DETERMINING IF LOWERING THE LEVEL OF DIETARY CALCIUM AND VITAMIN D IN AIN-93G DIET SUPPORTS NORMAL BONE DEVELOPMENT AND INTESTINAL INTEGRITY IN FEMALE CD-1 MICE

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ABSTRACT

Current levels of vitamin D (vit D) and calcium (Ca) in the reference AIN-93G rodent diet may be higher than required for healthy bone structure and bone mineral density (BMD). Other studies suggest that intestinal integrity may be altered by lowering levels of vit D or Ca. The study objective was to determine if lower diet levels of Ca and vit D support development of healthy bone structure and BMD in female CD-1 mice at 2 and 4 months of age without altering intestinal integrity. Lowering the levels of vit D (100 IU/kg) and Ca (3.5 g/kg) did not alter bone structure or BMD. Effects on intestinal integrity are less clear and requires further study using more comprehensive measures. Findings from this study suggest that dietary Ca and/or vit D at current levels in the AIN-93G reference diet may mask potential benefits of nutritional interventions aimed at promoting bone health.

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LIST OF ABBREVIATIONS

AI = adequate intake

AIN = American Institute of Nutrition

ANOVA = analysis of variance

BMC = bone mineral content

BMD = bone mineral density

BV = bone volume

BV/TV = bone volume to tissue volume

Ca = calcium

Conn.Dn = connectivity density

Ct.Ar = cortical bone area

Ct.Ar/Tt.Ar = cortical area fraction

Ct.Th = cross sectional thickness

DA = degree of anisotropy

DRI = dietary reference intakes

EAR = estimated average requirements

Ecc = mean eccentricity

E.Pm = endosteal perimeter

GIT = gastrointestinal tract

GM = gut microbiota

LPS = lipopolysaccharide

MA = medullary area

MMI-polar = mean polar moment of inertia

NIH = National Institutes of Health

NRC = National Research Council

Obj.Pm = mean total cross sectional object perimeter

PBM = peak bone mass

PTH = parathyroid hormone

RDA = recommended dietary allowance

REF = reference diet (AIN-93G)

ROI = region of interest

SEM = standard error mean

Tb.N = trabecular number

T.Pm = mean total cross sectional ROI perimeter

Tb.Sp = trabecular separation

Tb.Th = trabecular thickness

Tt.Ar = total cross-section area

TV = total volume

UL = upper intake level

UV = ultraviolet

VDR = vitamin D receptor

vit D = vitamin D

μCT = micro-computed tomography

25(OH)D_3 = 25-hydroxyvitamin D_3

CHAPTER 1: INTRODUCTION

According to Osteoporosis Canada, one in three women and one in five men will suffer from a fragility fracture in their lifetime (Osteoporosis Canada, 2016). These statistics identify a need for developing prevention strategies that target early life to improve bone health during aging. Preclinical models are commonly used to investigate the effects of nutrition on bone health. Our group routinely uses the CD-1 mouse model to study how dietary interventions modulate bone development (Ward, et al., 2016) and more recently, at key stages of trabecular and cortical bone development, 2 and 4 months, respectively (Sacco, et al., 2017). Currently, the AIN-93G reference diet is used to allow the comparison within studies and between studies of different research groups. This diet was formulated to support growth, pregnancy and lactation, however, the levels of vitamin D (vit D, 1000 IU/kg) and calcium (Ca, 5 g/kg) may be higher than required for healthy bone structure and bone mineral density (BMD) in rodents. Previous research has shown that lowering dietary vit D to 25 IU/kg diet has no significant effect on BMD or bone strength in mice (Glenn, et al., 2014; Villa, et al., 2016). Moreover, lowering dietary Ca below the standard level (5 g/kg) to 3 g/kg results in similar bone structure and strength in female rats (Hunt, et al., 2008). The effect of lowering the combination of vit D and Ca on bone structure and BMD has not been studied in growing female CD-1 mice.

CHAPTER 2: REVIEW OF THE LITERATURE

2.1 Bone formation, remodeling, and metabolism

Bone is a dynamic tissue that is conformed to various shapes and sizes. This multifunctional tissue plays significant roles in providing mechanical support and protection, mineral homeostasis, and hematopoiesis. Bones can be divided into two types, dense cortical bone and porous trabecular bone. These two different types of bone complement the actions of one another. For example, when a load stresses the bone the trabecular bone provides support against the load and redirects the stress to the stronger cortical bone, absorbing some of the energy from the external stess (Currey, 1970). On a microscopic level, the primary bone cells, osteoblasts, osteoclasts, and osteocytes, are integral for bone metabolism and mineral homeostasis. The coupled activities of osteoblasts, which form bone, and osteoclasts, responsible for degrading bone, constitute the basic multicellular unit of bone metabolism (Sims & Martin, 2014). Bone tissue is maintained when bone resorption and formation are in equilibrium (Jilka, 2003). Osteocytes are mature osteoblasts that become trapped in the bone matrix. Of the many functions of osteocytes, these cells play a key role in bone turnover (Aarden, et al., 1994).

Bone metabolism occurs throughout life to maintain structural integrity by reshaping or replacing bone via ossification and resorption processes. The formation of new bone occurs through one of two essential processes, intramembranous or endochondral ossification. Intramembranous ossification occurs during fetal skeletal development, predominately forming flat bones. Mesenchymal stem cells differentiate into osteoblasts and these cells secrete osteoid toward the ossification center. After

several days, the osteoid undergoes calcification to form trabecular bone, and the osteoblasts that remain trapped in the ossification center differentiate into osteocytes, responsible for maintaining the matrix formed around the bone. On the outer surface, mesenchyme differentiates into the periosteum and compact bone replaces the woven bone formed (Netter 1987, Brighton & Hunt, 1991). Unlike intramembranous ossification, endochondral ossification forms bone tissue in the presence of hyaline cartilage. Endochondral ossification is the primary bone formation process after fetal development, forming the majority of bones. This process is essential for the elongation of long bones at the epiphyseal plates and healing of bone fractures (Brighton & Hunt, 1986; Netter, 1987; Brighton, et al., 1973). Bone resorption is the process of removing mature bone tissue by monocyte derived multinucleated osteoclasts, initiating bone metabolism. Osteoclasts adhere to the bone surface, acidify, and degrade the mineralized matrix, ultimately releasing mineral content into the blood circulation. Trenches in the bone are formed and precursors recruit osteoblasts in order to lay down new matrix. Although osteoclasts are primarily responsible for degrading bone, there is evidence that these bone cells also play a role in regulating bone formation (Sims & Ng, 2014; Sims & Walsh, 2012).

Bone development occurs from infancy to approximately 20 to 30 years of age (Berger et al, 2010) and after peak bone mass (PBM), defined as the maximal amount of bone mass, is attained, there is a steady decline in bone mass until the end of life (Figure 1). In early life stages, osteoblast activity is greater than osteoclast activity and this results in bone growth. Steadily, there is an accumulation of bone mass, increasing bone thickness and density, and in time PBM is achieved. PBM is directly related to BMD,

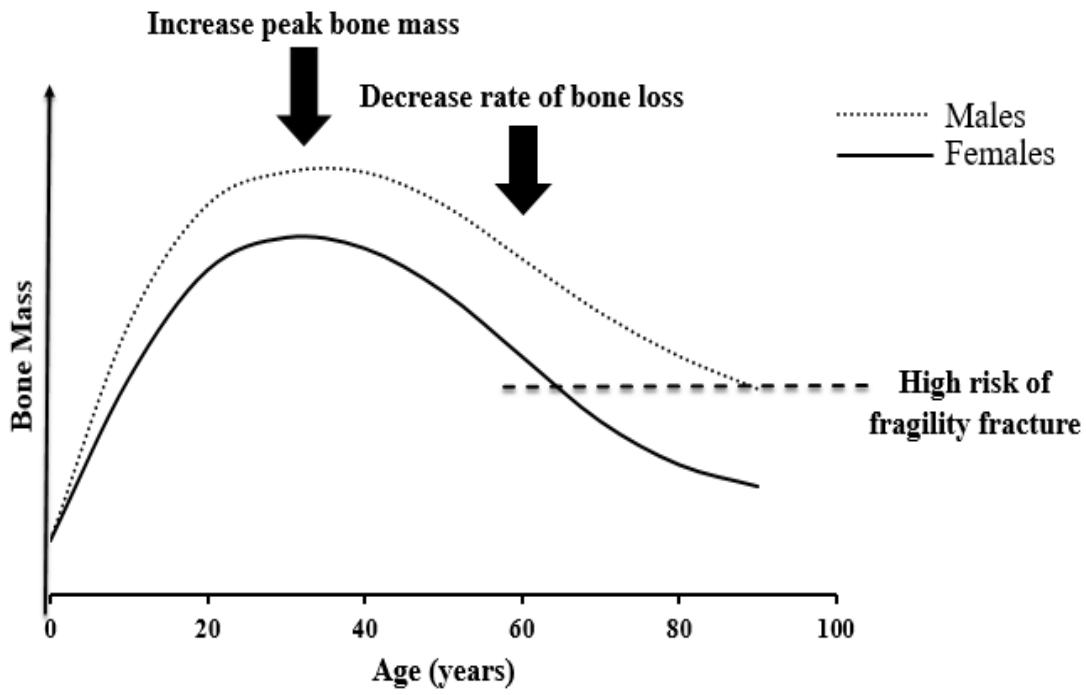


Figure 1. Age-related changes in bone mass of men and women. Black arrows represent prevention strategies to reduce the risk of osteoporosis, ensuring maximal peak bone mass (PBM) is reached and slowing the rate of age-related bone loss. Bone mass below the hatched line highlights a high risk of fragility fracture due to a loss of bone mass and compromised bone structure.

defined as the mass of mineral per area of bone (g/cm^2). As aging continues, the gradual loss of bone tissue is inevitable in both males and females. This occurs due to bone resorption exceeding bone formation, and ultimately, an individual often develops osteoporosis (Figure 2). In females, the rate of bone loss may increase exponentially after menopause, at around 50 years of age, because of the reduction of endogenous estrogen production. Estrogen directly promotes bone development through an increase in osteoblast formation and attenuation of osteoclast activity. During the perimenopausal transition, when there is a significant reduction in estrogen, a rapid decline in bone mass is evident (Heaney, et al., 1978a). This bone loss is also accompanied by negative changes in external Ca balance, due to a decrease in intestinal absorption, increase in urinary excretion of Ca, and a decrease in the circulating concentration of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) (Heaney, et al., 1978b). Strategies of building stronger bones during childhood and adolescence, attaining a higher PBM, and/or decreasing the rate of age-related bone loss, may reduce the risk of osteoporosis and related fragility fractures (Figure 1).

2.2 Osteoporosis

Osteoporosis is a silent epidemic in which the progression of the disease is gradual and absent of early symptoms (Rachner et al., 2011). This bone disease is characterized by low bone mass resulting in weak, fragile bones that are prone to fracture. Bone strength is determined by bone quantity (BMC, BMD), and bone quality (bone structure, properties of the collagen-mineral matrix and microdamage). In turn, a decrease

A)

B)

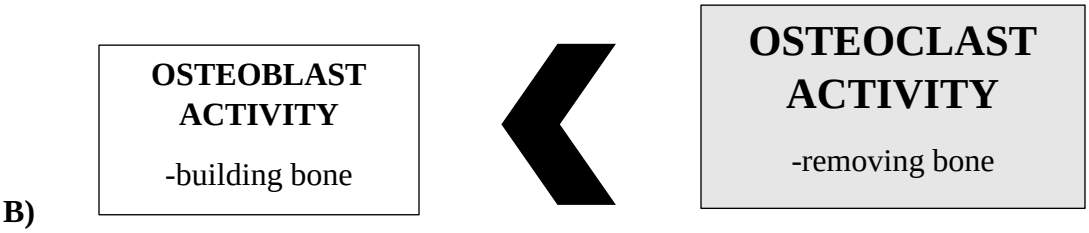


Figure 2. Relative activity of osteoblasts and osteoclasts in a healthy state and in osteoporosis. A) Through the process of bone remodelling in a healthy bone, the rate of bone formation by osteoblasts equals the rate of bone resorption by osteoclasts. B) When osteoclast activity exceeds osteoblast activity, bone mass declines (demonstrated by a reduction in bone mineral content (BMC) and BMD along with bone structure) and therefore becomes weak and brittle (osteoporosis).

in bone strength is associated with an increased risk for fragility fractures. Such fractures are most common at the hip, wrist or spine (Reid, 2013). Among Canadian women, the annual incidence of fragility fractures is greater than the incidence of heart attack, stroke, and breast cancer combined (Osteoporosis Canada, 2016). The onset of osteoporosis often leads to a reduction in quality of life, caused by a decrease or loss in mobility and independence as well as lowered self-esteem (Marottoli et al, 1992; Randell et al, 2000). Depending on the severity of the disease, osteoporosis-related discomfort may significantly impact daily activities (Ross et al, 1994). Healthcare services providing treatment for osteoporosis have resulted in an overall economic burden. In 2010, the Canadian healthcare system estimated that costs for acute care, outpatient care, prescription drugs and indirect costs were more than $2.3 billion and rose to $3.9 billion if long-term care facilities were utilized (Tarride et al, 2011). This number is projected to rise with an increase in the number of fractures and related complications (Johnell & Kanis, 2006; Brown & Josse, 2002) within the large aging population of baby boomers, individuals born between 1946 and 1964. Pharmacological agents have been studied for the prevention and treatment of osteoporosis. However, due to the potential adverse side effects of the pharmacological agents for treatment of osteoporosis, prevention strategies are also important to consider. Such strategies, using dietary interventions, may be a safe and effective approach for reducing the risk of fragility fracture.

2.2.1 Dietary prevention strategy: Dietary Reference Intakes for Ca and vit D

A healthy diet supports the development and maintenance of a healthy, strong skeleton by assisting with the attainment of PBM and attenuating age-related bone loss (Mitchell et al., 2015). Since bone mass is one of the main determinants of fragility

fracture, a significantly greater PBM will provide a larger reservoir of bone tissue to lose during aging, resulting in a lower risk of fragility fractures.

In humans, we have Dietary Reference Intakes (DRI) for Ca and vit D that have been established by the Institute of Medicine. The DRIs for Ca and vit D were largely based on bone health, including measures associated with bone accrual, maintenance and loss within the difference stages of life (Institute of Medicine, 2011). Meeting the required levels during growth and development stages is essential for modulating future bone health. There is a vast array of previous research demonstrating that consumption of specific foods, such as milk (Jones, 2011), and individual nutrients, particularly Ca (Huncharek, et al., 2008; Bonjour, et al., 1997; Johnston, et al., 1992) and vit D (Zamora, et al., 2000), during early life have a positive association with bone development in both human and animal models. Sex-specific responses to diet are important to consider, as males and females have different requirements for some nutrients. My thesis focuses on females as it is part of a larger research project in which another student has studied males. The DRIs for Ca and vit D for females are described in Table 1. The DRIs, which include a recommended dietary allowance (RDA) or an adequate intake (AI), an estimated average requirement (EAR), and a tolerable upper intake level (UL), were developed to act as a guide for assessing and planning diets in humans. According to the Institute of Medicine (1997), the RDA values were derived from the EAR and it is intended to meet the requirements for 97-98% of healthy individuals for each sex in a specific life stage. Currently, the RDAs for Ca and vit D are the same in males and females with the exception of Ca, which is increased at 71 years of age in males, compared to 51 years of age in females. The RDA for Ca increases later in life for males,

compared to females, because males tend to have a significantly greater amount of bone mass (Nieves, et al., 2005; Avdagic, et al., 2009). Furthermore, sex differences in bone quantity may be attributed to hormonal differences, as females often experience a more rapid decline of bone mass due to the cessation of estrogen during menopause, shown in both human and animal models (Reid, 2013; Richelson et al, 1984; Hedlund & Gallagher, 2009; Wronski et al, 1985). During infancy (0 to 12 months), no RDA is established for either Ca or vit D and therefore an AI, defined by the Institute of Medicine as the recommended average nutrient level, is used. This level is based on observed or experimentally determined estimates of intake in a group of healthy individuals assumed to be maintaining an adequate nutritional state (Institute of Medicine, 1997).

Ca and vit D are key nutrients for optimal bone health and function in combination to support a healthy skeleton, however, more is not always better. An UL is intended to act as a guide for ensuring the safety of individuals consuming these nutrients, identifying the maximal amount tolerable before adverse effects are demonstrated (Institute of Medicine, 1997). Although it is difficult to reach these levels through diet alone, excessive intake of Ca or vit D through use of dietary supplements may result in hypercalcemia (Institute of Medicine, 2011; Ozkan et al., 2012). Prolonged hypercalcemia can cause renal failure due to its association with calcification of kidney tissues (nephrocalcinosis) (Gillen, 2005), which occurs more commonly in women and elderly (Marangella, et al, 1990). Ca and vit D are essential nutrients for bone health, as approximately 80-90% of BMC, defined as the mass of mineral contained in the entire bone (g), is comprised of Ca and phosphorus, while other dietary components, including vit D, are required for normal bone metabolism (Institute of Medicine, 1997).

Table 1. DRIs: EAR, RDA or AI, and UL of Ca and vit D for healthy females (Institute of Medicine, 1997).

Age (years)	EAR	RDA or AI*	UL
Ca (mg/d)			
Females			
0 to 6 months	-	200*	1000
6 to 12 months	-	260*	1500
1-3	500	700	2500
4-8	800	1000	2500
9-18	1100	1300	3000
19-50	800	1000	2500
>50	1000	1200	2000
Vit D (IU/d)			
Females			
0 to 6 months	-	400*	1000
6 to 12 months	-	400*	1500
1-3	400	600	2500
4-8	400	600	3000
9-70	400	600	4000
>70	400	800	4000

*When RDA is unavailable, AI is presented as an alternative measurement

2.3 The role of Ca and vit D on bone metabolism

Ca is an essential nutrient which is abundant in dairy products, dark leafy greens and fish products (Dietitians of Canada, 2016a). Ca is the most abundant mineral in the body and is a highly recognized nutrient associated with bone health. Among the various roles in maintaining overall health and function, Ca is critical for providing mineral, strength and structure to bone (Black et al., 2002; French et al., 2000). Specifically, adequate Ca consumption during growth is important for future bone health. A longitudinal study in Chinese girls has demonstrated that Ca intake was a significant predictor of total bone mass (Zhu et al., 2005). In a three-year study using identical twins (6-14 years old) consuming on average just below the RDA (1 g/day compared to 1.3 g/day), an increase in calcium intake via supplementation of 1 g Ca citrate malate/day was positively associated with an increase in BMD at multiple sites (radius, hip and spine) compared to the twin receiving a placebo (Johnston et al., 1992). Ca, together with phosphate, is incorporated into hydroxyapatite that crystallizes around collagen fibers to form the matrix of the bone. During mineralization, as crystals become denser, they provide strength and rigidity to bones. In a preclinical model, mice that were Ca-deficient resulted in similar bone loss at the femur and vertebrae (Messer et al., 1981), which explains the significance of adequate Ca intake. When serum Ca levels are insufficient, the skeleton acts as a large reservoir for maintaining adequate concentrations. Throughout life, bone is continuously losing and gaining Ca deposits through the process of bone remodeling, regulated in part by vit D (Clarke, 2008; Lee et al., 2014; Anderson et al., 2011).

Vit D plays a significant role in stimulating bone matrix formation and regulating Ca absorption in the small intestine (Lips, 2012). Vit D, also known as the sunshine vitamin, can be synthesized by the body with exposure of ultraviolet (UV) beta radiation from the sun directly onto the skin. Sunlight on the skin converts 7-dehydrocholestrol to pre-vit D_3, which is metabolized to inactive vit D_3. Vit D_3 is transported to the liver where it is converted into 25 hydroxyvitamin D_3 ($25(OH)D_3$) and then to the kidney for activation, forming 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). $1,25(OH)_2D_3$ helps regulate Ca homeostasis and promotes Ca absorption and bone mineralization. Additionally, $25(OH)D_3$ can also promote bone mineralization via activation in bone cells (Lee et al., 2014; Anderson et al., 2011). However, UV beta radiation is not always a sufficient source for attaining optimal vit D levels, as there are various factors affecting this endogenous pathway, such as the high risk of skin cancer. Time of the day and year as well as location affects the level of UV beta energy available; as Canadians live in a northern climate, sun exposure is limited. In addition, darker skin absorbs less amounts of UV beta and requires a longer duration of sun exposure, unintentionally increasing the risk of skin cancer (Rivas, et al., 2015). Use of sunscreen and protective clothing prevents endogenous synthesis of vit D (Kim et al., 2010). Vit D can also be obtained from some foods, ingested in the form of ergocalciferol (vit D_2) from plant sources, including grains and fortified orange juice or milk, and cholecalciferol (vit D_3) from animal sources (Kulie et al., 2009; Kato, 2000), such as salmon, tuna, pork and beef liver (Dietitians of Caada, 2016b). Vit D_3 binds to transport proteins and $1,25(OH)_2D_3$ is formed similarly to the endogenous pathway (Suda et al., 1990; Kulie et al., 2009). Vit D plays a significant role in Ca homeostasis and bone health through both direct and indirect mechanisms via

upregulation of bone resorption and increasing VDR-dependent Ca absorption in the intestines and reabsorption in the kidneys, respectively (Eisman & Bouillon, 2014). During childhood, a lack of vit D is associated with inadequate bone mineralization, leading to rickets, abnormal bone development, and hypocalcaemia (Shore & Chesney, 2013). On the contrary, 12- and 15-year old girls with high vit D status (serum 25(OH)D >74 nmol/L) resulted in significantly greater BMD in their forearm, assessed using dual energy X-ray absorptiometry, and lower parathyroid hormone (PTH) levels (Cashman et al., 2008). The biological actions of vit D are primarily exerted through nuclear vit D receptor (VDR)-mediated control (Kato, 2000). Activated VDR prevents the release of Ca from bone to serum by stimulating intestinal Ca absorption and renal reabsorption (Yamamoto et al., 2013).

