The rat CGRP peptide could have been tested independently in cell culture to see if it indeed was biologically active by stimulating adenylate cyclase activity in striated muscle cells. This would have independently eliminated the possibility that CGRP was a biologically inactive batch. However, with the quality controls implemented by the company who manufactured the rat CGRP-α, and with our diligent handling, it is unlikely that it was a defective peptide.

It should also be noted as well that neither salmon calcitonin or rat CGRP-α are 100% homologous to mouse calcitonin and CGRP- α peptide and therefore caution should be used in interpreting the results.

D. Bone Microarchitecture

Considering the significant BMC loss seen in the *ctcgrp* null versus WT by DXA during lactation, bone microarchitecture was next assessed at the Centre for Bone and Periodontal Research, McGill University, to determine if there were any corresponding alterations in the trabecular structure. At baseline and throughout reproduction there were several notable micro architectural differences in the femora and vertebrae of *ctcgrp* nulls mice versus that seen in the WT siblings **(Table 2).** *Ctcgrp* null mice were found to have greater femora mean trabecular thickness (MTT) **(Figure 29)** and significantly lower mean trabecular spacing (MTS) **(Figure 30)** within the femora at baseline compared with the WT littermates at this time. In addition, *ctcgrp* null mice experienced significant

trabecular thinning in both the femora and vertebrae from baseline to late lactation, a trend that was not seen within WT mice (**Table 2**). Interestingly, despite obvious thinning in the trabecular region, MTT and MTS values in the *ctcgrp* null at late lactation were no different than that seen in the WT mice at this time (**Figure 29, 30**). μCT results indicated that *ctcgrp* null mice had a greater femora trabecular bone volume at baseline (0.47 $mm^3 \pm 0.07mm^3$) (n=3) versus that seen in the WT (0.19 $mm^3 \pm 0.07mm^3$) (n=3) at this time. WT trabecular bone volume, however, increased significantly from baseline to late lactation (this is a normal finding during this phase of reproduction (161)). These bone volume changes were blunted in *ctcgrp* null bones in the femora from baseline to late lactation. In the *ctcgrp* null vertebrae, however, no significant increases were seen in bone volume from baseline to late lactation (**Table 2**). To summarize, the normal change for WT mice is to increase bone volume during pregnancy perhaps to compensate for trabecular thinning. The *ctcgrp* null may not have needed to increase bone volume because of a higher baseline bone mass. After lactation, *ctcgrp* null mice had a bone volume and MTT that was no different than that seen in the WT siblings.

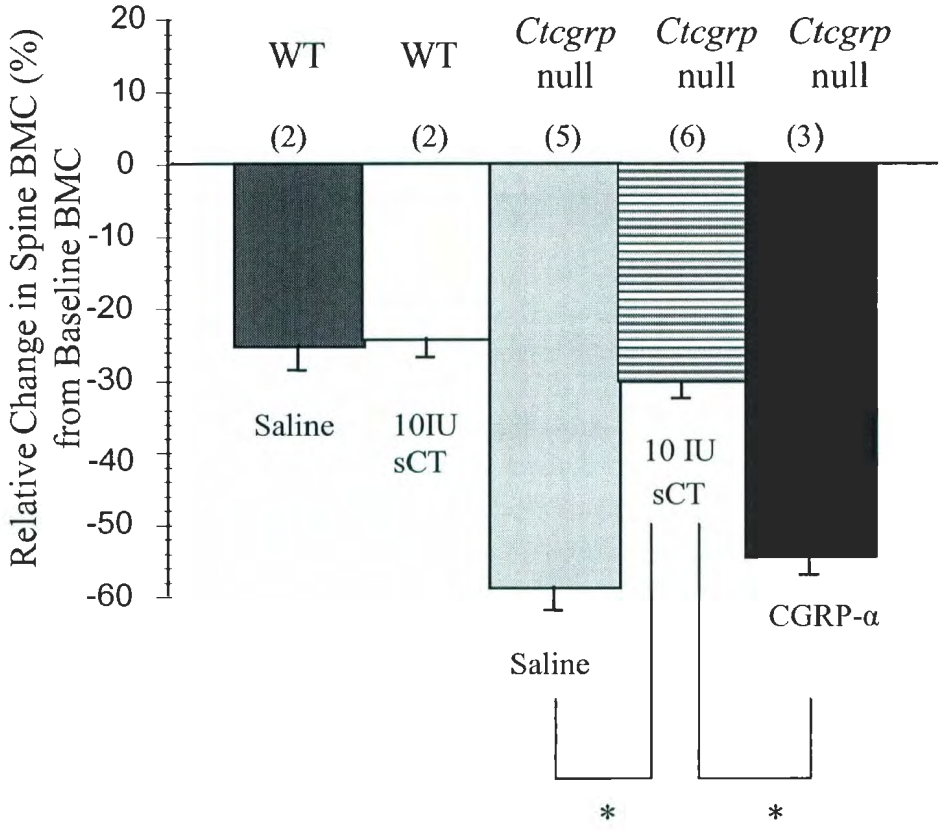


Figure 28: Calcitonin but Not CGRP-α Rescued the *Ctcgrp* null Phenotype. sCT administered i.m. once daily throughout lactation resulted in a loss of BMC (mean ± SE) in the *ctcgrp* null that was no different than that seen in the WT. In contrast, *ctcgrp* null mice that received equimolar doses of CGRP-α, or saline (controls) lost significantly more BMC than the *ctcgrp* null mice receiving calcitonin treatment. Numbers in parentheses represent the number of mice studied.

E. Bone Strength

Bone strength was next assessed to determine if significant BMC losses in the *ctcgrp* null mice, along with increased trabecular thinning and separation was associated with an increased risk of fracture (i.e. decreased load until failure) versus that of the WT. A standard 3-point bend test was first completed using femurs from *ctcgrp* null and WT mice at baseline and late lactation. This particular biomechanical testing method assesses primarily the cortical contributions to total strength, the fraction of bone that changes the least during lactation. The ultimate load required to cause bone breakage showed an apparent decrease from pregnancy to late lactation in both WT and *ctcgrp* null mice hindlimbs, but was not significantly different within or between groups **(Table 3)**. Not included in the calculations were two *ctcgrp* null femurs that broke upon bring placed into the device and thus could not be subjected to the 3-pont bend test. Results from this biomechanical test are shown in **Table 3** which illustrate that cortical bone strength was not significantly different between WT and *ctcgrp* nulls at any time point.

Next, using what others have considered to be a test of trabecular strength (i.e. the vertebral crush test), we found no difference in the ultimate load until failure using a 10 kN descending crush fixture at 10 mm/minute (1.86 kN± 0.20 kN WT, 1.78 kN ± 0.26 kN *ctcgrp* null) p=NS **(Figure 32)**. However, due to the strength of the walls of the cortical shell of the vertebral body (see methods and discussion) we designed two additional fixtures to focus on trabecular bone that could potentially bypass the strength of the outer cortical shell. This test proved very difficult to do because there was so little trabecular bone present at the end of lactation that the device encountered little or no resistance to its

Table 2: Micro Computed Tomographic Changes in WT and *Ctcgrp* null mice throughout reproduction

Femora	MTT(μm)	MTS(μm)	BV (mm3)	TV (mm3)	BV/TV (%)	BS (mm2)
Baseline WT (n=3)	53.3 ± 5.1^{b}	413 ± 26^{b}	0.19 ± 0.07^{a}	3.11 ± 0.22^{a}	$6.2 \pm 1.8^{a,b}$	15.0 ± 4.3^{a}
Lactation WT (n=5)	47.7 ± 3.6^{a}	$363 \pm 26^{a,c}$	0.42 ± 0.05	3.70 ± 0.16	11.6 ± 1.3^{b}	29.7 ± 3.0^{a}
Baseline *Ctcgrp* null (n=3)	$72.7 \pm 5.3^{a,b,c}$	$277 \pm 26^{a,b}$	0.43 ± 0.07^{a}	3.34 ± 0.22	12.8 ± 1.8^{a}	28.5 ± 4.3
Lactation *Ctcgrp* null (n=6)	47.7 ± 3.6^{a}	$363 \pm 26^{a,c}$	0.42 ± 0.05	3.70 ± 0.16	11.6 ± 1.3^{b}	29.7 ± 3.0^{a}
Vertebrae	**MTT(μm)**	**MTS(μm)**	**BV (mm3)**	**TV (mm3)**	**BV/TV (%)**	**BS (mm2)**
Baseline WT (n = 3)	49.0 ± 2.0	225 ± 11	$0.56 \pm 0.05^{a,b}$	2.92 ± 0.13^{c}	$19.0 \pm 1.1^{a,b,c}$	$29.5 \pm 2.1^{c,d}$
Lactation WT (n = 5)	44.6 ± 1.8^{b}	217 ± 11	0.77 ± 0.04^{a}	$3.54 \pm 0.12^{a,b,c}$	23.3 ± 1.1^{c}	$42.1 \pm 1.9^{a,d}$
Baseline *Ctcgrp* null (n = 3)	$54.5 \pm 2.0^{a,b}$	213 ± 12	0.65 ± 0.05	2.71 ± 0.15^{a}	23.7 ± 1.1^{a}	$31.4 \pm 2.1^{a,b}$
Lactation *Ctcgrp* null (n = 6)	46.2 ± 1.6^{a}	201 ± 9	0.82 ± 0.05^{b}	2.93 ± 0.12^{b}	25.2 ± 0.9^{b}	$38.2 \pm 1.7^{b,c}$

μCT analysis revealed higher trabecular bone volumes, greater trabecular thickness, and more densely packed trabeculae at baseline (mean ± SE) in the *ctcgrp* nulls. *Ctcgrp* null mice underwent more pronounced thinning of trabeculae than WT by the end of lactation but did not increase trabecular bone volumes. Lumbar spine showed a similar pattern of changes. Lumbar spine values include results from both the first and second lumbar vertebrae. MTT- mean trabecular thickness, MTS-mean trabecular spacing, BV- trabecular bone volume, TV - total volume including trabecular bone (but excluding cortical bone), BV/TV- trabecular bone volume relative to total volume; BS trabecular bone surface.$^{a-d}$ *Shared letters within columns* indicate statistical significance (i.e. $p < 0.05$).

passage. Ultimate load results indicated no difference in load until failure (kN) between WT and *ctcgrp* null mice during late lactation using these 2 specialized fixtures (i.e. pointed tip, and flattened tip fixtures) **(Figure 33, Table 4)**. Thus, despite significant BMC differences during lactation in the WT and *ctcgrp* null, no difference was seen in bone strength.

F. Bone Turnover

To investigate if the enhanced skeletal demineralization during lactation in the *ctcgrp* null versus WT, was associated with increased bone resorption, markers of bone turnover were assessed. Consistent with DXA results was the finding that urinary excretion of deoxypyridinoline (Dpd) / creatinine (Cr) (i.e. a marker of bone resorption corrected for creatinine concentrations) was increased significantly in *ctcgrp* nulls (8.9±1.02 nmol Dpd/mmol Cr) (n=7) versus that seen in the WT siblings (1.4 ± 1.3 nmol Dpd/mmol Cr) (n=4) during late lactation (p=0.003) **(Figure 34)**. Significant differences were also seen from early lactation (2.5±1.02 nmol Dpd/mmol Cre) (n=7) to late lactation (8.9 ±1.0 nmol Dpd/mmol Cr) (n=7) within the *ctcgrp* nulls ((p=0.0023) and between late lactation and post-weaning (3.3 ±1.2 nmol Dpd/mmol Cr) in the *ctcgrp* nulls (p=0.03).

In contrast, however, no difference was observed in the serum level of osteocalcin (a marker of bone formation) between the *ctcgrp* null and WT at any time point (p=0.413) **(Figure 35)**. The lack of change in osteocalcin is consistent with what others have observed about lactation, that there is little change in bone formation parameters but instead a large increase in bone resorption parameters (6-8).Thus it appears that lactation is dominated by bone resorption, and not bone formation.

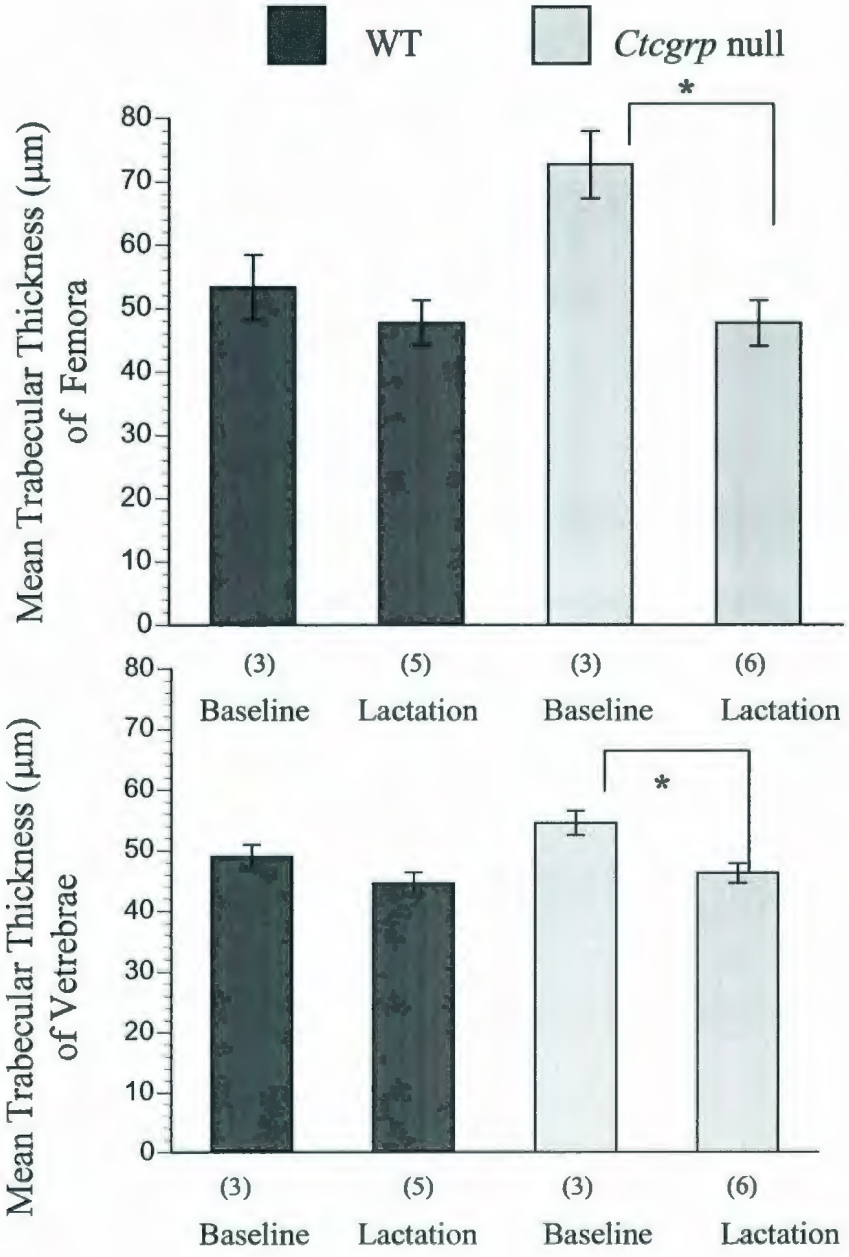


Figure 29: Mean Trabecular Thickness (MTT) in WT and *Ctcgrp* null mice Femora and Vertebrae at Baseline and Lactation. *Ctcgrp* null mice had a significant decrease in femoral and vertebral MTT (mean ± SE) as assessed by micro computed tomography from baseline to late lactation. In contrast, WT mice had a MTT value at baseline that was no different than that seen during lactation in both the femora and vertebrae. Numbers in parenthesis represent the number of mice studied.

G. Whole Blood Ionized Calcium

Ionized calcium is normally unchanged during pregnancy and lactation in both humans and mice (5-8). Ionized calcium was found to be normal in WT and *ctcgrp* null throughout reproduction with the sole exception being 24 hrs post lactation. Transient hypercalcemia was experienced during the first 24 to 48 hours postpartum in the *ctcgrp* null (1.40 ± 0.02 mmol/l) versus WT (1.26 ±0.02 mmol/l) ($p<0.001$) **(Figure 36)**. Our findings indicated, however, that after lactation was fully established both WT and *ctcgrp* null mice maintained a normal ionized calcium at throughout the remainder of lactation and the post-weaning period, as well at baseline and throughout pregnancy **(Figure 36)**. This hypercalcemia likely occurred as a result of the significant increase in bone resorption, and subsequent pooling of mineral within the blood before lactation was completely established.

H. Urine Calcium

Normally urine calcium decreases during lactation, likely due to the effect of PTHrP to stimulate renal tubular calcium reabsorption (6-8). Increased losses of calcium in the urine could have been one mechanism resulting in increased bone resorption in the *ctcgrp* null versus WT during lactation. Findings, however, indicated that both WT and *ctcgrp* null mice showed the same trend towards decreased urinary calcium excretion during lactation with no significant differences between the genotypes and reproductive stage (p=NS) **(Figure 37)**. Thus, the increased bone resorption seen in the *ctcgrp* null versus WT during lactation does not result in renal calcium wasting. Alternatively, it

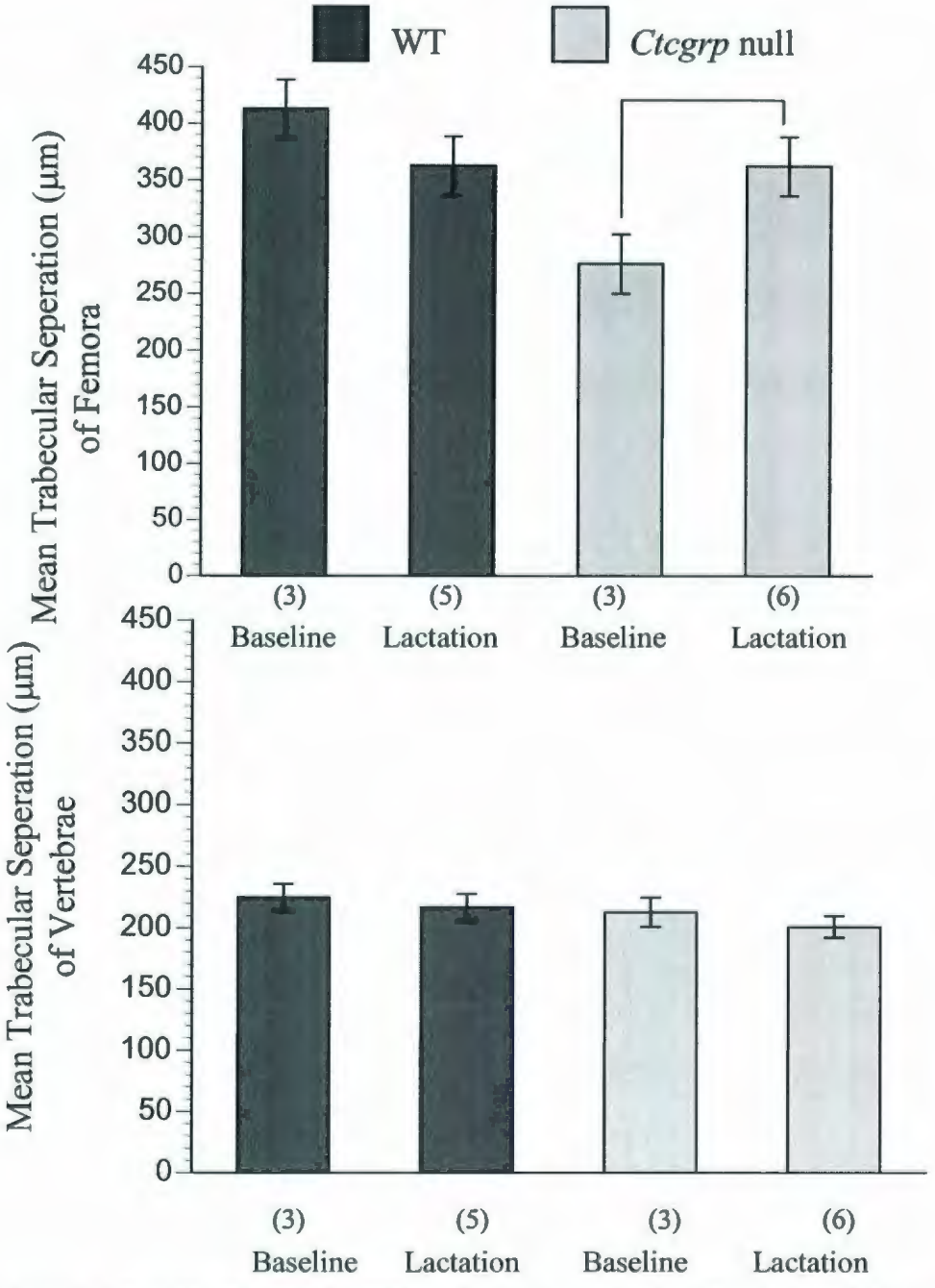


Figure 30: Mean Trabecular Spacing (MTS) in WT and *Ctcgrp* null mice Femora and Vertebrae at Baseline and Lactation. *Ctcgrp* null mice had a significant increase in both femoral MTS (mean ± SE) as assessed by µCT from baseline to late lactation. In contrast, *ctcgrp* null vertebrae did not have the same increase in MTS from baseline to late lactation mice. WT mice had a MTS value that was no no different in both femora and vertebrae from baseline to lactation. Numbers in parentheses represent the number of mice studied.

appears that the skeletal resorption in the absence of *ctcrrp* was not due to excess renal calcium losses.

I. Duodenal Calcium Absorption

Normally duodenal calcium absorption doubles during pregnancy primarily due to an increase in circulating 1, 25-dihydroxyvitamin D3. Intestinal calcium absorption subsequently returns to non-pregnant levels during lactation. A decrease in intestinal absorption of calcium (below normal) could be one way to cause increased bone resorption. Results indicated that duodenal ^{45}Ca absorption was significantly increased when comparing the non-pregnant WT (79.7 ± 3.1%) to mid-lactation in both the WT and *ctcgrp* null mice. ^{45}Ca absorption, however, was no different between WT and *ctcgrp* null at mid-lactation, when the BMC losses were significantly greater in the *ctcgrp* null **(Figure 38)**. Thus, increased calcium losses seen in the *ctcgrp* null versus WT during lactation did not occur as a result of decreased intestinal calcium absorption.

J. Milk Calcium

Due to the increase in bone resorption in the absence of a subsequent increase in urine Ca excretion, milk calcium content was examined to determine if calcium was going out in significant amounts into the milk supply. The calcium content of milk, corrected for protein, was assessed during early lactation (day 2-7) and again during mid-lactation (2 weeks). Initial findings during the first week of lactation indicated that *ctcgrp* nulls had a higher mean milk calcium value versus that of the WT mice during mid lactation **(Figure 39)**. This value, however, did not show statistical significance and the

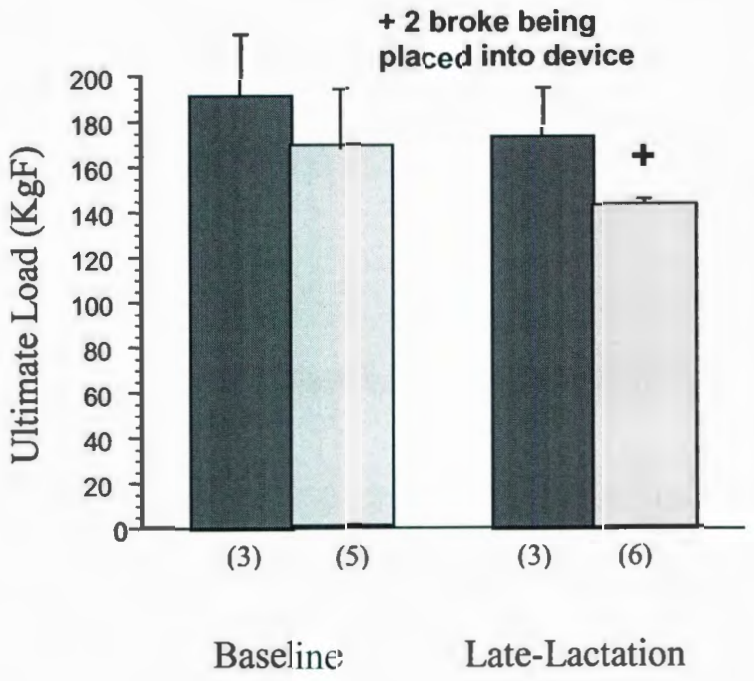


Figure 31: Ultimate Load until Failure using a Standard 3-Point Bend Test Fixture in WT and Ctcgrp null mice during Late Lactation. Ultimate load required to cause breakage showed an apparent decrease from baseline to the end of lactation in both WT and *ctcgrp* null mice (mean ± SE) but was not significantly different within or between groups. Not included in the calculations were two *ctcgrp* null femurs that were evidently fragile because they broke upon being placed into the device and thus could not be subjected to the three-point bend test. Numbers in parentheses represent the number of mice studied. See Table 3 for details.

variability between samples was quite large (**Figure 39**). Initially milk was collected and assayed in several different lots, leading to problems of combining data which in turn may have accounted for wide error bars on the first data set (**Figure 39**). In contrast, during mid-lactation milk calcium levels were significantly higher in *ctcgrp* nulls versus that seen in the WT siblings (**Figure 40**). Differences between early and mid-lactation could have been attributed to a variety of local or systemic mechanisms including increased bone resorption. As well, during mid-lactation milk samples were assayed and collected at same time, which may explain the tighter error bars and statistical significance being achieved. It is possible that increased milk calcium in the *ctcgrp* null versus WT could explain the cause (or consequence) of exacerbated BMC losses experienced. Increased milk calcium could be a consequence of more calcium flooding the mammary tissue from bone, or alternatively if absence of calcitonin was somehow stimulating milk production, then perhaps the bone resorption is occurring secondarily to a primary abnormality in the mammary tissue.

K. Serum Prolactin

Previous studies have shown that pharmacological administration and targeted overexpression of calcitonin in the pituitary lactotrophs caused hyperprolactinemia. Thus, to acknowledge the brain, as a physiological link potentially leading to significant BMC loss and bone resorption, prolactin levels were explored to see if absence of calcitonin would contribute to exaggerated BMC losses. As anticipated Prolactin levels were increased from baseline to late pregnancy in both WT and *ctcgrp* nulls in preparation for milk production. No significant differences however, were seen between WT and *ctcgrp*

Table 3. Three-point Bend Test Analysis of Bone in WT and *Ctcgrp* null mice at Baseline and Late Lactation

Genotype and Stage	Ultimate load (g)	Stiffness (N/mm)	Ultimate stress (mPA)	Work to failure (N x mm)	Moment of inertia (mm^4)	Toughness (J/mm^3)
WT Baseline (n = 3)	1914 ± 266	132 ± 14	208 ± 27	2.64 ± 0.39	0.088 ± 0.001	4.00 ± 0.65
WT Late Lactation (n = 5)	1737 ± 226	96 ± 18	3.78 ± 0.80	2.78 ± 0.48	0.120 ± 0.001	3.78 ± 0.80
Ctcgrp null Baseline (n = 3)	1678 ± 265	91 ± 14	189 ± 27	2.26 ± 0.39	0.086 ± 0.001	3.56 ± 0.65
Ctcgrp null Late Lactation (n = 6)	1430 ± 229	102 ± 14	179 ± 27	2.17 ± 0.39	0.100 ± 0.001	3.46 ± 0.65

This test of cortical strength showed a non-significant trend (mean ± SE) for decreased bone strength between baseline (non-pregnant) and end of lactation (trough), with no significant difference between groups at any time point. Two *Ctcgrp* null femurs were clearly fragile because they broke upon being placed into the device but could not be included in the data analysis. *Numbers in parentheses in the first column* indicate the numbers of mice studies.

nulls at any time point **(Figure 41)**.

