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**EFFECTS OF HIGH FAT FEEDING AND DIETARY VITAMIN D ON CALCIUM,
BONE, AND VITAMIN D METABOLISM IN MATURE MICE**

by

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ABSTRACT OF THE DISSERTATION

EFFECTS OF HIGH FAT FEEDING AND DIETARY VITAMIN D ON CALCIUM, BONE, AND VITAMIN D METABOLISM IN MATURE MICE

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Dietary fat sources containing high caloric energy are closely linked to energy overconsumption induced obesity leading to a variety of health problems, including compromised bone quality. High fat diets (HFD) also affect calcium metabolism. However, effects of HFD on intestinal calcium absorption and bone health in mature animals have not been addressed, and different types of dietary fatty acids may have differential effects (e.g. monounsaturated fatty acids (MUFA) vs. saturated fatty acids (SFA). In addition, low circulating vitamin D status is commonly found in obese individuals. However, the nature of the association between vitamin D deficiency and obesity remains unclear because an underlying mechanism for the association has not been delineated. Therefore, in the current dissertation, we sought to reveal the effects of dietary fat and vitamin D intake on calcium and vitamin D metabolism and bone health using a mature mouse model.

In study 1, the effects of HFD enriched with MUFA or with SFA on intestinal calcium absorption and bone health in mature lean mice were investigated. C57BL/6J mice were weight-controlled fed with either 10% normal fat diet (NFD) or a HFD (45% fat) enriched with either MUFA or SFA for 10wk. We found that high compared with normal fat intake induced higher fractional Ca absorption (Ca^{45} isotope method) and this did not differ with the type of dietary fatty acids (MUFA vs. SFA). In contrast, only the high fat feeding with SFA adversely affected total and femoral area bone mineral density (dual-energy X-ray absorptiometry), while MUFA was associated with greater femoral trabecular bone volume fraction (BV/TV) and thickness (micro-computerized tomography system).

In study 2, the objective was to determine whether high MUFA intake would have a neutral or even a beneficial effect on mature bone health under conditions of excess caloric intake and obesity, and whether high MUFA and SFA feeding would differentially affect vitamin D metabolism in obese mice. After *ad libitum* feeding C57BL/6J mice 10% NFD or 45% HFD enriched with MUFA or SFA for 10wk, we found that the HFD enriched with SFA, but not MUFA, resulted in greater energy intake, weight gain, total body fat mass (EchoMRI Body Composition Analyzer), and liver fat. High SFA intake, but not MUFA, also adversely affected femoral trabecular bone parameters, though no detrimental effects of SFA on bone mass were seen in mature mice under the condition of excess caloric intake and obesity. Moreover, high fat feeding lowered circulating 25OHD concentration (ELISA), which was also inversely associated with body fat percentage. However, this finding was not explained by differential effects of MUFA and SFA on gene (rt-PCR) and protein (western blotting) expression of hepatic vitamin D 25-hydroxylase Cyp2r1 in mature mice.

In study 3, the aim was to determine effects of dietary vitamin D on food intake and adiposity with and without high fat feeding. We found that supplemental vitamin D showed no beneficial effects on preventing HFD-induced obesity. Under conditions of high fat feeding, low vitamin D intake appeared to increase food intake, weight gain, and adiposity compared to the HFD-normal vitamin D diet, but the magnitude of the effect was inconsistent between two separate experiments. We also investigated the role of low vitamin D intake on serotonin production and major markers involved in the vitamin D and serotonergic pathways in the brain. It was found that low D intake, regardless of the presence of high fat feeding, lowered gene expression of vitamin D 1 α -hydroxylase *Cyp27b1* and frontal cortex serotonin concentrations in the brain.

In conclusion, this dissertation addressed the role of different amounts (high vs. normal) and types (MUFA vs. SFA) of dietary fat intake on Ca and vitamin D metabolism and bone health in mature female mice. Our findings support that high fat feeding increases intestinal Ca absorption and lowers circulating vitamin D status, and these effects were not dependent on the type of dietary fatty acids (MUFA vs. SFA). In contrast, dietary fat rich in SFA has detrimental effects on mature bone quality regardless of the presence of obesity, whereas high MUFA intake shows a neutral effect. Moreover, it was found that vitamin D deficiency combined with HFD potentially promotes food intake and weight gain that might be related to lowered cerebral serotonin concentration. Overall, the interaction of fatty acids on calcium and vitamin D metabolism and bone health is complex and deserves further attention in future studies.

