THE ROLE OF DIETARY VITAMIN D AND CALCIUM IN DETERMINING BONE HEALTH AND STRENGTH

A THESIS SUBMITTED IN TOTAL FULFILMENT OF THE REQUIREMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

Alice Ming Chin Lee, B. Health Sc. (Hons.)

Discipline of Physiology,

School of Medical Sciences,

The University of Adelaide

South Australia

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ABSTRACT

Adequate dietary vitamin D and calcium intake have shown to be important in regulating skeletal development and bone mineralization. Vitamin D insufficiency is associated with increased fracture risk suggesting that a minimum 25-hydroxyvitamin D (25D) production in bone may be essential for maintaining a healthy skeleton. However, the required level of vitamin D to maintain/improve bone quality is still undetermined. This thesis investigates the regulation of dietary vitamin D on bone in young adult rats as well as the interaction between vitamin D requirement and dietary calcium intake on bone structure and the mechanical measures of bone quality in aged rats. Bone mineral content in trabecular and cortical bones and a number of biochemical factors known to regulate renal metabolism of 1,25D hydroxyvitamin D3 (1,25D) such as PTH, calcium, 25D and 1,25D were examined. Enzymes responsible for the production of 1,25D, 25-hydroxyvitamin D3-1a-hydroxylase (CYP27B1) and the catabolism of 1,25D (25-hydroxyvitamin D-24-hydroxylase (CYP24)) mRNA expression in the kidney was also studied. Furthermore, bone mechanical quality was determined using 3-point bending on rat tibia with the aim to validate mechanical testing as well as determining the effects of varying levels of dietary vitamin D and calcium on bone strength.

We have previously shown that serum 25D levels, which represents the level of vitamin D status is a strong determinant of bone volume. Despite that vitamin D deficiency results in trabecular bone loss in the femur and vertebrae, further cortical bone analysis demonstrated that cortical bone volume, bone mineral distribution and strength was preserved in short term vitamin D deficiency suggesting that the effect of vitamin D deficiency in young adult rats varies between trabecular and cortical regions.

To further understand the reported effects on low dietary calcium induced bone loss, mRNA expressions of the renal enzymes were examined. High renal CYP27B1 mRNA expressions and

serum 1,25D levels in long term low dietary calcium animals suggested that the effects of bone loss may be due to 25D metabolism leading to the reduction in vitamin D status. CYP24 and other liver enzymes were not regulated by the low calcium diet.

We have reported that circulating levels of serum 25D are greater in animals fed a diet containing high levels of calcium. Thus, the effects of a high calcium diet to protect against bone loss may be due to the subsequent effects on the maintenance of circulating 25D levels. μ-CT analysis demonstrated that both femoral and tibial cortical bone volume as well as trabecular bone volume is higher in animals that are fed high calcium and vitamin D diet. Furthermore, 3-point bending demonstrated the greatest maximum load to failure was achieved in the same dietary group. Cortical bone volume and the sagittal loading are both strong determinants of ultimate load suggesting that mechanical forces and bone mineral content are crucial in maintaining the quality and function of bone strength. In addition, these results have validated our mechanical testing suggesting that bone strength is affected despite the subtle changes in cortical bone volume which may be the result of ovariectomy or dietary changes.

The studies of this thesis reveal a complex interaction between dietary calcium and vitamin D, and show that physiological changes in biochemical factors can affect structure and strength in different regions of bone. More importantly, it also demonstrate the optimal levels of dietary calcium and vitamin D that are required to prevent the development of osteoporosis

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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ACKNOWLEDGEMENTS

First I would like to thank my supervisors Dr. Peter O'Loughlin, Dr. Paul Anderson and Professor Howard Morris, for all the guidance and support they have given me and the time that they have invested in me. Peter, thank you for the inspiring talks during the difficult years of my phD, I still remember you told me that after I have finished my phD I will feel a sense of personal achievement more than just a triumphant degree. Paul, you are the greatest encouragement of all times, your understanding, support and patience have kept me going thru the years. Thank you for being a compassionate supervisor and you never gave up on me. Howard, thank you for your expert advice on presentations, papers and your continuous encouragement.

Special thanks to Dr. Mark Forwood and Dr. Roland Steck for teaching me a new technique and assistance in the project. Without your assistance I would have not have a complete project. Roland,thank you for answering all my questions and allowing me to your equipment and big thanks to the people in Institute of Health Biomedical Innovation (IHBI) for their help with my work when I was over there in Queensland. Thanks must also go to Dr. Phil Salmon for sitting down in front of the computer with me for hours and solving my problems.

Big thank you to all members of the Endocrine Research Laboratory, IMVS for their friendship and assistance over the years. In particular, thanks to Rebecca Sawyer, Juliette Tyson and Sarah Anderson for listening to my problems and complaints and deal with my hyperventilation during presentation times. Without you guys I wouldn't have made it thru those difficult times. In addition, I would like to thank the members in the Bone Growth and Repair group in Uni SA for their help and support, especially Rosa, Chia-Ming, Kristen, and Tristan. I forever treasure your friendship

Finally, I would like to thank Mum, Dad, my brother Cobden and his wife Siew Seen for your mental and financial support and encouragement throughout my years of study. I would especially like to thank you Hoang for your support and encouragement for and listen to my complaints days on end and put up with my stressful behavior. Without you guys I wouldn't be here where I am right now.

CHAPTER 1

1.1 INTRODUCTION

Vitamin D is a key hormone involved in calcium homeostasis. It is particularly important in regulating skeletal development and bone mineralization both indirectly by stimulating calcium absorption in the intestine and directly by its action on bone formation and bone resorption. Osteomalacia and osteoporosis are bone diseases characterized by low bone mineral content. While frank vitamin D deficiency leads to osteomalacia, it is hypothesised that a moderate depletion of vitamin D is a risk factor for the development of osteoporosis. However, the level of vitamin D required to prevent osteoporosis is controversial and is likely to be influenced by dietary calcium intake and age.

Vitamin D, largely originating from the conversion of 7-dehydrocholesterol in the skin when exposed to UV light from the sun is metabolized in the liver to 25-hydroxyvitamin D (25D) which is the major vitamin D metabolite in the circulation. When required, such as during periods of hypocalcemia, the metabolism of 25D occurs in the kidney to form the active hormone 1,25 dihydroxyvitamin D (1,25D). 1,25D mediates the majority of its actions via binding to vitamin D receptor (VDR) which is located in a number of cells and tissues throughout the body. In addition to its well described roles in the regulation of intestinal calcium absorption, 1,25D has been shown to be important in the process of both bone mineralization and resorption [1].

Vitamin D insufficiency, clinically defined by serum levels of 25D below 60 nmol/L is associated with increased fracture risk [2]. Clinical observations have demonstrated a significant reduction in hip fractures in institutionalised elderly with vitamin D and calcium supplementation [3]. Various other meta-analyses have demonstrated a reduction in the risk of falling in patients that were given a dose of 700IU to 1000 IU vitamin D per day [4-6].

As elderly hip fracture patients appeared to have decreased serum 25D levels rather than 1,25D levels [2], the findings suggest that a minimum 25D production in the bone may be essential for maintaining a healthy skeleton. Previous studies in our laboratory have demonstrated the occurrence of bone loss consistent with osteoporosis at moderately insufficient levels of 25D and osteomalacia at severe vitamin D deficiency. The data from this study suggest that a circulating level of 25D at 80 nmol/L is required to prevent the development of osteopenia in rats fed an adequate level of dietary calcium.

1.2 AIMS

The thesis aims to:

Aim 1: Investigate the effects of short term (3 months) vitamin D deficiency on cortical bone structure and strength in young male rats.

Aim 2: Determine in aged female rats whether there is an interaction between vitamin D status and dietary calcium intake for the maintenance of normal skeletal structure

Aim 3: Determine if there is interdependence between low vitamin D status, dietary calcium restriction and osteopenia on bone strength in aged female rats.

1.3 BONE BIOLOGY

1.3.1 Functions of the skeleton

Bones provide the body with essential structural support and act as levers for mechanical movement. They function to move, support and protect various parts of the body. They also act as a reserve of calcium and phosphorus. Bone is complicated but well-designed organ system responding to mechanical and physiological stimuli [7].

1.3.2 Composition of bone

The organic matrix of bone is composed of a protein scaffold of collagen which holds a ground substance made up of proteoglycans - complexes of carbohydrate and protein [8]. The collagen confers tensile strength to the connective tissue, while the ground substance binds substantial amounts of water. Cells in the connective tissue synthesise the collagen fibres and the ground substance. Bone differs from other types of connective tissue in that the organic matrix is calcified so that bone contains about 70% bone mineral in the form of hydroxyapatite [9].

1.3.3 Structure of bone

The basic unit of bone is the osteon (Harversian system) which is a hollow, laminated rod of collagen and calcium apatite. The hollow core is a nutrient channel, called the Harversian canal. Within the shaft of long bone, many of these osteons are bundled together in parallel, forming Harversian systems that form a mesh work of cancellous bone.

1.3.4 Cortical bone

Cortical bone plays an important role in fracture prevention [10] and seems to be responsible for most of the strength of whole bones [10-12]. Cortical bone is needed for long bones and is levers

for loading in locomotion [13]. It has a stiff structure which provides resistance against bending or torsion. There is high proportion of cell population completely surrounded by bone matrix in cortical bone [14]. Cells at the periosteal surface affect size and shape of the bone while cells at the endocortical surface mainly affect the thickness and position of the cortex [15]. Slight periosteal apposition can result in significant increase in the resistance of bone, which can be defined by mechanical loading environment.

1.3.5 Trabecular bone

The basic unit of trabecular bone is the trabeculum. Trabecular bone is a spongy-like lattice of bone encased by the cortical shell [16]. Cells of the trabecular bone are situated between lamellae on the surface of the trabeculae, thus they are directly influenced by marrow cells [14]. Trabecular bone is less stiff than cortical bone, it has the capability to deform and thus absorb energy [17]. Bone volume fraction, trabecular number, thickness and separation, form of plate or rod in architecture, connectivity density and degree of anisotropy that affects the strength of trabecular bone [18].

1.4 CELLS OF BONES

Bone is made up of four distinct cell types: the bone forming cell- the osteoblasts, the mature osteoblast- the osteocytes and the bone resorbing cell- the osteoclasts. Osteoblasts and osteocytes are all derived from a non-hematopoietic mesenchymal tissue whilst osteoclasts are differentiated from monocyte/macrophage lineage [19]. Osteoblasts are bone-building cells which synthesise and secrete vital components required for building the matrix of bone tissue [20]. Such components include various organic components such as osteocalcin, proteoglycans, as well as collagen fibres. Furthermore, osteoblasts initiate the process of calcification through the action of some of these bone matrix molecules. Osteocytes are the main bone cells in bone tissue, and they are the mature

osteoblasts entrapped within the matrix secretions. Although osteocytes do not secrete matrix materials like osteoblasts, they do play an integral role in maintaining the daily cellular activities of bone tissue, such as mechanical sensing through their long and interconnected cellular processes. Osteoclasts are of hematopoietic origin, resulting from the fusion of up to 50 monocyte-like cells [21]. Osteoclasts are responsible for the resorbing or degrading of the calcified bone or cartilage tissues required during bone growth, physiological bone remodelling, fracture repair or osteolytic diseases. Situated in the plasma membrane, these cells have distinct ruffles on the side where they are closest to the surface of the bone. Within these ruffles the osteoclasts release lysosomal enzymes and acids which digest the bone.

1.4.1 *The Osteoclast*

1.4.1.1 Osteoclast origin

Osteoclasts are terminally differentiated myeloid cells that are uniquely adapted to remove mineralized bone matrix. These cells have distinct morphologic and phenotypic characteristics such as its multinuclearity, expression of tartrate-resistant acid phosphatase (TRAP) and the calcitonin receptor [22]. A hematopoietic stem cell that is common to lymphocytes, red blood cells, platelets, granulocytes and mononuclear phagocytes, progress through the colony-forming unit for granulocytes and macrophages and differentiate into osteoclasts [23]. It is evident from chicken-quail chimera experiments [24], parabiosis experiments[25, 26], and marrow transplantation studies in osteopetrotic animals[25, 27] that osteoclasts are derived from circulating mononuclear precursors in hematopoietic tissues.

Osteoclasts are thought to differentiate from common mononuclear phagocytic precursor cells with macrophages. The hematopoietic stem cells (HSC) give rise to circulating mononuclear cells, termed colony forming unit granulocyte/macrophage (CFU-GM), or osteoclast precursors. CFU-GM then proliferates under the stimulation of macrophage/monocyte-colony forming factor (M-

CSF), followed by differentiation with the stimulation of M-CSF and Receptor activator of nuclear factor kappa B ligand (RANKL) to become perfusion osteoclasts (preosteoclasts), which express tartrate-resistant acid phosphatase (TRAP) and calcitonin receptor. Pre-osteoclasts will then fuse to become multinucleated cells under the stimulation of M-CSF and RANKL. However, the multinucleated osteoclasts are not functional due to lack of ruffled membrane for bone resorption, until further stimulation of RANKL to stimulate ruffled border formation [28, 29]

1.4.1.2 Osteoclast structure

Osteoclast is characterised by multiple nuclei and a cytoplasm with a homogeneous foamy appearance due to a high concentration of vesicles and vacuoles. At sites of active bone resorption, the osteoclast forms a specialized cell membrane or ruffled border that touches the surface of the bone tissue. The ruffled border, which increases surface area at the bone interface facilitating the removal of the bony matrix, is a morphologic characteristic of an active osteoclast [30]. The mineral ions are absorbed into small vesicles which move across the cell from the resorption surface to the extra-cellular surface where they are released to the extracellular fluid, thus increasing levels of the ions in blood. The clear zone surrounding the ruffle border contains bundles of actin-like filaments. It functions in osteoclast attachment to bone that result in the formation of a resorptive hemivacuole [31, 32]. Osteoclasts are also known to be acid phosphatase-positive. Its TRAP positive characteristics has been widely used as a histochemical marker for the identification of osteoclasts [33].

1.4.1.3 Osteoclast function

A morphological evaluation of osteoclastic bone resorption was established in 1984 by several groups [34, 35]. The osteoclast is required for the maintenance of sites of micro fracturing in the skeleton [36, 37]. In order for osteoclasts to resorb bone they attach to the bone surface and assume

a polarized morphology. The mononuclear precursors fuse to form the polykaryon at the target site which attaches to the mineralised bone matrix. After the formation of the ruffled border which is encircled by the sealing zone, acid and acidic collagenolytic enzymes are released into the space enclosed by the matrix, sealing zone and ruffled border. The fusion of the acidic vesicles with the ruffled border and an accumulation of the proton concentration result in a decrease in pH which dissolves the mineral phase [23]. The matrix is then degraded by lysosomal proteinases such as cathepsin K, the major matrix-degrading enzyme [38] and cathepsin D,B, and L [39]. Osteoclasts secrete both H+ ions and the cathepsin K which are important for osteoclastic bone resorption. Blair and co-workers (1989) and Vannanen and co-workers (1990) were the first who showed that it was vacuolar type proton ATPase in osteoclasts that was responsible for mineral dissolution. Protons were pumped into the resorption lacuna using vacuolar H+ATPase localised in the ruffled border of osteoclasts so that osteoclasts are capable of generating acidosis to cause mineral dissolution necessary for bone resorption [40]. Cathepsin K, which has high proteolytic activity and localises primarily in osteoclasts, is one of the few extracellular proteolytic enzymes capable of degrading collagen. Under low pH, cathepsin K exerts autocatalytic activation and collagenolytic activity [41, 42]. Inhibition of cathepsin K *in vitro* and *in vivo* inhibits bone resorption, and resultedinincreased trabecular number and thickness, which is a characteristic of osteopetrosis [43, 44]. Conversely, over expression of cathepsin K in mice resulted in thinning of the metaphyseal bone. Consequently, inhibitors of cathepsin K have been developed and trialed in rats and monkeys in the aim to treat postmenopausal osteoporosis in humans [45-48]

1.4.2 *The Osteoblasts*

1.4.2.1 Osteoblast origin

Osteoblasts arise from common progenitors with chondrocytes, muscle and adipocytes, and various hormones and local factors regulate their differentiation. These progenitors acquire specific

phenotypes depending on their maturation during differentiation. Osteoblasts express various phenotypic markers such as high alkaline phosphatase activity and synthesize collagenous and noncollagenous bone matrix proteins including osteocalcin [49]. Classical embryology has established that two different embryonic lineages, neural crest and mesoderm, form the early skeleton [50, 51]. The branchial arch derivatives of the craniofacial skeleton originate from neural crest, whereas the axial skeleton, ribs, appendicular skeletons, and the skull base arise from mesoderm. Among the skeletal tissues formed by the mesoderm, the axial skeleton originates from the sclerotome, and the appendicular skeleton arises from the lateral plate mesoderm.

In vivo and in vitro experiments have demonstrated the presence of osteoprogenitors in both bones and extraskeletal tissues in the postnatal state. In bone tissues, osteoprogenitors are present in bone marrow and the periosteum. Studies [52-54] have proved that osteoprogenitors are present in bone marrow and showed that bone marrow cells from in vitro cultures of marrow cells are able to from osteogenic tissues when cultured in vivo within diffusion chambers. They have also demonstrated that the single cell-derived fibroblastic colonies, termed CFU-F (colony forming units-fibroblastic) [54], retained osteogenic potential in various in vivo and in vitro experiments [55]. Other groups have also demonstrated that bone marrow cells contained mesenchymal progenitors, which can differentiate into osteogenic, chondrogenic, and adipogenic lineage cells [56-59]. Periosteum or periosteum- derived cells generate bone nodules in in vitro cultures [60].

1.4.2.2 Osteoblast structure

Differentiated, non-proliferating osteoblasts are characterized by an active synthesis of bone matrix proteins such as Type 1 collagen, proteoglycans, and osteocalcin [61-63]. The more mature cells immediately adjacent to the bone have little glycogen, while immature osteoblasts are glycogen-rich. Mature and immature osteoblastic cells, besides forming collagen fibrils are able to phagocytoze collagen fibers and mineralized bone matrix. The high amount of actin in osteoblasts

may be correlated with the synthesis and transport of new matrix components. Cytoplasm of the osteoblasts is basophilic largely due to the presence of a large amount of endoplasmic reticulum, golgi apparatus and a large spherical nucleus. Active osteoblasts synthesize and stain positively for type 1 collagen and alkaline phosphatase.

1.4.2.3 Osteoblast function

The osteoblast is the primary target cell in hormonal activation of the osteoclast. Osteoblasts were thought to possess specific surface receptors for PTH and PTH-related protein and nuclear receptors for 1,25D [64]. Osteoblastic cells comprise a diverse population of cells that include immature osteoblast lineage cells, differentiating and mature matrix producing osteoblasts. Phenotypic osteoblast heterogeneity is associated with cellular differentiation in vitro. Mice absent in osteoblasts are also deficient in osteoclasts. However, conditional depletion of mature osteoblasts in vivo only ablates bone formation while osteoclastic bone resorption persists [65]. These data suggest that immature osteoblasts direct osteoclastogenesis while mature osteoblasts perform the matrix production and mineralisation functions. Osteoblasts develop from pluripotent mesenchymal stem cells that have the potential to differentiate into adipocytes, myocytes, chondrocytes and osteoblasts under the direction of transcription factors. Osteoblast differentiation is controlled by the master transcription factor runt-related transcription factor-2 (RUNX2) [66]. RUNX2 null mice have a cartilaginous skeleton and completely lack mineralised tissue due to arrest of osteoblast maturation [67, 68]. Runx2 expression maintains a pool of immature osteoblasts to form the trabecular bone, whereas the suppression of Runx2 induces the expression of late stage differentiation, forming compact bone [69]. This was demonstrated in dominant negative-Runx2 mice, where the structure and composition of trabecular bone closely resembled compact bone, inducing late stage osteoblast differentiation, increasing the level of Runx2 expression regulates the level of bone maturity [69].

The need for the osteoblast to keep a close contact with other cell types is due to the requirements of other cells to access cell signals in the form of cytokines [70]. The protein secretion forms a thin layer of matrix (osteoid) between the osteoblast and the calcification front resulting in mineralisation of the newly secreted proteins. Osteoblasts that get encased within the newly laden matrix become osteocytes. Other cells including immature osteoblasts and earlier mesenchymal precursors, though are not directly associated with bone also play a role in cytokine presentation to osteoclast precursors [71]. Cytokines play a crucial role in cell to cell signalling between osteoclast and osteoblast/stromal cells involved in the activation of calcium mobilisation [71, 72]

1.4.3 *The Lining cells*

1.4.3.1 Lining cell origin

Bone lining cells (BLC) are osteoblasts left behind following mineralisation [73] are thought to regulate the movement of calcium and phosphate into and out of bone [73].

1.4.3.2 Lining cell structure

Bone surfaces which are not undergoing remodelling or modelling are covered by elongated, thin cells of the osteoblastic lineage. These bone lining cells are thought to be either inactive osteoblasts which perhaps can be activated to produce bone matrix or a cell type of its own. In Gardner staining, bone lining cells are seen as a row of nuclei along bone surfaces, since their cytoplasm does not contain many cell organelles and is therefore only weakly stained in this procedure. Retraction of lining cells exposes the underlying osteoid to proteolytic enzymes of osteoblasts [74] and further to osteoclastic resorption.

1.4.3.3 Lining cell function

The bone lining cells can be induced to proliferate and differentiate into osteogenic cells and may represent osteogenic precursors. BLC's and other cells of the endosteal tissues may be an integral part of the marrow stromal system and have important functions in hematopoiesis, perhaps by controlling the inductive microenvironment [75]. Because activation of bone remodelling occurs on inactive bone surfaces, BLC's may be involved in the propagation of the activation signal that initiates bone resorption and bone remodelling. Evidence also suggests that BLC's are important in the maintenance of the bone fluids and the fluxes of ions between the bone fluid and interstitial fluid compartments for mineral homeostasis [75].

1.4.4 *The Osteocyte*

1.4.4.1 Osteocyte origin

The osteocyte is the terminal differentiation stage of the osteoblast and supports bone structure and metabolic functions. Each osteocyte resides in lacunae and is connected to numerous cellular extensions of filapodia processes that lie in the canaliculi [76].

1.4.4.2 Osteocyte structure

Osteocytes are mature, non dividing bone cells that reside in a fluid-filled network made up of lacunae and are interconnected via cellular processes contained within the channels called canaliculi. They are derived from osteoblasts and they represent the final stage of maturation of the bone cell lineage. Osteocytes are metabolically and electrically coupled through gap junction protein complexes principally comprised of connexin 43. Gap junction formation is essential for osteocyte maturation, activity, and survival [77, 78].

1.4.4.3 Osteocyte function

Osteocytes are differentiated osteoblasts buried in the mineralized bone structure named lacunar-canalicular network that is bathed in interstitial fluid [79]. When bone is subjected to mechanical loading, osteocytes are thought to be able to sense the shear stress induced by interstitial fluid flow in lacunar-canalicular network, transduce the mechanical signals into biochemical signals, and finally regulate bone remodelling by affecting effectors [80]. Osteocytes are present throughout the mineralized matrix and are connected with each other via long slender cell processes which allow contacts with each other via gap junctions, therefore allowing direct cell to cell coupling [79]. The surrounding osteoid matrix then becomes mineralized under the control of enzymes. Matrix-metalloproteinase-2(MMP-2) has been demonstrated to play a role in the regulation of osteocyte production and the initiation of the canalicular system [81]. Mechanical stimulation was demonstrated to be an important factor in distributing sufficient extracellular fluid for oxygen and nutrient supply to maintain metabolic activity in the osteocytes. Osteocytes have been shown to induce osteoclastogenesis when co-cultured with osteoclast precursors [82]. Furthermore, co-culture and stromal cells and monocytes in media exposed to 2 hour of mechanical stimulation confirmed that osteocytes mediate stromal cells to induce osteoclastogenesis via diffusible factors [83].

Osteocyte density has been found to be significantly lower in ovariectomised rats compared to sham animals. Additionally, osteocyte density was positively correlated to bone mineral content and ultimate load [83]. Reduction in mineral content and bone strength is accompanied by increased osteocyte apoptosis due to a decrease in bone formation followed by an increase in resorption in weightlessness studies [84]. These observations suggest that osteocytes actively initiate and control bone remodelling possibly by releasing a signal to recruit osteoclasts and subsequently alter bone quality.

1.5 BONE REMODELLING

Bone is a specialized connective tissue composed of both mineral and organic phases that is designed for its role as the load bearing structure of the body. Bone is constantly undergoing bone remodelling which is a complex process, involving the resorption of bone on a particular surface followed by a phase of bone formation. There is a balance between the amount of bone resorbed by osteoclasts and the amount of bone formed by osteoblasts (Frost, 1964). Bone remodelling occurs in organised groups of cells, the basic multicellular unit (BMU), which turns bone over in multiple bone surfaces [85]. Each BMU is geographically and chronologically separated from other groups of remodelling suggesting that activation of the sequence of events responsible for remodelling is locally controlled by autocrine or paracrine factors generated in the bone microenvironment.

1.5.1 Activation Phase

The first stage of bone remodelling involves detection of an initiating remodelling signal. This signal can be a direct mechanical strain on bone that results in structural damage or hormone actions on bone in response to systemic changes. It has been suggested that osteocytes are important in instructing the initiation of remodelling. Activation of resorption was suggested to be mediated by osteocytes surrounding a micro-fracture [86] which may be due to the ongoing mechanical strain on the skeleton. An important role of osteocytes is to detect mechanical stress which leads to microcracks. Those osteocytes that are close to the microcracks are usually apoptotic. It has been suggested that osteocytes death or the increase in osteocytes apoptosis lead to increased osteoclastogenesis [87] and initiate resorption. Under basal conditions, osteocytes secrete TGF-β which inhibits osteoclastogenesis. A recent study demonstrated that osteoclasts resorb aged bones and few live osteocytes are present in older bones [88]. While osteocyte specific ablation mouse model demonstrated that the loss of osteocytes led to a large increase in osteoclast number and bone resorption, another in vitro study showed that live osteocytes secrete RANK and M-CSF which

support osteoclastogenesis [71, 89]. These contradiction studies suggested that although the role of osteocytes is becoming clearer, the exact mechanism is still yet to be solved.

1.5.2 Resorption Phase

The resorption phase involves the recruitment of osteoclast progenitors to bone. The progenitor cells are gathered from the haematopoietic tissues such as bone marrow via the circulating blood stream. They proliferate and differentiate into osteoclasts through the interaction between osteoblast stromal cells [90]. The lining osteoblasts remove the unmineralised osteoid layer on the bone surfaces and produce various proteolytic enzymes, such as MMPS, collagenase and gelatinase [91] to enable the movement of osteoclasts to the mineralised bone. The next step requires the binding of extracellular bone matrix proteins such as osteopontin to the vitronectin receptor to induce osteoclast polarisation [92]. The characteristic features of osteoclasts are the presence of ruffled borders and sealing zones which contain a thick band of actin which allow the attachment of osteoclasts to the bone [93]. The activated osteoclasts resorb bone by the production of hydrogen ions and proteolytic enzymes in the localised environment. Lysosomal cysteine proteinases such as cathepsin B L and K and MMPs are involved in the degradation of the matrix and the dissolution of the mineralised matrix [94]. Osteoclasts eventually undergo apoptosis by nuclear and cytoplasmic condensation and fragmentation of nuclear DNA into nucleosomal-sized units [95]. Osteoclasts have a limited life span of approximately 12.5 days; therefore the process of bone remodelling requires a continual supply of precursors to maintain its numbers.

1.5.3 Reversal Phase

The reversal phase involves the apoptosis of the osteoclasts followed by formation of the new bone by the osteoblasts activation. Initially, the reversal cell was proposed to be a monocytic phagocyte based on morphological assessment [96]. However, more recently, it was reported that the reversal

cell is from the osteoblast lineage based on cell morphology, positive expression for alkaline phosphatase [97] and the absence of the monocyte macrophage marker MOMA-2 [98] on these cells. Recent studies have suggested that both osteomacs and mesenchymal bone lining cells work together to facilitate events during the reversal phase. Osteomacs are likely responsible for the removal of matrix debris during the reversal phase. Macrophages produce the enzyme MMP [99] which is required for matrix degradation and osteopontin [100] which is incorporated into mineralised tissue. The final roles of the reversal cells are to produce coupling signals that allow bone formation to occur.

1.5.4 Formation Phase

Bone formation involves the proliferation of mesenchymal cells, the differentiation into osteoblast precursor cells, maturation of the osteoblasts and the formation and mineralisation of the matrix. Osteoblasts aggregate at the bottom of the resorptive cavity and form osteoid until the cavity is filled. Osteoblasts that lay down thick layer of osteoid at the bottom of the cavity gradually flatten and become inactive lining cells. Some osteoblasts differentiate into osteocytes and become embedded in the matrix. The chemotactic attraction of osteoblast precursors to the site of resorption involve in the initiation of bone formation that can be mediated by factors such as TFG-B, and collagen that are produced during resorption [101]. The subsequent proliferation and differentiation of osteoblast precursor is suggested to be mediated by osteoblast derived growth factors and growth factors released during the resorption process such as insulin-like growth factor I (IGFI) and II and TGF-β. The termination of the osteoblast activity may be due to the induction of osteoblast apoptosis by tumour necrosis factors released from neighbouring marrow cells [102].

1.5.5 Osteoclastogenesis

Osteoclasts differentiate from stem cells of the monocyte and macrophage lineages by a process known as osteoclastogenesis [103]. Formation of large multinucleated cells occurs by the fusion of precursor cells. The majority of cellular differentiation from precursors is likely to take place within the bone marrow. The differentiation of osteoclasts is a highly controlled process dependent on several hormonal signals such as 1,25D, PTH and IL-11. They act on osteoblasts and bone marrow stromal cells to activate the release of osteoclastogenesis inducing factors [104]. The inducing factors produced by osteoblast/stromal cells include RANKL and M-CSF [103]. The release of M-CSF and RANKL is crucial for the formation of osteoclasts while the release of the inhibitory OPG from the osteoblast is crucial for the control of osteoclastogenesis.

Receptor activator nuclear factor-kβ ligand (RANKL) is a member of the tumour necrosis factor super family that has been found to act as a regulatory protein in the differentiation of osteoblasts, T-cells and dendritic cells [103]. RANKL was identified and isolated by multiple research groups working on a variety of cell types in which RANKL plays a role in differentiation and maturation. Accordingly, RANKL is also referred to as TNF-related activated-induced cytokine- TRANCE [105], osteoprotegerin ligand (OPGL) [71] and osteoclast differentiation factor (ODF) [106]. All of these differentiation factors were shown to be the same molecule [103]. The majority of RANKL produced in vivo is an insoluble membrane bound protein, therefore cell to cell contact is required for osteoclastogenesis to occur. Osteoclast-like cells are not produced if stromal cells and osteoclast precursors are separated by a membrane in the absence of soluble RANKL [106]. RANKL not only acts in conjunction with M-CSF to stimulate differentiation but also acts to increase osteoclastic function and stimulate greater levels of resorption [106, 107].

RANK has a competitor for the binding of RANKL, osteoprotegerin (OPG) [108]. OPG is a water soluble receptor that binds RANKL resulting in inhibition of osteoclastogenesis as shown by severe

osteoporosis in OPG knockout mice [109]. OPG release is mediated by chemical signals such as interleukin-1 and is released by the osteoblasts. OPG has recently been shown to also bind to osteoclasts and osteoclast precursors directly increasing motility and inhibit resorption [110-112]. The current concept is that the RANKL/OPG expression ratio determines the degree of osteoclast differentiation and function [113].

1.6 HORMONES AND BIOCHEMICAL FACTORS KNOWN TO CONTROL BONE HOMEOSTASIS

1.6.1 *Parathyroid Hormone*

Parathyroid hormone (PTH) is secreted by the parathyroid glands as a polypeptide containing 84 amino acids [114]. PTH is the primary hormone responsible for maintaining serum calcium at a level sufficient for its wide range of functions which include bone mineralisation. A decrease in extracellular calcium levels is detected by the calcium sensing receptor in the parathyroid gland and initiates the secretion of PTH. PTH acts on the kidney to stimulate transcellular calcium reabsorption and decrease renal-phosphate reabsorption as well as upregulating CYP27B1 activity in the kidney. Furthermore, PTH also stimulates bone resorptive functions. These actions lead to a decrease in serum calcium [115]. In conditions of low serum calcium, the calcium sensing receptor of the parathyroid gland stimulates PTH secretion [116]. Action of PTH in the kidney is mediated by the activation of membrane-associated adenylate cyclase which leads to an increase in intracellular cAMP [117]. PTH increases circulating levels of 1,25D by increasing the gene expression of renal 1-a-hydroxylase [118, 119]. Subsequently, PTH and 1,25D act together to increase bone resorption and renal reabsorption of calcium [120]. As a form of negative feedback regulation, 1,25D decreases PTH expression by negatively regulating PTH gene [121]. In parathyroidectomised animals, no stimulation of renal CYP27B1 activity was detected in response

to hypocalcemia. However, the administration of PTH to these animals restored the induction of CYP27B1 enzyme activity [122, 123].

1.6.2 *1,25 Dihydroxyvitamin D3 (1,25D)*

1.6.2.1 Metabolism of Vitamin D

The two main forms are vitamin D_2 and vitamin D_3. Vitamin D_3 is synthesized in the skin after exposure to sunlight and vitamin D_2 is obtained by irradiation of plants and foods [124, 125]. Vitamin D is 25-hydroxylated in the liver, followed by 1a-hydroxylation to synthesise 1,25D in the kidney. Activation of 25D to 1,25D is catalysed by cytochrome P450 enzyme 25D-1α-hydroxylase (CYP27B1) [126]. CYP24 catabolises 1,25D to produce 1,24,25-trihydroxyvitamin D_3 (1,24,25D), which is the first step of 1,25D degradation, known as the C-24 oxidation pathway [127]. The synthesis of 1,25D by CYP27B1 and degradation of 1,25D by CYP24 are subject to a high degree of control, which confers specificity to 1,25D-mediated activity. The biological activity of 1,25D is mediated by a high affinity vitamin D receptor (VDR), which acts as a ligand-activated transcription factor. Un-liganded VDR/retinoid X receptor (RXR) heterodimer binds to vitamin D response elements (VDRE) of the promoter of target genes, recruiting a co-repressor complex resulting in repression of basal gene transcription. Upon VDR binding of 1,25D, the co-repressor is displaced allowing recruitment of a co-activator complex of proteins that interacts with RNA polymerase machinery and stimulates gene transcription of the target gene [128]

1.6.2.2 Endocrine actions of 1,25D

The involvement of 1,25D is also important in both bone formation and bone resorption. Active intestinal calcium absorption stimulated by 1,25D provides adequate supply of dietary calcium for optimal bone mineralisation or suppression of bone resorption [129]. In vitro studies have shown that in osteoblasts, the major bone cell containing VDR, 1,25D regulates genes involved in

proliferation [130], differentiation [131], and mineralization [132]. Atkins et al demonstrated that in vitro human osteoblasts respond to exogenous 1,25D by decreasing their rate of proliferation as well as increasing their expression of mRNA species encoding osteoblast-associated genes [133]. The primary function of the osteoblast is to secret a specialized organic extracellular matrix that consists of mainly type I collagen. This organic matrix is mineralized at discrete sites by the incorporation of calcium phosphate forming a mature bone matrix. Effects on matrix proteins such as collagen type I, osteopontin and osteocalcin are believed to be mediated via transcription VDREs within their promoter regions [134].

