

The Pennsylvania State University
The Graduate School
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**LIFE-LONG VITAMIN A SUPPLEMENTATION HAS NO DETRIMENTAL
EFFECTS ON TRABECULAR AND CORTICAL BONE OR BONE STIFFNESS**

A Thesis in
Nutrition
by
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Submitted in Partial Fulfillment
of the Requirements
for the Degree of
Master of Science

May 2010

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ABSTRACT

Vitamin A (VA) is a fat-soluble dietary component required for vision, cellular differentiation, proliferation, as well as normal growth and bone development. The principal biologically active metabolite of VA, retinoic acid (RA) acts through retinoid specific receptors to modulate gene expression.

More than 50% of US adults report use of a dietary supplement, which can result in VA intakes that are more than 200% of the recommended dietary allowance (RDA). Recent data suggest a much smaller changes in VA intake than previously suspected might result in alterations in skeletal fracture risk. VA is fat soluble and cleared by cells within bone marrow, and thus VA and its metabolically active metabolite RA have access to this highly sensitive hematopoietic environment. It is hypothesized that alterations to VA intake (both high and low) will result in changes in cortical and trabecular bone, and resistance to fracture.

Dosing of 25-day-old rat pups for 14 days with VA established that retinol does indeed travel to both the tibia and femur, with an increase in VA intake. Long-term evaluation in a chronic model of marginally VA-deficient, control and VA-supplemented animals, evaluated at three different ages (2-3, 8-10 and 20-22 months), provided a larger picture than previously described as to the effect of both marginally deficient VA intake and VA supplemented intake on bone health. Though both low and high VA resulted in increased medullary cavity size and decreased cortical thickness (as analyzed by micro-computed tomography) at 2-3 months, no changes were noted at 8-10 or 20-22 months. There was also no difference in resistance to fracture later in life, as analyzed by material testing system and bone loading. Taken together, these data indicate that alterations to

normal VA intake over the lifespan, within the range of marginal to supplemented intakes, do not have measurable detrimental effects on several parameters related to bone health.

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LIST OF ABBREVIATIONS

| | |
|--|--|
| u-CT : micro computed tomography | OM : old age marginally vitamin A deficient rats |
| AP : activator protein | OPG : osteoprotegerin |
| BM : bone marrow | OS : old age vitamin A supplemented rats |
| BMU : bone multicellular unit | PBS : phosphate buffered saline |
| BMD : bone mineral density | RA : all-trans-retinoic acid |
| C-FMS : M-CSF receptor | RANK : receptor activator of nuclear factor kB |
| CM : chylomicron | RANKL : receptor activator of nuclear factor kB ligand |
| CMR : chylomicron remnant | RDA : recommended dietary allowance |
| JNK : jun N-terminal kinase | REq : retinol equivalents |
| MC : middle age control diet rats | RE : retinyl esters |
| MCP-1 : monocytes chemoattractant protein 1 (also known as CCL2) | TMMP : trimethylmethoxyphenyl |
| M-CSF : macrophage colony stimulating factor | TRAF : tumor necrosis factor receptor associated factor |
| MIFT : microphthalmia-associated transcription factor | TRAP : tartrate resistant acid phosphatase |
| MM : middle age marginally deficient rats | VA : vitamin A |
| MS : middle age vitamin A supplemented rats | UL : tolerable upper intake level |
| MTS : mechanical testing system | YC : young age control rats |
| NFATc1 : nuclear factor of activated T-cells, calcineurin dependent 1 | YM : young age marginally vitamin A deficient rats |
| OC : old age control diet rats | YS : young age vitamin A supplemented rats |

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CHAPTER 1

INTRODUCTION TO THESIS

General Introduction to Vitamin A

Vitamin A (VA) is a fat-soluble dietary component required for vision, cellular differentiation, proliferation, as well as normal growth and bone development (Evans and Kaye 1999). The principal biologically active metabolite of VA, retinoic acid (RA) acts through retinoid specific receptors to modulate gene expression (Evans and Kaye 1999). Due to these nuclear regulatory properties, hypervitaminosis A often results in skeletal abnormalities, an increase in bone fragility, and, in extreme cases, death.

The recommended dietary allowance (RDA) for an adult male is 900 μg RAE/day, with a tolerable upper intake level (UL) of 3000 μg RAE/day (Genaro Pde and Martini 2004). According to data from the 1999-2000 National Health and Nutrition Examination Survey, 52% of US adults report use of a dietary supplement (Radimer, Bindewald et al. 2004). When all VA sources are considered, daily intakes of more than 2000 μg VA/day can be expected for nearly 50% of the population (Genaro Pde and Martini 2004). (Carpenter, Pettifor et al. 1987; Kneissel, Studer et al. 2005). Experimental dietary interventions in mice have suggested that a much lower VA intake than previously suspected might result in an increase in skeletal brittleness. However, few experiments have been done *in vitro* or have investigated moderately elevated intakes of VA longitudinally (Johansson, Lind et al. 2002).

VA, as a fat-soluble vitamin, is absorbed and transported with triacylglycerols and cholesterol in chylomicrons (CM), which also contain apoproteins A-I, A-IV, B-48, C

and E. Chylomicron triacylglycerols are lipolyzed in capillaries forming CM remnants (CMR), which then bind, primarily, to the hepatic apoprotein receptor, LDL receptor-related protein (LRP) (Shils and Shike 2006; Zemleni 2007). This receptor recognizes apolipoprotein E which is still incorporated in the CMR (Hussain, Mahley et al. 1989). It is well established that the major site of CMR clearance from circulation is the liver, but data also suggest bone marrow (BM) is the largest secondary site (Hussain, Mahley et al. 1989; Hussain, Mahley et al. 1989; Twining, Schulte et al. 1996; Niemeier, Niedzielska et al. 2008). The clearance of CMR by bone marrow may be explained by the recent discovery of LRP on osteoblasts (Niemeier, Kassem et al. 2005; Niemeier, Niedzielska et al. 2008). Though VA is not stored in the marrow cavity, the uptake of CMR by cells within the marrow cavity allows VA, a nuclear regulatory agent after VA is metabolized to RA, to gain access to this highly sensitive hematopoietic environment (Skrede, Olafsdottir et al. 1993; Skrede, Lie et al. 1994).

Bone Physiology

Bone is comprised of several specific types and regions of tissue (Figure 1A & B). The outer shell of bone is referred to as cortical bone, and is composed of two layers: the periosteum which comprises the outer portion, and the endosteum, which makes up the inner surface of the medullary cavity (Datta, Ng et al. 2008). Trabecular bone is the highly latticed scaffolding that fills the bone lumen at the ends of long bones, and is normally associated with the endosteal bone surface (Robling, Castillo et al. 2006).

In small spaces that traverse cortical bone and between trabeculae are osteons. Osteons are categorized into two types depending on whether the type of bone in which

they are located is newly forming (primary osteons) or the result of resorption of existing bone tissue (secondary osteons). Both primary and secondary osteons incorporate 16 cylindrical lamellae in layers radiating from the canal core, and are separated from surrounding bone tissue by an area called the cement line (Martin, Burr et al. 1998). The center of an osteon contains Haversian or Volkmann's canals that house blood vessels and nerves (Figure 1C; (Robling, Castillo et al. 2006).

Bone multicellular units (BMUs) are comprised of osteoblasts and osteoclasts and are located within these canals. BMUs act as a functional unit to remove and deposit collagen, causing both modeling and remodeling to occur depending on the signaling cascade initiated (Weiner & Wagner, 1998). Canaliculi are also present and are defined as small spaces within mineralized bone housing terminally differentiated osteoblasts. These cells, renamed osteocytes, are no longer able to generate collagen. Osteocytes are responsible for maintaining bone integrity (Weiner & Wagner, 1998). Osteocytes are also thought to sense stress and strain demands on bone, in order to initiate signaling cascades involved in remodeling of localized bone regions. This ability of bone effector cells to sense and respond to mechanical loads through a modification in structure is referred to as Wolff's law (Martin, Burr et al. 1998).

BMUs are activated through a complicated signaling cascade that can originate from multiple sources, most of which are focused on the activation of osteoblasts (Figure 2-3). Changes in BMU signaling result in alterations to the normal resorb/refill cycle and can lead to changes in BMD, bone strength and bone shape (Figure 2).

Under normal circumstances resting osteoblasts are activated and osteoclast precursors are recruited and activating forming functional, multinucleated osteoclasts

capable of matrix resorption. This process of recruitment and activation normally takes about three days (Martin, Burr et al. 1998). The BMU forms a cutting cone, headed by osteoclasts, which removes matrix at a rate of roughly 40 $\mu\text{m}/\text{day}$. Once signaling diminishes, a reversal phase occurs over several days where osteoclasts at the start of the BMU cutting cone stop resorption and undergo apoptosis. Osteoblasts are activated by compounds released from bone (TGF- $B_{1/2}$, B₂-microglobulin, PDGF, FGF, IGF-I and II). Filling resorption pits formed by osteoclasts at a rate of roughly 1-2 $\mu\text{m}/\text{day}$ (Terminie 1990).

After activation, osteoblasts polarize in a similar manner as osteoclasts, forming two distinct regions. The first is made up of two less defined regions deemed the nuclear region, which contains the nucleus, and the Golgi region, which characterized by Golgi sacules and centroiles complexes, as well as numerous rough endoplasmic reticulum cisternae (Leblond 1989; Mackie 2003). The second region is the frontal region made up of many thin processes several times the length of the cell.

The initial collagen precursors are poplypeptides, specifically pro- $\alpha 1(\text{I})$ and pro- $\alpha 2(\text{I})$ formed in a 2:1 ratio on ribosomes then released into the rough endoplasmic reticulum and finally transferred to spherical distensions of the Golgi (Leblond 1989). The filaments begin to align in parallel bundles causing the spherical distention to elongate, forming cylindrical distensions while moving from the cis- to the trans-side of the Golgi . Aggregation of the fibers continues until the distention has reached capacity, at which time the procollagen fibrils undergo a series of enzematic cleavages and the distensions release becoming secretory vacuoles, releasing newly formed collagen type-1 and allowing for the elongation of collagen fibrils on the bone surface (Leblond 1989).

This region of newly synthesized osteoid separates the osteoblast from collagen which has already been mineralized and is referred to as the osteoid seam (Mackie 2003).

Mineralization of collagen is a largely speculative process dependent upon tissue non-specific alkaline phosphatase (TNAP) and alkaline phosphatase (ALP) to convert inorganic pyrophosphate (PPi) into inorganic phosphate (Pi), a component necessary for the formation of hydroxyapatite. Calcium binding phospholipids and proteins promote matrix calcium accumulation (Orimo 2010). Cytosolic phosphatases produce phosphate and when the accumulation of calcium and phosphate exceeds the solubility point, deposition of CaPO₄ in the form of hydroxyapatite occurs, beginning with the space between fibrils then working outward (Orimo 2010).

This reduced rate of matrix formation compared to removal requires a much longer duration of time to fill the same amount of matrix the osteoclasts remove. After refilling is complete, osteoblasts are either encased in matrix or become lining cells. The whole process takes about 200 days to complete (Martin, Burr et al. 1998), so factors that influence the lag time between resorption and refilling, such as possibly VA, can have a dramatic effect on bone.

These alterations could potentially change both bone structure and both localized and total bone mineralization (Figure 2C). Net losses can occur when more osteoclasts, or BMUs are activated and therefore more resorption occurs than required, or when fewer osteoblasts are available or capable of refilling matrix losses. It is important to note that with ageing, the time between the activation and refilling increases, so modifiable environmental factors which could change any factor in this cascade could potentially have an effect on bone restoration and retention during the lifespan.