L. Serum Estradiol

Due to the fact that decreased estradiol levels increase bone resorption, and lactation causes a lowering of estradiol in the circulation, it was next explored if absence of calcitonin could cause an exaggerated decrease in estradiol levels during this time and therefore further stimulate bone resorption. Estradiol levels were subsequently examined to determine if levels were elevated in the *ctcgrp* null, thus exacerbating the BMC loss in the *ctcgrp* null versus WT during lactation. Results indicated that serum estradiol levels were at the level of detectability of the EIA used (8 pg/ml) (data not shown). Thus, because the levels were below detection, it was impossible to say if estradiol levels were lower in the *ctcgrp* null.

M. Mammary PTHrP mRNA

In contrast, real-time quantitative RT-PCR showed that PTHrP was significantly upregulated in the mammary tissue of *ctcgrp* nulls compared to the WT by a fold of 3.28 ± 0.09 SD at 21 days of lactation ($p<0.01$). Tests were performed in triplicate and normalized to a GAPDH control **(Figure 42)**.

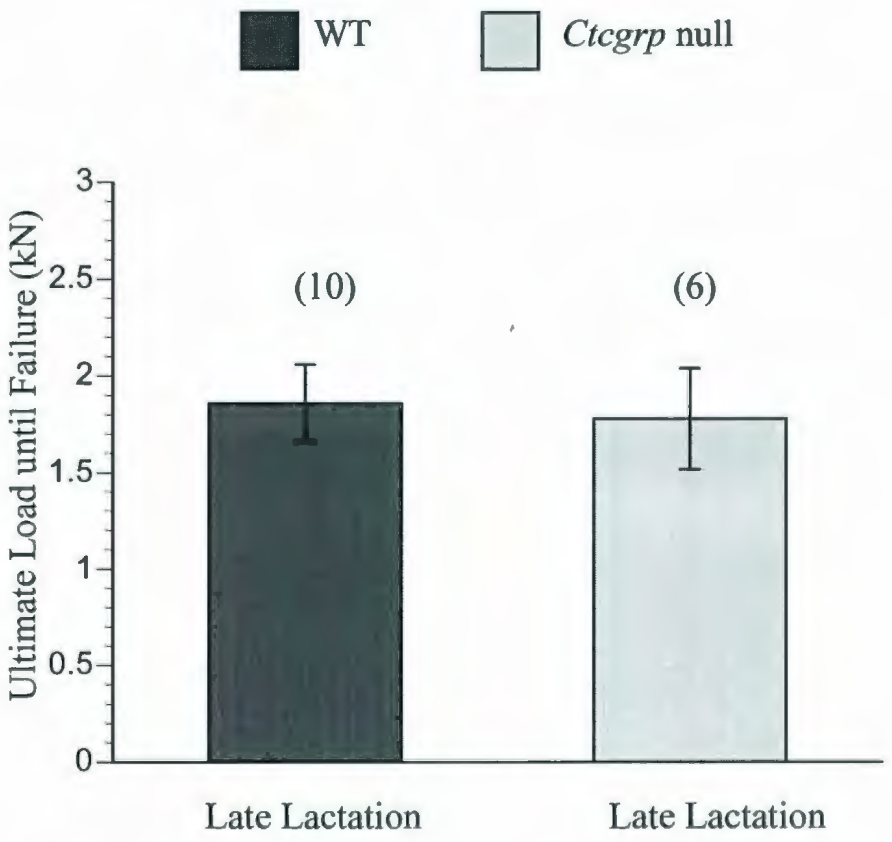


Figure 32: Ultimate Load until Failure using a Crush Test Fixture in WT and *Ctcgrp* null mice during Late Lactation. No significant differences were seen in the ultimate load until failure in WT and c*tcgrp* null mice (mean ± SE) during late lactation using a crush fixture attached to the Instron Biomech testing device. Numbers in parentheses represent the number of mice studied.

N. PTHrP Mammary Staining

It was shown via immunohistochemistry that PTHrP expression was visibly increased (as indicated by brown staining) in *ctcgrp* null mammary epitheilial tissue during lactation versus that in the WT during mid-lactation. WT and *ctcgrp* null mammary tissue without the primary antibody was used as a negative control (**Figure 43**).

O. Plasma PTHrP

Given the increased skeletal resorption during lactation, we anticipated that plasma PTHrP levels may be elevated in the *ctcgrp* null versus that seen in the WT siblings. Plasma PTHrP (1-40) levels, however, were found to be no different in WT and *ctcgrp* nulls at baseline, mid-lactation or late lactation (**Figure 44**). The mean PTHrP levels, however, did appear to be higher in the *ctcgrp* null versus WT during late lactation, but again these values were not significantly different. Thus, circulating PTHrP level were not conclusively demonstrated to be elevated but the increased mammary expression of PTHrP indicates that it should have been. Possible explanations for this could have been that PTHrP levels were all near the sensitivity of the assay; as well the samples were not drawn timed to specific suckling episodes which could have helped ensure that peak levels were obtained

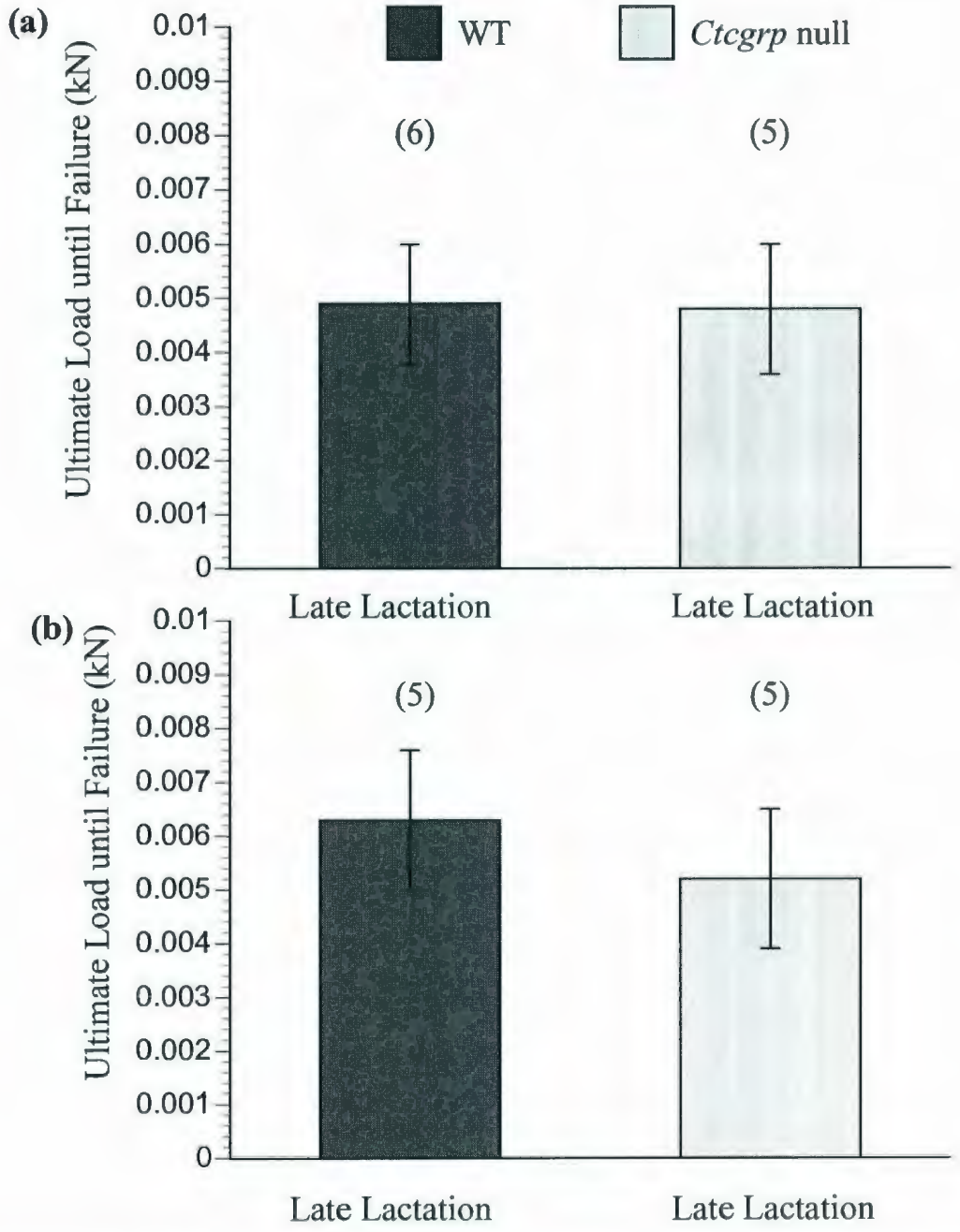


Figure 33: Ultimate Load until Failure using a Narrow Pointed Fixture and Narrow Flat Fixture in WT and *Ctcgrp* null mice during Late Lactation. No significant differences were seen in the ultimate load until failure in WT and *ctcgrp* null mice (mean ± SE) during late lactation using (a) Narrow pointed fixture or (b) Narrow Flat Fixture (b) attached to the Instron Biomechanical testing device. Numbers in parentheses represent the number of mice studied.

P. Serum Parathyroid Hormone (PTH)

Increased PTH could have also contributed to the significant BMC loss in the *ctcgrp* null during lactation. Previous unpublished work in the Calcium Research Laboratory at Memorial University, however, has shown that as in humans, WT mice in several inbred, outbred and mixed strains have PTH levels that are suppressed to the lower end of the normal range during pregnancy and lactation as compared to pre-pregnancy. In this experiment, serum PTH levels were no different at baseline in WT and *ctcgrp* null mice (**Figure 45**). Serum PTH was indeed suppressed by the WT mice during the study. Notably, however, during early lactation (7 days) PTH became significantly elevated in the *ctcgrp* nulls versus WT and versus all other time points ($p=0.05$). Also, PTH showed a trend for higher levels in *ctcgrp* nulls in late lactation when compared to WT. The elevation in PTH could have contributed to the enhanced skeletal demineralization seen in the *ctcgrp* nulls during lactation. It is important to note that the significant increase in PTH in the *ctcgrp* nulls occurred during the interval of normocalcemia, and not during the initial 24-48 hours postpartum in which the ionized calcium was transiently elevated. ($p<0.04$) (data not shown). In a separate analysis of serum obtained at 24–48 hrs (to correspond with the transient hypercalcemia), PTH levels were suppressed to the limit of detection (1.6 pg/ml) in both WT and *ctcgrp* null mice (p = not significant).

Table 4: Ultimate Load until Failure in WT and *Ctcgrp* Null Mice Vertebral Bodies at Mid-Lactation using Various Novel Fixtures

Type Fixture	Variable Assessed
Crush	**Ultimate load (kN) Until Failure**
WT Late Lactation (n = 10)	1.86 ± 0.20
Ctcgrp null Late Lactation (n = 6)	1.78 ± 0.26
Pointed Tip	
WT Late Lactation (n = 6)	0.0049 ± 0.0011
Ctcgrp null Late Lactation (n = 5)	0.0048 ± 0.0012
Flat Tip	
WT Late Lactation (n =5)	0.0063 ± 0.0013
Ctcgrp null Late Lactation (n = 5)	0.0052 ± 0.0013

Assessment of ultimate load until failure (kN) using a crush, pointed tip or flat tipped fixture (mean ± SE) showed no significant difference (p=NS) between WT and *Ctcgrp* null vertebral strength at mid lactation. The crush fixture assessed mainly the tough outer cortical shell and its contributions to failure, thus explaining the higher numbers with this analysis. The pointed fixture and flat tipped fixtures both assessed the inner trabecular contribution to bone strength. Numbers in parentheses indicate the numbers of mice studied.

Q. Expression of other Calcitropic Genes

Real-time quantitative RT-PCR results were analyzed using the comparative method, also referred to as the $2^{-\Delta\Delta C}T$ method, where the target and reference were amplified in separate wells (21). In this case, the mean C_t values of the *ctcgrp* null samples were compared to C_t values from normal WT mammary tissue. The C_t values of both the WT and *ctcgrp* null samples were normalized to respective C_t values from the GAPDH gene. The following comparisons were made:

- *Ctcgrp* null late lactation versus WT late lactation.
- *Ctcgrp* late lactation versus *ctcgrp* pregnancy.
- WT late lactation versus WT pregnancy.
- *Ctcgrp* null pregnancy versus WT pregnancy.

Mammary tissue expression ratios of mRNAs for: the calcium receptor, calcitonin receptor, 1-alpha-hydroxylase, vitamin D receptor, and the epithelial calcium channels (calcium transporter 1 and 2 [CaT1 and CaT2] were examined in triplicate. No significant differences, however, were seen between the WT and *ctcgrp* nulls during lactation (**Table 5**). ANOVA was used for the initial analysis; Tukey's test was used to determine which pairs of means differed significantly from each other.

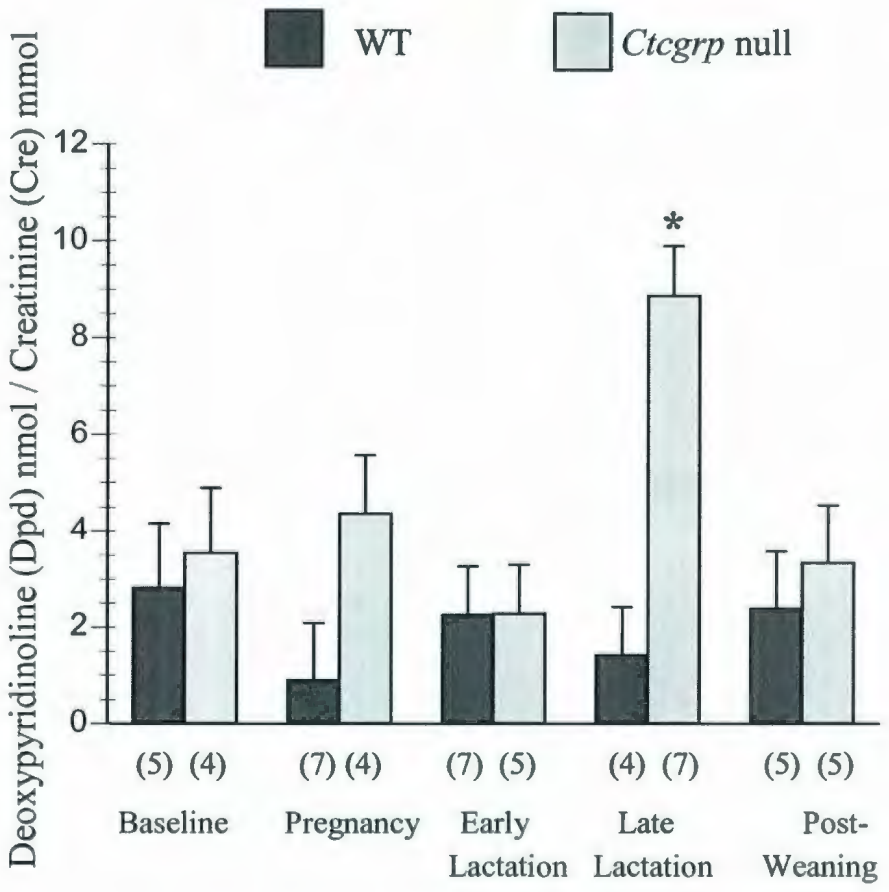


Figure 34: Urinary excretion of Deoxypyridinoline (Dpd)/creatinine (Cre) throughout reproduction in WT and Ctcgrp null mice. Dpd a marker of bone resorption (break-down of collagen) corrected for concentrations of Cre (mean ± SE) was increased significantly in *ctcgrp* nulls versus that seen in the WT siblings during late lactation (p=0.0025). Numbers in parentheses represent the number of mice studied.

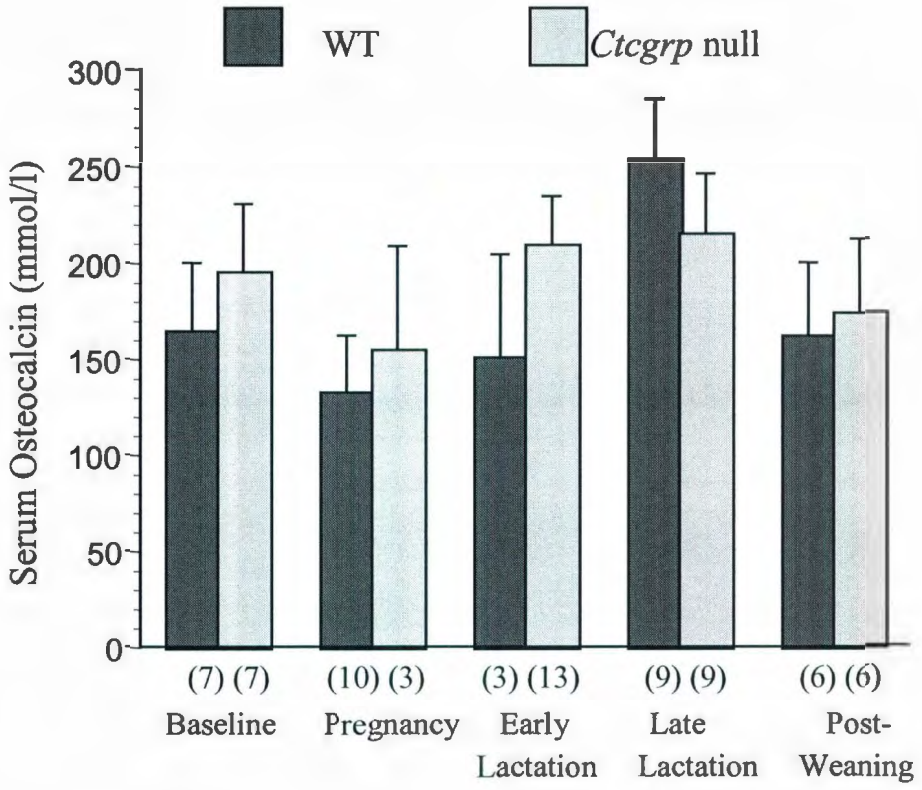


Figure 35: Serum Osteocalcin levels throughout Reproduction in WT and *Ctcgrp* null mice. No differences were observed in the serum level of osteocalcin (a marker of bone formation) (mean ± SE) between *ctcgrp* null and WT at any time point (p=0.413). Numbers in parentheses represent the number of mice studied.

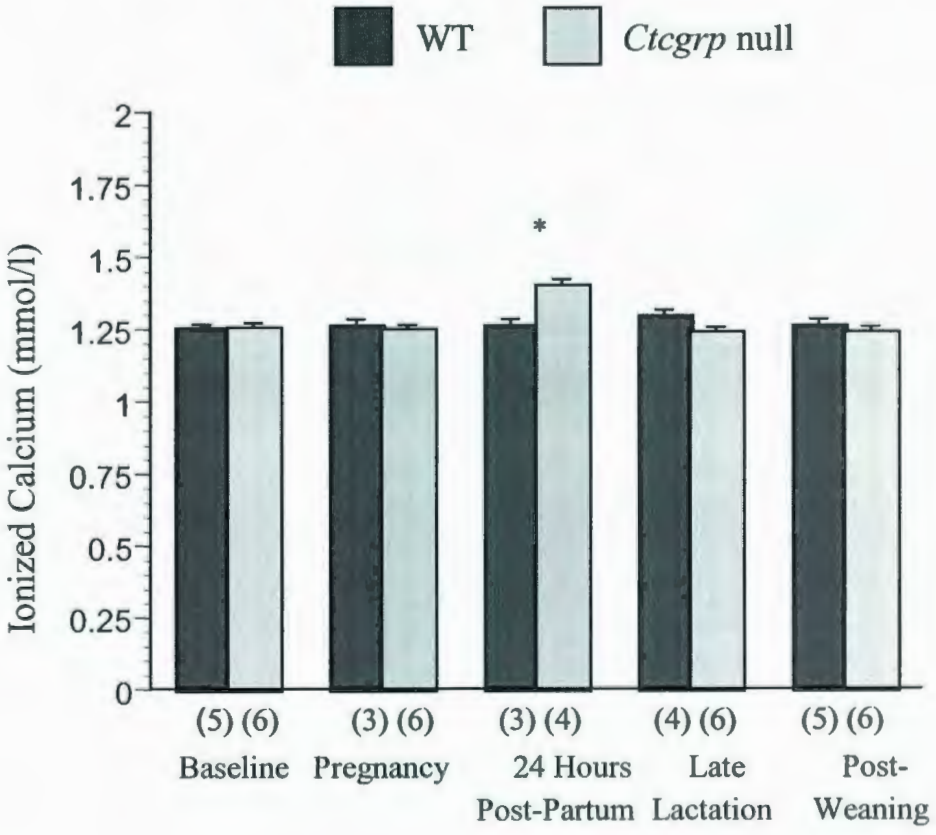


Figure 36: Maternal Whole Blood ionized Calcium in WT and *Ctcgrp* null mice throughout Reproduction. Blood ionized calcium (mean ± SE) remained constant throughout reproduction showing no differences between WT and *ctcgrp* nulls at the main time points. At 24 hours post-partum, however *ctcgrp* nulls experienced transient hypercalcemia versus that in WT and every other time point ($p<0.01$). Numbers in parentheses represent the number of mice studied.

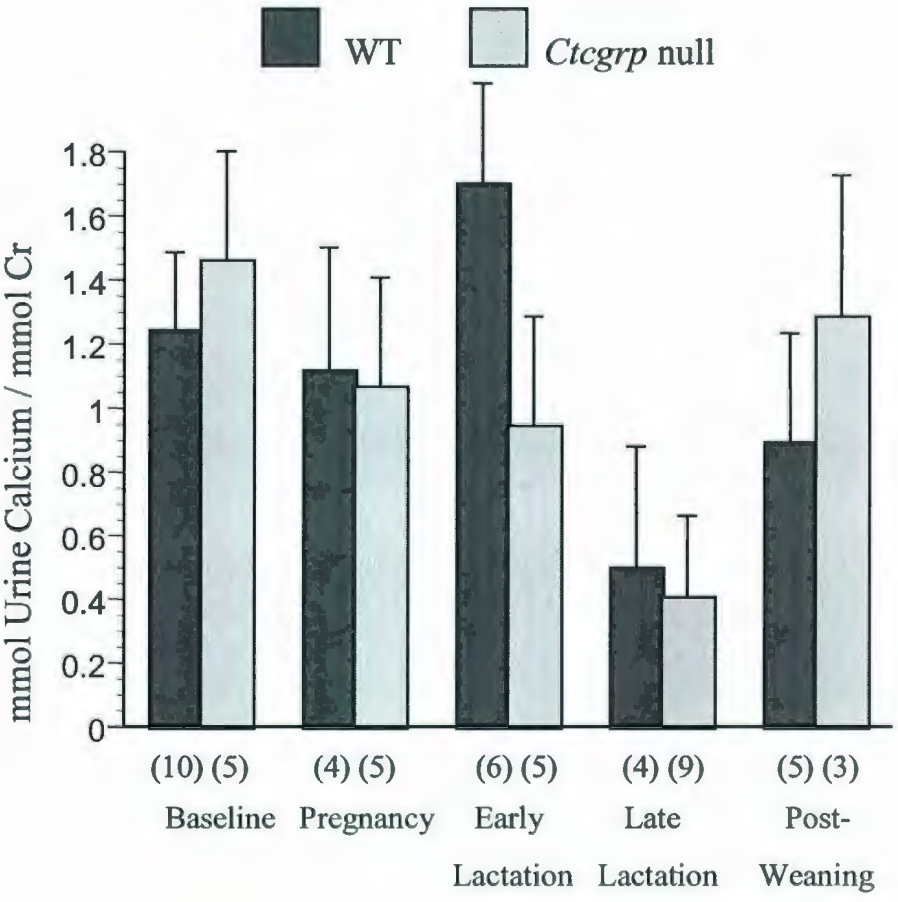


Figure 37: Maternal Urinary Calcium corrected for Creatinine concentration in WT and *Ctcgrp* null mice throughout Reproduction. *Ctcgrp* null and WT mice both experienced a decrease in urinary calcium excretion (mean ± SE) during lactation, but with no significant differences between the genotypes at any time point. Numbers in parentheses represent the number of mice studied.

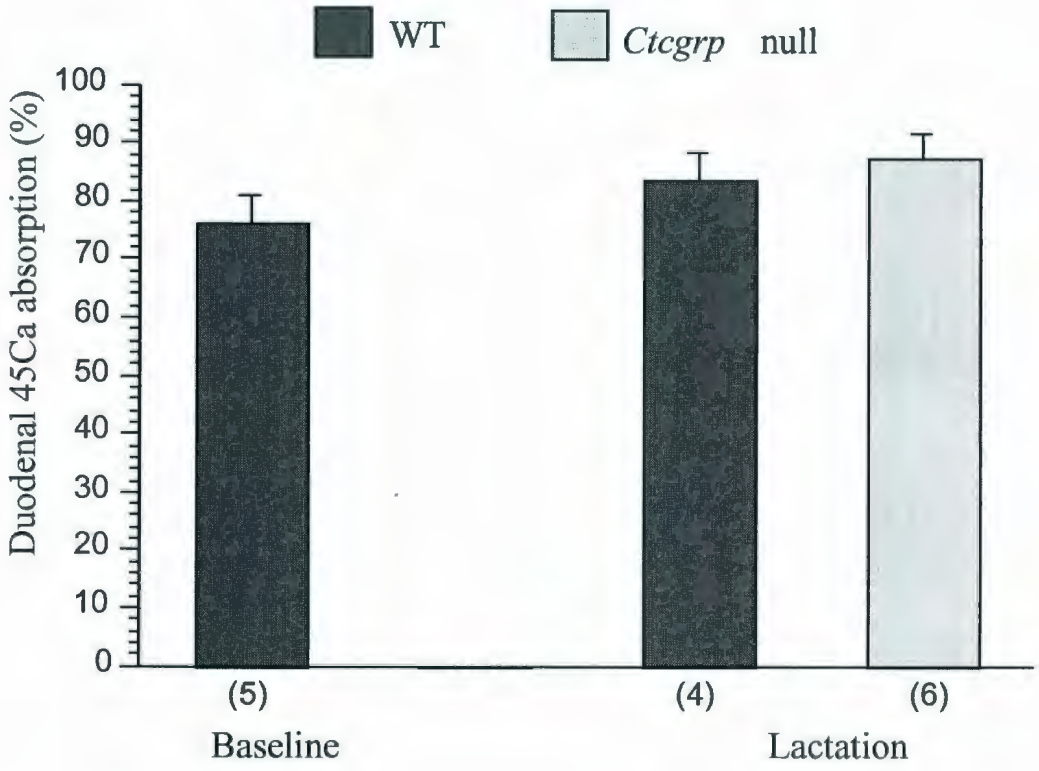


Figure 38: Intestinal Calcium Absorption in WT and *Ctcgrp* null mice during Lactation. Duodenal ^{45}Ca absorption (mean ± SE) was not significantly different at mid-lactation in WT and *ctcgrp* null mice. Efficiency of absorption was greater than 80% in both WT and *ctcgrp* null mice and not significantly different from the non-pregnant WT value. Numbers in parentheses represent the number of mice studied.