The osteoblast not only regulates bone synthesis but also mediates the regulatory signals that coordinate the resorbing osteoclasts. Continued alterations in expression of genes such as osteocalcin result in a mature osteoblast phenotype that is able to mineralise the collagen matrix [135, 136]. Osteoblasts activate osteoclasts through the induction of the osteoblast-membrane-associated protein, RANKL. The activation of osteoclastic bone resorption increases calcium flux from bone into the ECF. In vitro co-culture studies with immature bone marrow cells of the monocyte-macrophage lineage (containing pre-osteoclasts) and stromal cells (containing pre-osteoblasts) indicate that 1,25D acts on the stromal cells to induce production of factors stimulating differentiation of osteoclast precursors to mature osteoclasts [137, 138]. 1,25D promoted late stages of osteoblast differentiation indicated by in vitro mineralization and the in vitro surrogate of the osteogenic response and elicited RANKL transcriptional responses in primary osteoblastic cells, suggesting that 25D levels *in vivo* may regulate multiple facets of bone remodelling [139]. Osteoblasts from VDR knockout mice are unable to stimulate the differentiation of osteoclasts, demonstrating the requirement for VDR in the osteoblast for this 1,25D mediated effect [140].

The circulating levels of 1,25D rely also on adequate supply of the substrate, 25D. Vitamin D-deficiency may lead to altered regulation of bone cell activity due to an inadequate supply of the

substrate required for the local synthesis of 1,25D. Biochemical evidence of moderate vitamin D insufficiency occurring at circulating 25D levels between 25 and 75 nmol/L [141] showed an increase in the circulating markers of bone turnover, typical of which would be predicted to cause osteoporosis, a disease associated with an increase in the activation of BMU leading to perforation of trabeculae, reduced cortical bone and increased risk of fracture [142, 143].

1.6.2.3 Extra-renal Synthesis of 1,25D

Extra-renal synthesis of 1,25D was first documented in studies of vitamin D metabolism in human pregnancy [144] and the granulomatous disease sarcoidosis [145]. Studies on the cloning of the human for CYP27B1 revealed identical cDNA sequences in renal [146] and extra-renal tissues [147]. Analysis of bone mineral density showed that bone loss which is a prevalent complication of Crohn's disease was independently associated with serum 1,25D levels [148]. Furthermore, Vitamin D metabolites were detected in freshly isolated colon tumour cells [149] and colonocytes in cell culture was demonstrated to have the capacity to synthesize 1,25D from 25D [150].

In addition to the synthesis of 1,25D in various other tissues, the most clearly defined role for circulating 1,25D is its stimulation of intestinal calcium absorption. In the intestine, 1,25D exerts two effects: one dependent on gene expression and protein synthesis shown by calbindin synthesis [151] and a more rapid response by the increase in intracellular Ca^{2+} concentration and cyclic GMP levels [152]. Calcium enters from the intestinal lumen across the microvillar membrane and into the enterocyte down an electrochemical gradient. 1,25D was demonstrated to up-regulate expressions of transcelluar calcium transport genes such as calbindin D9k, plasma membrane Ca ATPase, epithelial calcium channels, TRPV5 and TRPV6 which are involved in apical membrane, transcellular and basolateral membrane calcium transport [153]. 1,25D was shown to up-regulate

calbindin D9k expression through the vitamin D response element in the proximal promoter of the gene [154].

1.6.3 Calcitonin

Calcitonin is a 32–amino acid peptide with an N-terminal disulphide bridge and a C-terminal prolineamide residue [155]. The actions of PTH and calcitonin are antagonistic on bone resorption but synergistic on decreasing the renal tubular reabsorption of phosphorus. The hypocalcemic effects of calcitonin are primarily the result of decreased entry of calcium from the skeleton into plasma, resulting from a temporary inhibition of PTH-stimulated bone resorption[156]. Calcitonin directly causes a loss of ruffled border of osteoclasts in bone sections and reduces osteoclast number in bone [157, 158]. It also reduces osteoclast acid secretion by changing Na^+-K^+-ATPase and inhibits H^+ATPase activity [159, 160].

Calcitonin was shown to enhance renal conversion of $25(OH)D_3$ into 1,25D in vitamin D-deficient rats [161, 162]. Contrary to that statement, other studies have also shown that calcitonin has no effect on vitamin D metabolism in thyroparathyroidectomized animals [162]. The major action of calcitonin appears to lower serum calcium levels via inhibition of bone resorption. Morel et al[163] and Kawashima et al[164] have demonstrated that calcitonin-sensitive adenylate cyclase is in the distal nephron and calcitonin stimulates CYP27B1 activity in proximal straight tubules in isolated – single-nephron segments. Furthermore, it was demonstrated that calcitonin stimulates the expression of CYP27B1 mRNA in the kidney in normalcalcemic rats [165]. They showed that while the PTH-mediated stimulation of the expression of CYP27B1 mRNA occurred during hypocalcemia, only calcitonin was able to stimulate the expression of CYP27B1 mRNA during

normocalcemia [165]. Although calcitonin has been shown to regulate CYP27B1 expression by some studies, the precise role of calcitonin in mediating vitamin D metabolism remains unclear.

1.7 BONE BIOMECHANICS

1.7.1 Stress and strain

The concepts of stress and strain are fundamental to bone biomechanics. Stress is defined as force per unit area and strain is the relative deformation. The relationship between stress and strain in bone follows a curve called the stress-strain curve which allows the extraction of various measures of stiffness within the bone [166-168]. The force can be compressive, tensile or shear depending on the method of loading. In loading, pure longitudinal axial compression force slightly shortens and widens the bone; Tension lengthens and narrows the bone. Shear stresses arise when one part of the material slide to its adjacent region [167, 168]. Stress is reported in units of Pascals and strain has no units. All the types of stresses mentioned above occur in combination even under simple loading methods [167, 168].

1.7.2 Elasticity and Plasticity

The degree of deformity in a material depends on not only the forces and moments applied to the structure but also the stiffness of the constituents [169]. Stiffness is determined by the proportions of the hydroxyapatite crystals and the collagen fibers in the specific material [170]. During the initial stages of a test, the elastic region is the linear portion of the stress-strain curve. If the load is removed during this phase of the test, the specimen will return to its original size and shape without incurring permanent damage.

The slope of the elastic region of the stress-strain curve reflects the stiffness of the material. Young's modulus is a measure of the intrinsic stiffness of the material. The trabecular bone

structure is less stiff than the compact bone structure, which increases its capability to deform and hence absorb energy [14, 171]. At a higher level of stress and strain, where the elastic limit is exceeded and permanent deformation occurs the region is referred to as the plastic region [167, 168].

1.7.3 Ultimate Failure

As loading continues in the plastic region, the material will eventually reach ultimate failure, at which point the specimen fails catastrophically [172]. The ultimate compressive strength of cortical bone can be ten times greater than in trabecular bone [14].The ability for a material to resist the propagation of cracks can be measured by the amount of post-yield strain that occurs before ultimate failure [173]. Ductile material has the ability to change its conformation without breaking. However, a material is considered brittle if it undergoes little post-yield behavior before ultimate failure. As osteoporosis is often referred to as the brittle-bone disease, in reality little experimental evidence supports an actual reduction in the ductility of bone material examined from patients diagnosed with this disease [168].

Loading creates complex strain and stress environments in a material. Maximum strains are achieved when axial loads are applied to a slightly curved beam compressing the concave side while causing tensile strain on the convex side. The strain is decreased to zero in the middle of the beam, which is known as the neutral axis [174]. The flexural rigidity of the material, EI, represents the amount of force per unit cross-sectional area required to deform the material a given amount, where E is elastic modulus, and I is the moment of inertia [168]. The moment of inertia reflects the contribution of each part of the material to the stiffness in each position in the cross-section of the beam. Therefore the further away the material is relative to the neutral axis; the more resistant it is to bending. In the elderly, it was shown that the subtle increases in the moment of inertia, which are

achieved through periosteal expansion, may structurally compensate for the bone loss and cortical thinning that parallels the aging process [175].

1.7.4 Mechanical forces on bone remodelling

The material of all bones has the same constituents. These include mineral, carbonated hydroxyapatite, type 1 collagen and other non-collagenous proteins and water. It is a graded material as its composition, structure, and mechanical properties vary continuously from one location to another [176]. Bone tissue seems to be able to detect changes in strain on a local base and then adapts accordingly. Internally, bone adapts by changing the density of trabeculae and osteons as well as their disposition. Externally it adapts by a change in shape and dimensions [177]. Bones have the capacity to adapt their architecture in relationship to changes in their habitual loading environment without causing extensive damages [178]. A key regulator of osteoblast and osteoclast activity in bone is mechanical strain. Bone formation and resorption are balanced under normal conditions. However, the equilibrium between formation and resorption shifts as the intensity of strain changes. When strain intensity is higher than the equilibrium strain, bone formation occurs. When strain intensity if lower than the equilibrium strain, bone resorption occurs. Dynamic equilibrium between resorption and deposition is again achieved when the equilibrium strain state is newly established [179]. The skeleton continues to adapt to the mechanical loading from daily activities through the adaptive remodelling by formation of new bones to withstand the increased amount of loading and the removal of bone in response to unloading [180, 181]. As discussed before, highly interconnected osteocytes, which exist in high numbers and are distributed throughout the bone matrix are suggested to be the main cells that are responsible for sensing mechanical strain and transducing their signals[182].

Studies have demonstrated that osteocyte selective markers such as dentin matrix protein 1(DMP1), MEPE and sclerostin are regulated by mechanical loading[183]. DMP1 was upregulated within a

few hours of loading in osteocytes in a tooth model [184]. Studies on DMP1-null mice show that DMP1 expression is required for osteoblast differentiation to mature osteocytes, demonstrating its importance in the extent of mineralisation [185, 186]. In weightlessness studies, reduced mechanical forces led to a decrease in bone formation followed by an increase in resorption causing a reduction in mineral content and bone strength accompanied by increased osteoblast and osteocyte apoptosis[84]. Other animal models have demonstrated that increased bone mass and size as a consequence of adaptation is dependent on the degree mechanical stimuli. In both rat and mouse studies, it was shown that osteoblast activity increased with an increase in mechanical strain, resulting in larger and stronger bones [187-189]. Furthermore, evidence of reduced loading due to long-term bed rest[190], hind limb unloading in animals [191] and space flights [192] have demonstrated significant bone loss and mineral changes emphasising the importance of mechanical forces required to maintain normal function. These studies support The Frost “mechanostat” model which states that bone adapts in ways dependent on the dynamic strain environment experienced by one tissue which indicated that low strain results in loss of bone and high strain results in gain of bone [193]. Biomechanics in bone is critical to an improved understanding of both the pathogenesis of metabolic bone disease and the promising breakthrough of controlling bone mass and structure through mechanical stimuli.

1.8 BONE MATERIAL PROPERTIES TESTING METHODS

Bone undergoes complex patterns of loading during lifetime. External and internal forces act to deform the bone. Mechanical testing of bone is a common way to determine its properties. Bone has an anisotropic structure and its structural properties vary with load direction [194]. Bone materials properties depend upon its mineral contents densities and collagenous contents. It is suggested that while performing tests, related materials and geometric dimensions affect the bone strength depending on load type and direction applied on the whole bone. Therefore, frequent testing

methods for other materials can not determine the bone structural characteristics and testing whole bone may be another way to study its properties [195].

Bending tests are useful for measuring the mechanical properties of bones from rodents and small animals. Bending can he applied to rodent bones and machined bone specimens using either three-point or four-point loading. Small bones are very difficult to machine tensile or compressive test specimens. In the bending test the long bone is loaded in bending until failure. Stress due to bending can be calculated using the beam bending formula. Bone strength and elastic modulus are functions of load applying direction and are stronger in compression comparing to tension. These varieties could be due to orthotropic properties of bone that cause its strength and modulus to be a function of tissue orientation exposed to load [168]. There are a number of reports in the literature using a variety of mechanical strength measures, with 3-point bending being the most common method for the characterization of long bone biomechanical properties in small animals [196-198]. The tibia has been favoured by some for mechanical testing because the metaphysis of the tibia is particularly predisposed to develop osteoporosis in rats [199]. However, the continually changing cross-sectional geometry makes it a less attractive bone for testing in terms of calculating Young's modulus.

1.8.1 Three- and Four-point bone bending tests

Usually, three-point bending [200-202] and four-point bending [201, 203] are used to determine whole bone mechanical strength. The three-point bending test was commonly used in the evaluation of bone strength in earlier studies and showed that bending breaking force and stiffness, as well as the intrinsic parameters, ultimate stress and elastic modulus are good indicators of the mechanical strength of cortical bone [200].

The main advantage of three-point bending is its simplicity and reproducibility making it the preferred method to use in small animals [168, 200]. The main disadvantage is that it creates high shear stresses near the midsection of the bone. Three point testing in the tibia was reported to have high reproducibility making it a desirable choice for three-point bending [196].

According to standard testing method, published by American Society of Agricultural Engineers, three-point bending test of animal bone should be used only when the bone is straight, has a symmetrical cross-section and has a support length to diameter ratio greater than 10 [204]. The testing machine used should be capable of applying constant rate of crosshead movement with reproducible speed and accuracy of ±1% (coefficient of variance). Adjustable fulcra should be used in order to obtain a support length to bone diameter ratio greater than 10. Details and previous histories about the animals from which the bones were taken also should be recorded [205].

Four-point bending produces pure bending between the two upper loading points which ensures that there are no transverse shear stresses. This means that there is a region of uniform stress distribution between the inner contacts[168]. However, in four-point bending, the forces at each loading point must be equal which is easy in regularly shaped specimens. Four-point bending is standard in large animals such as dogs, pigs, or sheep [206-208], therefore in rodent long bone testing, three- point bending is often preferred [196, 209].

1.9 MEASUREMENTS OF BONE STRUCTURE, CELLULAR ACTIVITY AND BONE MATERIAL PROPERTIES

1.9.1 *Static measures of bone structure*

1.9.1.1 2-Dimensional measurements

The primary measures for estimating bone remodelling and their nomenclature has been standardized [210]. The primary measurements for trabecular bone are area and perimeter and are often reported as volume and surface. Therefore the proportion of the sample area that contains bone will be shown as trabecular bone volume (BV/TV, %). Using standardized equations, the presence of trabecular bone can be also further described by the number (Tb. N) and thickness (Tb.Th, um).

1.9.1.2 3-Dimensional measurements

Micro-computed tomography (micro-CT) is an emerging technique for the nondestructive assessment and analysis of the three-dimensional bone structure. It has recently progressed from an invasive tool for ex-vivo bone specimens into an in vivo tool for small animals. The micro-CT machine has a superior X-ray tube and an image intensifier detector typically capable of resolving as low as um allowing the visualisation of individual trabecular elements. In addition, they have the appropriate software (a cone beam reconstruction algorithm (Feldkamp et al 1984) that allows the creation of a three-dimensional array of data. Trabecular thickness, trabecular spacing and trabecular number as well as bone volume and degree of anisotrophy can now be accurately derived due to its known 3D architecture. In terms of cortical bone, micro-CT allows a 3D reconstruction of the cortical bone region of interest, reduces the sample site variation and permits exact measurement of the bone volume. Studies comparing mechanical testing and computed tomography [200, 211] suggest that both mechanical tests and CT measurements are relevant in biomechanical studies on rodent bones.

Micro-CT has both strengths and limitations for assessing structural adaptation in bone. It has the capability to provide a three dimensional assessment of net mineral content of a specific region of the bone or throughout an entire bone. Quantitative micro- CT allows detection of differences in

bone volume fraction, mass and morphometric parameters [212]. The higher resolution of micro-CT is critical to localizing the mineral response in bony structures of small animals and facilitates paired analysis by allowing very accurate alignment of loaded versus contralateral bones [213]. However, micro CT lacks the ability to measure biological parameters such as the osteoid thickness, erosion depth, or osteoclast number and although it is a non-destructive method to quantify very accurately the structural properties of bone, harvesting bone biopsies for human studies is basically restricted to samples from the iliac crest and is highly invasive and inconvenient for the patient [214].

1.9.2 Dynamic measures of bone cell activity

1.9.2.1 Bone formation

The calculation of bone formation is achieved by the intravital labelling with tetracycline derivatives prior to biopsy [215]. Two doses of label are administered with a gap of 10 days between doses and a 3 day period before biopsy. These compounds can be visualised under UV light as florescent labels within the bone tissue. The extent of active surface mineralisation at a given time is estimated by incorporating only the surfaces covered by both labels and provide the product of double label surface/mineralising surface (%). Mineral apposition rate (MAR) is the distance between the corresponding edges of two consecutive labels, divided by the time between midpoints of the labelling periods. This gives a dynamic estimation of the activity of osteoblasts at the time of biopsy. Bone formation rate can then be derived from the product of double label surface and MAR [210].

To ensure an accurate measurement of continuous bone formation, only surfaces covered by both florescent labels are incorporated into the estimate of perimeter, namely double labelled surface (dLS). The perimeter may also include half of the single labelled surface to account for the

resorption of previously incorporated label. The calculation gives an estimate of osteoblastic activity at the time of biopsy. Bone formation rate (BFR) is the product of dLs and MAR [210].

1.9.2.2 Bone resorption

The primary measurements for bone turnover correspond to the perimeter of the bone surface undergoing by resorption or formation [216]. Resorption is estimated by identifying the presence of osteoclasts on the surface of the bone. The data is often expressed as the percentage of the bone covered by active osteoclasts called osteoclast surface (Oc.S., %).

1.10 SUMMARY

It is clear that the regulation of skeletal tissue is complex with effects of multiple regulatory factors being modulated to optimise both mechanical and homeostatic functions. These functions are not uniformly distributed across the skeleton. Particularly in lone bones, cortical and trabecular bone vary in response to different mechanical forces. While mechanical functions are preferentially performed by cortical bone, trabecular bone is more susceptible to vitamin D deficiency and calcium deficiency. This heterogeneity suggests an element of local control in bone response to systemic modulators of bone turnover. Previously our laboratory has developed a rat model for the assessment of nutritional and hormonal regulation of bone. We have demonstrated the occurrence of bone loss consistent with osteoporosis at moderately insufficient levels of 25D and osteomalacia at severe vitamin D deficiency. Our rat model provides the level on control necessary to investigate the interaction of these systemic factors such as vitamin D and calcium deficiency with local factors. Little is known about the level of vitamin D requirement for bone cell in both adult and aged rats, therefore our development of the vitamin D deplete rat model in which we have demonstrated the occurrence of osteopenia and osteomalacia under different conditions, will be able to provide us with the essential tool to further assess the vitamin D status for the maintenance of bone architecture and bone strength.

1.11 REFERENCES

1. Van Leeuwen, J.P., Et Al., *Vitamin D Control Of Osteoblast Function And Bone Extracellular Matrix Mineralization.* Crit Rev Eukaryot Gene Expr, 2001. 11(1-3): P. 199-226.
2. Chapuy, M.C., Et Al., *Vitamin D3 And Calcium To Prevent Hip Fractures In The Elderly Women.* N Engl J Med, 1992. 327(23): P. 1637-42.
3. Avenell, A., Et Al., *Vitamin D And Vitamin D Analogues For Preventing Fractures Associated With Involutional And Post-Menopausal Osteoporosis.* Cochrane Database Syst Rev, 2009(2): P. Cd000227.
4. Bischoff-Ferrari, H.A., Et Al., *[Effect Of Vitamin D On Muscle Strength And Relevance In Regard To Osteoporosis Prevention].* Z Rheumatol, 2003. 62(6): P. 518-21.
5. Bischoff-Ferrari, H.A., Et Al., *Fall Prevention With Supplemental And Active Forms Of Vitamin D: A Meta-Analysis Of Randomised Controlled Trials.* Bmj, 2009. 339: P. B3692.
6. Pfeifer, M., Et Al., *Effects Of A Short-Term Vitamin D And Calcium Supplementation On Body Sway And Secondary Hyperparathyroidism In Elderly Women.* J Bone Miner Res, 2000. 15(6): P. 1113-8.
7. Morgan, E.F., Et Al., *Mechanotransduction And Fracture Repair.* J Bone Joint Surg Am, 2008. 90 Suppl 1: P. 25-30.
8. Herring, G.M. And B.A. Ashton, *The Isolation Of Soluble Proteins, Glycoproteins, And Proteoglycans From Bone.* Prep Biochem, 1974. 4(2): P. 179-200.
9. Bonar, L.C., Et Al., *X-Ray Diffraction Studies Of The Crystallinity Of Bone Mineral In Newly Synthesized And Density Fractionated Bone.* Calcif Tissue Int, 1983. 35(2): P. 202-9.
10. Crabtree, N., Et Al., *Intracapsular Hip Fracture And The Region-Specific Loss Of Cortical Bone: Analysis By Peripheral Quantitative Computed Tomography.* J Bone Miner Res, 2001. 16(7): P. 1318-28.

11. Haidekker, M.A., R. Andresen, And H.J. Werner, *Relationship Between Structural Parameters, Bone Mineral Density And Fracture Load In Lumbar Vertebrae, Based On High-Resolution Computed Tomography, Quantitative Computed Tomography And Compression Tests.* Osteoporos Int, 1999. 9(5): P. 433-40.

12. Pistoia, W., B. Van Rietbergen, And P. Ruegsegger, *Mechanical Consequences Of Different Scenarios For Simulated Bone Atrophy And Recovery In The Distal Radius.* Bone, 2003. 33(6): P. 937-45.

13. Seeman, E. And P.D. Delmas, *Bone Quality--The Material And Structural Basis Of Bone Strength And Fragility.* N Engl J Med, 2006. 354(21): P. 2250-61.

14. Buckwalter, J.A., Et Al., *Bone Biology. I: Structure, Blood Supply, Cells, Matrix, And Mineralization.* Instr Course Lect, 1996. 45: P. 371-86.

15. Allen, M.R., J.M. Hock, And D.B. Burr, *Periosteum: Biology, Regulation, And Response To Osteoporosis Therapies.* Bone, 2004. 35(5): P. 1003-12.

16. Whitehouse, W.J., *Cancellous Bone In The Anterior Part Of The Iliac Crest.* Calcif Tissue Res, 1977. 23(1): P. 67-76.

17. Currey, J.D., *Bones. Structure And Mechanics.* New Jersey: Princeton University Press, 2002.

18. Eckstein, F., Et Al., *Sex Differences Of Human Trabecular Bone Microstructure In Aging Are Site-Dependent.* J Bone Miner Res, 2007. 22(6): P. 817-24.

19. Karsenty, G., *[Central Control Of Bone Formation].* Ann Endocrinol (Paris), 2002. 63(2 Pt 1): P. 145-53.

20. Yang, Y., Et Al., *Morphological Behavior Of Osteoblast-Like Cells On Surface-Modified Titanium In Vitro.* Biomaterials, 2002. 23(5): P. 1383-9.

21. Yang, T.T., Et Al., *Human Mesenchymal Tumour-Associated Macrophages Differentiate Into Osteoclastic Bone-Resorbing Cells.* J Bone Joint Surg Br, 2002. 84(3): P. 452-6.

22. Teitelbaum, S.L. And F.P. Ross, *Genetic Regulation Of Osteoclast Development And Function.* Nat Rev Genet, 2003. 4(8): P. 638-49.

23. Bar-Shavit, Z., *The Osteoclast: A Multinucleated, Hematopoietic-Origin, Bone-Resorbing Osteoimmune Cell.* J Cell Biochem, 2007. 102(5): P. 1130-9.

24. Kahn, A.J. And D.J. Simmons, *Investigation Of Cell Lineage In Bone Using A Chimaera Of Chick And Quial Embryonic Tissue.* Nature, 1975. 258(5533): P. 325-7.

25. Walker, D.G., *Control Of Bone Resorption By Hematopoietic Tissue. The Induction And Reversal Of Congenital Osteopetrosis In Mice Through Use Of Bone Marrow And Splenic Transplants.* J Exp Med, 1975. 142(3): P. 651-63.

26. Walker, D.G., *Osteopetrosis Cured By Temporary Parabiosis.* Science, 1973. 180(88): P. 875.

27. Marks, S.C., Jr., *Osteopetrosis In The Toothless (T1) Rat: Presence Of Osteoclasts But Failure To Respond To Parathyroid Extract Or To Be Cured By Infusion Of Spleen Or Bone Marrow Cells From Normal Littermates.* Am J Anat, 1977. 149(2): P. 289-97.

28. Suda, T., Et Al., *Modulation Of Osteoclast Differentiation And Function By The New Members Of The Tumor Necrosis Factor Receptor And Ligand Families.* Endocr Rev, 1999. 20(3): P. 345-57.

29. Feng, X., *Regulatory Roles And Molecular Signaling Of Tnf Family Members In Osteoclasts.* Gene, 2005. 350(1): P. 1-13.

30. Teitelbaum, S.L., *Osteoclasts: What Do They Do And How Do They Do It?* Am J Pathol, 2007. 170(2): P. 427-35.

31. Domon, T. And M. Wakita, *The Three-Dimensional Structure Of The Clear Zone Of A Cultured Osteoclast.* J Electron Microsc (Tokyo), 1991. 40(1): P. 34-40.

32. Teti, A., P.C. Marchisio, And A.Z. Zallone, *Clear Zone In Osteoclast Function: Role Of Podosomes In Regulation Of Bone-Resorbing Activity.* Am J Physiol, 1991. 261(1 Pt 1): P. C1-7.

33. Minkin, C., *Bone Acid Phosphatase: Tartrate-Resistant Acid Phosphatase As A Marker Of Osteoclast Function.* Calcif Tissue Int, 1982. 34(3): P. 285-90.

34. Chambers, T.J., Et Al., *Resorption Of Bone By Isolated Rabbit Osteoclasts.* J Cell Sci, 1984. 66: P. 383-99.

35. Jones, S.J., A. Boyde, And N.N. Ali, *The Resorption Of Biological And Non-Biological Substrates By Cultured Avian And Mammalian Osteoclasts.* Anat Embryol (Berl), 1984. 170(3): P. 247-56.

36. Roodman, G.D., *Advances In Bone Biology: The Osteoclast.* Endocr Rev, 1996. 17(4): P. 308-32.

37. Wise, G.E., Et Al., *Inhibition Of Osteoclastogenesis By The Secretion Of Osteoprotegerin In Vitro By Rat Dental Follicle Cells And Its Implications For Tooth Eruption.* Arch Oral Biol, 2002. 47(3): P. 247-54.

38. Gowen, M., Et Al., *Cathepsin K Knockout Mice Develop Osteopetrosis Due To A Deficit In Matrix Degradation But Not Demineralization.* J Bone Miner Res, 1999. 14(10): P. 1654-63.

39. Drake, F.H., Et Al., *Cathepsin K, But Not Cathepsins B, L, Or S, Is Abundantly Expressed In Human Osteoclasts.* J Biol Chem, 1996. 271(21): P. 12511-6.

40. Tuukkanen, J. And H.K. Vaananen, *Omeprazole, A Specific Inhibitor Of H+-K+-Atpase, Inhibits Bone Resorption In Vitro.* Calcif Tissue Int, 1986. 38(2): P. 123-5.

41. Mcqueney, M.S., Et Al., *Autocatalytic Activation Of Human Cathepsin K.* J Biol Chem, 1997. 272(21): P. 13955-60.

42. Konttinen, Y.T., Et Al., *Acid Attack And Cathepsin K In Bone Resorption Around Total Hip Replacement Prosthesis.* J Bone Miner Res, 2001. 16(10): P. 1780-6.

43. Saftig, P., Et Al., *Functions Of Cathepsin K In Bone Resorption. Lessons From Cathepsin K Deficient Mice.* Adv Exp Med Biol, 2000. 477: P. 293-303.

44. Saftig, P., Et Al., *Impaired Osteoclastic Bone Resorption Leads To Osteopetrosis In Cathepsin-K-Deficient Mice.* Proc Natl Acad Sci U S A, 1998. 95(23): P. 13453-8.

45. Yasuda, Y., J. Kaleta, And D. Bromme, *The Role Of Cathepsins In Osteoporosis And Arthritis: Rationale For The Design Of New Therapeutics.* Adv Drug Deliv Rev, 2005. 57(7): P. 973-93.

46. Close, P., A. Neuprez, And J.Y. Reginster, *Developments In The Pharmacotherapeutic Management Of Osteoporosis.* Expert Opin Pharmacother, 2006. 7(12): P. 1603-15.

47. Kumar, S., Et Al., *A Highly Potent Inhibitor Of Cathepsin K (Relacatib) Reduces Biomarkers Of Bone Resorption Both In Vitro And In An Acute Model Of Elevated Bone Turnover In Vivo In Monkeys.* Bone, 2007. 40(1): P. 122-31.

48. Teno, N., Et Al., *Effect Of Cathepsin K Inhibitors On Bone Resorption.* J Med Chem, 2008. 51(17): P. 5459-62.

49. Aubin Je, L.F., *The Osteoblast Lineage.* Principles Of Bone Biology, 1996(Bilezkian Jp, Raisz Lgm Rodan Ga): P. 51-67.

50. Aubin, J.E. And K. Turksen, *Monoclonal Antibodies As Tools For Studying The Osteoblast Lineage.* Microsc Res Tech, 1996. 33(2): P. 128-40.

51. Erlebacher, A., Et Al., *Toward A Molecular Understanding Of Skeletal Development.* Cell, 1995. 80(3): P. 371-8.

52. Friedenstein, A.J., R.K. Chailakhjan, And K.S. Lalykina, *The Development Of Fibroblast Colonies In Monolayer Cultures Of Guinea-Pig Bone Marrow And Spleen Cells.* Cell Tissue Kinet, 1970. 3(4): P. 393-403.

53. Friedenstein, A.J., *Precursor Cells Of Mechanocytes.* Int Rev Cytol, 1976. 47: P. 327-59.

54. Friedenstein, A.J., Et Al., *Precursors For Fibroblasts In Different Populations Of Hematopoietic Cells As Detected By The In Vitro Colony Assay Method.* Exp Hematol, 1974. 2(2): P. 83-92.

55. Friedenstein, A., *Osteogenic Stem Cells In The Bone Marrow.* Bone Miner Res, 1990. 7: P. 243–272

56. Owen, M., *Marrow Stromal Stem Cells.* J Cell Sci Suppl, 1988. 10: P. 63-76.

57. Caplan, A.I., *Mesenchymal Stem Cells.* J Orthop Res, 1991. 9(5): P. 641-50.

58. Beresford, J.N., *Osteogenic Stem Cells And The Stromal System Of Bone And Marrow.* Clin Orthop Relat Res, 1989(240): P. 270-80.

59. Pittenger, M.F., Et Al., *Multilineage Potential Of Adult Human Mesenchymal Stem Cells.* Science, 1999. 284(5411): P. 143-7.

60. Tenenbaum, H.C. And J.N. Heersche, *Dexamethasone Stimulates Osteogenesis In Chick Periosteum In Vitro.* Endocrinology, 1985. 117(5): P. 2211-7.

61. Ashton, B.A., Et Al., *Characterization Of Cells With High Alkaline Phosphatase Activity Derived From Human Bone And Marrow: Preliminary Assessment Of Their Osteogenicity.* Bone, 1985. 6(5): P. 313-9.

62. Mark, M.P., Et Al., *A Comparative Immunocytochemical Study On The Subcellular Distributions Of 44 Kda Bone Phosphoprotein And Bone Gamma-Carboxyglutamic Acid (Gla)-Containing Protein In Osteoblasts.* J Bone Miner Res, 1987. 2(4): P. 337-46.

63. Pritchard, J.J., *A Cytological And Histochemical Study Of Bone And Cartilage Formation In The Rat.* J Anat, 1952. 86(3): P. 259-77.

64. Brown, E.M., *Extracellular Ca2+ Sensing, Regulation Of Parathyroid Cell Function, And Role Of Ca2+ And Other Ions As Extracellular (First) Messengers.* Physiol Rev, 1991. 71(2): P. 371-411.

65. Corral, D.A., Et Al., *Dissociation Between Bone Resorption And Bone Formation In Osteopenic Transgenic Mice.* Proc Natl Acad Sci U S A, 1998. 95(23): P. 13835-40.

66. Franceschi, R.T. And G. Xiao, *Regulation Of The Osteoblast-Specific Transcription Factor, Runx2: Responsiveness To Multiple Signal Transduction Pathways.* J Cell Biochem, 2003. 88(3): P. 446-54.

67. Otto, F., Et Al., *Cbfa1, A Candidate Gene For Cleidocranial Dysplasia Syndrome, Is Essential For Osteoblast Differentiation And Bone Development.* Cell, 1997. 89(5): P. 765-71.

68. Komori, T., Et Al., *Targeted Disruption Of Cbfa1 Results In A Complete Lack Of Bone Formation Owing To Maturational Arrest Of Osteoblasts.* Cell, 1997. 89(5): P. 755-64.

69. Maruyama, Z., Et Al., *Runx2 Determines Bone Maturity And Turnover Rate In Postnatal Bone Development And Is Involved In Bone Loss In Estrogen Deficiency.* Dev Dyn, 2007. 236(7): P. 1876-90.

70. Woolf, A.D., Dixon, A., *Osteoporosis-A Clinical Guide.* Dunitz Ltd:Uk, 1998.

71. Lacey, D.L., Et Al., *Osteoprotegerin Ligand Is A Cytokine That Regulates Osteoclast Differentiation And Activation.* Cell, 1998. 93(2): P. 165-76.

72. Burgess, T.L., Et Al., *The Ligand For Osteoprotegerin (Opgl) Directly Activates Mature Osteoclasts.* J Cell Biol, 1999. 145(3): P. 527-38.

73. Miller, S.C. And W.S. Jee, *The Bone Lining Cell: A Distinct Phenotype?* Calcif Tissue Int, 1987. 41(1): P. 1-5.

74. Chambers, T.J. And K. Fuller, *Bone Cells Predispose Bone Surfaces To Resorption By Exposure Of Mineral To Osteoclastic Contact.* J Cell Sci, 1985. 76: P. 155-65.

75. Miller, S.C., Et Al., *Bone Lining Cells: Structure And Function.* Scanning Microsc, 1989. 3(3): P. 953-60; Discussion 960-1.