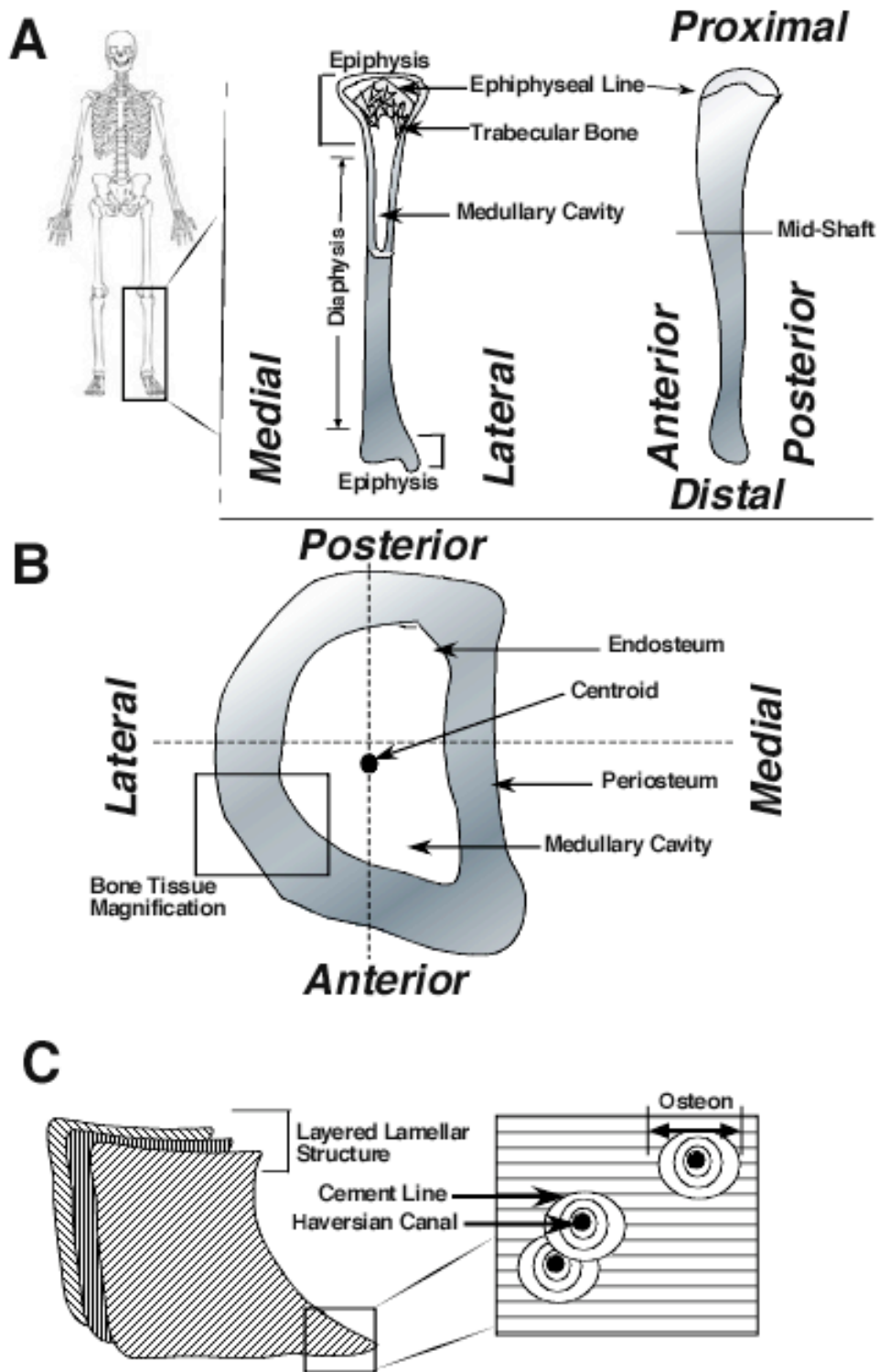


Figure 1: Bone physiology and basic structure. A) Bone nomenclature and anatomical position. B) Orientation and nomenclature associated with the mid-diaphyseal shaft. C) Composition of bone and functional remodeling units.

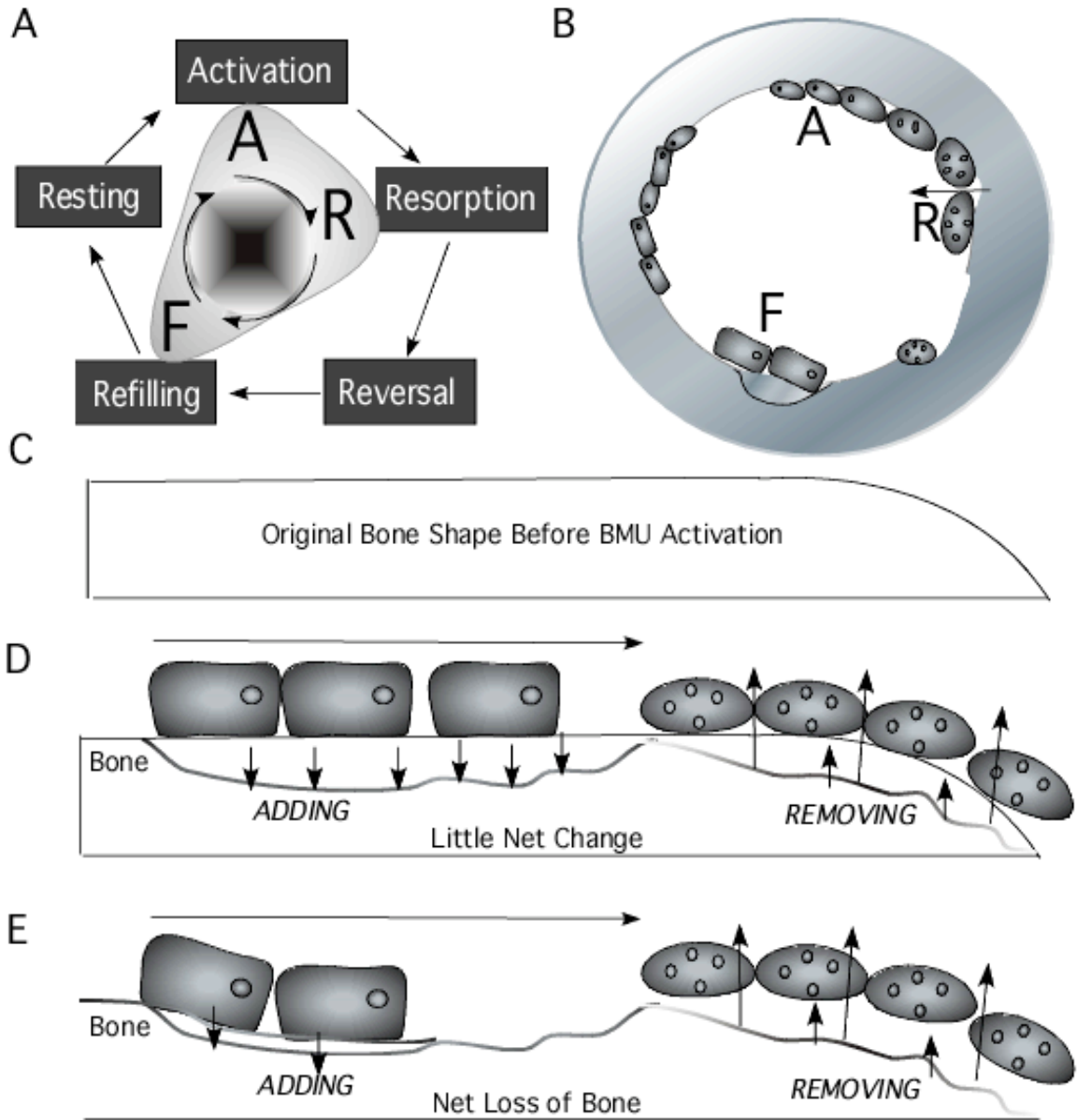


Figure 2: Bone Multicellular Units (BMUs) Activation-Resorption-Refilling Cycle. A) Activation-Resorption-Refilling cycle. B) Osteon remodeling via BMUs. C) Original shape of bone form before BMU activation. D) BMU activation after a complete cycle resulting in no net change in bone shape. E). BMU activation resulting in a net change in shape and/or removal of matrix. Illustrations based on information in *Skeletal Tissue Mechanics* by Martin, Burr & Sharkey (1998), 60-66, 79-101. See references for complete citation.

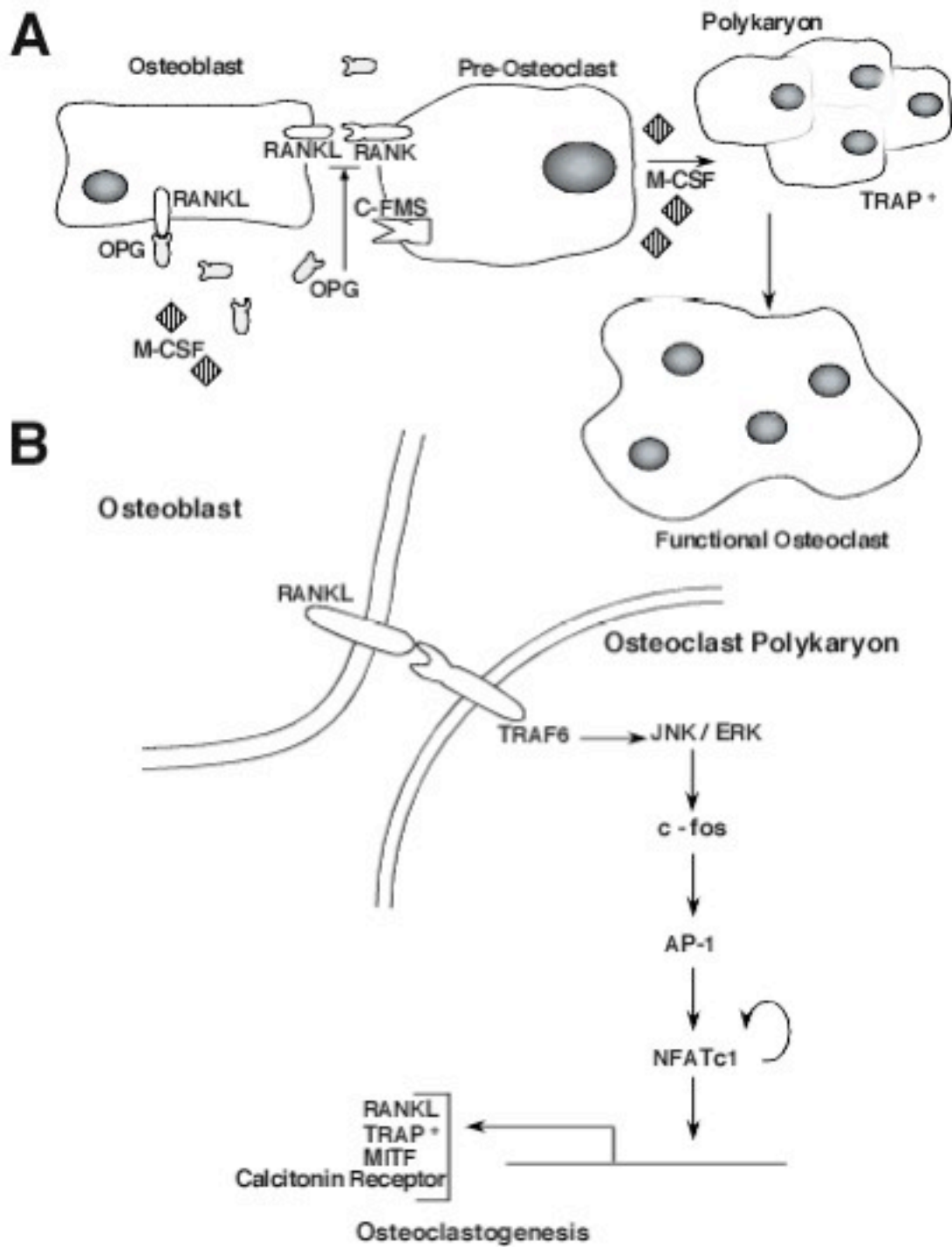


Figure 3: Osteoclastogenesis signaling pathway. A) Formation of functional osteoclast. B) Osteoblast/osteoclast interaction and purposed mechanism of osteoclastogenesis.

The cycle can be modified at any position and includes a number of signaling cascades. Beginning with activation, osteoblasts increase the production of membrane bound receptor activator of nuclear factor kB (RANKL) which binds to the receptor RANK located on pre-osteoclasts (Hadjidakis and Androulakis 2006). Osteoprotegrin (OPG), a soluble protein produced by stromal cells and osteoblasts, acts as a decoy receptor by binding RANKL and preventing osteoclast activation (Figure 3 B;(Conway, Persson et al. 2009). The ratio of RANKL to OPG is important when considering osteoclast activation. An increase in RANKL accompanied by a proportional increase in OPG would result in no net change in activation, because increased OPG could sequester the increased number of RANKL molecules and prevent the initiation of osteoclastogenesis (Robling, Castillo et al. 2006).

Pre-osteoclasts are recruited for activation by monocyte chemoattractant protein-1 (MCP-1, also known as CCL2) and stromal-derived chemotactic cytokine, stromal cell-derived factor (SDF-1, also known as CXCL12), produced by osteoblasts and stromal cells respectively (Li, Meng et al. 2007; Matsuo and Irie 2008). Osteoblasts also produce macrophage colony stimulating factor (M-CSF), a soluble protein that binds to the osteoclast receptor C-FMS causing lineage commitment and eventually aggregation of pre-osteoclasts into a multinucleated polykaryon, or immature osteoclast (Takayanagi 2005; Robling, Castillo et al. 2006).