2.3.1 Interplay between Ca and vit D in maintaining Ca homeostasis

Maintaining Ca balance occurs in a negative feedback loop, as the level of Ca in the blood facilitates the activity of the parathyroid glands. When blood Ca levels are low, the parathyroid cells increase the secretion of PTH that activates vit D in the kidney, increasing the reabsorption of Ca in the kidneys, absorption of Ca in the intestines through active transport, and stimulating the breakdown of bone by osteoclasts to release Ca into the bloodstream. This process results in an increase of blood Ca levels (Figure 3). When blood Ca levels are high, PTH is supressed by either the high concentration of Ca or vit D metabolites, binding to the membrane bound Ca receptor and nuclear VDR on the parathyroid cell, respectively (Lips, 2012). Furthermore, previous research has suggested that independent of VDR, bone mineralization and bone turnover can be maintained with sufficient Ca and phosphorus intake, suggesting a transport system that

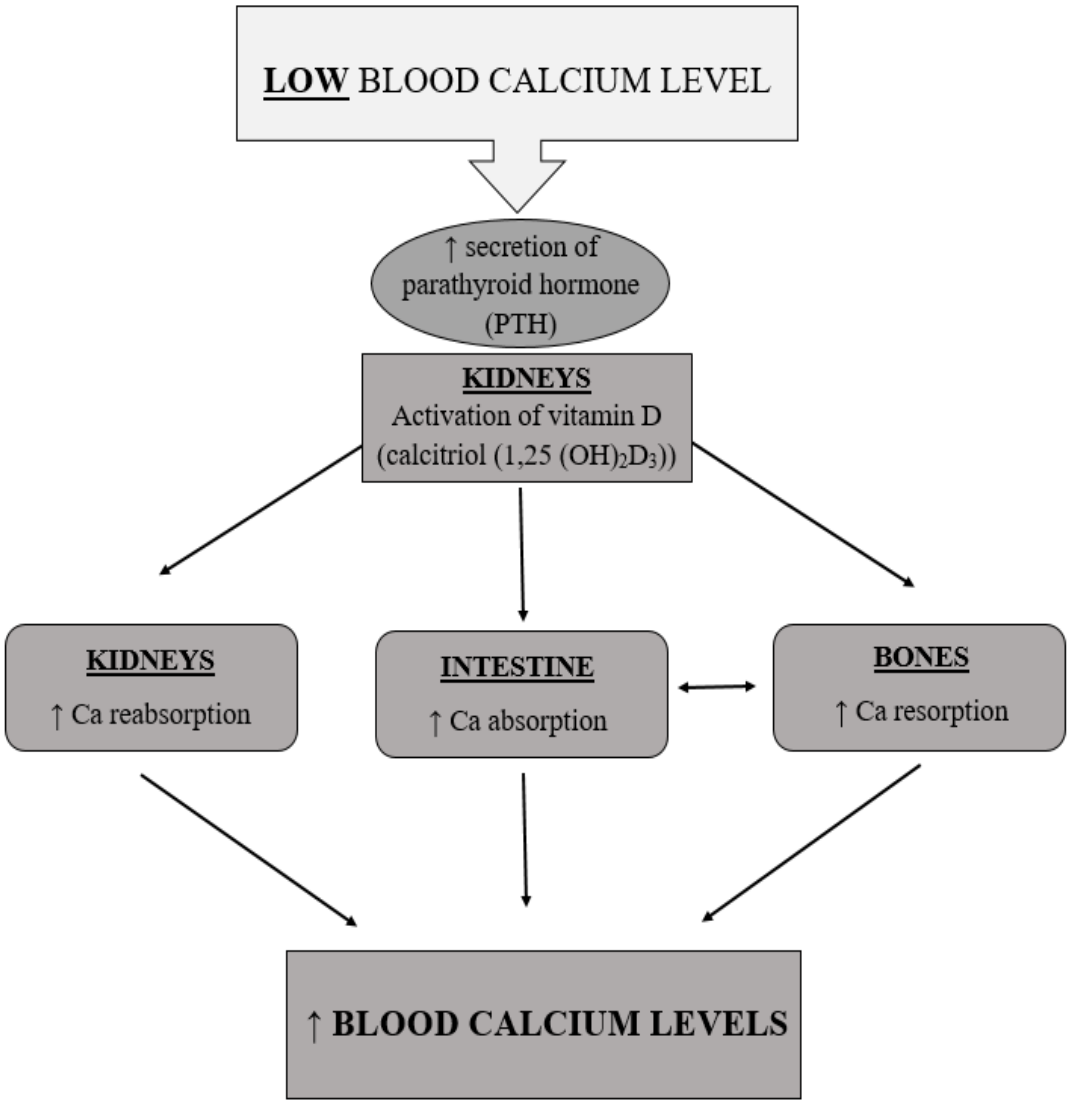


Figure 3. The interplay between Ca and vit D in the regulation of blood Ca homeostasis.

is dependent on the ratio of Ca and P may be present in the intestine (Masuyama et al., 2003). With consumption of a meal, vit D aids in Ca absorption from the intestine through both active and passive diffusion (Christakos, et al., 2015). Moreover, there is emerging evidence that gut microbiota may be altered by these specific nutrients, providing an indirect effect of Ca and vit D on bone metabolism (Villa, et al., 2015).

2.4 Intestinal integrity and the existence of a gut microbiota-bone axis

The intestines play an integral role in maintaining Ca homeostasis by absorbing essential nutrients as they pass through the digestive system. The digestive system is made up of the mouth, gastrointestinal tract (GIT), liver, pancreas, and gallbladder. Digestion plays a significant role in breaking down food into nutrients for the body to use for energy, growth, and repair. This occurs via the movement of food (peristalsis) through the GIT, a large, muscular tube extending from the mouth to the anus. When food and/or beverages are consumed, it enters the esophagus and it is carried from the mouth to the stomach. Within the stomach, the food and liquid are mixed with digestive juice and are then emptied into the small intestine. The walls of the small intestine absorb digested nutrients into the bloodstream, for distribution to the rest of the body, and waste products are further pushed into the large intestine. The large intestine is responsible for absorption of water and remaining nutrients and the production of stool from waste is excreted. In addition, to help with digestion, the GIT is home to bacteria, known as the gut microbiota (GM). The GM is harboured in the intestines and is composed of trillions of microorganisms, which can be divided into seven bacterial divisions with *Firmicutes* and *Bacteroidetes* being the most abundant phyla (Eckburg, et al., 2005). In a healthy GM, there is a state of homeostasis and

when a disturbance or imbalance in the GM (dysbiosis) occurs, health concerns may arise, including effects on bone health. Bone health is altered through the existence of a gut microbiota-bone axis, defined as the effect of the gut-associated microbial community or the molecules they synthesize on bone health (Villa et al, 2015).

2.4.1 Markers of intestinal integrity

The major role of the intestinal epithelium is to separate luminal contents from the interstitium. The function of the intestine is dependent upon its integrity, which can be characterized by intestinal permeability and inflammation. Intestinal permeability is defined as the regulation of solute and fluid exchange between the intestinal lumen and tissues (Odenwald & Turner, 2013). Albumin is a major protein in the plasma and fecal samples and a higher level of fecal albumin is a marker of compromised intestinal integrity (Wang, et al., 2015). Moreover, lipopolysaccharide (LPS) is derived from Gram-negative bacteria of the GM and can translocate to the bloodstream as a result of increased intestinal permeability. LPS is an inflammatory marker and induces osteoclast formation and thus stimulates bone resorption (Mundy & Roodman, 1987; Chambers, 1991). Studies have demonstrated that an increase in intestinal permeability accompanies various diseases such as inflammatory bowel disease, in both human and mouse models (Gomes, et al., 2015). Vit D plays a significant role in regulating the immune system. In Ca-deficient mice, administration of vit D resulted in improved inflammatory bowl disease (Cantorna, et al., 2004). Conversely, dietary interventions such as low dietary vit D generate a pro-inflammatory state and may modulate intestinal integrity (Jahani, et al., 2014).

Male CD-1 mice fed a low vit D diet (25 IU/kg) demonstrated an inflammatory-prone status at 3 months of age, as shown by elevated serum LPS levels. However, although bone structure was not measured, BMC, BMD, and bone strength were unaltered (Jahani, et al., 2014). Female mice were not studied. The lack of effect on bone health does not suggest that lowering Ca and vit D is adequate since there may be consequences of lowering these nutrients in other aspects of health, including intestinal integrity. In nutritional research, the levels of individual nutrients in a control or reference diet are important for ensuring that nutrient requirements are met. However, since these diets are also used to determine if a dietary intervention can have a positive effect on bone development, it is important that excesses of bone-supporting nutrients, which may be present in the current reference diet (AIN-93G), do not mask a potential benefit of the intervention.

2.5 Preclinical models: providing excess Ca and vit D may mask potential benefits of dietary interventions aimed at optimizing bone development

Rodent models have been commonly used to investigate the effects of nutrition on bone health to attain an understanding of the mechanisms that can ultimately be applied to human development (Reeves, 1989). The mouse model is often used because they are biologically similar to humans as we share a vast majority of the same genes (Waterston et al, 2002). Due to the fact that mice have accelerated lifespans, peak BMD and bone strength is reached at about 3 to 4 months of age in CD-1 mice (Ward, et al., 2007), equivalent to an adolescent life stage in humans (Flurkey, et al., 2007). However, bone structure has a different trajectory and has been studied in vivo at 2, 4 and 6 months of

age. Specifically, trabecular bone volume is higher at 2 compared to 4 and 6 months of age while cortical bone area increases from 2 to 6 months of age (Sacco, et al., 2017). This model allows for the potential to examine how specific food components modulate bone metabolism over a period of several months. Findings from our group have demonstrated that there is a window of opportunity to manipulate diet during early life stages and produce favourable bone outcomes later in life. CD-1 mice exposed to isoflavones during the first 21 days of life demonstrated improved bone health later in life, characterized by higher BMD at the lumbar vertebrae and whole femur as well as favourable outcomes in terms of structure and strength (Dinsdale, et al., 2012). Neonatal exposure to soy isoflavones, daidzein and genistein, during the first 5 days of life also resulted in higher BMD and improved bone structure and strength of female CD-1 mice at adulthood, when the treatment was given both individually and in combination (Kaludjerovic & Ward, 2009). Furthermore, this intervention during the first 5 days of life protected against the deterioration of bone tissue due to a decline in endogenous sex hormones after ovariectomy, a scenario which mimics changes in bone tissue after menopause (Kaludjerovic & Ward, 2010). All of the aforementioned studies used the AIN-93G diet containing 1000 IU vit D/kg and 5 g Ca/kg diet. If these nutrient levels are in excess, the benefical effect may have been diminished. For future programming studies in bone research, determining a reference diet without providing excess nutrients is warranted. Given that levels of bone supporting nutrients such as Ca and vit D may be in excess and may thereby mask potential benefits of test interventions with foods or food components, it is important to establish if a lower level of Ca and vit D similarly supports bone development as the levels of Ca and vit D in the current AIN-93G diet.

2.6 Formulation of the AIN-93G reference rodent diet

Over the years, standard rodent diets have been developed and modified to formulate a general rodent diet that can be used to simulate human dietary practices. It is important to have a consistent control diet to reduce the variation in nutrient levels when comparing findings within and between laboratories and to ensure that the effect of an intervention was actually due to the intervention and not a modification of the diet. The various types of diets for rodent models include cereal-based (unrefined or non-purified), purified, and chemically-defined diets. Cereal-based diets contain unrefined plant and animal materials and can be characterized as open or closed formula. In open formula diets the composition of the ingredients is readily available to the public and in closed formula diets the exact composition is unknown. Semi-purified diets are essentially the same as purified diets, however, technically they differ in terms of the types of ingredients used. Currently, the terms semi-purified and purified are used synonymously. Purified diets are composed of refined ingredients and as the concentrations of these ingredients are less variable, compared to the cereal-based diet. These diets are commonly used when examining nutritional excesses and deficiencies as the levels of nutrients are readily manipulated. Chemically-defined diets are the most controllable in respect to altering elemental ingredients, providing amino acids, mineral elements and vitamins are in maximally bioavailable forms (NRC, 1995). These diets are used for nutritional and metabolic experiments requiring high quantitative precision, however, it is not representative of human diet. Specifically, for my study, I used the AIN-93G purified rodent diet along with a modified version containing altered levels of vit D and Ca. Through years of modifications to the reference rodent diet, the current reference diet

(AIN-93G) was developed to support maternal nutritional needs required for fetal development and adequate nutrition to support early postnatal growth (Reeves, 1997). As this purified diet can be readily altered, it serves as an adequate reference diet for nutritional researchers to examine interventions during early life stages.

2.6.1 NIH-07 diet

Closed formula diets posed challenges for nutritional researchers due to the large amount of variability among batches and inability to be manipulated for the examination of specific nutrients. In 1972, the National Institutes of Health (NIH) developed the NIH-07 diet, which was a cereal-based open formula (Reeves et al., 1993a). This natural-ingredient diet was considered adequate for reproduction, lactation, and maintenance of tissues and metabolism in rodents and was intended to replace closed formula commercial diets, reducing experimental variability (Bieri et al., 1977). Advantages of this standard rodent diet included its relatively inexpensive cost to manufacture (NRC, 1995) and due to the known composition, it allowed for open purchasing competition, therefore reducing costs. However, the major challenge was that this diet could not be easily modified and manipulated to meet research needs. Natural-ingredient diets do not have identical batches as the composition of the individual ingredients have different levels of nutrients. This variation can be influenced by environmental factors such as soil, weather conditions, use of fertilizers, harvesting and storage procedures. Moreover, when adjusting an ingredient, the concentration of many other nutrients also require alterations in order to compensate for the ingredient of interest. In 1973, a committee of nutritional scientists was formed by the Council of the American Institute of Nutrition to develop

standardized diets for rodents. A few years later the AIN-76 purified rodent diet was developed to replace the NIH-07 (Bieri et al., 1977).

2.6.2 AIN-76 diet

The American Institute of Nutrition developed the AIN-76 diet to standardize nutritional studies using rodent models, ensuring consistency among laboratories (Reeves et al., 1993a). Overall, this purified diet was designed to meet, or exceed, the nutritional requirements and support growth, reproduction and lactation, comparable to the cereal-based diet (Reeves, 1997; Bieri et al.,1977). The purpose of this diet was to reduce the intrinsic variation of the natural ingredients in the cereal-based diet, NIH-07 (Reeves et al., 1993a). Shortly after, concerns regarding an increase number of hemorrhagic deaths (Roebuck et al., 1979) and shelf-life (Fullerton et al., 1982) provoked a revision designated AIN-76A. To address the increase in hemorrhagic deaths, 500 µg vit K/kg diet was added to the formulation and rectified this issue (Roebuck et al., 1979) and optimal storage was specified at below 4 degrees Celsius (Fullerton et al., 1982). The level of Ca met the NRC recommendations of 5 g/kg diet, which was demonstrated to be adequate to support a healthy pregnancy to lactation and the early life stages of offspring, in which growth is prominent (National Research Council, 1995; Bieri et al., 1977). Among the changes made to the diet, minerals, with few exceptions, were consistent with the NRC levels and vitamins greatly exceeded the NRC levels to allow for potential loss during mixing and storage (Bieri et al., 1977). Over time, a re-examination of the AIN-76A diet was warranted by a common problem, kidney calcification (nephrocalcinosis) in female rats fed the AIN-76A diet for a prolonged period of time, due to a low Ca:P molar ratio of ~0.75 (Schaafsma et al., 1985).

2.6.3 AIN-93 diet

In 1993, the AIN-93 purified diet was developed and largely replaced the AIN-76A diet (Reeves, 1989; Reeves, 1997; Reeves et al., 1993a; Reeves et al., 1993b). With the aim of achieving a Ca:P ratio of 1:3 or greater to prevent nephrocalcinosis, the forms of Ca and phosphorus were altered from $CaHPO_4$ to $CaCO_3$ and KH_2PO_4, respectively, and the amount of phosphorus was lowered (Reeves et al, 1993a). Major differences among the AIN-76, AIN-76A and AIN-93G diets are demonstrated in Table 2.

The AIN-93 diet, compared to the AIN-76A, contains an improved balance of essential nutrients, with major changes including the substitution of cornstarch, corn oil and DL-methionine for sucrose soybean oil (linolenic fatty acid) and L-Cystine (casein), respectively. Furthermore, the mineral and vitamin mixes were also altered to address nutritional and technical problems, such as nephrocalcinosis and anemia resulting from vitamin B12 deficiency (Reeves, 1997). The AIN-93 diet can be readily made from purified ingredients at a reasonable cost, meets or exceeds the nutrient requirements suggested by the NRC, is reproducible, and can be used for various nutritional studies (Reeves, 1997). The reformulated diet is comprised of two versions, AIN-93G diet, recommended for supporting healthy pregnancy to lactation and growth of offspring during early life stages, and AIN-93M diet, used to maintain tissue and metabolism during and after adulthood. My thesis study used the AIN-93G as I am studying female CD-1 mice from birth to young adulthood. There is evidence that a formulation, very similar to the AIN-93G rodent diet, provides comparable growth rate and body weight to rats fed a commercially available cereal-based, non-purified diet (Reeves et al., 1993b). Ca and vit D levels remained unaltered from its precursor AIN-76A diet, at the levels

recommended by the NRC (5 g Ca/kg and 1000 IU vit D/kg diet) (Reeves et al., 1993a; National Research Council, 1995; Bieri et al., 1977). These levels were established to meet the nutritional requirements of laboratory mice, however, many have identified the lack of strong scientific support for the levels of individual nutrients (NRC, 1995).

Table 2. Major modifications of the diet compositions among AIN-76, AIN-76A and AIN-93G.

	AIN-76	AIN-76A	AIN-93G
Ca and Phosphorus	5 g $CaHPO_4$/kg diet	5 g $CaHPO_4$/kg diet	5 g $CaCO_3$/kg diet 3 g KH_2PO_4/kg diet
Vit D	1000 IU/kg diet	1000 IU/kg diet	1000 IU/kg diet
Carbohydrate	Sucrose (500 g/kg diet)	(100 g/kg diet of any combination below) Glucose Glucose+Sucrose Cornstarch Glucose+Sucrose+Constarch	Sucrose (100 g/kg diet) Cornstarch (~400 g/kg diet) Dextrinized cornstarch (132 g/kg diet)
Fat	Corn oil (50 g/kg diet)	Corn oil (50 g/kg diet)	Soybean oil (70 g/kg diet)
Protein	Casein (200 g/kg diet)	Casein (200 g/kg diet)	Casein (200 g/kg diet) L-cystine (3 g/kg diet)
Antioxidant		Butylated hydroxytoluene/Ethoxyguin (5-10 g/kg diet)	Tertiary- butylhydroquinone (14 g/kg diet)

2.7 Dietary requirements for Ca and vit D in mice are not strongly based on evidence

In determining the nutritional requirements for mice, levels were established to promote rapid growth leading to maximum body size at maturity and it was assumed that it then would also be adequate for reproduction, lactation, and maintenance of tissues and metabolism. Due to the various genetic and environmental factors that influence nutrient requirements, there is a lack of support in the literature for establishing the requirements of individual nutrients such as Ca and vit D. The estimates were based on previous mouse data fed dietary ingredients that are currently unavailable or unidentifiable, results from studies that were not specifically aimed at establishing nutrient requirements, and the assumption that mouse and rats have similar nutrient requirements (NRC, 1995).

2.7.1 Ca requirements for mice

As discussed above, the standard level of Ca in AIN-93G for mice and rats may be significantly higher than what is required for bone development. Although there is limited research studying Ca at various levels in a CD-1 mouse model, in female Sprague-Dawley rats, Ca levels were manipulated by examining both lower and higher levels than in the reference rodent diet (5 g/kg), 1 through 7 g Ca/kg diet, respectively, at the reference level of vit D (1000 IU/kg diet). Indeed, results indicate that bone length and width, BMD, bone mineral retention, breaking force, flexibility, and trabecular number and spacing were optimal if Ca intakes met or exceeded approximately 2.5 g/kg diet, which is significantly lower than the requirement set forth by the NRC. Furthermore, a higher dietary Ca intake did not result in a higher BMD, better bone structure, or greater biomechanical strength compared to Ca intakes of 2.5 g/kg diet (Hunt, et al., 2008). As

these results were based on the normal vit D level (1000 IU vit D/kg diet), it is not known what happens to the Ca requirement when vit D is lowered simultaneously.