76. Bonewald, L.F., *Establishment And Characterization Of An Osteocyte-Like Cell Line, Mlo-Y4.* J Bone Miner Metab, 1999. 17(1): P. 61-5.

77. Schiller, P.C., Et Al., *Gap-Junctional Communication Is Required For The Maturation Process Of Osteoblastic Cells In Culture.* Bone, 2001. 28(4): P. 362-9.

78. Plotkin, L.I., S.C. Manolagas, And T. Bellido, *Transduction Of Cell Survival Signals By Connexin-43 Hemichannels.* J Biol Chem, 2002. 277(10): P. 8648-57.

79. Cheng, B., Et Al., *Expression Of Functional Gap Junctions And Regulation By Fluid Flow In Osteocyte-Like Mlo-Y4 Cells.* J Bone Miner Res, 2001. 16(2): P. 249-59.

80. Owan, I., Et Al., *Mechanotransduction In Bone: Osteoblasts Are More Responsive To Fluid Forces Than Mechanical Strain.* Am J Physiol, 1997. 273(3 Pt 1): P. C810-5.

81. Holmbeck, K., Et Al., *The Metalloproteinase Mt1-Mmp Is Required For Normal Development And Maintenance Of Osteocyte Processes In Bone.* J Cell Sci, 2005. 118(Pt 1): P. 147-56.

82. Zhao, S., Et Al., *Mlo-Y4 Osteocyte-Like Cells Support Osteoclast Formation And Activation.* J Bone Miner Res, 2002. 17(11): P. 2068-79.

83. You, L., Et Al., *Osteocytes As Mechanosensors In The Inhibition Of Bone Resorption Due To Mechanical Loading.* Bone, 2008. 42(1): P. 172-9.

84. Aguirre, J.I., Et Al., *Osteocyte Apoptosis Is Induced By Weightlessness In Mice And Precedes Osteoclast Recruitment And Bone Loss.* J Bone Miner Res, 2006. 21(4): P. 605-15.

85. Frost, H.M., *Some Abc's Of Skeletal Pathophysiology. 6. The Growth/Modeling/Remodeling Distinction.* Calcif Tissue Int, 1991. 49(5): P. 301-2.

86. Seeman, E., *Osteocytes--Martyrs For Integrity Of Bone Strength.* Osteoporos Int, 2006. 17(10): P. 1443-8.

87. Bitar, M., Et Al., *Effect Of Cell Density On Osteoblastic Differentiation And Matrix Degradation Of Biomimetic Dense Collagen Scaffolds.* Biomacromolecules, 2008. 9(1): P. 129-35.

88. Martin, T.J. And E. Seeman, *New Mechanisms And Targets In The Treatment Of Bone Fragility.* Clin Sci (Lond), 2007. 112(2): P. 77-91.

89. Kong, Y.Y., Et Al., *Opgl Is A Key Regulator Of Osteoclastogenesis, Lymphocyte Development And Lymph-Node Organogenesis.* Nature, 1999. 397(6717): P. 315-23.

90. Suda, M., Et Al., *C-Type Natriuretic Peptide As An Autocrine/Paracrine Regulator Of Osteoblast. Evidence For Possible Presence Of Bone Natriuretic Peptide System.* Biochem Biophys Res Commun, 1996. 223(1): P. 1-6.

91. Meikle, M.C., Et Al., *Human Osteoblasts In Culture Synthesize Collagenase And Other Matrix Metalloproteinases In Response To Osteotropic Hormones And Cytokines.* J Cell Sci, 1992. 103 (Pt 4): P. 1093-9.

92. Davies, J., Et Al., *The Osteoclast Functional Antigen, Implicated In The Regulation Of Bone Resorption, Is Biochemically Related To The Vitronectin Receptor.* J Cell Biol, 1989. 109(4 Pt 1): P. 1817-26.

93. Baron, R., *Molecular Mechanisms Of Bone Resorption: Therapeutic Implications.* Rev Rhum Engl Ed, 1996. 63(10): P. 633-8.

94. Everts, V., Et Al., *Degradation Of Collagen In The Bone-Resorbing Compartment Underlying The Osteoclast Involves Both Cysteine-Proteinases And Matrix Metalloproteinases.* J Cell Physiol, 1992. 150(2): P. 221-31.

95. Hughes, D.E., Et Al., *Bisphosphonates Promote Apoptosis In Murine Osteoclasts In Vitro And In Vivo.* J Bone Miner Res, 1995. 10(10): P. 1478-87.

96. Tran Van, P., A. Vignery, And R. Baron, *An Electron-Microscopic Study Of The Bone-Remodeling Sequence In The Rat.* Cell Tissue Res, 1982. 225(2): P. 283-92.

97. Heinemann, D.E., Et Al., *Alkaline Phosphatase Expression During Monocyte Differentiation. Overlapping Markers As A Link Between Monocytic Cells, Dendritic Cells, Osteoclasts And Osteoblasts.* Immunobiology, 2000. 202(1): P. 68-81.

98. Kraal, G., M. Rep, And M. Janse, *Macrophages In T And B Cell Compartments And Other Tissue Macrophages Recognized By Monoclonal Antibody Moma-2. An Immunohistochemical Study.* Scand J Immunol, 1987. 26(6): P. 653-61.

99. Newby, A.C., *Metalloproteinase Expression In Monocytes And Macrophages And Its Relationship To Atherosclerotic Plaque Instability.* Arterioscler Thromb Vasc Biol, 2008. 28(12): P. 2108-14.

100. Takahashi, F., Et Al., *Osteopontin Is Strongly Expressed By Alveolar Macrophages In The Lungs Of Acute Respiratory Distress Syndrome.* Lung, 2004. 182(3): P. 173-85.

101. Pfeilschifter, J., Et Al., *Chemotactic Response Of Osteoblastlike Cells To Transforming Growth Factor Beta.* J Bone Miner Res, 1990. 5(8): P. 825-30.

102. Hill, P.A., A. Tumber, And M.C. Meikle, *Multiple Extracellular Signals Promote Osteoblast Survival And Apoptosis.* Endocrinology, 1997. 138(9): P. 3849-58.

103. Takahashi, N., N. Udagawa, And T. Suda, *A New Member Of Tumor Necrosis Factor Ligand Family, Odf/Opgl/Trance/Rankl, Regulates Osteoclast Differentiation And Function.* Biochem Biophys Res Commun, 1999. 256(3): P. 449-55.

104. Nakagawa, N., Et Al., *Basic Fibroblast Growth Factor Inhibits Osteoclast Formation Induced By 1alpha,25-Dihydroxyvitamin D(3) Through Suppressing The Production Of Osteoclast Differentiation Factor.* Biochem Biophys Res Commun, 1999. 265(1): P. 45-50.

105. Wong, B.R., Et Al., *Trance (Tumor Necrosis Factor [Tnf]-Related Activation-Induced Cytokine), A New Tnf Family Member Predominantly Expressed In T Cells, Is A Dendritic Cell-Specific Survival Factor.* J Exp Med, 1997. 186(12): P. 2075-80.

106. Yasuda, H., Et Al., *Osteoclast Differentiation Factor Is A Ligand For Osteoprotegerin/Osteoclastogenesis-Inhibitory Factor And Is Identical To Trance/Rankl.* Proc Natl Acad Sci U S A, 1998. 95(7): P. 3597-602.

107. Fuller, K., Et Al., *Trance Is Necessary And Sufficient For Osteoblast-Mediated Activation Of Bone Resorption In Osteoclasts.* J Exp Med, 1998. 188(5): P. 997-1001.

108. Vidal, N.O., Et Al., *Osteoprotegerin Mrna Is Expressed In Primary Human Osteoblast-Like Cells: Down-Regulation By Glucocorticoids.* J Endocrinol, 1998. 159(1): P. 191-5.

109. Mizuno, A., Et Al., *Severe Osteoporosis In Mice Lacking Osteoclastogenesis Inhibitory Factor/Osteoprotegerin.* Biochem Biophys Res Commun, 1998. 247(3): P. 610-5.

110. Hakeda, Y., Et Al., *Osteoclastogenesis Inhibitory Factor (Ocif) Directly Inhibits Bone-Resorbing Activity Of Isolated Mature Osteoclasts.* Biochem Biophys Res Commun, 1998. 251(3): P. 796-801.

111. Wittrant, Y., Et Al., *Osteoprotegerin Differentially Regulates Protease Expression In Osteoclast Cultures.* Biochem Biophys Res Commun, 2002. 293(1): P. 38-44.

112. Theoleyre, S., Et Al., *The Molecular Triad Opg/Rank/Rankl: Involvement In The Orchestration Of Pathophysiological Bone Remodeling.* Cytokine Growth Factor Rev, 2004. 15(6): P. 457-75.

113. Hofbauer, L.C., Et Al., *The Roles Of Osteoprotegerin And Osteoprotegerin Ligand In The Paracrine Regulation Of Bone Resorption.* J Bone Miner Res, 2000. 15(1): P. 2-12.

114. Juppner H, G.T., Brown Em, Et Al, *Parathyroid Hormone And Parathyroid Hormone-Related Peptide In The Regulation Of Calcium Homeostasis And Bone Development.* Endocrinology, 2006: P. 1377-417.

115. Kemper, B., Et Al., *Parathyroid Secretion: Discovery Of A Major Calcium-Dependent Protein.* Science, 1974. 184(133): P. 167-9.

116. Brown, E.M., Et Al., *Calcium-Ion-Sensing Cell-Surface Receptors.* N Engl J Med, 1995. 333(4): P. 234-40.

117. Shigematsu, T., Et Al., *Human Parathyroid Hormone Inhibits Renal 24-Hydroxylase Activity Of 25-Hydroxyvitamin D3 By A Mechanism Involving Adenosine 3',5'-Monophosphate In Rats.* Endocrinology, 1986. 118(4): P. 1583-9.

118. Garabedian, M., Et Al., *Control Of 25-Hydroxycholecalciferol Metabolism By Parathyroid Glands.* Proc Natl Acad Sci U S A, 1972. 69(7): P. 1673-6.

119. Brenza, H.L., Et Al., *Parathyroid Hormone Activation Of The 25-Hydroxyvitamin D3-1alpha-Hydroxylase Gene Promoter.* Proc Natl Acad Sci U S A, 1998. 95(4): P. 1387-91.

120. Deluca, H.F. And C. Zierold, *Mechanisms And Functions Of Vitamin D.* Nutr Rev, 1998. 56(2 Pt 2): P. S4-10; Discussion S 54-75.

121. Halloran, B.P. And H.F. Deluca, *Appearance Of The Intestinal Cytosolic Receptor For 1,25-Dihydroxyvitamin D3 During Neonatal Development In The Rat.* J Biol Chem, 1981. 256(14): P. 7338-42.

122. Weisinger, J.R., Et Al., *Regulation Of 1,25-Dihydroxyvitamin D3 By Calcium In The Parathyroidectomized, Parathyroid Hormone-Replete Rat.* J Bone Miner Res, 1989. 4(6): P. 929-35.

123. Lobaugh, B., Et Al., *Parathyroidectomy Abolishes The Increase Of Renal 25-Hydroxyvitamin D-1 Alpha-Hydroxylase In Lactating Rats.* Am J Physiol, 1993. 264(6 Pt 1): P. E981-5.

124. Holick, M.F., *Environmental Factors That Influence The Cutaneous Production Of Vitamin D.* Am J Clin Nutr, 1995. 61(3 Suppl): P. 638s-645s.

125. Holick, M.F., *Mccollum Award Lecture, 1994: Vitamin D--New Horizons For The 21st Century.* Am J Clin Nutr, 1994. 60(4): P. 619-30.

126. Omdahl, J.L., Et Al., *Overview Of Regulatory Cytochrome P450 Enzymes Of The Vitamin D Pathway.* Steroids, 2001. 66(3-5): P. 381-9.

127. Henry, H.L., Et Al., *The Cellular And Molecular Regulation Of 1,25(Oh)2d3 Production.* J Steroid Biochem Mol Biol, 1992. 41(3-8): P. 401-7.

128. Omdahl, J.L., H.A. Morris, And B.K. May, *Hydroxylase Enzymes Of The Vitamin D Pathway: Expression, Function, And Regulation.* Annu Rev Nutr, 2002. 22: P. 139-66.

129. Cochran, D.L., Et Al., *Effects Of Platelet-Derived Growth Factor Isoforms On Calcium Release From Neonatal Mouse Calvariae.* Bone, 1993. 14(1): P. 53-8.

130. Atkins, G.J., Et Al., *Rankl Expression Is Related To The Differentiation State Of Human Osteoblasts.* J Bone Miner Res, 2003. 18(6): P. 1088-98.

131. Rickard, D.J., I. Kazhdan, And P.S. Leboy, *Importance Of 1,25-Dihydroxyvitamin D3 And The Nonadherent Cells Of Marrow For Osteoblast Differentiation From Rat Marrow Stromal Cells.* Bone, 1995. 16(6): P. 671-8.

132. Matsumoto, K., *Studies On Bone Marrow Cells In Experimental Animals: Bone Marrow Testing In The Safety Study.* Jikken Dobutsu, 1991. 40(1): P. 17-26.

133. Atkins, G.J., Et Al., *Metabolism Of Vitamin D3 In Human Osteoblasts: Evidence For Autocrine And Paracrine Activities Of 1 Alpha,25-Dihydroxyvitamin D3.* Bone, 2007. 40(6): P. 1517-28.

134. Aubin, J.E., Et Al., *Osteoblast And Chondroblast Differentiation.* Bone, 1995. 17(2 Suppl): P. 77s-83s.

135. Stein, G.S. And J.B. Lian, *Molecular Mechanisms Mediating Proliferation/Differentiation Interrelationships During Progressive Development Of The Osteoblast Phenotype.* Endocr Rev, 1993. 14(4): P. 424-42.

136. Stein, G.S., Et Al., *Transcriptional Control Of Osteoblast Growth And Differentiation.* Physiol Rev, 1996. 76(2): P. 593-629.

137. Vacher-Lavenu, M.C., Et Al., *[Tubular Filamentous Intranuclear Inclusions On Multinucleated Cells Of Bone Giant Cell Tumors]*. C R Seances Acad Sci Iii, 1981. 293(11): P. 639-44.

138. Yoshida, H., Et Al., *Enzyme Histochemical Study On Bone Tumors.* Acta Med Okayama, 1982. 36(6): P. 469-82.

139. Garimella, R., Et Al., *Nature Of Phosphate Substrate As A Major Determinant Of Mineral Type Formed In Matrix Vesicle-Mediated In Vitro Mineralization: An Ftir Imaging Study.* Bone, 2006. 38(6): P. 811-7.

140. Takeda, S., Et Al., *Stimulation Of Osteoclast Formation By 1,25-Dihydroxyvitamin D Requires Its Binding To Vitamin D Receptor (Vdr) In Osteoblastic Cells: Studies Using Vdr Knockout Mice.* Endocrinology, 1999. 140(2): P. 1005-8.

141. Anderson, P.H., Et Al., *Vitamin D Depletion Induces Rankl-Mediated Osteoclastogenesis And Bone Loss In A Rodent Model.* J Bone Miner Res, 2008. 23(11): P. 1789-97.

142. Nordin, B.E., *Bone Mass, Bone Loss, Bone Density And Fractures.* Osteoporos Int, 1993. 3 Suppl 1: P. 1-7.

143. Jesudason, D., Et Al., *Relationship Between Serum 25-Hydroxyvitamin D And Bone Resorption Markers In Vitamin D Insufficiency.* Bone, 2002. 31(5): P. 626-30.

144. Gray, T.K., G.E. Lester, And R.S. Lorenc, *Evidence For Extra-Renal 1 Alpha-Hydroxylation Of 25-Hydroxyvitamin D3 In Pregnancy.* Science, 1979. 204(4399): P. 1311-3.

145. Barbour, G.L., Et Al., *Hypercalcemia In An Anephric Patient With Sarcoidosis: Evidence For Extrarenal Generation Of 1,25-Dihydroxyvitamin D.* N Engl J Med, 1981. 305(8): P. 440-3.

146. Monkawa, T., Et Al., *Molecular Cloning Of Cdna And Genomic Dna For Human 25-Hydroxyvitamin D3 1 Alpha-Hydroxylase.* Biochem Biophys Res Commun, 1997. 239(2): P. 527-33.

147. Fu, G.K., Et Al., *Cloning Of Human 25-Hydroxyvitamin D-1 Alpha-Hydroxylase And Mutations Causing Vitamin D-Dependent Rickets Type 1.* Mol Endocrinol, 1997. 11(13): P. 1961-70.

148. Abreu, M.T., Et Al., *Measurement Of Vitamin D Levels In Inflammatory Bowel Disease Patients Reveals A Subset Of Crohn's Disease Patients With Elevated 1,25-Dihydroxyvitamin D And Low Bone Mineral Density.* Gut, 2004. 53(8): P. 1129-36.

149. Bareis, P., Et Al., *25-Hydroxy-Vitamin D Metabolism In Human Colon Cancer Cells During Tumor Progression.* Biochem Biophys Res Commun, 2001. 285(4): P. 1012-7.

150. Cross, H.S., Et Al., *Vitamin D Metabolism In Human Colon Adenocarcinoma-Derived Caco-2 Cells: Expression Of 25-Hydroxyvitamin D3-1alpha-Hydroxylase Activity And Regulation Of Side-Chain Metabolism.* J Steroid Biochem Mol Biol, 1997. 62(1): P. 21-8.

151. Wasserman, R.H., Et Al., *Intestinal Calcium Transport And Calcium Extrusion Processes At The Basolateral Membrane.* J Nutr, 1992. 122(3 Suppl): P. 662-71.

152. Xu, X., Et Al., *Depletion Of Intracellular Ca2+ Stores Activates Nitric-Oxide Synthase To Generate Cgmp And Regulate Ca2+ Influx.* J Biol Chem, 1994. 269(17): P. 12645-53.

153. Peng, J.B., E.M. Brown, And M.A. Hediger, *Apical Entry Channels In Calcium-Transporting Epithelia.* News Physiol Sci, 2003. 18: P. 158-63.

154. Darwish, H.M. And H.F. Deluca, *Analysis Of Binding Of The 1,25-Dihydroxyvitamin D3 Receptor To Positive And Negative Vitamin D Response Elements.* Arch Biochem Biophys, 1996. 334(2): P. 223-34.

155. Zaidi, M., B.S. Moonga, And E. Abe, *Calcitonin And Bone Formation: A Knockout Full Of Surprises.* J Clin Invest, 2002. 110(12): P. 1769-71.

156. Heersche, J.N., *Calcitonin Effects On Osteoclastic Resorption: The 'Escape Phenomenon' Revisited.* Bone Miner, 1992. 16(3): P. 174-7.

157. Chambers, T.J. And C.J. Magnus, *Calcitonin Alters Behaviour Of Isolated Osteoclasts.* J Pathol, 1982. 136(1): P. 27-39.

158. Kallio, D.M., P.R. Garant, And C. Minkin, *Ultrastructural Effects Of Calcitonin On Osteoclasts In Tissue Culture.* J Ultrastruct Res, 1972. 39(3): P. 205-16.

159. Akisaka, T. And C.V. Gay, *An Ultracytochemical Investigation Of Ouabain-Sensitive P-Nitrophenylphosphatase In Chick Osteoclasts.* Cell Tissue Res, 1986. 244(1): P. 57-62.

160. Moonga, B.S., Et Al., *Intracellular Regulation Of Enzyme Secretion From Rat Osteoclasts And Evidence For A Functional Role In Bone Resorption.* J Physiol, 1990. 429: P. 29-45.

161. Galante, L., Et Al., *Effect Of Calcitonin On Vitamin D Metabolism.* Nature, 1972. 238(5362): P. 271-3.

162. Lorenc, R., Et Al., *Lack Of Effect Of Calcitonin On The Regulation Of Vitamin D Metabolism In The Rat.* Endocrinology, 1977. 100(2): P. 468-72.

163. Morel, F., M. Imbert-Teboul, And D. Chabardes, *Distribution Of Hormone-Dependent Adenylate Cyclase In The Nephron And Its Physiological Significance.* Annu Rev Physiol, 1981. 43: P. 569-81.

164. Kawashima, H., S. Torikai, And K. Kurokawa, *Calcitonin Selectively Stimulates 25-Hydroxyvitamin D3-1 Alpha-Hydroxylase In Proximal Straight Tubule Of Rat Kidney.* Nature, 1981. 291(5813): P. 327-9.

165. Shinki, T., Et Al., *Calcitonin Is A Major Regulator For The Expression Of Renal 25-Hydroxyvitamin D3-1alpha-Hydroxylase Gene In Normocalcemic Rats.* Proc Natl Acad Sci U S A, 1999. 96(14): P. 8253-8.

166. Currey, J.D., *The Many Adaptations Of Bone.* J Biomech, 2003. 36(10): P. 1487-95.

167. Einhorn, T.A., *Bone Strength: The Bottom Line.* Calcif Tissue Int, 1992. 51(5): P. 333-9.

168. Turner, C.H. And D.B. Burr, *Basic Biomechanical Measurements Of Bone: A Tutorial.* Bone, 1993. 14(4): P. 595-608.

169. Einhorn, T.A., *Enhancement Of Fracture Healing.* Instr Course Lect, 1996. 45: P. 401-16.

170. Burr, D.B., Et Al., *Skeletal Change In Response To Altered Strain Environments: Is Woven Bone A Response To Elevated Strain?* Bone, 1989. 10(3): P. 223-33.

171. Currey, J.D., *How Well Are Bones Designed To Resist Fracture?* J Bone Miner Res, 2003. 18(4): P. 591-8.

172. Carter, D.R., Et Al., *Stress Fields In The Unplated And Plated Canine Femur Calculated From In Vivo Strain Measurements.* J Biomech, 1981. 14(1): P. 63-70.

173. Carter, D.R. And W.C. Hayes, *Bone Compressive Strength: The Influence Of Density And Strain Rate.* Science, 1976. 194(4270): P. 1174-6.

174. Rubin, C.R.A.J., *Bioemchanics And Mechanobiology Of Bone.* American Society For Bone And Mineral Research, 2006. Chapter 6.

175. Ruff, C.B. And W.C. Hayes, *Subperiosteal Expansion And Cortical Remodeling Of The Human Femur And Tibia With Aging.* Science, 1982. 217(4563): P. 945-8.

176. Sharir, A., M.M. Barak, And R. Shahar, *Whole Bone Mechanics And Mechanical Testing.* Vet J, 2008. 177(1): P. 8-17.

177. Brand, R.A., C.M. Stanford, And C.C. Swan, *How Do Tissues Respond And Adapt To Stresses Around A Prosthesis? A Primer On Finite Element Stress Analysis For Orthopaedic Surgeons.* Iowa Orthop J, 2003. 23: P. 13-22.

178. Frost, H.M., *Bone's Mechanostat: A 2003 Update.* Anat Rec A Discov Mol Cell Evol Biol, 2003. 275(2): P. 1081-101.

179. Cowin, S.C., Et Al., *On The Sufficiency Conditions For The Stability Of Bone Remodeling Equilibrium.* J Biomech, 1994. 27(2): P. 183-6.

180. Burr, D.B., A.G. Robling, And C.H. Turner, *Effects Of Biomechanical Stress On Bones In Animals.* Bone, 2002. 30(5): P. 781-6.

181. Ehrlich, P.J., Et Al., *The Effect Of In Vivo Mechanical Loading On Estrogen Receptor Alpha Expression In Rat Ulnar Osteocytes.* J Bone Miner Res, 2002. 17(9): P. 1646-55.

182. Lanyon, L.E., *Osteocytes, Strain Detection, Bone Modeling And Remodeling.* Calcif Tissue Int, 1993. 53 Suppl 1: P. S102-6; Discussion S106-7.

183. Narayanan, K., Et Al., *Dual Functional Roles Of Dentin Matrix Protein 1. Implications In Biomineralization And Gene Transcription By Activation Of Intracellular Ca2+ Store.* J Biol Chem, 2003. 278(19): P. 17500-8.

184. Gluhak-Heinrich, J., Et Al., *Mechanical Loading Stimulates Dentin Matrix Protein 1 (Dmp1) Expression In Osteocytes In Vivo.* J Bone Miner Res, 2003. 18(5): P. 807-17.

185. Feng, J.Q., Et Al., *Loss Of Dmp1 Causes Rickets And Osteomalacia And Identifies A Role For Osteocytes In Mineral Metabolism.* Nat Genet, 2006. 38(11): P. 1310-5.

186. Toyosawa, S., Et Al., *Dentin Matrix Protein 1 Is Predominantly Expressed In Chicken And Rat Osteocytes But Not In Osteoblasts.* J Bone Miner Res, 2001. 16(11): P. 2017-26.

187. Pedersen, E.A., Et Al., *Bone Response To In Vivo Mechanical Loading In C3h/Hej Mice.* Calcif Tissue Int, 1999. 65(1): P. 41-6.

188. Akhter, M.P., Et Al., *Characterization Of In Vivo Strain In The Rat Tibia During External Application Of A Four-Point Bending Load.* J Biomech, 1992. 25(10): P. 1241-6.

189. Turner, C.H., Et Al., *A Noninvasive, In Vivo Model For Studying Strain Adaptive Bone Modeling.* Bone, 1991. 12(2): P. 73-9.

190. Vico, L., Et Al., *Effects Of Long-Term Microgravity Exposure On Cancellous And Cortical Weight-Bearing Bones Of Cosmonauts.* Lancet, 2000. 355(9215): P. 1607-11.

191. Bloomfield, S.A., Et Al., *Site- And Compartment-Specific Changes In Bone With Hindlimb Unloading In Mature Adult Rats.* Bone, 2002. 31(1): P. 149-57.

192. Collet, P., Et Al., *Effects Of 1- And 6-Month Spaceflight On Bone Mass And Biochemistry In Two Humans.* Bone, 1997. 20(6): P. 547-51.

193. Frost, H.M., *The Mechanostat: A Proposed Pathogenic Mechanism Of Osteoporoses And The Bone Mass Effects Of Mechanical And Nonmechanical Agents.* Bone Miner, 1987. 2(2): P. 73-85.

194. Liu, D., S. Weiner, And H.D. Wagner, *Anisotropic Mechanical Properties Of Lamellar Bone Using Miniature Cantilever Bending Specimens.* J Biomech, 1999. 32(7): P. 647-54.

195. Van Der Meulen, M.C., K.J. Jepsen, And B. Mikic, *Understanding Bone Strength: Size Isn't Everything.* Bone, 2001. 29(2): P. 101-4.

196. Schriefer, J.L., Et Al., *A Comparison Of Mechanical Properties Derived From Multiple Skeletal Sites In Mice.* J Biomech, 2005. 38(3): P. 467-75.

197. Taylor, M.E., Et Al., *Stress And Strain Distribution Within The Intact Femur: Compression Or Bending?* Med Eng Phys, 1996. 18(2): P. 122-31.

198. Brzoska, M.M., K. Majewska, And J. Moniuszko-Jakoniuk, *Weakness In The Mechanical Properties Of The Femurs Of Growing Female Rats Exposed To Cadmium.* Arch Toxicol, 2005. 79(9): P. 519-30.

199. Sturmer, E.K., Et Al., *Standardized Bending And Breaking Test For The Normal And Osteoporotic Metaphyseal Tibias Of The Rat: Effect Of Estradiol, Testosterone, And Raloxifene.* J Bone Miner Res, 2006. 21(1): P. 89-96.

200. Jamsa, T., Et Al., *Comparison Of Three-Point Bending Test And Peripheral Quantitative Computed Tomography Analysis In The Evaluation Of The Strength Of Mouse Femur And Tibia.* Bone, 1998. 23(2): P. 155-61.

201. Jepsen, K.J., Et Al., *Bone Brittleness Varies With Genetic Background In A/J And C57bl/6j Inbred Mice.* J Bone Miner Res, 2001. 16(10): P. 1854-62.

202. Robling, A.G. And C.H. Turner, *Mechanotransduction In Bone: Genetic Effects On Mechanosensitivity In Mice.* Bone, 2002. 31(5): P. 562-9.

203. Brodt, M.D., C.B. Ellis, And M.J. Silva, *Growing C57bl/6 Mice Increase Whole Bone Mechanical Properties By Increasing Geometric And Material Properties.* J Bone Miner Res, 1999. 14(12): P. 2159-66.

204. Kaveh Pourakbar Saffar, N.J.A.S.M.R., *How Does The Bone Shaft Geometry Affect Its Bending Properties?* American Journal Of Applied Sciences 6 (3): 463-470, 2009, 2009. 6(3): P. 463-470.

205. Kaveh Pourakbar Saffar, N.J., Seyed Mohammad Rajaai *How Does The Bone Shaft Geometry Affect Its Bending Properties?* American Journal Of Applied Sciences, 2009.

206. Bramer, J.A., Et Al., *Representative Assessment Of Long Bone Shaft Biomechanical Properties: An Optimized Testing Method.* J Biomech, 1998. 31(8): P. 741-5.

207. Peter, C.P., Et Al., *Effect Of Alendronate On Fracture Healing And Bone Remodeling In Dogs.* J Orthop Res, 1996. 14(1): P. 74-9.

208. Lewandrowski, K.U., Et Al., *Incorporation Of Perforated And Demineralized Cortical Bone Allografts. Part Ii: A Mechanical And Histologic Evaluation.* Biomed Mater Eng, 2001. 11(3): P. 209-19.

209. An, Y.H.A.R.A.D., *Mechanical Testing Of Bone And The Bone Implant Interface.* Crc Press, Boca Raton, Fl., 1999.

210. Parfitt, A.M., Et Al., *Bone Histomorphometry: Standardization Of Nomenclature, Symbols, And Units. Report Of The Asbmr Histomorphometry Nomenclature Committee.* J Bone Miner Res, 1987. 2(6): P. 595-610.

211. Cuppone, M., Et Al., *The Longitudinal Young's Modulus Of Cortical Bone In The Midshaft Of Human Femur And Its Correlation With Ct Scanning Data.* Calcif Tissue Int, 2004. 74(3): P. 302-9.

212. Muller, R., Et Al., *Morphometric Analysis Of Human Bone Biopsies: A Quantitative Structural Comparison Of Histological Sections And Micro-Computed Tomography.* Bone, 1998. 23(1): P. 59-66.

213. Fritton, J.C., Et Al., *Loading Induces Site-Specific Increases In Mineral Content Assessed By Microcomputed Tomography Of The Mouse Tibia.* Bone, 2005. 36(6): P. 1030-8.

214. Muller, R., T. Hildebrand, And P. Ruegsegger, *Non-Invasive Bone Biopsy: A New Method To Analyse And Display The Three-Dimensional Structure Of Trabecular Bone.* Phys Med Biol, 1994. 39(1): P. 145-64.

215. Tam, C.S. And W. Anderson, *Tetracycline Labeling Of Bone In Vivo.* Calcif Tissue Int, 1980. 30(2): P. 121-5.

216. Vedi, S., J.R. Tighe, And J.E. Compston, *Measurement Of Total Resorption Surface In Iliac Crest Trabecular Bone In Man.* Metab Bone Dis Relat Res, 1984. 5(6): P. 275-80.

CHAPTER 2: MATERIALS AND METHODS

2.1 INTRODUCTION

Although many studies have performed mechanical testing on animals models, few have looked at the combined effects of dietary vitamin D and calcium on the bone structure and strength in rat models. This chapter describes a modified technique for mechanical testing; in particular, three-point bending as the most commonly used mechanical testing in rodents. Previous studies have examined the effects of vitamin D deficiency and ovariectomy by using a three point cantilever bending technique. They showed a reduction in ultimate stress in the femoral neck. This chapter describes three point bending at the anterior posterior position on the rat tibia. Furthermore, the dietary treatments, specimen collection procedures and the techniques used to measure the biochemical factors that are associated with calcium homeostasis and vitamin D metabolism are described.

2.2 MATERIALS

All chemicals and consumables used in the experiments were purchased from Sigma Chemical Company (Milwaukee, USA), unless otherwise stated.

2.3 ANIMALS

All animals used in the experiment were Sprague-Dawley rats obtained from the University of Adelaide animal house. Animals used to investigate the effects of varying levels of dietary vitamin D were male rats. Animals used to investigate the effects of dietary vitamin D and calcium were female rats. All animal procedures were approved by the Institute of Medical and Veterinary Science Animal Ethics Committee.

2.3.1 Housing

All animals were housed at 24°C with a 12-hour light/dark cycle. Animals that were raised as vitamin D-deplete (fed 0 IU/vitamin D) were exposed to incandescent lighting. All other animals were exposed to standard (is this fluorescent) lighting.

2.3.2 Diet

Rats were fed either a commercial rat chow containing 0.76% calcium, 0.46% available phosphorus and 2000U/Kg vitamin D (Milling Industries Pty Ltd., Adelaide, Australia) or tap water *ad libitum* unless otherwise stated. In dietary vitamin D and calcium studies, 40g per day of a modified AIN-93-VX semi-synthetic diet (American Institute of Nutrition 1977, American Institute of Nutrition 1980) where levels of dietary calcium and vitamin D were modified as specified in the relevant chapters.

2.3.3 Semi-synthetic diet

The semi synthetic diets were prepared in the laboratory according to a standard formula (Table 1). The components of the mineral mix were weighed, crushed and thoroughly mixed. Cornstarch, casein and cellulose were mixed for 30 minutes in a dough mixer (OEM, VE201, Bozzolo (MN) Italy). The remaining dry ingredients were added slowly and the mixture combined for a further 30 minutes. The ingredients of the mineral mix were weighed and crushed and manually mixed for 5 minutes before adding with the rest of the dry ingredients. Subsequently, the corn oil was drizzled into the mixture and combined for another 30 minutes. Finally distilled water was added to form a thick paste, which was spread 3 cm thick in trays and allowed to partially solidify before being cut into blocks and frozen at -20C.

Table 2.1 Components of the Semi-Synthetic Diet

Ingredient	(g/Kg mix)
Casein	200
Corn Starch	650
Cellulose	50
DL-Methionine	3
Choline Bitartrate	2
Mineral Mix*	35
AIN-93-VX Vitamin Mix[#]	10
Calcium Carbonate (0.1%-1%)	25-250
Corn oil	50

Table 2.2 Components of the Mineral Mix*

Ingredient	(g/Kg mix)
Sodium di-hydrogen phosphate ($2H_20$)	197
Potassium di-hydrogen phosphate	275
Potassium sulphate	52
Magnesium oxide	24
Manganous carbonate	3.5
Ferric citrate	6
Zinc carbonate	1.6
Cupric carbonate	0.3
Potassium iodate	0.01
Sodium selenite	0.01
Chromium potassium sulphate	0.55
Sucrose (finely powdered)	440.03

2.3.4 Fluorochrome Labelling Injections

Demeclocycline and calcein injections were used as the two flurochrome labels throughout all studies. All solutions were prepared on the day of administration. Demeclocycline (Lederle, Sydney, Australia) was prepared by adding 0.5g of declomycin, containing 0.3g of demeclocyline, to 10 mL of normal saline and stirring for 2 hours. The supernatant was decanted and 0.1mL/100g body weight was injected intraperitoneally at the dose of 30mg/kg 6 days prior to killing. Calcein (Sigma Chemical Co., St Louis, USA) was prepared by adding 0.2g of calcein and 0.4g of sodium bicarbonate to 10 mL of normal saline and stir for 30 minutes. 0.1mL/100g body weight of this solution was injected intraperitoneally at the dose of 20mg/kg 2 days prior to killing.