Osteoclast polykaryons are tartrate-resistant acid phosphatase positive cells, but are not yet considered functional (Takayanagi 2005; Robling, Castillo et al. 2006). Repeated stimulation of the osteoclast by RANKL binding to RANK initiates tumor necrosis factor receptor-associated factor-6 (TRAF6), activating the Jun N-terminal

kinase (JNK) pathway (Figure 3 B). RANKL induces c-Fos expression, where it forms a complex known as activating protein-1 (AP-1), which in turn can cause the auto-amplification of nuclear factor of activated T-cell calcineurin dependent-1 (NFATc1; (Robling, Castillo et al. 2006). NFATc1 then signals to initiate transcription, resulting in the formation of a functional calcitonin receptor, and up-regulation of RANKL, microphthalmia transcription factor (MITF), osteoclast associated receptor (OSCAR) and other less known proteins (Takayanagi 2005). This pathway results in a functional osteoclast.

The functional osteoclast, as part of a BMU, attaches to the bone matrix through the membrane bound alpha v beta 3 ($\alpha v\beta 3$) integrin. The matrix bound spleen tyrosine kinase (SYK) and c-SRC kinase form a ternary complex with $\alpha v\beta 3$ resulting in the polarization of the cell (Yavropoulou and Yovos 2008). Polarization of the sealing zone signals for acidifying vesicles within the cells to fuse, creating the characteristic ruffled border of the osteoclast. The osteoclast then demineralizes the collagen matrix through secretion of hydrochloric acid (HCl) through the cell's ruffled border (Vaananen, Zhao et al. 2000). The solubilized mineral is then transported through transcytotic vesicles, leaving the osteoclast's opposite side through the functional secretory domain (Vaananen, Zhao et al. 2000; Yavropoulou and Yovos 2008).

Though it might seem detrimental, remodeling of bone is essential for material strength and is necessary for repair of naturally occurring microcracks. It is also a response to environmental loading of bone tissue (Figure 4 (Frost 2001). This is a dynamic process, in that as part of a BMU, the osteoclasts resorb bone, and osteoblasts follow behind and generate new collagen to be mineralized. As people age, the duration

of time between the removal of mineralized bone and the addition of mineralized collagen begins to increase, causing a net reduction in mineralization (Frost 2000; Frost 2001; Frost 2003). The area where these losses are most evident is the endosteal surface. The inner-radius (from the centroid to the endosteum) increases, as does the medullary cavity size, but the distance to the periosteal surface (outer-radius) does not necessarily change (Figure 5). This change in timing is responsible for the decrease in cortical thickness associated with aging. The ability to influence the threshold at which remodeling occurs, or the lowest level of stimulation possible to elicit the desired response, has a large potential impact on the prevention of fracture. Age-related bone loss affects 44 million people in the US alone (Sharkey and Lang 2007).

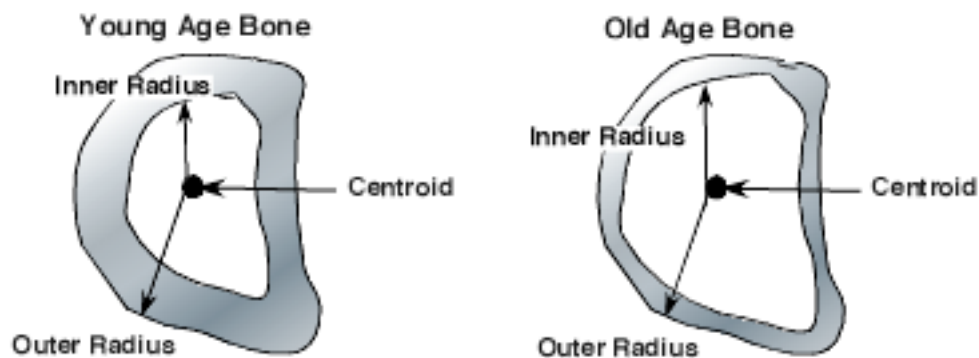


Figure 4: Age-related changes in bone. Though the outer (periosteal) radius has not changed, the medullary cavity has increased in size, and the inner (endosteal) radius has increased in length.

Material and Mechanical Properties of Bone

A major factor that influences changes in bone strength is mechanical loading, through increased stress (σ) and strain (ϵ). Stress is defined as force per unit area (see formula 1), while strain is the percent change in length. Strain is commonly used to

determine relative deformation of a material. Both σ and ϵ can be compressive, tensile or shear in nature (Figure 5; (Turner and Burr 1993). Material testing calculations are based on: (F) the yield force needed to cause a non-linear slope, (L) the length of the support span in millimeters, (d) the displacement at the yield point, (c) the cross-sectional distance from the centroid to the periosteal tensile surface (Figure 1), and (I) a constant generated by material testing.

$$\sigma = F L c / 4 I \quad (1)$$

$$\epsilon = 12 c d / L^2 \quad (2)$$

A stress-strain curve can be generated from these calculated values, allowing for an evaluation of the material properties of bone tissue (Figure 5). Bone tissue is a term used when describing material property, and it encompasses the structural composition of the material itself. With bone, the major structural components are the following: collagen, which provides flexibility; hydroxyapatite mineral $[Ca_{10}(PO_4)_6(OH)_2]$, which provides stiffness; water; and small amounts of noncollagenous proteins and proteoglycans (Martin, Burr et al. 1998). In contrast, the term “whole bone” commonly refers to bone as an organ or relates to the strength properties allowed by the shape and structure of the bone itself (Turner and Burr 1993). A commonly used example would be the comparison of maximal load before deformation of two support beams; one made of putty and the other of rubber. Though both substances (putty and rubber) can be molded into the same shape (an example of structural stiffness, paralleling whole bone properties), the resistance to deformation is a material property based on the composition of that structure (Martin, Burr et al. 1998). In this example, the material property refers to how the different composites (putty or rubber) respond to increased loading. The distinction between material and structural properties is important when discussing the

properties of bone because it can influence the lens through which one sees a certain problem and therefore how one attempts to solve it.

When a stress-strain curve is generated, it is separated into distinct regions based on the permanence of bone deformation (Figure 5 B). The slope of the linear line formed by stress and strain can be calculated, resulting in Young's modulus, which is also known as the modulus of elasticity (E), which is a measure of the intrinsic stiffness of bone (F, L, d and I are described previously). This area under the curve is referred to as the elastic or pre-yield region, since the removal of the load leaves no permanent structural damage [Hooke's Law; (Turner and Burr 1993)]. An example would be a compressed spring: it undergoes change in both size and shape during compression (Figure 5A), but after the force is removed, it returns to its preload form (Figure 5 B).

$$E = F L^3 / d 48 I \quad (3)$$

The separation point for these two regions is the yield point, or the point at which the slope becomes non-linear. The stress level at the yield point is referred to as the yield strength. The area under the curve, starting after the yield point, is called the plastic strain or post-yield region. This area marks the region at which there is lasting damage to the material. Returning to the spring example, this region would be indicative of removing the compressive forces, but finding the spring unable to return to its original form due to lasting, irreversible damage (Figure 5 B). The point at which the bone breaks is referred to as the ultimate load or the maximal stress before failure and often refers to breaking strength of a material. The post-yield area also determines whether the material is ductile or brittle in nature by the materials ability to undergo a large or small amount of post-yield strain before material failure is reached (Turner and Burr 1993).

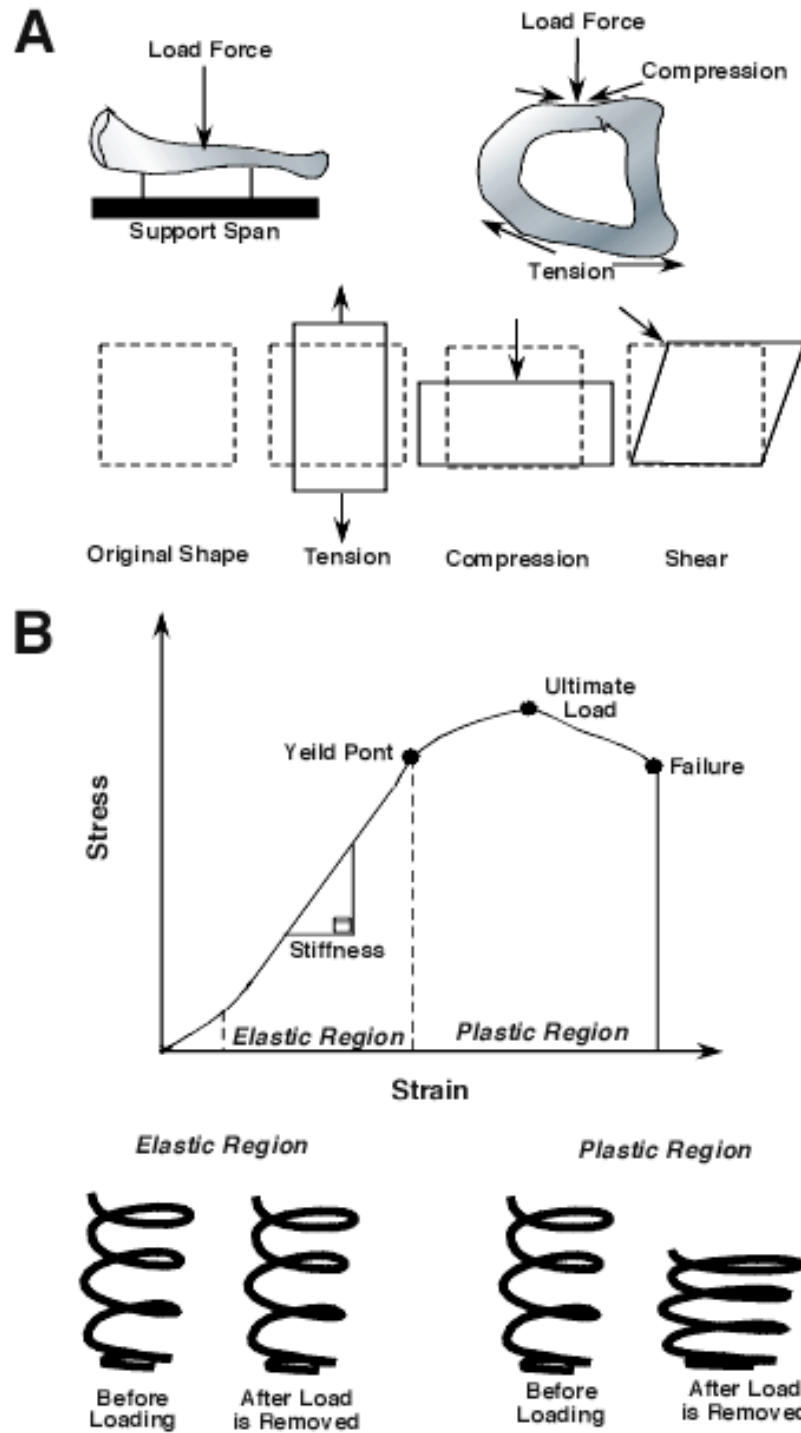


Figure 5: Mechanical properties of bone. A) Types of forces and loading of bones for 3-point bending. B) Stress-strain curve and spring illustration of elastic and plastic regions. Illustrations based on information in *Skeletal Tissue Mechanics* by Martin, Burr & Sharkey (1998), 127-178. See references for complete citation.

Tissue failure occurs in three different stages. The first stage is crack initiation and accumulation, and is typically associated with a decrease in material stiffness. The second stage is the longest and deals with the propagation of cracks formed in stage one. Stage three is the final stage and is marked when the cracks increase to critical size, causing mechanical failure. Bone is an anisotropic material (Figure 1C) because it is composed of different “layers” of structural tissue oriented in different directions, and several factors can influence the ability of bone to undergo stress without spontaneous fracture (entering into stage three of the model above). Bone quality is the largest influential factor, and is relatively independent of bone mineral density (BMD).

Modeling drifts refer to the thresholds (activation or inactivation) at which changes in bone shape and mineralization occurs (Frost 2001). Research in the field of bone physiology suggests non-mechanical modulators of modeling drifts could change whole bone properties through an alteration in the threshold required to initiate modeling and remodeling, a phenomenon commonly referred to as “modeling formation drift” (Frost 2000). This occurs through an unknown pathway, and typically results in increased bone strength, though the exact mechanism as to how this occurs despite bone loss has yet to be discovered. VA could be a non-mechanical modulator of modeling formation drifts.

Bone strength is not exclusively associated with BMD since bone quality cannot be inferred from BMD alone (Frost 2001). Instead, whole bone strength is determined by mechanical bone competence and mechanotransduction (Frost 2001; Frost 2003). Mechanical bone competence refers to the ability of bone as both a tissue and an organ to

resist fracture, while mechanotransduction refers to signaling pathways that influence modeling formation drift.