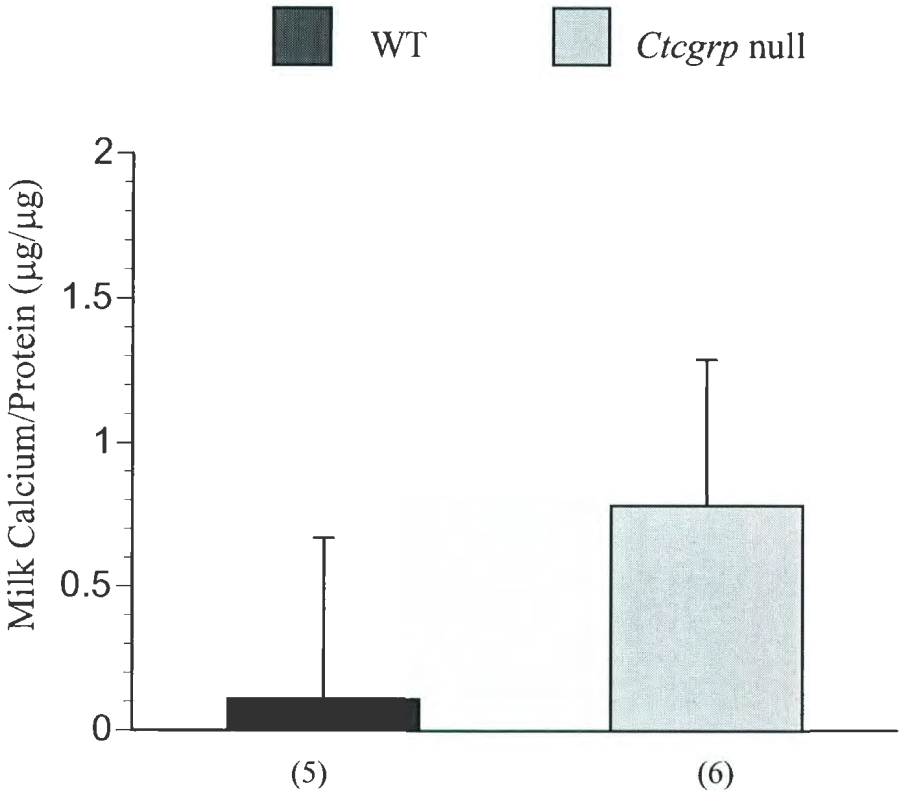


Figure 39: Milk Calcium levels in WT and *Ctcgrp* null mice Early Lactation.

Milk calcium, corrected for protein (mean ± SE) was not significantly different between *ctcgrp* nulls versus that of the WT mice during early lactation (day 2-7). Numbers in parentheses represent the number of mice studied.

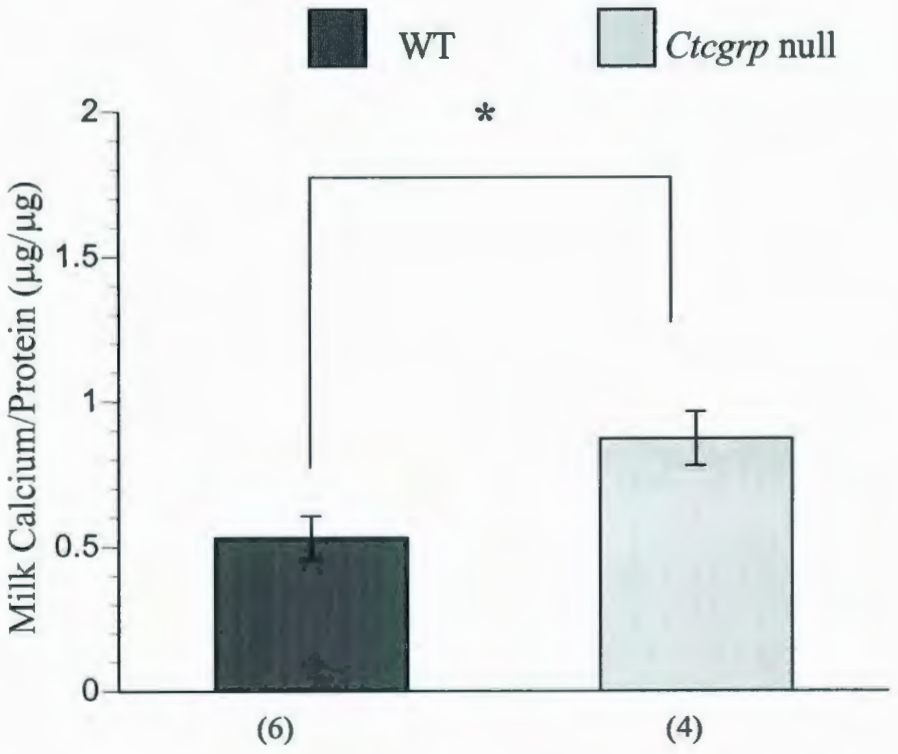


Figure 40: Milk Calcium levels in WT and *Ctcgrp* null mice during Mid-Lactation

Milk calcium, corrected for protein (mean ± SE) was significantly higher (p=0.0213) in *ctcgrp* null mice versus that of the WT mice during mid-lactation (week 2). Numbers in parentheses represent the number of mice studied.

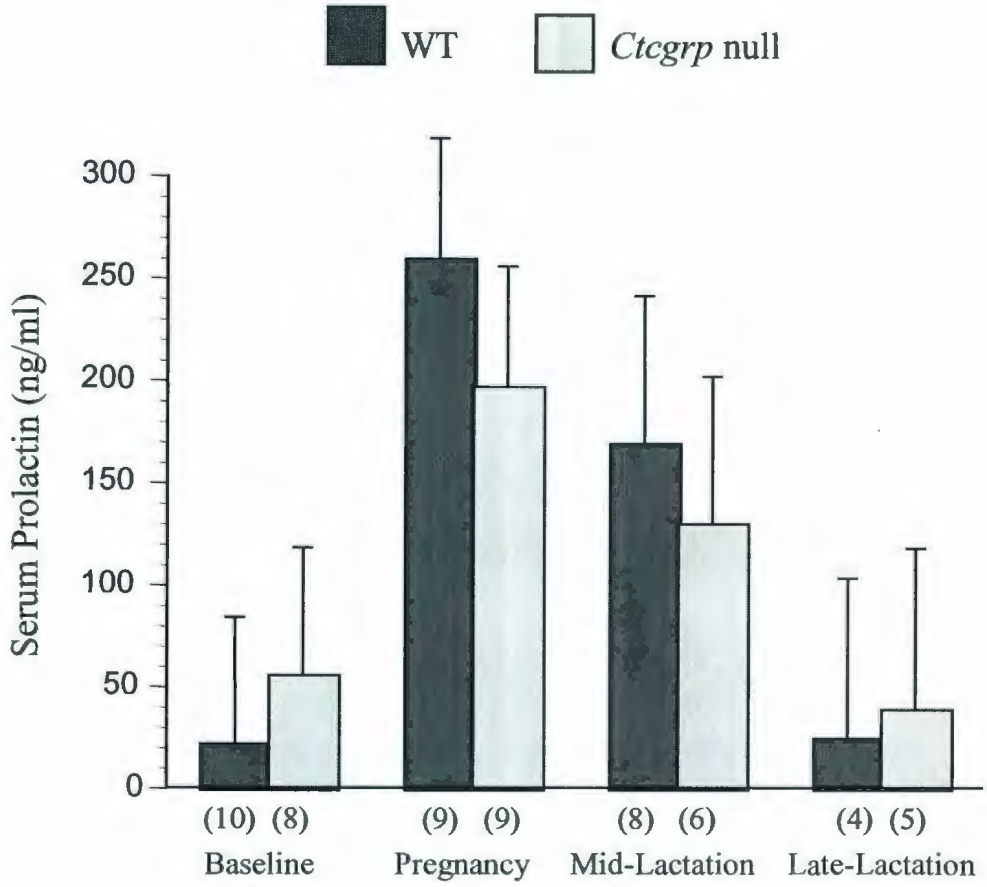


Figure 41: Maternal Serum Prolactin levels in WT and *Ctcgrp* null mice throughout Reproduction. Prolactin levels (mean ± SE) were increased from baseline to late pregnancy in both WT and *ctcgrp* nulls in preparation for milk production. No significant differences however, were seen between WT and *ctcgrp* nulls at any time point. Numbers in parentheses represent the number of mice studied.

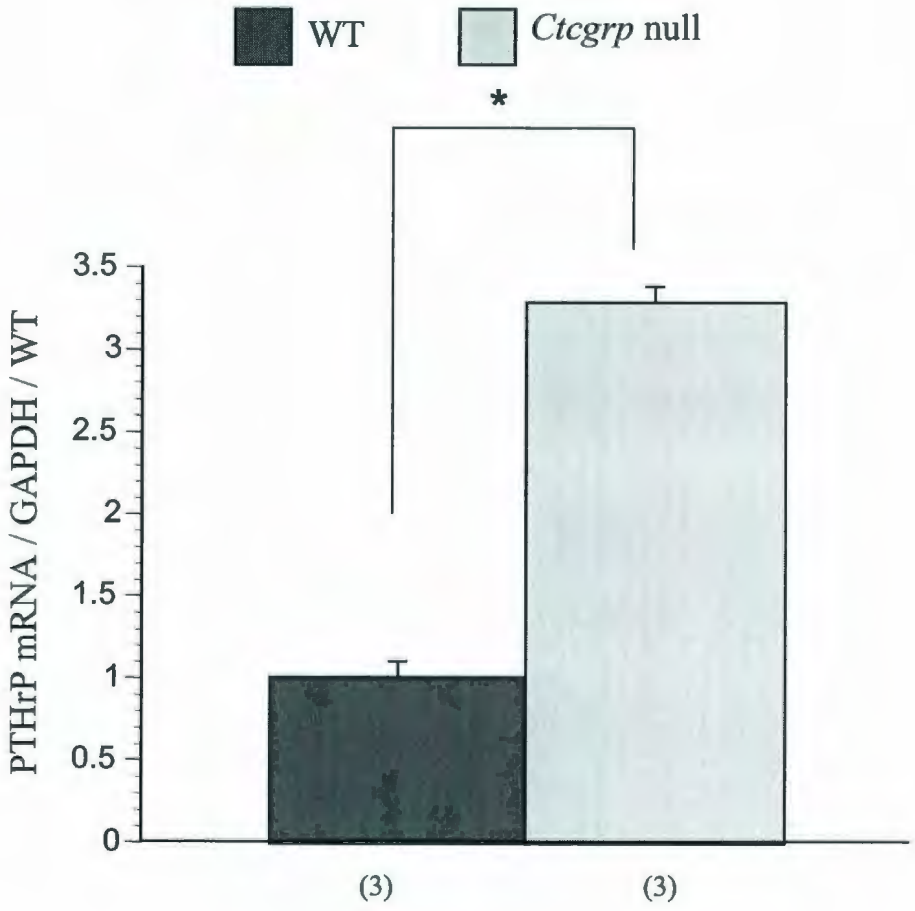


Figure 42: Mammary Tissue PTHrP mRNA in WT and *Ctcgrp* null mice during Late Lactation. Real-time quantitative RT-PCR showed that PTHrP was significantly upregulated in the mammary tissue of *ctcgrp* nulls compared to the WT by a fold of 3.28 ± 0.09 SD at 21 days of lactation. Tests were performed in triplicate and normalized to a GAPDH control.

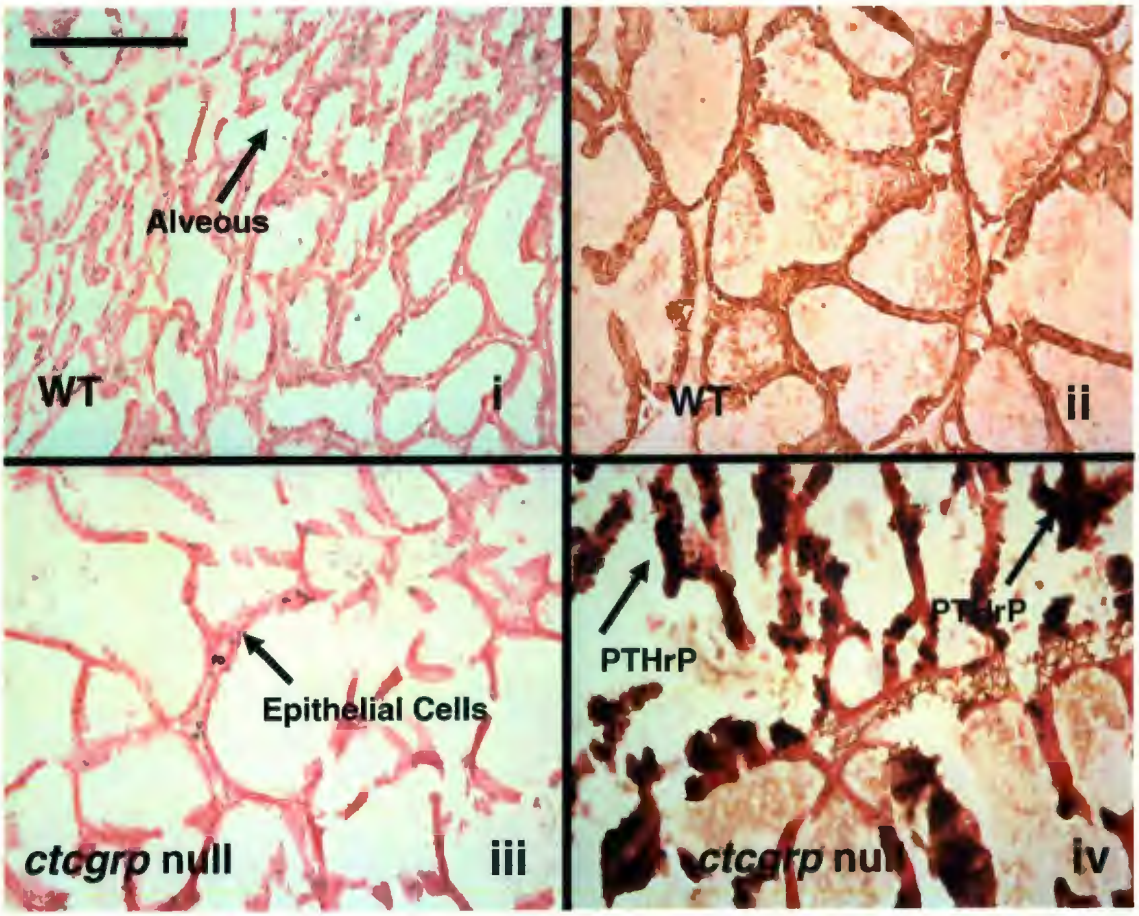


Figure 43: Immunohistochemistry of mammary epithelium using a Rabbit Anti-PTHrP Antibody (1-40). PTHrP staining was visibly increased (dark pink) (iv) in *ctcgrp* null mammary epithelium during lactation compared to that in WT mammary epithelium during lactation (ii). WT and *ctcgrp* null mammary epithelium was used as a negative control without the primary antibody (i, iii) (*scale bar*, 100 μm).

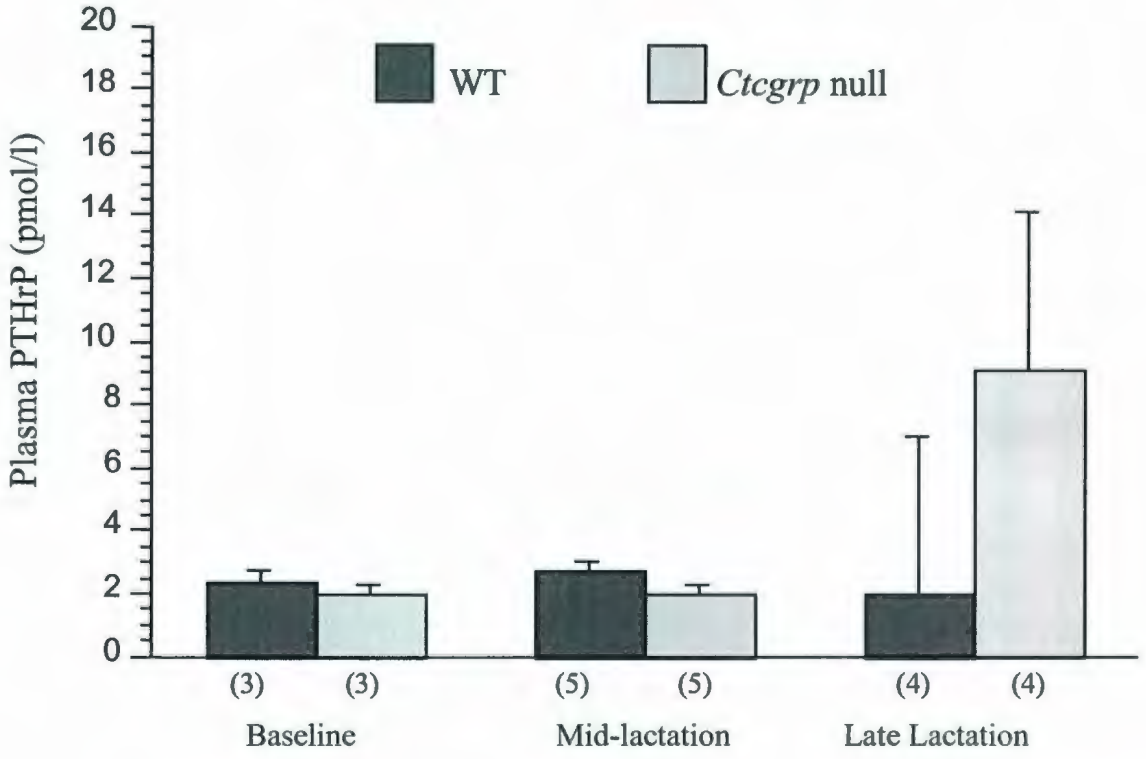


Figure 44: Maternal Plasma PTHrP levels in WT and *Ctcgrp* null mice throughout Reproduction. Within the circulation, the plasma PTHrP (mean ± SE) levels showed no significant difference was seen between WT and *ctcgrp* nulls at any time point. Numbers in parentheses represent the number of mice studied.

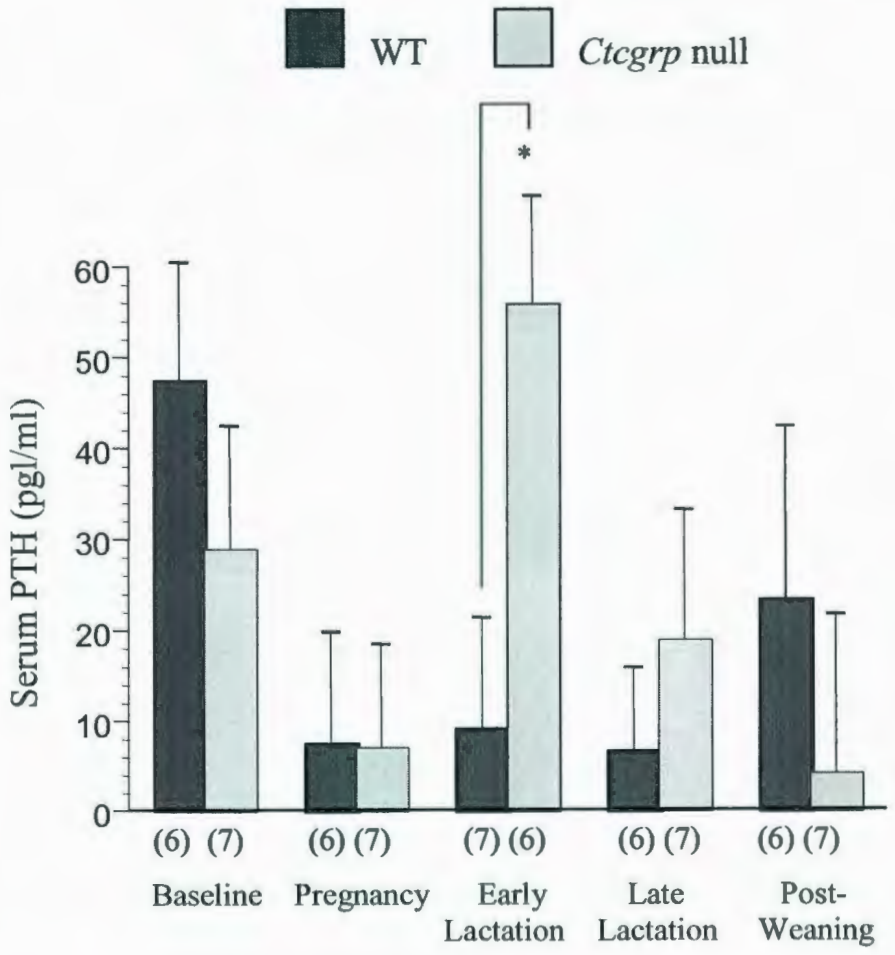


Figure 45: Serum PTH levels in WT and *Ctcgrp* null mice throughout Reproduction. During early lactation (7 days) PTH (mean ± SE) became significantly elevated in the *ctcgrp* nulls (77.9 ± 17.6) (n=8) versus WT and all other time points (ANOVA p=0.05). In a separate analysis of serum obtained at 24–48 hrs (to correspond with the transient hypercalcemia), PTH levels were suppressed to the limit of detection (1.6 pg/ml) in both WT and *ctcgrp* null mice (*p* = not significant), (not included in graph). Numbers in parentheses represent the number of mice studied.

Table 5: Real-Time RT PCR Relative C_t* Expression Ratio of Calcium Regulating Genes in the Mammary Gland of WT and *Ctcgrp* null mice

Relative C_t* Expression Ratio	PTHrP	CaR	CTR	1αOH	VDR	CaT1	CaT2
***Ctcgrp* null late lactation relative to WT late lactation**	3.28±0.095	Undetectable	Undetectable	0.766±1.635	1.82±0.227	Undetectable	Undetectable
***Ctcgrp* null late lactation relative to *Ctcgrp* null Peak**	4.272±0.072	Undetectable	Undetectable	0.834±1.584	0.567±0.231	Undetectable	Undetectable
WT late lactation relative to WT peak	1.202±0.124	Undetectable	Undetectable	1.07±0.776	0.391±0.242	Undetectable	Undetectable
***Ctcgrp* null peak relative to WT peak**	0.923±0.107	Undetectable	Undetectable	0.986±0.662	1.250±0.246	Undetectable	Undetectable

Mammary PTHrP mRNA was upregulated over 3-fold in the *Ctcgrp* null versus WT at late lactation. No differences, however, were seen in the relative expression of mammary gland vitamin D receptor (VDR) and 1-alpha- hydroxylase (1αOH). The calcitonin receptor (CTR), the calcium receptor (CaR), and the epithelial calcium channels (calcium transporter 1 and 2 [CaT1 and CaT2] were undetectable within the mammary samples in both normal and *Ctcgrp* null mice. Samples were examined in triplicate and expressed relative to GAPDH as a control. Multiple analysis were completed on triplicate samples, the chart above represents one set.

**C_t (threshold cycle)*: *Threshold cycle reflects the cycle number at which the fluorescence generated within a reaction crosses the threshold. It is inversely correlated to the logarithm of the initial copy number. The C_t value assigned to a particular well thus reflects the point during the reaction at which a sufficient number of amplicons have accumulated.*

V Discussion

A. *Foreword*

In this doctoral work, *ctcgrp* null mice were compared to WT siblings at four main time periods: *pre-pregnancy* (baseline), *pregnancy*, *lactation*, and the *post-weaning* period. Of these stages, the most noteworthy phenotypic differences were found in the spine region during lactation and during the post-weaning period. *Ctcgrp* null mice lost more than double the amount of skeletal mineral content after 21 days of lactation (i.e. 51.6%) versus that seen in the WT siblings (24.4%). Despite these significant losses *ctcgrp* nulls regained BMC completely after just 3 weeks. Consequently, this discussion will focus on the periods of lactation and post-weaning recovery in the absence of calcitonin. The broader context concerning the role of calcitonin in bone metabolism will also be discussed.

B. *Thesis Statements*

In light of earlier studies that suggested the possibility that calcitonin may have a physiological role in mammals during the reproductive period (89, 90, 150, 153), this doctoral research was carried out to test the thesis that: *calcitonin is required physiologically during reproduction to protect the maternal skeleton against excessive resorption of mineral*. In addition, because of the location of the CTR within key regions potentially responsible for mineral regulation during reproduction (i.e. mammary epithelial cells, osteoclasts, and pituitary lactotrophs) and the production of calcitonin by thyroidal c-cells and pituitary lactotrophs, the second thesis is that: *lactational bone*

losses, in the absence of calcitonin, involves linked physiological pathways between the bone and breast.

C. *Thesis 1: Calcitonin is required physiologically during reproduction to protect the maternal skeleton against excessive resorption of mineral.*

The reproductive period clearly illustrates the dynamic nature of the skeletal system. Previous reports have indicated that after 6 months of exclusive lactation up to 5-10% of BMC can be lost in humans (5-8) and up to 35% of BMC can be lost after 3 weeks of lactation in mice (168). Remarkably, despite these significant lactational BMC deficits, the postweaning period brings about a rapid regain of all BMC losses with no long-term adverse effects on bone health (5-8, 168). The reproductive skeleton has the unique capacity to fully gain back what it has lost, an ability that the adult skeleton does not have after the achievement of PBM. By understanding the mechanisms involved in BMC cycling during reproduction, one could explore how this relates to BMC loss and possible regain during the post-menopausal years when osteoporosis and subsequent fracture incidence increase significantly (11, 12). Current pharmacological therapies help sustain exiting BMC values or, at best aid in modest re-gains over time (52, 211, 218).

In the past, animal studies suggested that calcitonin, a 32 amino acid peptide predominantly of thyroid origin, could possibly play a role in the cycling of BMC during reproduction (89, 90, 150, 152). Sheep, rats and goats with total thyroidectomy, parathyroid gland autotransplantation and thyroid hormone replacement were used to represent calcitonin 'deficiency' in these particular studies (89, 150 -152). Some researchers determined that there was a reduction in BMC at the time of weaning in the

so-called calcitonin deficient animals. Other investigators, however, did not find such an effect (89, 150-152). Therefore, these studies were largely inconclusive and the role of calcitonin in skeletal BMC cycling during lactation was left unanswered. It is important to highlight also that in these animals, serum calcitonin was not measured to determine if they were truly calcitonin-deficient. As well, extrathyroidal sources of calcitonin (e.g. breast, pituitary, placenta, etc) were not appreciated at the time and this might have possibly confounded the interpretation of the experimental results. Therefore, the use of the *ctcgrp* null model (103) in this research provided the first insights into the physiological effects of *true* lack of calcitonin during the reproductive period.

Using the *ctcgrp* null model, we found that mice lacking calcitonin lost significantly more *total* body BMC (32.3%) via DXA after a cycle of lactation versus that experienced by the WT sibling controls (19.9%). This difference in BMC between the WT and null first became apparent after just 7 days of lactation, indicating that ablation of calcitonin in mice had an effect early on and was necessary to sustain normal BMC cycling during the full duration of lactation. Calcium resorption during lactation is the predominant adaptation in which increased requirements for calcium are met (5-8). The release of calcium from skeletal storage ensures an adequate supply of mineral to the breastmilk for neonatal growth and development (5-8).

The finding of a significant lactational BMC loss in the *ctcgrp* null versus that in the normal WT controls was consistent with some of the earlier studies using surgical models (150, 152). Subsequent to this finding the following questions were asked: what is driving this significant loss of BMC in the null? Is the loss driven by maternal metabolic alterations in the absence of calcitonin (and/or CGRP-α) or fetal demands? Where is the

calcium going? Where is the BMC loss primarily occurring from? Is the BMC loss predominantly trabecular or cortical in nature? Furthermore, what are the implications of losing such a significant amount of BMC during reproduction?