2.3.5 Blood sample collection

All blood samples were taken from the tail vein under halothane anaesthesia. 2.5mLs of blood was collected by removing 2mm from the tip of the tail with a scalpel. The blood was collected in tubes with a clotting activator and centrifuged at 3,500 rpm for 15 minutes to collect the serum. A 250µl aliquot of serum from each blood sample collected was stored separately for parathyroid hormone analysis. All serum samples were frozen at –20°C until required for analysis.

2.4 BLOOD BIOCHEMISTRY

2.4.1 Serum calcium and phosphate

Serum calcium was measured according to the method of Moorehead and Biggs (1974). Calcium reacts with cresolphthalein complexone in alkaline solution to form a purple coloured complex. The intensity of the purple colour formed is proportional to the calcium concentration and can be measured photometrically at 575nm. Serum calcium was measured on a Clinical chemistry analyser (Cobas Bio, IN, USA), using reagents manufactured by Trace Scientific (Melbourne, Australia).

2.4.2 Serum 1,25-dihydroxyvitamin D_3

Serum 1,25 dihydroxyvitamin D_3 (1,25D) was measured by a ^{125}I radioimmunoassay (RIA) (Immunodiagnostic Systems Ltd, Bolden, UK). The serum samples were delipidated and 1,25D extracted from potential cross-reactants by incubation with a highly specific, solid phase, monoclonal anti-1,25D antibody. The purified 1,25D eluate was incubated with a highly specific sheep anti-1,25D antibody. Separation of the antibody-bound tracer from the free tracer was achieved by a short incubation with anti-sheep IgG cellulose. The bound radioactivity was inversely proportional to the concentration of 1,25D. The minimum detectable concentration of the assay was 5pmol/L and at 118pmol/L, the inter-assay coefficient of variation was 5% at what concentration.

2.4.3 Serum 25-hydroxyvitamin D3

Serum 25 hydroxyvitamin D_3 (25D) was measured by a ^{125}I radioimmunoassay (RIA) (Immunodiagnostic Systems Ltd, Bolden, UK). This method involved the extraction of 25D, followed by incubation with both ^{125}I-25D and a highly specific sheep anti-25D-antibody. Separation of the antibody-bound tracer from the free tracer was achieved during a short incubation step with anti-sheep IgG cellulose. The bound radioactivity was inversely proportional to the concentration of 25D. The minimum detectable concentration of the assay was 3.0ηmol/L and at 136nmol/L. The inter-assay coefficient of variation was 7.3%.

2.4.4 Serum parathyroid hormone

Serum parathyroid hormone (PTH) was measured using a rat-specific, two-site immunoradiometric assay (IRMA) (Immutopics, Inc., San Clemente, CA USA). Both intact PTH (1-84 amino acids) and N-terminal PTH (1-34 amino acids) are immunologically bound by an immobilised antibody and a radiolabelled antibody, to form a sandwich complex. The levels of the radioactively bound

complex are then measured in a gamma counter (Crystal II, Multidetector RIA System, Packard Instruments Inc, Illinios, USA). The minimum detectable concentration of the assay was at 1.0 pg/mL and 50 pg/mL, the inter-assay coefficient of variation was 4%.

2.5 BONE HISTOLOGY

2.5.1 *Bone preparation*

Rat femora were removed from animals and defleshed before being placed in 10% formalin at 4°C for 2 hours. Femora were then cut using a slow speed saw (Beuhler, Ltd, Lake Bluff, USA) equipped with a diamond tipped blade bathed in 70% ethanol (van Moppes, Gloucester, UK), to expose the epiphyseal and metaphyseal regions. Samples were dehydrated in graded ethanol (70%, 90%, 2 x 100%) for 1 hour each, then transferred into two changes of methyl methacrylate (MMA) and 10% w/v K-Plast plasticiser (Medim, Giessen, Germany) each for 10-14 days. 4mL of the final embedding mixture containing MMA, 10% w/v plascticiser and 0.9% (w/v) K-Plast initiator (peroxydicarbonate – perkadox 16) (Medim, Giessen, Germany) was poured into 25mL polypropylene tubes and the cut bone surface facing down. The tubes were tightly capped and transferred to a 37°C water bath 24 hours polymerization in the fume hood. The embedded samples were cut from the tubes using a band saw and fixed to aluminium block holders (Bio-Rad, Sydney, Australia) with araldite glue (Selleys, Sydney, Australia).

Following embedding, the samples were trimmed to expose the sample area by removing and discarding 10um sections with a motorised microtome (Jüng K, Reichert, and Heidelberg, Germany). To expose the maximal epiphyseal and metaphyseal area, the samples were trimmed to the midline of the femur and tibia. The exposed sample area was then moistened with demineralised water and three consecutive 5um sections were cut and placed onto glass slides. Sections were flattened onto the slides following immersion in a 30:70 mixture of ethylene glycol mono-ethyl

ether (Merck, Kilsyth, Australia) and 70% ethanol (spreading solution), heated to 65-70ºC. Polyethylene plastic was dipped into the spreading solution before clamping to the slide with the cartridge paper. The slides were placed in the 37°C oven overnight. Prior to staining, MMA was removed by 15 minute immersions in 100% acetone. Sections were then dehydrated in ethanol (2 x 100%), cleared in xylene (2 x 100%) and mounted in xylene-based moutant Eukill (Kinder GmbH and co., Freiburg, Germany).

2.5.2 Von Kossa staining for calcium deposition with a haemotoxylin and eosin counterstain

MMA was removed from bone sections in acetone (see 2.5.1), which were then washed twice with distilled water and transferred to an aqueous solution of 1% silver nitrate and exposed to UV light for 60 minutes. Sections were then rinsed in distilled water and bathed in an aqueous solution of 2.5% sodium thiosulphate for 5 minutes. Following the final distilled water wash of the Von Kossa staining protocol, sections were stained in Lillie Mayer alum haematoxylin for 8 minutes and rinsed in demineralised water. The next step involved a differentiation in acid alcohol solution and bluing in a saturated aqueous solution of lithium carbonate. The appropriate end-point for this step was blue nuclei and basophilic cytoplasm over a colourless background, overstaining was reduced by further exposure to acid alcohol. Sections were then rinsed in running tap water for 30 seconds and stained with a 1% aqueous eosin solution for 4 minutes before mounting. The end point of this step was pink osteoid and strong staining of acidophilic cytoplasm, such as osteoclasts.

2.5.3 Tartrate resistant acid phosphatase (TRAcP) staining of osteoclasts

Sections were dewaxed 3 times in histolene for 10 minutes and rehydrated in graded ethanol (100%x2, 95%, 90%, 75%, 50%) and demineralised water for 3 minutes each. Fixative solution (25mL of citrate solution, 65mLs of acetone, 8mLs of 37% Formaldehyde) was prepared in a glass bottle. Fix slides by immersing in room temperature fixative solution for 30 seconds and rinse

thoroughly in prewarmed demineralised water. Diazotised Fast Garget GBC solution was made by adding 0.5mL of Fast Garnet GBC base solution to 0.5mL of sodium nitrite solution and inverted for 30 seconds. The next step involves adding 1mL of the above solution to a 100mL beaker with prewarmed demineralised water (45mLs), naphthol AS-BI phosphate solution (0.5mL), acetate solution (2mLs) and tartrate solution (1mL). After mixing the solutions were transferred to coplin jars in which slides were incubated for 1 hour at 37 degrees in water bath protected from light. Rinse the slides thoroughly in demineralised water and counterstain in Haematoxylin solution for 2 minutes and rinse in alkaline tap water for several minutes to blue nuclei. Finally air dry and evaluate microscopically.

2.6 BONE HISTOMORPHOMETRY

2.6.1 *Static and dynamic bone mineral measures*

Sequential sagittal sections cut to 5μm thickness were stained either by a modified von Kossa silver technique, Haematoxylin and Eosin stain (2.5.2), or left unstained for fluorochrome label (2.3.4). An Olympus BH-2 microscope (Olympus, Tokyo, Japan) interfaced with a Quantimet 520 Image Analysis System (Cambridge Instruments, Cambridge, UK) was used to measure the percentage of epiphyseal and metaphyseal trabecular bone volume within the bone marrow cavity (BV/TV%), trabecular thickness (Tb.Th) and trabecular number (Tb.N). Haematoxylin and Eosin stained sectioned were used to measure osteoid surface (O.Sur %) and osteoid thickness (O.Th). Unstained sections were used to measure inter-label distance and to calculate mineral apposition rate (MAR) and bone formation rate (BFR). Mineralization Lag Time (MLT) was derived from the measurements of O.Th and MAR.

2.6.2 *Three-dimensional analyses using micro-computed tomography*

Tibiae were removed from the -20 freezer and placed in a plastic tube for micro-CT analysis. The tibial micro-architecture was analysed using a high resolution micro-CT system (Skyscan 1076, Belgium) to obtain multiple x-ray transmission images. Cross sectional images of the object were then reconstructed by a modified Feldkamp cone-beam algorithm, creating a complete 3-D representation of internal microstructure with ring artefact reduction set at level 12 and beam hardening set to 20%. The cortical bone analysis was limited to a region of interest of ±4mm from the midpoint of the bone. The resolution of scanning was 18µm/pixel which provides sufficiently detailed information to calculate cortical bone volume.

In dietary vitamin D and calcium studies (Chapters 5 and 6), the micro-architecture of both femur and tibia were evaluated. Tibia and femur were analysed using the same method as detailed above with an alternate high resolution micro-CT system (Skyscan 1174, Belgium) at 12 um/pixel. The cortical bone analysis for femur was limited to a region of interest of ±2mm from the midpoint of the bone. The trabecular bone analysis for the proximal tibia and femur were limited to a region of interest of trabecular network 4mm below the intertrochanteric line. Bone BV/TV, Trabecular number, trabecular thickness, periosteal surface area (mm^2) and endosteal surface area (mm^2 were calculated from CTan software (v1.7, Skyscan, Belgium). Global threshold was used in the cortical bone analysis while adaptive threshold with the radius of 13 and constant of 10 were used in the trabecular bone analysis. Tibial sagittal cortical thickness (mm) is derived by measuring the cortical thickness in the mid shaft of the bone in the plane of mechanical loading. Tibia length was measured using digital callipers.

2.7 MECHANICAL TESTING OF BONE PROPERTIES

The mechanical properties of the tibiae were assessed by three-point bending method, performed by a miniature Instron materials testing machine (Instron 5848 MicroTester) with a 500N load cell. Prior to testing, tibiae were thawed in PBS at room temperature for 30 minutes and mid- point of the tibia determined using digital callipers. The lower anvil points were set at 20mm apart, equidistant from the mid-point for each bone. The bones were all pre conditioned at 1N of force before testing. The upper anvil was lowered on to the mid-point at a rate of 1.0mm/min for up to 5mm maximum deflection in the bone or until the bone failed. Results were collected in Wavemaker (version 9.1.00, Instron, Instron Corp., Canton, MA). From the force (F) versus displacement (D) curve we calculated, ultimate force to failure (ULF) and Young's modulus (E) (reference). E, defined as the intrinsic stiffness of the bone was based on the following calculation:

$$E = (F / D) \times (L3 / 48 \times I)$$

where: L = length of span

I = cross sectional moment of inertia, derived using Image J (Java 1.6.0_10, USA)

ULF was the maximum force required to break the bone. Breaking energy was obtained by calculating the area under the stress-strain curve, which defines the amount of energy needed to cause a fracture. Yield point is a boundary above which stress causes permanent damage to the bone structure and is defined as the point when the stress-strain curve become non-linear. In dietary vitamin D and calcium studies (Chapter 5 and 6) , three-point bending was performed on Test Resources 800LE4 test machine (Minnesota, USA) following the same procedure as discussed above. Results were collated using Microsoft office excel 2007.

2.8 TISSUE MESSENGER RNA ANALYSES

The kidney and soft tissues surrounding the organs were removed. The kidneys were placed in 5mL collection pots and snap frozen in liquid nitrogen and stored at –70°C until required for analysis. Slice of the kidney was removed and homogenised in 1mL of Trizol in a 2mL sterile eppendorf tubes.

2.8.1 *Extraction of total RNA*

The extraction of RNA from the tissue lysate was based on a modified method of Chomczynski and Sacchi (1987) and Davey et al (2000). The lysate in each tube was mixed by inversion, with 200uL of Chloroform for 15 seconds and incubated again at room temperature for 3 minutes. The aqueous phase, containing the RNA, was removed after centrifuging the mixture at 12,000 rpm at 4ºC for 15 minutes and mixed in a new tube with 500uL of isopropanol. The samples were left at room temperature for 10 minutes before centrifuging them at 12,000rpm at 4ºC for 15 minutes. The supernatant was removed and the RNA pellet washed with 1mL of 75% ethanol. The samples were re-centrifuged at 7,500 rpm at 4ºC for 5 minutes, dried at room temperature, and finally dissolved in 200μL of diethylpyrocarbonate (DEPC)-treated water.

The integrity of the extracted RNA was determined by firstly combining 4μL of RNA with 2μL of 0.2% bromophenol blue dye mix, 10uL of deionized formamide, 4uL of formaldehyde, 2uL of 10 x 3-(N-morpholino) propanesulfonic acid (MOPS) buffer and 2uL of diluted sybr gold. This mixture was then heated at 65°C for 10 minutes to denature the ribosomal RNA. The samples were then cooled immediately on ice and separated by electrophoresis in a 1% agarose gel (Promega Company, Annadale, Australia) in 10X MOPS running buffer and at 80 volts for 30 minutes. The gel was stained in a 1mg/L ethidium bromide solution and imaged by FluorImager 595 (Molecular Dynamics Inc., CA, USA) and ImageQuant version 3.3 software (Molecular Dynamics Inc., CA,

USA) to determine the integrity of the RNA. Pure, undegraded RNA is indicated by the clear presence of the ribosomal RNA bands 26s, 18s and 4s.

2.8.2 Quantification of messenger RNA

Purified RNA was quantified on a Nanodrop spectrophotometer (Beckman Instruments, CA, USA). 1uL of the RNA samples were measured at wavelengths of 260 and 280nm. The RNA concentration was automatically calculated using the following formula:

$$\text{RNA concentration } (\mu g/\mu L) = \text{Absorbance at 260nm} \times \frac{40}{1000}$$

Where absorbance at 260nm = 1 for a 40μg/mL RNA solution

The RNA was considered to be adequately pure when the ratio between A_{260} and A_{280} was 1.8 or higher (Sambrook et al 1989).

2.8.3 Synthesis of cDNA

Messenger RNA was reverse-transcribed to generate first strand cDNA. 4ug RNA was incubated with 1uL of 200ng oligo-dT primer (Geneworks, Adelaide, Australia) 1uL of reaction mixture of 1nM of dATP, dTTP, dGTP and dCTP (Geneworks, Adelaide, Australia) and deionised H_2O in a total volume of 14μL. The mixture was flickspinned and placed on the PCR machine at 65ºC for 5 minutes and quickly placed on ice until the machine has reached 4ºC. The volume of calculated RNA and deionised H_2O was added to reaction buffer (250mM Tris-HCL, pH 8.3, 375mM potassium chloride, 15mM $MgCl_2$), 10nM DTT and Superscript III reverse-transcriptase enzyme were and heated at 50°C for 60 minutes followed by 70°C for 5 minutes.

2.8.4 Quantitative Real Time Polymerase Chain Reaction (RT-PCR)

The mRNA of genes of interest was analysed by real-time RT-PCR using the SYBR Green incorporation technique. All PCR reactions were carried out in a final volume of 25 µL and were performed in duplicate for each cDNA. The reaction mix consisted of 12.5 µL of iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), cDNA specific forward and reverse primers (0.5 µl) (Geneworks, Australia), and dH2O (µL) and cDNA. Included in each PCR run were a no template control and DNA negative control for each primer set. Primers were designed using GenBank NIH genetic database sequences, Primer-BLAST and BLAST web-based software (NCBI, Bethseda MD, USA) to achieve mRNA specific amplification at consistent annealing temperatures and constant PCR conditions across all primer sets. Primer sets used are summarised in Table 2.3. PCR condition were as follows: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 sec and 60°C for 1 min; 72°C for 5 min followed by a melt-curve analysis where temperature gradually rose from 72°C to 95°C over 5 min. All PCR reactions were validated by the presence of a single predicted peak in the melt curve analysis. Relative expression of mRNA was determined using the comparative cycle threshold (CT) method.

Table 2.3: Primer sequences of Sense (S) and anti-sense (A) for each messenger RNA species of interest.

Gene	Sequence 5' →3'	Genbank® accession no.
CYP27B1	*S: TGCAGAGACTGGAATCAGATGTTTG* *A: CACTATGGACTGGACAGACACC*	*NM053763*
CYP24	*S: TTGAAAGCATCTGCCTTGTGT* *A: GTCACCATCATCTTCCCAAAC*	*NM201635*
CYP27A1	*S: ATGTGGCACATCTTCTCTACC* *A: GGGAAGGAAAGTGACATAGAC*	*NM178847*
CYP2R1	*S: CTTGGAGGCATATCAACTGTG* *A: ATCCATCCTCTGCCATATCTG*	*NM001108499*
CYP2J3	*S: CCTGGATTTTGCTAACATTC* *A: CTAAGCTCTTCTTTCCTAGT*	*NM175766*
CYP3A1	*S: GGAAATTCGATGTGGAGTGC* *A: AGGTTTGCCTTTCTCTTGCC*	*X64401*
CYP3A2	*S: AGTAGTGACGATTCCAACATAT* *A: TCAGAGGTATCTGTGTTTCCT*	*NM153312*
CYP3A9	*S: GGACGATTCTTGCTTACAGG* *A: ATGCTGGTGGGCTTGCCTTC*	*NM147206*
CYP3A11	*S: GACAAACAAGCAGGGATGGAC* *A: CCAAGCTGATTGCTAGGAGCA*	*NM007818*
β-*Actin*	*S: ATCATGTTTGAGACCTTCAAC* *A: CTTGATCTTCATGGTGCTAG*	*AF541940*

2.9 STATISTICAL ANALYSES

2.9.1 *One-way analysis of variance*

One-way analysis of variance was used to analyse the effect of varying levels of vitamin D on bone strength. The data were analysed in Microsoft Excel 2000 (version 9.0.2720), which was run on a personal computer. A value of $p<0.05$ was considered to be statistically significant.

2.9.2 *Two-way analysis of variance*

Two-way analysis of variance was used to analyse the interactions of the effects of dietary calcium and Vitamin D treatment on bone structure and strength. The data were analysed in Statistical Package for the Social Sciences (SPSS) (version 9.0.2720), which was run on a personal computer. A value of $p<0.05$ was considered to be statistically significant.

2.9.3 *Tukey's post-hoc test*

A Tukey's post-hoc test was used to identify the mean values that were significantly different from each other within a data set that had been found to be statistically significant with either a one- or two-way analysis of variance. The data were analysed in SPSS program, which was run on a personal computer.

2.9.4 *Linear and multiple-linear regression analysis*

Linear regression analysis was used, based on the "least squares" method to fit a line of-best-fit through a set of observations and to obtain a coefficient of determination (R^2) for the correlation between the observations recorded. A multiple linear regression analysis was used to identify the determinants of a particular dependent variable with two or more other variables when single variable demonstrated a significant linear relationship. The data were analysed in Microsoft Excel

2000, which was run on a personal computer. A value of $p<0.05$ was considered to be statistically significant.

CHAPTER 3: EVALUATION OF THE METHODOGIES FOR MECHANICAL STRENGTH TESTING OF RODENT TIBIA BONE.

3.1 INTRODUCTION

The continual innovation of new research tools for the evaluation and characterization of the skeleton has greatly broadened our understanding of bone physiology. Since it was suggested that bone structure and structural rigidity and strength represent the ultimate phenotype of the skeleton, structural strength testing becomes an important role in skeletal research [1]. The primary function of the skeleton is locomotion of the body and only adequate rigid and strong bones are functional. The most commonly used method for the characterization of lone bone biomechanical properties of the skeleton is method known as three point bending [2]. The primary end-point measure of this type of analysis is the failure load (strength) of given bone, where as the structural rigidity (stiffness) and the post-yield behaviour and fatigue characteristics are secondary measures [3]. The advantages for using this test include its ability to use small sized long bones for testing and the technical ease to reproduce each test. Given the long bones are generally irregularly shaped, none have the geometry and morphology of an ideal, straight forward mechanical test specimen. Previously, a study found that in terms of mechanical testing, the breaking force of tibia bone had the lowest coefficient of variation (7.3%) which may be attributed to fact that the shape of the tibia includes a relatively flat lateral surface along the shaft, which allows the ease of positioning during testing [4]. Despite this, the methods and the machinery for testing are likely to provide varied results with variances in the preparation of bones for testing trough to the positioning and loading of bones. Thus, this chapter focus on two aspects of validation of mechanical testing as based on the protocols described previously [5]. Firstly, as a study suggested that there were no significant differences in the bone mineral densities of the left and right proximal femur in women, we are further investigating whether the testing of one tibia could be used to predict the mechanical properties of the

contralateral tibia with sufficient accuracy. Secondly, we want to further investigate the relationship between bone mineral content and strength measurements. Effects of ovariectomy and vitamin D deficiency have been shown to have significant reductions in trabecular bone volume as well as bone mineral density when compared with sham animals. Furthermore, the loss of bone mineral density is accompanied by a significant reduction in ultimate moment and stiffness in the left femoral necks of the vitamin D deficient ovariectomised animals. Therefore, we want to test the quality of the bone by 3 point mechanical testing and subsequently investigate the effects of ovariectomy on bone volume and its relationship to strength.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Thirty male Sprague–Dawley rats were raised in an incandescent-lighted environment, and were maintained on vitamin D deficient semi-synthetic diet containing 1% calcium and 0.6% phosphorus as previously published. All animals were fed on their assigned diets from weaning until 10 weeks of age, at which point the animals were allocated to groups and pair-fed a modified AIN-93 diet (ICN Biomedicals, Aurora, OH, USA) containing 0.4% calcium and either 0, 2, 4, 8, 12 or 20 IU/day vitamin D. The animals were fed their assigned diets until 30 weeks of age and sacrificed. Both tibiae were removed and processed for micro-CT analysis and 3-point mechanical strength testing at time of death. All animal procedures were approved by the Institute of Medical and Veterinary Science Animal Ethics Committee.

In the validation of mechanical testing section, nine-month old female Sprague-Dawley rats (n=66) were either sham or ovariectomised and allocated to varying levels of vitamin D_3 (D) (0, 2, 12 and 20 IU/day) and either low (0.1%, LCa) or high (1%, HCa) dietary calcium based on the recommended semi-synthetic diet for rodents (AIN-93-VX, ICN, CA, USA).

3.2.2 Micro-computed topographical analyses

The micro-architecture of the tibia was evaluated using a high resolution micro-CT system (Skyscan 1076, Brussels, Belgium) to obtain multiple X-ray transmission images. Transverse CT slices were acquired at the tibial mid-shaft using 18 μm slice increment. An 8 mm region of cortical bone, located 4 mm above and below the mid-point of the tibia was used for cortical bone analyses.

3.2.3 Three-point mechanical strength testing of bone

The mechanical properties of the tibiae were assessed by three-point bending method, performed by a miniature Instron materials testing machine (Instron 5848 MicroTester) with a 500 N load cell. Prior to testing, tibiae were thawed in PBS at room temperature for 30 min and mid-point of the tibia determined using digital callipers. The lower anvil points were set at 20 mm apart, equidistant from the mid-point for each bone. The upper anvil was lowered on to the mid-point at a rate of 1.0 mm/min for up to 5 mm maximum deflection in the bone or until the bone failed. Results were collected in Wavemaker (version 9.1.00, Instron, Instron Corp., Canton, MA, USA). From the force (F) versus displacement (D) curve we calculated, ultimate force to failure (ULF) and Young's modulus (E). E, defined as the intrinsic stiffness of the bone was based on the following calculation: $E = (F/D) \times (L3/48 \times I)$, where L = length of span and I = cross-sectional moment of inertia, derived using Image J (Java 1.6.0_10, USA). ULF was the maximum force required to break the bone. Breaking energy was obtained by calculating the area under the stress–strain curve, which defines the amount energy needed to cause a fracture. Yield point is a boundary above which stress causes permanent damage to the bone structure and is defined as the point when the stress–strain curve become non-linear. Flexural Rigidity is the extrinsic stiffness of the bone and is equal to EI. The reproducibility of the biomechanical testing of the rat tibia was determined by comparing the data from the right and left tibia of sham rats.

3.2.4 Data expression and statistical analysis

One-way analysis of variance was used to analyse the effect of varying levels of dietary vitamin D on bone structure and strength. A value of $p<0.05$ was considered to be statistically significant. Multi-Linear regression analyses were performed on biochemical, morphological and biomechanical measures to determine interactions.

3.3 RESULTS

3.3.1 Reproducibility of measures of mechanical testing

Reproducibility of the mechanical strength parameters was tested by using both left and right tibiae of the rat. The R^2 value for flexural rigidity was the highest at 0.96. For Youngs Modulus and cross sectional moment of inertia the R^2 value was above 0.7. For ultimate load, the R^2 value was lower at 0.48. The plots for the left and right tibial are: ultimate load, $R^2=0.48$, $p<0.01$(Figure1), cross sectional moment of inertia $R^2=0.75$. $p<0.01$ (Figure 2), Young's Modulus $R^2=0.78$, $p<0.01$ (Figure 3) and Flexural Rigidity $R^2=0.96$, $p<0.01$ with a statistically excluded outlier (Figure 4). There was a high degree of reproducibility when analysed by the linear regression analysis. The intercept of the line-of-best-fit was not statistically different from 0 and the slope of the line-of-best-fit was not statistically different from 1. Therefore, we concluded that one tibia is enough to determine the biomechanical strength in rats.

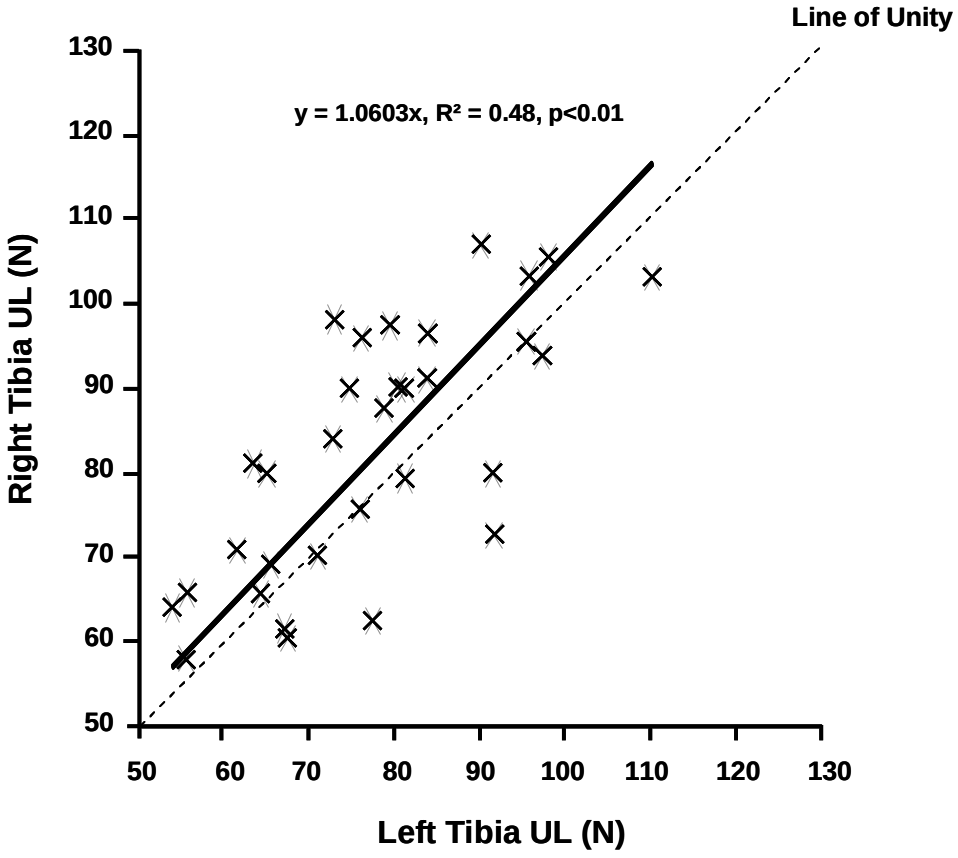


Figure 3.1 Reproducibility of Ultimate Load to Failure between measures of left and right tibia. The horizontal axis is the measure of left tibia UL and the vertical axis is the measure of right tibia UL. The equation for the line-of-best-fit, y= slope (±S.D.)x + y-intercept (±S.D.), and the coefficient of determination (R^2) are shown for the regression analyses. All linear regression analyses were not statistically different form the line-of-unity. UL, Ultimate Load to Failure; n = 30.

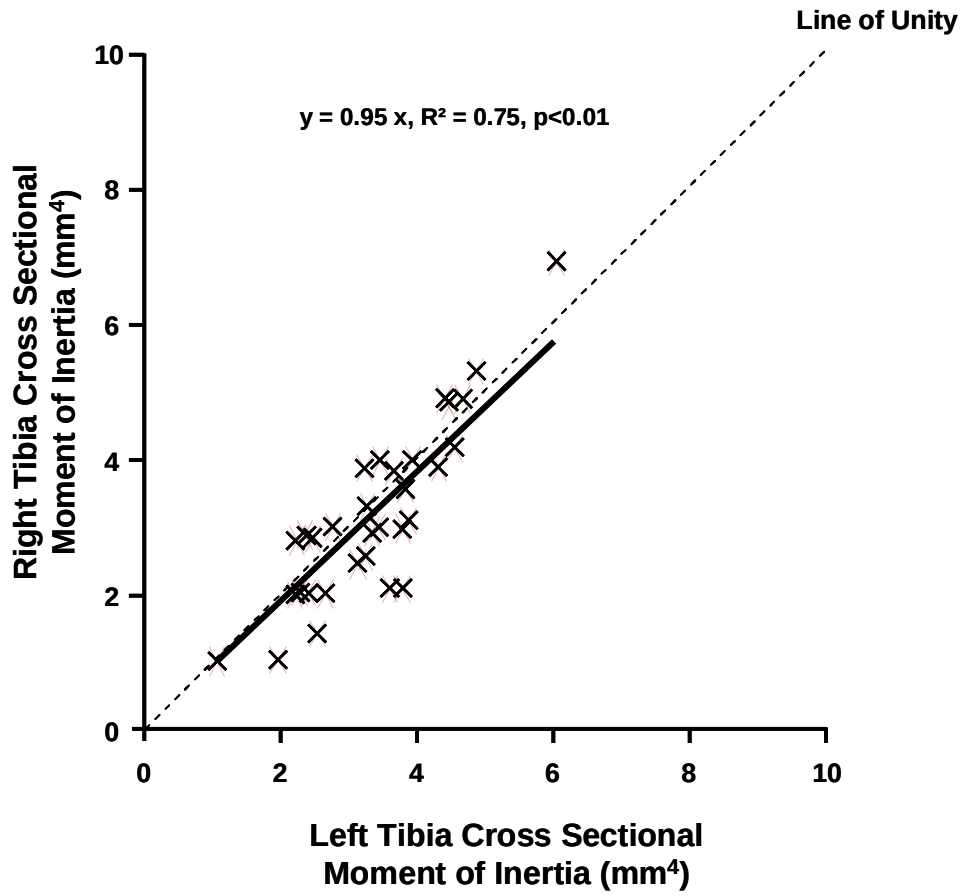


Figure 3.2 Reproducibility of cross sectional moment of inertia (I) between measures of left and right tibia. The horizontal axis is the measure of left tibia I and the vertical axis is the measure of right tibia I. The equation for the line-of-best-fit, y= slope (±S.D.)x + y-intercept (±S.D.), and the coefficient of determination (R^2) are shown for the regression analyses. All linear regression analyses were not statistically different form the line-of-unity. UL, Ultimate Load to Failure; n = 30.

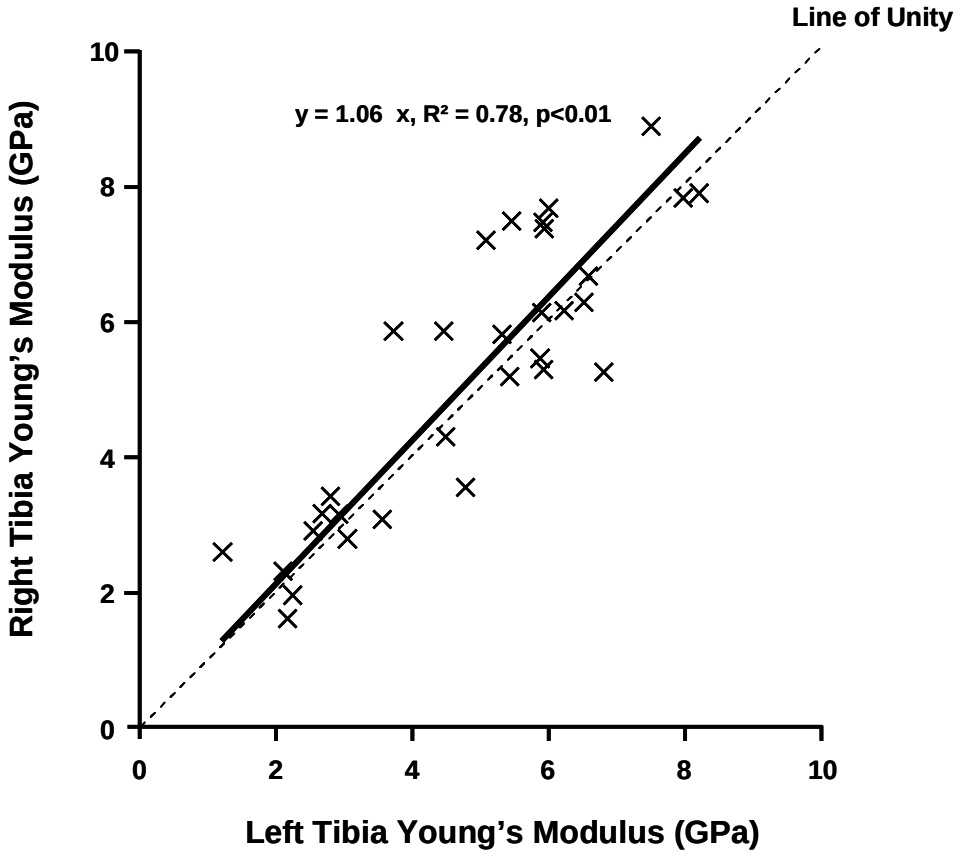


Figure 3.3 Reproducibility of Young's Modulus (E) between measures of left and right tibia. The horizontal axis is the measure of left tibiae and the vertical axis is the measure of right tibia E. The equation for the line-of-best-fit, y= slope (±S.D.)x + y-intercept (±S.D.), and the coefficient of determination (R^2) are shown for the regression analyses. All linear regression analyses were not statistically different form the line-of-unity. UL, Ultimate Load to Failure; n = 30.