Bone and Vitamin A

Current information regarding the effect of VA on bone tissue and whole bone resistance to fracture is summarized in Table 1. Data have been compiled from clinical trials, human and animal intervention studies, as well as national dietary collection studies. The results regarding the effect of VA on bone are not consistent among all types of studies. VA supplementation was reported to cause a decrease in BMD, accompanied with decreased strength (Melhus, Michaelsson et al. 1998; Johansson, Lind et al. 2002; Michaelsson, Lithell et al. 2003; Macdonald, New et al. 2004). Others have claimed there is only a change in BMD, but it does not influence the material properties of bone (Houtkooper, Ritenbaugh et al. 1995; Johansson, Lind et al. 2002; Kneissel, Studer et al. 2005). Still other reports have concluded VA supplementation actually results in a decreased risk for fracture, independent of BMD, or there is no change in bone strength or BMD (Sowers, Wallace et al. 1985; Yano, Heilbrun et al. 1985; Freudenheim, Johnson et al. 1986; Sowers and Wallace 1990; Kaptoge, Welch et al. 2003; Lim, Harnack et al. 2004; Barker, McCloskey et al. 2005; Lind, Johansson et al. 2006; Penniston, Weng et al. 2006; Caire-Juvera, Ritenbaugh et al. 2009). In other situations, citations are made that a particular study done previously supports a certain claim, but VA was not even evaluated (Sigurdsson and Franzson 2001; Ribaya-Mercado and Blumberg 2007). Thus, overall, the literature regarding the interaction between VA and bone is inconsistent.

Several factors may explain the heterogeneity of results. First, is the difficulty in obtaining VA status markers in human trials; the lack of reliability of reported dietary intakes exacerbates this problem. Supplement use is a major contributing factor to micronutrient intake, and carotenoids are sometimes but not always included in the total reported VA intake. Another possible contributing factor is the extrapolation of bone quality when only BMD was measured as a marker. BMD is not always associated with changes in the mechanical properties of bone; thus, one factor does not necessarily predict the other (Frost 2000; Frost 2001; Frost 2003; Lind, Johansson et al. 2006). An additional gap in current knowledge is a lack of understanding of the effects of slight elevations in VA intake over prolonged periods of time, since the majority of studies have administered very high, pharmacological doses of VA for a short duration of treatment (Johansson, Lind et al. 2002). Research related to vitamin D has suggested that alterations in both the level and the duration of dosing could change the observed effect on both osteoclast and osteoblast activation (Takasu, Sugita et al. 2006; Sato, Nakamichi et al. 2007). The changes could alter the compositional and potentially the mechanical properties of bone tissue over time (Takasu, Sugita et al. 2006). This concept is particularly intriguing since VA is known to influence both c-fos and $\alpha\beta3$ transcription (Zhao and Patrick Ross 2007; Yavropoulou and Yovos 2008; Conway, Persson et al. 2009).

Hypotheses

It is hypothesized that exposure to VA *in vivo* results in changes in osteoclast signaling, which then result in an increase in trabecular bone number and trabecular bone

fraction (number of trabeculae compared with void space). It is also thought that VA influences collagen ossification, causing increased tensile strength of bone tissue and thereby decreasing the risk of fracture. Thus, a long-term intake of elevated VA, even if not causing hypervitaminosis A, may also result in an increase in BM VA levels, changes in bone physiology, and resistance to mechanical strain.

This thesis addresses the following hypotheses in a rat model:

H₁: Acute VA dosing over a two-week period will result in higher bone retinoid concentrations.

H₂: Elevated dietary VA over the lifetime (VA supplemented diet in old-age animals) will result in positive changes in BMD and increased resistance to fracture, measured by mechanical testing.

H₃: VA will slow endosteal bone loss with age, causing increased cortical thickness, and a reduction in the inner (endosteal) radius when compared to age matched control animals.

H₄: Increased dietary VA will increase the number and size of trabeculae within the medullary cavity through retention throughout the lifespan.

Table 1: Vitamin A and Bone: Humans

| Study Design | Assessment | Outcome Evaluated | VA Reported ¹ | Conclusions | Reference |
|---|---|--|--|--|---------------------------|
| Cross-sectional study in 324 postmenopausal women, age 55-80 years | 24-hour recall and supplement use interview | Radial bone mass by single photon absorptiometry | 2068 ± 2281 (SD) µg RAE/day, with those consuming low calcium diet, 2497.5 ± 2265 µg RAE/day with those consuming high calcium diet | No association of VA intake with bone mass | Sowers et al. (1985) |
| Cross-sectional study of 1208 and 912 elderly Japanese men and women living in Hawaii | 24-hour recall interview and supplement use in previous week | BMC/BMD at five sites of the radius and ulna using single photon absorptiometry | Mean intake for supplement users was 4383 µg RAE/day, for non-supplement users (diet alone) was 1815.9 µg RAE /day | BMD was not different in VA supplement users and non-users. Positive association of BMC and VA in women but not men | Yano et al. (1985) |
| Cross-sectional, longitudinal analysis of 3 year data of pre- and postmenopausal women (35-65 years old) in a clinical trial of calcium supplements | Up to 72 daily food records over the 3 year intervention (two daily records per month on assigned days, with alternating days of the week per record) | BMC in radius, ulna and humerus by single photon absorptiometry every 6 months (6 total scans) | Mean intakes ± SD. Those receiving calcium supplements 1256 ± 4325.4 µg RAE /day; those receiving calcium placebo 972 ± 4046.7 µg RAE /day | No correlation of BMC with VA. Those not taking calcium supplements showed a beneficial relationship of VA supplements slowing BMC loss. | Freudenheim et al. (1986) |

| Study Design | Assessment | Outcome Evaluated | VA Reported ¹ | Conclusions | Reference |
|--|--|--|---|---|---------------------------|
| Cross-sectional study of 246 postmenopausal women between the ages of 55-80 years | 24-hour recall and supplement use interview | Radial bone mass by single photon absorptiometry and self reported fracture occurrence | Mean \pm SD. Supplemental VA was 309 ± 537 , dietary VA was 594 ± 681 μg RAE /day | No association of VA intake and bone mass or fractures | Sowers and Wallace (1990) |
| Longitudinal study of 66 women (ages 28-39 years), who participated in a clinical calcium supplement and resistance training trial. Mean age 34.4 ± 2.7 years. 56 women completed all 18 months. | At least 4 and up to 12 daily food records over 18 months | BMD of whole body, spine and three sites on the femur at 5-, 12- and 18-months using DXA | 1220 ± 472 μg RE/day from diet | VA from food was associated with slowing total bone loss and increasing trabecular bone | Houtkooper et al. (1995) |
| Swedish women (40-76 years old). 247 with first hip fracture and 873 controls | FFQ with 60 food items for intakes during the past 6 months and four 1-week diet records | Hip fracture based on hospital discharge records | Intakes are mean \pm SD. Those with a history of fracture, 960 ± 480 μg RAE/d; without fracture, 880 ± 430 μg RAE/d | >1500 $\mu\text{g}/\text{d}$ versus ≤ 500 $\mu\text{g}/\text{d}$. Higher VA associated with increased fracture | Melhus et al. (1998) |
| Cross-sectional study. Swedish women age 28-74 years | FFQ with 60 food items for intakes during the past 6 months and four 1-week diet records | BMD of lumbar spine, total body and three sites of the femur using DXA | Mean \pm SD. 780 ± 420 μg RAE/d | BMD was reduced by 6-14% with retinol intakes >1500 $\mu\text{g}/\text{d}$ when compared to ≤ 500 $\mu\text{g}/\text{d}$ | Melhus et al. (1998) |
| Cross-sectional study of 232 Icelandic women, age 70 years | FFQ with 130 food items for intakes during the past 3 months | BMD of total body, lumbar spine, femoral neck and hip using DXA | ² Though sited by other sources, no VA intake was actually recorded, or reported | No association of retinol intake and BMD claimed by other sources, but retinol was not measured. | Sigurdsson et al. (2001) |

| Study Design | Assessment | Outcome Evaluated | VA Reported ¹ | Conclusions | Reference |
|--|---|--|---|---|-------------------------|
| Postmenopausal women in the Nurses' Health Study. N=72337 with 18 year follow up. Mean age ~60 years at conclusion of study. | Average of 5 FFQs with 61-130 food items taken over an 18 year time frame | Self-reported hip fracture | 500-3000 µg RE/d | Increased risk associated with intakes ≥3000 µg RE/d when compared to intakes of <1200 µg RE/d and < 500 µg RE/d. Supplement users had higher intakes than non-users. | Feskanich et al. (2002) |
| Randomized clinical study in 18-58 year old men taking either 25000 IU retinyl palmitate (N=40) or a placebo (N=40) for 6 weeks | Clinical Trial | Serum markers of skeletal turnover (BSAP, osteocalcin and NTx) | 7500 µg RAE | No effects of supplementation on serum markers | Kawahara et al. (2002) |
| 570 post menopausal women (mean age 71.3 years) and 288 men (mean age 70.0 years) in the Rancho Bernardo Study with a 4-year follow-up. Mean age at follow up, 71 years) | Self administered Harvard Willett Diet Assessment Questionnaire (FFQ), carotenoids excluded | BMD of hip, femoral neck and spine at baseline and after 4 years using DXA | Total dietary retinol: 1246.77 ± 1573.32 µg RAE /d (Women), 1242.39 ± 1441.92 µg RAE /d (Men) | High and low retinol intakes were associated with lower BMD at baseline and after 4 years, as well as increased BMD loss. Maximal BMD occurred at intakes of 2000-2800 IU/d (or 600-800 µg RAE /d). Supplement use increased risk for bone loss. Increased BMD with food source retinol than supplemental derived retinol | Promislow et al. (2002) |

| Study Design | Assessment | Outcome Evaluated | VA Reported ¹ | Conclusions | Reference |
|---|--|--|--|--|---------------------------|
| White men (N=470) and women (N=470) in Norfolk England. Age 67-79 years at baseline | 7-day food records | BMD of the hip using DXA with an average of two scans three years apart | Mean intake for men was 358 µg RAE/d, with a 95% range intake of 3836 µg RAE/d. Mean intake for women was 289 µg RAE/d, with a 95% range intake of 3517 µg RAE/d | No association of VA and BMD loss | Kaptoge et al. (2003) |
| Nested longitudinal cohort of 70 year old Swedish men (n=1221) with 30 year follow up (diet data taken at 70 years of age) | 7-day diet record | Fractures based on hospital radiographic and outpatient records | Serum retinol status was the main determinant, only two ranges of dietary VA listed: > 1500 µg /d versus <530 µg /d | Increased risk with retinol intakes > 1500 µg /d versus <530 µg /d | Michaelsson et al. (2003) |
| Postmenopausal women from the Iowa Women's Health study (N=34703) with incident of hip fractures (n=525) or all fractures (n=6502) during a mean 9.5 year follow-up | FFQ with 127 food items. Includes supplements | Self reported hip fracture and non-hip fractures | Total VA intake range of 1533.9-8771.7 µg RAE/d | No association of VA intake and incident of fracture | Lim et al. (2004) |
| Mainly postmenopausal women ages 45-55 years at baseline (N=891) from the Aberdeen Prospective Osteoporosis Screening Study in Scotland with a 5-7 year follow-up | FFQ with 98 food items for intakes over the past 12 months. Obtained at baseline and after 5 years. Includes supplements | BMD of lumbar spine and femoral neck at baseline and after 5-7 years using DXA | Mean ± SD. 924 ± 666 µg RAE/d; Range was 85-4354 µg RAE/d, median was 4354 µg RAE/d | VA from food sources increase bone loss. No association of bone loss and VA primarily from supplement use. | MacDonald et al. (2004) |

| Study Design | Assessment | Outcome Evaluated | VA Reported ¹ | Conclusions | Reference |
|--|--|---|--|---|----------------------------|
| Peri-menopausal Danish women in the DOPS study. 163 fracture cases identified after 5-year follow-up, matched to 6 controls. | 4-day or 7-day food records at baseline | BMD of lumbar spine and femoral neck at baseline and after 5 years using DXA. Self-reported fractures validated by hospital discharge records | > 1500 µg/d or less than 500 µg/d | No association of retinol and BMD, change in BMD or number/ frequency of fractures when comparing retinol intakes of > 1500 µg/d to <500 µg/d | Rejnmark et al. (2004) |
| 312 cases, 934 controls from a cohort of >75 year old women in the United Kingdom with a mean follow-up age of 3.7 years | Detailed medical and lifestyle history at baseline | Hip and other fractures validated with medical records and home visits by nursing staff | Serum retinol as a measure of VA intake | Multivitamin and cod liver oil supplements was associated with a significantly lower risk of all types of fracture | Barker et al. (2005) |
| Case-control study of postmenopausal women ages 48-83 years) with (N=27) or without (N=24) osteoporosis | 3-day food records from two weekdays and one weekend day | Osteoporosis, defined by a BMD T-score ≤-2.5 at the lumbar spine or proximal femur using DXA | Total VA intake from those with osteoporosis, 1336 ± 631 µg RAE /d, or control subjects, 1424 ± 661 µg RAE /d (mean ± SD). | No differences in VA intake of women with or without osteoporosis | Penniston et al. (2006) |
| 75747 postmenopausal women 50-79 years of age, from the Women's Health Initiative Observational Study | FFQ with 122 food items taken at baseline and at 3 year follow-up compared to a 4-day food record and four 24-hour recalls | Self-reported fracture. Hip-fractures validated using medical and radiological reports (71% confirmed) | Total retinol intake range of 412-3234 µg RAE /day | No association between VA and risk of fracture as long as the individual is not vitamin D deficient | Caire-Juvera et al. (2009) |

¹ VA intakes standardized to the same unit [µg Retinol Activity Equivalent (RAE)] as described in Modern Nutrition in Health and Disease (10th edition). For more information see references.