To begin addressing these questions, we first explored *where* the BMC loss was occurring in both the WT and *ctcgrp* null mice. Both the hind limb and spine were singled out as key regions of interest to examine if losses were predominantly cortical or trabecular. The trabecular rich spine region has a greater surface area per bone volume ratio and thus is considered more metabolically active (i.e. there are more mineral fluxuations between the bone and blood in this region) (3).The hindlimb, in contrast, is made up a relatively larger proportion of cortical bone, and thus has a lower bone surface to bone volume ratio (3).

Results indicated that the most dramatic of the BMC losses were those seen within the trabecular-rich spine region, to the extent that 51.6% of the baseline BMC was lost in the *ctcgrp* nulls versus 24.4% in the WT, relative to the respective pre-pregnancy baseline BMC values **(Figure 26)**. Losses within the hind limb were also over double in the *ctcgrp* null (28.4%) versus WT (12.9%), but the degree of BMC loss was proportionately less in the hind limb versus that seen in the spine, which is not surprising due to the lower area and bone volume ratio.

These findings indicated that calcitonin, in mice, is indeed required from early lactation as well as throughout the full lactation process because, in its absence, BMC losses were more profound when compared to WT controls. Also, BMC losses appear to be predominantly trabecular in nature, as the trabecular rich spine region lost proportionally more mineral versus that seen in the cortical rich hind limb region. This

increased loss in the spine is likely due to the metabolically active nature of this region of the skeleton. The absence of calcitonin and possibly secondary effects subsequent to this genetic alteration (e.g. increased PTHrP) could have also contributed to the increase in trabecular BMC loss during lactation. The exploration of these other pathways will also be discussed.

When considering regional decreases in BMC during lactation, it is relevant to point-out obvious skeletal differences and potential bone resorption patterns in humans versus rodents. Unlike humans, rodents largely remodel their cortical skeletons and their growth plates remain open during most of their lifespan (6-8). Also, in rodents and humans there are notable postural differences, and thus biomechanical loading patterns and possibly lactational BMC cycling patterns may differ. Thus, significant regional lactational losses seen in the rodents may not necessarily reflect what may occur in other species. Despite these obvious anatomical and physiological differences, trabecular bone is generally more actively resorbed than cortical bone during lactation in all mammals that have been studied (6-8). In any instance of increased bone resorption, the loss is always detected first in the trabecular compartment because that is the bone with the greatest surface area and thus osteoclasts can resorb it quickly.

To summarize, these findings support the first thesis by showing that calcitonin is indeed required physiologically during lactation, because in its absence significant BMC losses occur. Furthermore, these results provided the justification for supplementary investigation of the *ctcgrp* null, as compared to WT siblings, in hopes of better understanding mechanisms underlying the significant BMC loss during lactation and more generally, the role of calcitonin in mammalian physiology.

After the aforementioned DXA analysis in which *ctcgrp* null mice experienced an exaggerated BMC cycling pattern during lactation, it was next called into question whether these findings resulted subsequent to the absence of calcitonin, CGRP-α or both. Because this genetically engineered mouse colony has both calcitonin and CGRP-α ablated, absence of either or, or both gene products, could have resulted in the BMC pattern observed during lactation. To address this question, a 'rescue' experiment was designed in which sCT was provided via daily injections to a level that would result in BMC losses that were no different between the WT and *ctcgrp* null during lactation (i.e the null would experience *normal* losses). Then, equimolar doses of rat CGRP- α peptide were provided to see if it would have same effect on BMC loss. It was found that daily injections of 10 IU of calcitonin peptide prevented significant BMC losses in the *ctcgrp* null to levels that were no different than that seen in the WT siblings (i.e. normal BMC lactational losses) (Figure 26). *Ctcgrp* null mice, however that received equimolar daily doses of CGRP-α peptide throughout lactation had BMC losses were no different that that seen in saline controls. Thus, based on these findings, replacement of circulating levels of calcitonin, and not CGRP- α, prevented the significant loss of BMC during lactation.

There were several limitations to this 'rescue' experiment that should be noted. First, CGRP-alpha peptide was expensive and therefore could only be tested at a fixed dose in 4 mice. Second, the numbers of mice injected with CGRP-alpha were low due to the fact that a mouse died during the analysis, leaving only 3 mice. Third, CGRP-alpha was not tested independently to confirm if it was indeed biologically active. Such testing would have eliminated the possibility that CGRP was a bad batch of peptide. Fourth, it is possible that pharmacological doses of sCT could have independently inhibited lactation

through the suppression of prolactin gene transcription and secretion (234-236). However, this is unlikely as pups were fine and mothers appeared to lactate normally. Fifth, pharmacological doses may not be physiologically relevant. Sixth, compensatory mechanisms in the absence of calcitonin and CGRP-α may have contributed to, or even masked the skeletal phenotype. Such compensatory mechanisms could possibly include the upregulation or downregulation of other calcium regulating hormones such as PTH, PTHrP, or CaR.

Nevertheless, experimental results support the first thesis, by further illustrating the fact that calcitonin is needed during lactation. Provision of sCT resulted in less BMC loss during lactation, whereas provision of CGRP-α had no effect. Absence of calcitonin could have resulted in: decreased osteoclast number in the presence of sCT and thus less bone resorption (77), decreased osteoclast function (i.e. resorptive capacity) in the presence of supplementary calcitonin (77), or possibly the presence of sCT could have counteracted the catabolic effects of another peptide on bone (e.g. PTHrP, which has been suggested to promote bone resportion during lactation) (146). Exploration of these options will be discussed in detail further along.

There is some evidence that DXA changes are also a measure of changes in bone microarchitecture, an important determinant of biomechanical strength (229-231). Considering this, we next examined both the trabecular structure and the relative strength of WT versus *ctcgrp* null bones at baseline and late lactation. With increased bone resorption and significant losses seen by DXA in the *ctcgrp* null versus WT during lactation, we hypothesized that *ctcgrp* null bones would have significant alterations in

microarchitecture (e.g. increased porosity, trabecular thinning, etc) and thus greater susceptibility to fracture.

Consistent with the aforementioned findings and supporting the hypothesis was the discovery that *ctcgrp* null mice underwent more pronounced thinning of trabeculae versus that seen in the WT controls by the end of lactation in the femora and lumbar spine regions (Figure 27, 28). Also, within the femora there was an increase in spacing between trabeculae in the *ctcgrp* null that was not seen in the WT mice. The main biological effect of calcitonin is to inhibit osteoclastic bone resorption (77), and thus the finding of increased trabecular thinning and spacing during a time of increased calcium demand, in the absence of calcitonin, does not come as a surprise. With less control over osteoclast activity and number, one would expect there to be exaggerated bone resportion. Also, in the absence of calcitonin the activity of other key calcium regulating hormones may also be altered. For example, increases in PTHrP and decreases in estrogen have been associated with increased bone resorption (5-8, 60, 175, 193). By removing calcitonin from the equation, a key calcium regulating hormone, other calcium regulating hormones (e.g. PTHrP and estrogen) could have been altered such that bone resorption was increased and subsequently trabecular thinning and spacing were also increased.

It was observed that despite significant losses in BMC via DEXA during lactation in the *ctcgrp* nulls, μCT results showed that the amount of trabecular bone volume relative to total volume (BV/TV) did not significantly decrease in the null at this time. In fact, the BV/TV slightly increased in the null vertebrae during lactation versus baseline (23.7 ±1.1% vs. 25.2 ±0.9% respectively). Inconsistencies could reflect differences in the two techniques (μCT performed at McGill University versus DEXA completed at

Memorial University) as well as the fact that the samples were drawn at several different time points (i.e. samples sent to McGill were obtained a much earlier time point during lactation than the DEXA analysis at Memorial University). Also, due to the expense associated with performing the μCT analysis at McGill the sample number for this analysis was lower than that used within the DEXA analysis.

Interestingly, however, μCT analysis revealed higher trabecular bone volume, greater trabecular thickness, and more densely packed trabeculae at baseline in the *ctcgrp* nulls versus that seen in the WT. In fact, the baseline bone volume within the *ctcgrp* null femora was no different than that seen during late lactation in this region in the WT mice **(Table 2)**. These baseline results were consistent with Hoff's (104, 105) initial findings illustrating higher bone mass in the *ctcgrp* null (103). Interestingly, using much older mice Gagel found an osteopenic phenotype with age in the *ctcgrp* null versus WT mice (104, 105).

The issue of bone mass in the *ctcgrp* nulls has been an obvious area of scientific contention in the past. Using older mice R.F. Gagel (104, 105) found osteopenia and trabecularization in the *ctcgrp* null mice. The current data, using younger mice, illustrates increased trabecular thickness, a baseline BMC that trended higher and μCT data that indicated a higher baseline bone volume in the *ctcgrp* nulls that was no different than that found in the WT siblings during late lactation. The latter of these results may suggest that *ctcgrp* nulls lack (or do not require) the ability to naturally increase the bone volume during lactation, especially in the cortical rich femur region. Normally, WT mice experience a doubling in femoral bone volume from baseline to late lactation, perhaps to support the fetus (161-163). This data, however, supports the finding that bone mass is

indeed higher in the *ctcgrp* nulls versus WT. Unlike Hoff's initial experiments (103) however, in which *ctcgrp* nulls were mated to each other, these finding were based on matings first-degree relatives generated from the same HET x HET matings, and they were mated to the same HET males. Therefore, despite the possibility that the initial findings of increased bone mass and volume was an inherited mutation, due to ongoing matings of homozygous mice with homozygous mice (i.e. *ctcgrp* null x *ctcgrp* null) in Hoff's experiments, this data actually supports the suggestion that *ctcgrp* nulls actually have a higher baseline bone mass phenotype in the context of sound animal husbandry (i.e. nulls compared to related WT littermates and backcrossed for 10 generations into a BLKSW colony).

Despite the hypothesis that *ctcgrp* nulls would have weaker bones during lactation (i.e. because of significantly lower BMC values at this time) the biomechanical results indicated that there were no significant differences in the strength of *ctcgrp* null bones versus that seen in the WT siblings. Several factors may explain why no differences were seen in the biomechanical properties. Most standard bone biomechanical tests, such as the 3PBT, focus primarily on cortical strength. During these experiments lactational bone losses via DXA were most exaggerated in the spine, which consists of a large ratio of trabecular bone content. BMC alterations in the cortical rich hindlimb were not as exaggerated, and thus might explain why there were no significant differences seen biomechanically in this area. Another possibility could have been that the test was not sensitive enough to detect any biomechanical differences in such small fragile samples. It is also quite possible that because the *ctcgrp* nulls ended up with a bone volume and trabecular thickness that was no different than that seen in the WT siblings, this might

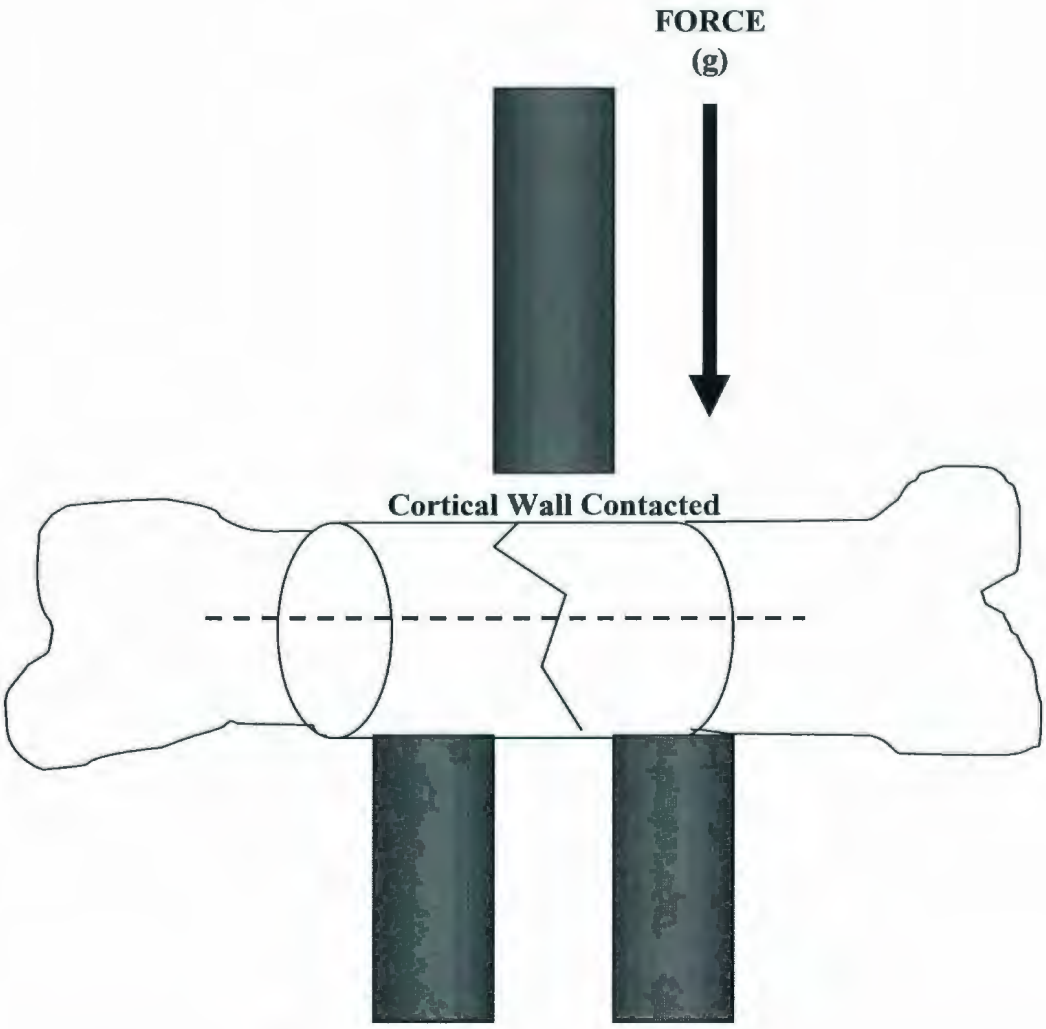


Figure 46: Break in a Cylinder as Demonstrated in a 3-Point Bend Test on a Long Bone. All three points on the fixture initially come into contact with the tough outer cortical shell of the long bones because it is the length of the cylinder, not the interior that will resist bending and breaking.

have accounted for lack of a difference biomechanically at late lactation between genotypes. Furthermore, the higher baseline bone volume in the *ctcgrp* null and the increased bone volume changes during pregnancy in the WT may have offset any discrepancies that may have been detected in bone strength between genotypes.

The theory and practical reality of biomechanical testing and the novel approaches that were taken to overcome existing limitations in these experiments are also relevant to discuss. A standard three-point bend test (3PBT) was initially used to examine bone strength within the hind limb region. Like many biomechanical tests, this particular method is very sensitive to specimen and loading geometry and strain rate (i.e. a material property that reflects the change in strain, or displacement of an object, over a specified period of time) (24). As well, the 3PBT mainly reflects the strength of cortical bone (because all three points on the fixture initially come into contact with the tough outer cortical shell of the long bones and because it is the shell of the cylinder, not the interior that will resist bending and breaking) **(Figure 46)**. Also, cortical bone is relatively avascular and undergoes more modest resorption compared to trabecular bone (3). During lactation it is likely the trabecular fraction of bone that is more rapidly mobilized due to its large surface area and vascularity. These factors enable the spine region to be resorbed by osteoclasts on multiple surfaces (5-8, 113, 114, 116, 121). Thus, a vertebral test was subsequently deemed as a more appropriate model for determining the strength of WT and *ctcgrp* null bones during lactation to overcome these limitations. Next a crush fixture was constructed in an effort to focus more specifically on the trabecular rich vertebral samples **(Figure 47)**. Small, disk-shaped metal plates were crafted, one side comprising the lodging surface for the vertebral sample to be centered on, and the other

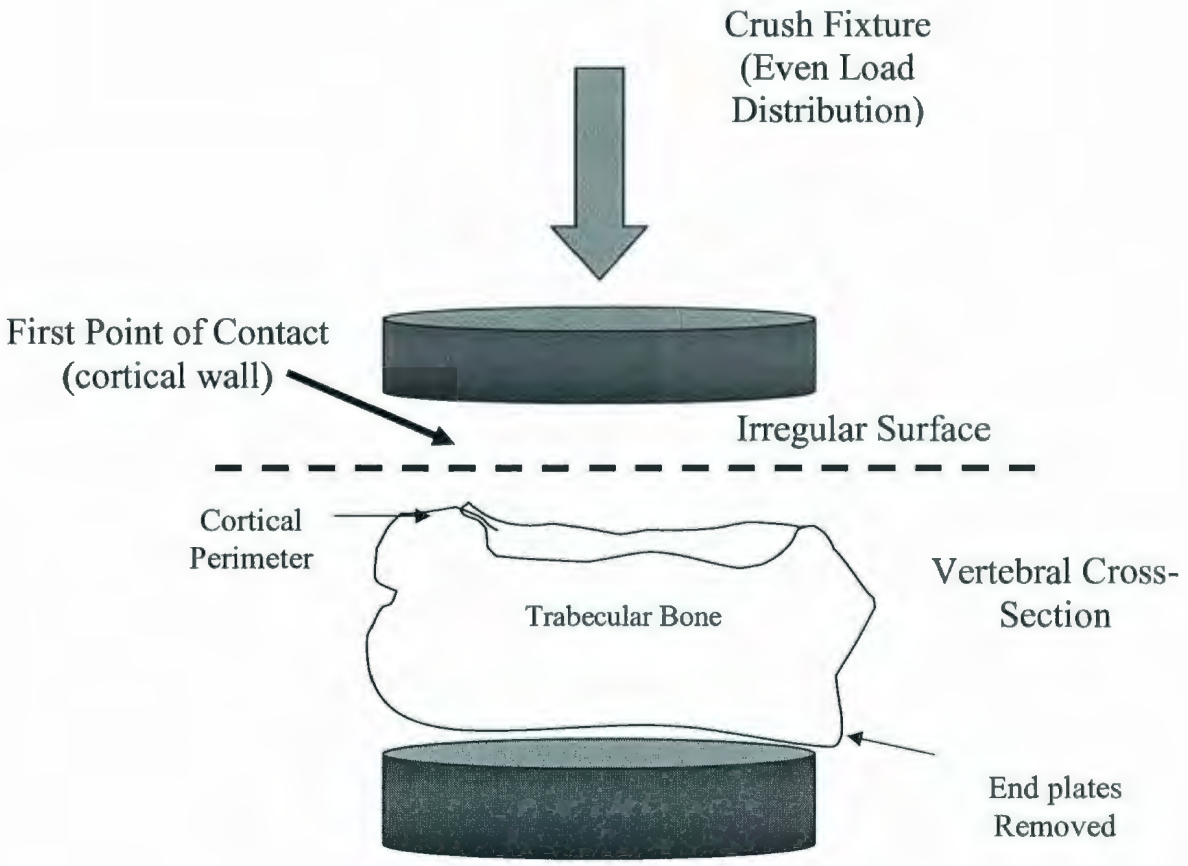


Figure 47: The Vertebral Crush Test Considers Cortical Contributions to Strength. As the cross-head compresses a single vertebrae, it comes into contact with high points on the surface. These high points are comprised of the outer cortical shell, and not the trabelcular framework contained deeper within the bone sample.

serving as the attachment site for the moving biomechanical crosshead which compresses the sample **(Figure 20b)**. Software was programmed to move the crosshead (10 mm/min) until adequate force was provided to serially compress each vertebral body minus the end plates **(Figure 20b)**. Samples were carefully centered on the lower fixture. Tests results indicated that, regardless of the blinded sample being tested, the maximum load (10kN) was achieved for every specimen. Thus, the load/displacement curve appeared exponential making it challenging to extrapolate any differences in strength between samples. It was decided that the second spike on the curve would serve as the ultimate failure point for the sample because there was less probability that it represented contact with the cortical perimeter and more likely indicated the inner trabecular contributions to strength. The first spike occurred when the cross-head contacted the outer spherical cortical shell. Thus, the first spike was disregarded because of the probability of a cortical contribution. It was observed, however, that despite using the second spike on the curve as the selected failure point, that the tough outer cortical shells of the vertebrae were so irregular in shape that erratic spikes on the load/ displacement curve made it difficult to extrapolate information even from the second peak on the curve. By contacting the uneven cortical shell of the bone first and subsequently causing flexion on the standard curve, the crush fixture did not accurately assess the trabecular bone contributions to strength, yet focused more on the tough outer cortical surface. No differences, however, were seen between WT and *ctcgrp* null at the BMC trough point of lactation. To overcome the limitations of the crush fixture, another biomechanical attachment was designed. This next fixture was comprised of a generic base that fit into the biomechanical testing devise, and a narrow pointed tip **(Figure 21a)**. The metal point

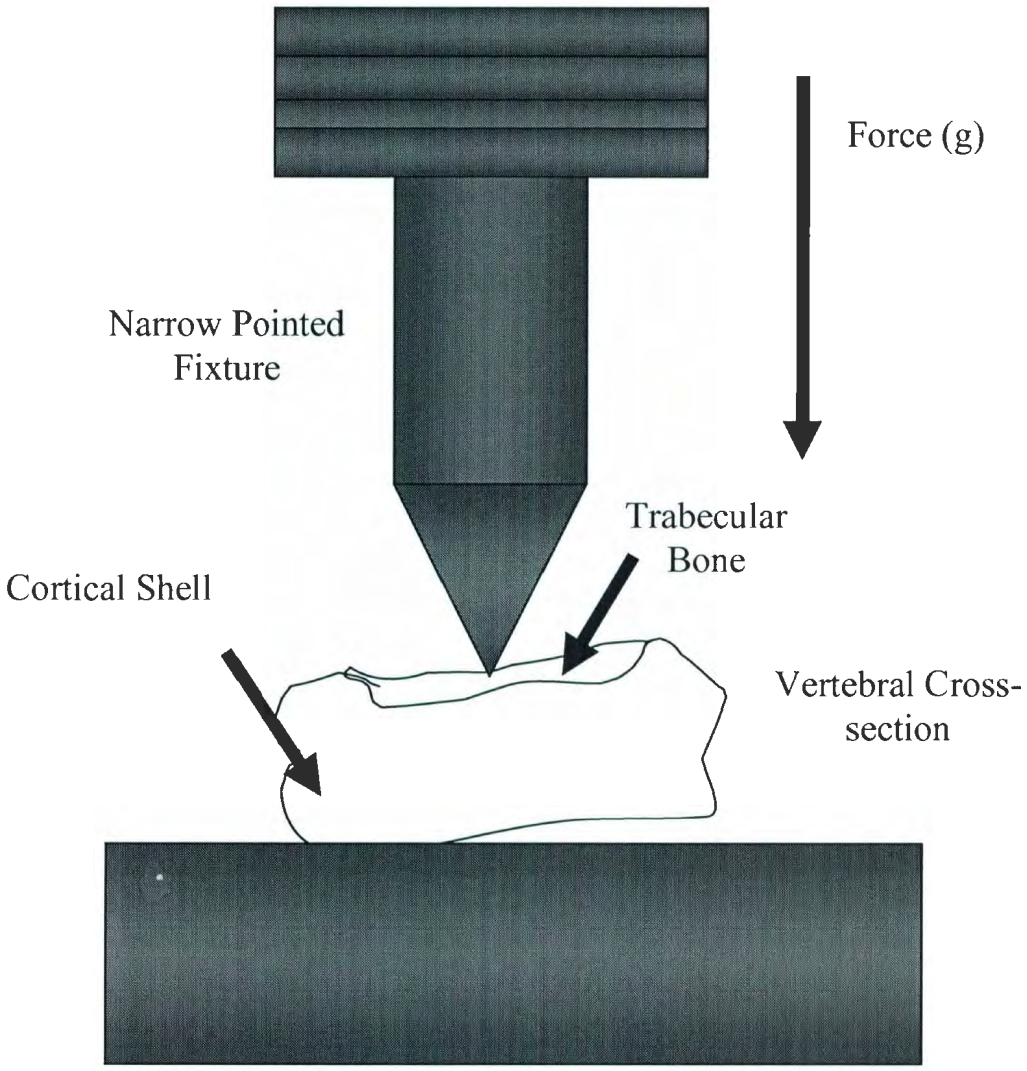


Figure 48: Pointed Fixture Testing Trabecular Contributions to Bone Strength.

As the cross-head compresses a single vertebrae, it bypasses the outer cortical rim and compresses the trabecular framework within the bone sample.

was created to bypass the cortical shell of the vertebrae and simply pass through compressing the trabecular framework until it reached a failure point (i.e. level of resistance to a controlled load) of only the inner trabecular fraction of the sample. Results however, again showed no difference between the load/displacement curve between *ctcgrp* null and WT bones during lactation. This could be explained simply because of the fact that there was so little trabecular bone remaining during late lactation that the fixture did not consistently register a signal presence. It was observed that the limitation of this fixture was that (because of its narrow pointed tip) it came into contact with only a fraction of the trabecular surface as it was lowered onto the bone sample, leaving an area of trabecular bone on both sides not accounted for within the biomechanical analysis. Finally, in an effort to overcome the problem faced with the pointed tip, a flat tipped elongated fixture was created (**Figure 21b**). The full trabecular surface of the vertebrae was contacted by the flat tip as the cross-head descended through the cortical shell of the vertebrae (i.e. the outer cortical shell was bypassed). Despite the ability of the flat tipped elongated fixture to fully contact the trabecular surface, no differences were seen in load until failure in the WT versus *ctcgrp* null during lactation. It is possible that there was no actual difference in biomechanical strength between genotypes, even in the presence of significant BMC loss in the *ctcgrp* null during lactation. However, it may also be considered that there's probably too little trabecular bone present to be tested with existing biomechanical technology and thus, that may be why we couldn't resolve a difference in strength between WT and *ctcgrp* null.

There are, of course, a multitude of limitations when assessing animal bone biomechanically that must be discussed. Trabecular bone is physically challenging to

obtain from small animals, and thus bone strength tests are typically limited to larger animals (e.g. rats, pigs, sheep, primates). Sampling during lactation, with such significant reductions in trabecular BMC, makes it even harder to assess strength because there is less bone surface available to provide resistance. In two *ctcgrp* null vertebral samples only the outer cortical shell was visible, with very little trabecular bone present inside. These samples crumbled while placing them in the fixture and thus were not considered in the final analysis. This may infer that these samples may quite possibly have had a decrease in bone strength at this time. Finally, the general destructive nature of biomechanical testing ultimately precludes any further analysis of the samples being used, and thus repeat tests could not be performed on the samples. Therefore, contrary to DXA findings, biomechanical testing illustrated that skeletal strength was not significantly different at baseline or the end of lactation in WT and *ctcgrp* null mice.

In summary, the first thesis that *calcitonin is required physiologically during reproduction to protect the maternal skeleton against excessive resorption of mineral* is supported by the fact that *ctcgrp* null mice experience significantly greater BMC losses by DXA and trabecular thinning and separation by μCT when compared to the WT counterparts, thus defining calcitonin physiologically important. Also, the results of the primary analysis and supplementation ("rescue") experiment support the primary hypothesis that absence of calcitonin leads to the observed bone phenotype during lactation and that absence of CGRP-α does not contribute. Admittedly, CGRP-α is thought to function predominantly as a neuropeptide and thus a once-daily subcutaneous injection might not be an adequate means of reversing CGRP-α deficiency in these mice,

and thus a role for CGRP-α in contributing to the bone phenotype of *ctcgrp* null mice cannot be completely ruled out.