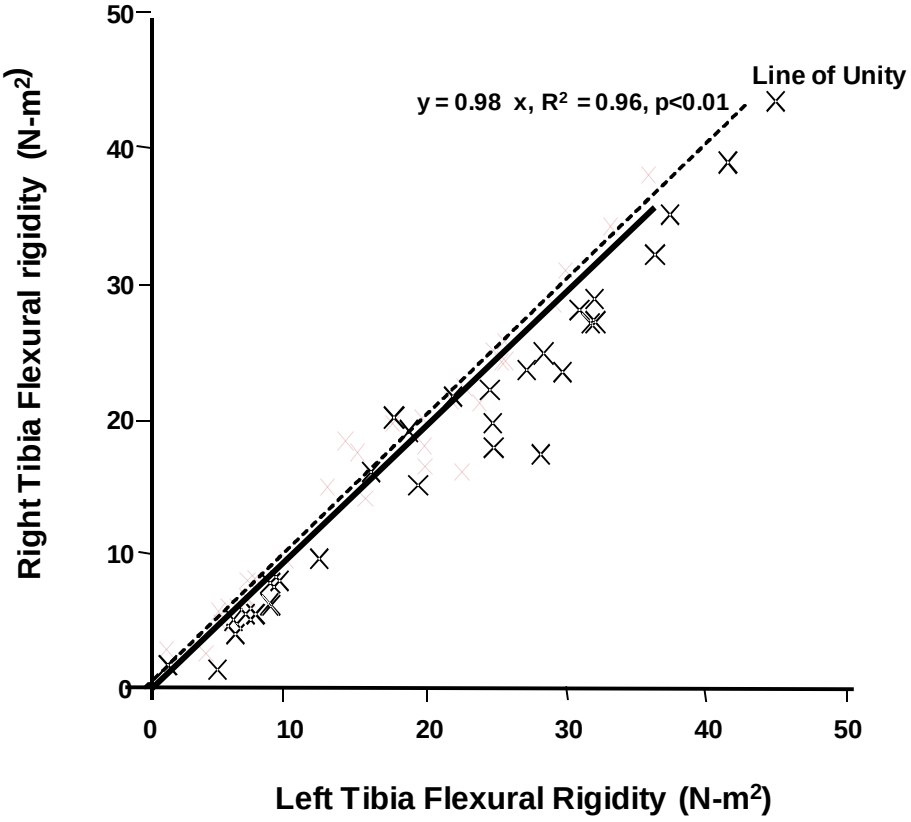


Figure 3.4 Reproducibility of flexural rigidity between measures of left and right tibia. The horizontal axis is the measure of left tibia flexural rigidity and the vertical axis is the measure of right tibia flexural rigidity. The equation for the line-of-best-fit, y= slope (±S.D.)x + y-intercept (±S.D.), and the coefficient of determination (R^2) are shown for the regression analyses. All linear regression analyses were not statistically different form the line-of-unity. UL, Ultimate Load to Failure; n = 30.

3.3.2 Validation of mechanical strength measures with bone histomorphometric analyses

3.3.2.1 Correlation between Cortical bone volume and Three-Point Testing

The tibial cortical bone volume ranged from 30.31mm3 to 42.85 mm3 in sham animals and from 28.19mm3 to 40.43mm3 in ovariectomised animals. To investigate the quality for our 3 point bending, we investigated the relationship between cortical bone volume and different mechanical parameters with both sham and ovariectomised animals. Cortical bone volume was significantly correlated to ultimate load (Figure 3.5) and flexural rigidity (Figure 3.6). There was no significant relationship observed between cortical bone volumes and cross sectional moment of inertia or Young's Modulus (Figure 3.7 and 3.8 respectively). These results suggested that although there was only a subtle reduction in tibial cortical bone volume in the ovariectomised animals, it can potentially impose significant effects on the mechanical strength. We have further verified that the three point bending test is a sensitive test in measuring the mechanical strength of long bones.

3.3.2.2 Evaluation of the Predictors of the Mechanical Parameters

Linear regressions with both sham and ovariectomised animals showed a statistically significant correlation between bone volume and ultimate load as well as flexural rigidity (Table 3.1). Both ultimate load and flexural rigidity were also shown to be predicted by sagittal cortical thickness as well as average cortical thickness. While there was no relationship between bone volume and the calculated Young's modulus, Young's modulus of tibia was best predicted by average cortical thickness (Table 3.1). Cross sectional moment of inertia was not predicted by any of the structural measurements.

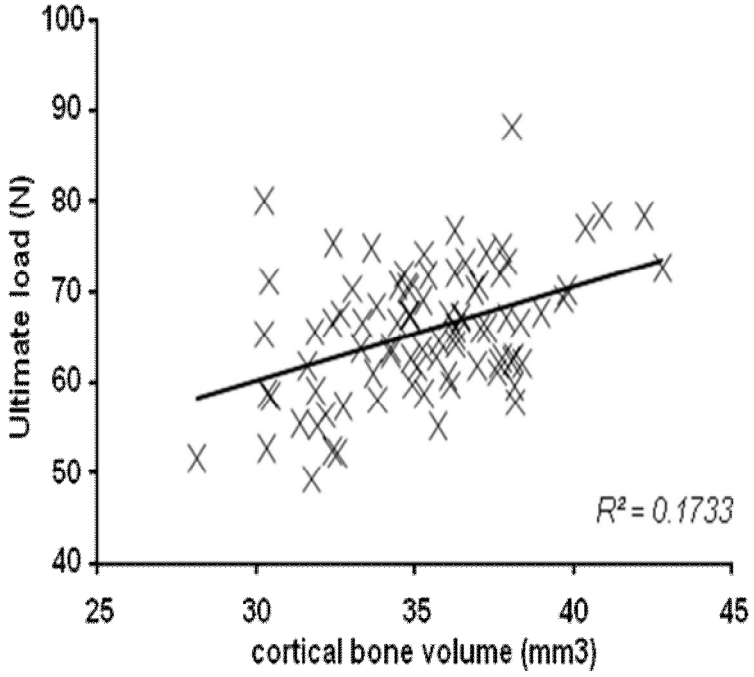


Figure 3.5 The relationship between tibial cortical bone volume and ultimate load

The horizontal axis is the measure of cortical bone volume and the vertical axis is the measure of ultimate load. The coefficient of determination (R^2) are shown for the regression analyses, $p<0.05$, n=66.

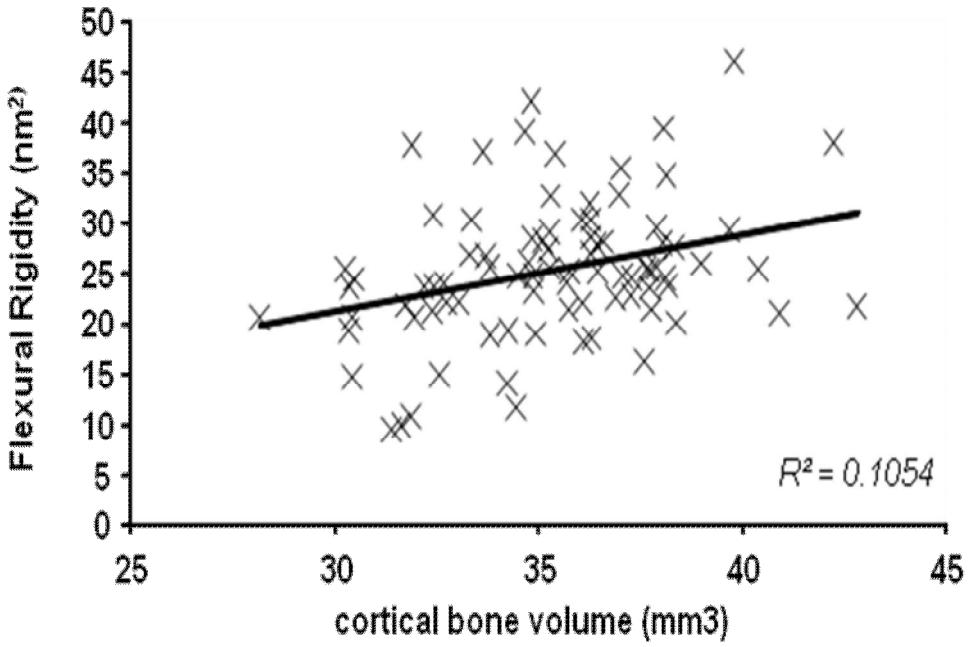


Figure 3.6 The relationship between tibial cortical bone volume and flexural rigidity

The horizontal axis is the measure of cortical bone volume and the vertical axis is the measure of flexural rigidity. The coefficient of determination (R^2) are shown for the regression analyses, $p<0.05$,n=66.

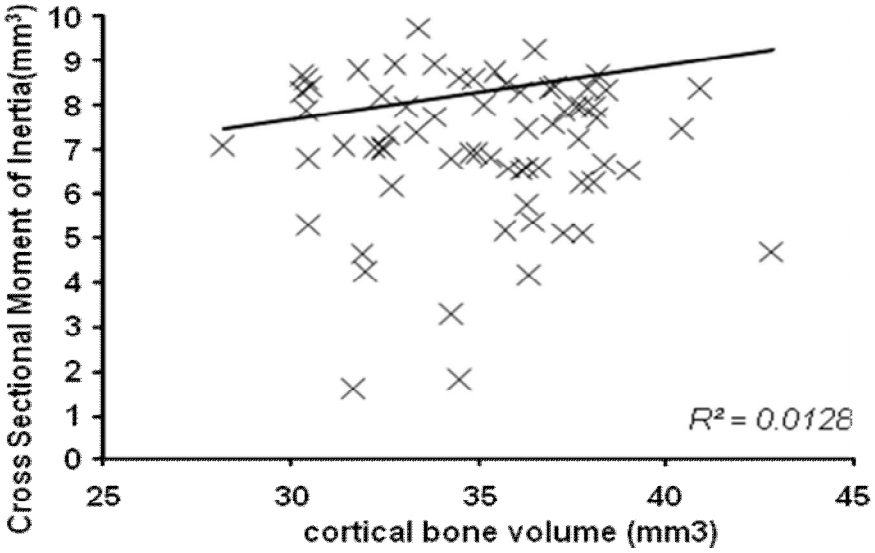


Figure 3.7 The relationship between tibial cortical bone volume and cross sectional moment of inertia.The horizontal axis is the measure of cortical bone volume and the vertical axis is the measure of cross sectional moment of inertia. The coefficient of determination (R^2) are shown for the regression analyse, n=66.

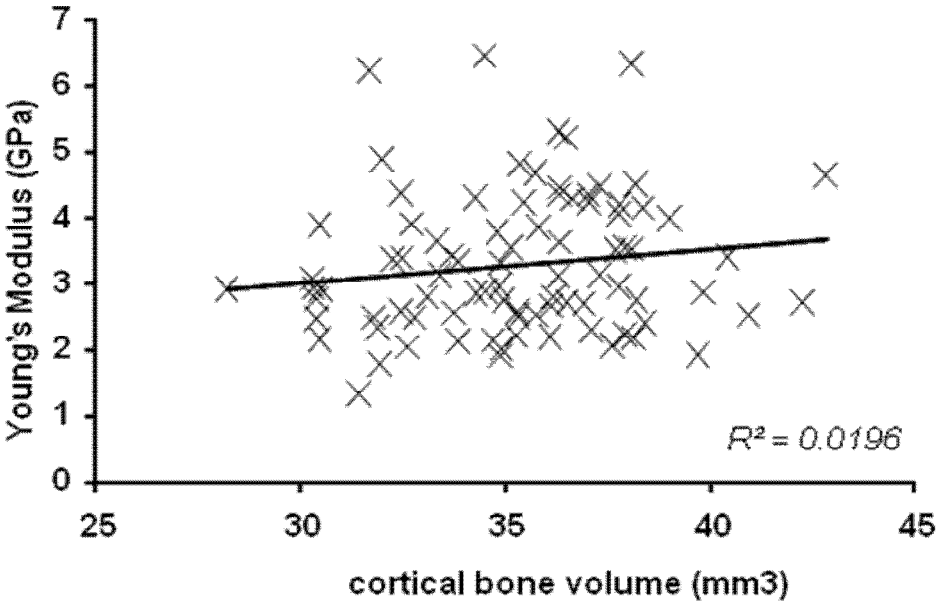


Figure 3.8 The relationship between tibial cortical bone volume and Young's Modulus.The horizontal axis is the measure of cortical bone volume and the vertical axis is the measure of cross sectional moment of inertia. The coefficient of determination (R^2) is shown for the regression analysis, n=66

Table 3.1 Summary of linear regression analyses between cortical bone mineral measurments and measures of mechanical properties of the tibia

	Ultimate Load		Young's Modulus		Flexural Rigidity		X-Sectional Moment of Inertia	
	R	P-value	R	P-value	R	P-value	R	P-value
Cortical BV	0.42	<0.01	0.14	0.2	0.32	<0.01	0.11	0.3
Cortical Th	0.21	0.05	0.19	0.08	0.3	0.19	0.09	0.41
Sagittal Cortical Th	0.57	<0.01	0.22	0.04	0.14	<0.01	0.01	0.89

3.4 DISCUSSION

The mechanical competence of bone represents the ultimate measure of bone quality. In this study, we have sought to determine if bone strength and strain measurements which represent the extrinsic and intrinsic qualities of bone strength differs between left and right tibia of the same animal. Using the 3 point bending procedure [3] with the mid-point of the tibia shaft as the focus for strength measures, we found that there was high reproducibility between left and right tibia in terms of all major measures of bone strength. Thus, our data provides significant evidence that 3-point mechanical strength testing on tibia from rodents is a reproducible and sensitive method which is not compromised by differences in sidedness under conditions tested in this thesis.

Consistent with our findings, previous studies have demonstrated no systematic differences in bone mineral density measurements between left and right femora [6]. Non-statistical differences in bone mineral density of less than 0.061 $g.cm^{-2}$ between left and right proximal femora have been reported and it was concluded that it is not necessary to measure both femurs. In addition, in another study [2], it has also been demonstrated that in general there is no systematic difference between the structural and mechanical characteristics of the right and left femur determined by 95% limits of agreement as well as the absolute measure of reproducibility. For example, the coefficient of variation for breaking load was determined to be 3.8%, which is consistent with data from our current study on the tibia. However, the coefficient of variation for energy absorption was 18.7% and it was concluded that energy absorption and stiffness showed poor repeatability when compared to breaking load. This is consistent with our current study which demonstrates that energy absorption, which showed a coefficient of determination value of 0.001(data not shown).

The fundamental principles in the execution of biomechanical testing have been extensively reviewed by Turner and Burr [7]. The reproducibility of any measurement is important as a poor reproducibility leads to excess variability in the data and thus jeopardise the validity of the data obtained. Therefore, the goal of this study was to compare the reproducibility of the biomechanical testing measurements between the left and right tibia. The coefficient of determination values we obtained for ultimate load (R^2=0.75), cross sectional moment of inertia (R^2=0.75), E (R^2=0.78) and flexural rigidity (R^2=0.96) between the left and right tibia have demonstrated that we were able to execute the testing with high reproducibility.

It is established that the strength and rigidity of the whole bone are the interaction of material properties, amount of material, morphological, organizational, and the whole [1]. Naturally, the degree of loading on a long bone largely determines whether the bone will suffer a break. If the magnitude of the stresses within the bone structure exceeds the capacity of bone material to withstand those stresses, the structure will fail and even relatively small deterioration of the bone structure or material can be detrimental. We have assessed the mechanical competence of rat tibial midshaft using three point bending. Although biomechanical testing of bones over simplifies the complexity of the actual in vivo situation, the intention is to test the skeletal structure of interest as closely as possible in terms of biological destruction. For the purpose of examining the effects of changes in structure in relation to its strength, we have subjected animals to various levels of dietary vitamin D (ranging from absolute deficiency to replete), dietary calcium (ranging from moderately low to replete) and estrogen depletion by ovariectomy to establish conditions known to alter bone structure and mineralisation states. The physiological roles of vitamin D and calcium in determining bone structure are discussed in chapter 5 and 6. Estrogen depletion is well described to reduce bone volume by Li and Wronski showing a significant reduction in cancellous bone volume in ovariectomised animals. The reduction was mainly due to the loss of trabecular number with no

significance in trabecular width which was shown to be very similar of the aged related loss of iliac cancellous bone in women [8]. Histomorphometric indices demonstrated that the cancellous bone loss induced by estrogen depletion was associated with an increase in bone resorption [9]. Furthermore, the changes in the cancellous bone were accompanied by cortical osteopenia and resulted in a decrease in breaking strength of the femoral neck in estrogen depleted animals. Another study demonstrated that 12 weeks of estrogen depletion led to a significant decrease in bone mineral density as well as trabecular number due to high bone turnover [10]. These studies suggested that the ovariectomised animal model is a sensitive and suitable model for detecting changes in bone structure and strength.

Utilizing the left tibia from the animals which have been subjected to these physiological challenges of calcium and bone mineral homeostasis, we have shown that changes to cortical bone volume correlates positively and significantly with changes to ultimate load and to flexural rigidity. Flexural rigidity and ultimate load are structural properties of the bone as they provide information on bone as a structural element within the body. Thus, they depend highly on geometry and bone quantity. If the geometry of the bone is known, the material properties of the bone can be determined to provide information on the mechanical quality of bone [11, 12]. The significant correlation between bone volume and the two mechanical strength measurements validated our mechanical testing suggesting that bone strength is affected despite the subtle changes in cortical bone volume which may be the result of ovariectomy or dietary changes. Furthermore, sagittal cortical thickness has also shown to be a strong determining factor in flexural rigidity and ultimate load. Sagittal cortical thickness was determined by the measure of the thickness in the axis of the mechanical loading which demonstrated that it is also an important factor in detecting the changes in mechanical strength. The complex physiological relationship between sagittal cortical thickness and mechanical strength will be further elucidated in chapter 6.

Unlike ultimate load and flexural rigidity, Young’s modulus, is poorly correlated measure with cortical bone volume. Flexural rigidity is a measure for the bending stiffness of a bone. It's the product of its material properties (ie Young's modulus, "E") and its geometry (ie cross sectional moment of inertia, "I"). The Young's modulus is normally correlated to the bone mineral density, ie if the bone contains more mineral, it will become stiffer. If at the same time the geometry remains the same, then the flexural rigidity will increase as well. The poor correlation between Young’s Modulus and cortical volume suggest that the Young's modulus of each bone is very similar, and the increase in rigidity is mainly caused by adaptation of the geometry, but not the material.

In conclusion, we have validated our mechanical testing method for assessing bone strength in rat tibia in our laboratory. Our results not only show that the method is highly sensitive and biologically valid but also that, in the structural testing of rat bones, the determination of bone break load displays clearly superior reproducibility of flexural rigidity, ultimate load, Young’s Modulus and cross sectional moment of inertia.

3.5 REFERENCES

1. Jarvinen, T.L., et al., *Revival of bone strength: the bottom line.* J Bone Miner Res, 2005. **20**(5): p. 717-20.
2. Leppanen, O., et al., *Three-point bending of rat femur in the mediolateral direction: introduction and validation of a novel biomechanical testing protocol.* J Bone Miner Res, 2006. **21**(8): p. 1231-7.
3. Turner, C.H. and D.B. Burr, *Basic biomechanical measurements of bone: a tutorial.* Bone, 1993. **14**(4): p. 595-608.
4. Jamsa, T., et al., *Comparison of three-point bending test and peripheral quantitative computed tomography analysis in the evaluation of the strength of mouse femur and tibia.* Bone, 1998. **23**(2): p. 155-61.
5. Mashiba, T., et al., *Suppressed bone turnover by bisphosphonates increases microdamage accumulation and reduces some biomechanical properties in dog rib.* J Bone Miner Res, 2000. **15**(4): p. 613-20.
6. Bonnick, S.L., et al., *Right and left proximal femur analyses: is there a need to do both?* Calcif Tissue Int, 1996. **58**(5): p. 307-10.
7. Lacey, D.L., et al., *Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation.* Cell, 1998. **93**(2): p. 165-76.
8. Parfitt, A.M., et al., *Relationships between surface, volume, and thickness of iliac trabecular bone in aging and in osteoporosis. Implications for the microanatomic and cellular mechanisms of bone loss.* J Clin Invest, 1983. **72**(4): p. 1396-409.
9. Li, M. and T.J. Wronski, *Response of femoral neck to estrogen depletion and parathyroid hormone in aged rats.* Bone, 1995. **16**(5): p. 551-7.
10. Kaastad, T.S., et al., *Vitamin D deficiency and ovariectomy reduced the strength of the femoral neck in rats.* Calcif Tissue Int, 2001. **69**(2): p. 102-8.

11. Carter, D.R. and W.C. Hayes, *Bone compressive strength: the influence of density and strain rate.* Science, 1976. **194**(4270): p. 1174-6.

12. Ruff, C.B. and W.C. Hayes, *Subperiosteal expansion and cortical remodeling of the human femur and tibia with aging.* Science, 1982. **217**(4563): p. 945-8.

STATEMENT OF AUTHORSHIP

VITAMIN D DEFICIENCY CAUSES IN TRABECULAR BONE LOSS WHILE PRESERVING CORTICAL BONE ARCHITECTURE AND STRENGTH IN GROWING RODENTS

Text in manuscript

Lee AMC (Candidate)

Participated in tissue collection, histology, μ-CT analysis, mechanical testing, interpreted data and wrote manuscript.

I hereby certify that the statement of contribution is accurate.

Paul H Anderson

Supervised development of work and critical manuscript evaluation; acted as corresponding author

I hereby certify that the statement of contribution is accurate.

Rebecca K Sawyer

Participated in animal trials, tissue collection, analyzing serum and 2D histomorphometric analysis

I hereby certify that the statement of contribution is accurate.

Alison J Moore.

Performed and collected 2D histomorphometric analysis

I hereby certify that the statement of contribution is accurate.

Mark R Forwood

Provided advice in mechanical testing and assisted in collaborations interstate.

I hereby certify that the statement of contribution is accurate.

Roland Steck

Provided advice in mechanical testing and assisted in analyzing mechanical testing results.

I hereby certify that the statement of contribution is accurate.

Howard A Morris

Supervised development of work and critical manuscript evaluation

I hereby certify that the statement of contribution is accurate.

Peter D O'Loughlin

Supervised development of work and critical manuscript evaluation; acted as corresponding author. I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the manuscript in the thesis.

CHAPTER 4: VITAMIN D DEFICIENCY CAUSES IN TRABECULAR BONE LOSS WHILE PRESERVING CORTICAL BONE ARCHITECTURE AND STRENGTH IN GROWING RODENTS

Alice MC Lee [1,2], Paul H Anderson [1,2], Rebecca K Sawyer[2], Alison J Moore[2], Mark R Forwood[3], Roland Steck[4], Howard A Morris [1,2,5], Peter D O'Loughlin [1,2]

[1]School of Medical Sciences, Faculty of Health Sciences, University of Adelaide, Adelaide, Australia

[2]Endocrine Bone Research, Chemical Pathology, SA Pathology, Adelaide, Australia

[3]Department of Anatomy, School of Medical Science, Griffith University, Gold Coast, Australia

[4]Dept of Orthopaedics & Trauma, Institute of Health and Biomedical Innovation, Queensland University of Technology, Queensland, Australia

[5]School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, Australia

NOTE: The work presented in this chapter has been published in the Journal of Steroid Biochemistry and Molecular Biology. A reprint of the published manuscript can be found in the appendix.

4.1 ABSTRACT

We have previously shown that vitamin D deficiency in young male rats results in significant reduction in femoral trabecular bone volume (BV/TV). However, the effects of vitamin D deficiency and its impact on other relevant skeletal sites remain unclear. Ten week old male Sprague-Dawley rats were fed various levels of vitamin D_3 (2, 4, 8, 12 IU/day) with standard Ca (0.4%) until 30 weeks of age and achieved stable serum 25 hydroxyvitamin D_3 (25D) levels between 16 and 117 nmol/L.. At time of death, femora, L2 vertebrae and tibiae were processed for bone histomorphometric analyses and tibial cortical strength by 3-point mechanical testing. A significant association between serum 25D and trabecular bone occurred for both the distal femoral metaphysis (R^2=0.34, P<0.05) and L2 vertebrae (R^2=0.24, P<0.05). Tibia mid-shaft cortical bone was not, however, changed in terms of total volume, periosteal surface or endosteal surface as a function of vitamin D status. Furthermore, no changes to mechanical and intrinsic properties of the cortices were observed. We conclude that cortical bone is maintained under conditions of vitamin D deficiency in preference to cancellous bone in young growing rats

4.2 INTRODUCTION

Although it has been well documented that vitamin D insufficiency, clinically defined by serum 25 hydroxyvitamin D_3 (25D) levels below 60nmol/L is associated with increased fracture risk [1], the level of vitamin D required to maintain bone strength is controversial. The incidence of hip fracture in the elderly has been associated with decreased serum 25D levels rather than 1,25-dihydroxyvitamin D_3 (1,25D) levels suggesting that maintaining an adequate level of 25D is important for the prevention of osteoporosis [2]. Previously, we demonstrated trabecular bone loss in a rat model consistent with osteoporosis at moderately insufficient levels of 25D and osteomalacia at severe vitamin D deficiency [3]. Results from these finding suggest that while a

circulating levels of 25D of 80 nmol/L is required to prevent the development of osteoporosis in rats, a positive linear relationship between serum 25D levels ranging between 20nmol/L and 115nmol/L and femoral trabecular bone volume was demonstrated. Furthermore, these changes in trabecular bone volume were shown to be independent of changes to serum calcium, parathyroid hormone (PTH) and 1,25D levels [4].

While the effects of vitamin D deficiency on trabecular bone structure are well described, the effects on cortical bone volume and strength have not been reported in the vitamin D-deficient normocalcaemic model. A number of studies using various rodent models have reported changes to bone strength with various effects on cortical and trabecular bone volume. For example, the effects of ovariectomy combined with a diet containing low levels of calcium in rats caused a significant reduction of femoral shaft strength with only a reduction in trabecular bone volume and no change in cortical bone volume [5]. In contrast, no changes in the strength or other biomechanical properties of the femoral shaft were observed in other vitamin D deficient or ovariectomised rats, despite showing a similar pattern of trabecular bone loss and maintenance of femoral and tibial cortical bone area in the mid-shaft [6]. Consistent with these findings, femoral neck strength was shown to be unchanged in aged ovariectomised rats which had significant loss of trabecular bone. However, no report was made of cortical bone volume in this study [7]. Furthermore, long-term ovariectomy studies in young rats were able to demonstrate maintenance[8] or gain [9]in cortical bone volume despite a rapid trabecular bone loss in the distal femoral metaphysis. Together these findings suggest that while characteristics of bone quality other than density and size may play important contributions to overall bone strength, the results may vary due to the conditions of hormone and calcium deprivation. The aim of the current study was to investigate the effects of vitamin D deficiency on cortical bone structure and strength in young male rats. We have utilised

both micro-CT and the three-point bending test in our vitamin D-deficient model of osteopenia to investigate tibial mid-shaft structure and strength.

4.3 MATERIALS AND METHODS

4.3.1 *Animals*

Twenty-four male Sprague-Dawley rats were raised in an incandescent-lighted environment, and were maintained on vitamin D-deficient semi-synthetic diet containing 1% calcium and 0.6% phosphorus as previously published [4]. All animals were fed on their assigned diets from weanling until 10 weeks of age, at which point the animals were allocated to groups and pair-fed a modified AIN-93 diet (ICN Biomedicals, Aurora, Ohio, USA) containing 0.4% calcium and either 2, 4, 8 or 12 IU/day vitamin D (n = 5-6/grp). The animals were fed their assigned diets until 30 weeks of age and sacrificed. Fasting blood were collected and femora, tibiae and lumbar spine were removed and processed for micro-CT analysis and 3-point mechanical strength testing at time of death. All animal procedures were approved by the Institute of Medical and Veterinary Science Animal Ethics Committee.

4.3.2 *Biochemical analyses*

Serum calcium was measured using a chemistry analyser (Trace Scientific reagents, Vic, Australia; Hitachi 911 automated analyser, Roche, IN, USA). Serum 1,25D and 25D were measured by a ^{125}I radioimmunoassay (RIA) (Immunodiagnostic Systems Ltd, Bolden, UK). Serum PTH was measured using rat-specific; two-site immunoradiometric assays (Immutopics, Inc., San Clemente, CA, USA).

4.3.3 Micro Computed Topographical analyses

The micro-architecture of the tibia was evaluated using a high resolution micro-CT system (Skyscan 1076, Brussels, Belgium) to obtain multiple x-ray transmission images. Transverse CT slices were acquired at the tibial mid-shaft using 18-µm slice increment. An 8mm region of cortical bone, located 4mm above and below the mid-point of the tibia was used for structural analyses. Periosteal surface area (mm^2) and endosteal surface area (mm^2) were assessed using CTan software (v1.7, Skyscan, Belgium). For 3-D analysis, using the same technique as above in an 8mm region of cortical bone, we assessed the total cross-sectional volume, cortical bone volume and medullary volume (TV, BV and MV, respectively, mm^3). Tibia length was measured using digital callipers.

4.3.4 Three Point Mechanical Strength Testing

The mechanical properties of the tibiae were assessed by three-point bending method[10], performed by a miniature Instron materials testing machine (Instron 5848 MicroTester) with a 500N load cell. Prior to testing, tibiae were thawed in PBS at room temperature for 30 minutes and mid point of the tibia determined using digital callipers. The lower anvil points were set at 20mm apart, equidistant from the mid-point for each bone. The upper anvil was lowered on to the mid-point at a rate of 1.0mm/min for up to 5mm maximum deflection in the bone or until the bone failed. Results were collected in Wavemaker (version 9.1.00, Instron, Instron Corp., Canton, MA). From the force (F) versus displacement (D) curve we calculated, ultimate force to failure (ULF) and Young's modulus (E). E, defined as the intrinsic stiffness of the bone was based on the following calculation: **E= (F / D) x (L3 / 48 x I),** where **L** = length of span and **I** = cross sectional moment of inertia, derived using Image J (Java 1.6.0_10, USA). ULF was the maximum force required to break the bone. Breaking energy was obtained by calculating the area under the stress-strain curve, which defines the amount energy needed to cause a fracture. Yield point is a boundary above which stress

causes permanent damage to the bone structure and is defined as the point when the stress-strain curve become non-linear.

4.3.5 Statistical analyses

One-way analysis of variance was used to analyse the effect of varying levels of dietary vitamin D on bone structure and strength. A value of $p<0.05$ was considered to be statistically significant. Multi-Linear regression analyses were performed on biochemical, morphological and biomechanical measures to determine interactions.

4.4 RESULTS

4.4.1 Biochemistry

Stable levels of 25D were achieved by 18 weeks of age and were maintained until time of death at 30 weeks of age. Treatment groups achieved serum 25D levels ranging from 16 nmol/L to 117 nmol/L with the highest in animals fed with 12 IU/day. There were no changes in serum PTH or serum Ca associated with varying vitamin D status (Table 1).

4.4.2 Bone structure and strength

Serum 25D levels were positively associated with both femoral metaphyseal trabecular bone (R^2 = 0.34, $P<0.05$) and L2 vertebral bone ($R^2=0.24$, $P<0.05$) (Fig 1A and 1B). No changes to tibial length, cortical bone volume or measures of cortical bone distribution were observed between dietary vitamin D groups (Table 2). Furthermore, no statistically significant relationship occurred between serum 25D levels and midshaft tibial cortical bone volume ($R^2=0.24$, $P=0.15$) (Fig 1C), periosteal or endosteal surface area (data not shown). While cortical bone volume and distribution of bone was not altered due to vitamin D depletion, it was important to test the quality of the bone by 3-point mechanical testing. Cross sectional moment of inertia (data not shown), ultimate load,

yield point, breaking energy and young's modulus were all unaffected by changes to dietary vitamin D (Table 3) or changes to serum 25D levels (data not shown).

Table 4.1 Serum levels of 25 hydrocyvitamin D, parathyroid hormone and total calcium in each dietary group. Values are mean (SEM)

Dietary vitamin D (IU/day)	2	4	8	12
25D, nmol/L	20 # (1.6)	46 # (4.4)	83 # (3.0)	93 # (8.0)
1,25D, pmol/L	162.8 (13.2)	168.7 (14.1)	234.0 # (30.6)	224.5 # (16.4)
PTH, pmol/L	6.0 (1.1)	6.2 (0.3)	6.6 (1.2)	5.1 (0.2)
Ca, mmol/L	2.45 (0.01)	2.52 (0.02)	2.57 (0.07)	2.58 (0.09)

**Serum biochemistry from these animals are adapted from reference (2). 25D, 25-hydroxyvitamin D; 1,25D, 1,25 dihydroxyvitamin D_3;PTH, parathyroid hormone; Ca, calcium.# P<0.05 when compared with lower vitamin D treatment groups.*

Table 4.2 Body weights and bone structure measurements in the mid-shaft of the tibia. Values are mean (SEM).

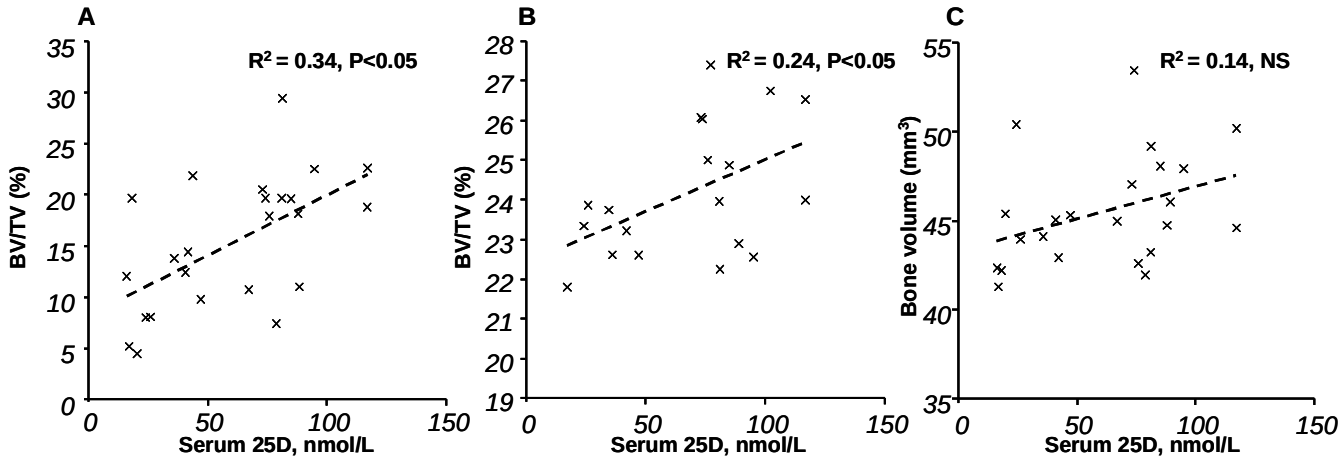
Dietary vitamin D (IU/day)	2	4	8	12
Tibia length, mm	45 (0.4)	44 (0.4)	45 (0.3)	44 (0.6)
Cortical BV, mm3	44 (1.4)	44 (0.4)	47 (1.7)	46 (1.1)
Periosteal SA, mm2	121 (2.4)	120 (3.0)	124 (3.2)	122 (5.2)
Endosteal SA, mm2	55 (3.5)	50 (2.0)	62 (3.1)	52 (3.2)

BV, bone volume; SA, surface area.