² Table modified from the work of Ribaya-Mercado, J & Blumberg J, 2007 (see references for complete citation).

CHAPTER 2

EXPERIMENTAL METHODS

ANIMALS, DIETS AND EXPERIMENTAL DESIGN

Acute Vitamin A Treatment (Study 1)

Male and female Sprague-Dawley rat pups obtained through mating within our facility were used to determine bone marrow VA content. Rat dams were fed a purified VA deficient (0.35 mg REq/kg) diet prepared by Research Diets (New Brunswick, NJ) to prevent hepatic VA accumulation in pups. The rats had free access to food and their weight was monitored daily. Pups were weaned at 25 days, and then given the same VAD diet as the dam. Rats were randomly assigned to either an all-trans retinyl palmitate (Sigma-Aldrich) treatment (n = 5; 6 mg retinyl palmitate dispersed in 150 μ l canola oil, for a concentration of 0.21 M) or the same volume of oil vehicle alone as a control (n = 4). Treatments were given orally daily for 14 days, at which time the animals were then euthanized via carbon monoxide asphyxiation.

Longitudinal Vitamin A Treatment (Study 2)

The tibia of male Lewis rats from planned mating within the animal facility were obtained from a previous study (Dawson, Li et al. 1999) and were used in this experiment. In this study, a normal 12 hour:12 hour light:dark cycle and a climate controlled environment had been maintained. Before weaning, the dams had been fed a VA deficient diet based on AIN-93 diet to reduce hepatic VA storage in the rat pups

(Reeves, Nielsen et al. 1993; Reeves, Rossow et al. 1993). Rats were fed the AIN-93 purified diet, modified to contain 0.35, 4.0 or 50.0 mg retinol equivalents (RE; added as retinyl palmitate) per kilogram of diet as described in Dawson et al. (1999). All diets were prepared by Dyets (Bethlehem, PA). The diets were designated marginally VA deficient (0.35 mg RE/ kg diet), control (VA sufficient, 4.0 mg RE/ kg diet) and VA-supplemented (50.0 mg RE/kg diet). The diets contained 0.2, 3.3 or 42 times the RE found in a standard AIN-93 diet.

All animals had free access to food at all times during the experiment, and food intake and weight gain were monitored periodically (Dawson, Li et al. 1999). Rats were euthanized in groups by carbon monoxide asphyxiation, with one animal from each VA diet treatment and age group (total of 9 groups) per euthanasia batch, resulting in nine rats per batch over a seven week period (Dawson, Li et al. 1999). The ages of the animals at the end of the study were designated as young, middle age, and old corresponding to 2-3, 8-10, and 20-22-months, respectively. Bones were removed and cleaned of excess tissue while samples were on ice, submersed in ice-cold phosphate buffered saline (PBS). After samples were thoroughly cleaned, they were stored individually at -20°C in sealed Whirl-pak containers until they were analyzed in this study.

RETINOID ANALYSIS

In study 1, blood was taken from the vena cava of euthanized rats in the acute treatment protocol, using heparinized needles and syringes. Samples were kept at 4°C until centrifugation at 1500 rpm for 20 minutes. The plasma fraction of all samples was

then recovered, leaving behind the buffy coat. Plasma was then frozen at -20°C until analyzed. To determine the VA content of plasma, the plasma sample was thawed at 4°C , then $200\ \mu\text{l}$ of each sample was removed into a clean glass screw-cap tube. A standard saponification mixture consisting of 5% potassium hydroxide dissolved in an equal amount of distilled water, to which was added ethanol to achieve the desired total volume (95% ethanol) and pyrogallol at 1% of total volume. The sample was mixed by inversion three times and heated at 55°C for 30 minutes in a water bath to achieve hydrolysis of lipid esters. After heating, the samples were allowed to cool while covered for 5 minutes until they were cool to the touch. Then 4 ml hexanes and 2 ml distilled-deionized water were added to the saponified mixture, mixing by inversion 3 times between the hexanes and water additions. Samples were then centrifuged at 1500 rpm for 5 minutes at room temperature and 3.5 ml of the retinoid containing hexanes upper fraction was removed. The removed fraction was dried under argon 3x at 55°C with decreasing amounts of methanol to ensure that all retinoids had been concentrated in the bottom of the container. After the sample was dried, it was reconstituted with $150\ \mu\text{l}$ methanol, and 7-10 μl (218.4 pmol per sample) trimethylmethoxyphenyl-retinol (TMMP-ROH) was added depending on treatment group as an internal standard, then $20\ \mu\text{l}$ of the TMMP-ROH/ plasma sample was analyzed by reverse-phase HPLC using a photodiode array detector at a wavelength of 325 nm for detecting retinol content. Results were calculated as μM retinol.

Bone VA was extracted from one tibia and femur from each acutely VA-treated rat. A wet weight was first obtained, then VA was extracted using a chloroform:methanol 2:1 v:v Folch lipid extraction (Folch, Lees et al. 1957). The extract was dried under argon

at 55°C with repeated reductions in chloroform:methanol 1:1, v:v, to concentrate the lipids which were then saponified, extracted, and dried under argon as described above for analysis of plasma. The sample was then reconstituted with 150 µl chloroform:methanol, and TMMP-ROH was added as previously described to serve as an internal standard. Reverse-phase HPLC was performed by the same method as for rat plasma, and data were calculated as retinol in pmol/ gram bone weight.

BONE MORPHOLOGY AND PHYSIOLOGY ANALYSIS

All samples from the long-term study were stored at -20°C until analyzed. Samples were re-hydrated by complete submersion in 10 ml sterile PBS at 4°C overnight. The researcher was blinded to each sample's dietary treatment group to prevent bias. Caliper measurements were taken for total length, epiphyseal, saggital and coronal widths, to detect changes in bone size or shape.

Trabecular reconstructions, periosteal and endosteal radii, cortical thickness, BMD, trabecular number and bone fraction. The proximal epiphysis was scanned using a desktop micro-computed tomography (µCT; ICT 40; Scanco Medical, Bassersdorf, Switzerland). A five-centimeter section was scanned at the proximal metaphysis of the right tibia (Shahnazari, Lang et al. 2007). An additional section was scanned for mid-diaphyseal shaft analysis using a MATLAB program (version 6.5, release 13; MathWorks, Natick, MA), as described previously by Shahnazari, Lang et al., (2007).

Mechanical testing. After scanning, tibias were stored in PBS for no more than 48 hours at 4°C. Tibial three-point bending tests were conducted using a mechanical testing system (MTS) MiniBionix 858 apparatus (Eden Prairie, MN) with a 16 mm

support span attached to a 50 lb load cell (Shahnazari, Lang et al. 2007). Bones were consistently oriented with the load applied on the posterior side of the midshaft (Figure 5), producing compression on the posterior surface and tension on the anterior surface. Force/ displacement curves were generated to obtain yield load, ultimate load, and stiffness.

STATISTICAL ANALYSIS

Statistical analysis was performed (Graphpad Prism version 5) using two-way ANOVA or t-tests where appropriate. Values of $p < 0.05$ were considered statistically significant.

CHAPTER 3

EXPERIMENTAL RESULTS

ACUTE VA TREATMENT

VA treated animals have increased plasma and bone ROH

Male and female S-D rat pups fed a vitamin A deficient diet were treated with either a supplemental level of RE (using canola as a vehicle) or an equal volume of vehicle (control) for 14 days starting at 25 days of life. Rat plasma results indicated that VA treated animals had higher circulating ROH levels than control (vehicle only) animals (Figure 6).

There was nearly a two-fold increase in bone VA in VA-treated animals irrespective of bone origin (both tibia and femur, Figure 6). Both control tibiae and femurs showed similar retinoid levels per gram weight ($p > 0.05$). Femur retinoid content was statistically lower than that of the tibia ($p < 0.05$) in all groups. Bones from VA treated rats had a higher retinoid content than those from oil-treated controls ($p < 0.002$). The tibia of VA treated animals had a 25% higher ROH concentration (pmol/g weight) than the femur. Animal weights and sample weights were similar between groups. Thus, VA treatment increased retinoid levels in both the tibia and femur.

LONGITUDINAL VA TREATMENT

VA treatment does not alter animal weight until old age

Male Lewis rats were fed one of three standardized diets (marginal, control or supplemented) until 2-3, 8-10, and 18-20-months of age, respectively. Animal weights were taken at the time of euthanasia for each dietary treatment group and age group.

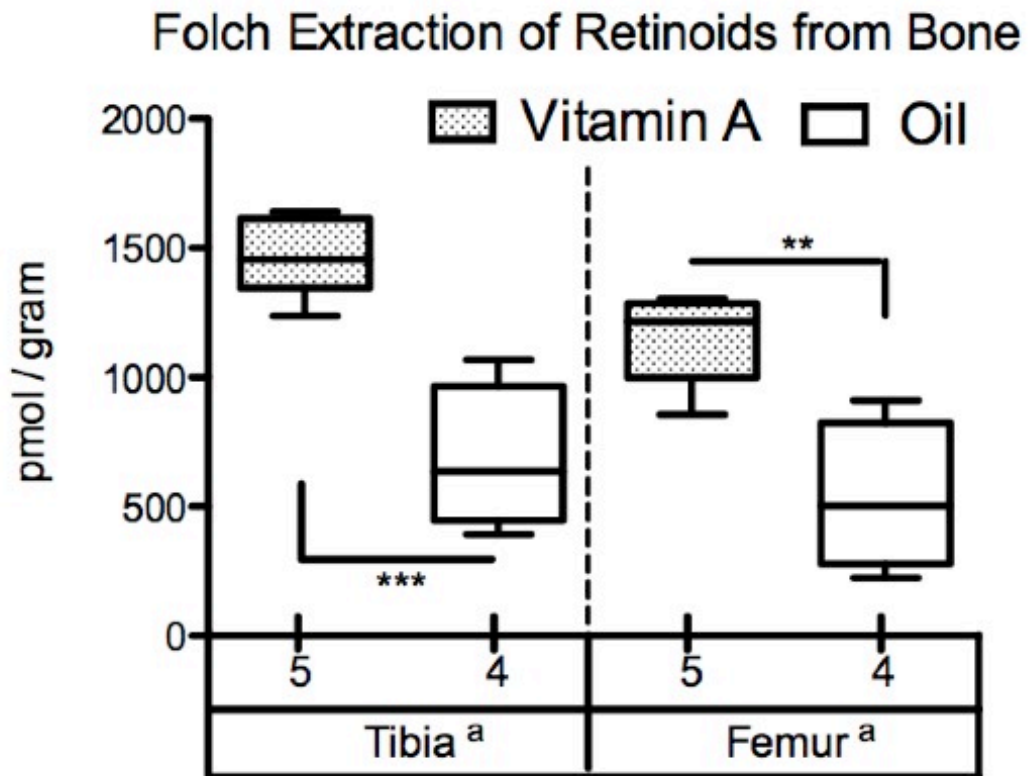


Figure 6: Acute VA results in detectable increases in VA in long bones. VA is higher in VA treated tibia when compared to oil treated controls and VA and oil treated femurs. Numbers on the x-axis indicate the number of animals per group (ANOVA, * p < 0.05, ** p < 0.001)

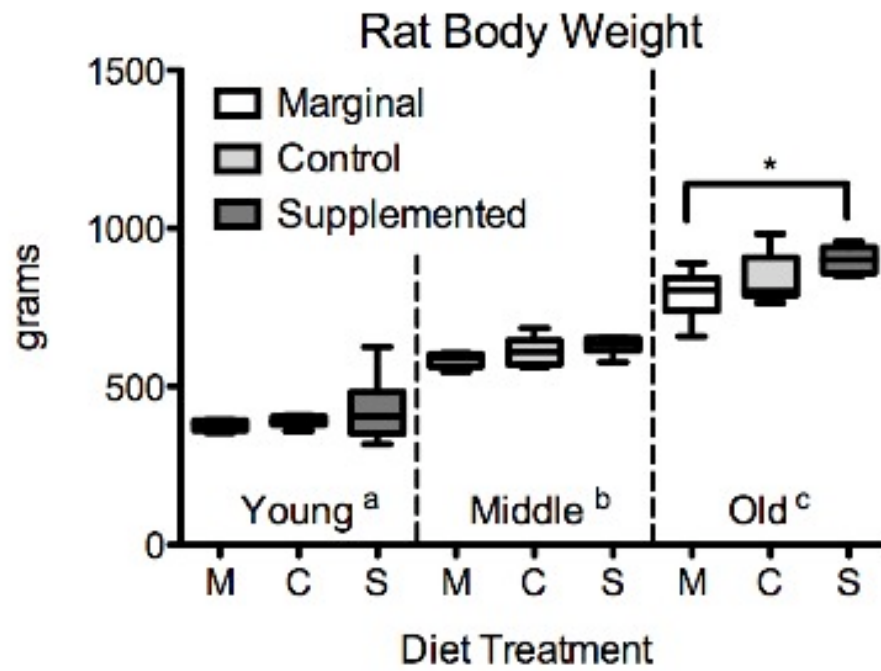


Figure 7: Animal weights for long-term VA alteration study. Letters indicate differences between groups ($a < b < c$; ANOVA, * $p < 0.05$, ** $p < 0.001$)

(Figure 7). Each age group differed significantly from each other group ($p < 0.001$), but there was no statistically significant difference due to diet until the animals reached old age ($p < 0.05$). Neither the marginally VA deficient nor VA supplemented groups differed from the control group, but the marginal and supplemented rats differed from each other (Figure 7). Since it is known that body weight can initiate changes in bone tissue and the mineralization process, the subsequent results are shown as both data uncorrected for body weight as well as corrected for body weight.