DEDICATION

I dedicate this work to my beloved parents, for their lifelong love and faith and the constant support and encouragement in every step of my life.

To Joe and Grace, for being you and knowing how to be there for me, always.

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LIST OF ABBREVIATIONS

1,25(OH)₂D, 1,25-dihydroxycholecalciferol

25OHD, 25-hydroxycholecalciferol

BMC, bone mineral content

BMD, bone mineral density

BV, bone volume

BV/TV, bone volume fraction

Ca, calcium

CalbD_{9k}, calbindin-D_{9k}

Ct.Ar, cortical total cross-sectional bone area

Ct.Po, cortical porosity

Ct.Th, cortical cross-sectional thickness

CNS, central nervous system

Cyp2r1, cytochrome P450 2r1

Cyp24, cytochrome P450 24

Cyp27b1, cytochrome P450 27b1

EF1 α , elongation factor 1 α gene

FCA, fractional calcium absorption

FGF23, fibroblast growth factor 23

Gapdh, glyceraldehyde-3-phosphate dehydrogenase

HD, high vitamin D content

HF, high fructose

HFD, high fat diet

HS, high sucrose

Htr2c, 5-hydroxytryptamine receptor 2C

J , polar moment of inertia

LD, low vitamin D content

MUFA, monounsaturated fatty acid

NFD, normal fat diet

ND, normal vitamin D content

PTH, parathyroid hormone

PBS, phosphate buffered saline

Sert, serotonin transporter

SFA, saturated fatty acids

SMI, structure model index

TLC, thin layer chromatography

Tb.N, trabecular number

Tb.Sp, trabecular separation

Tb.Th, trabecular thickness

Tph2, tryptophan hydroxylase-2

Trpv5, transient receptor potential cation channel subfamily V member 5

Trpv6, transient receptor potential cation channel subfamily V member 6

TV, total volume

vBMD, volumetric bone mineral density

Vdr, vitamin D receptor

VitD, vitamin D

CHAPTER I: INTRODUCTION

1. Obesity as a public health issue

Obesity is worldwide epidemic and a severe public health issue. By definition, overweight and obesity refers to abnormal or excessive amount of body fat accumulation that may impair health (National Institutes of Health 1998). In adults (18 years and older), body mass index (BMI) serves as a useful population-based measure or estimation of overweight and obesity, and it can be used for both women and men across different age groups. By definition, normal weight is defined as a BMI between 18.5-24.9, overweight is BMI between 25-29.9, and obesity is BMI equal to or greater than 30 (National Institute of Diabetes and Digestive and Kidney Disease). In the United States, the obesity rate has dramatically increased since 1985 (**Figure 1**), and to date, more than two-thirds (68%) of the adult population in the United State is considered to be overweight or obese (National Institute of Diabetes and Digestive and Kidney Diseases). The estimated direct medical cost of treating obesity is more than \$100 billion per year (CDC 2012). Obesity greatly reduces quality of life and life span, and is a significant risk factor of various chronic diseases such as metabolic syndrome, type II diabetes, cardiovascular disease, stroke, and cancers (breast, colon, and kidney). Moreover, obesity impairs musculoskeletal functions and contributes to the pathogenesis of osteoporosis that is characterized by low bone density and quality. Osteoporosis greatly increases the risk of fracture in older populations. According to the National Osteoporosis Foundation (2011), more than 50 million people who are 50 years and older are suffering from osteoporosis, and the mortality risk associated with osteoporotic fracture is greater than 6%.