2.7.2 Vit D requirements for mice

In mice, previous research has demonstrated that a diet low in vit D (25 IU/kg) has no negative effect on BMC, BMD, and bone strength when Ca levels were normal (5 g Ca/kg diet). Pregnant female CD-1 mice were fed a modified AIN-93G diet, containing either high (5000 IU/kg diet) or low (25 IU/kg diet) vit D level. At weaning, a subset of male offspring was given their mothers' respective diet or the other diet until sacrifice. Results suggested low vit D induces an inflammatory-prone status, demonstrated by an increase in colonic pro-inflammatory genes. However, although bone structure was not measured, BMC, BMD, and strength of bone was not altered (Jahani et al., 2014). Females were not studied. Similarly, in an interleukin (IL)-10 knock-out mice model, prone to intestinal inflammation, females at 3 months of age developed intestinal inflammation and offspring exposed to the low vit D diet had higher levels of inflammation, as seen by higher serum IL-6. Furthermore, there were no differences in femur or vertebral BMC, BMD, or bone strength between the groups (Glenn et al, 2014). This is further supported by a study in which long-term vit D deficiency, induced by feeding a diet devoid of vit D (0 IU vit D/kg diet) to adult CD-1 mice had no negative effect on bone health. Results indicated serum $25(OH)D_3$ levels were below 4 nmol/L, and at 24 months of age, micro-computed tomography (μCT) and histomorphometric analysis revealed no differences in bone structure, remodeling and BMD between the two groups (van der Meijden et al, 2015). In all these studies, Ca was at the normal level in the AIN-93G diet.

In summary, it is important to determine the levels of Ca and vit D that support bone development without providing excess to test the effectiveness of dietary strategies to support bone development as well as bone health throughout the lifespan. Modification of Ca and vit D levels in the AIN-93G diet need to be considered together to determine how altering both nutrients affect bone development. My thesis research studied Ca levels of 5, 3.5, 3, and 2.5 g/kg diet, at two levels of vit D, 100 or 400 IU/kg diet – both levels being lower than current 1000 IU/kg in the AIN-93G diet. While previous research has investigated the effect of varying levels of vit D or Ca, there is limited research performed using a CD-1 female mouse model. However, our group consistently uses a CD-1 mouse model to study the effect of nutritional interventions on bone development. Thus, the CD-1 mouse model was used for this research. Moreover, CD-1 mice are an outbred strain and differences in genetic variation are closely relatable to human populations (Aldinger, et al., 2009). Our laboratory group has demonstrated that in female CD-1 mice, reduction in trabecular structure occurred from 2 to 4 months of age, while cortical bone structure continued to increase from 2 to 6 months of age. Therefore, analyzing bone structure and BMD at 2 and 4 months allows for comparison among dietary groups at key bone developmental stages (Sacco, et al., 2017; Ward, et al., 2007).

CHAPTER 3: STATEMENT OF THE RESEARCH QUESTION, OBJECTIVES AND HYPOTHESES

3.1 Overall research question

To determine if Ca and vit D levels that are markedly lower than the levels in current reference diet (REF, AIN-93G) can support healthy bone development in mice. Key outcomes of bone development including bone structure and BMD at skeletal sites rich in trabecular or cortical bone will be specifically studied. This is important to determine as a diet that provides an excess of Ca and/or vit D may mask potential benefits of nutritional interventions aimed at promoting bone health.

3.2 Objectives

In female CD-1 mice, the specific aims of this study are to determine: i) if a lower level of Ca and vit D than is in the REF diet, supports normal bone development at 2 and 4 months of age, as determined by measuring bone structure and BMD and ii) if the integrity of the intestine is altered with varying levels of dietary Ca and vit D, as determined by measuring fecal albumin and serum LPS.

3.3 Hypotheses

Based on existing literature, it is hypothesized that the level of Ca and vit D found in REF, 5 g Ca/kg diet and 1000 IU vit D/kg diet, respectively, is higher than what is

required for supporting healthy bones in female CD-1 mice. I hypothesize that lowering vit D and Ca levels to 100 IU/kg and 2.5 g/kg, respectively (but not other combinations of vit D and Ca), will have a negative effect on bone structure and BMD. With respect to bone developmental changes, trabecular bone will begin to deteriorate between 2 and 4 months of age, while cortical bone will continue to develop until 4 months. Furthermore, intestinal integrity will not be altered with lower levels of vit D and Ca.

CHAPTER 4: MATERIALS AND METHODS

4.1 Animals and diets

A total of twenty-two timed-pregnant CD-1 mice were purchased from Charles River Laboratories (St. Constant, QC, Canada) and the arrival of the mothers were staggered with 2 batches (n=5 mothers/batch x 2 batches). Mice were acclimatized for one week and kept on a 12h:12h light-dark cycle in accordance with the standard operating procedures *Care and maintenance of mice* and *Environmental enrichment for mice* at the animal facility of Brock University. Considering that vit D can be endogenously produced with exposure to UV beta radiation, LED lighting with zero UV emissions was used in the animal housing rooms. Mothers were fed AIN-93G (REF) and water ad libitum throughout pregnancy and lactation. Following delivery, sex is identified within the first 2-4 days after birth by measuring anogenital distance. I specifically studied female offspring, while my colleague studied the male offspring. Due to the variability in bone development, studying females separately is integral for establishing adequate nutrient levels of vit D and Ca in the REF diet for future bone research.

As shown in Figure 4, weanling mice (21 days of age) (n=118) were randomly allocated to 1 of the 4 diets (within each of the two trials) until necropsy at 4 months of age: Trial 1: 1000 IU vit D/kg and 5 g Ca/kg diet (AIN-93G diet, REF) (TD.94045); 100 IU vit D/kg and 3.5 (TD.160266), 3 (TD.160267), or 2.5 g Ca/kg diet (TD.160268) (3 experimental diets) and Trial 2: 1000 IU vit D/kg and 5 g Ca/kg diet (AIN-93G diet, REF) (TD.94045), 400 IU vit D/kg and 3.5 (TD.160269), 3 (TD.160270), or 2.5 g Ca/kg

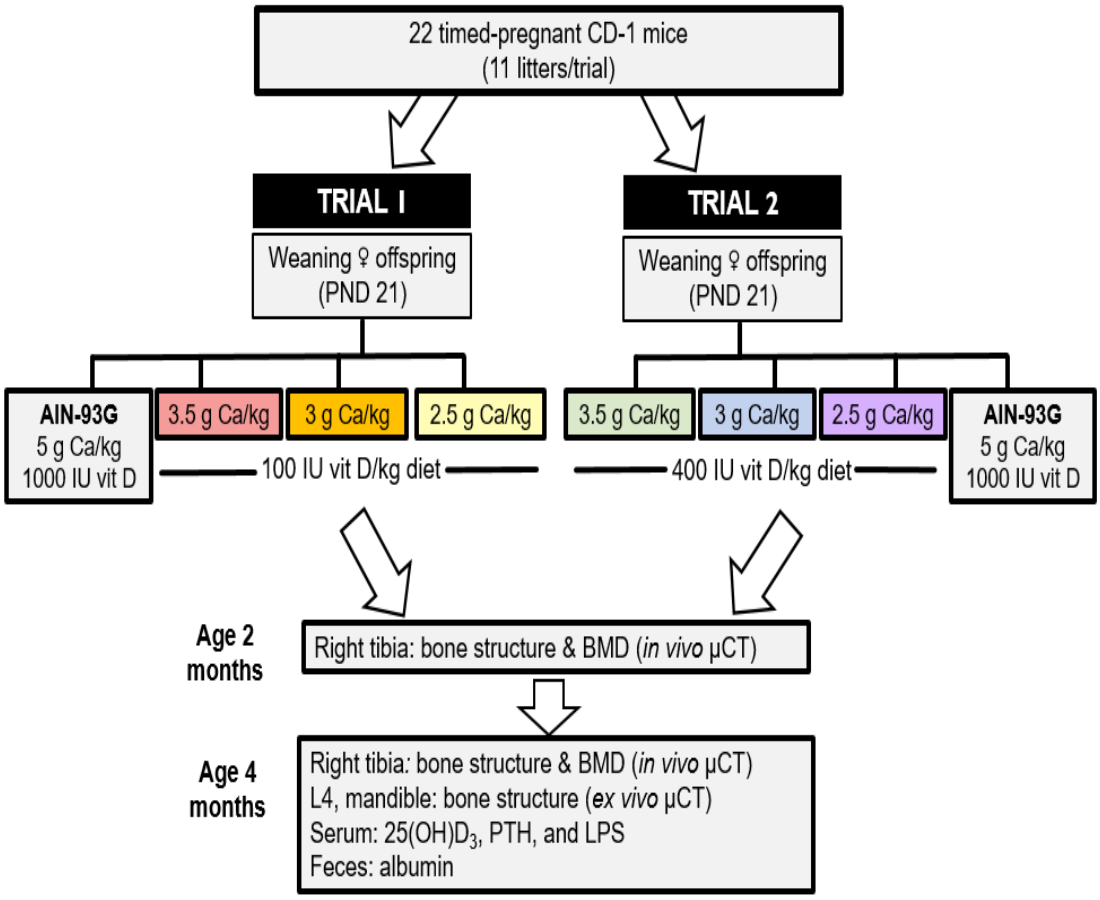


Figure 4. Experimental design.

diet (TD.160271) (3 experimental diets). All diets were prepared by Envigo, Madison, WI. The level of Ca and vit D in the diets were confirmed by a third party (Maxxam Analytics, Mississauga, ON, Canada) (Table A1).

Our approach of altering both vit D and Ca simultaneously has not been thoroughly used. We based our levels on previous literature in which vit D or Ca was modified independent of each other. Because our previous studies using 25 IU vit D/kg diet have not shown problems with bone development, but intestinal inflammation was present, we chose to study a slightly higher level of vit D (100 IU/kg) with the intention of not inducing intestinal inflammation (Jahani et al., 2014). 400 IU vit D/kg diet was chosen for the higher vit D level as previous studies have demonstrated no effect on bone development (Fleet et al., 2008). With respect to Ca levels, at reference vit D level (1000 IU/kg diet), lowering Ca to 3 g/kg diet does not compromise bone development, however, diet containing 2.5 g Ca/kg diet lowered BMD, compromised bone morphology and weakened bone strength (Hunt et al., 2008; Fleet et al., 2008). To eliminate bias, diets were colour-coded so I was unaware of which groups received a specific dietary intervention. Throughout the study, body weight was measured once weekly and food intake was measured 2 to 3 times per week using an electronic scale (Denver Instrument, MXX-5). The right tibia was scanned at 2 and 4 months of age using a high resolution μCT (SkyScan 1176, Bruker-microCT, Belgium). Freshly passed fecal pellets of female mice were collected at 2 and 4 months of age. The steps varied slightly based on the age of mouse due to size and animal behaviour differences. Generally, mice were placed in a large beaker enclosed by a paper towel punctured with holes, which provides the mouse

with a sense of safety. Once fresh stool was passed, it was placed in an Eppendorf tube using forceps and stored at -80◦C.

At 4 months of age, mice were anaesthetized in an induction chamber using isoflurane inhalant gas at a rate of 3-5% for 1 to 3 minutes (*SOP HEAL25 Use of isoflurane for anesthesia in rodents' surgeries using a vaporizer)*. The mouse was then placed in a supine position on the surgical bench and a nose cone was placed over the face of the mouse to continuously administer the isoflurane at a rate of 2.5-3.5% for the duration of the cardiac puncture procedure (*SOP TECH01 Intracardiac blood collection in mice and rats*). Blood obtained by cardiac puncture was placed in Eppendorf tubes for serum 25(OH)D_3 and PTH analyses and LPS-free Eppendorf tubes for serum LPS analysis. Blood was centrifuged at 1000 or 21130 relative centrifugal force for 15 minutes at room temperature, for serum 25(OH)D_3 and PTH or LPS collection, respectively. All serum samples were stored at -72◦C. Following exsanguination, euthanasia was completed by cervical dislocation, after which the lumbar vertebrae 4 (L4) and mandible were removed for *ex vivo* analysis.

4.2 Bone Measurements by µCT

4.2.1 *In vivo* bone structure and BMD of the tibia

µCT was used to assess bone structure and BMD of the tibia through morphometric and density measurements, respectively. Bone morphometric analysis of trabecular and cortical bone provides information for bone structure, with key parameters

identified in Table 3. X-ray projection images are taken incrementally from many angles and the rotation of the images are reconstructed to produce a precise 3D output.

The right tibia of the offspring was scanned at 2 and 4 months of age using a high resolution μCT (SkyScan 1176, Belgium) (Table 4). Mice were anesthetized in an induction chamber using isoflurane inhalant gas at a rate of 3-5% for 1-3 minutes (*SOP HEAL25 Use of isoflurane for anesthesia in rodents' surgeries using a vaporizer*). Ophthalmic gel was placed on the eyes of the mice to protect their cornea and prevent potential irritation from the vaporized isoflurane. Furthermore, their tail and left hind limb were secured to the scan bed, using masking tape, to prevent movement at the hips during the scan (Figure 5). The specific regions of interest are the metaphysis of the proximal tibia to analyse trabecular bone and the midshaft of the tibia for cortical bone analysis (Campbell & Sophocleous, 2014). A nose cone was used to provide a continuous administration of the isoflurane at a rate of 2.5-3.5%. For the duration of the scan, breathing rate and temperature of the scan chamber were closely monitored.

To make certain for a safe and comfortable recovery from the anesthesia, mice were held and gently rocked until consciousness was regained and then placed in an individual cage. The cage was kept warm by placement of a heating pad under a portion of the cage. Once normal behaviour resumed, the mouse was transferred back to their regular cage. Mice were monitored for 2 hours post-sedation to ensure a safe and full recovery (*SOP HEAL25 Use of isoflurane in rodent surgery using a vaporizer*).

Table 3. Key outcomes of bone structure for trabecular and cortical bone, based on 3D image data acquired using μCT (Bouxsein, et al., 2010; Badea, et al., 2008, Manual for CTan v.1.10).

Variable	Description	Units
Trabecular bone morphology		
Bone volume (BV)	Volume of the region segmented as bone	mm^3
Total volume (TV)	Total volume of the ROI (2D)	mm^3
Bone volume fraction (BV/TV)	Ratio of the bone volume to the total volume of the ROI	%
Trabecular thickness (Tb. Th)	Mean thickness of the trabeculae	mm
Trabecular separation (Tb. Sp)	Mean distance between the trabeculae	mm
Trabecular number (Tb. N)	Average number of trabeculae per unit length	1/mm
Degree of anisotropy (DA)	Determinant of mechanical strength. Measure of 3D symmetry or the presence/absence of preferential alignment of structures.	N/A
Connectivity density (Conn.Dn)	Degree of connectivity of trabeculae normalized by total volume (TV)	$1/mm^3$

Table 3 continued.

Cortical bone morphology		
Cortical bone area (Ct. Ar)	Cortical volume divided by the product of the number and thickness of slices	mm^2
Total cross-sectional area (Tt. Ar)	Total cross-sectional area inside the periosteal envelope	mm^2
Cortical area fraction (Ct. Ar/Tt. Ar)	The ratio of the cortical bone area to the total cross-sectional area	%
Mean total cross sectional ROI perimeter (T.Pm)	Mean of the cross sectional ROI perimeter for all sites of the VOI	mm
Mean polar moment of inertia (MMI-polar)	Basic strength index. Indicates resistance to rotation of a cross section about a chosen axis (2D)	mm^4
Mean eccentricity (Ecc)	2D analysis of the object shape (higher=elongated; lower=circular)	N/A
Cross sectional thickness (Ct.Th)	thickness excluding the vertical surfaces between pixels of adjacent cross sections (2D)	mm
Medullary area (MA)	The cross-sectional area of the medullary cavity	mm^2
Mean total cross sectional object perimeter (Obj.Pm)	The mean of the cross sectional object/bone perimeter for all slices in the selected volume of interest	mm
Endosteal perimeter (E.Pm)	Object perimeter subtract the mean total cross sectional ROI perimeter (2D)	mm

*Bold variables indicate critical outcomes that should be reported by all studies as per published guidelines (Bouxsein, et al., 2010)

Table 4. *In vivo* scanning parameters for right tibia of mice.

Parameter	**Value**
Resolution	High (9 μm)
Filter	Al 1mm
Voltage	40 kV
Amperage	300 uA
Exposure time	3350 ms
Rotation Step	0.8 degrees
Type of scan frame	180 degrees
Averaging	N/A

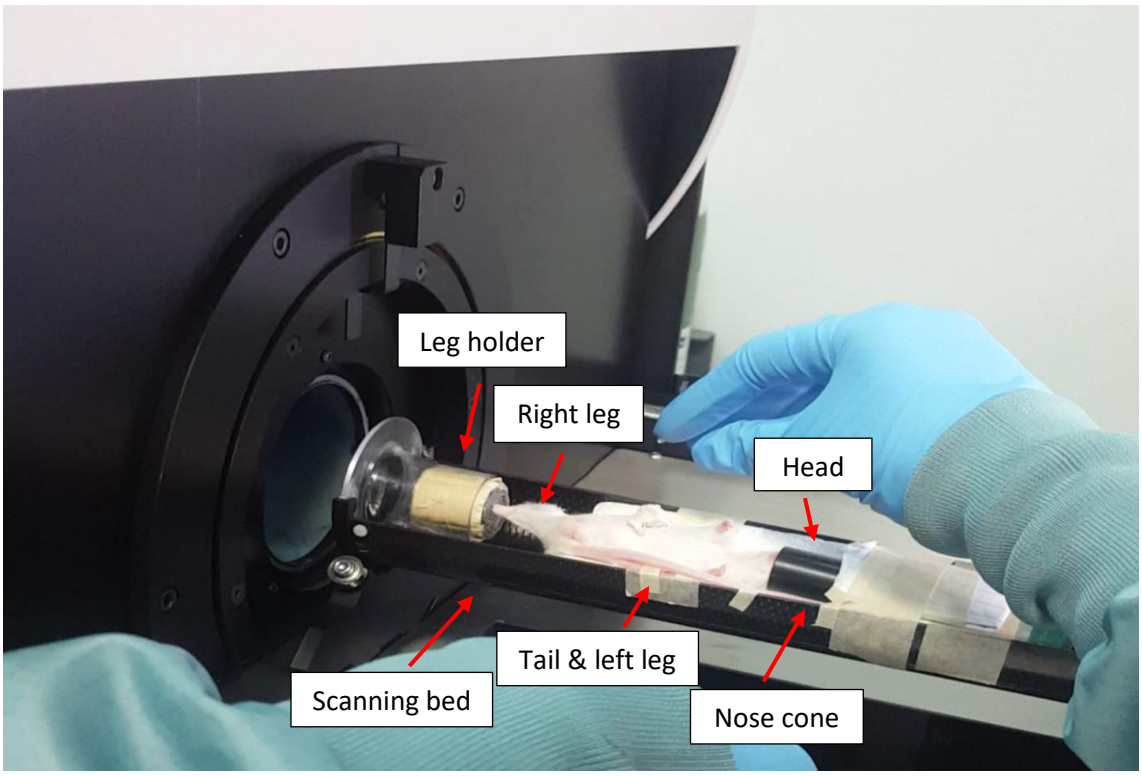


Figure 5. *In vivo* scanning set-up for μCT imaging of the right tibia of a female CD-1 mouse. The mouse is restrained with right leg extended in order to avoid movement during scanning of the right tibia.

Image reconstruction

Images were reconstructed with NRecon software, version v.1.9.6.10 (Bruker-microCT, Belgium). A Gaussian filter was applied to the images with the parameters described in Table 5. Parameters were determined based on a random sample of five male and five female mice, representing the spectrum of weights among both sexes.

Image analysis

The regions of interest (ROI) for trabecular and cortical bone were delineated at the proximal tibia and midpoint of the diaphysis (Figure 6). The reference point was set using the point at which the primary spongiosa disconnects and the formation of the growth plate occurs. An offset of 75 and height of 70 slices were used to analyze trabecular bone at the proximal tibia metaphysis. Analysis of cortical bone occurred at the tibia midpoint was calculated based on the

total length of the tibia. From the midpoint, 50 slices towards the proximal end and 50 slices towards the distal end (total height=100), represented the region of cortical analysis. Within the ROIs, trabecular and cortical bone were defined using a threshold of 105. Key outcomes, described in Table 3, were quantified using 2D and 3D analysis of the selected ROI (CT Analyzer v.1.14.4.1 + (64-114 bit), Bruker-microCT, Belgium).

4.2.2 *Ex vivo* bone structure of L4 and mandible

μCT has become the gold-standard for analysing *ex vivo* bone morphology and structure in mice (Harrison & Cooper, 2015; Clark & Badea, 2014; Bouxsein, et al., 2010). Female offspring *ex vivo* analyses occurred at the body of L4 and inter-radicular septum of the first molar. Prior to scanning, the samples were wrapped in parafilm to

Table 5. NRecon parameters for *in vivo* analysis of the right tibia of mice (SkyScan NV, NRecon User Manual, 2011).