Dietary VA supplementation throughout the lifespan does not alter bone length, epiphyseal or mid-diaphyseal bone width.

Total tibia length and epiphyseal, coronal, and saggital widths were measured to determine alterations in physiological bone dimensions. An age dependent difference was found at all time points (young, middle age, old age), as indicated by a difference in letter in Figure 8 ($p < 0.0001$) for bone length and epiphyseal width (Figure 8 A-D), but there was no difference between middle age and old age animals for coronal width or saggital width (Figure 8 E-H).. There was no diet related difference within any of the three age groups, before or after body weight adjustment (Figure 8). There was an age related difference between each of the young, middle age and old age groups ($p < 0.0001$)

Dietary VA causes alternate changes in mid-diaphyseal bone depending on dose amount and duration of exposure

Medullary area has an age dependent difference between young age and the other two time points (middle age and old age), but there was no difference between middle age and old age animals (Figure 9). Though there were no age dependent differences in

medullary area in the two later time points, there is a diet dependent difference.

Regardless of age, alterations (both low and high) to dietary VA resulted in differences from control animals for medullary area (Figure 9 A-B). Though at 2-3 months of age (young age time point) the short duration of VA alterations resulted in an increase in luminal size, by 10-12 months (middle age) this picture has changed in a VA dependent manner. Increasing dietary VA increases luminal/medullary area. The VA supplemented group had a 20% increase in area compared to the control group, but due to a small sample size, statistical significance was not reached ($p > 0.05$).

To determine if this change in mid-diaphyseal area was due to alterations of the endosteal or periosteal surfaces, the radius was determined from the centroid to each of these surface areas (Figure 8 C-F, see Figure 1 & 3 for diagram). There was an age related reduction in the endosteal radius in the young versus the middle age group, and the young versus the old group ($p < 0.0001$), but no difference was detected between middle age and old age animals regardless of diet.

Young control animals had reduced endosteal radii when compared to marginally VA deficient and VA supplemented animals ($p < 0.0001$ and $p < 0.05$ respectively). These results mirrored the decreased medullary area (Figure 8 A-B), indicating that the change in area might be influenced by an alteration at the endosteal surface.

Marginally VA deficient and VA supplemented animals at both the middle age and old age time points had differing endosteal radii ($p < 0.05$, and $p < 0.001$ respectively) but neither group differed from the control animals (Figure 8 C-D), though the same trend noted in Figure 8 A-B can be seen here. With a reduction in medullary

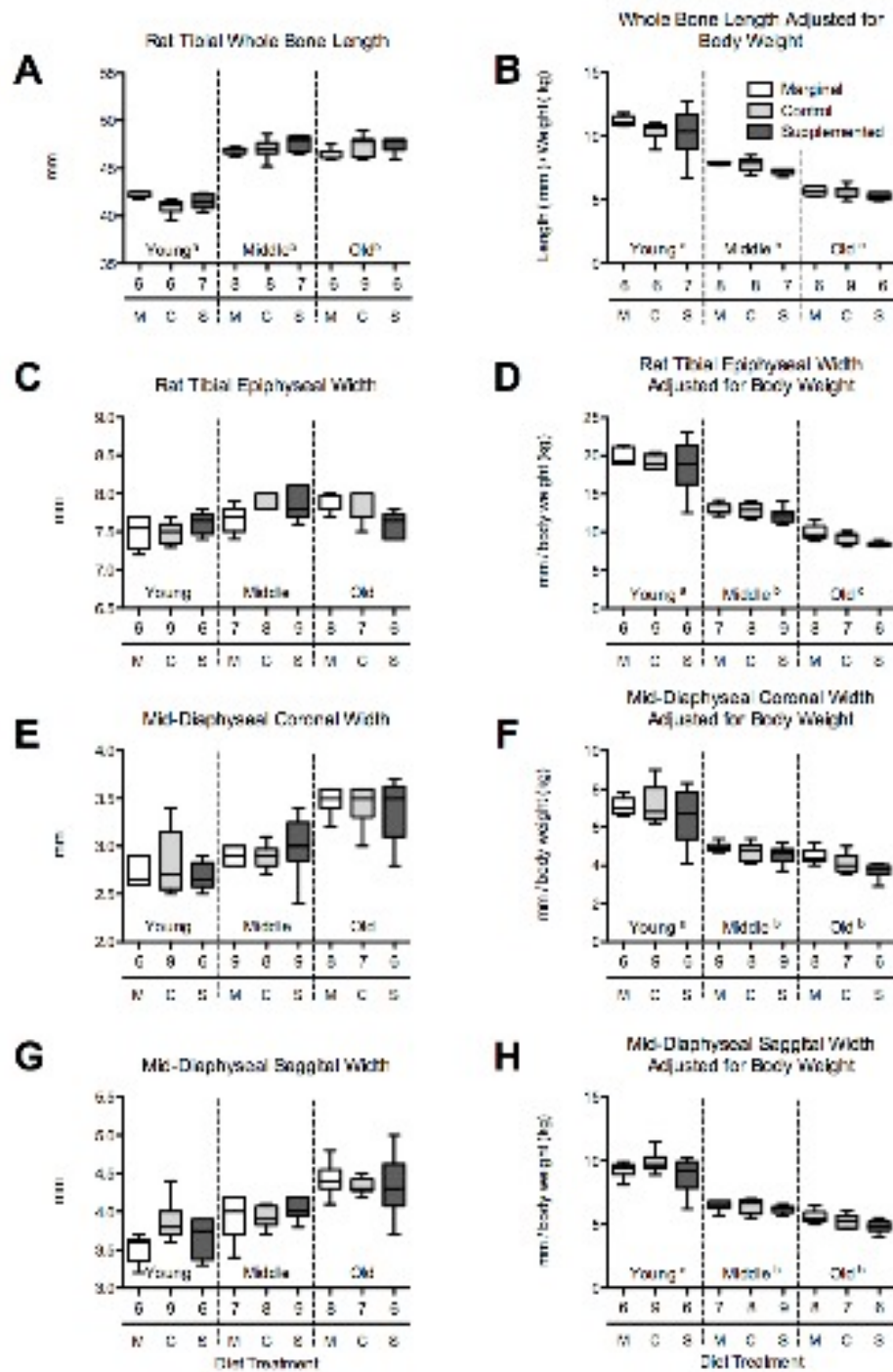


Figure 8: Differences in dietary VA intake (low or high) had no effect on bone measurements. Shown are both raw and weight corrected data for each measurement. Numbers on the x-axis indicate the number of animals per group, and uppercase letters correspond with the dietary treatment groups, M = marginally VA deficient, C = control, S = VA supplemented. Lowercase letters indicate a difference between ages (ANOVA, * $p < 0.05$, ** $p < 0.001$)

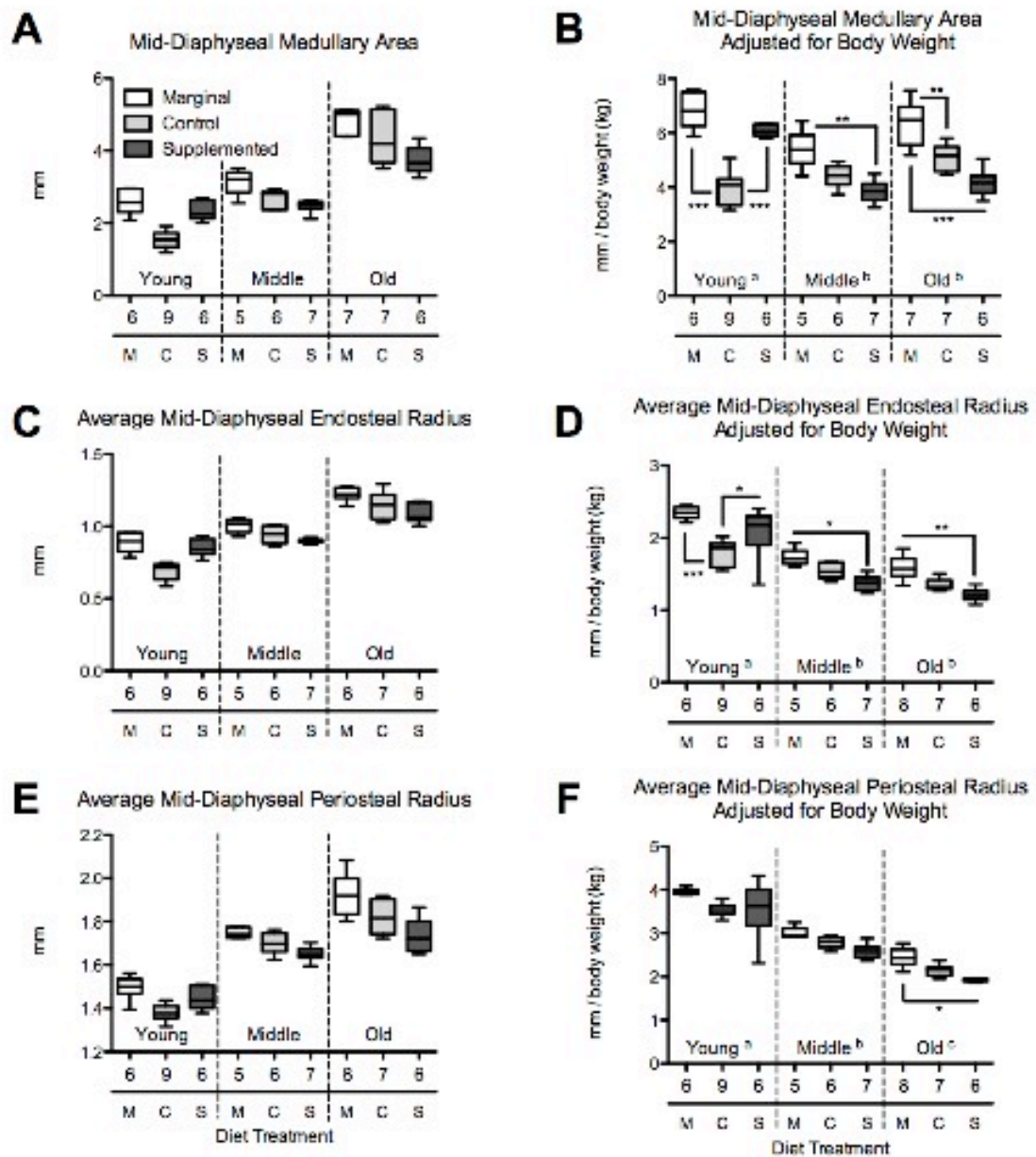


Figure 9: Midshaft medullary area is smaller with VA supplementation due to changes in endosteal and periosteal radii. Numbers on the x-axis indicate the number of animals per group, and uppercase letters correspond with the dietary treatment groups, M = marginally VA deficient, C = control, S = VA supplemented, respectively. Lowercase letters show differences between groups (ANOVA, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$)

area, an increase in endosteal radius would be expected in order to account for the difference in luminal size.

To assure the difference in medullary area was not compounded with a change in bone shape, the periosteal radius was also measured (Figure 9 E-F). The mid-diaphyseal periosteal radius differed in an age related manner between all three age groups ($p < 0.0001$) but there were no dietary differences for either the young or middle aged rats (Figure 9 E-F). There was a difference between marginally VA deficient and VA supplemented old animals ($p < 0.05$). The same trend mentioned earlier is once again evident.

These results when taken together show there might have been fundamental differences in bone shape, or mid-shaft width that was not evident with this relatively small number of animals. The periosteal and endosteal radii of marginally VA deficient animals was larger than that of the other diet treatments, which leads one to believe the bone itself was larger, though these results are not mirrored in the caliper measurements shown in Figure 8.