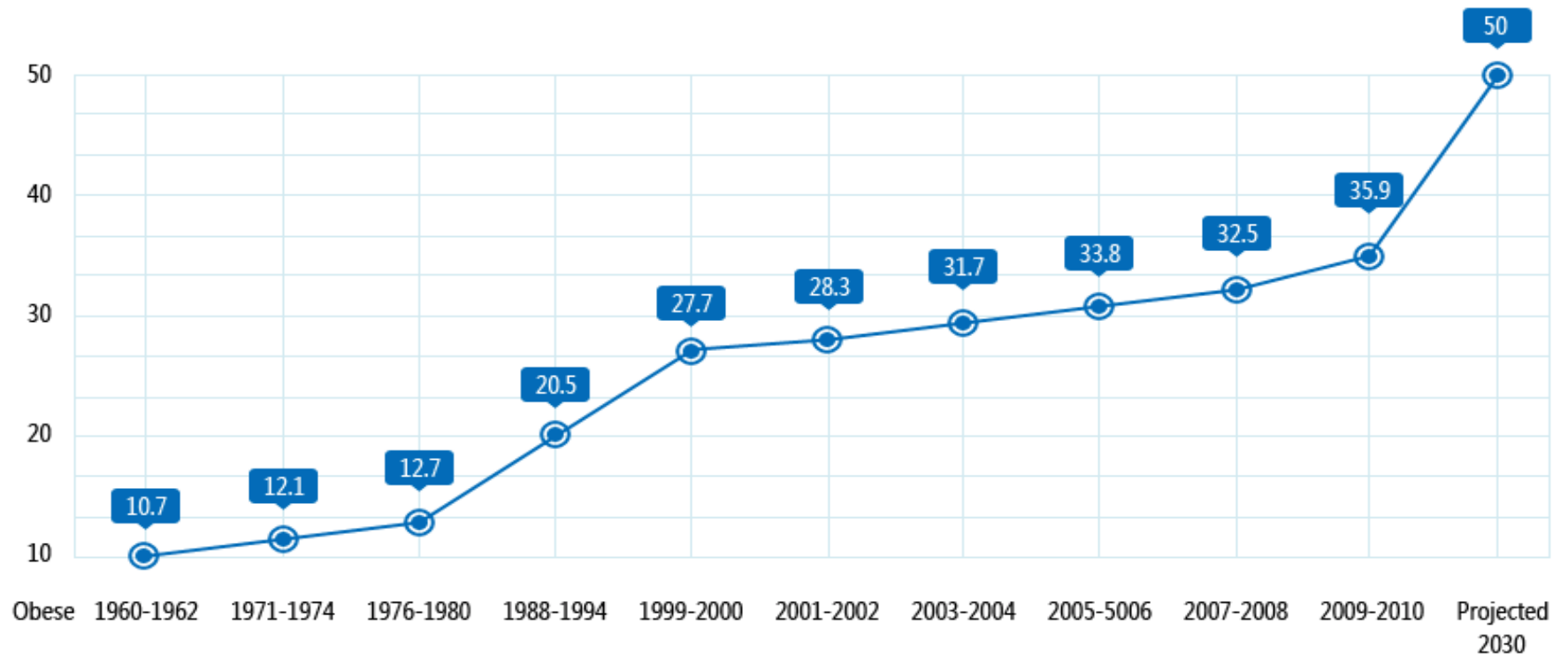


Figure 1. Obesity Epidemic in the United States¹.

http://healthyprotocols.com/2_weight_loss.htm

¹From 1960-1962 through 2009-2010, the prevalence of obesity significantly increased in adults ages between 20-74y in the United States (NHANES data).

1.1 Type and structure of dietary fat

Fats, also known as triglycerides, are esters containing three fatty acid chains and one alcohol, glycerol. Dietary fat is one of the three main macronutrients, and it is important for providing energy to the body and absorption of fat-soluble vitamins. Dietary fat falls into three major categories based on the degree of saturation in the carbon-carbon bonds among fatty acid chains (**Figure 2**) (American diabetes association, ADA). Saturated fatty acids (SFA) are hydrocarbon chains with no double bonds. SFA are commonly found in dairy products (cream, cheese, butter), red meats, and many processed and fast foods. In contrast, unsaturated fat such as monounsaturated fatty acids (MUFA) contain only one double bond within the fatty acid chain. Palmitoleic acid and oleic acid are two major MUFA that are commonly found in nuts, olive oil, and avocados (ADA). In addition, polyunsaturated fatty acids (PUFA) contain two or more carbon-carbon double bonds, and are found in seafood, fish, algae, leafy greens, and krill (ADA).