Parameter	Description	Correction value
Smoothing	Removal of noise, blurring the image, at the expense of reducing contrast	4
Ring artifact reduction	Reduction of ring artifacts (blurring may occur with high values)	10
Beam hardening	Places a filter in the x-ray path to narrow the energy spectrum	35%
Defect pixel masking	Removal of "hard" rings	5%
Dynamic image range	Converting real numbers into integers	0.0000000-0.0939204

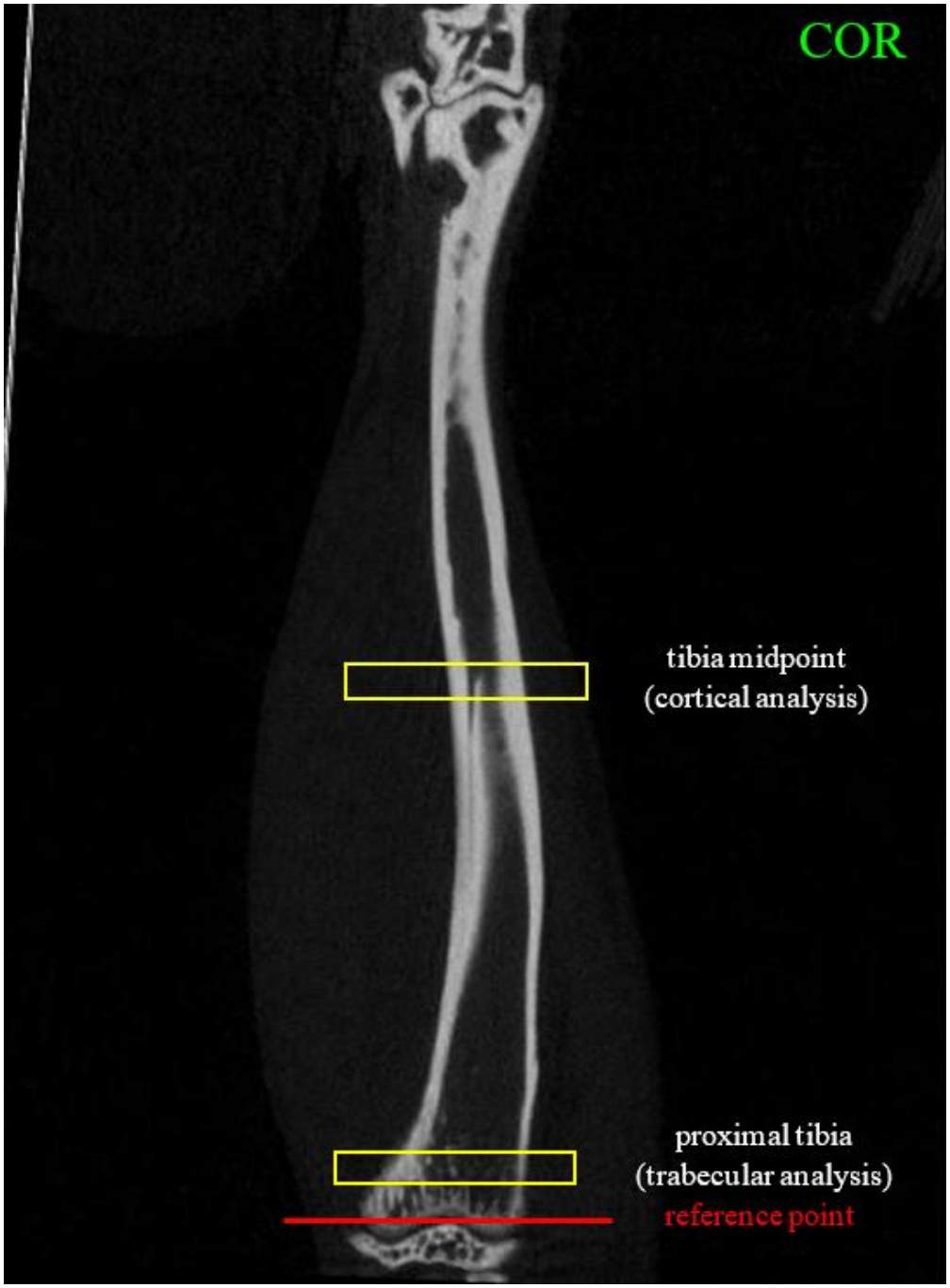


Figure 6. *In vivo* image analysis: coronal (COR) view of the right tibia of a female CD-1 mouse. The reference point (red) indicates the start of the growth plate. Yellow boxes represent the ROIs for cortical bone analysis at midpoint tibia (100 slices) and trabecular bone analysis at proximal tibia (70 slices).

prevent moisture loss. Sequentially, the samples were placed in a polystyrene foam tube and secured to the scan bed using tape (Figure 7). The output parameters for analysing bone quality are listed for the *ex vivo* μCT scans of L4 and mandible bones (Table 6). Due to the ROI drawn, cortical bone parameters of the L4 included cortical bone area and cross-sectional thickness.

Image reconstruction and reorientation

Ex vivo images was reconstructed with NRecon software, version v.1.9.6.10 (Bruker-microCT, Belgium). A Gaussian filter was applied to the images with the parameters described in Table 7. Due to the variable curvature of bones and position during scanning, all images were rotated using DataViewer v.1.5.1.3 (Bruker-mircoCT, Belgium) to allow similar regions of interest to be compared among the samples.

Image analysis

The procedures for image analyses of trabecular and cortical bone were the same as described for *in vivo* analysis (CT Analyzer v.1.14.4.1 + (64-114 bit), Bruker-microCT, Belgium). With regards to L4, the top and bottom slices of the body were identified and 80 slices from the top and bottom set the boundaries for the ROI (Figure 8A). The ROI of the mandible bones included 90 slices, which started from 10 slices inferior to the bifurcation roof of the first molar roots, defined by the slice number where the roots became visibly independent structures (Figure 8B).

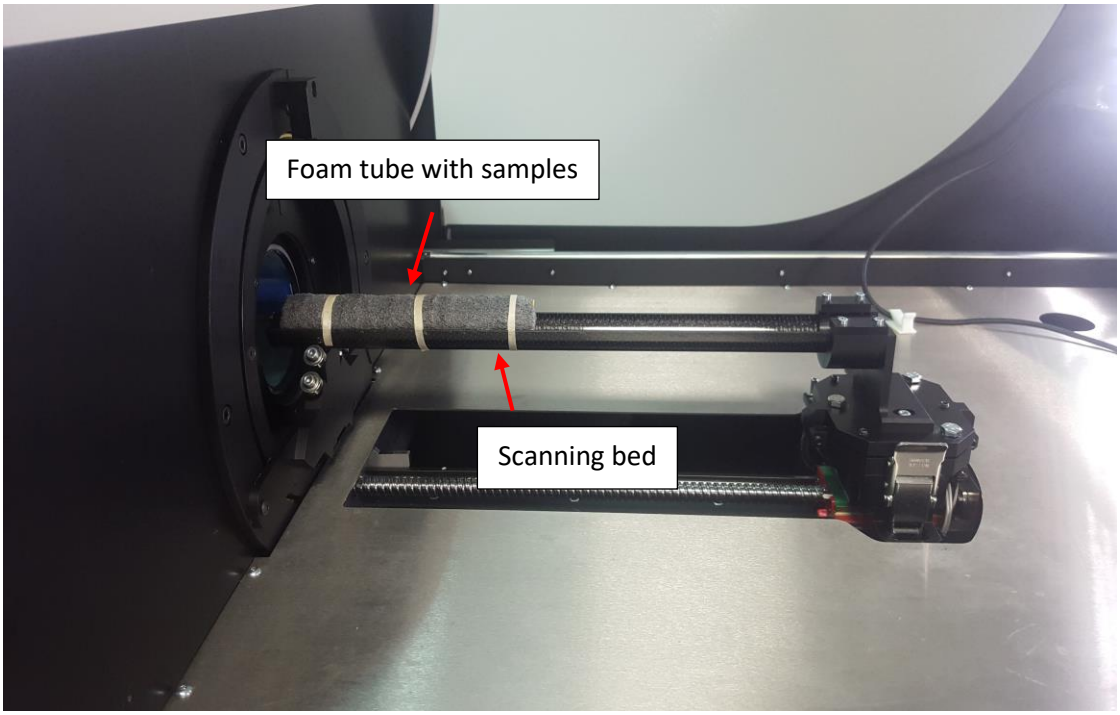


Figure 7. *Ex vivo* scanning setup for μCT imaging of L4 and mandible of female CD-1 female mice. Within the foam tube, L4 and mandible are oriented horizontally, with the anterior direction towards the μCT chamber.

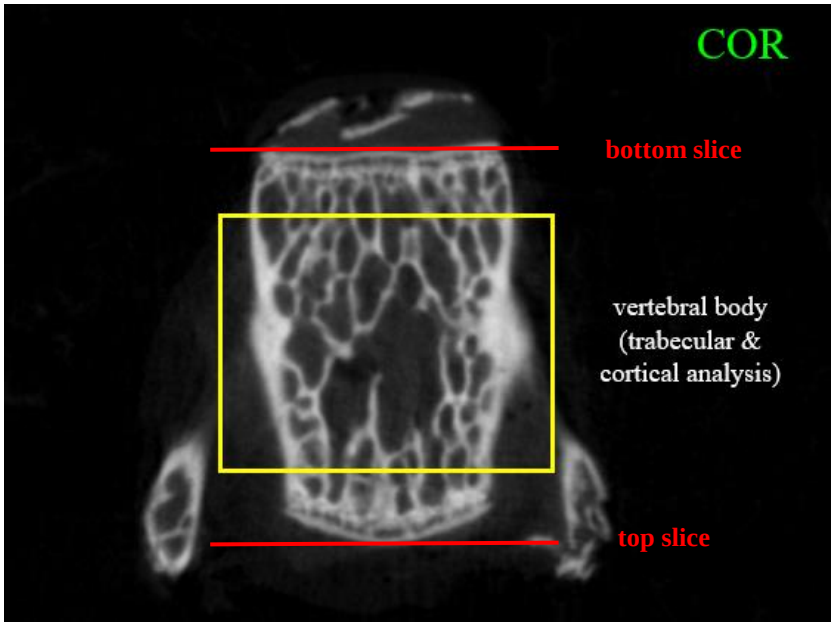
Table 6. *Ex vivo* scanning parameters for mouse tibia, L4 and mandible.

Parameter	**Value**
Resolution	High (9 µm)
Filter	Al 0.25mm
Voltage	45 kV
Amperage	545 uA
Exposure time	850 ms
Rotation Step	0.2 degrees
Type of scan frame	180 degrees
Averaging	N/A

Table 7. NRecon parameters for *ex vivo* analysis of L4 and mandible of mice.

Parameter	Description	L4 Value	Mandible Value
Smoothing	Removal of noise, blurring the image, at the expense of reducing contrast	3	5
Ring artifact reduction	Reduction of ring artifacts (blurring may occur with high values)	8	10
Beam hardening	Places a filter in the x-ray path to narrow the energy spectrum	35%	35%
Defect pixel masking	Removal of "hard" rings	5%	5%
Dynamic image range	Converting real numbers into integers	0.0000000-0.108731	0.0000000-0.204095

A)



B)

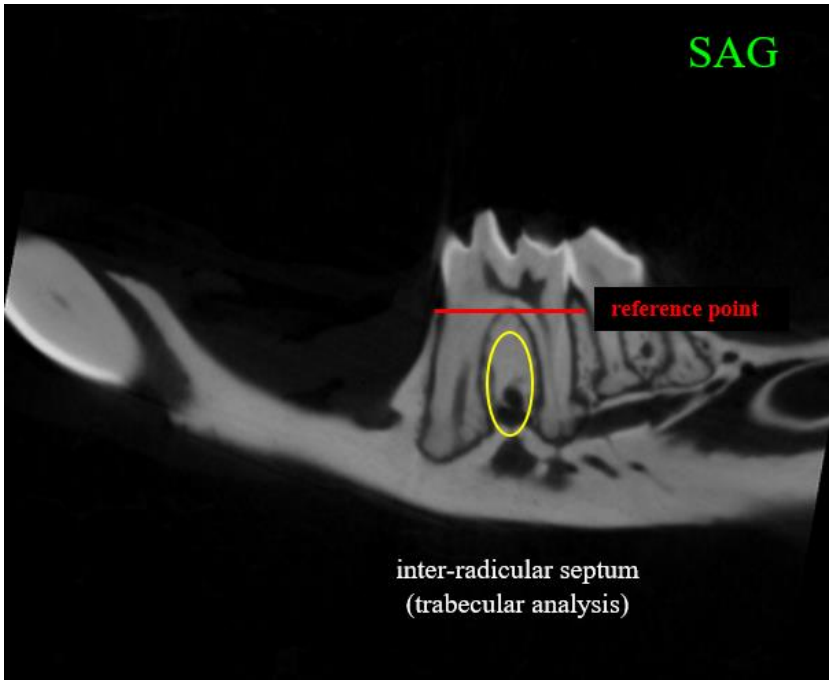


Figure 8. *Ex vivo* image analysis: A) coronal (COR) view of L4 of a female CD-1 mouse; yellow box represents the ROI for trabecular and cortical bone. B) sagittal (SAG) view of the mandible; yellow circle represents the ROI for trabecular bone.

4.3 Serum 25(OH)D_3 and PTH

Serum 25(OH)D_3 concentrations were determined via liquid chromatography-mass spectrometry at the Analytical Facility for Bioactive Molecules of the Center for the Study of Complex Childhood Diseases, The Hospital for Sick Children (Toronto, ON). Serum PTH concentration was analyzed using Mouse PTH 1-84 ELISA, Immunotopics (REF 60-2305) and read by BIO-TEK Synergy HT-1 at a wavelength of 450nm on the computer software Gen5 v2.09.2.

4.4 Markers of intestinal integrity

Serum LPS concentration was determined using Mouse LPS ELISA, Cusabio (REF CSB-E13066m) following manufacturer's instructions and read at a wavelength of 450nm. Prior to fecal albumin analysis, the fecal sample was diluted with 1mL of 1X dilution buffer. Fecal albumin concentration was determined using Mouse Albumin ELISA Kit, Bethyl (E99-134) following the manufacturer's instructions and read at a wavelength of 450nm (Villa, et al., 2016).

4.5 Statistical Analyses

Statistical analyses were performed using IBM SPSS Statistics 24 and results are presented as mean ± standard error mean (SEM). Data was cleaned through the exclusion of outliers 3.0 or more inter-quartile range (IQR). Sequentially, assumptions are checked respective to the statistical test performed. Independence of observations was accounted for in the study design. Equal cell sizes were examined within the descriptive statistics. Normal distribution was analyzed at both independent variables, time point and

treatment, by examining the Shapiro-Wilk test of normality and skewness, kurtosis, and mean, median and mode values. Finally, homogeneity of variance or sphericity was checked via a Levene's test or Mauchly's test, respectively.

At age 2 and 4 months, the effects of the various Ca levels, within each vit D trial, on bone development, was determined using a 2 (age) by 4 (diet) mixed analysis of variance (ANOVA). A repeated measures ANOVA was used for body weight and food intake data to compare among intervention groups. A one-way ANOVA was used to examine *ex vivo* bone outcomes of L4 and mandible, as well as serum $25(OH)D_3$, serum PTH, fecal albumin and serum LPS concentrations. Differences were considered significant if $p < 0.05$ and a Bonferroni *post hoc* was used to identify the specific differences.

CHAPTER 5: RESULTS

5.1 Trial 1: Intervention diets containing 100 IU vit D/kg and 3.5, 3 or 2.5 g Ca/kg diet had no effect on bone development or intestinal integrity at 2 and 4 months of age in female CD-1 mice

Body weight

There was no effect on body weight due to the diets ($p > 0.05$). There was a main effect of age ($p < 0.001$) as body weight increased from weaning to 4 months of age. No significant diet x age interaction ($p > 0.05$) was observed (Figure 9). Furthermore, there was no difference in daily food intake among groups ($p > 0.05$) (Figure A11).

In vivo tibia structure

There was no main effect of diet ($p > 0.05$) (Figures 10 and A1-A3). Developmental changes were observed for all trabecular and cortical bone structure outcomes measured. There was an effect of age ($p < 0.05$) at the proximal tibia and midpoint of the tibia, respectively, and an increase in tibia length with age ($p < 0.05$) was also observed. Furthermore, there was no significant diet x age interaction ($p > 0.05$). BMD of the proximal tibia did not differ among dietary intervention groups (Table 8 and Figure A2).

Ex vivo structure of L4 and mandible

There were no significant differences in trabecular and cortical bone outcomes of L4 among the diet groups ($p > 0.05$) (Figures 9, A1 and A4). There were no significant

differences in trabecular bone structure of the mandible at 4 months of age ($p > 0.05$) (Tables 9 and A5).

Biochemical Analyses

Serum 25(OH)D_3 concentrations were significantly lower in the 100 IU vit D/kg dietary groups compared to the REF group ($p < 0.05$). Serum PTH concentrations did not differ among diet groups ($p > 0.05$) (Table 10).

Serum LPS and fecal albumin

No data was collected for serum LPS due to the limited quantity of serum available. There were significant differences in fecal albumin among diet groups ($p > 0.05$) (Figure 11).

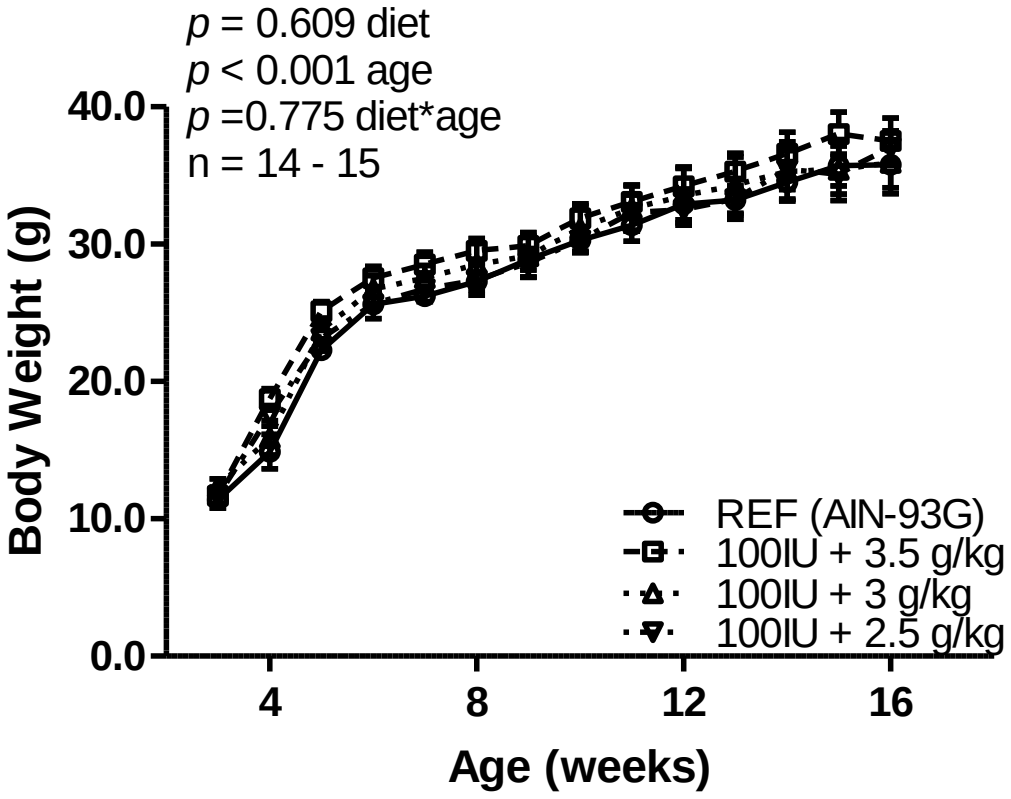


Figure 9. Body weight of female CD-1 mice in Trial 1 (100 IU vit D/kg diet). Data was reported as mean ± SEM and no significance difference ($p > 0.05$) is reported among dietary interventions.