Dietary VA does not alter average cortical bone thickness over the lifespan

There was an age dependent decrease in average mid-diaphyseal cortical bone thickness at each of the three time points ($p < 0.0001$, Figure 10 A-B), but a diet related difference only between young control animals and the two VA diets. Marginally VA deficient had a p-value of $p < 0.05$ and VA supplemented had a p-value of $p < 0.0001$ when compared to the controls. These results combined with the results shown in Figure

indicate the changes seen in radii are not the result of a decrease in bone thickness but are instead are bone dimensional changes. In Figure 10 C, variability between different specific points around the mid-diaphyseal shaft could be noted, but these differences could be attributed to the shape of the bones themselves.

Dietary VA over the lifespan does not BMD, total number of trabeculae or trabeculae thickness

Though there was an age dependent reduction in BMD at all three time points ($p < 0.01$, Figure 10) there were no diet induced changes in BMD regardless of dietary VA treatment. This was mirrored in the total number of trabeculae when adjusted for body weight (Figure 11 D). The dramatic differences seen in the tibial reconstructions (Figure 13), is most likely attributed to the weight differences noted in Figure 6 because of the change noted between the un-weight adjusted data (Figure 11 C) and the weight adjusted data (Figure 11 D).

Trabeculae thickness was also affected by animal weight, and though there is an age depended change at all time points, controlling for animal body weight eliminates changes seen between VA supplemented and control diet animals (Figure 11 E-F).

Short term dietary VA alterations reduces bones resistance to mechanical loading, but this difference is ameliorated with longer exposure.

There is neither an age nor a diet related change in the yield point or stiffness of bone when mechanically loaded (Figure 12). Age alters the ultimate load and maximal stress a bone can withstand before failure, but diet had no effect.

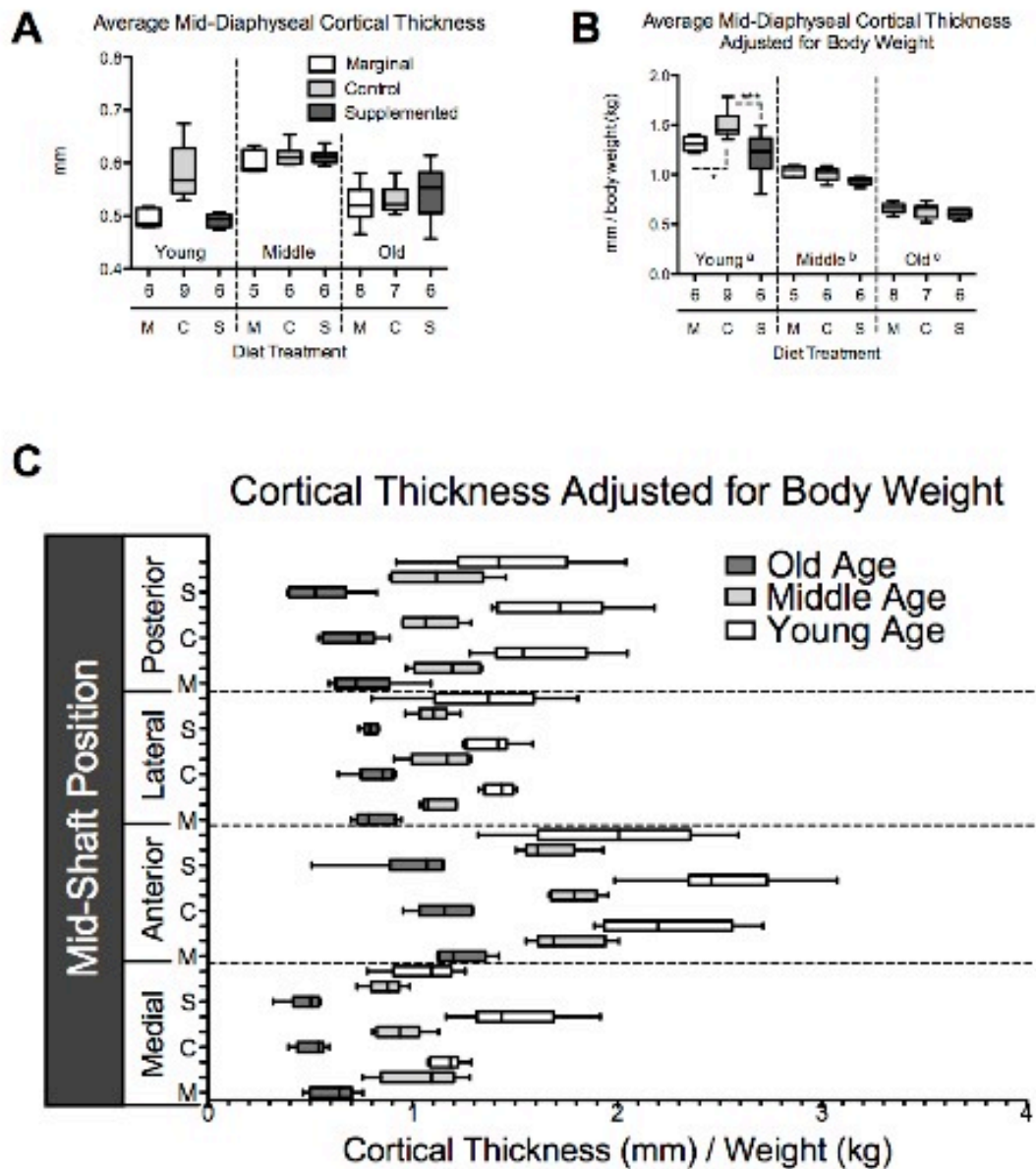


Figure 10: Differences (both low and high) in dietary VA intake results in short-term, but not long-term changes to cortical thickness. A-B) Unadjusted and adjusted average midshaft cortical thickness. C) Cortical thickness by age, diet and four different mid-shaft locations (see Figure 1 for locations). Numbers on the x-axis indicate the number of animals per group, and uppercase letters correspond with the dietary treatment groups, M = marginally VA deficient, C = control, S = VA supplemented, respectively. Letters show differences between groups (ANOVA, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$)

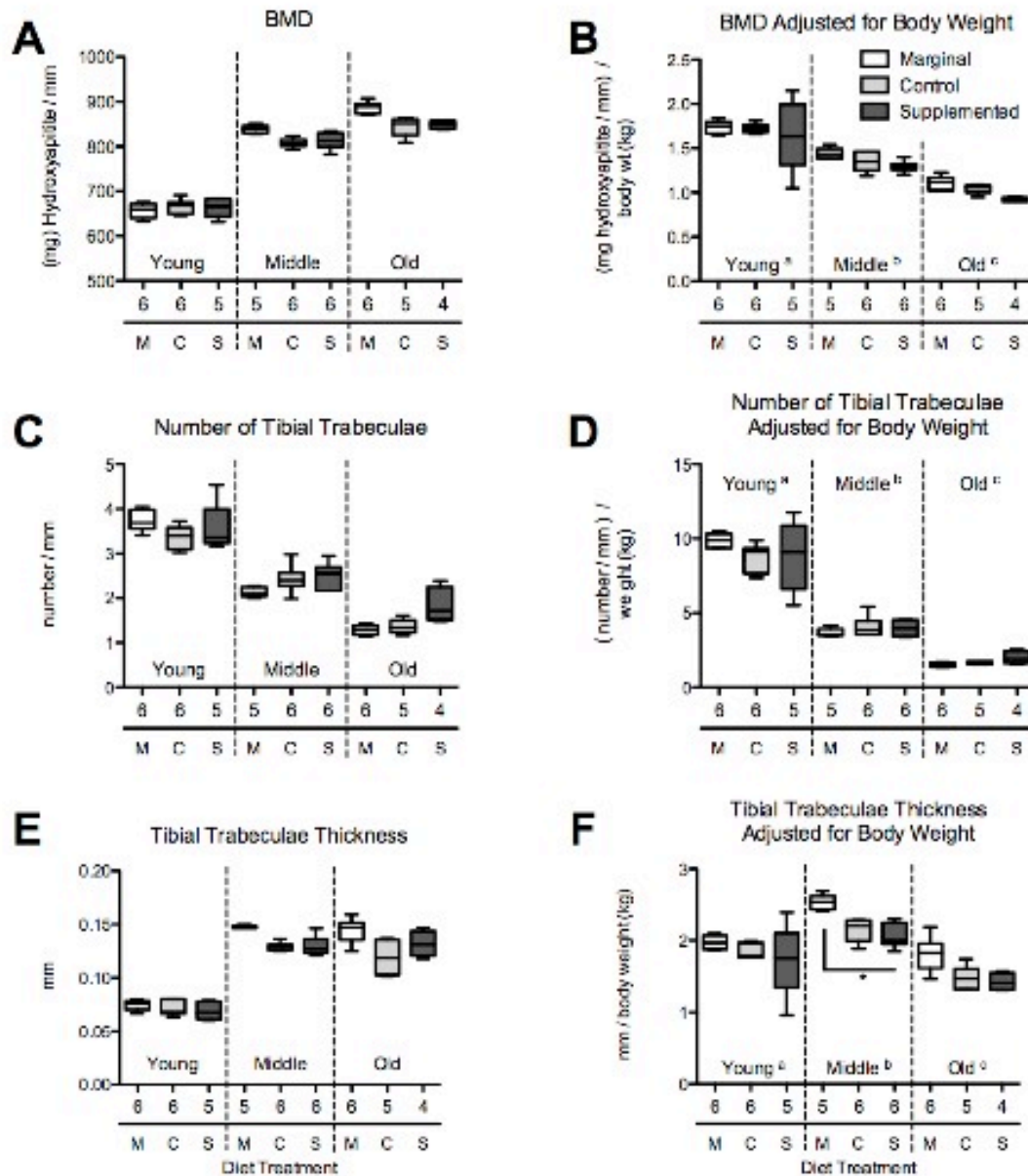


Figure 11: Long-term dietary differences in VA intake (both low and high) have no effect on bone mineral density (BMD), trabeculae number or trabeculae thickness. A-B) BMD both adjusted for body weight and raw data. C-D) Number of trabeculae adjusted and not adjusted for body weight. E-F) Thickness of trabeculae both adjusted and not adjusted for body weight. Numbers on the x-axis indicate the number of animals per group, and uppercase letters correspond with the dietary treatment groups, M = marginally VA deficient, C = control, S = VA supplemented, respectively. Lower case letters show differences between groups (ANOVA, * $p < 0.05$, ** $p < 0.001$).

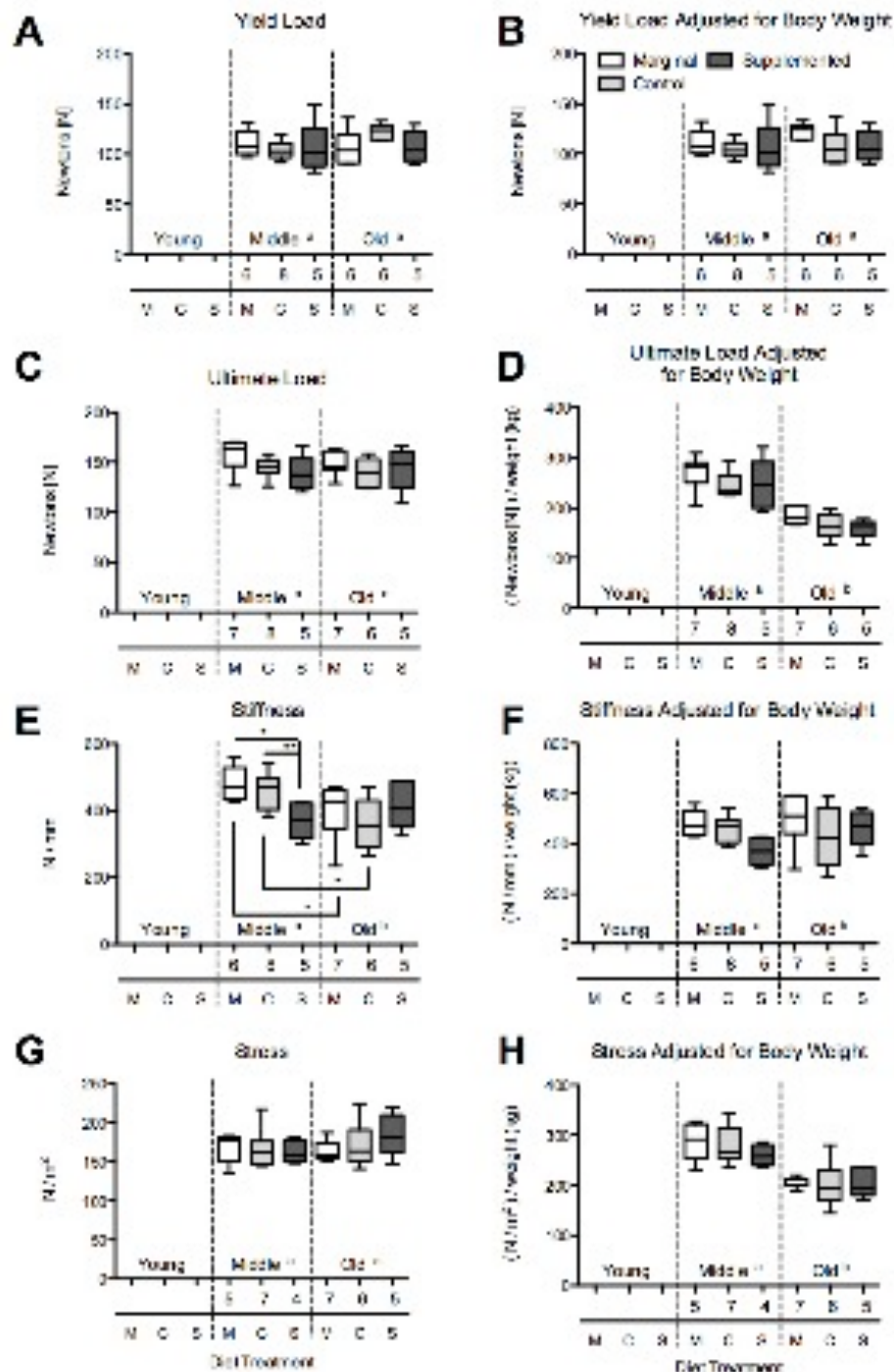


Figure 12: Differences (both high and low) in dietary VA have no effect on mechanical properties of bone. A-B) Load at yield point. C-D) Ultimate load achieved. E-F) Material stiffness of bone tissue. G-H) Stress. All figures show both adjusted and not adjusted for body weight differences. Numbers on the x-axis indicate the number of