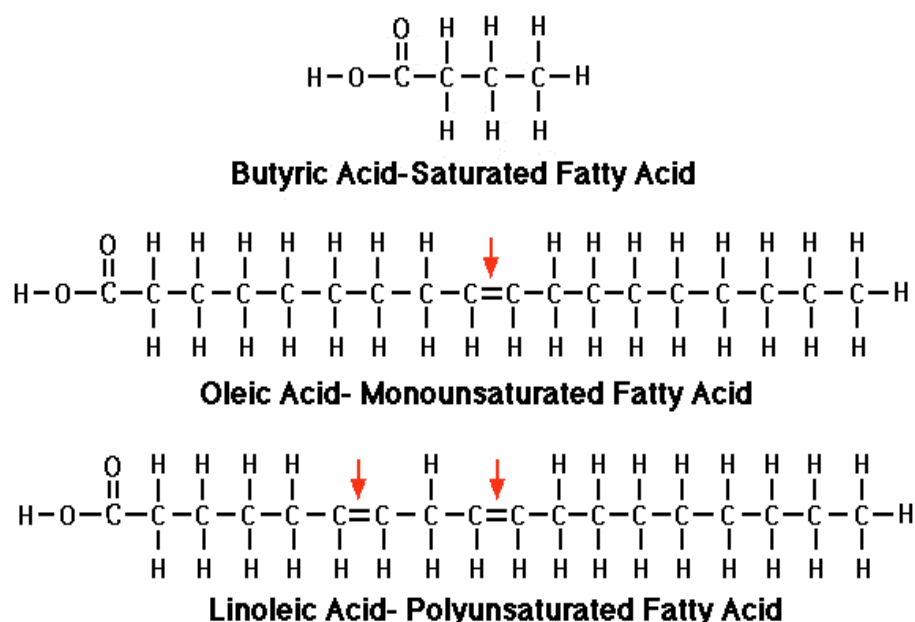


Figure 2. Structures of saturated and unsaturated fatty acids.

<http://foodscienceacademy.org/2013/06/15/fats-explained/>

1.2 Energy intake, dietary fat, and obesity

The imbalanced relationship between energy intake and expenditure, as well as factors such as age-associated hormonal changes, and genetic, dietary, environmental and social factors, contribute to excess energy deposition and lead to obesity (Doucet 1998). High fat feeding is a significant contributor to weight gain and adiposity (Boozer 1995, Storlien 1986). There is evidence from studies in rodents that despite having a relatively normal or low food intake, high fat diets (HFD) dramatically increase total body fat mass deposition and increase weight gain when compared with low fat fed mice (Lin 2000). Excess fat intake can rapidly result in white adipose tissue accumulation in the body and induce obesity (Lissner 1995, Bray 2004). Dietary fat promotes obesity via different mechanisms. For example, diets rich in fat are high in palatability and promote excess food consumption. Dietary fat is energy dense and contains 9 kcal/g diet (Rolls 2006, Stubbs 2004). Therefore, calorie overconsumption from dietary fat is easily achieved as compared with the other macronutrients, protein and carbohydrate, when consuming the same amount of food by weight. Also, metabolic utilization of fat is more efficient and creates less thermogenic effects than carbohydrate and protein (Astrup 1993).

In recent years, an extensive amount of research has focused on how each type of fatty acid differentially affects physiological functions. Notably, saturated fat has been shown to have distinct effects on weight gain, adipose accumulation, and other metabolic functions compared with unsaturated fat (de Wit N 2012, Astrup 2000). In general, when total energy from fat is controlled, a higher proportion of total fat intake as saturated fat promotes obesity and fatty liver, and adversely affects health (Field 2007, Cavaliere 2016). Rats fed with a high lard (SFA) diet had greater weight gain, fat accumulation, and body energy level when compared with rats fed a normal fat diet or a high PUFA diet (Cavaliere 2016). Moreover, dietary SFA negatively affects the serum lipid profile (i.e. lowers HDL), and promotes insulin resistance, systemic and local inflammation, and oxidative stress, which in turn

promote the development of cardiovascular disease, fatty liver, metabolic syndrome, and type II diabetes (Ward 1994, Viggiano 2016). In contrast, dietary long chain ω 3-PUFA intake increases the fluidity of cell membranes, increases HDL cholesterol levels, and enhances mitochondrial energy efficiency. Thus, high intake of ω 3-PUFA not only benefits weight management and obesity, but also protects against the pathogenesis of several chronic diseases such as cardiovascular disease, stroke, and diabetes (Cavaliere 2016). Substituting dietary saturated fat with unsaturated fat helps obesity and diabetes management, and promotes overall metabolic functions (Kim 2016,).