D. ***Thesis 2: Lactational bone loss in the absence of calcitonin involves linked physiological pathways between breast and bone.***

Having confirmed that calcitonin is physiologically important to protect the maternal skeleton against excessive absorption of mineral during lactation, the mechanisms driving this significant lactational BMC losses seen in the *ctcgrp* null versus the of the WT were subsequently explored. Lactational bone loss typically occurs as a result of an uncoupling in the bone cells, such that ostoclastic bone resorption increases over that of bone formation (11-14). Therefore, loss of calcitonin was hypothesized to accentuate BMC loss simply by enabling more active osteoclasts to resorb bone, thus releasing the *'brake'* that controls this process. Significant lactational BMC losses in the *ctcgrp* null could have occurred as a consequence of alterations within the bone microenvironment (i.e. increased bone resorption over bone formation). Consistent with this hypothesis, was the finding of significantly increased urinary Dpd levels during late lactation in the *ctcgrp* null versus that of the WT. Dpd is a crosslink product of collagen molecules found in bone and excreted in urine during bone degradation. In contrast, serum osteocalcin, which is secreted by osteoblasts and thought to play a role in bone mineralization, was no different between genotypes or at any time point. These results supported the hypothesis that lactation in the absence of calcitonin is dominated by bone resorption and not bone formation.

Bone turnover markers provide a broad picture of whole body bone formation and resorption, an assessment that does not lend much information regarding the bone cells. Histomorphometric analysis however, unlike bone markers, can provide a quantification of the osteoclasts, osteoblasts, osteocytes, as well as information describing the activity along the bone surface. The adult skeleton is continuously broken down and reformed by the coordinated activities of osteoblasts and osteoclasts on trabecular surfaces and in Haversian systems. Similar to the importance of considering the total bone volume when assessing the biomechanical properties of bone, assessment of calcium flux along the surface of bone is critical to reveal differences in bone cell activity during times of high turnover, such as during reproduction. In light of this, bones were collected and prepared for histomorphometric analysis in the *ctcgrp* null versus WT. It was hypothesized that absence of calcitonin would result in increased osteoclast number and function when compared to the WT controls, thus contributing to the rapid skeletal demineralization during lactation.

Due to lack of local expertise and training in bone histomorphometry this analysis was completed by my supervisor (CS Kovacs) while visiting the University of Melbourne (237). The work by C.S. Kovacs illustrated that lactation samples had decreased osteoblast surface (OcS) and number (OcN) (as well as osteoid), and increased osteoclast number (OcN) and surface (OcS) in the nulls versus WT. This fits with the excess bone loss during lactation seen via DEXA in the *ctcgrp* nulls. In contrast, during late lactation and early weaning there was upregulation in osteoblast surface and number (and osteoid) in both the *ctcgrp* null and WT mice. The relative increase was a quadrupling in the null versus doubling in the WT, but the absolute level attained was the same in both

genotypes. Osteoclast parameters were reduced significantly from earlier in lactation, but the null still had greater osteoclast surface and number than the WT. Overall the pattern suggests that bone recovery has begun, and the normal osteoblast surface and number explains why the mice are able to recover from such a substantial loss of bone, taking just a few more days versus the WT.

It was also observed illustrated that *ctcgrp* null vertebral bone volume as a expression of total bone volume during lactation was lower than that seen in the WT siblings at this time (i.e. WT 14.3±1.1% (n=4) vs. ctcgrp null 11.3±1.3% (n= 3)). This was also seen in the tibia (i.e. WT 5.1 ±1.2% (n=5) vs. 2.9±1.2% (n=5)), These findings were also consistent with the bone phenotype in the ctcgrp at this time as seen via DEXA.

Hypercalcemia Post Partum

Blood ionized calcium, the physiological important fraction of calcium, typically remains unaltered by reproduction in humans and rodents with a few exceptions in rodents with larger litter sizes (5-8). Results indicated that transient hypercalcemia was experienced in the *ctcgrp* null at 24-48 hours post-partum, an effect that was not observed in the WT control siblings at this time.

The two main hormones responsible for regulating calcium in the blood are PTH and calcitonin. When blood calcium falls, the CaR detects this change and the body produces more PTH and subsequently levels of calcium rise. On the other hand, if the calcium in the blood rises too high, the thyroid gland produces calcitonin, which slows the release of calcium from bone (2, 90-92). In the *ctcgrp* null, calcitonin production from the thyroid (as well as all other extrathyroidal sites) was completely absent and thus this

part of the regulatory feedback loop was not present to compensate for rapid increases in blood calcium. It is also possible that this early lactational hypercalcemic spike may have occurred as a consequence of increased mineral flooding the maternal circulation at a time when milk production or let-down was not fully established. The absence of calcitonin to counter this sudden flux of calcium to the system could have exacerbated this hypercalcemia.

Hypercalcemia can also be caused by increased activity of the parathyroid glands (i.e. hyperparathyroidism). This however, was not the case in the *ctcgrp* nulls, as levels of serum PTH obtained at 24 - 48 hrs post-partum were actually suppressed to the limit of the detection of the assay (1.6 pg/ml) in both WT and *ctcgrp* null mice (i.e the time of the hypercalcemic spike in the *ctcgrp* null). Excessive dietary intake of calcium or vitamin D may also raise blood calcium levels above normal. Both WT and *ctcgrp* null mice were both on the same standard mouse chow with 1% calcium and 0.75% phosphorus by weight.

After the second day post-partum maternal blood ionized calcium returned to normal and stayed there for the remainder of lactation. Thus, beyond the 24 - 48 hour period post-partum the absence of calcitonin did not have any long term effects on circulating blood calcium levels.

Systemic Calcium Handling

Calcium balance involves the activity of multiple physiological compartments including: storage of calcium in bones, blood and muscle, dietary intake, intestinal absorption, fecal excretion, renal excretion and reabsorption as well as bone resorption

(Figure 4). All of these pathways work synergistically to maintain the calcium levels in the blood, which is necessary for survival. Systemic balance of calcium can shift if one, or multiple, of these parameters increase or decrease (e.g. if dietary calcium is low, intestinal calcium absorption may increase to compensate). In the case of the *ctcgrp* null, in which BMC was significantly reduced during lactation compared to the WT control siblings, there were several possible routes that warranted exploration to determine how calcium balance was altered such that mineral losses were exacerbated.

Hypercalciuria in the *ctcgrp* null was hypothesized as one of the possible explanations for the significant loss of mineral during lactation versus that seen in the normal WT siblings. 'Leakage' of calcium into the urine (i.e. increased excretion) could have caused a shift in calcium balance resulting in mineral breaking down from within the skeleton to sustain systemic calcium levels. Previous studies, however, in both humans and rodents have shown that the glomerular filtration rates typically decrease during lactation versus that seen in pregnancy and thus renal excretion of calcium is often times reduced (5-8). In this study we examined whether urine calcium levels were different in the *ctcgrp* null versus that seen in the WT. If levels were found to be higher in the urine of the *ctcgrp* null this would warrant further exploration of calcium handling by the kidneys to determine how calcitonin affects the active and passive processes involved in renal calcium handling. Findings indicated however, that in both WT and *ctcgrp* null mice, urinary calcium levels were decreased during lactation. More importantly, no differences were seen between the *ctcgrp* null and WT. Therefore significant losses of mineral in *ctcgrp* null were likely not attributed to a renal axis in which calcium excretion was exaggerated and systemic calcium balance was thrown off. With this pathway

eliminated, we next explored what was happening in the intestine in the *ctcgrp* null and WT.

Intestinal calcium absorption is increased during pregnancy in both humans and rodents to meet the needs of the growing fetus (5-8). During lactation, however, intestinal calcium absorption typically decreases to a level that is no different than that during non-pregnancy (5-8). In contrast, the predominant mechanism in which the additional needs for calcium are met during this time is through enhanced resorption of bone. It was hypothesized, however, that the absence of calcitonin could cause a substantial decrease in intestinal absorption of calcium, thus resulting in exaggerated bone resportion during lactation to sustain blood calcium levels. Duodenal ^{45}Ca absorption, however, was no different at mid-lactation between WT and *ctcgrp* null mice. Thus, mice lacking calcitonin experienced a calcium absorption rate that was no different than normal during late lactation and thus this particular hypothesis was proven incorrect (**Figure 36**).

During lactation the breast also actively participates in maintaining systemic calcium balance (5-8). The suckling neonate uses the mother's reservoir of skeletal mineral to nourish its growth and development. Another hypothesis was that the significant BMC loss in the *ctcgrp* null occurred as a result of calcium being drained into the milk supply during lactation. Indeed, despite the lack of a change in urinary calcium excretion or intestinal calcium absorption during lactation, *ctcgrp* null mice were found to have a higher mean milk calcium concentration during mid-lactation versus that of the WT siblings. Therefore increased skeletal resorption may have increased the loss of calcium into milk.

Initially, considerable variation was seen in milk calcium in both genotypes with standard error values being quite large **(Figure 37)**. In an attempt to control for some of the variability, pups were separated from the lactating mothers for an hour before milking, and milk was collected at similar times each day. Milk calcium concentrations can vary not only from time of day, but within a particular feed. Controlling for all of these inconsistencies, as well as dealing with small milk volumes for analysis created experimental road blocks that were challenging given the physical size of the animals and the numbers of lactating mice. At two weeks of lactation, however, milk calcium corrected for protein was significantly higher in *ctcgrp* null mice versus that of the WT mice **(Figure 40)**. Excess calcium losses during lactation seen in the *ctcgrp* null mice (versus WT) appear to be drained into the milk supply. The cause of this drainage could be mammary tissue creating more calcium rich milk (possibly through increased local production of PTHrP) and therefore causing increased bone resorption through the actions of systemic PTH and/or PTHrP. Or it is possible that the mammary tissue could be draining off the increased calcium that was initially created by increased bone resorption.

Hormonal Milieu during Lactation

During lactation osteoclast-mediated bone resorption is enhanced due to a unique hormonal milieu characterized by low circulating PTH and estradiol and elevated PTHrP and calcitonin. This particular microenvironment allows for the production of milk and thus the provision of nutrients required for neonatal growth and development. Thus, in further exploring the skeletal phenotype in the *ctcgrp* null versus WT, it was hypothesized that exaggerated BMC losses may result in high PTHrP levels that, in the setting of low

estrogen, plays a major role in the regulation of skeletal demineralization during lactation (6 – 8, 174). Supporting this fact is the finding that mice lacking PTHrP in mammary tissue during lactation lose only a fraction of mineral as their WT counterparts (193). Considering these previous findings, it is possible that exaggerated BMC losses in the *ctcgrp* nulls may result from high PTHrP levels. Evidence refuting this theory, however, included lack of a difference in plasma PTHrP levels between WT and *ctcgrp* null mice **(Figure 44)**. It is important to note, however, that plasma PTHrP levels fluctuate relative to suckling (174) and samples in this analysis were not timed to control for suckling patterns. Also, it is possible that the sensitivity and performance characteristic of the PTHrP RIA may have made it unable to detect a difference in circulating PTHrP. Despite lack of a detectable difference in circulating PTHrP levels, however, PTHrP mRNA was increased over 3 fold during late lactation in mammary tissue in *ctcgrp* null mice versus that seen in the WT controls at the same time point. Consistent with these finding as well was a qualitative increase in immunohistochemical staining of PTHrP in the mammary epithelial tissue using an anti-PTHrP antibody and normal WT lactating mammary tissue as a negative control. Thus, evidence suggests that increased mammary derived PTHrP in the *ctcgrp* null contributed to increased bone resorption.

It is relevant to note that PTH and PTHrP are equivalent in their ability to activate the PTH/PTHrP receptor on osteoblasts and communicate through the RANK-RANKL-OPG pathway to stimulate osteoclast-mediated bone resorption; thus, the mechanism for enhanced bone resorption in the *ctcgrp* null mice may simply occur as a result of exaggerated stimulation of PTH/PTHrP receptor-mediated bone resorption by PTH and PTHrP, coupled with absence of the effect of calcitonin to serve as a systemic break to

inhibit the activity of osteoclasts. Thus, because of increased mammary PTHrP in the *ctcgrp* null versus WT, it appears that the breast is an important physiological pathway regulating bone loss during lactation in the absence of calcitonin.

High PTH levels (i.e. hyperparathyroidism, a group of conditions characterized by an overproduction of PTH by the parathyroid glands) is a known cause of hypercalcemia and bone loss (34, 43). Findings, however, indicated that PTH levels were no different in the WT and *ctcgrp* null at 24-48 hours post-partum (i.e. at the time of hypercalcemia in the *ctcgrp* null). Thus, the early lactational hypercalcemia in the *ctcgrp* null was not due to hyperparathyroidism. PTH levels, however, did spike after 1 week of lactation in the *ctcgrp* null. This was not seen in the normal WT siblings, as PTH levels in these animals remained on the lower side of normal, a finding anticipated from previous studies in humans and rodents at this time (7, 8, 168). The cause of the elevated PTH may have been subsequent to the significant loss of calcium in the milk. Hypocalcemia resulting from an excess loss of calcium in the milk is a condition called 'milk fever'. Milk fever, characterized by a drop in blood calcium, paralysis, etc, is most commonly seen in dairy cows after calving. Milk fever could be a possible explanation for this secondary hyperparathyroidism occurring after the first 24 hrs post partum in the *ctcgrp* null. Milk calcium content was doubled during lactation in the *ctcgrp* nulls versus that in the WT mice, these changes could have been compensated for by elevations in PTH. This compensatory increase in PTH possibly prevented any hypocalcemia from occurring or, it is possible that the increase in PTH could have primarily contributed to the exaggerated skeletal mineral losses seen in the *ctcgrp* null. Other considerations include, larger litter size or a restricted calcium diet may also induce maternal secondary hyperparathyroidism

during lactation (6-8, 33). In these cases, loss of calcium into the milk may compromise the mother's ability to maintain her own blood calcium.

E. Breast and Bone Circuitry

Evidence from this research has pointed to both the breast and bone as key players in the cycling of bone during reproduction. In the absence of calcitonin mammary tissue stimulates skeletal resorption during lactation by producing a temporary spike in PTH, excess PTHrP during lactation and resorption becomes exaggerated in the bone microenvironment **(Figure 49)**. The data collected for this dissertation clearly indicates that calcitonin plays a role by modulating the degree of skeletal resorption during lactation. Absence of calcitonin in the systemic circulation leads to increased bone resorption (likely due to an increase in OcS/BS (237)). However, since the CTR is also expressed in mammary tissue it is possible that calcitonin acts at both sites to modulate lactation – possibly to inhibit osteoclasts during mid-lactation, and within mammary tissue to modulate PTHrP or milk production.

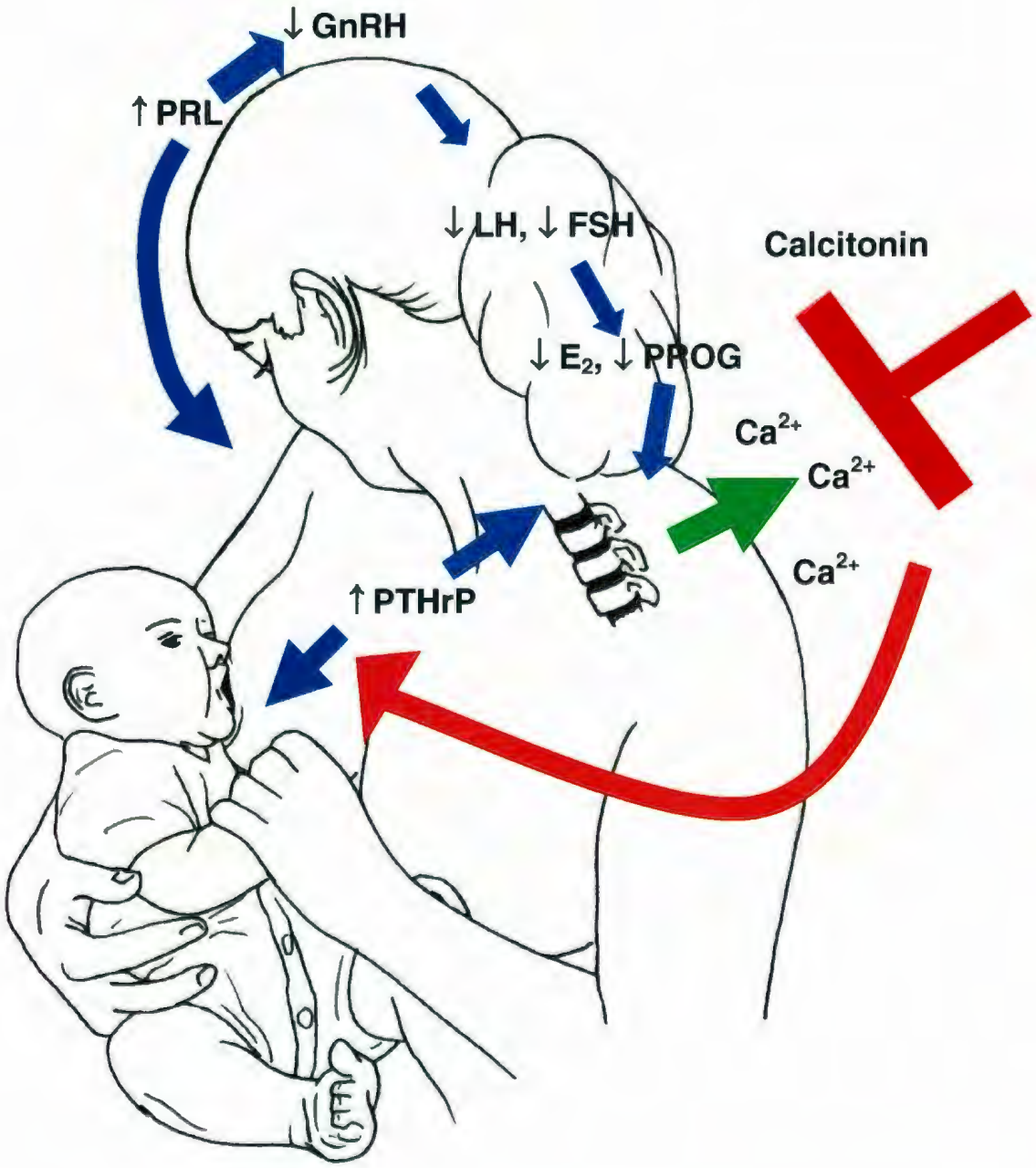


Figure 49: Breast, Brain and Bone Circuitry during Lactation. Enhanced skeletal resorption of calcium in the *ctcgrp* null versus WT during lactation is due mainly to increased mammary production of PTHrP and increased bone resorption (red arrows).

F. Limitations of Using Animal Models

A scientific model (whether animal or cell) is a useful starting point in research. The process, however, of extrapolating findings and ultimately applying them to the etiology of human disease is (recognizably) a convoluted process. The work completed in this dissertation, although useful in its suggestion of a physiological role for calcitonin, is certainly not exempt from scientific critique. At the end of the day, it must be recognized that there are many notable differences between human and mouse physiology, and limitations of using genetically engineered animal knockout models.

The surge of sex hormones at puberty terminates linear growth in humans by fusing the growth plates of the long bones (3, 13, 14). Rodents, in contrast, do not experience this fusion of the long bones, and thus have continual bone growth throughout life. This lack of growth cessation may infer drastic differences in the bone remodeling process of mice versus that of humans, especially during lactation. As well, unlike humans, mice go through continual estrus cycles, and thus could theoretically lactate almost continuously throughout much of their adult life. Number of offspring in mice averages from 8-12 pups per litter, versus mainly singleton births in humans. Mice and humans have obvious postural differences which make biomechanical loading patterns quite different and comparing biomechanical results across species may be difficult. Furthermore, rodents may exhibit different remodeling patterns in cortical versus trabecular bone.

Use of gene ablation techniques in mice permits the exploration of mechanisms underlying the regulation of calcium and bone metabolism that would otherwise be impossible (at this point in time) to examine in humans. These techniques however, like

most in science, have notable limitations. Notable challenges include: 1) transferability of outcome from mouse to human 2) secondary, tertiary, etc effects of gene ablation and 3) effect of mouse background variability on phenotype.

Global knock-out of a gene, or any genetic variation thereof (i.e. conditional knockout, knock-in, etc) can have various unexpected outcomes. In some cases (e.g. PTHrP null, CTR null) ablation of the gene of interest may result in embryonic or neonatal lethality. Thus, if the HET survives, it would be considered the *experimental* animal, used to compare to the normal WT littermate. As well, one must always remember that modifying the genetic structure of an organism may cause other genes to have compensatory effects. Thus, the outcome may not be straightforward, and may involve multiple pathways.

The genetic background is another necessary consideration when completing work in animals. Knowing the phenotype will allow the researcher to use caution in making conclusions about the outcome of a genetic manipulation based on the background characteristics of the colony (e.g. BLKSW mice have higher blood calcium and larger litter sizes when compared to some inbred strains of mice and therefore larger little size may not be the result of the ablation).

G. Clinical Implications

A few case series have examined male and female thyroidectomized individuals taking thyroid hormone replacement and reported conflicting results as to whether such patients develop a long-term deficit in bone mass or an increase in fracture risk relative to matched individuals with intact thyroids (82-84). No study, however, has examined the

bone mass of thyroidectomized individuals after a cycle of pregnancy and lactation, and of course such individuals would retain the ability to make calcitonin in pituitary and mammary tissue and therefore would not be truly calcitonin-deficient. Vertebral compression fractures occasionally occur during lactation in otherwise healthy young women, a condition termed osteoporosis of lactation (203-206). In such cases excess bone resorption during lactation, and possibly a low baseline BMC, is presumed to have led to increased fragility that resolved after weaning. Our results raise the possibility that some of these human cases might represent the human equivalent of absence of calcitonin.

H. Future Considerations

Pituitary Axis

Due to the presence of the CTR within the pituitary lactotrophs, and published evidence showing that pharmacological administration or targeted overexpression of calcitonin in this area of the brain causes hypoprolactemia (200, 201) preliminary data was collected to provide the basis for future exploration of this intricate physiological loop. Based on these findings, one could hypothesize that absence of calcitonin would result in increased prolactin production and secretion. This in turn would cause more profound ovarian suppression, lower estradiol, and thus significant bone resorption. Also, increased prolactin could also result in increased PTHrP, thus perpetuating bone resorption from this angle.

Preliminary results indicated that there was no difference in mean circulating prolactin levels at any time point in *ctcgrp* null mice versus WT (**Figure 41**). It is

important to note, however, like many hormones, prolactin is released into the system in a pulsatile fashion throughout lactation (194). Ideally multiple serum samples would need to be collected from one mouse to assess peak levels of prolactin. The proposed role of calcitonin to regulate pituitary lactotrophs and systemic prolactin levels may indeed be a physiologically important pathway. Pooled pituitary samples could also be assessed to determine if prolactin, LH, FSH or markers of apoptosis in lactotrophs are altered in *ctcgrp* nulls. Altered production of these brain peptides could result in markedly increased or decreased milk production, translating into associated skeletal changes linking calcitonin with the brain during lactation. Lack of a role of these peptides, will reinforce the mammary-tissue as the main calcitonin physiological loop during lactation.

In addition to the possibility of alterations in prolactin, exaggerated BMC losses may also be caused by decreased estradiol production during lactation, or increased sensitivity to estradiol. Preliminary experiments indicated that estradiol levels for all genotypes and time points were barely at the limit of delectability for the commercial EIA (data not shown). An assay with more acute sensitivity may have detected differences in the genotypes or it could be possible that estradiol levels were extremely low during lactation in WT mice or were no different than the *ctcgrp* nulls. To overcome the commercial assay detection limitations, large volumes of serum were collected and sent to Dr. A.F. Parlow (National Hormone & Peptide Program, Torrance, California, USA) for analysis using an in-house highly sensitive 17β- estradiol assay. Unfortunately, after the samples were received by the Parlow lab they were stored at room temperature and thus rendered un-analyzable. Due to the work involved in pooling multiple serum samples

to achieve 300µl samples (i.e. up to 5-6 mice per sample, for at least 20 samples), it was not possible to collect enough additional serum in the given time frame to be assessed.

Role of the Calcitonin Receptor

It is a possibility that the CTR may also control BMC losses during lactation. Unfortunately, studies suggest that CTR null mice die at mid-gestation and therefore cannot be studied (92, 93, 107). Mice HET for the CTR, however, are healthy, fertile and have been shown in one study to have a higher bone mass with age (107). Proof of role of the receptor for calcitonin during lactation could facilitate additional *in vitro* studies regarding the role of calcitonin.

Exploration of the Post-weaning Period

The weaning period is a physiological paradox in that the cessation of lactation in the adult skeleton can stimulate the skeleton to completely and rapidly reverse the significant mineral losses that occurred during that time. This challenges the classic catabolic skeletal response seen throughout adulthood in that significant BMC losses typically cannot be regained. In our studies, we have found that during the post-weaning period *ctcgrp* null mice completely regain the mineral lost after 21 days of lactation, as per that seen in the normal, WT siblings (**Figure 24**). It is important to note however, that *ctcgrp* nulls, take several days longer to remineralize their skeletons post weaning, a finding that was not unexpected due to the greater BMC loss experienced versus that seen in the WT. Therefore, in contrast to the physiological requirement for calcitonin during

the lactation period, calcitonin does not appear to be required for the skeletal recovery process post-weaning, as mice recover completely for lactational BMC losses.

Despite the finding that calcitonin does not appear to be of physiological importance in the long-term (i.e. post lactation) the aforementioned results nonetheless may remain clinically relevant. Normally the adult skeleton is unable to significantly regain mineral that has been lost through such things as: prolonged periods of weightlessness, the use of corticosteroids, metabolic disease, etc. Understanding the means which the mouse skeleton completely and rapidly rebuilds itself post lactation, could provide useful insight into anabolic therapies for bone disease. *Ctcgrp* null mice may be an ideal model to examine skeletal recovery since the factors stimulating recovery are likely to be upregulated further in these mice compared to normal as these mice are able to recover from over a 50% deficit in spine BMC.

Furthermore, perhaps the most useful, albeit ambitious, future study could focus on the mechanisms underlying the skeletal recovery of mineral during the post-weaning period in the *ctcgrp* null versus WT. A genome-wide microarray analysis could be completed on bone samples specifically during the recovery period. More obvious calcitropic candidate genes (e.g. RANKL, OPG, osteocalcin) would be included (i.e. those associated with skeletal anabolism), as well as other genes of interest. Differentially expressed candidates could be further explored.

I. Conclusions

Osteoporosis Canada has estimated that 1.4 million Canadians suffer from this

disease. The associated financial costs are estimated to be $1.9 billion each year in Canada alone. Considering the aging demographic, this number will likely rise in the years to come. In light of these statistics, future research contributing to the etiology and treatment of this disease is critical. The *ctcgrp* null mouse model fits in this picture by offering a physiological context in which to study the anabolic behavior of bone. The post weaning skeleton of the *ctcgrp* null is capable of rebuilding over half of its vertebral mineral content within a relatively short period of time. Our evidence has pointed to two important physiological pathways in bone flux during reproduction: the breast and bone. Mammary tissue enhances skeletal resorption of mineral during lactation through the production of excess PTHrP and bone resorption is increased in the absence of the calcitonin gene. Further work is needed to determine how the pituitary axis is involved during reproduction in the absence of calcitonin.