Table 4.3 Bone strength measurements in the tibial mid-shaft measured by 3-point mechanical testing. Values are mean (SEM)

Dietary vitamin D(IU)	2	4	8	12
Ultimate load, N	90 (4.8)	83 (2.4)	80 (3.3)	84 (4.6)
Yield point, n	80.0 (4.7)	72.1 (2.1)	72.6 (3.1)	75.5 (5.6)
Breaking energy, mJ	62.4 (6.9)	65.6 (7.6)	56.6 (2.8)	57.5 (13.1)
Young's modulus, GPa	6 (0.6)	5.9 (0.1)	6 (0.6)	6 (0.4)

Figure 4.1: The relationship between serum 25D and (A) femoral trabecular bone volume, (B) L2 vertebral trabecular bone volume and (C) mid-shaft tibial cortical bone volume in animals with serum 25D ranging between 16 and 117 nmol/L.



4.5 DISCUSSION

In contrast to our previous and current findings of vitamin D deficiency-related trabecular bone loss [4], our present findings indicate that tibial cortical bone volume and measures of cortical architecture were preserved even when growing rats were reduced to serum 25D levels as low as 16 nmol/L. This is consistent with previous studies, where trabecular bone loss due to other factors did not also result in cortical bone loss [4,5,6]. Furthermore, others have demonstrated an absence of cortical bone loss [8] or some evidence of bone gain [9] during ovariectomy which occurs regardless of age and stage of bone growth. The findings suggest the stimulus for maintenance or increased cortical bone following ovariectomy may be an adaptive process in response to increasing mechanical forces on those structural elements that remain after initial bone loss. Thus, while we show marked effects of vitamin D depletion of trabecular bone loss in both the femur and vertebrae, the lack of effect of vitamin D depletion of cortical bone suggests that other factors such as mechanical strain determine the maintenance of its structure.

In addition to our observation that cortical bone volume did not change, we observed no redistribution of bone mineral. In particular, there was no change in either endosteal or periosteal surface indicating that overall bone turnover in this region was maintained at a constant level for all vitamin D diet groups. Cortical bone, which constitutes the majority of the skeleton has a predominantly mechanical function, while trabecular bone has both mechanical and metabolic functions as a store for calcium and phosphorus [11]. The ability of cortical bone to resist load, as a function of its geometry and intrinsic material properties is important. An important factor in the determination of bone structure and volume is mechanical strain. The application of mechanical forces results in the response of osteogenic/antiresorptive bone cells to maximise the load resisting properties [12]. The preservation of cortical bone structure and strength in growing rats in spite of the bone losing state of vitamin D deficiency highlights the dominance of mechanical forces.

Another possible explanation for the differential effect of vitamin D deficiency on cortical bone compared to trabecular bone is simply that cortical bone is less sensitive to conditions that lead to bone loss. The lower surface area to volume ratio inherent to cortical bone compared to the more complex trabecular architecture itself will contribute to the much slower changes seen in the cortical bone compare to the trabecular bone. An ovariectomy study in rats suggested that although indices of bone turnover were observed in the endocortical surface 1-2 months post-ovariectomy, significant reduction in cortical thickness in the femoral neck only occurred 1 year post-ovariectomy despite a significant change in the trabecular bone in the proximal tibial metaphysis as early as 14 days after ovariectomy [13]. The observation suggests loss of cortical bone may only be observed under more extreme bone loss conditions, such as marked calcium deprivation for an extended period of time or with ageing.

Although vitamin D depletion leads to a loss of trabecular bone, our present demonstration of preserved cortical bone volume, distribution and strength, indicates that the effect of vitamin D deficiency varies at least between the trabecular and cortical regions, further investigations of the mechanisms that lead to trabecular bone loss while preserving cortical bone is warranted which may include the investigations into the distribution and activity of osteocytes in these bone compartments.

4.6 REFERENCES

1. Jesudason, D., A.G. Need, M. Horowitz, P.D. O'Loughlin, H.A. Morris, and B.E. Nordin, *Relationship between serum 25-hydroxyvitamin D and bone resorption markers in vitamin D insufficiency*. Bone, 2002. 31(5): p. 626-30.
2. Chapuy, M.C., M.E. Arlot, F. Duboeuf, J. Brun, B. Crouzet, S. Arnaud, P.D. Delmas, and P.J. Meunier, *Vitamin D3 and calcium to prevent hip fractures in the elderly women.* N Engl J Med, 1992. 327(23): p. 1637-42.
3. Anderson, P.H., R.K. Sawyer, B.K. May, P.D. O'Loughlin, and H.A. Morris, *25-Hydroxyvitamin D requirement for maintaining skeletal health utilising a Sprague-Dawley rat model*. J Steroid Biochem Mol Biol, 2007. 103(3-5): p. 592-5.
4. Anderson, P.H., R.K. Sawyer, A.J. Moore, B.K. May, P.D. O'Loughlin, and H.A. Morris, *Vitamin D depletion induces RANKL-mediated osteoclastogenesis and bone loss in a rodent model*. J Bone Miner Res, 2008. 23(11): p. 1789-97.
5. Kaastad, T.S., O. Reikeras, J.E. Madsen, S. Narum, J.H. Stromme, K.J. Obrant, and L. Nordsletten, *Effects of clodronate on cortical and trabecular bone in ovariectomized rats on a low calcium diet*. Calcif Tissue Int, 1997. 61(2): p. 158-64.
6. Kaastad, T.S., O. Reikeras, V. Halvorsen, J.A. Falch, K.J. Obrant, and L. Nordsletten, *Vitamin D deficiency and ovariectomy reduced the strength of the femoral neck in rats.* Calcif Tissue Int, 2001. 69(2): p. 102-8.
7. Peng, Z.Q., H.K. Vaananen, H.X. Zhang, and J. Tuukkanen, *Long-term effects of ovariectomy on the mechanical properties and chemical composition of rat bone.* Bone, 1997. 20(3): p. 207-12.
8. Waarsing, J.H., J.S. Day, J.A. Verhaar, A.G. Ederveen, and H. Weinans, *Bone loss dynamics result in trabecular alignment in aging and ovariectomized rats.* J Orthop Res, 2006. 24(5): p. 926-35.

9. Jiang, Y., J. Zhao, H.K. Genant, J. Dequeker, and P. Geusens, *Long-term changes in bone mineral and biomechanical properties of vertebrae and femur in aging, dietary calcium restricted, and/or estrogen-deprived/-replaced rats.* J Bone Miner Res, 1997. 12(5): p. 820-31.

10. Turner, C.H. and D.B. Burr, *Basic biomechanical measurements of bone: a tutorial.* Bone, 1993. 14(4): p. 595-608.

11. Bagi, C.M., N. Hanson, C. Andresen, R. Pero, R. Lariviere, C.H. Turner, and A. Laib, *The use of micro-CT to evaluate cortical bone geometry and strength in nude rats: correlation with mechanical testing, pQCT and DXA.* Bone, 2006. 38(1): p. 136-44.

12. Mosley, J.R. and L.E. Lanyon, Growth rate rather than gender determines the size of the adaptive response of the growing skeleton to mechanical strain. Bone, 2002. 30(1): p. 314-9.

13. Li, M., Y. Shen, and T.J. Wronski, *Time course of femoral neck osteopenia in ovariectomized rats.* Bone, 1997. 20(1): p. 55-61.

STATEMENT OF AUTHORSHIP

THE EFFECT OF DIETARY CALCIUM ON 1,25(OH)2D3 SYNETHESIS AND SPARING OF SERUM 25(OH)D3 LEVELS

Paul H Anderson

Conducted animal trials, interpreted data and wrote manuscript

I hereby certify that the statement of contribution is accurate.

Lee AMC. (Candidate)

Performed the majority of RNA extractions and real-time PCR and contributed strongly to all stages of the interpretation of the data and preparation of the manuscript.

I hereby certify that the statement of contribution is accurate.

Rebecca K Sawyer

Participated in animal trials, tissue collection, analyzing serum and 2D histomorphometric analysis

I hereby certify that the statement of contribution is accurate.

Sarah M Anderson

Participated in RNA extractions and real-time PCR

I hereby certify that the statement of contribution is accurate.

Peter D O'Loughlin

Supervised development of work and manuscript evaluation

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the manuscript in the thesis.

Howard A Morris

Supervised development of work and critical manuscript evaluation; acted as corresponding author.

I hereby certify that the statement of contribution is accurate.

CHAPTER 5: THE EFFECT OF DIETARY CALCIUM ON 1,25(OH)2D3 SYNTHESIS AND SPARING OF SERUM 25(OH)D3 LEVELS

Paul H Anderson[1,2], **Alice M Lee**[1,2], Sarah M Anderson[1], Rebecca K Sawyer[1], Peter D O'Loughlin[1,2], Howard A Morris[1,2,3].

[1] Chemical Pathology, SA Pathology, Frome Rd, Adelaide, SA, Australia, 5000

[2] School of Medical Sciences, Faculty of Health Sciences, University of Adelaide, North Tce, Adelaide, SA, Australia, 5005

[3] School of Pharmacy and Medical Sciences, University of South Australia, SA Australia 5001

NOTE: The work presented in this chapter has been published in the Journal of Steroid Biochemistry and Molecular Biology. A reprint of the published manuscript can be found in the appendix. Alice is a key contributor to this study having performed the majority of RNA extractions and real-time PCR analyses for this chapter. She has also contributed strongly to all stages of the interpretation of the data and preparation of the manuscript.

5.1 ABSTRACT

Vitamin D depletion in rats causes osteopenia in at least three skeletal sites. However it is unclear whether modulation of dietary calcium intake impacts on the relationship between the level of serum 25-hydroxyvitamin D (25D) and bone loss. Nine-month-old female Sprague-Dawley rats (n=5-6/grp) were pair-fed a semi-synthetic diet containing either 0 or 20IU vitamin D3/day with either low (0.1%) or high (1%) dietary Ca for 6 months. At 15 months of age, fasting bloods were collected for biochemical analyses. Serum 25D levels were lowest in the animals fed 0 IU vitamin D and 0.1% Ca. The animals fed 1% Ca had significantly higher serum 25D levels when compared to animals fed 0.1% Ca ($P<0.05$). The major determinants of serum 25D were dietary vitamin D and dietary calcium (Multiple $R=0.75$, $P<0.05$)). Animals fed 0.1% Ca had higher renal CYP27B1 mRNA expression and 12 to 18-fold increased levels of serum 1,25D. Hence, the reported effects of low calcium diets on bone loss may be, in part, due to the subsequent effects of 25D metabolism leading to reduction in vitamin D status. Such an interaction has significant implications, given the recent evidence for local synthesis of active vitamin D in bone tissue.

5.2 INTRODUCTION

We have previously reported that vitamin D depletion in rats causes osteopenia in at least three skeletal sites including the distal and proximal femoral metaphyses and vertebrae. Furthermore, serum 25 hydroxyvitamin D (25D) levels ranging from 20-115nmol/L were a positive and independent determinant of femoral trabecular bone volume (1). It is clear that a diet containing high levels of calcium protects against bone loss, presumably by reducing PTH secretion, which in turn reduces osteoclastic activity. The question of whether, high dietary calcium also results in reduced renal vitamin D metabolism, resulting in maintenance of serum 25D levels is less certain. Such an interaction between dietary calcium and vitamin D has significant implications, given the recent evidence for local synthesis of active vitamin D in bone tissue (2-4). We propose that the

supply of 25D to the skeleton is an important factor for autocrine and paracrine activities of 1,25dihydroxyvitamin D (1,25D) via the activity of 25 hydroxyvitamin D 1alpha-hydroxylase (CYP27B1) within osteoblasts (2-4) and other bone cells. A limited supply of serum 25D to the bone may impair osteoblastic 1,25D synthesis as the CYP27B1 enzyme activity does not appear to be induced to levels that have been observed in the kidney under the stimulation of PTH (2). Previously, the role of calcium deprivation on serum 25D levels was not shown to be attributed the synthesis of picomolar levels of 1,25D as it was considered not to have the capacity to greatly influence nanomolar levels of 25D (5). Rather, the influence of calcium deprivation on 25D catabolism within the liver implicated either PTH or 1,25D itself in the enhanced degradation of 25D by an unknown hepatic oxidative enzyme. Regardless, sustained production of 1,25D during calcium deprivation over an extended period of time, in combination with a vitamin D-deficient diet, has not been previously studied to determine its effects on serum 25D levels, particularly with regards to effects on gene expression of key vitamin D metabolising enzymes. The identification of an interaction between calcium deficiency promoting vitamin D deficiency could be important in understanding the pathogenesis of rickets and osteoporosis.

5.3 MATERIAL AND METHODS

5.3.1 *Animals*

Nine-month old female Sprague-Dawley rats (n=24) were allocated to either vitamin D-replete (20IU D3/day) or vitamin D-deplete diets containing either 0.1% or 1% calcium, based on the recommended semi-synthetic diet for rodents (AIN-93-VX, ICN, CA, USA). All animals were maintained on their assigned diets for six months, at which point they were killed. All animal procedures were approved by the Institutional Animal Ethics Committee.

5.3.2 Biochemical analyses

Fasting blood samples were collected at time of death for analyses. A chemistry analyser was used to measure serum calcium (Cobas Bio, Roche, IN, USA) and inorganic phosphate (Trace Scientific reagents, Vic, Australia; Hitachi 911 automated analyser, Roche, IN, USA). Serum 1,25D and 25D were measured by RIA (Immunodiagnostic Systems Ltd, Bolden, UK).

5.3.3 Messenger RNA analyses

The isolation of total RNA from whole rat kidney, liver and duodenum of 15 month old rats were performed using Trizol (Invitrogen). First strand cDNA synthesis was performed as previously described (6). Real-time RT-PCR was performed using primers designed to span an intronic sequence (Table 5.1) (Geneworks, SA, Australia). Target mRNAs of interest were expressed relative to the levels of β-Actin mRNA using a comparative method of analysis. Multivariate statistical analysis of variance and Tukey's post hoc test analysis were performed of biochemical and mRNA measures.

Table 5.1 Primer sequences of Sense (S) and anti-sense(A) for each messenger RNA species of interest.

Table 1: Primer sets used for real time RT-PCR

Gene	Sequence 5' →3'	Genbank® accession no.
CYP27B1	*S: TGCAGAGACTGGAATCAGATGTTTG* *A: CACTATGGACTGGACAGACACC*	*NM053763*
CYP24	*S: TTGAAAGCATCTGCCTTGTGT* *A: GTCACCATCATCTTCCCAAAC*	*NM201635*
CYP27A1	*S: ATGTGGCACATCTTCTCTACC* *A: GGGAAGGAAAGTGACATAGAC*	*NM178847*
CYP2R1	*S: CTTGGAGGCATATCAACTGTG* *A: ATCCATCCTCTGCCATATCTG*	*NM001108499*
CYP2J3	*S: CCTGGATTTTGCTAACATTC* *A: CTAAGCTCTTCTTTCCTAGT*	*NM175766*
CYP3A1	*S: GGAAATTCGATGTGGAGTGC* *A: AGGTTTGCCTTTCTCTTGCC*	*X64401*
CYP3A2	*S: AGTAGTGACGATTCCAACATAT* *A: TCAGAGGTATCTGTGTTTCCT*	*NM153312*
CYP3A9	*S: GGACGATTCTTGCTTACAGG* *A: ATGCTGGTGGGCTTGCCTTC*	*NM147206*
CYP3A11	*S: GACAAACAAGCAGGGATGGAC* *A: CCAAGCTGATTGCTAGGAGCA*	*NM007818*
β-*Actin*	*S: ATCATGTTTGAGACCTTCAAC* *A: CTTGATCTTCATGGTGCTAG*	*AF541940*

5.4 RESULTS

Low mean levels of serum 25D occurred only in animals fed 0 IU D3/day and 0.1% calcium (Table 2. Serum 25D levels were significantly higher in animals fed 1% calcium in both the 0 IU and 20 IU D3/day fed animals when compared to animals fed 0.1% calcium ($P<0.05$), suggesting that a diet containing high levels of calcium is capable of increasing, or preserving serum 25D levels, relative to animals fed a low calcium diet. The animals fed 20 IU D3/day and 1% calcium recorded the highest mean serum 25D ($P<0.05$). In addition to dietary vitamin D levels, dietary calcium was a major determinant of serum 25D levels ($P<0.05$, $R^2=0.23$, Multiple R=0.75) (data not shown). Despite the relative low serum 25D levels in animal fed 0 IU D3/day and 0.1% calcium, serum 1,25D levels were significantly increased and comparable to levels in vitamin D-replete animals fed 0.1% calcium. Mean serum calcium levels were marginally but significantly elevated in the animals fed 20 IU D/day and 1% calcium.

Kidney levels of mRNA for CYP27B1 were significantly increased in animals fed 0.1% calcium in both the animal groups fed 0 or 20 IU D3/day when compared to animals fed 1% calcium (Figure 2) ($P<0.05$). In contrast, kidney CYP24 mRNA levels were highest only in animals fed 1% and 20 IU D3/day calcium ($P<0.05$).

In the liver, there were no statistically significant differences in levels for three mRNA species, CYP27A1, CYP2R1 and CYP2J3, known to be important for the conversion of vitamin D to 25-hydroxyvitamin D in rodents (Figure 3), suggesting that changes in serum 25D levels are not due to changes in liver 25D synthesis. Furthermore, levels of mRNA for the CYP3A isoforms, CYP3A1, CYP3A2, CYP3A9 and CYP3A11 were not determinants of serum 25D levels nor were associated with serum 1,25D levels. No detectable levels of liver CYP24 mRNA levels were observed in any group (data not shown).

In the proximal intestine, the levels of TRPV6 mRNA were greater in the animals fed the 0.1% calcium diet (Figure 3A, $P<0.01$), consistent with the elevated serum 1,25D levels in these groups. The level of CYP24 mRNA was 4-fold higher in animals fed 0 IU D3/day and 0.1% calcium when compared to animals fed 20 IU D3/day. In contrast, CYP27B1 mRNA levels were approximately two-fold higher in the animals fed 20 IU D3/day and 1% calcium.

Table 5.2 Serum levels of 25 hydrocyvitamin D, 1,25 dihydroxyvitamin D3, parathyroid hormone, total calcium and phosphate in each dietary group.

Vitamin D, IU/kg	**0**	**0**	**20**	**20**
Dietary Ca, (%)	**0.1**	**1.0**	**0.1**	**1.0**
25D, nmol/L	*21.7*	*84.5**	*90.9*	*161.3*#*
(sem)	*(2.4)*	*(6.5)*	*(11.8)*	*(35.3)*
1,25D, pmol/L	*246.5*#*	*19.5*	*248.6*#*	*13.8*
(sem)	*(24.7)*	*(6.5)*	*(68.1)*	*(3.5)*
Ca, mmol/L	*2.8*	*2.6*	*2.6*	*3.2*#*
(sem)	*(0.1)*	*(0.1)*	*(0.2)*	*(0.2)*
Phos, mmol/L	*1.4*	*1.6*	*1.6*	*1.9*
(sem)	*(0.1)*	*(0.1)*	*(0.1)*	*(0.1)*

*Values are mean (sem), n=6. 25D, 25-hydroxyvitamin D_3; 1,25D, 1,25 dihydroxyvitamin D_3; * P<0.05 when compared with other 0.1%Ca fed animals; # P<0.05 when compared with other 0 IU/kg vitamin D fed animals.*

Figure 5.1. Levels of CYP27B1 and CYP24 mRNA (relative to β-Actin mRNA) in kidney tissue from each dietary treatment group. Values are mean ± SEM. (n=6). * P<0.05 when compared with other 0.1%Ca fed animals; # P<0.05 when compared with other 0 IU/kg vitamin D fed animals; CYP27B1, 25-hydroxyvitamin D-1α-hydroxylase; CYP24, 25-hydroxyvitamin D-24-hydroxylase.

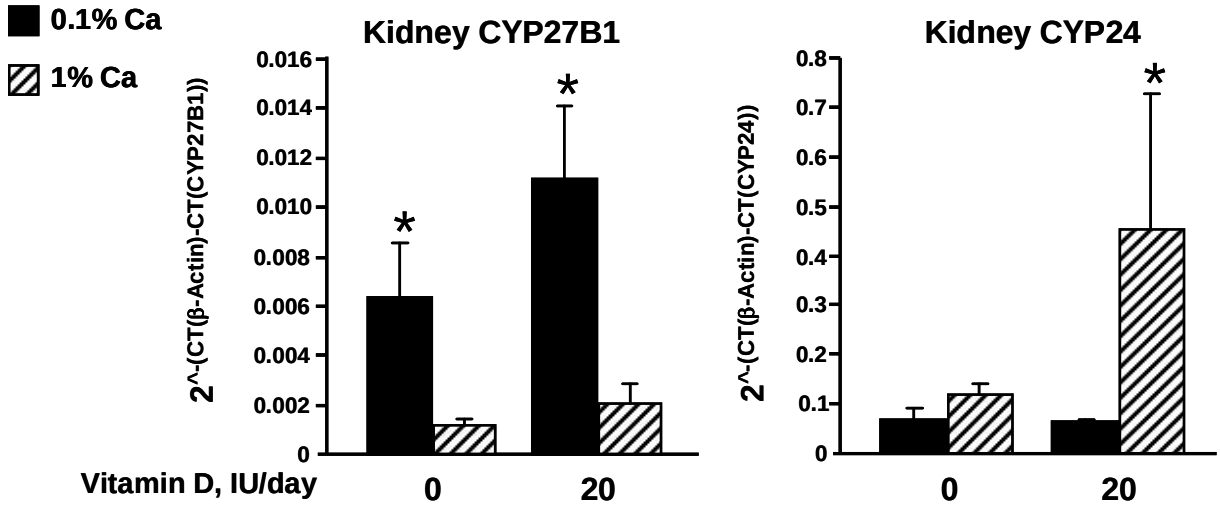


Figure 2. Levels of CYP27Al, CYP2R1 and CYP2J3 mRNA (relative to β-Actin mRNA) in liver tissue from each dietary treatment group. Values are mean ± SEM. (n=6).

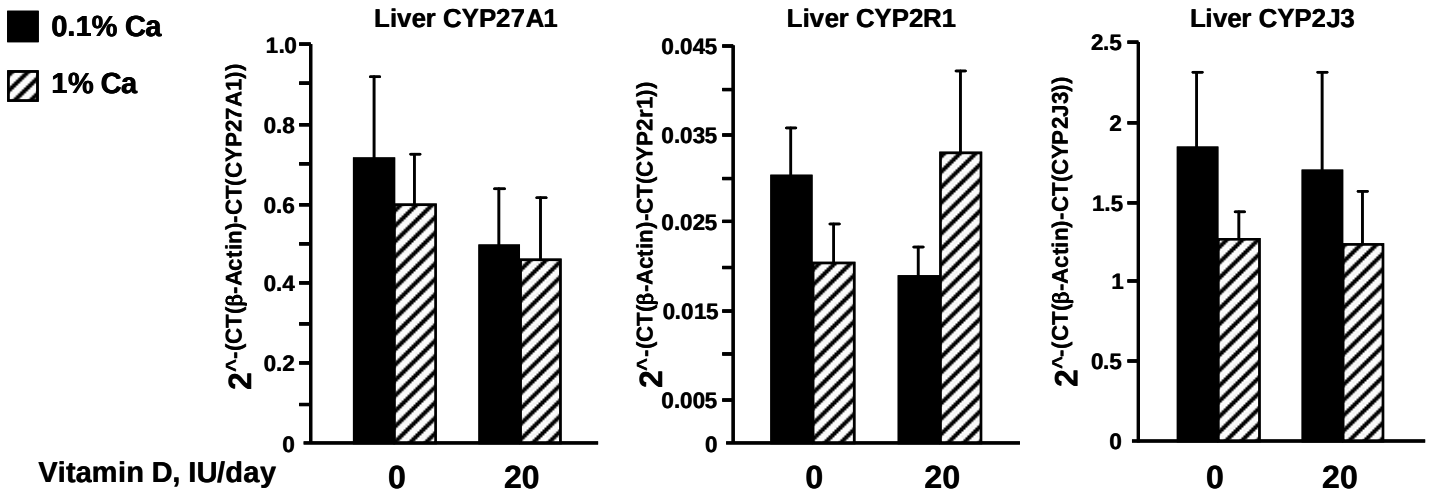


Figure 3. Levels of CYP3A1, CYP3A2, CYP3A9 and CYP3A11 mRNA (relative to β-Actin mRNA) in liver tissue from each dietary treatment group. Values are mean ± SEM. (n=6).

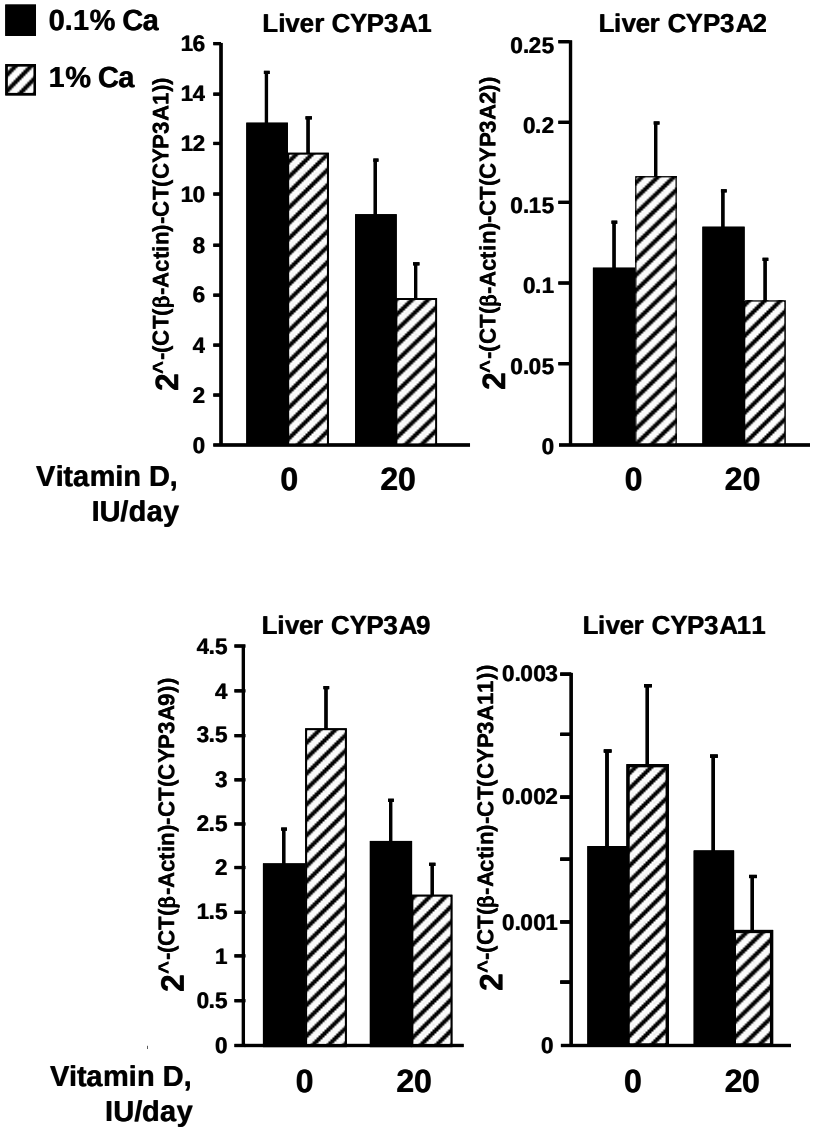
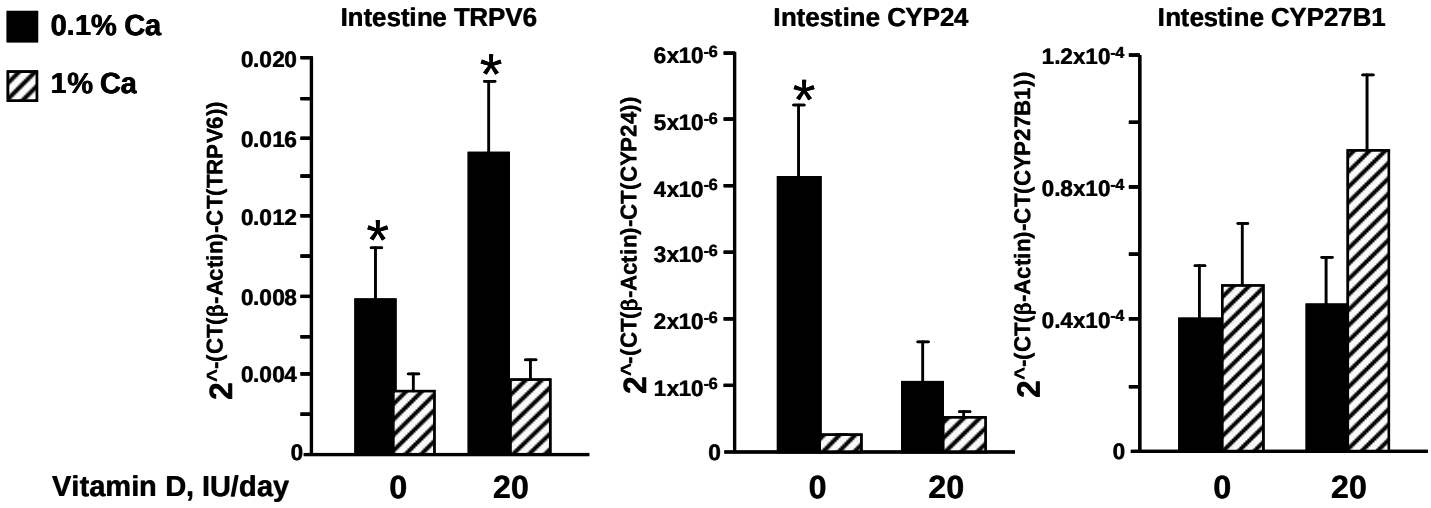


Figure4. Levels of TRPV6, CYP24 and CYP27B1 mRNA (relative to β-Actin mRNA) in proximal intestinal tissue from each dietary treatment group. Values are mean ± SEM. (n=6). * P<0.05 when compared with other 0.1%Ca fed animals; # P<0.05 when compared with other 0 IU/kg vitamin D fed animals.



5.5 DISCUSSION

The data obtained during the present study demonstrate that reduction in serum 25D levels can be induced by feeding rats low diet calcium, regardless of the vitamin D content. The increase in serum 25D levels in animals fed high dietary calcium was not to be due to changes in the mRNA levels of known liver 25D synthetic enzymes, CYP27A1, CYP2R1 and CYP2J3. It is also unlikely that changes in serum 25D levels are due to catabolism of serum 25D via CYP24 activity, as CYP24 was undetectable in the liver and CYP24 in the kidney was only induced in the animals with the highest serum 25D levels which were vitamin D-replete animals fed high dietary calcium. It is possible however that increased expression and activity of renal CYP27B1 in response to calcium deprivation, with increased production of 1,25D may be responsible for the consumption of 25D stores providing the explanation for decreased 25D levels with low dietary calcium intake. Previously, it has been postulated that the relatively low levels of 1,25D production is unlikely to account for the reduction in the larger pool of 25D (5). However this study was conducted over 6 months of calcium deprivation and thus the subsequent 1,25D production and its short half-life in the circulation may account for a larger fraction of the decline of 25D levels. This observation is consistent with clinical observations that during primary and secondary hyperparathyroidism, the half-life of serum 25D is inversely correlated to serum 1,25D (7).

That renal CYP27B1 activity influences the metabolic clearance 25D does not, however, rule out the possibility that a low dietary calcium and subsequent high serum 1,25D may lead to an increased catabolism of 25(OH)D by hepatic oxidative enzymes, as has been previously described (5). However, the ability for 1,25D to directly stimulate the regulation of hepatic oxidative enzymes is presumably dependent on the expression of the vitamin D receptor (VDR). There are conflicting reports regarding the expression of VDR within human and rat liver tissue. Part of the confusion may be explained by general acceptance that VDR is absent in rat hepatocytes, but present in non-parenchymal cells and biliary epithelial cells (8). Given that the major site of the target oxidative

enzyme genes in hepatocytes, it appears less likely that 1,25D could directly regulate oxidative enzyme genes via a classical genomic response. There are numerous reports demonstrating that 1,25D can regulate hepatocyte cell processes. For example, Baran et al. (9) have shown that 1,25D increases intracellular calcium levels in rat hepatocytes. Furthermore, other studies have shown that the liver responds to 1,25D as indicated by its control of DNA polymerase activity as well as cytoplasmic and nuclear protein kinases. Also in human liver slices, CYP3A4 is unregulated by 1,25D, but only in liver samples that showed expression of VDR (10). Interestingly, in separate experiments, while CYP3A4 has been shown to stimulate the 25-hydroxylation of vitamin D (11,12), CYP3A4 has also been shown to be stimulated by treatment with 1,25D and was able to generate three major metabolites of 1,25D catabolism (13), suggesting that CYP3A4 in humans may be a target enzyme responsible for 25D catabolism as well. While rats do not possess CYP3A4, several other isoforms of CYP3A are expressed in liver tissue with reported similar actions to CYP3A4 (14-16). In the current study, however, none of these CYP3A isoforms appear to be regulated in such a way as to explain the differences in serum 25D levels observed.

An alternative explanation for the role of high 1,25D regulating liver processes may involve the interaction of vitamin D with calcium on hepatic intracellular stores of calcium, [Ca2+]i. Gascon-Barre and colleagues reported that both resting and stimulated [Ca2+]i are sensitive to changes in serum 25D levels as revealed in short-term primary culture of hepatocytes isolated from livers of rats depleted in 25D levels. The conclusions that hypocalcemia, secondary to low 25D levels, is a determinant of [Ca2+]i levels, raises the possibility that a lower than normal [Ca2+]i may be linked to changes in calcium signaling pathways, such as those shown to be involved in CYP3A induction of mouse hepatocytes (17).