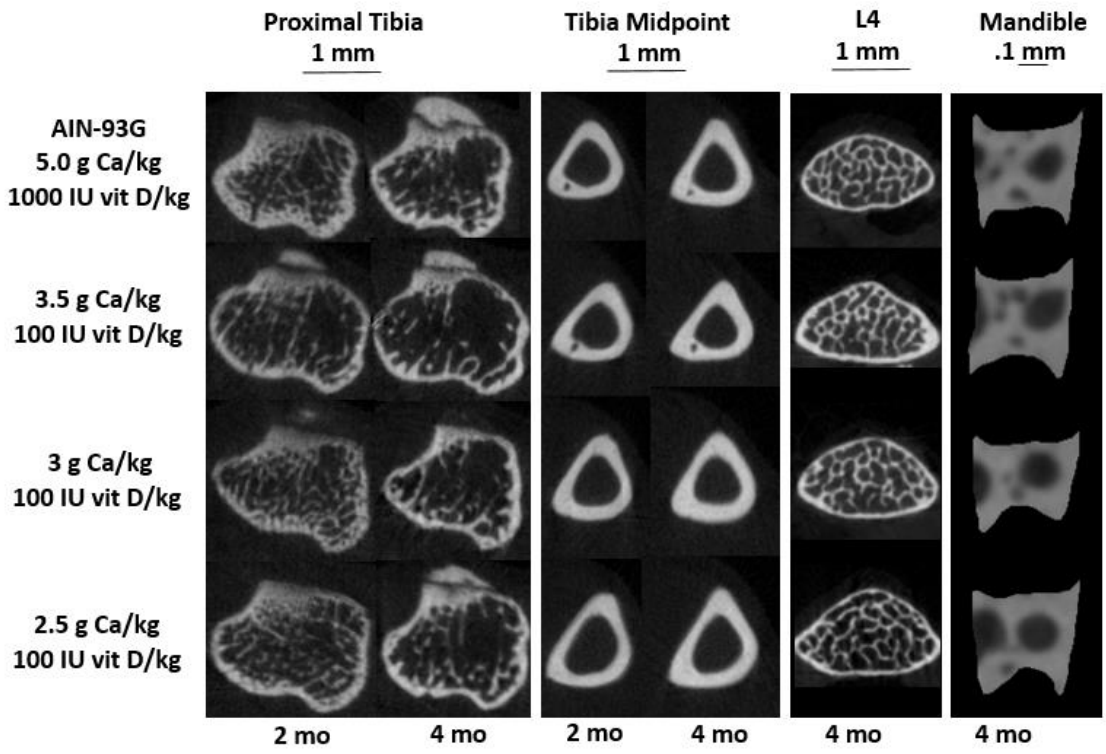


Figure 10. Trial 1 (100 IU vit D/kg diet): representative grayscale transaxial images of the right tibia, L4 and mandible of female CD-1 mice. No visual differences among dietary groups were observed for bone structure of the tibia, L4 and mandible.

Table 8. Trial 1 (100 IU vit D/kg diet): *in vivo* analysis of trabecular and cortical bone morphology, of the proximal and midpoint tibia, respectively, in female CD-1 mice.

		Dietary Intervention				Mixed ANOVA, P-values		
			100 IU vit D/kg					
	Age (months)	REF n = 15	3.5 g Ca/kg n = 15	3 g Ca/kg n = 14	2.5 g Ca/kg n = 12	Diet	Age	Diet x Age
Tibia Length, *mm*	2	17.40 ± 0.12	17.57 ± 0.11	17.62 ± 0.10	17.41 ± 0.13	NS	0.000	NS
	4	18.77 ± 0.12	18.81 ± 0.10	18.90 ± 0.09	18.74 ± 0.13[a]			
Tibia (Trabecular)								
BMD, *g/cm²*	2	0.208 ± 0.008	0.200 ± 0.007[a]	0.206 ± 0.007	0.219 ± 0.010	NS	0.000	NS
	4	0.177 ± 0.009[a]	0.163 ± 0.005	0.174 ± 0.012[a]	0.182 ± 0.010			
BV/TV, *%*	2	13.87 ± 1.35	13.12 ± 0.99[a]	13.88 ± 1.03	15.76 ± 1.75	NS	0.000	NS
	4	10.25 ± 1.35[a]	9.77 ± 1.07	11.19 ± 1.85[a]	11.52 ± 1.47			
Tb.Th., *mm*	2	0.073 ± 0.002	0.073 ± 0.002[a]	0.071 ± 0.001	0.074 ± 0.002	NS	0.000	NS
	4	0.089 ± 0.002	0.088 ± 0.002	0.090 ± 0.003	0.089 ± 0.002			
Tb.Sp., *mm*	2	0.317 ± 0.017	0.323 ± 0.016	0.304 ± 0.014	0.292 ± 0.014	NS	0.000	NS
	4[b]	0.431 ± 0.009	0.442 ± 0.015	0.441 ± 0.018	0.431 ± 0.007			
Tb.N, *mm⁻¹*	2	1.879 ± 0.154	1.789 ± 0.108[a]	1.932 ± 0.117	2.081 ± 0.172	NS	0.000	NS
	4	1.122 ± 0.125	1.096 ± 0.106[a]	1.205 ± 0.148[a]	1.259 ± 0.137			
DA, *no units*	2	2.162 ± 0.091	2.070 ± 0.056	2.255 ± 0.075	2.013 ± 0.063	NS	0.000	NS
	4	1.808 ± 0.044	1.987 ± 0.071	2.053 ± 0.099	1.891 ± 0.120[a]			
Conn.Dn, *mm⁻³*	2	117.10 ± 9.43	95.92 ± 7.58	101.18 ± 6.15	118.88 ± 9.34	NS	0.044	NS
	4[b]	93.17 ± 8.16	93.79 ± 9.06	110.16 ± 18.59	88.88 ± 13.21			

Table 8 continued.

Tibia (Cortical)								
Ct.Ar/Tt.Ar, *%*	2	59.48 ± 0.82	58.91 ± 0.58	58.75 ± 0.76	58.82 ± 0.74	NS	0.000	NS
	4	67.97 ± 0.87	66.29 ± 0.77	67.25 ± 0.77	66.63 ± 0.80			
Ct.Th, *mm*	2	0.204 ± 0.003	0.209 ± 0.003	0.207 ± 0.004	0.204 ± 0.003	NS	0.000	NS
	4	0.247 ± 0.004	0.245 ± 0.002	0.249 ± 0.004	0.245 ± 0.004			
Ps.Pm, *mm*	2	7.387 ± 0.150	7.645 ± 0.110	7.595 ± 0.127	7.590 ± 0.171	NS	0.000	NS
	4	7.090 ± 0.151	7.502 ± 0.144	7.336 ± 0.116	7.342 ± 0.147			
Ec.Pm, *mm*	2	2.966 ± 0.071	3.048 ± 0.053	3.015 ± 0.062	3.039 ± 0.072	NS	0.000	NS
	4	2.596 ± 0.070	2.775 ± 0.067	2.678 ± 0.050	2.715 ± 0.064[a]			
Ma.Ar, *mm^2*	2	0.518 ± 0.025	0.558 ± 0.017	0.553 ± 0.021	0.545 ± 0.026	NS	0.000	NS
	4[b]	0.417 ± 0.023	0.472 ± 0.022	0.447 ± 0.020	0.454 ± 0.022			
Ecc, *no units*	2	0.666 ± 0.012	0.666 ± 0.010	0.671 ± 0.013	0.692 ± 0.013	NS	0.000	NS
	4	0.655 ± 0.011	0.655 ± 0.015[a]	0.644 ± 0.018	0.655 ± 0.017			

[1] Values are mean ± standard error of the mean (SEM), NS = not significant

[a] Data were not normally distributed and violated the Shapiro-Wilks test, but had homogeneous variance

[b] Data violated Levene's homogeneity of variance test; but were normally distributed

Table 9. Trial 1 (100 IU vit D/kg diet): *ex vivo* analysis of trabecular and cortical bone morphology of L4 and bone volume fraction of the mandible in female CD-1 mice.

	Dietary Intervention				
		100 IU vit D/kg			
	REF n = 10	**3.5 g Ca/kg n = 10**	**3 g Ca/kg n = 9**	**2.5 g Ca/kg n = 10**	**One way - ANOVA P-value**
L4 (Trabecular)					
BV/TV, *%*	25.51 ± 1.754	22.88 ± 1.064	25.39 ± 2.274	23.31 ± 1.367	NS
Tb.Th., *mm*	0.08 ± 0.001	0.08 ± 0.002[a]	0.08 ± 0.002	0.08 ± 0.002[a]	NS
Tb.Sp., *mm*	0.30 ± 0.020	0.31 ± 0.014	0.29 ± 0.020	0.30 ± 0.011	NS
Tb.N, mm^{-1}	3.23 ± 0.190	2.97 ± 0.102	3.23 ± 0.199	2.99 ± 0.116	NS
DA, *no units*	1.81 ± 0.069	1.77 ± 0.033	1.77 ± 0.030	1.70 ± 0.053	NS
Conn.Dn, mm^{-3}	140.04 ± 12.011	127.31 ± 9.544	101.18 ± 6.145	118.88 ± 9.341	NS
L4 (Cortical)					
Ct.Ar, (mm^2)	0.46 ± 0.009	0.48 ± 0.021	0.47 ± 0.024	0.48 ± 0.013	NS
Ct.Th, *mm*	0.10 ± 0.002	0.10 ± 0.003	0.10 ± 0.004	0.10 ± 0.002	NS
Mandible					
BV/TV, *%*	90.91 ± 2.173	93.28 ± 1.019	89.79 ± 2.354	93.10 ± 1.768	NS

[1] Values are mean ± standard error of the mean (SEM), NS = not significant

[a] Data were not normally distributed and violated the Shapiro-Wilks test, but had homogeneous variance

Table 10. Serum 25(OH)D_3 and PTH concentrations for Trial 1 and Trial 2. Mean ± SEM and no significance differences ($p < 0.05$) is reported between dietary interventions.

	Dietary Intervention				One-way ANOVA
	AIN-93G	**3.5 g Ca/kg**	**3 g Ca/kg**	**2.5 g Ca/kg**	**P-value**
Trial 1: 100 IU vit D/kg					
25(OH)D_3, ng/mL (n=3)	15.49 ± 4.32	5.79 ± 0.83*	5.02 ± 0.31*	5.66 ± 1.05*	0.0322
PTH, pg/mL (n=6-11)	185.09 ± 35.88	224.37 ± 23.40	233.34 ± 36.15	234.97 ± 76.41	NS
Trial 2: 400 IU vit D/kg					
25(OH)D_3, ng/mL (n=3)	13.40 ± 1.97	16.97 ± 0.69	12.50 ± 1.70	14.53 ± 2.43	NS
PTH, pg/mL (n=6-8)	296.39 ± 39.03	299.87 ± 75.31	198.28 ± 52.38	116.73 ± 26.00*	0.0483

[1] Values are mean ± standard error of the mean (SEM), NS = not significant

* Significant difference compared to AIN-93G group ($p < 0.05$)

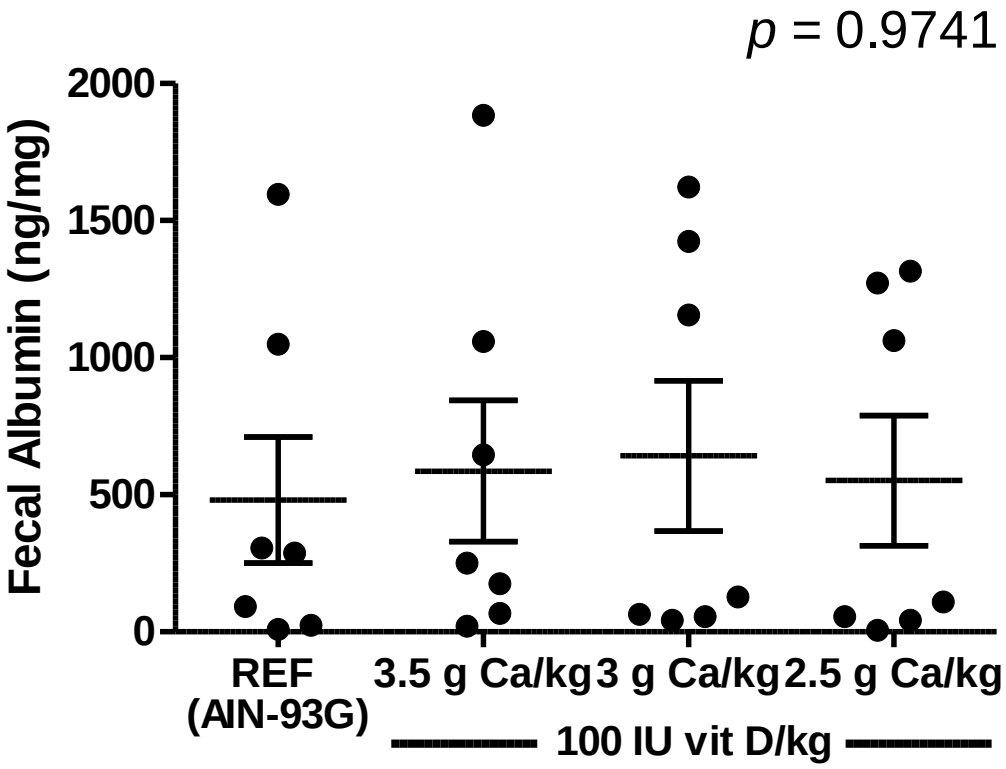


Figure 11. Trial 1 (100 IU vit D/kg diet): measure of intestinal integrity; fecal albumin concentrations. No significant difference among dietary groups.

5.2 Trial 2: Intervention diets containing 400 IU vit D/kg and 3.5, 3 or 2.5 g Ca/kg diet had a modest effect on bone development at 2 months of age, while intestinal integrity was unaltered in female CD-1 mice

Body weight

Similar to Trial 1, diet had no significant effect on body weight ($p > 0.05$) while there was an effect of age due to growth ($p < 0.001$). No diet x age interaction was present ($p > 0.05$) (Figure 12). Furthermore, there was no difference in daily food intake among groups ($p > 0.05$) (Figure A12).

In vivo tibia structure

There were no main differences in trabecular and cortical bone structure among diet groups (Figures 13 and A6-A8). Developmental effects occurred in both trabecular and cortical bone outcomes ($p < 0.05$), with few significant diet x age interactions ($p < 0.05$). At the midpoint of the tibia, there was an effect of diet at 2 months of age in which the REF group had greater Ct.Ar/Tt.Ar than the 400 IU vit D/kg, 2.5 g Ca/kg diet group ($p < 0.05$) (Figure A8). At 4 months of age, in the REF and 400 IU vit D, 3.5 g Ca/kg diet groups, BV/TV and BMD were greater at L4 compared to 2 months ($p < 0.05$) (Figure A9). Furthermore, an increase in tibia length with age ($p < 0.05$) was also observed. There was a significant diet x age interaction ($p < 0.05$) in which BMD was greater at 4 months, compared to 2 months, in the REF and 2.5 g Ca/kg diet groups (Table 11 and Figure A7).

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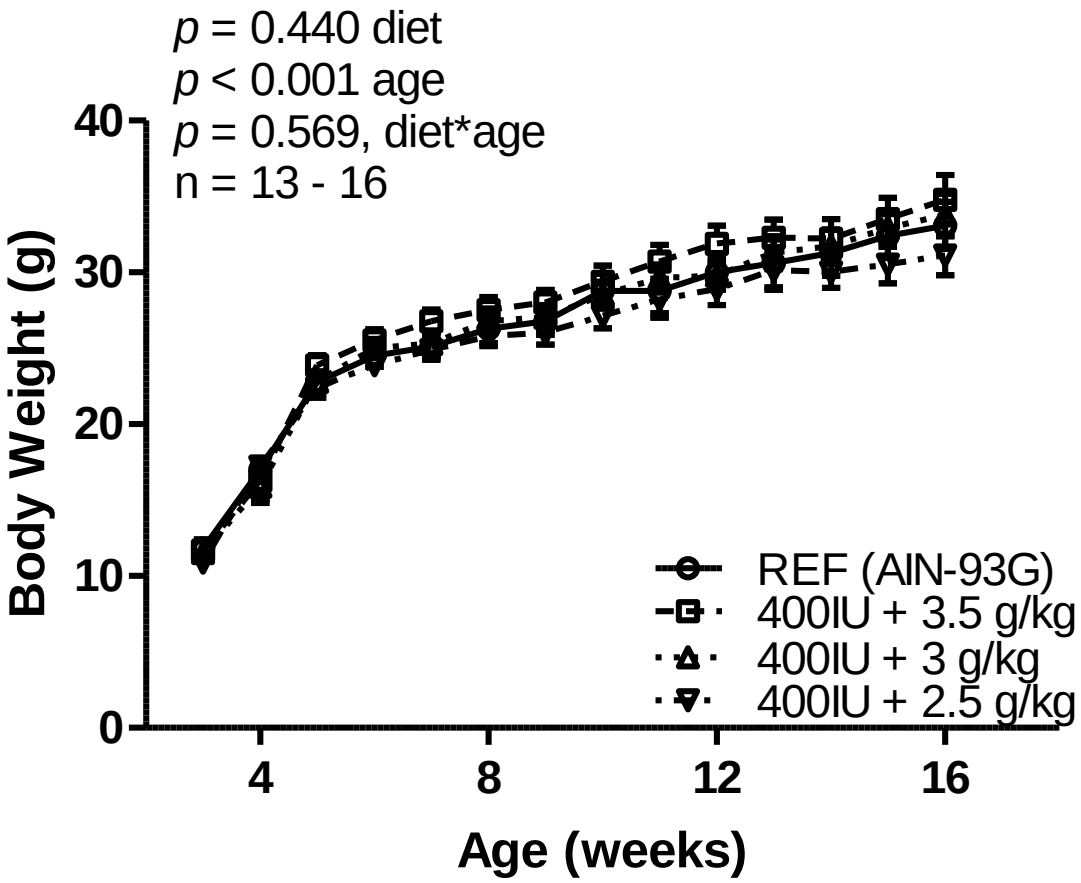


Figure 12. Bodyweight of female CD-1 mice in Trial 2 (400 IU vit D/kg diet). Data are reported as mean ± SEM and no significance difference ($p > 0.05$) is reported among dietary interventions.

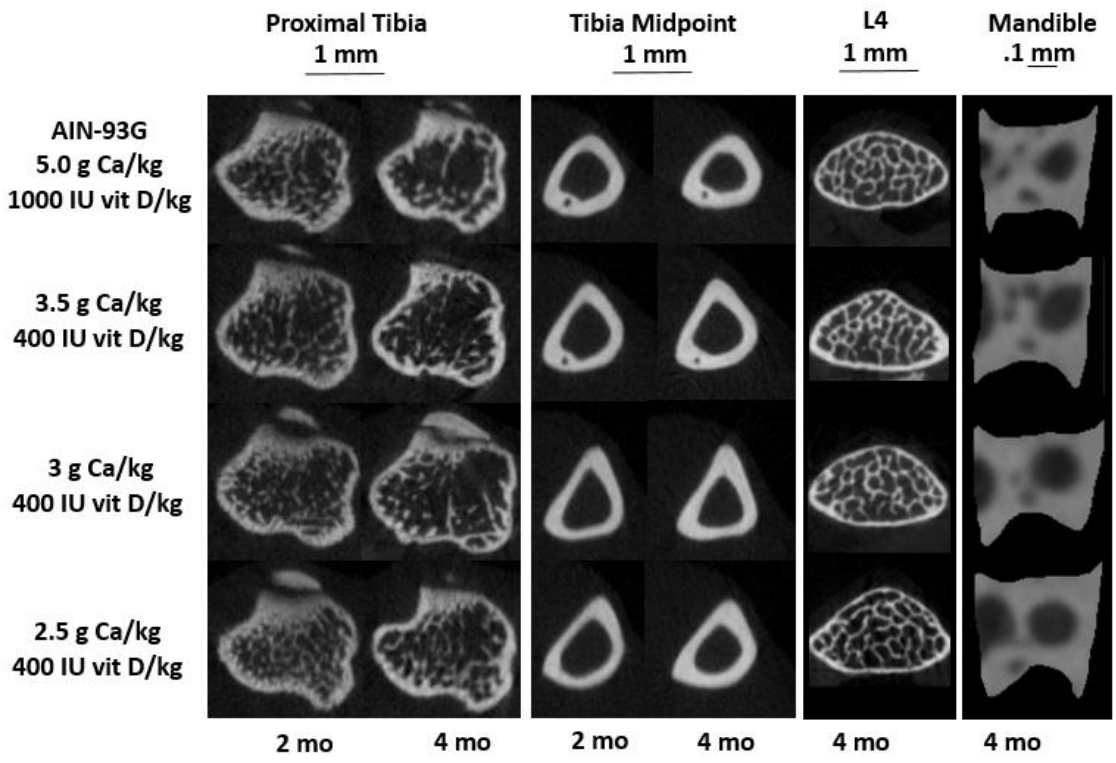
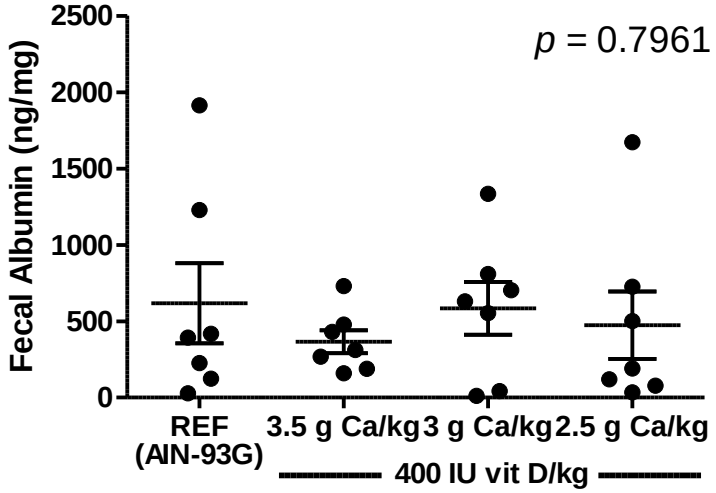
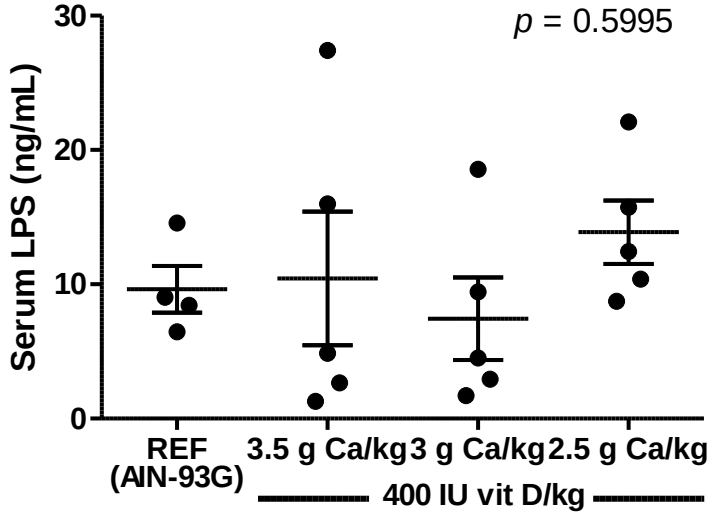


Figure 13. Trial 2 (400 IU vit D/kg diet): representative grayscale transaxial images of the right tibia, L4 and mandible of female CD-1 mice. No visual differences among dietary groups were observed for bone structure of the tibia, L4 and mandible.