animals per group, and uppercase letters correspond with the dietary treatment groups, M = marginally VA deficient, C = control, S = VA supplemented, respectively. Lowercase letters show differences between groups (ANOVA, * $p < 0.05$, ** $p < 0.001$).

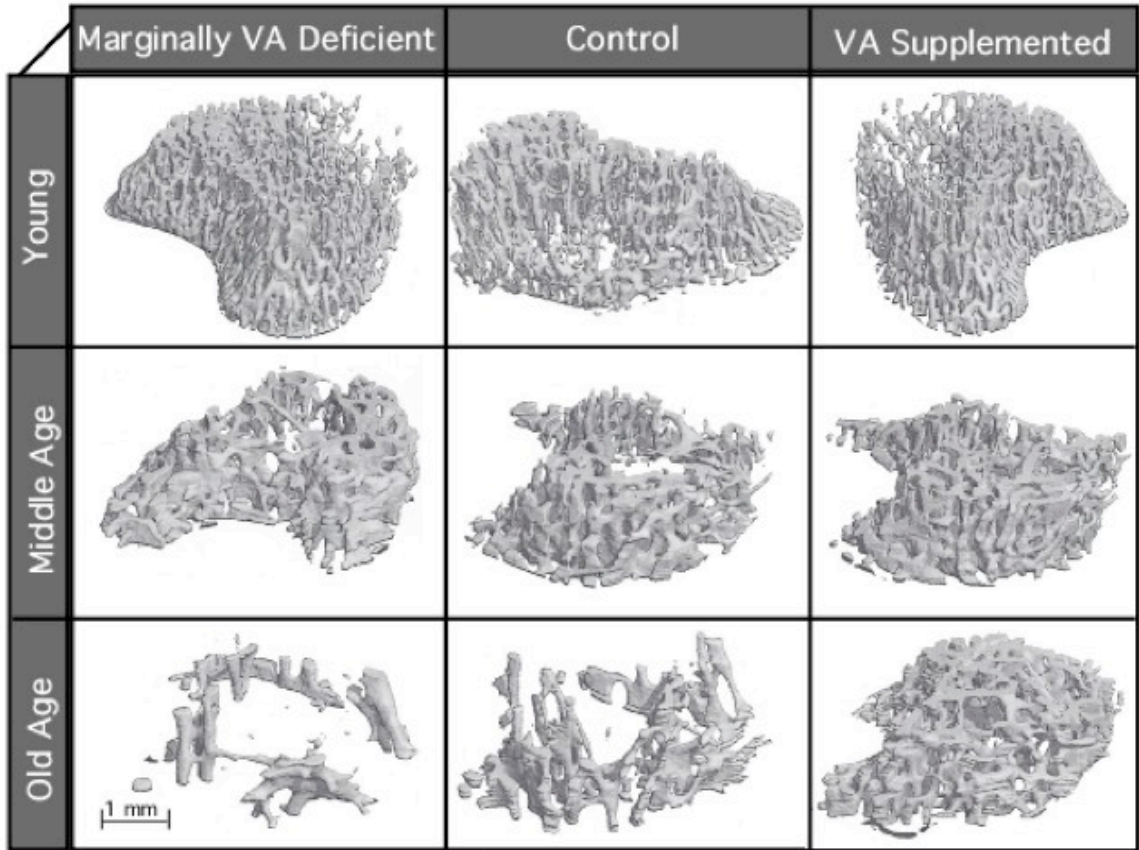


Figure 13: Micro-CT reconstructions of proximal epiphyseal trabecular bone by diet and age. Each reconstruction is representative of the group. Quantification of these results can be found in Figure 11.

CHAPTER 4

DISCUSSION

The experiments outlined earlier provide new evidence in a unique way for a possible biphasic response of bone to VA treatment. Unlike most published research in this field, both low and high levels of dietary VA were evaluated over the lifespan, instead of the more common time frame of 2-4 weeks. Multiple bone parameters were evaluated, including methods not available in human studies, such as material testing of the mechanical and material properties of bone tissue. All data was shown as both raw data and corrected for weight, since mechanical factors such as increased loading involved with increased weight causes changes to bone tissue and can encourage mineralization and changes to micro-architecture (Frost 2000; Frost 2001; Frost 2003).

VA is known to cause alterations in material and mechanical properties of bone tissue, but the mechanism as to how this occurs, and how VA is exposed to bone tissue is unknown. Figure 5 demonstrates that VA does travel to the marrow of both the femur and the tibia and can be detected when ingesting levels above that of the RDA. These data also show the tibia is a more active clearance site than the femur, with higher retinoid pmol/ gram bone weight amounts being detected. This is a reasonable assumption since it has been well established that the tibia has a higher luminal adipose content than the femur (Gimble, Zvonic et al. 2006).

Surprisingly, there was no effect of VA on any of the bone measures seen in Figure 7 once differences in body weight are accounted for. Though a larger number of animals would strengthen the power of the results, these experiments provide a clear

picture to the long-term effects of VA intake on bone. As stated previously, many studies report VA intake to be detrimental to bone health (Table 1) but these studies are normally done in over a very short time span with near pharmacologic doses of VA. The ambiguities in the diet data from elderly human trials would not support this unless someone accounts for the duration of supplement use and the VA intake of an individual over time. Supplement use is more common in the elderly, so it would be interesting to evaluate the duration of time the individual has been consuming supplements to age matched controls who have taken either multivitamin or multivitamin and multimineral supplements the majority of their life.

Results from short term dosing (Figure 9, 10, 12, 13) shows a very different picture than dosing over the span of an animals lifetime so it stands to reason that an individual who begins taking VA supplements recently would have a different outcome when DXA measurements are taken or incidences of fracture are reported.

The differences in short-term and long-term dosing lend more evidence to the idea that VA signaling in bone is biphasic and dependent on the duration of exposure. Though no cellular analyses were possible, this could be attributed to two main factors. The first is a decrease in the c-Fos protein shown in figure 3, preventing NFAT amplification which prevents the formation of a functional osteoclast. Short-term dosing is known to decrease the formation of OPG, causing increased RANK-RANKL signaling and an increase in osteoclast formation, though it is interesting to note here that the marker used to determine a mature, terminally differentiated osteoclast is TRAP, which is not necessarily indicative of a functional osteoclast.

The second purposed mechanism is osteoblast derived. As described earlier, osteoblasts depend on TNAP to convert PPI to Pi, which allows for matrix mineralization. RA, a derivative of VA, is known to up-regulate TNAP mRNA, which would allow for a greater conversion of PPI to Pi and potentially an increased rate of osteoid mineralization (Orimo and Shimada 2005; Orimo 2010). No differences in BMD or cortical thickness were noted at any age, which further compounds the need of a clearer understanding of VA-osteoblast interactions. If TNAP is upregulated, allowing for more of the substrates needed in mineralization, why were there no increases in BMD or cortical thickness later in life. This also does not account or the decrease in cortical thickness seen in young animals. Could the biphasic picture shown in the results be an explanation? This is an area which warrants more research before a explanation ca be determined.

Small sample sizes (power <0.80) prevented trends from reaching statistical significance, so the inclusion of more animals would strengthen the tentative results found in both the material testing (Figure 12) and the mid-shaft analyses (Figure 9). In these experiments, the highest number of animals used was 9 per group but with current variability 28 animals per group would be needed to detect a difference. Large variation within each diet treatment, combined with small sample size makes the material testing data seen in Figure 12 tentative, but promising. If variability was decreased from the current level to around 15%, as few as 8 animals per group could be used.

Although the expected outcome that VA supplementation would cause alterations in the mechanical properties of bone were not observed, the observance of no change

from that of the control is also an important point, since there is currently controversy regarding VA RDA amounts in populations at risk for fracture.

Increased sample size would also help to clarify the changes seen in the mid-diaphyseal region. As stated earlier in Figure 9, VA decreases the size of the medullary area through a reduction in the endosteal radius. The accompanied decrease in the periosteal radius would indicate changes in bone shape and total size that did not reach significance in Figure 8.

Increases in the number of trabeculae were not expected since it is currently thought VA has no effect on osteoblasts, but instead directly stimulate osteoclasts (Figure 13). Or results indicate this might not be the case, and though little research has been done in the area, osteoblasts-VA interactions would be interesting to evaluate and further research involving TNAP and other signaling factors involved in osteoid production and mineralization is needed. This increase in trabeculae could be explained by the differences in weight between the diet groups or other unknown mechanisms (Figure 11), since the increase is normalized when trabeculae number is adjusted for body weight.

The ability of VA to both signal decreases in cortical bone thickness and an increase in medullary area with short term intake, and the reversal and recovery by long-term supplementation is a novel and interesting aspect of VA signaling only beginning to emerge. Further investigation into the mechanism of VA signaling and the mechanical consequences of these experiments is warranted. This finding could help to reduce the number of hip fractures seen in vulnerable populations each year.

This experiment provides supporting evidence that CMR transports VA to the bone marrow cavity where it was cleared from circulation. It also demonstrates that long term VA supplementation does not have detrimental consequences to bone.

FUTURE STUDIES

The research presented here offers a unique starting point to complete the picture of VA-bone interaction and interdependence. Since the specific types of retinoids were not evaluated, HPLC could be performed on bone lipid extracts to determine what forms and in what quantities of VA are found in bone. From these findings it could be established if the ester storage form of VA is found in bone. This would lead to a second kinetic experiment in which a radioisotope labeled dose of VA could be given to rats and the transport of VA throughout the body could be evaluated, including the duration of time it took for VA to appear within the marrow and how long it remains. The evidence provided from this experiment could be the groundwork for the duration of exposure of the hematopoietic environment to VA and would give optimal time points for subsequent dosing studies. From here we could evaluate changes to the bone microenvironment, including cell population shifts and gene regulation changes. Changes found at this level could then be translated into further studies of immune function and material properties of bone.

From here we could evaluate what changes in adiposity of an organism could do to VA transport and signaling within bones. Since it is known that obese individuals have a higher bone adipose content than normal weight individuals, it would stand to reason that this increase in lipid transfer would also be accompanied by an increase in VA

transport. Mechanical factors would be difficult to measure, since bone is sensitive to the mechanical stimuli such as increased loading through increased weight, but changes in cellularity independent of weight could be evaluated.

If cell populations become more or less numerous, it would also be interesting to note the timing of these events, and a second kinetic study could be justified by the two distinct pictures seen in the above results. Protein and RNA extracts could be evaluated to determine if c-Fos really is a regulatory component in the osteogenic cycle.

Duration of exposure is an important consideration, as evident from the findings from the experiments discussed earlier. It would be interesting if this discrepancy could account for some of the variation between findings in many of the large-scale nutritional reports. If supplement use increases with age, would the duration of intake play a role in the differences in results between studies? The forms of VA being consumed are also important. Few of the reports distinguish between food sources and intakes that are predominantly from supplement use.

The experiments briefly outlined above would provide a clearer and more complete picture of the interaction of VA with bone and bone derived cells and allow for understanding from the most basic genetic and molecular signaling level, upwards to the whole bone level or even into the interaction of VA and bone with other pathways, such as the immune response and adiposity.

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