Regardless of the mode of mechanism by which calcium deprivation may lead to reduced levels of 25D, the role for dietary calcium in the regulation of serum 25D levels is under-appreciated and may explain some of the beneficial effects on bone for the combination of vitamin D with calcium

supplementation. Furthermore, to improve 25D status by vitamin D supplementation, one may need to consider the levels of dietary calcium, when recommending an effective regimen.

5.6 REFERENCES

1. Anderson PH, Sawyer RK, Moore AJ, May BK, O'Loughlin PD, Morris HA 2008 *Vitamin D depletion induces RANKL-mediated osteoclastogenesis and bone loss in a rodent model.* Journal of Bone and Mineral Research 23(11):1789-97.
2. Anderson PH, Atkins GJ, Findlay DM, Morris HA 2005 *Target genes: bone proteins.* In: Feldman D, Pike JW, Glorieux FH (eds.) Vitamin D, 2nd edition ed., vol. 1. Elsevier Academic Press, San Diego, pp 711-720.
3. Anderson PH, Atkins GJ, Findlay DM, Oloughlin PD, Welldon K, Vincent C, Morris HA 2007 *RNAi-mediated silencing of CYP27B1 abolishes 1,25(OH)2D3 synthesis and reduces osteocalcin and CYP24 mRNA expression in human osteosarcoma (HOS) cells.* J Steroid Biochem Mol Biol 103(3-5):601-5.
4. Atkins GJ, Anderson PH, Findlay DM, Welldon KJ, Vincent C, Zannettino AC, O'Loughlin P D, Morris HA 2007 *Metabolism of vitamin D(3) in human osteoblasts: Evidence for autocrine and paracrine activities of 1alpha,25-dihydroxyvitamin D(3).* Bone 40(6):1517-28.
5. Clements MR, Johnson L, Fraser DR 1987 *A new mechanism for induced vitamin D deficiency in calcium deprivation.* Nature 325(6099):62-5.
6. Anderson PH, O'Loughlin PD, May BK, Morris HA 2003 *Quantification of mRNA for the vitamin D metabolizing enzymes CYP27B1 and CYP24 and vitamin D receptor in kidney using real-time reverse transcriptase- polymerase chain reaction.* J Mol Endocrinol 31(1):123-32.
7. Davies M, Heys SE, Selby PL, Berry JL, Mawer EB 1997 *Increased catabolism of 25-hydroxyvitamin D in patients with partial gastrectomy and elevated 1,25-dihydroxyvitamin D levels.* Implications for metabolic bone disease. J Clin Endocrinol Metab 82(1):209-12.

8. Gascon-Barre M, Demers C, Mirshahi A, Neron S, Zalzal S, Nanci A 2003 *The normal liver harbors the vitamin D nuclear receptor in nonparenchymal and biliary epithelial cells.* Hepatology 37(5):1034-42.

9. Baran DT, Sorensen AM, Honeyman TW, Ray R, Holick MF 1990 *1 alpha,25-dihydroxyvitamin D3-induced increments in hepatocyte cytosolic calcium and lysophosphatidylinositol: inhibition by pertussis toxin and 1 beta,25-dihydroxyvitamin D3*. J Bone Miner Res 5(5):517-24.

10. Drocourt L, Ourlin JC, Pascussi JM, Maurel P, Vilarem MJ 2002 *Expression of CYP3A4, CYP2B6, and CYP2C9 is regulated by the vitamin D receptor pathway in primary human hepatocytes.* J Biol Chem 277(28):25125-32.

11. Gupta RP, He YA, Patrick KS, Halpert JR, Bell NH 2005 *CYP3A4 is a vitamin D-24- and 25-hydroxylase: analysis of structure function by site-directed mutagenesis.* J Clin Endocrinol Metab 90(2):1210-9.

12. Gupta RP, Hollis BW, Patel SB, Patrick KS, Bell NH 2004 *CYP3A4 is a human microsomal vitamin D 25-hydroxylase*. J Bone Miner Res 19(4):680-8.

13. Xu Y, Hashizume T, Shuhart MC, Davis CL, Nelson WL, Sakaki T, Kalhorn TF, Watkins PB, Schuetz EG, Thummel KE 2006 *Intestinal and hepatic CYP3A4 catalyze hydroxylation of 1alpha,25-dihydroxyvitamin D(3): implications for drug-induced osteomalacia*. Mol Pharmacol 69(1):56-65.

14. Li Y, Ross-Viola JS, Shay NF, Moore DD, Ricketts ML 2009 *Human CYP3A4 and murine Cyp3A11 are regulated by equol and genistein via the pregnane X receptor in a species-specific manner*. J Nutr 139(5):898-904.

15. Nehring JA, Zierold C, DeLuca HF 2007 *Lithocholic acid can carry out in vivo functions of vitamin D*. Proc Natl Acad Sci U S A 104(24):10006-9.

16. Robertson GR, Field J, Goodwin B, Bierach S, Tran M, Lehnert A, Liddle C 2003 *Transgenic mouse models of human CYP3A4 gene regulation.* Mol Pharmacol 64(1):42-50.

17. Galisteo M, Marc N, Fautrel A, Guillouzo A, Corcos L, Lagadic-Gossmann D 1999 *Involvement of cyclic nucleotide- and calcium-regulated pathways in phenobarbital-induced cytochrome P-450 3A expression in mouse primary hepatocytes.* J Pharmacol Exp Ther 290(3):1270-7.

CHAPTER 6: DIETARY CALCIUM AND VITAMIN D ARE BOTH REQUIRED TO OPTIMISE BONE REMODELLING AND STRENGTH IN ADULT RATS

Alice MC Lee[1], Paul H Anderson[1,2,5], Roland Steck[3], Mark Forwood[4], Rebecca K Sawyer[2], Alison J Moore[2], , Howard A Morris[1,2,5] Peter D O'Loughlin[1,2]

[1]Discipline of Physiology, Faculty of Health Sciences, University of Adelaide, North Tce, Adelaide, SA, Australia, 5005

[2]Endocrine Bone Research, Chemical Pathology, SA Pathology, Frome Rd, Adelaide, SA, Australia, 5000

[3]Dept of Orthopaedics & Trauma, Institute of Health and Biomedical Innovation, Queensland University of Technology, Queensland, Australia

[4]Department of Anatomy, School of Medical Science, Griffith University, Gold Coast, Australia

[5]School of Pharmacy and Medical Sciences, University of South Australia, SA Australia 5001

NOTE: The work presented in this chapter is currently being submitted to the Journal of Bone and Mineral Research. The data included in the manuscript may be condensed to comply with the journal authourship preparation rules.

6.1 ABSTRACT

We have previously reported that while short-term vitamin D depletion in rats caused osteopenia in the distal femoral metaphysis, no change was observed in the tibial cortices with respect to bone loss, geometry and strength. The question, however, of whether the effects of longer-term dietary vitamin D deficiency in adult rats impacts cortical bone structure remain unclear. Hence, nine-month-old female Sprague-Dawley rats (n=5-6/grp) were pair-fed a semi-synthetic diet containing varying levels of vitamin D^3 (D) (0,2,12 and 20 IU/d) with either low (0.1%) or high (1%) dietary calcium (LCa or HCa) for 6 months. At 15 months of age, animals were killed, tibia and femora retrieved for cortical and trabecular bone analyses. Cortical bone volume (CBV) at the mid-point of the femora and tibia were analysed as well as the proximal femoral metaphyses. CBV and the proximal metaphysis were analysed using 3D micro-CT scans (Skyscan) at a resolution of 12.6µm/pixel. Bone strength parameters, Young's modulus (E) and Ultimate load, were obtained by performing 3-point bending on the mid-shaft of the tibia. Serum 25D levels ranged from 22(±2.9) nmol/L to 161(±38.8) nmol/L and serum calcium levels ranged from 2.5(±0.05) mmol/L to 3.2(±0.2) mmol/L in the animals fed 0D/LCa diet up to animals fed 20IUD/HCa diet. In mechanical testing, ultimate load was shown to be positively correlated to CBV across all dietary groups. Both Femur and Tibia achieved the highest cortical bone volume in animals fed high vitamin D and calcium. Hence, a diet containing high levels of both vitamin D and calcium are required for optimal bone structure and highest resistance to fracture.

6.2 INTRODUCTION

Osteoporosis is a preventable bone disease which can occur as a consequence of prolonged depletion of circulating levels of 25 hydroxyvitamin D (25D). The level of 25D required in humans to prevent osteoporosis has been reported to be 75 nmol/L which is necessary to maintain normal bone mineral density and reduce the incidence of hip fracture in the elderly as determined by meta-analyses of a clinical trials when vitamin D supplementation is 800 IU per day or greater [1, 2]. We have previously reported that vitamin D depletion in rats causes osteopenia in at least three skeletal sites including the distal and proximal femoral metaphyses and vertebrae. Furthermore, serum 25 hydroxyvitamin D (25D) levels ranging from 20-115nmol/L were a positive and independent determinant of femoral trabecular bone volume [3]. However, the level of vitamin D required to prevent bone loss is likely to be dependent on factors such as the measured bone parameters, the subject population , and also influenced by other factors such as the level of calcium intake [4]. It has been shown that administration of vitamin D and calcium in combination reduces the incidence of hip fracture in elderly nursing home residents [5]. It is clear that a diet containing high levels of calcium protects against bone loss, at least in part by reducing PTH secretion, which in turn reduces osteoclastic activity. However, we have recently reported that circulating levels of serum 25D are greater in animals fed a diet containing high levels of calcium [6]. The reduced need for active duodenal calcium absorption in animals fed high calcium diet results in a decrease in the renal 25D metabolism to active 1,25 dihydroxyvitamin D3 (1,25D), which may explain the conservation of serum 25D levels (Chapter 5). Thus, the effects of a high calcium diet to protect against bone loss may also be, in part, due to the subsequent effects on the maintenance of circulating 25D levels. Such an interaction has significant implications, given the recent evidence for local synthesis of active vitamin D in bone tissue (Chapter 5).

The mechanism by which vitamin D-depletion gives rise to osteoporosis is somewhat controversial. A partial depletion of vitamin D is associated with increases in markers of bone resorption due to

secondary hyperparathyroidism [7, 8]. As serum 25D levels fall, an increase in serum parathyroid hormone (PTH) is attributed to decreased calcium absorption [9, 10]. In postmenopausal women, however, no relationship between serum PTH and calcium absorption levels has been observed [11]. In addition, although the seasonal reduction in serum 25D levels in humans is not associated with changes in calcium absorption there is an increase in osteoid volume and an increase in mineralisation lag time [12]. Although it is not yet fully known how serum 25D directly influences bone, a direct role for vitamin D in bone has been suggested as shown by the osteoblast-specific vitamin D receptor (VDR) over-expression mouse model in which there is increased cortical cross-sectional area and strength due to reduced osteoclast activity and increased bone formation [13]. It is also clear that osteoblasts, through the activity of 25-hydroxyvitamin D 1alpha hydroxylase (CYP27B1), can convert 25D to 1,25D which regulates the expression of genes associated with bone formation, consistent with the effects of 25D in promoting mineralisation and reducing osteoblast proliferation [14]. Hence it is possible that vitamin D-depletion may directly affect bone by reducing the availability of substrate for the local production of 1,25D in bone which supposedly has autocrine and paracrine actions.

In order to quantify the serum 25D level required for optimal mineralization, we have previously developed an animal model which demonstrates the effects of frank vitamin D-deficiency as well as graded vitamin D-depletion on the skeleton [15]. With this model we have shown that levels of 25D, well above the level which invokes a PTH response, are required to improve bone mineral volume. The question as to whether changes to dietary calcium influence the relationship between vitamin D status and bone mineral volume and strength remains to be elucidated.

Bone mineral content and bending moments were significantly lower in pigs that were fed low (0.4%) and very low (0.1%) calcium diet compared to control pigs [16]. Furthermore, another study looked at combined calcium and phosphorus diet and demonstrated no significant differences in length, volume, bone mineral density, bone mineral content and maximum load in the femur

when given 0.5% calcium with various levels of phosphorous. However, they observed a significant reduction in bone mineral content and maximum load in L5 of vertebrae, suggesting that bone loss occurred where greater amount of cancellous bone exists [17]. In a vitamin D deficient rat study, no changes in the strength or other biomechanical properties of the femoral shaft were observed despite showing a similar pattern of trabecular bone loss and maintenance of femoral and tibial cortical bone area in the mid-shaft [18]. The current study aims to further elucidate the effects of various levels of dietary vitamin D and calcium on bone structure and cortical bone strength in aged female rats.

6.3 MATERIALS AND METHODS

6.3.1 *Animals*

Nine-month old female Sprague-Dawley rats (n=5-6/group) were allocated to either vitamin D-diets (0,2,12,20 IU/day) containing either 0.1% or 1% calcium, based on the recommended semi-synthetic diet for rodents (AIN-93-VX, ICN, CA, USA) [19]. All animals were maintained on this diet for 6 months, at which time animals were killed. All animal procedures were approved by the Institutional Animal Ethics Committee.

6.3.2 *Biochemical analyses*

Food was withdrawn from animals 16 hours before blood samples were collected via the tail vein at time of death. Serum 1,25D and 25D were measured by RIA (Immunodiagnostic Systems Ltd, Bolden, UK). Serum PTH was measured using rat-specific, two-site immunoradiometric assays (Immutopics, Inc., CA USA). Levels of serum C-terminal telopeptide α1 chain of rat type I collagen (Cross-laps) were measured by ELISA (Nordic Bioscience Diagnostics, Denmark). Serum Calcium, Phosphate, and alkaline phosphatase have been reported previously in our laboratory [15].

6.3.3 Micro-Computed Topographical analyses

6.3.3.1 Trabecular bone

The proximal femur and tibia bone micro-architecture was analysed using a high-resolution micro-CT system (Skyscan 1072, Belgium) to obtain multiple x-ray transmission images. Cross sectional images of the object were then reconstructed by a modified Feldkamp cone-beam algorithm, creating a complete 3-D representation of internal microstructure and density. The analysis was limited to a region of interest of trabecular network 2 mm (for femur) and 4mm (for tibia) below the intertrochanteric line. The resolution of scanning was 5µm/pixel which provides sufficiently detailed information to calculate bone BV/TV, Tb.N and Tb.Th.

6.3.3.2 Cortical bone

The micro-architecture of the tibia was evaluated using a high resolution micro-CT system (Skyscan 1174, Brussels, Belgium) to obtain multiple x-ray transmission images. Transverse CT slices were acquired at the tibial mid-shaft using 12.6-µm slice increment. For Femur; a 4mm region of cortical bone, located 2mm above and below the mid-point of femur was used for structural analyses. For Tibia; an 8mm region of cortical bone, located 4mm above and below the mid-point of the tibia was used for structural analyses. Periosteal surface area (mm2) and endosteal surface area (mm2) were assessed using CTan software (v1.7, Skyscan, Belgium). For 3-D analysis, using the same technique as above in an 8mm region of cortical bone, we assessed the total cross-sectional volume, cortical bone volume and medullary volume (TV, BV and MV, respectively, mm3). Cross sectional moment of inertia was derived using Image J (Java 1.6.0_10, USA) by calculating the distribution of bone around the neutral axis. Tibial sagittal cortical thickness (mm) is derived by measuring the cortical thickness in the mid shaft of the bone in the plane of mechanical loading.Tibia length was measured using digital callipers.

6.3.4 Three Point Mechanical Strength Testing

The mechanical properties of the tibiae were assessed by three-point bending method [20], performed by a miniature Instron materials testing machine (Instron 5848 MicroTester, Canton, MA) with a 500N load cell. Prior to testing, tibiae were thawed in PBS at room temperature for 30 minutes and midpoint of the tibia determined using digital callipers. The lower anvil points were set at 20mm apart, equidistant from the mid-point for each bone. The upper anvil was lowered on to the mid-point at a rate of 1.0mm/min for up to 5mm maximum deflection in the bone or until the bone failed. Results were collected in Wavemaker (version 9.1.00, Instron, Instron Corp., Canton, MA). From the force (F) versus displacement (D) curve we calculated, ultimate load (UL) and Young's modulus (E). E, defined as the intrinsic stiffness of the bone was based on the following calculation: E= (F / D) x (L3 / 48 x I), where L = length of span and l = cross sectional moment of inertia, derived using Image J (Java 1.6.0_10, USA). UL was the maximum force required to break the bone. Flexural Rigidity is determined base on the calculation: E x I.

6.3.5 Data expression and statistical analyses

The effects of diet on histological measurements and biochemical markers were statistically analyzed by multivariate analysis of variance and Tukey's post hoc test analysis. Multiple linear regression analyses were used to identify determinants of bone mineral volume. A p-value of 0.05 or less was considered to be statistically significant.

6.4 RESULTS

6.4.1 Serum Biochemistry

Low mean levels of serum 25D occurred only in animals fed 0 IU vitamin D_3/day and 0.1% calcium (Table 1). Serum 25D levels were significantly higher in animals fed 1% calcium in the 0,2 and 20

IU vitamin D_3/day fed animals when compared to animals fed 0.1% dietary calcium ($P<0.05$). The animals fed 20 IU D3/day and 1% calcium recorded the highest mean serum 25D ($P<0.05$). Despite the relative low serum 25D levels in animal fed 0 IU D_3/day and 0.1% calcium, serum 1,25D levels were significantly increased and comparable to levels in vitamin D-replete animals fed 0.1% calcium. Mean serum calcium levels were significantly elevated in the animals fed 20 IU D/day and 1% calcium.

6.4.2 *Body weights and bone length*

There were no significant differences across the group in body weights, femur lengths and tibial lengths as shown in table 2.

6.4.3 *Bone Structure, Content and Strength*

6.4.3.1 Bone Structure and Content

The trabecular bone volume, trabecular thickness and number in the rat femoral and tibial metaphysis are shown in table 3. Using µ-CT analysis program CTan software demonstrated no significant differences in bone mineral volume (BV/TV) in the proximal femoral metaphysis. However, proximal tibial BV/TV was significantly higher in animals fed with 20IU vitamin D_3, 1% Ca when compare to animals fed with 20IU vitamin D_3, 0.1% Ca. The increase in BV/TV was caused by the increase in Tb.N with no change in Tb. Th.

Micro-CT analysis of the mid shaft of the femoral and tibial cortical bone is shown in table 4.

Femoral cortical bone volume was significantly higher in the animals fed with 20IU D_3/day and 1% dietary to animals with fed with 0.1% dietary calcium($p<0.01$). No significant differences were found in tibial cortical bone volume or endosteal and periosteal surface areas. Tibial sagittal cortical thickness showed no significant difference across the dietary groups.

6.4.3.2 Bone strength

Biomechanical measurements of the different treatment groups are shown in Table 5. Ultimate load (N) and flexural rigidity (N-m2) are representations of the structural properties of the cortical bone. The load at which failure occurred was 10% higher in animals fed 20IU D_3/day with 1% dietary calcium compared to animals fed 0 and 20IU D_3/day with 1% dietary calcium. There was no difference in flexural rigidity, cross sectional moment of inertia or E.

6.4.3.3 Relationships between structure and strength

To further examine the relationships between structure and strength, linear and multiple regressions were carried out to evaluate the level of significance as well as finding out the determinants of the mechanical measurements. There was a positive relationship between serum 25D levels and femoral BV/TV ($p<0.01$, Figure 1A) and femoral cortical bone volume ($p<0.01$, Figure 1B) across all dietary groups. Moreover, even when the three highest individual serum 25D values were removed, the relationship between serum 25D levels and cortical bone volume remained significant ($R^2=0.21$, $p<0.001$). The coefficient of determination for the relationship was 0.23 and 0.18 respectively. The positive relationship can also be found between serum 25D levels and tibial BV/TV ($p<0.05$,Figure 1C) but not tibial cortical bone volume (Figure 1D).

Although there was no positive relationship between serum 25D level and tibial cortical bone volume, there was a positive relationship between serum 25D levels and sagittal cortical thickness ($p<0.01$, Figure 2). To identify the determinants of the changes in mechanical strength, further regression analysis was completed. Sagittal cortical thickness as well as tibial cortical bone volume were both significantly correlated to ultimate load ($p<0.01$, Figure 3A and $p<0.05$, Figure 3B respectively) with the coefficient of determination of 0.3 and 0.1 respectively. No other significant relationships were found between the structural and strength parameters.

Vitamin D (IU/day)	0		2		12		20	
Dietary Ca(%)	0.1	1	0.1	1	0.1	1	0.1	1
Serum 25D, nmol/L	21.7	84.5*	69.5	86.2	87.8	119.4*	90.9	161.3*
(sem)	*(2.9)*	*(6.5)*	*(9.4)*	*(7.8)*	*(10.7)*	*(11.6)*	*(11.8)*	*(38.8)*
Serum Ca, mmol/L	2.8	2.6	2.7	2.5	2.9	2.8	2.6	3.2*
(sem)	*(0.1)*	*(0.1)*	*(0.2)*	*(0.1)*	*(0.2)*	*(0.2)*	*(0.2)*	*(0.2)*
Serum PTH, pg/ml	114	104	135	106	140	111	114	127
(sem)	*(22.5)*	*(23.2)*	*(32.3)*	*(24)*	*(12.2)*	*(21.5)*	*(23.6)*	*(6.1)*
Serum 1,25D, pmol/L	246.6	19.5*	226	48.0*	216.6	9.0*	248.6	13.8*
(sem)	*(30.2)*	*(6.5)*	*(32.4)*	*(15.3)*	*(31.8)*	*(4.5)*	*(68.1)*	*(3.5)*

Table 1. Serum levels of 25 hydroxyvitamin D, calcium, parathyroid hormone and 1,25D in rats fed varying levels of vitamin D and either 0.1% or 1% dietary calcium. Serum biochemistry from these animals is adapted from previous published paper [21] Values are mean (sem), n=5-6. 25D, 25-

hydroxyvitamin D_3; 1,25D, 1,25 dihydroxyvitamin D_3; PTH, parathyroid hormone;* $P<0.05$ when compared with 0.1% dietary calcium in the same vitamin D_3 group

Vitamin D (IU/day)	0		2		12		20	
Dietary Ca(%)	0.1	1	0.1	1	0.1	1	0.1	1
Body weight, grams	321.9	311.0	342.5	334.8	302.6	323.9	292.8	327.2
(sem)	*(22.6)*	*(12.8)*	*(19.5)*	*(6.8)*	*(12.0)*	*(17.3)*	*(7.0)*	*(6.2)*
Femur length, mm	35.5	35.0	35.5	35.3	35.2	36.0	35.6	35.6
(sem)	*(0.3)*	*(0.3)*	*(0.5)*	*(0.1)*	*(0.6)*	*(0.3)*	*(0.4)*	*(0.4)*
Tibia length, mm	40.3	40.1	40.8	40.8	40.0	41.0	40.5	40.9
(sem)	*(0.7)*	*(0.3)*	*(0.5)*	*(0.8)*	*(0.5)*	*(0.3)*	*(0.7)*	*(0.4)*

Table 2. Body weight, and bone lengths of the femur and tibia in rats fed varying levels of vitamin D_3 and either 0.1% or 1% dietary calcium. Values are mean (sem), n=5-6.

Vitamin D (IU/day)		0		2		12		20	
	Dietary Ca(%)	0.1	1	0.1	1	0.1	1	0.1	1
Femur	BV/TV, %	25.7	30.1	29.4	30.0	28.2	32.4	28.9	32.4
	(sem)	*(1.9)*	*(1.9)*	*(1.7)*	*(0.5)*	*(0.9)*	*(1.1)*	*(1.5)*	*(1.8)*
	Tb.N, /mm	2.84	3.03	2.83	2.95	2.85	2.93	2.96	3.17
	(sem)	*(0.1)*	*(0.12)*	*(0.12)*	*(0.05)*	*(0.13)*	*(0.07)*	*(0.11)*	*(0.11)*
	Tb.Th, m	91.4	98.3	90.5	101.7	95.9	107.7	99.5	97.5
	(sem)	*(3.7)*	*(1.7)*	*(3.8)*	*(1.9)*	*(2.1)*	*(2.7)*	*(0.5)*	*(2.5)*
Tibia	BV/TV, %	17.6	18.9	16.0	19.0	18.0	18.6	16.9	21.3*
	(sem)	*(0.5)*	*(0.6)*	*(1.1)*	*(1.1)*	*(0.4)*	*(1.6)*	*(0.8)*	*(0.9)*
	Tb.N, /mm	2.11	2.47	1.90	2.49	2.20	2.45	2.01	2.55*
	(sem)	*(0.16)*	*(0.22)*	*(0.15)*	*(0.18)*	*(0.11)*	*(0.18)*	*(0.27)*	*(0.07)*
	Tb.Th, m	84.2	83.3	84.3	81.1	79.3	79.5	83.7	83.9
	(sem)	*(3.1)*	*(1.1)*	*(1.1)*	*(1.2)*	*(1.0)*	*(1.5)*	*(2.4)*	*(1.7)*

Table 3. Static bone histomorphometry of the proximal femoral and tibial metaphysis (bv/tv, trabecular number and trabecular thickness). Values are mean (sem), n=5-6. $^{*}P<0.05$ when compared to 0.1% dietary calcium in the same vitamin D_3 group

	Vitamin D (IU/day)	0		2		12		20	
	Dietary Ca(%)	0.1	1	0.1	1	0.1	1	0.1	1
Femur	Cort BV, mm^3	27.2	28.6	27.3	28.8	27.3	30.8*	27.1	31.7*
	(sem)	*(0.8)*	*(0.6)*	*(1.1)*	*(0.4)*	*(1.4)*	*(1.2)*	*(0.9)*	*(1.2)*
	Peri. Sur, mm^2	2.84	3.03	2.83	2.95	2.85	2.93	2.96	3.17
	(sem)	*(0.1)*	*(0.12)*	*(0.12)*	*(0.05)*	*(0.13)*	*(0.07)*	*(0.11)*	*(0.11)*
	Endo. Sur, mm^2	91.4	98.3	90.5	101.7	95.9	107.7	99.5	97.5
	(sem)	*(3.7)*	*(1.7)*	*(3.8)*	*(1.9)*	*(2.1)*	*(2.7)*	*(0.5)*	*(2.5)*
Tibia	Cort BV, mm^3	35.8	35.6	35.7	35.2	34.5	37.7	35.2	37.8
	(sem)	*(0.9)*	*(0.6)*	*(1.7)*	*(0.9)*	*(1.4)*	*(1.4)*	*(0.6)*	*(1.4)*
	Peri. Sur, μm^2	155.8	120.2	106.9	103.6	105.1	111.0	103.8	118.2
	(sem)	*(2.2)*	*(12.2)*	*(2.4)*	*(1.7)*	*(3.4)*	*(2.4)*	*(2.6)*	*(7.8)*
	Endo. Sur, mm^2	54.8	52.1	56.9	53.3	53.9	58.0	52.1	58.9
	(sem)	*(3.7)*	*(2.6)*	*(3.1)*	*(1.6)*	*(3.2)*	*(2.0)*	*(1.7)*	*(1.1)*

Table 4. Static bone histomorphometry of the femoral and tibial cortical bone (cortical bone volume, periosteal surface and endosteal surface). Values are mean (sem), n=5-6. * $P<0.05$ when compared to 0.1% dietary calcium in the same vitamin D_3 group**.**

Vitamin D (IU/day)	0		2		12		20	
Dietary Ca(%)	0.1	1	0.1	1	0.1	1	0.1	1
Ultimate load (N)	**70.1**	**64.6***	**67.8**	**63.9***	**63.5**	**66.9**	**66.8**	**71.8**
(sem)	*(2.3)*	*(1.3)*	*(3.1)*	*(2.2)*	*(2.6)*	*(3.3)*	*(3.8)*	*(0.5)*
E(Gpa)	**4.3**	**4.2**	**3.6**	**3.1**	**3.2**	**2.7**	**2.6**	**3.3**
(sem)	*(0.3)*	*(0.7)*	*(0.7)*	*(0.6)*	*(0.6)*	*(0.3)*	*(0.2)*	*(0.5)*
I (mm4)	**5.8**	**6.8**	**8.4**	**9.4**	**8.7**	**10.2**	**9.3**	**8.7**
(sem)	*(1.1)*	*(0.9)*	*(2.2)*	*(2.8)*	*(1.1)*	*(1.5)*	*(0.8)*	*(2.0)*
Flexural Rigidity ($N \cdot m^2$)	**27.3**	**23.4**	**25.3**	**24.7**	**24.3**	**22.8**	**22.2**	**22.4**
(sem)	*(1.6)*	*(3.0)*	*(3.2)*	*(3.5)*	*(4.3)*	*(2.4)*	*(2.6)*	*(0.6)*
Sagittal Cortical thickness (mm)	**0.99**	**1.02**	**0.97**	**1.03**	**0.97**	**1.08**	**0.98**	**1.09**
(sem)	*(0.04)*	*(0.02)*	*(0.05)*	*(0.02)*	*(0.04)*	*(0.06)*	*(0.08)*	*(0.03)*

Table 5. Biomechanical strength measurements in the tibial cortical bone (Ultimate load, E, I, flexural rigidity and sagittal cortical thickness. Values are mean (sem), n=5-6. * P<0.05 when compared to 20IU vitamin D_3/day with 1% dietary calcium.

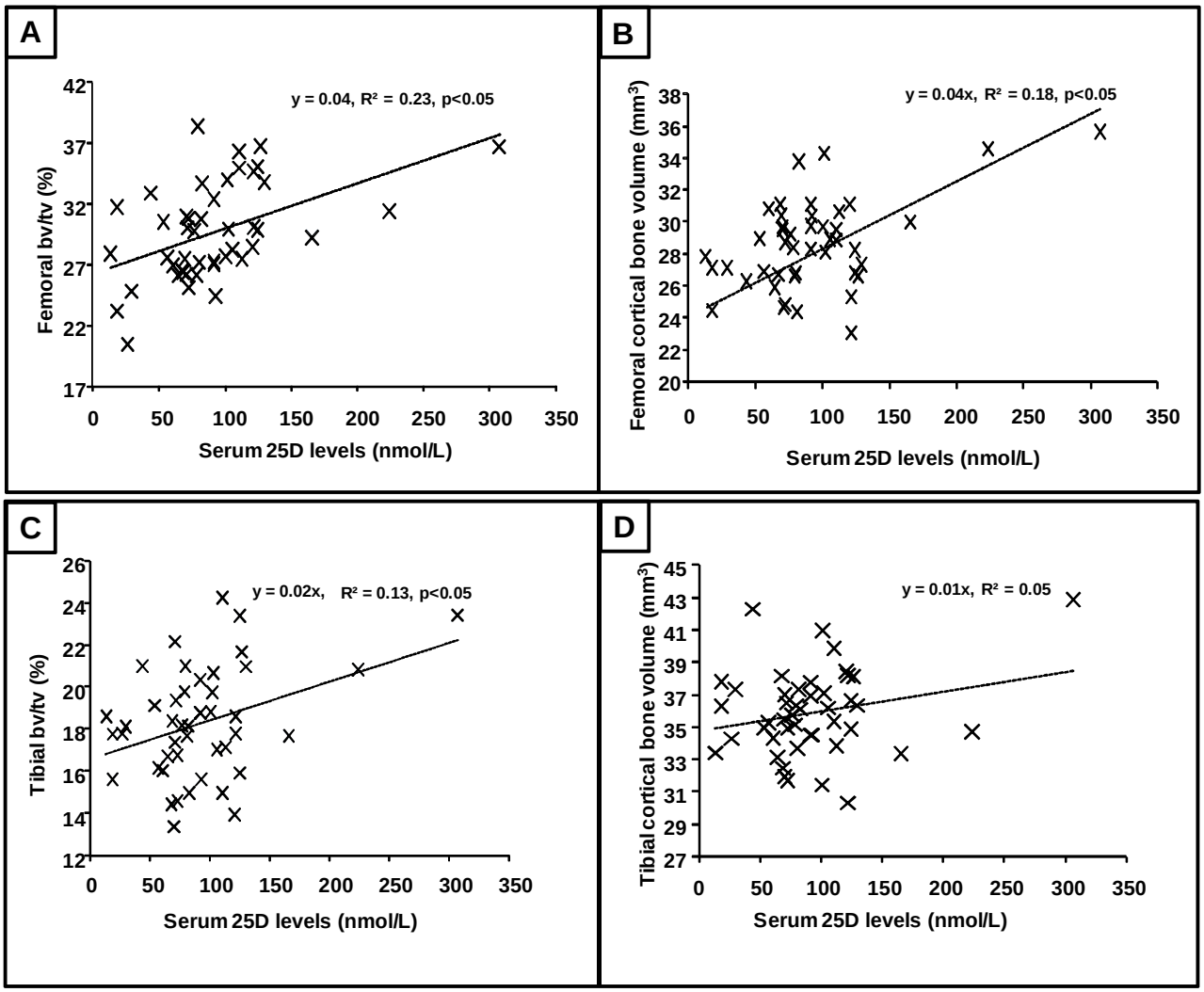


Figure 1. The relationship between serum 25D and A) Femoral trabecular bone volume (%) B) Femoral cortical bone volume (mm^3) C) Tibial trabecular bone volume (%) D) Tibial cortical bone volume (mm^3) in animals with serum 25D level ranging from 22 nM to 161 nM. The coefficient of determination (R^2) and p values are shown for the regression analysis.

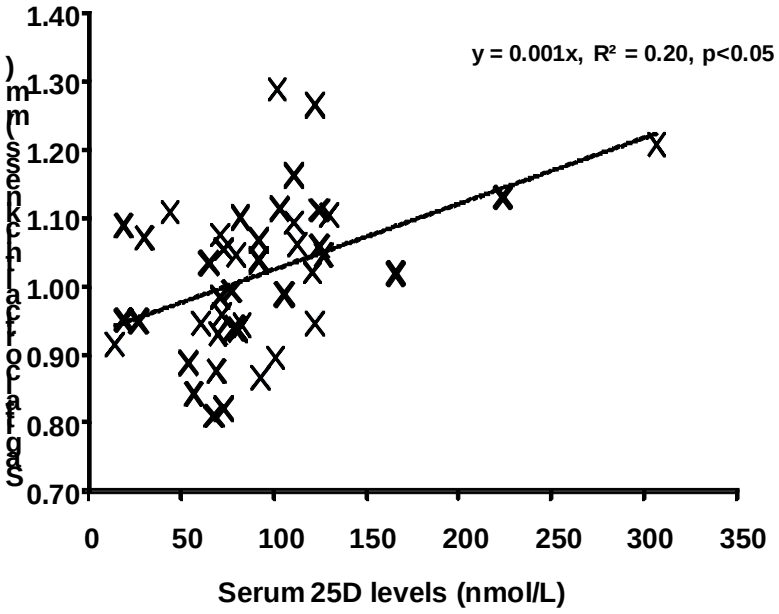


Figure 2. The relationship between serum 25D levels and sagittal cortical thickness (mm) of the tibial mid shaft in animals with serum 25D levels ranging from 21.7 to 161.3 nM. The regression coefficient for the positive linear relationship is 0.20.