A)



B)

Figure 14. Trial 2 (400 IU vit D/kg diet): measures of intestinal integrity; A) fecal albumin concentrations and B) serum LPS concentrations. No significant difference among dietary groups.

Table 11. Trial 2 (400 IU vit D/kg diet): *in vivo* analysis of trabecular and cortical bone morphology, of the proximal and midpoint tibia, respectively, in female CD-1 mice.

		Dietary Intervention				Mixed ANOVA, P-values		
			400 IU vit D/kg					
	Age (months)	REF n = 15	3.5 g Ca/kg n = 13	3 g Ca/kg n = 13	2.5 g Ca/kg n = 14	Diet	Age	Diet x Age
Tibia Length, *mm*	2	17.21 ± 0.13	17.44 ± 0.10	17.36 ± 0.14	17.24 ± 0.11	NS	0.000	0.020
	4	18.36 ± 0.09	18.75 ± 0.11	18.50 ± 0.13	18.71 ± 0.14			
Tibia (Trabecular)								
BMD, *g/cm^2*	2	0.220 ± 0.011	0.227 ± 0.008[a]	0.202 ± 0.008	0.218 ± 0.010	NS	0.000	0.017
	4[b]	0.188 ± 0.012	0.199 ± 0.005	0.178 ± 0.008	0.209 ± 0.009			
BV/TV, *%*	2[b]	16.53 ± 1.71	14.05 ± 0.93	13.04 ± 1.30	15.20 ± 1.73	NS	0.019	0.000
	4[b]	11.71 ± 1.58	13.47 ± 0.56	10.78 ± 1.02	15.19 ± 1.35			
Tb.Th., *mm*	2	0.079 ± 0.001	0.073 ± 0.002	0.073 ± 0.001	0.075 ± 0.001	NS	0.000	NS
	4	0.094 ± 0.003	0.092 ± 0.003[a]	0.090 ± 0.003	0.092 ± 0.001			
Tb.Sp., *mm*	2	0.302 ± 0.022	0.303 ± 0.025	0.320 ± 0.020	0.298 ± 0.021	NS	0.000	NS
	4	0.411 ± 0.023	0.403 ± 0.017[a]	0.430 ± 0.014	0.383 ± 0.015			
Tb.N, *mm^{-1}*	2	2.086 ± 0.190	1.917 ± 0.107	1.793 ± 0.159	1.914 ± 0.167	NS	0.000	0.004
	4[b]	1.247 ± 0.153	1.460 ± 0.048	1.190 ± 0.106	1.618 ± 0.130			
DA, *no units*	2	2.133 ± 0.081	2.055 ± 0.065	2.056 ± 0.074[a]	2.043 ± 0.076	NS	NS	NS
	4	2.261 ± 0.134	1.839 ± 0.071	2.180 ± 0.108	2.040 ± 0.090			
Conn.Dn, *mm^{-3}*	2	112.85 ± 11.49	104.43 ± 8.04	111.33 ± 12.27	126.91 ± 10.03	NS	0.000	NS
	4[b]	64.62 ± 7.46	67.48 ± 4.80	61.87 ± 7.74	73.48 ± 5.68			

Table 11 continued.

Tibia (Cortical)								
Ct.Ar/Tt.Ar, *%*	2	61.53 ± 0.67*	60.33 ± 0.82	59.84 ± 0.97	57.28 ± 0.93*	0.046	0.000	0.046
	4	68.85 ± 0.57	67.43 ± 1.05	66.44 ± 0.85	66.43 ± 1.00			
Ct.Th, *mm*	2	0.214 ± 0.003	0.208 ± 0.003	0.204 ± 0.003	0.196 ± 0.004	NS	0.000	NS
	4[b]	0.249 ± 0.003	0.248 ± 0.004	0.240 ± 0.002	0.244 ± 0.006			
Ps.Pm, *mm*	2	7.368 ± 0.088	7.571 ± 0.145	7.444 ± 0.141	7.633 ± 0.123	NS	0.000	NS
	4	7.237 ± 0.089	7.346 ± 0.164	7.307 ± 0.149	7.347 ± 0.095			
Ec.Pm, *mm*	2	2.892 ± 0.050	3.065 ± 0.076	2.981 ± 0.075	3.101 ± 0.071	NS	0.000	NS
	4	2.611 ± 0.040[a]	2.723 ± 0.090	2.632 ± 0.048	2.710 ± 0.062			
Ma.Ar, *mm^2*	2	0.494 ± 0.014	0.521 ± 0.024	0.513 ± 0.025	0.559 ± 0.023	NS	0.000	NS
	4	0.413 ± 0.011	0.445 ± 0.027	0.428 ± 0.014	0.454 ± 0.017			
Ecc, *no units*	2	0.704 ± 0.010	0.682 ± 0.003[a]	0.703 ± 0.010	0.699 ± 0.015	NS	0.000	NS
	4	0.704 ± 0.014	0.648 ± 0.013[a]	0.683 ± 0.013	0.678 ± 0.016			

[1] Values are mean ± standard error of the mean (SEM), NS = not significant

[a] Data were not normally distributed and violated the Shapiro-Wilks test, but had homogeneous variance

[b] Data violated Levene's homogeneity of variance test; but were normally distributed

* Significant difference between dietary groups ($p < 0.05$)

Ex vivo structure of L4 and mandible

For both the L4 and mandible (Figures 13, A5, A9 and A10), there were no significant differences in trabecular or cortical structure among the diet groups ($p > 0.05$), with the exception of a lower L4 BV/TV ($p < 0.05$) in the 400 IU vit D, 3 g Ca/kg diet group compared to REF (Figures 13 and A9, Table 12).

Biochemical Analyses

There were no significant differences for serum 25(OH)D_3 concentrations among the dietary interventions. However, Serum PTH was significantly greater in the REF group compared to the 2.5 g Ca/kg diet group ($p < 0.05$) (Table 10).

Serum LPS and fecal albumin

Serum LPS and fecal albumin concentrations did not differ among diet groups ($p > 0.05$) (Figure 14).

Table 12. Trial 2 (400 IU vit D/kg diet): *ex vivo* analysis of trabecular and cortical bone morphology of L4 and bone volume fraction of the mandible in female CD-1 mice.

	Dietary Intervention			One way - ANOVA P-value
		400 IU vit D/kg		
	REF n = 9	3.5 g Ca/kg n = 10	3 g Ca/kg n = 10	2.5 g Ca/kg n = 10	
L4 (Trabecular)					
BV/TV, *%*	29.27 ± 1.692*	26.74 ± 1.623	22.16 ± 1.291*	24.06 ± 1.675	0.043
Tb.Th., *mm*	0.08 ± 0.002	0.08 ± 0.002[a]	0.07 ± 0.001	0.08 ± 0.002	NS
Tb.Sp., *mm*	0.26 ± 0.011	0.26 ± 0.017	0.30 ± 0.013	0.29 ± 0.014	NS
Tb.N, mm^{-1}	3.65 ± 0.158	3.47 ± 0.235	2.98 ± 0.158	3.17 ± 0.168	NS
DA, *no units*	1.75 ± 0.035	1.85 ± 0.068	1.86 ± 0.084	1.72 ± 0.067	NS
Conn.Dn, mm^{-3}	150.76 ± 12.516	132.88 ± 19.221	131.63 ± 14.640	136.01 ± 11.679	NS
L4 (Cortical)					
Ct.Ar, (mm^2)	0.48 ± 0.015	0.45 ± 0.012	0.46 ± 0.016	0.48 ± 0.022	NS
Ct.Th, *mm*	0.10 ± 0.002	0.09 ± 0.002	0.09 ± 0.001	0.10 ± 0.003	NS
Mandible					
BV/TV, *%*	90.84 ± 1.832	88.82 ± 2.333	90.14 ± 2.345	87.11 ± 2.211	NS

[1] Values are mean ± standard error of the mean (SEM), NS = not significant

[a] Data were not normally distributed and violated the Shapiro-Wilks test, but had homogeneous variance

* Significant difference between dietary groups ($p < 0.05$)

CHAPTER 6: DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Discussion

This study demonstrated that lowering both vit D and Ca to 100 or 400 IU vit D/kg, in combination with 3.5, 3 or 2.5 g Ca/kg, had little to no effect on bone development in terms of bone structure and BMD measured *in vivo* at age 2 and 4 months in female CD-1 mice. As expected, independent of dietary intervention female mice grew over time, which was supported by the increase in body weight and tibia length. Moreover, there were few differences in skeletal sites measured *ex vivo* at age 4 months. When vit D was lowered to 100 IU/kg, there were no differences at any of the sites of interest, including lumbar spine and mandible. When vit D was lowered to 400 IU/kg diet, the combination with lowered Ca had modest effects on trabecular and cortical bone structure at the proximal and midpoint tibia, respectively. Few interactions were observed between diet and age; however, results were inconsistent and may be due to the multiple comparisons performed, thus increasing type 1 error. With respect to L4 and the mandible, dietary intervention had no effect on bone development, with exception to the 400 IU vit D, 3 g Ca/kg diet group having lower BV/TV at L4 compared to the REF group. The fact that these same differences were not evident in both the trials, and particularly that this effect was not observed at the lower level of vit D, suggests that these are not biologically meaningful differences. But nonetheless, should be further studied. Analyzing multiple sites gives us a better understanding of the effect of the intervention diets on overall bone structure and BMD. While most women in Canada do not consume the recommended levels of vit D and Ca (Government of Canada, 2012),

arguably, it is important for the standard rodent diet to reflect this in order to allow for generalizability to humans.

$25(OH)D_3$ is the predominate form in circulation to evaluate vit D nutritional status. Interestingly, when vit D was lowered to 100 IU/kg, serum $25(OH)D_3$ concentration was significantly lowered with no effect on bone structure or BMD. Our results are within the range of normal circulating $25(OH)D_3$ levels in mice that use the gold standard technique, LC-MS, for measuring serum $25(OH)D_3$ (Kaufmann, et al., 2014). That serum PTH levels were not elevated in the intervention groups compared to the REF group suggests the lowered levels of Ca and vit D did not perturb overall Ca balance. However, lower serum PTH concentration in the 400 IU vit D/kg, 2.5 g Ca/kg diet group compared to REF group may be a result of the small sample size analyzed, as limited serum samples were available. The lack of differences in serum PTH concentrations may be due to adaptations to active Ca absorption mechanisms that are vit D-dependent (Lieben, et al., 2010).

It has been identified that both strain and species of rodents play an integral role in establishing minimal nutritional requirements of vit D and Ca for bone development. Replogle et al. (2014) examined multiple inbred strains of mice and showed that these strains responded differently with respect to changes in femur BMD content when fed a low Ca diet (2.5 g/kg diet). With respect to different species, there is evidence that mice or rats of the same strain adapt differently to low levels of Ca (2 g/kg diet), characterized by Ca absorption and the rate of bone loss (Wolynsky & Guggenheim, 1974). While previous research has investigated the effect of varying levels of vit D or Ca, there is limited research performed using a CD-1 female mouse model. Given the strain-specific

effects shown by others (Replogle et al., 2014), characterizing the response of a specific mouse model is prudent if planning to use this model consistently within a research program. In this study, CD-1 mice were studied as we have routinely used this strain to study nutritional programming effects on bone development (Ward, et al., 2016). Moreover, CD-1 mice are an outbred strain and differences in genetic variation are closely relatable to human populations.

Our findings were consistent with the results obtained from lowering vit D or Ca independently (Glenn, et al., 2014; Villa, et al., 2016; Hunt et al., 2008). Glenn et al. (2014) observed that female 129/SvEvIL-10 KO mice fed low (25 IU/kg) or high (5000 IU/kg) vit D levels had no differences in femur or vertebral BMC, BMD, or bone strength at 3 months of age. Using the same study design, in a C57BL/6J mouse model, Villa et al. (2016) extended the study period to a lifespan of 7 months and demonstrated at 25 IU vitD/kg diet, BMD, BMC, strength and structure of the femur and L2 was not compromised. When Ca was modified within a range of 1-7 g/kg diet (and vit D levels were held constant at 1000 IU/kg diet), Hunt et al. (2008) suggested a bone threshold response of the tibia is approximately 2.5 g Ca/kg diet in female Sprague-Dawley rats, with respect to bone strength and structural properties (Hunt et al., 2008). Although bone was not compromised, lowering level of vit D has been associated with intestinal inflammation in male CD-1 mice (Jahani et al., 2014), however, the response in female CD-1 mice has not been thoroughly studied.

That there were no significant differences between dietary interventions in regards to serum LPS and fecal albumin measures suggests lowering the levels of Ca and vit D does not compromise intestinal integrity. LPS is derived from Gram-negative bacteria of

the GM and can translocate to the bloodstream as a result of increased intestinal permeability. LPS induces osteoclast formation which is involved in bone resorption (Mundy & Roodman, 1987; Chambers, 1991). Furthermore, albumin is a major protein in the plasma and in fecal samples. Increased loss of albumin through the feces is indicative of a disrupted intestinal barrier (Wang, et al., 2015). If less than 1 mL of serum was able to be collected, it was used for 25(OH)D_3 and PTH analysis as these outcomes related directly to the primary objective. Unfortunately, there was an insufficient quantity of serum collected for LPS analysis in Trial 1. Interestingly, the intestinal barrier was not compromised with consumption of 400 IU vit D/kg and 3.5, 3 or 2.5 g Ca/kg diet. When vit D was lowered to 100 IU/kg diet, based on fecal albumin measures, no inflammatory response was observed regardless of Ca level. In a previous study in male mice, serum LPS was increased when vit D was lowered to 25 IU/kg (Jahani et al., 2014) which may be a result of sex-specific response or the fact that further lowering vitamin D below 100 IU/kg in females might compromise intestinal integrity.

In determining the nutritional requirements for mice, recommended by the NRC, levels were established to promote rapid growth leading to maximum body size at maturity and it was assumed that it then would also be adequate for reproduction, lactation, and maintenance (NRC, 1995). While it is recognized that the formulation of a REF diet is dependent of the study objective this study suggests that the current AIN-93G provides excessive levels required for normal development of bone structure and BMD. In the formulation of the diet, the estimates were based on manufacturing and storage loss, bioavailability of the nutrients and potential nutrient interactions (NRC, 1995). Due to the predicted loss, the American Institute of Nutrition kept the excessive nutrient levels

consistent with precursor diets, however, this presents the limitation of nutrient compensation when the diet is manipulated or an intervention is administered. Previously, determining Ca requirements for mice was largely based on preventing kidney calcification, commonly observed with the precursor AIN-76 diet. In the reformulation of the diet, Reeves et al. (1993b) compared the current level of Ca (5 g/kg diet) to both a lower and higher level, 3 and 6.7 g/kg, respectively. Regardless of Ca within this range, no differences in tibia Ca content was observed. Although the measured outcome of bone health was bone mineral content, the lack of difference suggests the current level of Ca may be in excess, with respect to bone development. However, with the development of more sophisticated methods, we can more fully discern levels of Ca and vitD needed for bone development.

Strengths and Limitations

The ability to study the same mouse over time was advantageous for reducing variability and decreasing the sample size needed. With respect to limitations, due to the time frame of the study, we were unable to observe the effects of the dietary intervention on bone health during and after late adulthood. Furthermore, it is acknowledged that that CD-1 is only one species of mice and using other species may result in different outcomes at the levels studied. There are also possible sex-specific responses to diet, due to differences in growth rates and absolute growth, however, this study focused on bone development in female mice as a first step. The limited sample available for measurement of LPS and not using the gold standard technique for measuring intestinal integrity are limitations of this study. Further investigation of intestinal integrity in the context of

these lower levels of Ca and vit D are required to more conclusively determine the effects on intestinal integrity.

6.2 Conclusions

In summary, when vit D and Ca are lowered to 100 or 400 IU vit D/kg and 3.5, 3 or 2.5 g Ca/kg, there were little to no changes with *in vivo* bone structure and BMD at proximal tibia at 2 and 4 months of age or *ex vivo* bone structure measured at age 4 months . Further study is required to confirm if intestinal integrity is compromised. The lack of effect on bone structure, BMD and intestinal integrity suggests that the current levels of Ca and vit D in the AIN-93G diet are higher than required for normal bone development in female CD-1 mice. Thus, there may be an opportunity for the AIN-93G diet to be re-evaluated to contain levels of Ca and vit D adequate for future bone research without being in excess. The implications of having high levels of these essential nutrients is the masking of potential effects from an intervention in study which may present favourable bone outcomes and prevent debilitating fragility fractures.

6.3 Future directions

Given that outcomes of bone structure and BMD of all groups were similar to those fed AIN-93G diet suggests that the current levels of vit D and Ca in the reference rodent diet are higher than that required for bone development in female CD-1 mice. A next step would be to test an intervention in both the context of the AIN-93G or a diet containing 100 IU vit D/kg diet and 3.5 g Ca/kg diet, as a proof of principle, that the

current AIN-93G diet masks or attenuates the positive effect of a dietary intervention. Specifically, future studies can investigate if this modified diet results in enhances the known nutritional programming effect of soy isoflavones on bone health (Dinsdale, et al., 2012; Kaludjerovic & Ward, 2009; Kaludjerovic & Ward, 2010). Contrary to analyzing early life stages such as 2 and 4 months, this study can also be extended over time to analyze the effect of 100 IU it D/kg and 3.5 g Ca/kg in late adulthood to elderly life stages. Such a study would more clearly identify any potential long-term problems of these lower levels of vit D and Ca for bone health during aging. Ultimately, the modified diet may be used as a reference diet for bone researchers to continue to develop dietary strategies for lowering the risk or preventing osteoporosis.

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APPENDIX

Table A1. Dietary levels of vit D and Ca measured by Maxxam Analytics.

	Intervention Diets							
	Trial 1 100 IU vit D/kg				Trial 2 400 IU vit D/kg			
	REF	3.5 g Ca/kg	3 g Ca/kg	2.5 g Ca/kg	REF	3.5 g Ca/kg	3 g Ca/kg	2.5 g Ca/kg
vit D_3[1], *IU/kg*	880	ND	ND	ND	880	370	340	360
Ca[2], *g/kg*	0.44	0.31	0.27	0.23	0.44	0.32	0.27	0.23

[1] Reportable Detection Limit: vit D_3 = 20 IU/100g
[2] Reportable Detection Limit: Ca = 0.02 g/kg

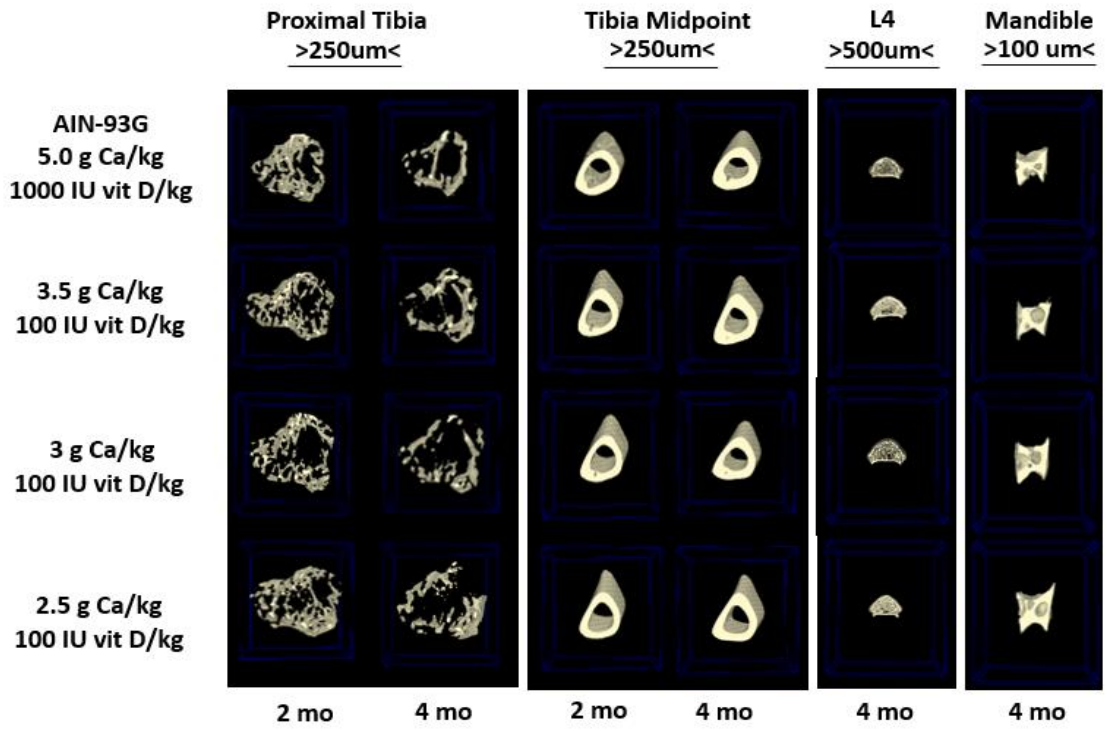

Figure A1. Trial 1 (100 IU vit D/kg diet): representative 3D images of the right tibia, L4 and mandible of female CD-1 mice. Images have been constructed using CTVox. There were no visual differences among dietary interventions at each skeletal site.