2. Bone structure and osteoporosis

Bones are hard tissues having complex hierarchical structures (Rho 1998). Under normal physiological conditions, bone fulfills two principal functions: a mechanical function and a metabolic function (Weiner 1999). Mechanically, bones serve as the structural framework of the body. They are the levers for muscle function, and are responsible for protecting and supporting the internal soft organs. Bones facilitate locomotion and serve as the protective housing for the blood-forming bone marrow (Loveridge 1999). Metabolically, bones are the largest reservoir of the major body minerals including: Ca^{2+} , Mg^{2+} , and PO_4^{3-} (McClean 1958). Bones release ionic forms of Ca needed for normal physiological functions such as muscle contraction, cell signaling, and neurotransmission (Szent-Györgyi 1975, Ghosh 1995).

Despite their tough and inert appearance, bones are dynamic tissues (Hadjidakis 2006, Rodan 2000). Bone remodeling, bone metabolism or turnover, constantly occurs to sustain the mechanical strength and integrity of skeletons and to maintain mineral homeostasis (Weilbaecher 2011, Ross 2006). Two major bone cells include bone-resorptive osteoclasts and bone-formative osteoblasts, which function integrally to accomplish the bone remodeling process (Hadjidakis 2006,

Boyle 2003, Ross 2006). Briefly, during the bone resorption phase, multinuclear osteoclasts derived from haematopoietic stem cells (HSC) accumulate on the surface of the bone and release calcified minerals such as Ca to form a bone resorption pit (Hadjidakis 2006, Weilbaecher 2011). In reversal, during the bone formation phase, mononuclear osteoblasts derived from mesenchymal stem cells (MSC) lay down new bone matrix consisting of type I collagen and minerals and bone-secreting factors at the site of the resorption pit until it is completely replaced (Weilbaecher 2011, Ross 2006) (**Figure 3**).