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APPENDIX A

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Journal: Endocrine Reviews
Author Name: C. Kovacs
Title: Maternal-fetal calcium and bone metabolism during pregnancy, puerperium, and lactation
Year: 1997
Volume: 18
Page Range: 832-872
Figure Modification:
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Prior Approval: Specific modification would be adding a 'post-weaning' addition to Figure 5: "Schematic illustration contrasting the adaptive processes of calcium homeostasis in human pregnancy and lactation, as compared with normal. The thickness of arrows indicates a relative increase or decrease with respect to the normal, nonpregnant state". Permission has been obtained from PhD supervisor (the author) Dr. C.S. Kovacs. If formal written permission is required pls contact me and I can fax documentation'

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APPENDIX B

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APPENDIX C

In-House Mouse PTHrP Radioimmunoassay

Reagents

Standard: Recombinant PTHrP (1-84), reconstitute 2 µg/10µl aliquot in 1 ml assay buffer which gives a concentration of 200,000 pmol/L, Make up several dilutions from 0.8 to 400 pmol/L concentrations (i.e. 0, 0.8, 2, 4, 8, 15, 20, 30, 40, 80, 200, 400pmol/L).

Antibody: INCSRAR antihuman PTHrP (1-40) Goat number 906. Dilute 1/100 stock solution to 1/10, 000 and use 100 µl/tube. Final dilution 1/40,000.

Tracer: ^{125}I Tyr^{0}PTHrP (1-34), 10, 000 cpm in 100 µl

Assay Buffer: 0.05 M barbitone pH 8.6 with 0.5% BSA. 0.02% Tween, 0.05% Sodium Azide, 0.5 ml (20TIU) Aprotinin (Trasylol)/100 ml assay buffer

.

Antigoat Sac Cel: SAC-CEL Anti-sheep/goat code AA-SAC2.

Aprotinin: Protease inhibitor from bovine lung (Sigma A-1153), reconstitute 100 mg with 10 ml 0.05M barbital buffer, pH 8.6.

Plasma samples: Collect in 0.5 M EDTA 100 µl (or EDTA coated tube) and Aprotinin 100 µl (4TIU)/10 ml of blood.

Pooled Normal Plasma: EDA plasma with aprotinin from haemochromatosis patients are pooled and used as control for bonding changes observed in the presence of plasma. 10 µl is added to each standard tube.

Controls: C1, C2, C3. Low, medium and high levels of recombinant human PTHrP (1-84) are added to normal human plasma.

Assay Procedure:
DAY 1
Incubate the following:
Standard or sample (100µl)
Antibody (100µl)
Assay buffer/Pooled normal plasma (100µl)

Non-specific Binding Tube Containing:
Assay buffer (200µl)
Normal plasma (100µl)
At room temperature for 4 hours

Add tracer 10,000 cpm (100µl) and incubate overnight at room temperature. Final incubation volume is 400µl per tube.

DAY 2
Add anti0goat Sac-Cel (100µl) and incubate for 30 minutes at room temperature to weight protein down. Add 1 ml distilled water to each tube. Centrifuge and aspirate supernatant. Count pellet using multigammer counter.

NOTE: For mini assay use half the amounts of standard, sample, assay buffer, and tracer.

APPENDIX D

Structural parameters of trabecular bone measured by the Skyscan micro-CT.

Relative bone volume

Symbol: BV/TV

This is bone volume / tissue volume, the proportion of a studied volume of trabecular bone occupied by bone as opposed to marrow. This parameter is only relevant if the studied volume is fully contained within a trabecular bone region, and does not extend into or beyond the bounding cortical wall of the bone. Unit: % or fraction of 1.

Bone surface to volume ratio

Symbol: BS/BV

This parameter can be measured from 3D micro-CT images of bone regions after a surface has been fitted to the image. BS/BV is a useful basic parameter in characterising the complexity of structures and is the basis of model-dependent estimates of trabecular thickness. Unit: mm^{-1}.

Trabecular thickness

Symbol: Tb.Th

Calculation – or estimation – of Tb.Th from 2D measurements requires an assumption about the nature of the bone structure. Two simple bone structure models, the parallel plate and the cylinder rod model, provide the boundary values within which a hypothetical "true" Tb.Th will be located. Thickness defined by these two models (Parfitt *et al.* 1987) is given:

Parallel plate model:

$$Tb.Th = \frac{2}{(BS/BV)}$$ Unit: mm or μm

Cylinder rod model:

$$Tb.Th = \frac{4}{(BS/BV)}$$ Unit: mm or μm

Where *BS/BV* is the surface to volume ratio, mm^{-1}.

With 3D image analysis by micro-calcitonin a true 3D thickness can be measured which is model-independent. This is determined as an average of the local thickness at each voxel representing bone (Ulrich *et al.* 1999). Local thickness for a point in bone is defined by Hildebrand and Ruegsegger (1997a) as the diameter of a sphere which fulfils two conditions:

(a) the sphere encloses the point (but the point is not necessarily the centre of the sphere);

(b) the sphere is entirely bounded within the bone surfaces.

Therefore for this direct Tb.Th parameter to be measured it is necessary for a bone surface to be fitted to the micro-calcitonin image of bone.
Histomorphometrists typically measure a single mean value of Tb.Th from a trabecular bone site. However a trabecular bone volume can also be characterised by a distribution of thicknesses. The Skyscan micro-CT outputs a histogram of trabecular thicknesses with variable interval to allow flexible and powerful thickness analysis of bone – or of any other structure.

Trabecular separation

Symbol: Tb.Sp
Trabecular separation is essentially the thickness of the marrow spaces between trabecular structures. It can also be calculated either from 2D images with model assumptions or directly in 3D. Applying the two models as for Tb.Th, trabecular separation is calculated:

Parallel plate model:

$$Tb.Sp = \left(\frac{1}{Tb.N}\right) - Tb.Th$$ Unit: mm or μm

Cylinder rod model:

$$Tb.Sp = Tb.Th \times \left(\left[\left(\frac{4}{\pi}\right) \times \left(\frac{TV}{BV}\right)\right] - 1\right)$$ Unit: mm or μm

where TV is total volume of the volume analysed and BV is bone volume (Parfitt *et al.* 1987). Note that each of the above definitions takes the Tb.Th value derived from the corresponding plate or rod model.
In practice it is undesirable to employ inaccurate 2D model assumptions, especially because the "plate-like" or "rod-like" character of trabecular bone can change from one end of a studied volume to the other (see "structure model index", below). Therefore Skyscan analysis software can measure Tb.Sp directly and model-independently in 3D from micro-CT images by the same method used to measure trabecular thickness (see above), with the analysis applied to the marrow spaces instead of the bone.

Trabecular number

Symbol: Tb.N

Trabecular number implies the number of traversals across a trabecular structure made per unit length on a linear path through a trabecular bone region. Plate and rod model 2D-based definitions of Tb.N again take the corresponding Tb.Th values:

Parallel plate model:

$$Tb.N = \frac{(BV/TV)}{Tb.Th}$$ Unit: mm^{-1}

Cylinder rod model:

$$Tb.N = \frac{\sqrt{\left(\left(\frac{4}{\pi}\right)\times\left(\frac{BV}{TV}\right)\right)}}{Tb.Th}$$ Unit: mm^{-1}

Again the complexities of model dependence are eliminated by true 3D calculation of Tb.N from micro-CT images. Trabecular structure is calculated from micro-calcitonin 3D images of trabecular bone as the inverse of the mean distance between the middle axes of trabecular structures (Ulrich *et al.* 1999). These axes are identified by a skeletonisation process.

Trabecular pattern factor

Symbol: TBPf

This is an index of connectivity of trabecular bone, which was developed and defined by Hahn *et al.* (1992). It was applied by these authors to 2D images, and calculates an index of relative convexity or concavity of the total bone surface, on the principle that concavity indicates connectivity, and convexity indicates isolated disconnected structures. TBPf is calculated in 2D (slice-by-slice) and in 3D by Skyscan software. TBPf is calculated by comparing bone area and perimeter before and after an image dilation. It is defined:

$$TBPf = \left(\frac{P_1 - P_2}{A_1 - A_2}\right)$$ Unit: mm^{-1}

Where P and A are bone area and perimeter, and the subscript numbers 1 and 2 indicate before and after image dilation.

Where trabecular connectedness results in enclosed marrow spaces, then dilation of trabecular surfaces will contract the perimeter. By contrast, open ends or trabecular nodes will have their perimeter expanded by surface dilation. As a result, lower TBPf signifies better connected trabecular lattices while higher TBPf means a more disconnected trabecular structure.

Loss of trabecular connectivity can lead to loss of strength in bone disproportionate to bone volume loss, and is a particular concern regarding osteoporosis.

Euler-Poincare number

The Euler-Poincare – or the abbreviated "Euler number" – is also an indicator of connectedness of a 3D complex structure. The Euler number is characteristic of a three-dimensional structure which is topologically invariant (it is unchanged by inflation or compression of the structure). It measures what might be called "redundant connectivity" – the degree to which parts of the object are multiply connected (Odgaard *et al.* 1993). The components of the Euler number are the three Betti numbers: β_0 is the number of objects, β_1 the connectivity, and β_2 the numner of enclosed cavities. The Euler-Poincare formula for a 3d object X is given:

$$\chi(X) = \beta_0 - \beta_1 + \beta_2$$

Connectivity in the Euler sense of "redundant connectivity" is a measure of how many connections in a structure can be severed before the structure falls into two separate pieces.
Euler analysis provides a measure of connectivity density, indicating the number of redundant connections between trabecular structures per unit volume. To calculate this, divide the connectivity β_1 (a unitless number) by the analysed volume. Trabecular connectivity may contribute significantly to structure strength although this relation is a question requiring further research (Odgaard 1997)
Unit: none.

Degree of anisotropy

Symbol: DA
Isotropy is a measure of 3d symmetry or the presence or absence of preferential alignment of structures along a particular directional axis. Apart from percent volume, DA and the general stereology parameters of trabecular bone are probably the most important determinants of mechanical strength (Odgaard 1997). Mean intercept length (MIL) and Eigen analysis are used to calculate DA, and these involve some quite advanced engineering mathematics. However the essentials of the MIL eigen analysis can be summarised in normal English.
Consider a two phase complex object, like a region of trabecular bone, where there are two phases (bone and marrow) and the shape of both phases is complex. We can study a volume selected within the object to be representative of the whole object, to determine isotropy of the object. If the object is isotropic, then a line passing through the object at any angle will pass through a similar length of solid phase as a proportion of its total length. A bag of marbles would be isotropic. However a packet of spaghetti would be non-isotropic, or anisotropic, since lines going along the direction of the spaghetti would pass through spaghetti only while lines crossing at right-angles would alternate between spaghetti and air.
Mean intercept length (MIL) analysis measures isotropy (it is usual to talk of measurement of the negative quantity anisotropy). Mean intercept length is found by sending a line through a region of trabecular bone, and recording what part of the line's length passes through bone and what part through non-bone. The length passing through

bone is the MIL for bone for that line. In a 3d MIL analysis, you need to send multiple lines through the volume over the full range of 3d angles. Also, for each angle, many parallel lines are sent covering the whole test volume, and the MIL for that angle is calculated as an average for all these lines. This requires that a spherical region is first defined within which the MIL analysis will be done and isotropy measured, since the test lines must all cross the sphere centre and have an equal distribution of lengths, covering all 3d angles but distributed at random.

Say we start a MIL analysis of a spherical region of trabecular bone by sending multiple lines through the sphere as described above. The next step involves visualisation of the 3d distribution of MIL lengths. All the MIL lines are drawn passing through the sphere centre, but the length of each line is reduced to the bone phase MIL for that line. This process is called a polar plot of MIL. In 3d this creates a dense pin-cushion like effect with lines in all directions at different lengths.

Figure 1 shows in a simple diagram the appearance of an MIL distribution in 3d. Any asymmetry in the MILs with respect to 3d angle - which will represent the anisotropy of the bone in the spherical region - will make the line distribution depart from an overall spherical shape and become elongated in the direction where the solid structures have the longest MIL (such as the axis of the spaghetti packet).

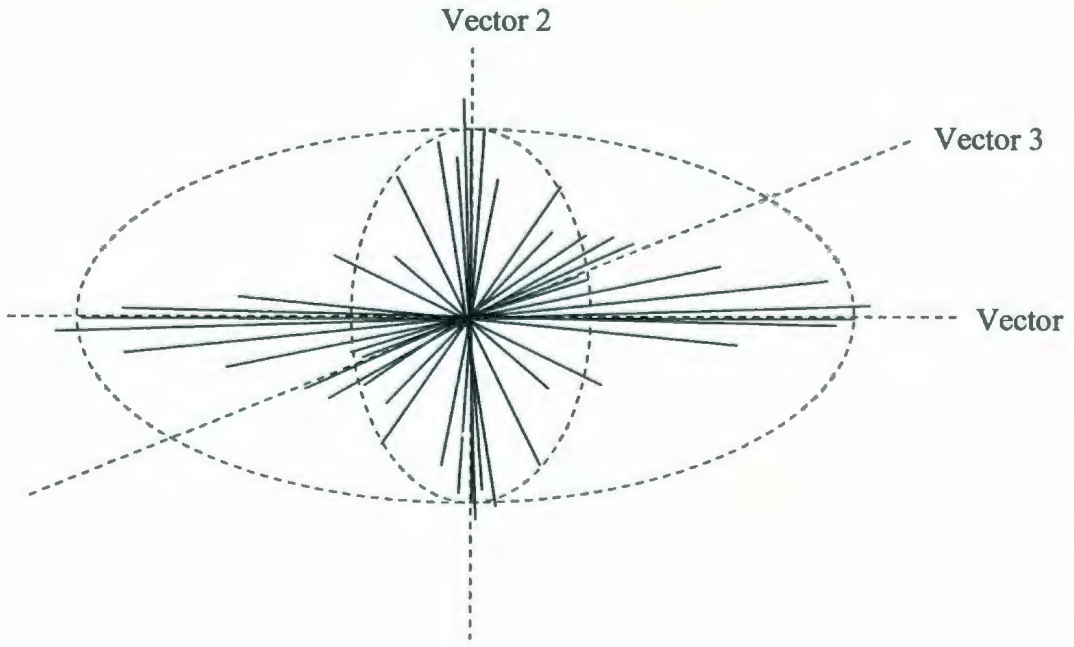

Figure 1. An ellipsoid (3D ellipse) is fitted to the 3D distribution of MILs (mean intercept lengths) measured over a full range of 3D stereo-angles. This ellipsoid is fitted statistically and has 3 vectors which are orthogonal (at right-angles to each other). A tensor (matrix) of 9 (3x3) eigenvectors describes the directions of the three vectors - one for each column. The three eigenvalues are the ellipsoid lengths (representing the mean MIL) in each vector direction.

Clearly the MIL "pin-cushion" is a complex object, and a method is needed to extract some summary numerical parameters defining the orientation and isotropy / anisotropy of the MIL distribution. This is where the anisotropy tensor analysis steps in. (Tensor means matrix.) This method is probably best attributed to Harrigan and Mann (1984) and essentially describes the MIL distribution as an ellipsoid. An ellipsoid is a 3d ellipse. As shown in figure 1, an ellipsoid has three axes. These describe the longest orientation, and the length and width (major and minor axes) of the ellipse section at right-angles to the longest orientation. The ellipsoid can be asymmetric in one axis only, like a rugby ball, or in two axes, like a bar of soap.
An ellipsoid is fitted to the MIL "pin-cushion" 3d polar plot. This is a statistical fit, finding the ellipsoid which most closely describes the 3d shape of the MIL distribution. MIL analysis therefore should also output values indicating the strength of fit of the ellipsoid and associated error, such as the correlation coefficient.
A tensor or matrix is a way of describing an ellipse by a 3x3 matrix of numbers. Technically this is a second order tensor. The tensor (or matrix) describing the anisotropy ellipsoid is in the class of orthogonal tensors, since it describes the ellipsoid axes which are orthogonal (at right angles) to each other. The end result of the anisotropy tensor analysis is called the eigen analysis, eigen meaning characteristic. This comes in two parts. You have the 3x3 matrix of eigenvectors which describe the 3d coordinates of the three axes of the ellipsoid as described above - one column of 3 numbers for each vector. And the three eigenvalues are each an index of the relative length or amount of bone present in each of the three axes described by the eigenvectors.
Finally, you can derive from the tensor eigenanalysis a single parameter measuring anisotropy: this is the degree of anisotropy (DA), and is simply the maximum eigenvalue divided by the minimum eigenvalue. A value of 1 for DA means a fully isotropic object - a large bag of small marbles for example. Increasing anisotropy gives increasing values above 1. (This is somewhat unhelpful in mathematic indices - it would be better if 0 meant isotropic and 1 meant totally anisotropic. However we are stuck for the time being with a DA having values from 1 to infinity.)
It should be noted that stereology analysis can also identify principal orientations that are not orthogonal to eachother. Each of these directions has its own associated degree of anisotropy, which should not be confused with the "global" DA derived from the MIL ellipsoid eigenanalysis.
Unit: none.

Structure model index

Symbol: SMI
Structure model index indicates the relative prevalence of rods and plates in trabecular bone – of importance in osteoporosis. An infinite parallel plate and cylindrical rod have SMI values of 0 and 3 respectively. A sphere has a SMI of 4. This scale of SMI values applies where the structures occupy a relatively small volume fraction of space – less than half. However, where a structure such as bone occupies more than half of the volume of interest, then the SMI parameter result becomes inverted and negative SMI values are obtained.

For this reason, SMI applied to trabecular bone in practice varies sensitively with relative bone volume (BV/TV). This might give strong differences between experimental groups in *in-vivo* bone studies, but in this situation SMI cannot be said to be giving information purely about surface topography. For this reason another parameter can also be employed to give an index of the prevalence of plates and rods in trabecular bone – the "percent plate" parameter described next.
SMI is calculated by a differential analysis of the triangulated surface of trabecular bone and involves a measurement of surface convexity (Hildebrand and Ruegsegger 1997b).
Unit: none.

Percent plate

Symbol: P.Pl
Percent plate is a parameter devised by Borah *et al.* (2002) which compares the true 3D thickness measured by micro-calcitonin (as described above) with thickness measurements based on 2D plate and rod models. Note that the BS/BV value on which the model-dependent Tb.Th values are based is itself obtained in 3D by micro-calcitonin surface-fitting. The parameter essentially assesses whether the measured direct 3D thickness is closer to the plate or the rod model-based thickness estimate. Percent plate is calculated as:

$$P.Pl = \left[1 - \left(\frac{(Th_{direct} - Th_{plate-model}}{Th_{rod-model} - Th_{plate-model}}\right)\right] \times 100 \qquad \text{Unit: \%}$$

where *Th* is trabecular thickness.

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Appendix E

Immunohistochemistry Protocol

Peroxidase antioperoxidase protocol

1. Fix tissue in 10% buffered formalin as briefly as possible. Embed in paraffin wax. Cut 5 μm sections.
2. Dry slides overnight in a 37° C oven.
3. Dewax slides in xylene; 2 changes x 5 minutes.
4. Wash in absolute ethanol for 30-60 seconds.
5. Put slides in methanol with 1% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity of the sections. Make up 1 litre phosphate buffered saline.
6. Wash in phosphate buffered saline for 3 x 1 minute. Wipe slide dry around section.
7. Carefully pipette over the section 1/10 normal goat antiserum in phosphate buffered saline to block nonspecific background binding. Incubate in a humid box at room temperature for 30 minutes.
8. Tip off phosphate buffered saline and drain.
9. Pipette on the primary antiserum at the required dilution e.g., rabbit anti PTHrP at 1/200 in phosphate buffered saline and incubate for 1 hour at room temperature or overnight at 4 °C at a lower dilution. Include a negative control of non-immune rabbit serum at the same dilution.
10. Rinse sections with a stream of phosphate buffered saline then was in 3 changes of phosphate buffered saline (3 x 10 minutes).
11. Pipette on the secondary antibody e.g. goat anti rabbit immunoglobulin diluted 1/40 in phosphate buffered saline. Incubate for 30 minutes.
12. Rinse sections with a stream of phosphate buffered saline then wash again 3 x 5 minutes in phosphate buffered saline.
13. Incubate sections for 7 minutes in Diaminobenzidine tris, 100 mg 3,3 diminobenzidine, 180 ml water, 20 ml Tris buffer pH 7.6. Mix well and just before incubation, add 0.01 ml hydrogen peroxide (30%).
14. Rinse in distilled water 3 x 5 minutes.
15. Stain with Mayer's haematoxylin 30-60 seconds.
16. Wash in distilled water 3 – 5 minutes.
17. Put in tap water for 10 minutes.
18. Dehydrate through graded absolute ethanol ethanol 75%, 90%, 100% x 2.
19. Move through two rinses of xylene.
20. Mount samples.

Appendix F

Endocrinology publication

0013-7227/06/$15.00/0
Printed in U.S.A.

Endocrinology 147(9):4010–4021

doi: 10.1210/en.2006-1616

Calcitonin Plays a Critical Role in Regulating Skeletal Mineral Metabolism during Lactation

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The maternal skeleton rapidly demineralizes during lactation to provide calcium to milk, responding to the stimuli of estrogen deficiency and mammary-secreted PTH-related protein. We used calcitonin/calcitonin gene-related peptide-α (*Ctcgrp*) null mice to determine whether calcitonin also modulates lactational mineral metabolism. During 21 d of lactation, spine bone mineral content dropped 53.6% in *Ctcgrp* nulls *vs.* 23.6% in wild-type (WT) siblings ($P < 0.0002$). After weaning, bone mineral content returned fully to baseline in 18.1 d in *Ctcgrp* null *vs.* 13.1 d in WT ($P < 0.01$) mice. Daily treatment with salmon calcitonin from the onset of lactation normalized the losses in *Ctcgrp* null mice, whereas calcitonin gene-related peptide-α or vehicle was without effect. Compared with WT, *Ctcgrp* null mice had increased circulating levels of PTH and up-regulation of mammary gland PTH-related protein mRNA. In addition, lactation caused the *Ctcgrp* null skeleton to undergo more trabecular thinning and increased trabecular separation compared with WT. Our studies confirm that an important physiological role of calcitonin is to protect the maternal skeleton against excessive resorption and attendant fragility during lactation and reveal that the postweaning skeleton has the remarkable ability to rapidly recover even from losses of over 50% of skeletal mineral content. (*Endocrinology* 147: 4010–4021, 2006)

THE SKELETON FULFILLS several obvious and important roles; it protects the vital organs, it is the scaffold from which organs and tissues are hung, it enables locomotion on land in a biped or quadruped posture, and it houses the bone marrow. Common clinical concerns about the skeleton include traumatic fractures, fragility fractures from osteoporosis, and a multitude of disorders that crowd the marrow space. Far less well appreciated is the function of the skeleton to serve as a storehouse of minerals and alkali, even to the point of compromising its integrity and strength. Among the times of great demand for calcium and other minerals are lactation, egg laying, and antler formation (1, 2). In each of these time periods, the skeleton undergoes a significant, rapid, and reversible demineralization to provide mineral, respectively, to the neonate, egg shell, and antlers.

Clinical studies have demonstrated (by dual x-ray absorptiometry) that a woman will lose an average of 5–10% of trabecular mineral content during 6 months of near-exclusive lactation and then fully regain it within several months of weaning (reviewed in Refs. 1, 3, and 4). Depending upon a woman's peak bone mass, these losses during lactation are sufficient enough to temporarily reduce her bone density into the osteopenic or osteoporotic range. In contrast, mice and rats will lose approximately 30% of skeletal mineral content (by dual x-ray absorptiometry or by ash weight) during 3 wk of lactation and then fully restore it within 2–3 wk after weaning (1). The rapid and complete recovery from the large losses of skeletal mineral induced by lactation is a clinically important finding, because the adult skeleton normally recovers slowly and incompletely from insults induced by glucocorticoids, prolonged bed rest, menopause, GnRH analog treatment, and weightlessness (1, 5–10).

The mechanism by which the skeleton demineralizes during lactation is not fully understood (1, 11–13). Mammary tissue dramatically up-regulates the expression and release of PTH-related protein (PTHrP), which enters the mother's bloodstream to stimulate bone resorption (12, 14, 15) and, in so doing, mimics the actions of sustained high levels of PTH. At the same time, hyperprolactinemia and other factors suppress the release of GnRH within the hypothalamus, thereby suppressing in sequence the pituitary gonadotropins, ovaries, ovulation, and the sex steroids (especially estradiol) (16). In addition, low estradiol levels of lactation permit enhanced osteoclast-mediated bone resorption and, thereby, potentiate the effect of PTHrP on the skeleton (13).

Calcitonin was originally thought to be an important regulator of calcium and bone metabolism in adults (17), but evidence gathered over the years has largely forced the conclusion that it is a vestigial hormone in mammals (18). The effects of calcitonin to reduce osteoclast-mediated bone resorption (to treat osteoporosis or Paget's disease) or to enhance urinary calcium excretion (to treat hypercalcemia of malignancy) require pharmacological doses of the hormone and not physiological levels (19, 20). However, several investigators had originally proposed that calcitonin might protect the skeleton against excessive resorption during pregnancy and lactation (21–24). The experimental approach

First Published Online May 4, 2006

Abbreviations: BMC, Bone mineral content; BMD, bone mineral density; CaT1, calcium transport protein subtype 1; CGRPα, calcitonin gene-related peptide-α; μCT, microcomputed tomography; WT, wild type.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

to test this hypothesis had been to remove the thyroid glands from sheep, goats, or rats, to replace thyroid hormone, and to determine the effect of a full cycle of pregnancy and lactation on the maternal bone mineral content (BMC) or ash weight of these thyroidectomized *vs.* sham-operated animals (21–24). Although some investigators determined that these presumably calcitonin-deficient animals had reduced BMC at the time of weaning, other investigators found no such effect (21–24). It wasn't appreciated at the time that thyroidectomized animals were not devoid of calcitonin because of extrathyroidal sources of calcitonin in mammary tissue (25, 26), placenta (27, 28), and pituitary (29). Thyroidectomized women experience increases in circulating calcitonin during lactation and express normal levels of calcitonin in milk (26).

We have retested the hypothesis that physiological levels of calcitonin are required to protect the maternal skeleton against excessive resorption during pregnancy and lactation. To do this, we used an established murine model in which exons 3–5 of the calcitonin/calcitonin gene-related peptide-α gene (*Ctcgrp*) have been ablated (30). Calcitonin gene-related peptide-α (CGRPα) is an alternative splice product of the calcitonin gene, whereas a second gene produces CGRPβ. Therefore, *Ctcgrp* null mice completely lack calcitonin in all tissues but still produce CGRP through the CGRPβ gene. Using this model, we determined that ablation of the gene doubled the losses of maternal mineral content during lactation and that the effect was specific to loss of calcitonin and not CGRPα.

Materials and Methods

Animal husbandry

Ctcgrp null mice were obtained by targeted ablation of exons 3–5 of the murine gene and genotyped by PCR as previously described (30). The original strain was back-crossed into Black Swiss (Taconic, Germantown, NY) for at least six generations, and the colony was maintained by breeding heterozygous-deleted mice together. Virgin first-degree relative pairs of wild-type (WT) and *Ctcgrp* null females were selected for study after 10 wk of age (attainment of peak bone mass). Mice were mated overnight, and the presence of a vaginal mucus plug on the morning after mating marked gestational d 0.5. Normal gestation in these mice is 19 d. We planned to cull litters within 48 h of delivery if needed to maintain similar numbers of pups (8 ± 1) per mouse, and weaning was forced at 21 d by removing the pups from the mother. All mice were given a standard chow (1% calcium, 0.75% phosphorus) diet and water *ad lib*. All studies were performed with the previous approval of the Institutional Animal Care Committee of Memorial University of Newfoundland.