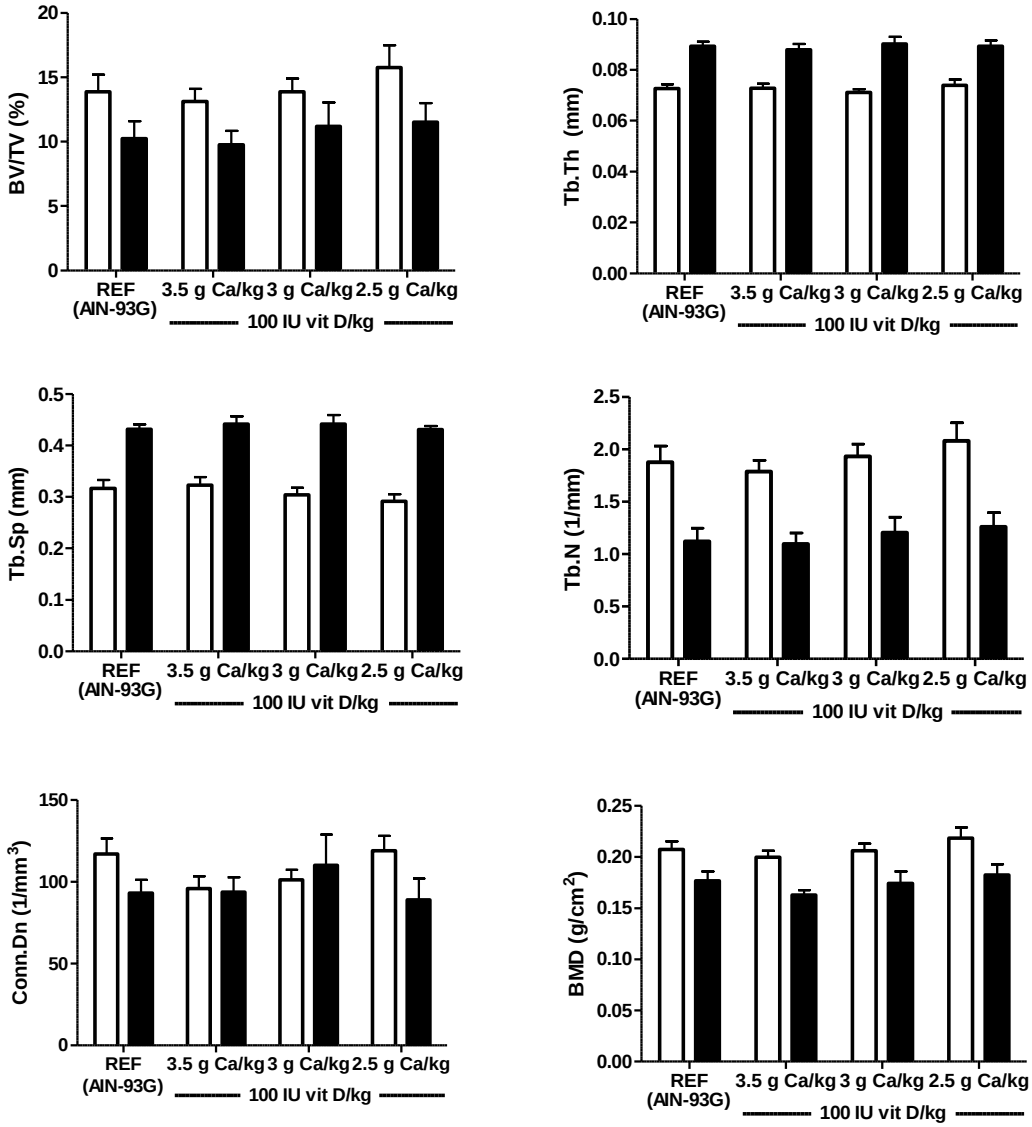

Figure A2. Trial 1 (100 IU vit D/kg diet): critical trabecular bone outcomes and BMD of the right proximal tibia at 2 (white columns) and 4 (black columns) months of age in female CD-1 mice. n = 12-15 mice per group. Mean ± SEM. Significant age effect ($p < 0.05$) observed for all outcomes within each dietary group.

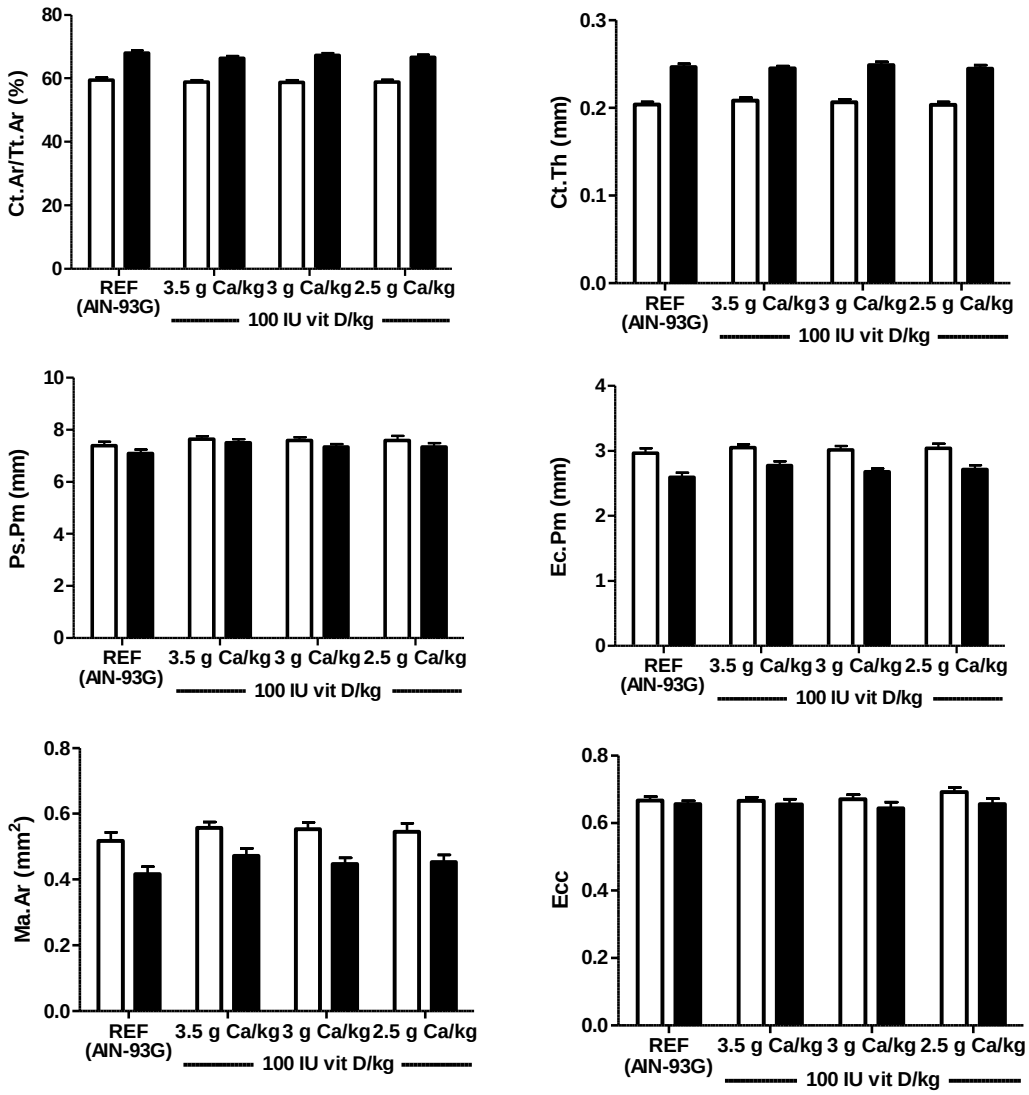

Figure A3. Trial 1 (100 IU vit D/kg diet): critical cortical bone outcomes of the tibia midpoint at 2 (white columns) and 4 (black columns) months of age in female CD-1 mice. n = 12-15 mice per group. Mean ± SEM. Significant age effect ($p < 0.05$) observed for all outcomes within each dietary group.

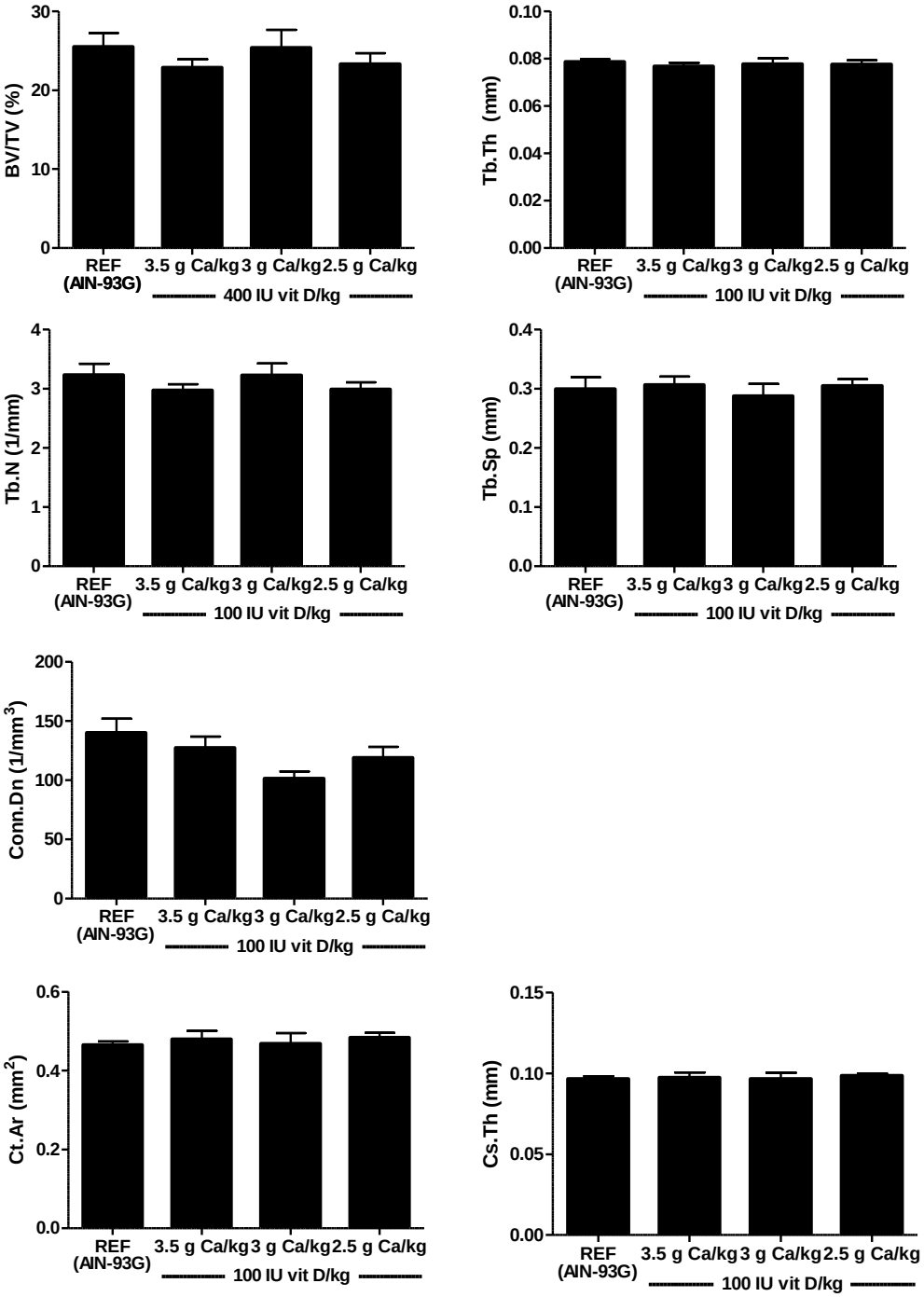

Figure A4. Trial 1 (100 IU vit D/kg diet): critical trabecular and cortical bone outcomes of L4 at 4 months of age in female CD-1 mice. n = 9-10 mice per group. Mean ± SEM.

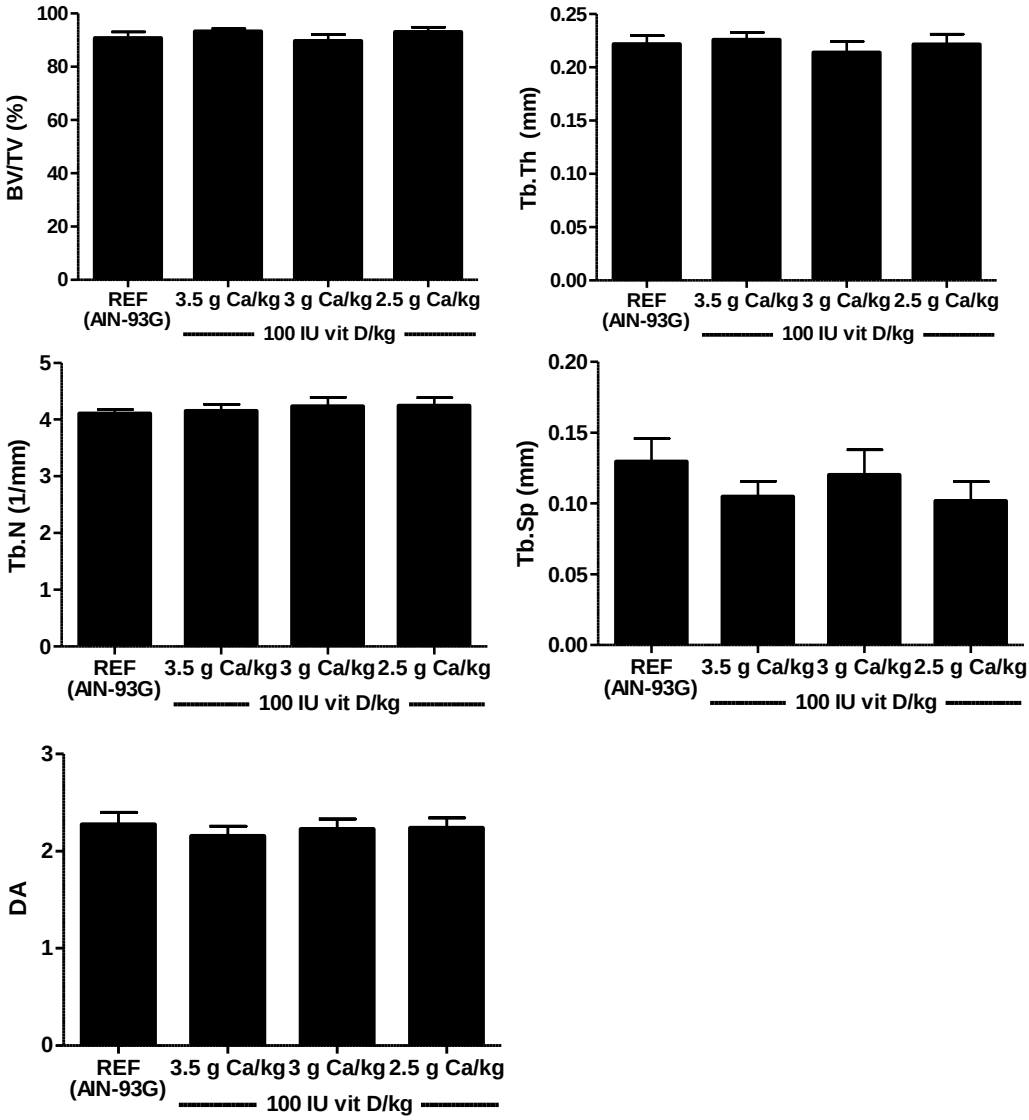

Figure A5. Trial 1 (100 IU vit D/kg diet): critical trabecular bone outcomes of the mandible at 4 months of age in female CD-1 mice. n = 9-10 mice per group. Mean ± SEM.

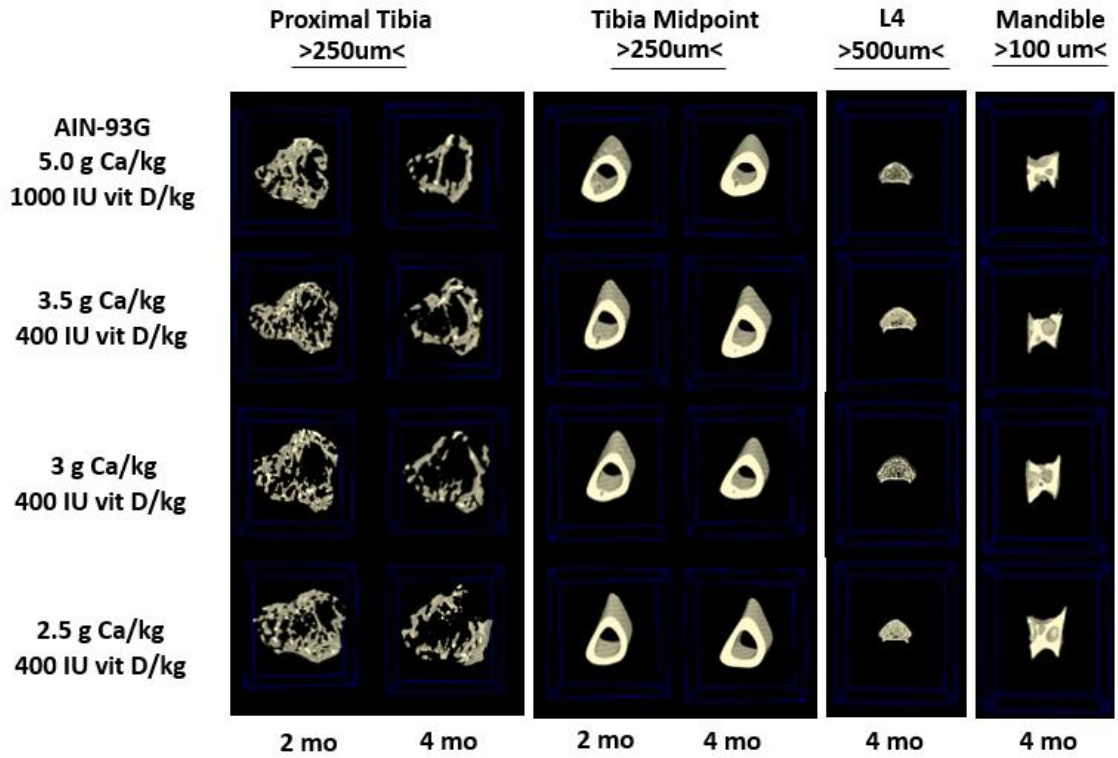

Figure A6. Trial 2 (400 IU vit D/kg diet): representative 3D images of the right tibia, L4 and mandible of female CD-1 mice. Images have been constructed using CTVox. There were no visual differences among dietary interventions at each skeletal site.