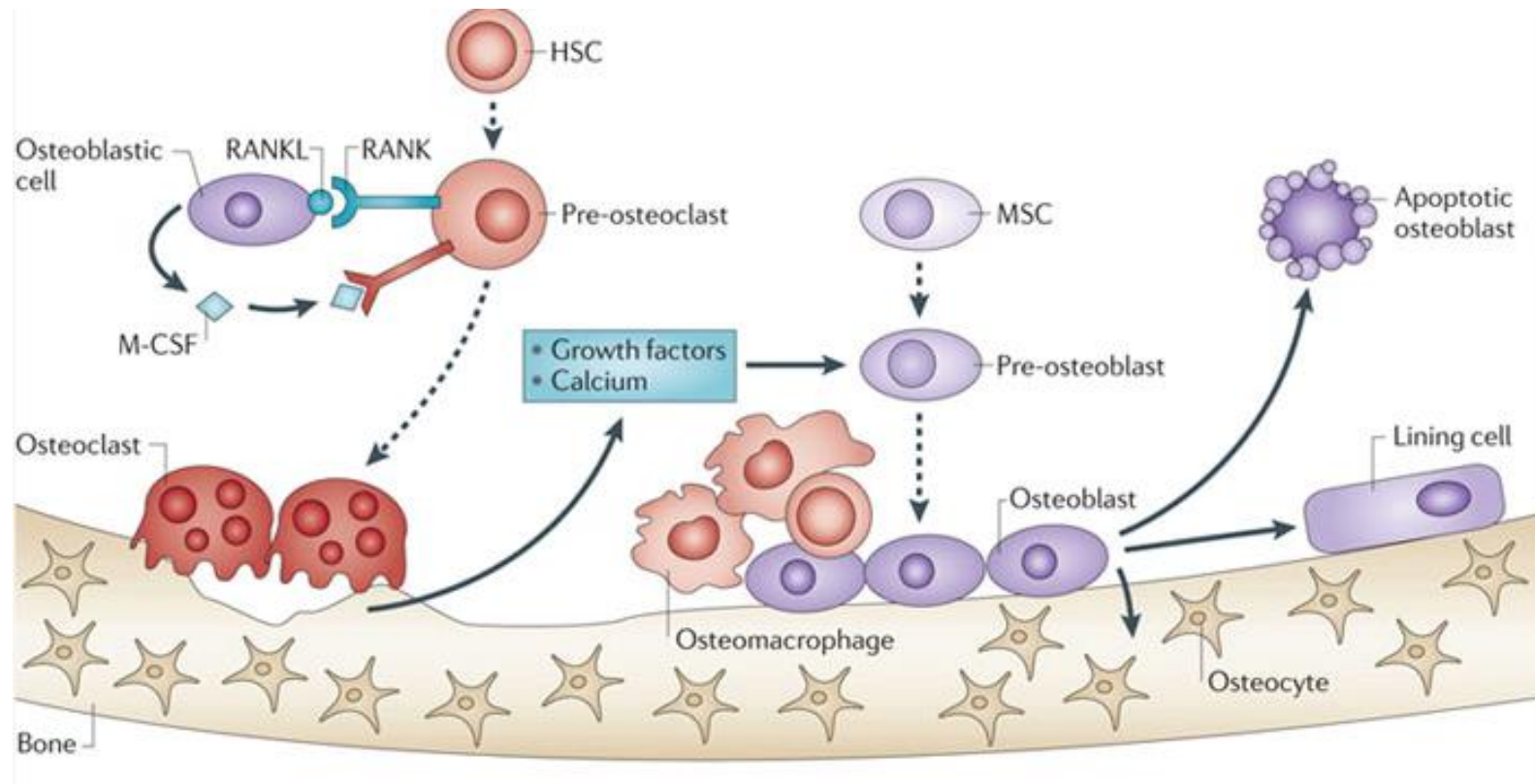


Figure 3. Bone remodeling model¹.

http://www.nature.com/nrc/journal/v11/n6/fig_tab/nrc3055_F1.html

¹During the bone resorption phase, activated osteoclasts accumulate on the surface of bones, remove minerals to form resorption pits. During the bone formation phase, osteoclasts build new bones at the same resorption pit by depositing new bone matrix and minerals (Weilbaecher 2011).

Regulation of bone remodeling can occur systemically and locally (Hadjidakis 2006). Several endocrine hormones such as 1,25-dihydroxycholecalciferol ($1,25(\text{OH})_2\text{D}$), parathyroid hormone (PTH), thyroid hormone, and sex hormones systemically regulate bone turnover by directly affecting gene expression and activity of osteoblasts and osteoclasts (Chow 1992, Karsenty 2006, Teitelbaum 2000, Teitelbaum 2003). Studies have reported that elevated PTH and $1,25(\text{OH})_2\text{D}$ and low estradiol status up-regulate osteoclastogenesis and down-regulate osteoblastogenesis leading to bone resorption. In addition, local synthesized factors from bone, including cytokines and several growth factors, affect bone cell fate and metabolism (Hadjidakis 2006). For example, proinflammatory cytokines such as interleukin-6 (IL-6), monocyte chemoattractant protein-1, C-reactive protein (CRP) and tumor necrosis factor α (TNF α) appear to modulate osteoclastogenesis and bone resorption. Also, osteoblasts and adipocytes originate from a common precursor, MSC, and excess adipogenesis in bone marrow may compromise osteoblastogenesis. Abnormal functions of bone cells (osteoclasts vs. osteoblasts) can lead to rapid bone turnover and pathological bone loss (Raisz 2005). An initial and mild bone loss that leads to low bone density below that of healthy bone mass is called osteopenia (t-score between -1.0 and -2.5) (National Osteoporosis Foundation, NOF) (**Figure 4**). Chronic loss of bone minerals causes bones to become increasingly porous and fragile, which eventually destroys the morphology and mechanical supporting functions (Rachner 2011). This is known as osteoporosis, indicated by a t-score less than -2.5 (NOF). Osteoporosis is characterized by compromised bone quality (Kanis 2005, Wilson 2011), and it threatens a large amount of women and men (~44 million) and their quality of life in the United States (Eghbali-Fatourehchi 2003). In older women and men aged 50yr and older, osteoporosis greatly increases the risk of falling and fractures at all bone sites of the body (Premaor 2010, Nielson 2012).