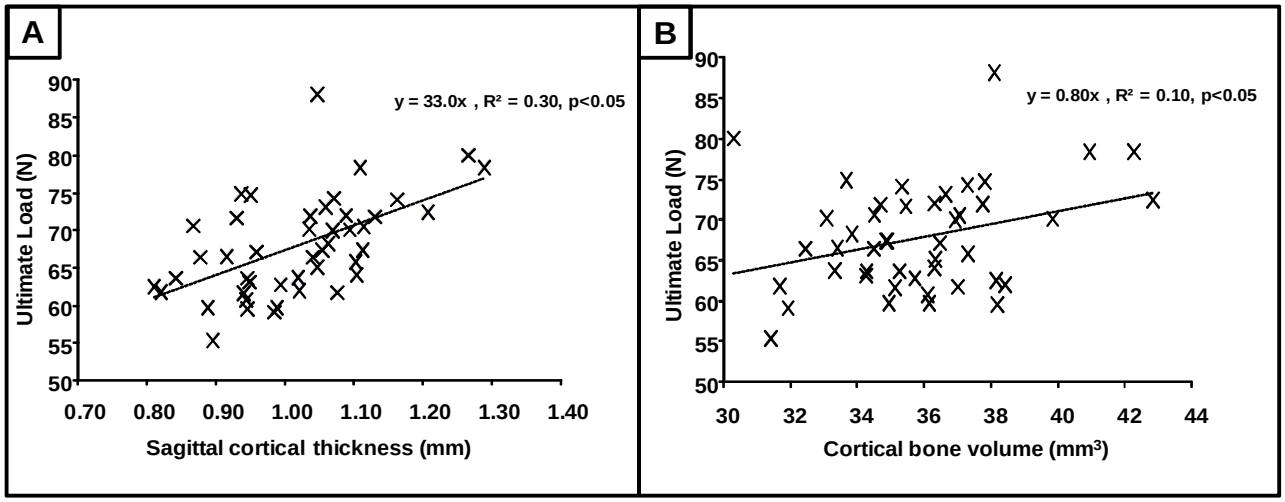


Figure 3 The relationship between A) sagittal cortical thickness and ultimate load and B) cortical bone volume and ultimate load in animals with serum 25D ranging between 22 and 161 nM. The coefficient of determination (R^2) and p values are shown for the regression analysis

6.5 DISCUSSION

This comprehensive study of the effects of dietary vitamin D and calcium in rodents is provided by the measurement and subsequent comparison of a wide spectrum of direct (mineral contents, breaking strength, and remodeling) and indirect (bone-related plasma markers) bone assessment variables. We have shown a significant association between circulating 25D levels, with individual 25D values ranging between 21nM and 161 nM and values of sagittal cortical thickness in the midshaft of the tibial cortical bone on 0.1% and 1% calcium diet. Improvement in bone mineral volume was observed in the tibial proximal metaphysis and femoral diaphysis with higher levels of serum 25D. There were also significant relationships between bone structure and bone mechanical measurements.

We have previously reported that, in animals with serum 25D levels between 20 and 115nM on a 0.4% calcium diet, there were no significant changes in serum calcium, serum phosphate or PTH, with value remaining within normal ranges [21]. Furthermore, we have shown that an adequate circulating 25D levels is important for preventing loss of trabecular structures and maintaining bone strength in young adult rats [22]. The current study showed that the reduction in serum 25D levels can be induced by feeding rats low diet calcium, regardless of the vitamin D content. As discussed in chapter 5, the rise in serum 1,25D levels and reduction in serum 25D levels with animals fed low calcium diet may be due to the anabolic conversion of the precursor, serum 25D [6]. Although serum calcium levels were significantly elevated, there were no significant changes in the levels of PTH, indicating that this level of hypercalcemia was not sufficient to suppress PTH. The animals with the highest serum 25D levels have shown to have significantly high femoral cortical bone volume and tibial metaphyseal trabecular bone volume. The results are not surprising as we discussed in chapter 3 that there are site specific responses to diet manipulations which may

contribute to the variation in observations between cortical bone of the femur and tibia in aged animals. Further studies would be required to establish this proposal. A study using senescence-accelerated mouse model found a 15%-19% reduction in femoral and tibial BV/TV in the senescent mice at 5 months and 12 months of age when compared to the control mice and no marked difference in the mid-tibial and mid-femoral cortical bones [23]. It is generally understood that there are three major processes that lead to bone loss for senile osteoporosis. The first and most important is trabecular bone loss. The second process is continued net bone resorption at the endocortical surface and the last one is a decrease of cortical bone. In the current study, animals that were fed the replete vitamin D and 1% calcium diet had the highest femoral but not tibial cortical bone structure. This is consistent with the published data in chapter 4, suggesting that significant changes in the tibial cortical bone structure may only be observed with prolonged diet/hormone manipulations.

Interestingly, serum 25D levels were significantly related to metaphyseal bone volume in femur and tibia as well as cortical bone in the femur. This is consistent with our previous published data showing that serum 25D levels are significantly correlated to trabecular bone mineral volume in the distal femoral metaphysis [21]. The previous data showed that the loss of trabecular bone at the lower serum 25D levels is the consequence of a marked rise in osteoclast surface. However, the range of serum 25D levels in the present study, are higher and peaked at 161nM, where a significant increase in bone volume was observed. More interestingly, even when the extreme serum 25D levels were removed from the analysis, the relationship remained significant. This suggests that when given high amounts of vitamin D with 1% calcium in the diet, femoral and tibial bone structure are preserved through the prevention of bone loss. Meta-analysis of eight double blind randomised controlled trials found a 38% reduction in the risk of falling for the higher dose range of vitamin D with treatment for 2-5 months and sustained 17% fall reduction with treatment for 12-36 months [24]. WHI trials with vitamin D and calcium (up to 600IU/ day and 1000mg calcium/day)

supplementation showed that women received calcium with vitamin D supplement had greater preservation of total hip bone mineral density than women assigned to placebo. Other studies have shown that vitamin D supplementation at the dose of 600IU or higher are required to observe beneficial effects [25]. Furthermore, a large population based regression analysis confirmed that higher serum 25D levels were associated with higher bone mineral density values throughout the entire range from 22.5 to 92.6 nM. Chapuy et al reported that calcium with vitamin D (1000mg of calcium and 800 IU of vitamin D per day) significantly reduced the risk of hip fractures among elderly woman who were vitamin D deficient [5].

To maintain a healthy skeleton, maintenance and/or improvement of both bone structure and strength are crucial. We have found a significant relationship between serum 25D levels and sagittal cortical thickness. Sagittal cortical thickness is calculated as the thickness of the cortical bone in the axis of loading. Many studies have looked at the bone strength in the axis of loading known as the anterior-posterior (AP) position [26-29]. At the tibial midshaft, where the primary mode of bending is in the AP direction, the optimal cross sectional shape is one where the greatest moment area is aligned with the AP bending plane [29]. This information allows us to investigate the distribution of bone in a certain axis even if there is no change in the overall bone mineral content. Bone quality is determined by mass, microstructure, and material properties. According to the primary locomotive function of the skeleton, mechanical competence of bones represents the ultimate measure of bone phenotype [30]. The structural rigidity of the skeleton is principally maintained by a mechanosensory feedback system that senses the loading induced deformations within the bones and copes with the locomotive challenges through modifications in bone size and shape (ie through geometry, structural and architectural adaptation) [31]. New bone is laid on regions that are subject to loading that exceeds the customary loading range, whereas bone is removed from regions experiencing reduced loading well below the customary loading range [32]. The positive

association between serum 25D levels and sagittal cortical thickness suggests that higher amounts of dietary vitamin D and calcium may be beneficial to the distribution of bone mineral in the region of daily mechanical loading, without dramatic changes in bone mineral content.

We have found positive correlations between sagittal cortical thickness and ultimate load as well as cortical bone volume and ultimate load. Bone tissue is composed of primarily collagen and mineral, with mechanical properties determined largely by amounts, arrangement, and molecular structure of these constituents. An osteoporosis animal model showed a clear positive correlation between the maximal load of the femoral neck and the trabecular bone volume in the distal femur as well as the femoral ash weight [33]. Ultimate load in rats supplemented with 1,25D and alendronate have shown to be significantly correlated to bone mineral density demonstrating that the increase in bone strength is mainly due to the increase in bone mass [34]. Vitamin D deficiency in ovariectomised rodents led to a decrease in bone mineral density and femoral neck strength but not in the mid shaft [18]. While the present study demonstrated that 25D is a determinant of cortical bone volume and sagittal thickness, each of which in turn are determinants of strength the study was under powered to demonstrate a relationship between 25D and bone strength per se. Further studies of this important relationship are warranted.

More recently, studies have used area moment of inertia also known as the cross sectional moment of inertia to characterize the resistance of a long bone to bending around a particular axis [35]. Essentially, it is how material is distributed about a defined axis. When ultimate load and stiffness (structural properties) are normalized to cross-sectional geometry, the resulting indices yield information about material properties of the tissue independent of cross sectional moment of inertia. There were no significant differences in E with increase in dietary vitamin D and calcium. If bone material properties are unchanged in the face of increasing whole bone stiffness, the remaining

possibility is that the quantity and/or geometry of the bone changed with changes in diet. An immobilisation and calcium deficiency study showed a significant 19% reduction in ultimate load in immobilised animals compared to control animals ($p<0.05$) and no difference in E [36]. They have also found a more dramatic bone loss at sites within the tibia that have a higher content of cancellous bone. Furthermore, in hindlimb unloading in female rats, there were no differences in the ultimate load but a 13% drop in E when compared to control rats in the femoral mid diaphysis. The drop in E was compensated for by a larger femoral mid diaphysis in the control animals [37]. It is estimated that in humans, 70-80% of the variance in the ultimate strength of bone tissue is accounted for by an aged related decrease in bone mineral density [38]. Adaptation by changes in bone geometry from cortical expansion or periosteal apposition results in the maintenance of mechanical properties due to increase in the areal and polar moment of inertia properties of the whole bone [39-42]. In rats, modelling occurs in cortical bone and remodelling occurs primarily in cancellous bone. Bone modelling changes the size and shape of cortical bone by resorption and formation drifts [43]. A similar adaptive change was observed in the current study. Our study showed no significant differences in the cross sectional moment of inertia or E suggesting that in a state of vitamin D calcium deficiency, the geometry of the bone in the mid diaphyses may compensate for the bone loss in the metaphysis. However, more detailed work on dynamic cell activity is required to elucidate the mechanism of adaptation in the cortical bone.

The present study of various levels of dietary vitamin D and calcium on structure in different regions of the rat limb and strength in the tibial midshaft has shown that optimal amount of dietary vitamin D and calcium are required to maintain bone mineral content and prevent aged-related bone loss. The high trabecular bone volume in the femoral and tibial metaphysis with higher serum 25D levels is consistent with our previous published data. Of particular interest are the results obtained from the regression analysis looking at the relationships between serum 25D levels, bone structure

and subsequently strength. Although there were no significant differences in strength measurement between the dietary groups, we found a positive correlation between serum 25D and sagittal cortical thickness suggesting that high serum 25D levels improve cortical bone structure. Furthermore, sagittal cortical thickness as well as cortical bone volume are determinants of ultimate load. Taken together, the study suggests serum 25D levels of 90nM or more are required to improve the quality and quantity of a healthy bone and prevent age-related bone loss.

This study has demonstrated for the first time, the effects of various levels of dietary vitamin D with either low (0.1%) or high (1%) calcium on bone structure and strength. Although the exact cellular mechanism behind the unchanged cross sectional moment of inertia and material properties is unclear, this finding is a continued study from our previous published data showing that a serum 25D levels of 90nM of more is required to prevent vitamin D deficiency bone loss. The current study showed that a serum 25D levels of 90nM or more is required to improve bone structure and bone strength. Further studies should investigate the major mechanosensor cell, osteocytes and its activities in response to dietary manipulations and mechanical loading.

6.6 REFERENCES

1. Bischoff-Ferrari, H.A., et al., *Fracture prevention with vitamin D supplementation: a meta-analysis of randomized controlled trials.* Jama, 2005. 293(18): p. 2257-64.
2. Bischoff-Ferrari, H.A., et al., *Estimation of optimal serum concentrations of 25-hydroxyvitamin D for multiple health outcomes.* Am J Clin Nutr, 2006. 84(1): p. 18-28.
3. Anderson, P.H., et al., *Vitamin D depletion induces RANKL-mediated osteoclastogenesis and bone loss in a rodent model.* Journal of Bone and Mineral Research, 2008. 23(11): p. 1789-97.
4. Bailey, R.L., et al., *Estimation of total usual calcium and vitamin D intakes in the United States.* J Nutr. 140(4): p. 817-22.
5. Chapuy, M.C., et al., *Vitamin D3 and calcium to prevent hip fractures in the elderly women.* N Engl J Med, 1992. 327(23): p. 1637-42.
6. Anderson, P.H., et al., *The effect of dietary calcium on 1,25(OH)2D3 synthesis and sparing of serum 25(OH)D3 levels.* J Steroid Biochem Mol Biol. 121(1-2): p. 288-92.
7. Need, A.G., *Bone resorption markers in vitamin D insufficiency.* Clin Chim Acta, 2006. 368(1-2): p. 48-52.
8. Jesudason, D., et al., *Relationship between serum 25-hydroxyvitamin D and bone resorption markers in vitamin D insufficiency.* Bone, 2002. 31(5): p. 626-30.
9. Eastell, R., et al., *Interrelationship among vitamin D metabolism, true calcium absorption, parathyroid function, and age in women: evidence of an age-related intestinal resistance to 1,25-dihydroxyvitamin D action.* J Bone Miner Res, 1991. 6(2): p. 125-32.
10. Parfitt, A.M., S. Qiu, and D.S. Rao, *The mineralization index--a new approach to the histomorphometric appraisal of osteomalacia.* Bone, 2004. 35(1): p. 320-5.

11. Need, A.G., et al., *The effects of age and other variables on serum parathyroid hormone in postmenopausal women attending an osteoporosis center.* J Clin Endocrinol Metab, 2004. 89(4): p. 1646-9.

12. Need, A.G., et al., *Seasonal change in osteoid thickness and mineralization lag time in ambulant patients.* J Bone Miner Res, 2007. 22(5): p. 757-61.

13. Gardiner, E.M., et al., *Increased formation and decreased resorption of bone in mice with elevated vitamin D receptor in mature cells of the osteoblastic lineage.* Faseb J, 2000. 14(13): p. 1908-16.

14. Atkins, G.J., et al., *Metabolism of vitamin D(3) in human osteoblasts: Evidence for autocrine and paracrine activities of 1alpha,25-dihydroxyvitamin D(3).* Bone, 2007. 40(6): p. 1517-28.

15. Anderson, P.H., et al., *25-Hydroxyvitamin D requirement for maintaining skeletal health utilising a Sprague-Dawley rat model.* J Steroid Biochem Mol Biol, 2007. 103(3-5): p. 592-5.

16. Eklou-Kalonji, E., et al., *Calcium-regulating hormones, bone mineral content, breaking load and trabecular remodeling are altered in growing pigs fed calcium-deficient diets.* J Nutr, 1999. 129(1): p. 188-93.

17. Koshihara, M., et al., *Reduction in dietary calcium/phosphorus ratio reduces bone mass and strength in ovariectomized rats enhancing bone turnover.* Biosci Biotechnol Biochem, 2005. 69(10): p. 1970-3.

18. Kaastad, T.S., et al., *Vitamin D deficiency and ovariectomy reduced the strength of the femoral neck in rats.* Calcif Tissue Int, 2001. 69(2): p. 102-8.

19. Reeves, P.G., F.H. Nielsen, and G.C. Fahey, Jr., *AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet.* J Nutr, 1993. 123(11): p. 1939-51.

20. Turner, C.H. and D.B. Burr, *Basic biomechanical measurements of bone: a tutorial.* Bone, 1993. 14(4): p. 595-608.

21. Anderson, P.H., et al., *Vitamin D depletion induces RANKL-mediated osteoclastogenesis and bone loss in a rodent model.* J Bone Miner Res, 2008. 23(11): p. 1789-97.

22. Lee, A.M., et al., *Discordant effects of vitamin D deficiency in trabecular and cortical bone architecture and strength in growing rodents.* J Steroid Biochem Mol Biol. 121(1-2): p. 284-7.

23. Chen, H., et al., *Site-specific bone loss in senescence-accelerated mouse (SAMP6): a murine model for senile osteoporosis.* Exp Gerontol, 2009. 44(12): p. 792-8.

24. Bischoff-Ferrari, H.A., et al., *Fall prevention with supplemental and active forms of vitamin D: a meta-analysis of randomised controlled trials.* BMJ, 2009. 339: p. b3692.

25. Jackson, R.D., et al., *Calcium plus vitamin D supplementation and the risk of fractures.* N Engl J Med, 2006. 354(7): p. 669-83.

26. Jarvinen, T.L., et al., *Dual-energy X-ray absorptiometry in predicting mechanical characteristics of rat femur.* Bone, 1998. 22(5): p. 551-8.

27. Jarvinen, T.L., et al., *Randomized controlled study of effects of sudden impact loading on rat femur.* J Bone Miner Res, 1998. 13(9): p. 1475-82.

28. Pajamaki, I., et al., *The bone gain induced by exercise in puberty is not preserved through a virtually life-long deconditioning: a randomized controlled experimental study in male rats.* J Bone Miner Res, 2003. 18(3): p. 544-52.

29. Macdonald, H.M., D.M. Cooper, and H.A. McKay, *Anterior-posterior bending strength at the tibial shaft increases with physical activity in boys: evidence for non-uniform geometric adaptation.* Osteoporos Int, 2009. 20(1): p. 61-70.

30. Jarvinen, T.L., et al., *Revival of bone strength: the bottom line.* J Bone Miner Res, 2005. 20(5): p. 717-20.

31. Frost, H.M., *Bone's mechanostat: a 2003 update.* Anat Rec A Discov Mol Cell Evol Biol, 2003. 275(2): p. 1081-101.

32. Kimmel, D.B., *A paradigm for skeletal strength homeostasis.* J Bone Miner Res, 1993. 8 Suppl 2: p. S515-22.

33. Peng, Z., et al., *The mechanical strength of bone in different rat models of experimental osteoporosis.* Bone, 1994. 15(5): p. 523-32.

34. Ito, M., et al., *Curative effect of combined treatment with alendronate and 1 alpha-hydroxyvitamin D3 on bone loss by ovariectomy in aged rats.* Jpn J Pharmacol, 2002. 89(3): p. 255-66.

35. Lieberman, D.E., J.D. Polk, and B. Demes, *Predicting long bone loading from cross-sectional geometry.* Am J Phys Anthropol, 2004. 123(2): p. 156-71.

36. Inman, C.L., et al., *Mechanical loading attenuates bone loss due to immobilization and calcium deficiency.* J Appl Physiol, 1999. 87(1): p. 189-95.

37. Allen, M.R. and S.A. Bloomfield, *Hindlimb unloading has a greater effect on cortical compared with cancellous bone in mature female rats.* J Appl Physiol, 2003. 94(2): p. 642-50.

38. Smith, C.B. and D.A. Smith, *Relations between age, mineral density and mechanical properties of human femoral compacta.* Acta Orthop Scand, 1976. 47(5): p. 496-502.

39. Enlow, D.H., *A study of the post-natal growth and remodeling of bone.* Am J Anat, 1962. 110: p. 79-101.

40. Faulkner, K.G., et al., *Simple measurement of femoral geometry predicts hip fracture: the study of osteoporotic fractures.* J Bone Miner Res, 1993. 8(10): p. 1211-7.

41. Boonen, S., et al., *Measurement of femoral geometry in type I and type II osteoporosis: differences in hip axis length consistent with heterogeneity in the pathogenesis of osteoporotic fractures.* J Bone Miner Res, 1995. 10(12): p. 1908-12.

42. Genant, H.K., C.C. Gluer, and J.C. Lotz, *Gender differences in bone density, skeletal geometry, and fracture biomechanics.* Radiology, 1994. 190(3): p. 636-40.

43. Jiang, S.D., et al., *Differences of bone mass and bone structure in osteopenic rat models caused by spinal cord injury and ovariectomy.* Osteoporos Int, 2007. 18(6): p. 743-50.

CHAPTER 7: SUMMARY AND CONCLUSIONS

7.1 SUMMARY

This thesis investigated the effects of various levels of dietary vitamin D and calcium on bone structure and strength in young adult rats as well as senescent rats. The broad aim was to determine whether the high levels of dietary vitamin D and/or calcium are maintaining/improving bone mineral content and mechanical strength. Three point bending showed no systematic differences between the left and right limb indicating this technique was able to detect changes in bone strength in a highly reproducible and sensitive manner. The measurement of the serum levels of 25D, 1,25D, PTH and calcium was used to determine the effects of age, dietary calcium and vitamin D status on the metabolism of vitamin D as well as the mRNA expressions of CYP27B1 and CYP24 in the kidney. The data derived from the studies on age and dietary manipulations have provided valuable insight into the levels of serum 25D and dietary Ca intake required to maintain a healthy skeleton.

7.2 THE ROLE OF MECHANICAL TESTING OF LONG BONES TO ASSESS BIOLOGICAL FUNCTION

Chapter 3 investigated the effectiveness and sensitivity of three-point bending as a measure of mechanical strength. This study demonstrated high reproducibility in both extrinsic and intrinsic qualities of bone strength measurements; ultimate load, cross sectional moment of inertia flexural rigidity and Young's Modulus that are not compromised by differences in sidedness. Furthermore, significant correlation between bone volume and two mechanical measurements were found when animals were subjected to various levels of dietary vitamin D, dietary calcium and estrogen depletion by ovariectomy. The results suggested that three-point bending is a sensitive technique in testing bone strength despite the subtle change in cortical bone volume which may be the result of

ovariectomy and/or dietary changes. The poor correlation of E and cortical volume suggested a further physiological explanation and not a sensitivity issue.

7.3 THE EFFECT OF VITAMIN D AND DIETARY CALCIUM ON BONE STRUCTURE AND STRENGTH

Based on the previous published paper in our laboratory which showed that vitamin D deficiency in young male rats results in significant reduction in femoral trabecular bone volume (BV/TV) in the distal and proximal metaphyses as well as the vertebrae [1], chapter 4 investigated the effects of various levels of dietary vitamin D on bone structure and strengh in the cortical bone of young adult rats [2]. A significant association between serum 25D and trabecular bone occurred for both the distal femoral metaphysis and L2 vertebrae. However, tibial cortical bone mineral content and architecture were preserved even when serum 25D levels were reduced to 16nmol/L. Furthermore, there was no evidence of redistrution of bone mineral across different diet regimes, suggesting that overall bone turnover was maintained at a constant level in the cortical bone. These observations are consistent with other published studies where there was a significant reduction in the BV/TV but not cortical bone until 1 year post ovariectomy [3]. In addition, the lower surface area to volume ratio inherent to cortical bone may contribute to the much slower changes observed in cortical bone. Together, these results demonstrate site-specific responses to vitamin D deficiency and that an extended period of calcium and/or vitamin D deprivation maybe required to observe significant changes in the cortical bone.

The previous chapter stated that vitamin D depletion in rats causes osteopenia in at least three skeletal sites. Chapter 5 investigates whether modulation of dietary calcium intake impacts on the relationship between the levels of serum 25D and bone loss. Animals fed high calcium diet showed higher serum 25D levels when compared to animals fed low dietary calcium. The increase in serum

25D levels was not due to the changes in the mRNA levels of liver 25D synthetic enzymes or the catabolism of serum 25D via CYP24 activity. A possible explanation for the reduction in serum 25D levels in low calcium diet animals could be that in response to long period of calcium deprivation, renal CYP27B1 expression is up-regulated accompanied by an increase in 1,25D production which may be responsible for the consumption of 25D stores. The major determinants of serum 25D were dietary vitamin D and dietary calcium. Hence, the reported effects of low calcium diets on bone loss may be due to the subsequent effects of 25D metabolism leading to reduction in vitamin D status. CYP24 and a range of liver enzymes were not regulated by the low calcium diet [4].

As the published data in chapter 5 indicated that circulating levels of serum 25D are greater in animals fed a high calcium diet, chapter 6 further investigated the potential role of high calcium diet in protecting bone loss and maintaining bone strength. Micro-CT analysis demonstrated that femoral cortical bone volume as well as tibial trabecular bone volume was significantly higher in animals with the highest serum 25D levels. Furthermore, the greatest maximum load to failure was achieved in the same dietary group. To further investigate the effects of various dietary vitamin D and calcium on bone structure and strength, chapter 6 discussed those effects in bone structure, particularly in the weight bearing bones of the skeleton, femur and tibia in senescent animals. We showed that animals that achieved serum 25D levels of 90nM or more maintained bone mineral content in the femoral and trabecular metaphysis as well as femoral cortical bone. Despite the lack of significance observed between the dietary groups in either cross sectional moment of inertia, or sagittal cortical thickness, there was a positive association between serum 25D and sagittal cortical thickness, demonstrating that serum 25D levels may be an important regulator in influencing the distribution of bone mineral in the loading axis. Interestingly, regression analysis showed that sagittal cortical thickness is a determinant of ultimate load further suggesting that any subtle

changes in bone structure can consequently cause dramatic changes in bone mechanical function. Ultimate load is a measurement of the mechanical properties of the bone without taking geometry changes into consideration. Although we observed no significant changes in any of the mechanical strength measurements between the dietary groups, there was a positive association between tibial cortical bone volume and ultimate load. The lack of change in tibial cortical bone volume can be explained by the maintenance of cross sectional moment of inertia, as well as periosteal and endosteal surface areas (data not shown). Data imply that there may be a subtle redistribution of bone without a change in the total cortical area/volume suggesting that this change may be better detected in a study conducted over a longer time.

Similar to our previous study where we observed a dramatic drop in the distal femoral BV/TV, we observed no changes in the cortical bone or cross sectional moment of inertia. Therefore, the positive association between cortical bone volume and ultimate load suggest that with the subtle increase in the cortical bone volume, which is due to increase in vitamin D and calcium diet, maybe causing a significant increase in the structural properties of the bone. However, when taking the shape or geometry of the bone into account, the lack of overall changes in the material properties of the bone is masked by the adaption of cortical bone.

In summary, the findings of this study indicate that serum 25D levels may be an important determinant of cortical thickness and subsequently, structural properties of bone in rodents. When serum 25D levels were higher than our previously published paper [1], there was a significant effect on cancellous bone as well as cortical bone. Our data suggested that in animals a serum 25D level of >90nM is sufficient to protect against bone loss provided dietary calcium is not restricted. This value is consistent with our previous data suggesting that serum 25D levels of 80nM or more are required to prevent vitamin D deficiency induced bone loss. Furthermore, optimal levels of dietary vitamin D and calcium are required to improve bone quality and quantity. The role of 25D in bone

cell metabolism should be further investigated and may provide an indication of clinical requirement of dietary vitamin D and calcium that are required to prevent osteoporosis

7.4 LIMITATIONS

The design of the experiments in this thesis was such that it is difficult to directly compare the results of 3 months vitamin D deficiency and 6 months of vitamin D and calcium deficiency. The first study used young adult male rats to investigate 3 months of vitamin D deficiency by giving animals various levels of dietary vitamin D plus standard 0.4% calcium diet whilst the second study investigated 6 months of vitamin D deficiency in aged female rats fed various levels of dietary vitamin D plus low (0.1%) or high (1%) calcium diet. Animals from the first study were assigned to various dietary groups at 10 weeks of age while the second study started diet regimes at 9 months of age. If serum 25D levels were measured before the animals were assigned to their diet regimes then it would be a more accurate measurement of how much serum 25D levels are left in the circulation. Furthermore, further studies should investigate various level of calcium content in the diet. Our previous data suggested that a diet containing 12 IU of vitamin D per day plus 0.4% calcium is sufficient enough to achieve serum 25D levels that are required to prevent vitamin D deficiency induced bone loss. The question as to whether that is the minimum required amount of calcium in the diet to prevent bone loss is still unclear. Studies of dietary calcium below 0.4% are required to establish a more definite explanation.

We have demonstrated the regulation of the transcriptional activity of CYP27B1 and CYP 24 in the kidney and vitamin D metabolising enzymes in liver in response to varying dietary vitamin D and calcium. The increase in production of serum 1,25D levels was accompanied by an upregulation of renal CYP27B1 in response to calcium deprivation. It is less clear, however, whether the levels of

the mRNA levels of CYP27B1 and CYP24 in the bone directly relate to the activity of these enzymes and the production of 1,25D within bone. Further studies, however, investigating the specific regulation of CYP27B1 in the bone would be required to identify the molecular signals that give rise to the changes we observed in bone structure. The activity of CYP27B1 and CYP24 can be determined in primary cell culture experiments of cells harvested from treated animals. Furthermore, western blot analysis may be utilised to measure the protein levels of VDR in response to altered vitamin D status and dietary calcium intake. Immunohistochemistry could also be used to identify VDR protein in the kidney and bone

This thesis validated and used three-point bending as a method of biomechanical testing. Three point bending was employed by many other studies as the simple and reproducible mechanical testing method used on rodents due to the limitations that small bones are very difficult to machine tensile or compressive test specimens. In the bending test, high shear stress is created near the mid section of the bone which may result in an underestimation of the Young's Modulus [5] . Another limitation to three point bending is the use of cross section moment of inertia, which is a measure of the distribution of material around a give axis. This value is usually estimated using formulae defined for a perfect elliptical cross section from data collected by peripheral quantitative computed tomography (pQCT) [6]. As bone is anisotropic, the continual change in cross sectional geometry of tibia makes it an inaccurate bone to test. Although the tibia is commonly used because its unique shape allows for reproducible alignment in the testing equipment, there are no formulae that can accommodate its triangular cross sectional shape. Therefore, although the tibia can be a precise bone to test, its cross sectional and longitudinal morphology does not lend itself to accurate results.

The hypotheses generated in this thesis are based on our previous data demonstrating a significant reduction in femoral metaphyseal BV/TV in response to vitamin D deficiency. The identification of

bone mechanical strength is limited by the sensitivity of the site of testing. In this thesis, we found insignificant subtle changes in the cortical bone in response to vitamin D deficiency as well as combined vitamin D and calcium deficiency. As discussed in chapter 4 and chapter 6, the lack of changes observed in the cortical bone indicate that cortical bone may be less sensitive to metabolic disturbances compared to trabecular bone. Further studies should conduct mechanical testing on areas that have shown significant bone loss such as the compression testing of femoral neck or L2 vertebrae to directly correlate bone loss and strength

7.5 FUTURE DIRECTIONS

We have demonstrated that vitamin D status is a major determinant in femoral cancellous bone volume and we have identified structural factors that contribute to the maintenance of cortical bone volume and strength. However, it is postulated that the signals that direct resorption or formation as required by bone in regards to mechanical strain are translated by osteocytes. Studies have shown that the ability of bone to remodel efficiently and maintain its homeostasis is associated with the presence of viable osteocytes [7, 8]. Osteocyte density was found significantly lower in ovariectomised rats when compare to sham animals. Additionally, osteocyte density was positively correlated to bone mineral content and ultimate load [9]. Reduction in mineral content and bone strength is accompanied by increased osteocyte apoptosis due to a decrease in bone formation followed by an increase in resorption in weightlessness studies [10]. These observations suggest that osteocytes actively initiate and control bone remodelling possibly by releasing a signal to recruit osteoclasts and subsequently alter bone quality. Further studies should investigate osteocyte density as well as the amount of osteocyte apoptosis in vitamin D and calcium deficiency studies.

Furthermore, 1,25D is thought to play an important role in stimulating the differentiation of osteoclasts from monocyte-macrophage stem cell precursors in vitro [11] and is a key regulator of

RANKL expression [12]. Therefore, genetic models where vitamin D metabolism and activity have been deleted in specific bone cell types need to be undertaken to establish the importance of synthesis of 1,25D at the level of bone.

7.6 REFERENCES

1. Anderson, P.H., et al., *Vitamin D depletion induces RANKL-mediated osteoclastogenesis and bone loss in a rodent model.* J Bone Miner Res, 2008. 23(11): p. 1789-97.
2. Lee, A.M., et al., *Discordant effects of vitamin D deficiency in trabecular and cortical bone architecture and strength in growing rodents.* J Steroid Biochem Mol Biol. 121(1-2): p. 284-7.
3. Li, M., Y. Shen, and T.J. Wronski, *Time course of femoral neck osteopenia in ovariectomized rats.* Bone, 1997. 20(1): p. 55-61.
4. Anderson, P.H., et al., *The effect of dietary calcium on 1,25(OH)2D3 synthesis and sparing of serum 25(OH)D3 levels.* J Steroid Biochem Mol Biol. 121(1-2): p. 288-92.
5. van der Meulen, M.C., G.S. Beaupre, and D.R. Carter, *Mechanobiologic influences in long bone cross-sectional growth.* Bone, 1993. 14(4): p. 635-42.
6. Turner, C.H. and D.B. Burr, *Basic biomechanical measurements of bone: a tutorial.* Bone, 1993. 14(4): p. 595-608.
7. Qiu, S., et al., *Reduced iliac cancellous osteocyte density in patients with osteoporotic vertebral fracture.* J Bone Miner Res, 2003. 18(9): p. 1657-63.
8. Hernandez, C.J., R.J. Majeska, and M.B. Schaffler, *Osteocyte density in woven bone.* Bone, 2004. 35(5): p. 1095-9.
9. Ma, Y.L., et al., *Quantitative associations between osteocyte density and biomechanics, microcrack and microstructure in OVX rats vertebral trabeculae.* J Biomech, 2008. 41(6): p. 1324-32.
10. Aguirre, J.I., et al., *Osteocyte apoptosis is induced by weightlessness in mice and precedes osteoclast recruitment and bone loss.* J Bone Miner Res, 2006. 21(4): p. 605-15.
11. Nijweide, P.J., E.H. Burger, and J.H. Feyen, *Cells of bone: proliferation, differentiation, and hormonal regulation.* Physiol Rev, 1986. 66(4): p. 855-86.

12. Yasuda, H., et al., *Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL*. Proc Natl Acad Sci U S A, 1998. 95(7): p. 3597-602.

APPENDIX

Anderson, P.H., Lee, A.M., Anderson, S.M., Sawyer, R.K., O'Loughlin, P.D. & Morris, H.A. (2010)
The effect of dietary calcium on $1,25(OH)_2D_3$ synthesis and sparing of serum $25(OH)D_3$ levels.
Journal of Steroid Biochemistry & Molecular Biology, v. 121 (1-2), pp. 288 -292