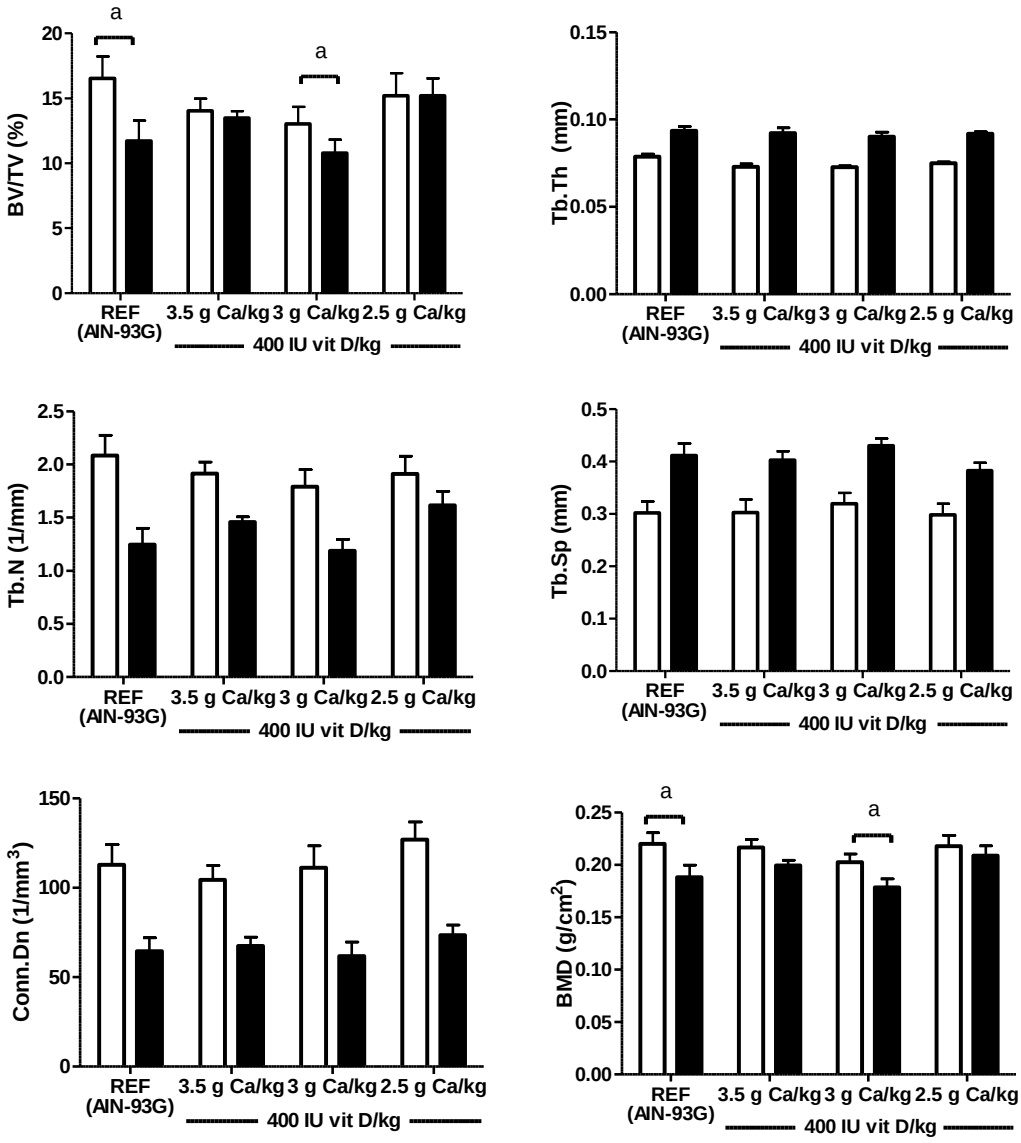

Figure A7. Trial 2 (400 IU vit D/kg diet): critical trabecular bone outcomes and BMD of the right proximal tibia at 2 (white columns) and 4 (black columns) months of age in female CD-1 mice. n = 13-15 mice per group. Mean ± SEM. Significant age effect ($p < 0.05$) observed for all outcomes within each dietary group. a denotes significant difference ($p < .05$) in the presence of a diet x age interaction ($p < 0.05$).

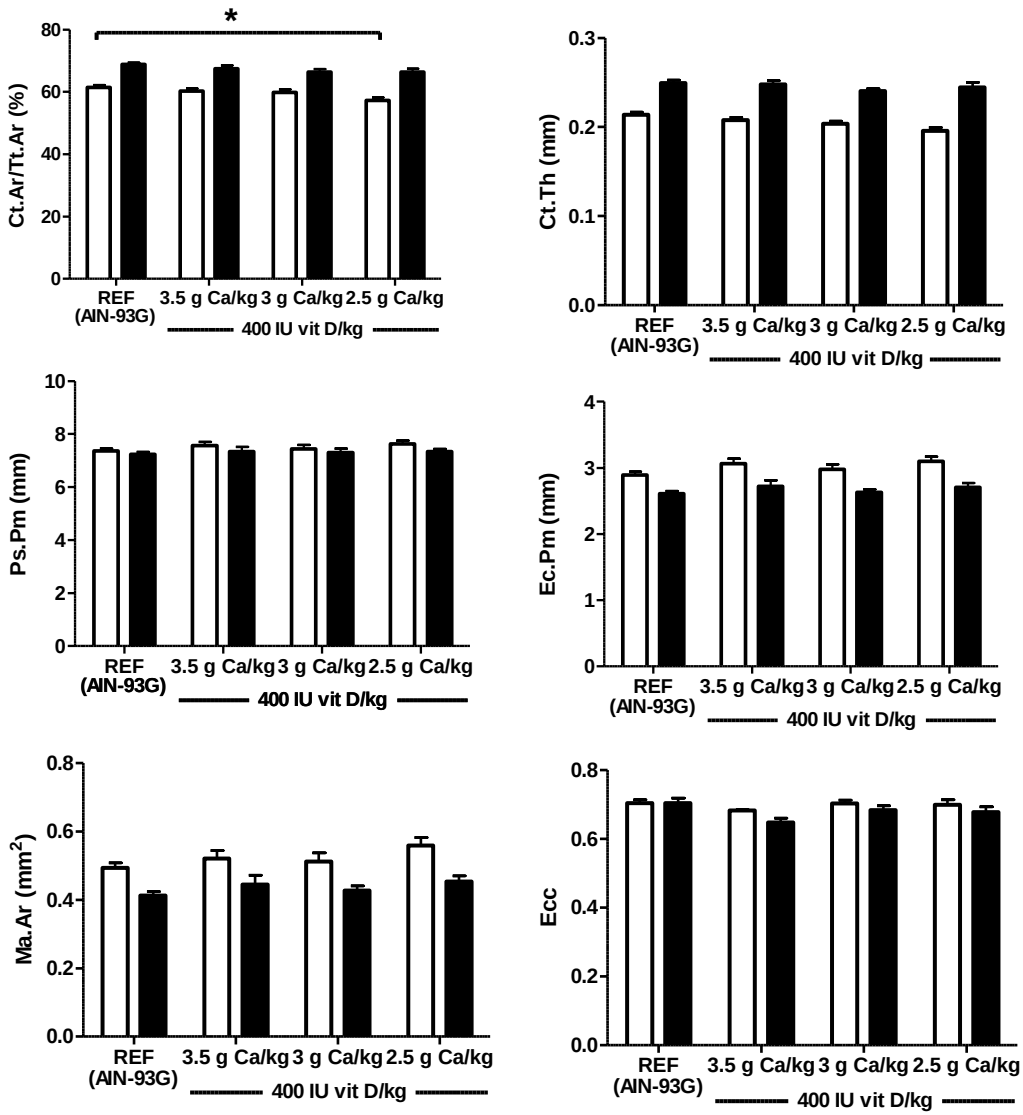

Figure A8. Trial 2 (400 IU vit D/kg diet): critical cortical bone outcomes of the tibia midpoint at 2 (white columns) and 4 (black columns) months of age in female CD-1 mice. n = 13-15 mice per group. Mean ± SEM. Significant age effect ($p < 0.05$) observed for all outcomes within each dietary group. * denotes significant difference ($p < 0.05$) between dietary groups.

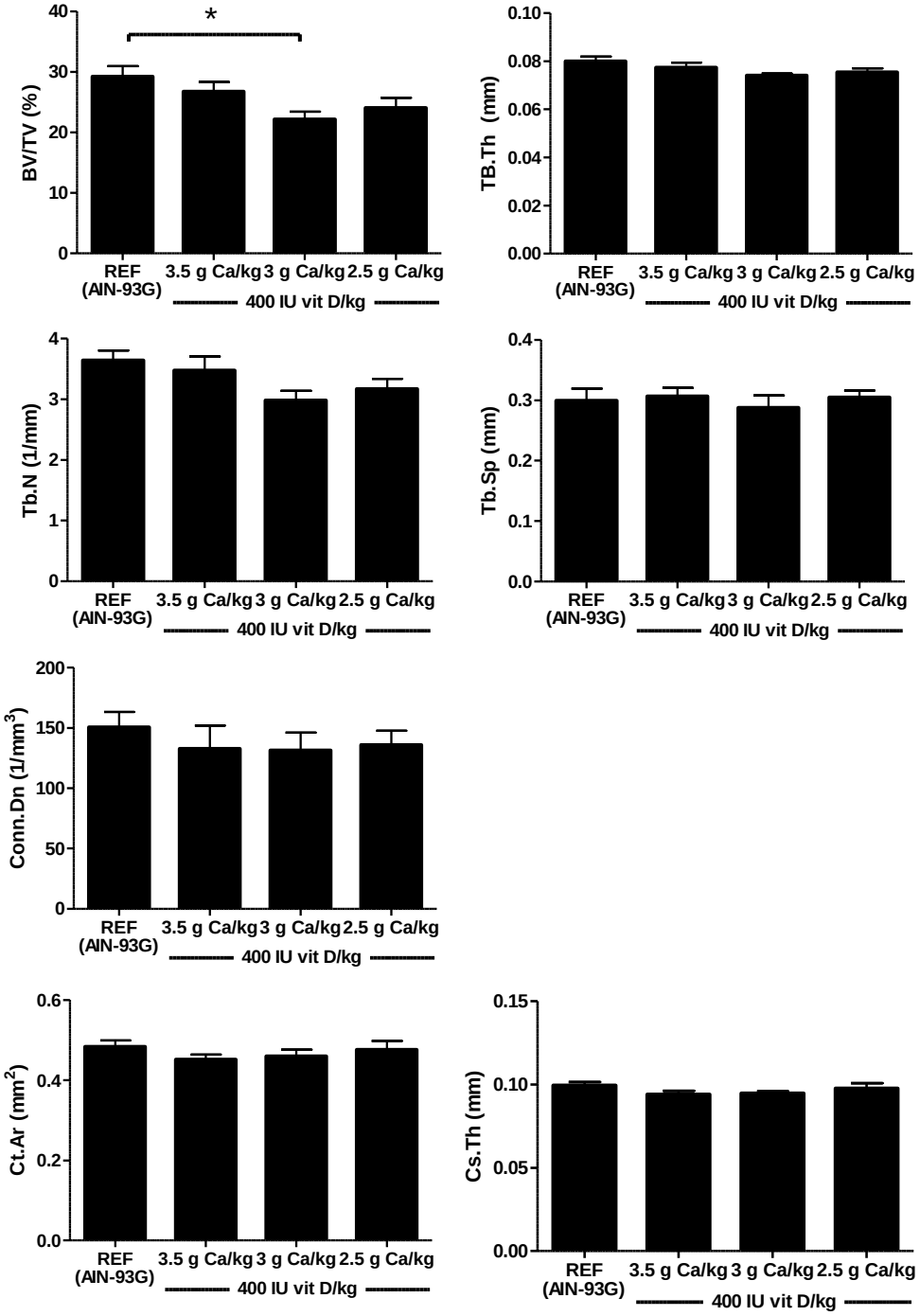

Figure A9. Trial 2 (400 IU vit D/kg diet): critical trabecular and cortical bone outcomes of L4 at 4 months of age in female CD-1 mice. n = 9-10 mice per group. Mean ± SEM. * denotes significant difference ($p < 0.05$) between dietary groups.

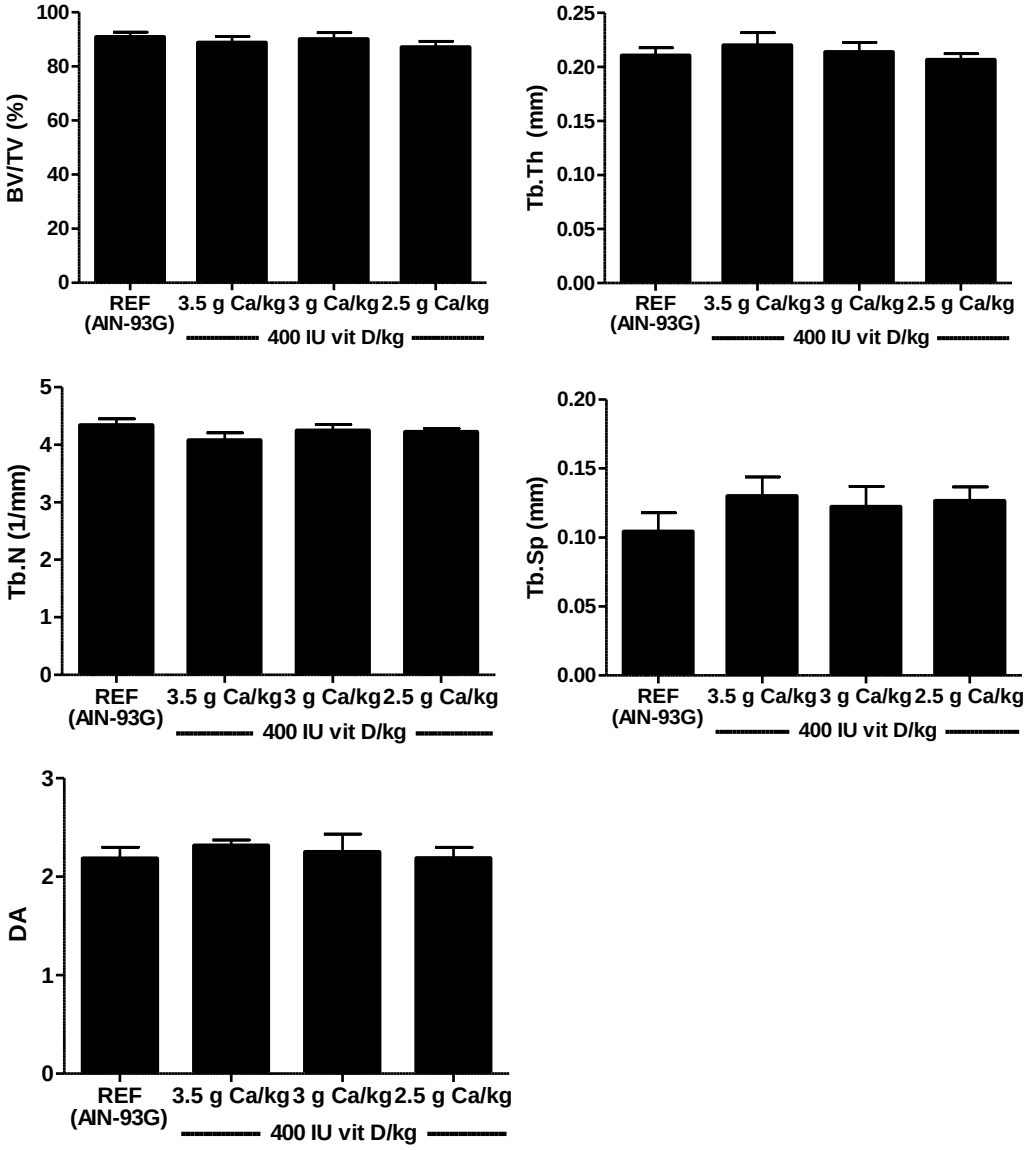

Figure A10. Trial 2 (400 IU vit D/kg diet): critical trabecular bone outcomes of the mandible at 4 months of age in female CD-1 mice. n = 9-10 mice per group. Mean ± SEM.

Table A2. Sample size calculation for bone and intestinal integrity analyses.

	Outcome	**Standard deviation**	**Alpha (α)**	**Power**	**Effect size (cohen's d)**	**Sample size (n)***
Bone	BMD of LV	4 mg/cm^2	0.05	0.80	5 mg/cm^2	12
Intestine	Serum LPS	0.97 ng/mL	0.05	0.80	1.54 ng/mL	5

*sample size calculation based on $n = 2(\sigma/d)^2$, where σ =2.80 is a function of α and powe

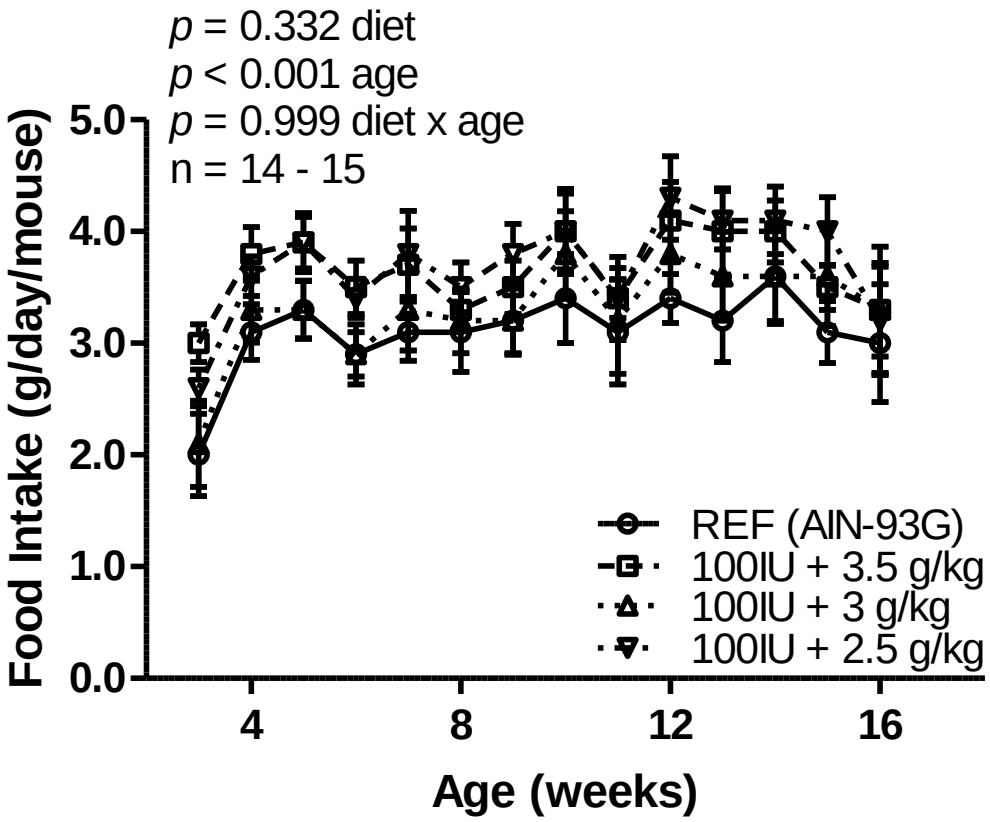

Figure A11. Average daily food intake of female CD-1 mice in Trial 1 (100 IU vit D/kg diet). Data are reported as mean ± SEM. There was no significance difference ($p > 0.05$) in food intake among dietary interventions.

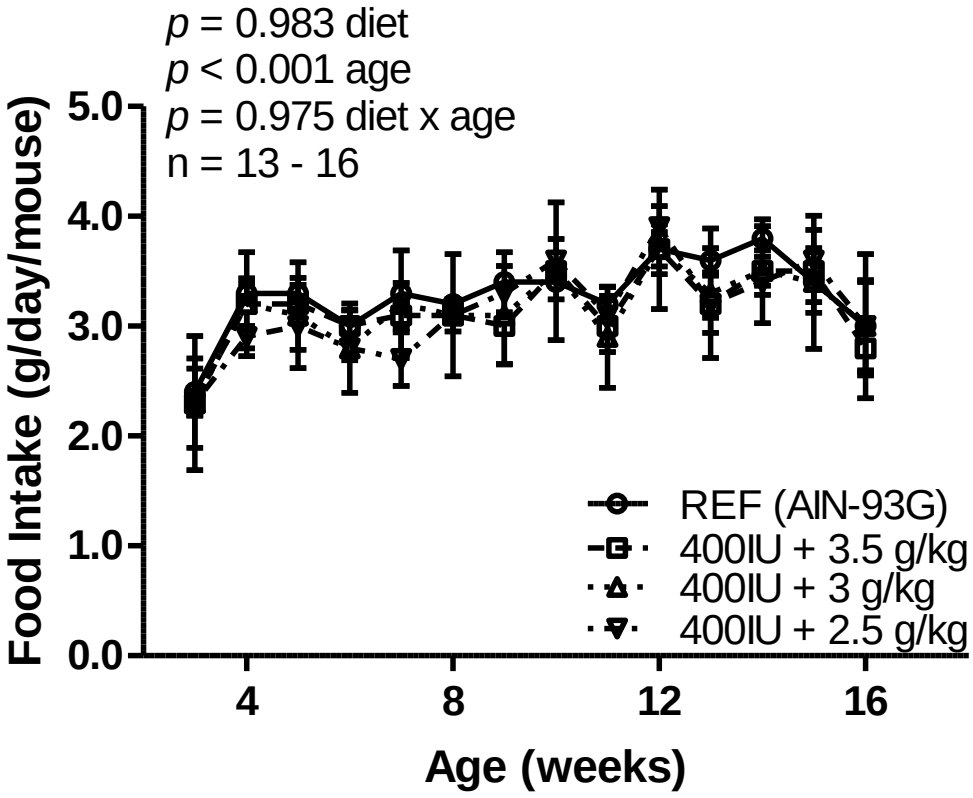

Figure A12. Average daily food intake of female CD-1 mice in Trial 2 (400 IU vit D/kg diet). Data are reported as mean ± SEM. There was no significance difference ($p > 0.05$) in food intake among dietary interventions.