Reproductive cycles and data collection time points

These studies were completed according to an approximate 70-d reproductive cycle. Baseline scans were collected for 5–10 d before mating and subsequent pregnancy (prepregnancy interval). Pregnancy lasted 19 d, lactation lasted 21 d, and postweaning recovery was 21 d. The four main data collection points included prepregnancy, end of pregnancy (day before expected delivery), end of lactation (forced weaning at d 21 of lactation), and end of postweaning recovery of the skeleton (d 21 after weaning). Additional time points included start of lactation (24–48 h), early lactation (5–7 d), and mid-lactation (10–14 d).

BMC and bone mineral density (BMD)

BMC and BMD were measured using the PIXImus 2 Bone Densitometer (GE Lunar, Madison, WI) and analyzed with PIXImus software version 2.1. A standard phantom (fat 11.9% and BMC 0.063 g) was used to calibrate the PIXImus daily. Anesthesia was induced with isoflurane (Baxter Corp., Toronto, Ontario, Canada) and maintained with a single ip injection of either thiopental sodium (Abbot Laboratories, Toronto, Ontario, Canada) or a 5:1 combination of ketamine hydrochloride (Wyeth Canada, Guelph, Ontario, Canada) and xylazine (Bayer Inc., Toronto, Ontario, Canada). The anesthetized mice were immobilized prone on a plastic tray with the spine straightened, and to maintain reproducibility with less than 1% precision error, the head was excluded in all scans. In additional quality control studies, mice on d 18.5 of pregnancy were scanned immediately before and after a cesarean section in which the pups were removed; this determined that the fetal skeletons contributed 1% or less to the apparent maternal BMC or BMD and were, therefore, negligible. Total body (minus head) and regional (spine and hind limb) BMC and BMD measurements were obtained for each mouse, and the absolute values were normalized to the nonpregnant baseline measurements. In the initial studies to determine the pattern of BMC and BMD change (comprising data in Fig. 1), each mouse was scanned every 2 d throughout the entire reproductive cycle. In later studies in which tissue would be harvested at specific time points, the mice were scanned at the main time points to ensure that the expected BMC and BMD changes were occurring. There were a few anesthetic related deaths, but otherwise, the outcomes of the reproductive cycle (live pups and magnitude of BMC loss and recovery) were no different between dams that had been scanned every 2 d *vs.* those that had been scanned at the main time points. Pups that had received *in utero* exposure to x-rays were euthanized at weaning, whereas the dams were euthanized at the end of the postweaning recovery phase.

Chemical and hormone assays

Serial blood samples were taken from tail veins, whereas a cardiac puncture was done to obtain larger samples at the time that each mouse was euthanized. Urine was collected by allowing the mice to void into a clean, empty cage. Ionized calcium was measured on whole blood using a Chiron Diagnostics 634 Ca^{2+}/pH Analyzer (Chiron Diagnostics, East Walpole, MA). Urine calcium was measured using a colorimetric assay (Sigma-Aldrich, Oakville, Ontario, Canada). PTH was measured using a rat PTH 1–34 ELISA kit (Immutopics, San Clemente, CA). Osteocalcin was assessed using a two-site immunoradiometric assay (Immutopics). Prolactin was assessed using a RIA by Dr. A. F. Parlow (National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA). Estradiol was measured with an enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI). Plasma PTHrP was measured using a sensitive RIA with an antibody directed to an amino-terminal epitope on samples that had been collected in a cocktail of aprotinin and EDTA (31). Deoxypyridinoline was measured using the METRA DPD enzyme immunoassay kit (Quidel Corp., San Diego, CA). Urinary measurements of deoxypyridinoline and calcium were expressed relative to creatinine to correct for variations in urine concentration. Creatinine was measured using an alkaline picrate colorimetric assay (Diagnostic Chemicals Ltd., Charlottetown, Prince Edward Island, Canada).

Milk collection and analysis

On d 7 of lactation, anesthetized mice were injected with 1.2 U oxytocin (Hospira Healthcare Co., Montreal Quebec, Canada). After 1–2 min, milk was obtained using a self-designed pump consisting of a 1-ml pipette tip (Mandel Scientific Co. Inc., Guelph, Ontario, Canada) inserted into thick rubber tubing that in turn was connected to vacuum suction. The wide end of the pipette tip was placed over a nipple, and a gentle pumping action was used to obtain milk, which was then stored at −20 C until assayed. The milk was diluted 1:100 with distilled water and analyzed for calcium content using a Model 2380 Atomic Absorption Spectrophotometer (PerkinElmer, Norwalk, CT). Because much of the calcium content of milk is bound to proteins (32), and to control for differing milk concentrations, the protein content of milk was measured using the BCA Protein Assay Kit (Pierce, Rockford, IL). Final results are expressed as micrograms calcium per microgram protein.

Duodenal calcium absorption

We adapted the method of Fleet and colleagues (33) to measure duodenal calcium absorption *in vivo* during mid-lactation and in non-

pregnant mice. Food was removed from the cages 3 h before the experiment to empty the duodenum and jejunum. Under anesthesia, an abdominal incision was made and a closed duodenal loop was created by tying two ligatures, one at the pyloric juncture and the second one about 1.5 cm distal to the first one. Forty microliters of ^{45}Ca buffer (150 mM NaCl, 2 mm $CaCl_2$, 30 mM Tris-HCl, and 16 μCi ^{45}Ca/ml at pH 7.4) were injected into the closed loop (^{45}Ca was obtained from Promega/Fisher Scientific Ltd., Burlington, Ontario, Canada). After 10 min, the loop was inspected to ensure that no areas of ischemia were evident. The loop was excised, solubilized in Scintigest (Promega/Fisher), and then counted on a liquid scintillation counter. To measure the total ^{45}Ca activity administered, and to control for the effect of biological tissue to quench the apparent level of radioactivity, additional scintillation vials were prepared in which the same amount of ^{45}Ca and an identical length of nonradioactive duodenum were added. Efficiency of ^{45}Ca absorption was expressed as (1 − ^{45}Ca remaining in each loop/amount of ^{45}Ca injected into the loop) × 100.

Biomechanical analysis

Left hind limbs were stored at −20 C after first removing the soft tissues and then thawed to room temperature in normal saline before analysis. Cortical bone strength was assayed using an Instron Series 3340 electromechanical test instrument (Instron, Norwood, MA). In brief, the femur was immobilized at each end in a fixture, and a load cell with maximum capacity of 10 N of force was positioned 2–3 cm above the mid-shaft of the tibia. The force required to break the femur (failure) was recorded, as was the load-displacement curve from which other biomechanical parameters could be derived.

Rescue experiments

Beginning on the day of delivery and for 21 d of lactation, selected WT and *Ctcgrp* null mice received once daily im injections of 0.9% saline (vehicle), salmon calcitonin (Rhone-Poulenc Rorer, Collegeville, PA), or rat CGRPα (American Peptide Co., Sunnyvale, CA). Mice were briefly anesthetized with isoflurane to receive the injection. Bone densitometry was used every second day throughout the experiment to precisely determine the excursion of BMC.

Microcomputed tomography (μCT) analysis

Right hind limbs and lumbar spines were harvested, adjacent flesh was removed, and then the samples were fixed in paraformaldehyde for 18 h. The bones were then rinsed and stored in 10× PBS at room temperature until analysis. The quantitative and qualitative μCT analysis for each femur and for the first and second lumbar vertebrae was completed at the Centre for Bone and Periodontal Research at McGill University using the SkyScan 1072 (SkyScan, Aartselaar, Belgium), as previously described (34).

Mammary tissue collection and storage

Mammary glands were harvested after a rapid cervical dislocation during late pregnancy, mid-lactation, and late lactation. Lymph nodes were removed and mammary tissue was washed with water containing 0.1% diethylpyrocarbonate. Tissues were either snap-frozen in liquid nitrogen and then placed at −70 C or placed immediately in 10% formalin [37% formaldehyde, 10× PBS (pH 7.3), and distilled water].

RNA extraction and cDNA synthesis

RNA was extracted from whole mammary glands using an RNeasy Midi Kit (QIAGEN, Mississauga, Ontario, Canada). Approximately 100 g of mouse mammary gland yielded 0.6–1.3 μg RNA/μl solution. cDNA synthesis was carried out with the SuperScript III First-Strand Synthesis System (Invitrogen Canada Inc., Burlington, Ontario, Canada).

Real-time quantitative RT-PCR

cDNA synthesis was performed using the Superscript III First-Strand Synthesis System (Invitrogen) as previously described (35). Quantitative real-time RT-PCR data and analysis were obtained using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), also as previously described (35). For the calcitonin receptor, the calcium receptor, and the vitamin D receptor, TaqMan Gene Expression Assays were used that included predesigned primers and probes for optimal amplification (Applied Biosystems) along with TaqMan Universal PCR Master Mix (Applied Biosystems). All gene expression assays have a FAM reporter dye at the 5′ end of the TaqMan MGB probe and a nonfluorescent quencher at the 3′ end of the probe. Other gene-specific primers used for this study were as follows: for PTHrP, forward 5′-TCCACACAGCCGAAATCAGAGCTA-3′ and reverse 5′-TTCTCCTGTTCTCTGCGTTTCCCA-3′; for 1-α-hydroxylase, forward 5′-CCAGAGCGCTGTAGTTTCTCATCA-3′ and reverse 5′ATGAAGGTTTCTGTGTCAGGAGGG-3′; for calcium transport protein subtype 1 (CaT1), forward 5′-ATCGATGGCCCTGCGAACT-3′ and reverse 5′-CAGAGTAGAGGCCATCTTGTTGCTG-3′ (36); and for epithelial calcium channel (ECaC or CaT2), forward 5′-ATTGACGGACCTGCCAATTACAGAG-3′ and reverse 5′-GTGTTCAACCCGTAAGAACCAACGGTC-3′ (36). These primers were used along with SYBR Green PCR Master Mix (Applied Biosystems). The real-time PCR thermal cycler protocol included one cycle at 50 C for 2 min, one cycle at 95 C for 10 min, and 40 cycles of 95 C for 15 sec and 1 min at 60.0 C. For primers using SYBR Green PCR Master Mix, the real-time PCR program was the same but without the first cycle at 50 C for 2 min. All samples were analyzed in triplicate. Relative expression ratios were representative of the threshold cycle (the PCR cycle at which an increase in reporter fluorescence is above a baseline signal) normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and compared with the WT.

Statistical analysis

Data were analyzed using SYSTAT 5.2.1 for Macintosh (SYSTAT Inc., Evanston, IL). ANOVA was used for the initial analysis; a *post hoc* test (Tukey's) was used to determine which pairs of means differed significantly from each other. Real-time PCR results were analyzed by the $2^{-\Delta\Delta C}T$ method where the target and reference are amplified in separate wells (37). Two-tailed probabilities are reported, and all data are presented as mean ± SE.

Results

BMC during pregnancy, lactation, and recovery

At the baseline age of 10–12 wk, and consistent with an earlier report (30), *Ctcgrp* null mice had a BMC of 0.397 ± 0.02 g *vs.* 0.383 ± 0.02 g in WT ($P = 0.61$). At the level of the whole body, WT and *Ctcgrp* null mice gained a similar degree of BMC during 18 d of pregnancy, but *Ctcgrp* null mice lost approximately twice the amount of BMC as WT during 21 d of lactation (Fig. 1A). The difference was present from early in lactation; WT mice lost 4.5 ± 3.6 and 15.7 ± 3.7% of baseline BMC by 7 and 14 d of lactation, respectively, whereas *Ctcgrp* null mice lost 10.1 ± 3.1 and 29.6 ± 3.1% of baseline BMC at the same time points. Both genotypes restored their BMC to their respective baseline values after weaning (Fig. 1A), although full recovery took several more days for *Ctcgrp* null mice (Fig. 1B). Closer examination of the BMC excursion indicated that the lactational losses were much more profound in the trabecular-rich vertebral bodies, such that *Ctcgrp* null mice lost more than half of their baseline BMC in the spine (Fig. 1C). At the hind limb, which contains relatively more cortical bone than the vertebral bodies, the losses were proportionately less, but *Ctcgrp* null mice still lost twice the WT value (Fig. 1D). The losses were not a result of any difference in litter sizes, which did not require any culling and were 8.9 ± 0.9 in WT and 7.6 ± 1.2 in *Ctcgrp* null (P = not significant).

The enhanced skeletal demineralization during lactation indicates increased bone resorption in the *Ctcgrp* null mice.

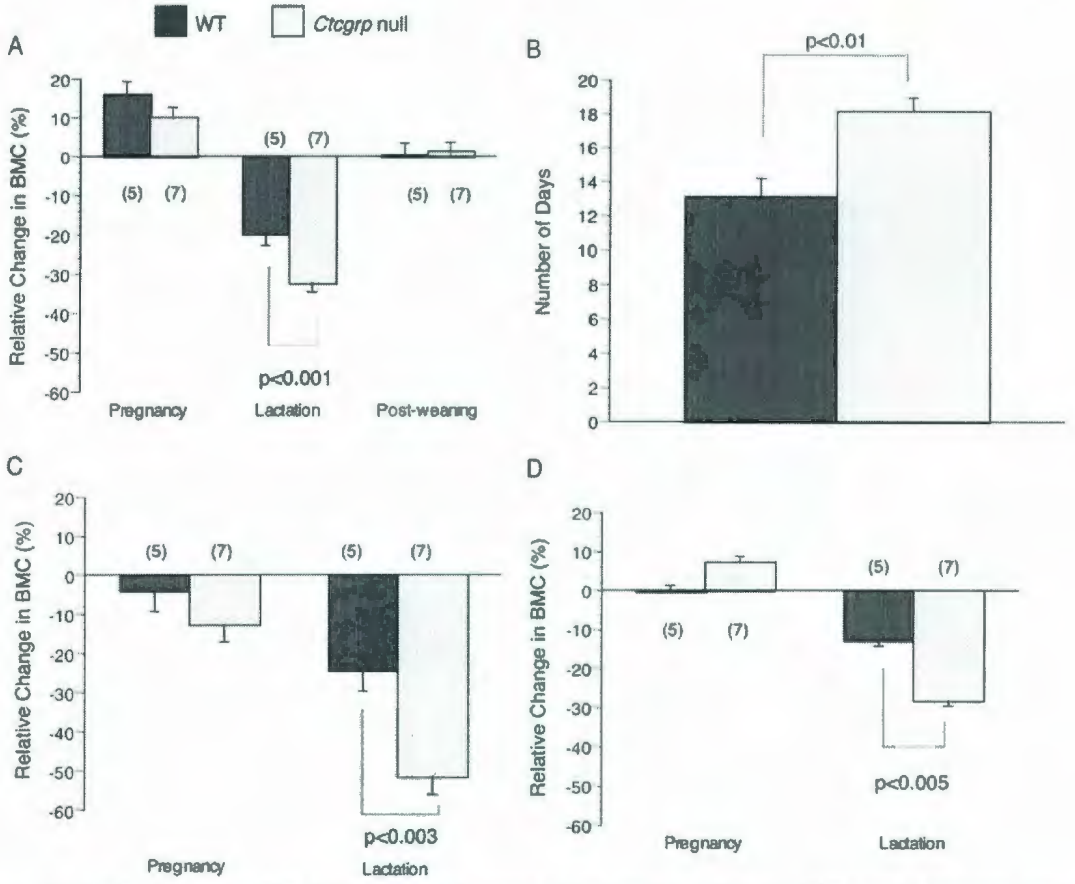

FIG. 1. *Ctcgrp* null mice lose more BMC during lactation but recover fully post weaning. A, Total-body BMC at end of pregnancy, lactation, and post weaning expressed as a percentage of prepregnancy baseline BMC; B, mean days taken to recover to baseline BMC post weaning; C, spine BMC; D, hind limb BMC. All values are mean ± SE. The *numbers in parentheses* indicate the numbers of mice studied.

Consistent with this, the urinary excretion of deoxypyridinoline/creatinine (a marker of bone resorption) was increased significantly in *Ctcgrp* null *vs.* WT mice during lactation (Fig. 2A). On the other hand, no difference was observed in the serum level of osteocalcin (a marker of bone formation) between *Ctcgrp* null and WT mice at any time point (Fig. 2B).

Mineral and hormones

We measured mineral and hormonal indices to examine possible mechanisms and consequences of the enhanced bone demineralization observed in *Ctcgrp* null mice. In our laboratory, WT mice in several inbred, outbred, and mixed strains maintain a normal ionized calcium during pregnancy and lactation and have suppressed levels of PTH during the same intervals compared with prepregnancy (not shown). Both WT and *Ctcgrp* null mice generally maintained a normal ionized calcium throughout pregnancy, lactation, and post weaning (Fig. 3A); however, *Ctcgrp* null mice were transiently hypercalcemic during the first 24–48 h postpartum at 1.40 ± 0.02 mmol/liter *vs.* 1.26 ± 0.02 mmol/liter in WT mice ($P < 0.001$). Urinary excretion of calcium normally decreases during lactation, likely because of the effect of PTHrP to enhance urinary calcium reabsorption. *Ctcgrp* null and WT mice both showed the same trend to decreased urinary calcium excretion during lactation but with no significant differences between the genotypes at any time point (Fig. 3B).

Serum PTH is normally suppressed to the lower end of the normal range during pregnancy and lactation, and this was observed in the WT mice (Fig. 3C). Although PTH was also reduced during pregnancy in *Ctcgrp* null mice, PTH became significantly elevated in *Ctcgrp* null mice during lactation and may have contributed to the enhanced demineralization observed in these mice (Fig. 3C). The elevation in PTH occurred during the interval of normocalcemia and not during the initial 24–48 h postpartum in which the ionized calcium was transiently elevated. In a separate analysis of serum

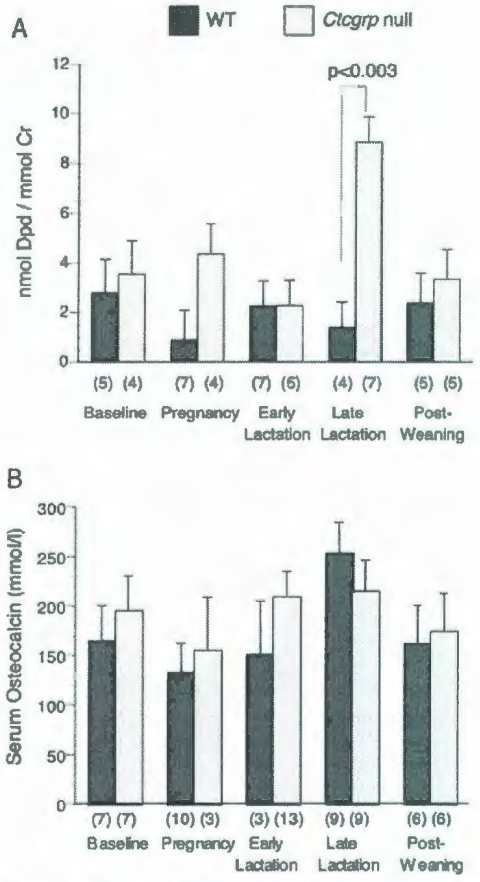

FIG. 2. Bone formation and resorption markers during the reproductive cycles. A, Urine deoxypyridinoline/creatinine (bone resorption marker) was elevated in *Ctcgrp* null mice *vs.* WT during lactation; B, serum osteocalcin (bone formation marker) showed no significant differences between genotypes at any time point. The *numbers in parentheses* indicate the numbers of mice studied.

obtained at 24–48 h (to correspond with the transient hypercalcemia), PTH levels were suppressed to the limit of detection (1.6 pg/ml) in both WT and *Ctcgrp* null mice (P = not significant). Estradiol is normally suppressed during lactation as a consequence of hyperprolactinemia, and low estradiol contributes to the skeletal resorption during this time period. We found that estradiol levels at mid-lactation were suppressed to the level of sensitivity of the estradiol assay (8 pg/ml) in all samples from both WT and *Ctcgrp* null mice (P = not significant). Prolactin levels (Fig. 3D) were also not significantly different between *Ctcgrp* null and WT mice at any time point.

We assayed duodenal ^{45}Ca absorption at mid-lactation in WT and *Ctcgrp* null mice to rule out impaired intestinal absorption as an indirect cause of the enhanced skeletal resorption in *Ctcgrp* null mice. Efficiency of absorption was greater than 80% at 10 min in both WT and *Ctcgrp* null mice and not significantly different from the nonpregnant WT value (Fig. 4A).

Given that the *Ctcgrp* null mice are resorbing more bone than WT but are not losing the excess calcium in urine and do not have impaired intestinal calcium absorption, we expected to find increased calcium content in milk. There are several challenges to measuring milk content in mice: milk content is highly variable within and between feeds (38, 39), much of the calcium is bound to proteins (32), and collecting milk is technically difficult. Despite these issues, we found an apparent (but not statistically significant) increase in milk calcium content corrected from the protein content (Fig. 4B).

PTHrP in lactating mammary tissue and circulation

Given the established importance of mammary-derived PTHrP to stimulate skeletal demineralization during lactation, we measured both mRNA and protein levels of this hormone. Within mammary tissue, PTHrP mRNA was significantly elevated as assessed by real-time quantitative RT-PCR (Fig. 5A), and the protein level was visibly increased by immunohistochemistry (Fig. 5B). Within the circulation, the plasma PTHrP level showed no significant difference during lactation compared with WT, with most values clustered near the assay detection limit of 2 pmol/liter (Fig. 5C).

Other calciotropic hormones and receptors within lactating mammary tissue

We also examined the expression of other calciotropic genes that are known to be expressed in mammary tissue, including the mRNAs for the calcium receptor, calcitonin receptor, 1-α-hydroxylase, and the vitamin D receptor. In addition, mRNAs for the epithelial calcium channels (CaT1 and CaT2) were also examined. There was no difference in the expression of any of these mRNAs between WT and *Ctcgrp* null mice, whereas CaT1 and CaT2 were not detected (data not shown).

Skeletal strength and microarchitecture

We also examined the consequences of enhanced demineralization during lactation on skeletal microarchitecture of the femur and lumbar vertebrae and on the strength of the tibia. μCT measurements demonstrated that *Ctcgrp* null mice had greater trabecular thickness and more closely spaced trabeculae at baseline compared with WT in both femora and lumbar vertebrae (Table 1). *Ctcgrp* null mice experienced more marked trabecular thinning and reached an equivalent level to WT at the end of lactation (Table 1). Trabecular spacing increased significantly in *Ctcgrp* null femora and approached the level of the WT at baseline, whereas trabecular spacing did not change significantly within vertebrae (Table 1). The μCT results also indicate a greater trabecular bone volume at baseline in *Ctcgrp* null mice *vs.* WT. Whereas WT trabecular bone volume increased significantly during lactation (a normal finding), the changes were blunted in *Ctcgrp* null bones at both the femora and vertebrae (Table 1).

Bone strength was assessed by the three-point bend test of the femur, a measurement that reflects cortical bone strength more than trabecular strength. Ultimate load required to

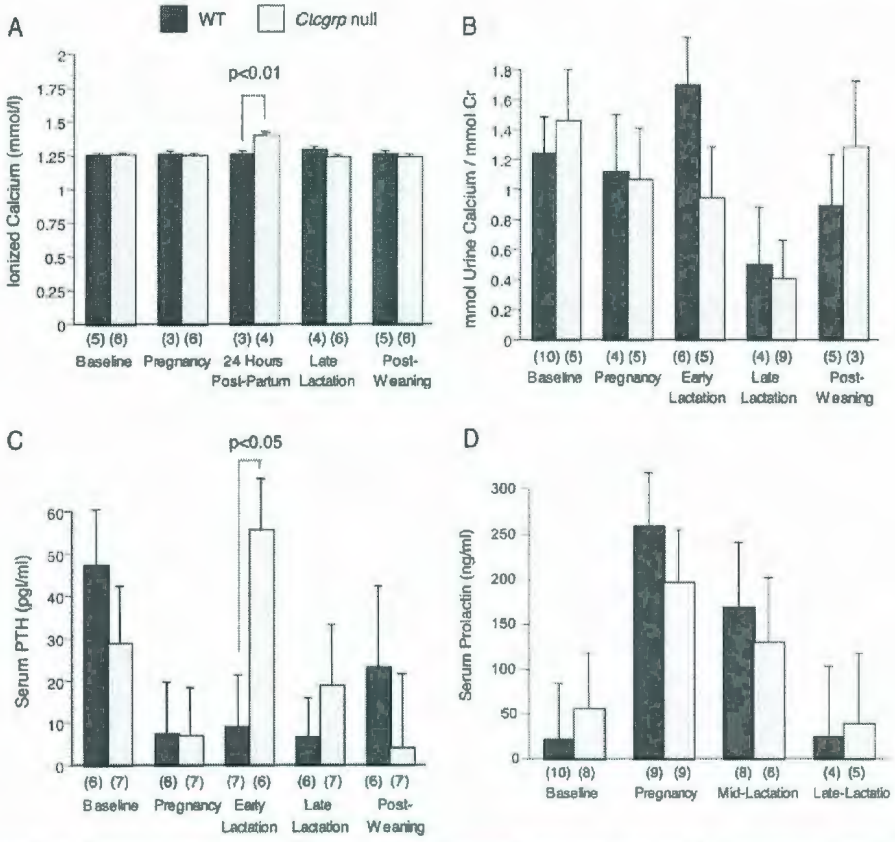

FIG. 3. Chemistry and hormone measurements. A, Ionized calcium remained constant throughout the reproductive cycle and showed no difference between *Ctcgrp* null and WT at the main time points apart from 24–48 h postpartum; B, urine calcium/creatinine showed a trend to decreased calcium excretion during lactation as expected, but no significant difference between genotypes; C, serum PTH was elevated during lactation in *Ctcgrp* null *vs.* WT but suppressed at 24–48 h postpartum (see text); D, serum prolactin was increased during pregnancy and early lactation but was no different between the genotypes. The *numbers in parentheses* indicate the numbers of mice studied.

cause breakage showed an apparent decrease from baseline to the end of lactation in both WT and *Ctcgrp* null mice but was not significantly different within or between groups (Table 2). Not included in the calculations were two *Ctcgrp* null femurs that were evidently fragile because they broke upon being placed into the device and thus could not be subjected to the three-point bend test. Additional intrinsic and extrinsic bone parameters are shown in Table 2 that suggest that cortical bone strength is not significantly different between WT and *Ctcgrp* null mice at any time point.

Rescue of skeletal phenotype during lactation

Lastly, we determined whether the enhanced skeletal losses during lactation were a consequence of absence of calcitonin, CGRPα, or both. Preliminary dose-finding experiments indicated that a mean dose of 10 IU salmon calcitonin daily would prevent the excessive losses of lactation in *Ctcgrp* null mice; larger doses reduced the normal losses during lactation, whereas 1 IU daily had minimal effect (not shown). Starting at the day of delivery and continuing for 21 d, WT and *Ctcgrp* null mice were treated with daily injections of calcitonin or vehicle, and several *Ctcgrp* null mice were treated with an equimolar dose (255 μg) of CGRPα. The mean losses of BMC observed in saline or CGRPα-treated mice were similar, showing enhanced losses approximately double that of WT (Fig. 6). In contrast, treatment with calcitonin resulted in lactational losses that were no different from those observed in saline-treated WT mice (Fig. 6). Calcitonin treatment was without visible effect in WT mice (Fig. 6).

Discussion

We have compared *Ctcgrp* null mice to their WT siblings to test the hypothesis that calcitonin protects the maternal skeleton from excessive resorption during pregnancy and lactation. We found that calcitonin was not required during pregnancy, which is an interval of net bone formation as

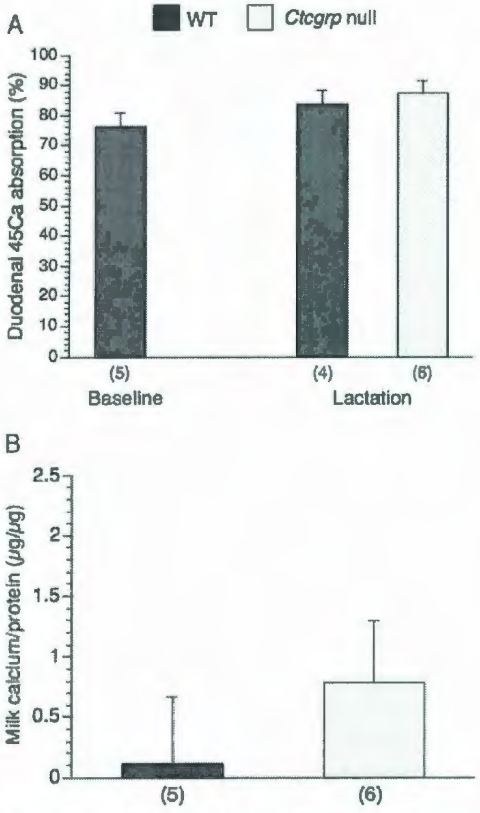

FIG. 4. Duodenal calcium absorption and milk calcium content. A, The efficiency of ^{45}Ca absorption at 10 min was no different between WT and *Ctcgrp* null during mid-lactation, and both showed a nonsignificant increase over the nonpregnant WT value; B, the calcium content of expressed milk, corrected for protein content, showed a higher mean value that was not statistically different from the WT value. *Numbers in parentheses* indicate the numbers of mice studied.

indicated by the rise in maternal BMC between mating and delivery. On the other hand, we found that calcitonin is required during lactation because *Ctcgrp* null mice lost about twice the amount of BMC during lactation as their WT relatives, with more profound losses at the trabecular-rich spine to the extent that over half of baseline BMC was lost. The enhanced losses were accompanied by increased circulating PTH and mammary expression of PTHrP, transient hypercalcemia in the first 24 h postpartum, increased bone resorption, normal intestinal calcium absorption, and apparently increased milk calcium content. Despite the dramatic losses in BMC, cortical bone strength was not significantly different between *Ctcgrp* null and WT mice at the end of lactation. The excessive resorption in *Ctcgrp* null mice was prevented by treatment with daily injections of calcitonin but not by treatment with CGRPα, confirming that the skeletal phenotype is specific to loss of calcitonin and not to loss of CGRPα. Finally, *Ctcgrp* null mice fully regained to their baseline BMC after weaning, demonstrating the skeleton's tremendous ability to repair itself from a greater than 50% deficit.

Physiological role for calcitonin during lactation

Our confirmation of a physiological role for calcitonin during lactation brings closure to a hypothesis that had been raised more than 30 yr ago but left unresolved (21–24). The inconsistent results among these early investigations likely reflected the confounding effects of thyroid ablation and thyroid hormone replacement, but especially the previously unrecognized fact that extrathyroidal sources of calcitonin existed (25, 27, 29). Only through the use of a gene ablation model has it become possible to examine the effect of complete absence of calcitonin on skeletal metabolism during pregnancy and lactation.

Ctcgrp null mice have enhanced skeletal resorption as demonstrated by the large drop in BMC from either prepregnancy or peak of pregnancy to end of lactation, the trabecular thinning and increased trabecular separation on μCT, and the elevated excretion of urinary deoxypyridinoline/creatinine. The transient hypercalcemia in the first 24 h postpartum may be a consequence of enhanced bone resorption flooding the maternal circulation with calcium at a time when milk production is not fully established; the ionized calcium was normal throughout the rest of lactation.

Calcitonin evidently plays a central role in modulating skeletal responsiveness during lactation, a role that may not be limited to its direct actions on osteoclasts. The presence of calcitonin and its receptor in mammary tissue (25) and pituitary (29, 40) indicates that there are several possible routes through which absence of calcitonin may lead to enhanced bone resorption in *Ctcgrp* null mice. We will consider the osteoclast, pituitary, and mammary tissue in turn.

Calcitonin, osteoclasts, and increased trabecular bone resorption

During lactation, enhanced osteoclast-mediated bone resorption is driven by a hormonal milieu in which circulating PTH is low, PTHrP is elevated, estradiol is low, and calcitonin is elevated (1, 3, 4). In *Ctcgrp* null mice, circulating PTH was elevated, mammary gland expression of PTHrP was up-regulated above normal, estradiol was low, and calcitonin was absent. The circulating level of PTHrP was not significantly increased in *Ctcgrp* null mice, but the assay sensitivity (2 pmol/liter) was likely insufficient to detect a true difference given that most values were clustered near the detection limit. PTH and PTHrP are equivalent in their ability to activate the PTH/PTHrP receptor on osteoblasts and communicate through the RANK-RANKL-OPG pathway to stimulate osteoclast-mediated bone resorption (41, 42); in contrast, calcitonin acts directly on osteoclasts to inhibit bone resorption (18). Thus, the mechanism for enhanced bone resorption in the *Ctcgrp* null mice may be simply a result of exaggerated stimulation of PTH/PTHrP receptor-mediated bone resorption by both PTH and PTHrP, coupled with absence of the effect of calcitonin to directly inhibit the activity of osteoclasts. In turn, pituitary and mammary tissue may

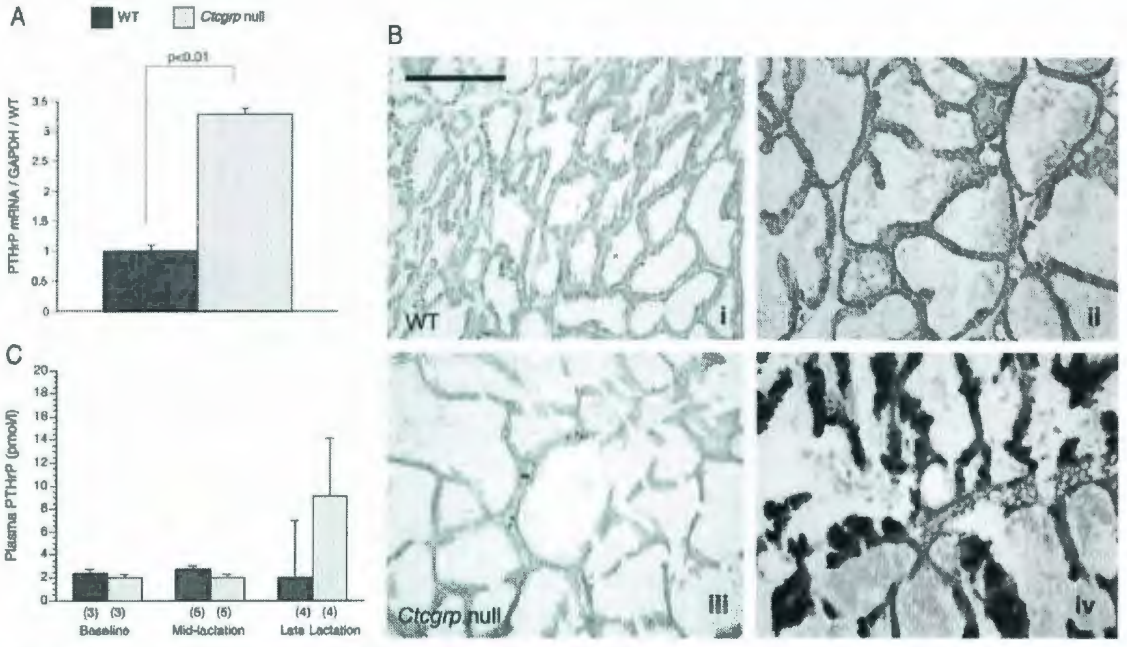

FIG. 5. Mammary gland production of PTHrP. A, Real-time quantitative RT-PCR demonstrated a significant increase in PTHrP mRNA of *Ctcgrp* null mammary tissue *vs.* WT; B, immunohistochemistry of mammary tissue using a rabbit anti-PTHrP antibody. PTHrP was significantly increased in *Ctcgrp* null mammary tissue (*scale bar*, 100 μm); C, plasma PTHrP showed no significant difference between WT and *Ctcgrp* null mice at any time point studied. The *numbers in parentheses* indicate the numbers of mice studied. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

both contribute to the up-regulation in PTH and PTHrP as described below.

Calcitonin, pituitary, and prolactin

With respect to the pituitary, both pharmacological administration of calcitonin (43) and targeted overexpression of calcitonin within pituitary lactotrophs have been found to inhibit production of prolactin (44). Therefore, loss of calcitonin might be expected to lead to enhanced synthesis and release of prolactin, which in turn would cause more profound ovarian suppression, lower estradiol, and enhanced bone resorption. Low estradiol (45) and high prolactin (15, 46–49) are factors that stimulate PTHrP production by mammary tissue *in vivo*. However, we found no significant difference in mean prolactin levels at any time point in *Ctcgrp* null *vs.* WT mice, and estradiol levels were undetectable in both genotypes during mid-lactation. Prolactin is released in pulsatile fashion during lactation (16), but it was not possible

TABLE 1. μCT analysis of trabecular bone parameters within femora and lumbar vertebrae

	BV (mm^3)	TV (mm^3)	BV/TV (%)	BS (mm^2)	SMI	Tb.Th. (μm)	Tb.Sp. (μm)
Femora							
Ctcgrp null baseline (n = 3)	0.43 ± 0.07^{a}	3.34 ± 0.22	12.8 ± 1.8^{a}	28.5 ± 4.3	1.83 ± 0.09^{a}	$72.7 \pm 5.3^{a,b,c}$	$277 \pm 26^{a,b}$
Ctcgrp null trough (n = 6)	0.42 ± 0.05	3.70 ± 0.16	11.6 ± 1.3^{b}	29.7 ± 3.0^{a}	1.79 ± 0.06^{b}	47.7 ± 3.6^{a}	$363 \pm 26^{a,c}$
WT baseline (n = 3)	0.19 ± 0.07^{a}	3.11 ± 0.22^{a}	$6.2 \pm 1.8^{a,b}$	15.0 ± 4.3^{a}	$2.21 \pm 0.08^{a,b,c}$	53.3 ± 5.1^{b}	413 ± 26^{b}
WT trough (n = 5)	0.34 ± 0.06	3.90 ± 0.17^{a}	8.8 ± 1.4	24.5 ± 3.3	1.92 ± 0.07^{c}	52.0 ± 3.9^{c}	495 ± 32^{c}
Vertebrae							
Ctcgrp null baseline (n = 3)	0.65 ± 0.05	2.71 ± 0.15^{a}	23.7 ± 1.1^{a}	$31.4 \pm 2.1^{a,b}$	1.11 ± 0.06	$54.5 \pm 2.0^{a,b}$	213 ± 12
Ctcgrp null trough (n = 6)	0.77 ± 0.04^{a}	2.93 ± 0.12^{b}	25.2 ± 0.9^{b}	$38.2 \pm 1.7^{b,c}$	1.10 ± 0.05^{a}	46.2 ± 1.6^{a}	201 ± 9
WT baseline (n = 3)	$0.56 \pm 0.05^{a,b}$	2.92 ± 0.13^{c}	$19.0 \pm 1.1^{a,b,c}$	$29.5 \pm 2.1^{c,d}$	1.31 ± 0.06^{a}	49.0 ± 2.0	225 ± 11
WT trough (n = 5)	0.82 ± 0.05^{b}	$3.54 \pm 0.12^{a,b,c}$	23.3 ± 1.1^{c}	$42.1 \pm 1.9^{a,d}$	1.15 ± 0.06	44.6 ± 1.8^{b}	217 ± 11

μCT analysis of femora and lumbar vertebrae was performed at baseline (nonpregnant) and at the end of lactation (trough). Within femora, *Ctcgrp* null mice showed higher trabecular bone volumes, greater trabecular thickness, and more densely packed trabeculae at baseline. *Ctcgrp* null mice underwent more pronounced thinning of trabeculae than WT by the end of lactation but did not increase trabecular bone volumes. Lumbar spine showed a similar pattern of changes. Lumbar spine values include results from both the first and second lumbar vertebrae. BS, Trabecular bone surface; BV, trabecular bone volume; BV/TV, trabecular bone volume relative to total volume; SMI, structure-model index; Tb.Sp., trabecular spacing; Tb.Th., trabecular thickness; TV, total volume including trabecular bone (but excluding cortical bone).

$^{a-d}$ *Shared letters within columns* indicate statistical significance of $P < 0.05$ (or lower) by Tukey analysis.

TABLE 2. Biomechanical parameters derived from the three-point bend test of femurs

	Ultimate load (g)	Stiffness (N/mm)	Toughness (J/mm^3)	Ultimate stress (mPA)	Work to failure (N × mm)	Moment of inertia (mm^4)
Ctcgrp null baseline (n = 3)	1678 ± 265	91 ± 14	3.56 ± 0.65	189 ± 27	2.26 ± 0.39	0.086 ± 0.001
Ctcgrp null trough (n = 6)	1430 ± 229	102 ± 14	3.46 ± 0.65	179 ± 27	2.17 ± 0.39	0.100 ± 0.001
WT baseline (n = 3)	1914 ± 266	132 ± 14	4.00 ± 0.65	208 ± 27	2.64 ± 0.39	0.088 ± 0.001
WT trough (n = 5)	1737 ± 226	96 ± 18	3.78 ± 0.80	143 ± 34	2.78 ± 0.48	0.120 ± 0.001

This test of cortical strength showed a nonsignificant trend for decreased bone strength between baseline (nonpregnant) and end of lactation (trough), with no significant difference between groups at any time point. Two *Ctcgrp* null femurs were clearly fragile because they broke upon being placed into the device but could not be included in the data analysis. *Numbers in parentheses in the first column* indicate the numbers of mice studied.

to collect multiple serum samples from each mouse to assess peak levels of this hormone. Our results indicate that the proposed role of calcitonin to regulate pituitary lactotrophs and systemic prolactin levels may not be a physiologically important pathway.

Calcitonin in mammary tissue

The presence of calcitonin and its receptor within mammary tissue (25) may indicate a local regulatory role for calcitonin in which the loss of it leads to enhanced PTHrP production. The calcium receptor has been shown to locally regulate the production of PTHrP within mammary tissue as well as the calcium and fluid content of milk (50). We did not observe a change in calcium receptor mRNA expression by real-time quantitative RT-PCR, and thus the pathway by which absence of calcitonin leads to stimulation of PTHrP may be independent of the calcium receptor.

The factors that control milk production are not well understood, although it is known that the calcium receptor, PTHrP, prolactin, and suckling all contribute to the regulation of the calcium and fluid content of milk (12, 50, 51). The numerous calcium regulatory genes that are expressed by lactating mammary tissue include the calcium receptor, calcitonin receptor, 1-α-hydroxylase, and vitamin D receptor

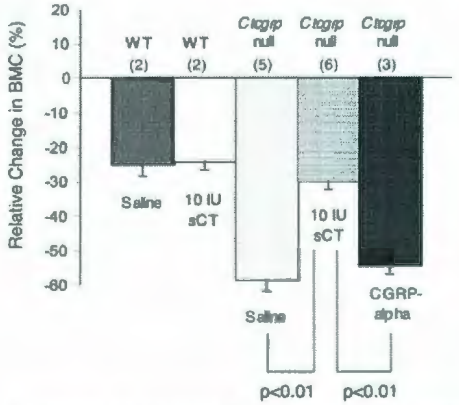

FIG. 6. Calcitonin but not CGRP rescues the *Ctcgrp* null phenotype. Salmon calcitonin administered im once daily throughout 21 d of lactation resulted in a loss of BMC in the *Ctcgrp* null mice that was similar to WT. *Ctcgrp* null mice that received saline (controls), as well as *Ctcgrp* null mice that received an equimolar dose of CGRPα, lost significantly more BMC than the *Ctcgrp* null mice receiving calcitonin treatment. The *numbers in parentheses* indicate the number of mice studied.

(25, 50, 52). We did not observe any alteration in the expression of these genes within mammary tissue; the only consistent finding was an elevation in PTHrP in mammary tissue that lacks calcitonin. No previous publications have examined CaT1 or CaT2 mRNA expression in lactating mammary tissue, although CaT1 protein had previously been detected by immunohistochemistry in nonlactating human breast tissue (53). We did not detect CaT1 or CaT2 mRNA in lactating mammary tissue of WT or *Ctcgrp* null lactating mice, which suggests that the epithelial calcium channels are not involved in milk production.

Secondary hyperparathyroidism in Ctcgrp null mice

Normally PTH is suppressed during lactation in humans and rodents (1), but we observed a significant elevation in PTH in *Ctcgrp* null mice during mid-lactation and to a lesser degree during late lactation. The cause of the elevated PTH may be an indirect consequence of loss of calcitonin. It has previously been demonstrated that larger litter sizes or a restricted calcium intake will induce maternal hypocalcemia and secondary hyperparathyroidism during lactation (54–56). In such cases, loss of calcium into milk may overwhelm the mother's ability to maintain her own blood calcium, resulting in hypocalcemia that in turn acts through the calcium receptor to induce PTH release. *Ctcgrp* null mice have low urinary calcium excretion, normal duodenal calcium absorption, and enhanced skeletal resorption; these factors should obviously lead to increased availability of calcium for milk production. The fact that PTH is elevated in *Ctcgrp* null mice may indicate that the loss of calcium into milk exceeds the requirement to maintain a normal blood calcium in the mother. Milk production by mammary tissue is controlled by both local and systemic factors (16, 57) and can induce hypocalcemia and tetany (milk fever) in lactating animals (58, 59); therefore, it is conceivable that loss of calcitonin within mammary tissue leads to an up-regulation in milk production that, in turn, challenges the ability of the mother to maintain her own serum calcium.

Confounding influences on bone strength

Ctcgrp null mice lost twice as much mineral content as WT siblings, and yet skeletal strength (as assessed by the three-point bend test) was not significantly different from WT at the end of lactation. This likely reflects several factors. First, the three-point bend test examines the strength of cortical bone, which undergoes very modest resorption during lactation (1, 60). Second, it is trabecular bone that is rapidly

mobilized during lactation because of its large surface area and vascularity, factors that enable it to be resorbed by osteoclasts on multiple surfaces (60, 61). Third, absence of calcitonin from conception leads to increased trabecular bone mass, as indicated in the original report (30) and in our study in which the *Ctcgrp* null skeleton had thicker and more densely packed trabeculae compared with WT. Thus, even a 50% drop in skeletal mass was well tolerated by the *Ctcgrp* null mice because the large losses resulted in a trabecular thickness that was equivalent to the level of WT mice at the end of lactation. Finally, pregnancy and lactation are normally associated with other changes in skeletal architecture (increased bone volume and expansion of periosteal surfaces while endosteal bone is being lost) that will offset the weakening effect that skeletal demineralization during lactation would be expected to induce (62). *Ctcgrp* null mice have greater trabecular and total bone volumes than WT at baseline; although these volumes increased substantially in WT mice, the changes were comparably modest in *Ctcgrp* null mice. The larger bone volumes of the *Ctcgrp* null mice likely compensated for the loss of trabecular bone during lactation.

Relevance to lactation in humans

A few case series examined male and female thyroidectomized individuals who received thyroid hormone replacement and reported conflicting results as to whether such patients developed a long-term deficit in bone mass or an increase in fracture risk relative to matched individuals with intact thyroids (63–67). No study has examined the bone mass of thyroidectomized women after a cycle of pregnancy and lactation, and of course such women would retain the ability to make calcitonin in pituitary and mammary tissue and would not be fully calcitonin deficient (26). Vertebral compression fractures occasionally occur during lactation in otherwise healthy young women, a condition termed osteoporosis of lactation (68). In such cases, excess bone resorption during lactation is presumed to have led to increased fragility that resolves after weaning. Our results raise the possibility that at least some of these cases might represent the human equivalent of absence of calcitonin or its receptor.

Skeletal recovery after weaning

In contrast to the demineralization that occurs during lactation, the factors that are required to stimulate recovery of the skeleton after weaning remain unknown. Our results indicate that calcitonin is not required for skeletal recovery to be achieved in full, although its absence may contribute to a delay in recovery. Calcitonin is required in the short term during lactation to prevent excessive resorption of the skeleton and likely attendant fragility, but it is not required in the long term because the skeleton recovers fully despite its absence and despite the increased mineral deficit induced by lactation. Resolving the mechanism by which the skeleton recovers remains a clinically important and relevant task because the skeleton is able to recover so completely and rapidly from the large losses of skeletal mineral content that are induced by lactation. *Ctcgrp* null mice may be an ideal model to examine skeletal recovery because the factors stimulating recovery are likely to be up-regulated more in these mice compared with normal.

Breast, brain, and bone circuit

In summary, evidence from recent studies has pointed to the central role of mammary tissue to stimulate skeletal resorption during lactation by producing PTHrP under the control of the calcium receptor and also to the central role of the pituitary to secrete prolactin and, thereby, suppress estradiol and stimulate PTHrP (the breast, brain, and bone circuit, Fig. 7). The data in our manuscript indicate that calcitonin plays a key role by modulating the extent of skeletal resorption during lactation. Absence of calcitonin in the systemic circulation will lead to increased osteoclast activity through loss of its direct effects via the calcitonin receptor to inhibit osteoclast number and function. However, because the calcitonin receptor is also expressed in mammary tissue and pituitary (two tissues that also produce calcitonin), it is possible that calcitonin acts at all three sites to modulate lactation: directly to inhibit osteoclasts, within pituitary to

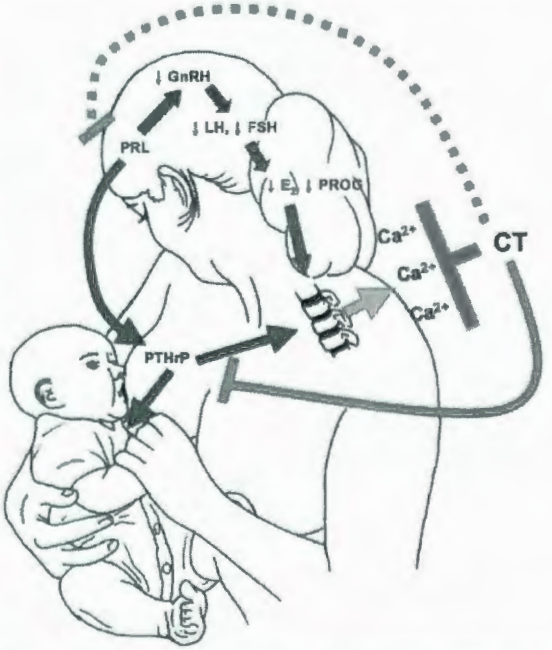

FIG. 7. Breast, brain, and bone circuit. High prolactin (PRL) levels during lactation stimulate PTHrP production by mammary tissue, and suppress the GnRH pulse center in the hypothalamus, in turn suppressing the pituitary gonadotropins (LH and FSH) and then the ovaries [ovulation, estradiol (E_2), and progesterone (PROG) production]. Low estradiol and mammary-derived PTHrP act synergistically to enhance osteoclast-mediated bone resorption. The presence of calcitonin (CT) and its receptor in mammary tissue, pituitary, and bone (*red lines*) allows for at least three pathways by which calcitonin could modulate BMC losses during lactation. Our observation that serum prolactin levels were no different between *Ctcgrp* null and WT indicates that the postulated role of calcitonin to inhibit pituitary lactotrophs (*dashed red line*) may not be physiologically important.

inhibit prolactin release, and within mammary tissue to modulate PTHrP or milk production. Additional work is needed to determine the relative importance of these three physiological loops.

Acknowledgments

Received December 19, 2005. Accepted April 21, 2006.

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This work was supported by an operating and equipment grant (to C.S.K.), a 5-yr Scholarship/New Investigator Award (to C.S.K.), and a doctoral research award (to J.P.W.), all from the Canadian Institutes of Health Research.

Results from this work were presented in part at the 2003 American Society for Bone and Mineral Research annual conference for which J.P.W. received a Young Investigator Award and at the Canadian Society for Endocrinology and Metabolism conferences for which J.P.W. received the Antoni Nalecz Award, the Harold Copp Award for best abstract, and two travel awards.

The authors have no conflicts of interest to disclose

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Appendix G

American Society for Bone and Mineral Research Abstract

Session:	Growth Factors and Cytokines: Calcitonin and Related Peptides
Presentation Number:	Sa194
Title:	Lack of Calcitonin Accentuates Bone Loss During Lactation by Enhanced Osteoclast Formation and Reduced Osteoblast Formation
Category:	F. Calciotropic and Phosphotropic Hormones and Mineral Metabolism
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Lactation in mice induces substantial reductions in bone mineral content (BMC, assessed by DXA) that are reversed rapidly after weaning. *Ctcgrp*-null mice, with a global deletion of the gene encoding calcitonin and calcitonin gene-related peptide, suffer a 55% reduction in BMC during lactation (versus 25% in wild type [WT] mice) followed by complete restoration post-weaning. *Ctcgrp*-null mice also demonstrate upregulated mammary gland PTHrP and serum PTH during lactation, which may increase osteoclast-mediated bone resorption during lactation even further above normal. To assess this, we performed histomorphometry in WT and *Ctcgrp*-null mice during lactation and weaning. Pairs of sister WT and *Ctcgrp*-null mice were sacrificed at day 7 of lactation and at weaning (day 21 after parturition) and histomorphometry was carried out on toluidine blue stained undecalcified sections of vertebrae and tibiae.

During lactation, both WT and *Ctcgrp*-null mice demonstrate reduced trabecular thickness and increased trabecular separation, compared to non-pregnant mice of the same genotype, as well as high osteoclast and osteoid surfaces (OcS/BS and OS/BS). In lactating *Ctcgrp*-null vertebrae and tibiae, OcS/BS was more than double that of WT (vertebrae: 13.9 ± 2.2 vs 4.9 ± 0.6%, $p<0.003$; tibiae: 24.5 ± 4.3 vs 9.6% ± 0.5, $p<0.01$). In contrast, osteoblast parameters were halved in *Ctcgrp*-null vertebrae and tibiae compared to WT (vertebral osteoblast surface [ObS/BS]: 10.7 ± 2.0 vs 20.4 ± 2.9%, $p < 0.03$; OS/BS: 11.7 ± 2.3 vs 21.0 ± 3.0, $p < 0.04$). Large osteocytic lacunae consistent with osteocytic osteolysis were evident in both genotypes.

At weaning, the very high OcS/BS persisted in *Ctcgrp*-null mice. However, between day 7 and 21 ObS/BS quadrupled in *Ctcgrp*-null to 40.0 ± 4.8%, and doubled in WT to 46 ± 2.9%; similar changes were observed in osteoblast number and OS/BS.

Thus, we have confirmed upregulated osteoclast-mediated bone resorption and osteocytic osteolysis during normal lactation. Absence of calcitonin led to a greater increase in OcS/BS, consistent with elevated circulating PTH, but also led to a milder increase in ObS/BS vs WT. The sum of these differences may explain the more substantial bone loss in lactating *Ctcgrp*-null mice. Moreover, *Ctcgrp*-null mice retain the ability to upregulate osteoblast generation to a maximal level at the onset of weaning. This suggests that calcitonin normally dampens the effect of lactation on osteoclasts but not on osteocytic osteolysis, yet enhances the effect of lactation on osteoblasts, and is not required for skeletal recovery after lactation.