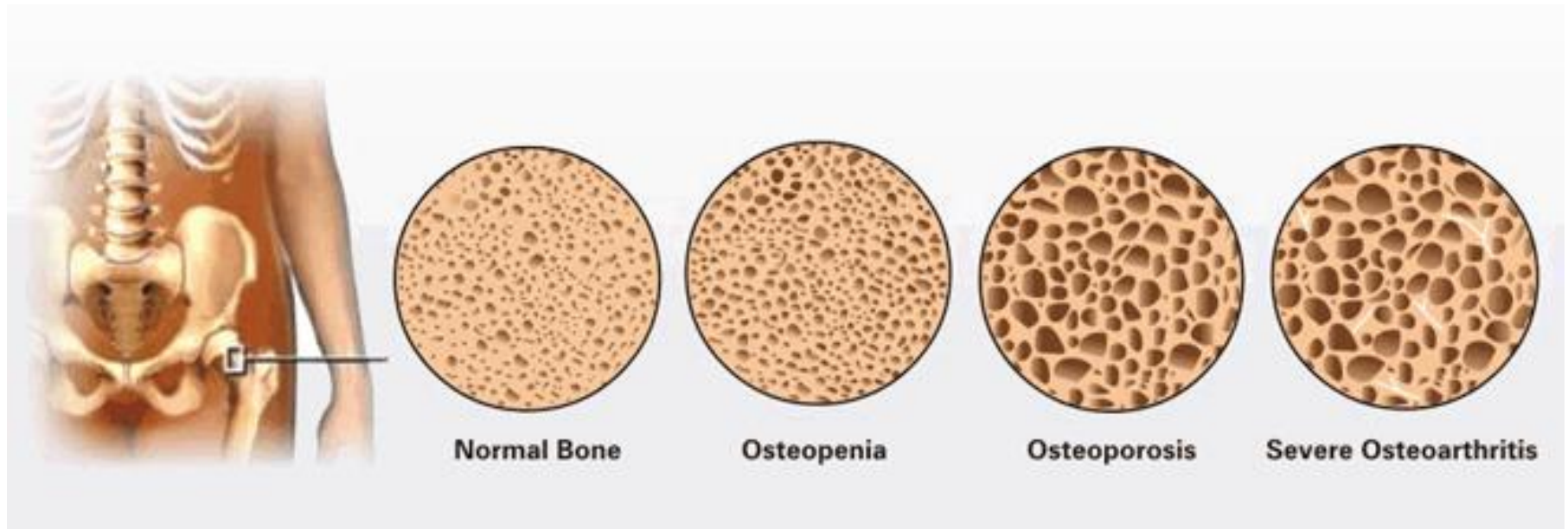


Figure 4. Osteoporosis.

Source: <http://osteotide.org/osteoporosis.html>

2.1 Body weight and bone health

Total body weight affects bone mass. Greater body weight is believed to have beneficial effects on bone health in humans. There is a direct relationship between body mass index (BMI) and bone mineral density (BMD). Also, there is some evidence that caloric restriction and weight loss negatively affect bone mass in older women (Shapses 2012, Cifuentes 2002). This could be due to the effects of mechanical loading on bone attributed to higher body weight (Felson 1993, Ravn 1999). Mechanical loading reduces apoptosis and increases proliferation and differentiation of osteoblasts and osteocytes. Therefore mechanical loading stimulates bone formation possibly via the Wnt/ β -catenin signaling pathway (Ehrich 2002, Sawakami 2006). Moreover, higher body weight is positively associated with greater adipose tissue, which may benefit bone mass by increasing the secretion of bone-active hormones. Adipose derived hormones such as leptin, adiponectin, and estradiol (Bolland 2006) are particularly important in preserving bone mass and prevent bone loss in postmenopausal women (Shapses 2012, Riggs 2002). However, the positive association between body fat mass and bone mass becomes less significant after total body weight is adjusted (Yamaguchi 2009, Hsu 2006). Despite the positive effects of body weight on bone mass, an increasing amount of evidence from clinical trials have shown that diabetic obesity compromises bone quality (Peng 2011, William 2009, Shapses 2012). A negative association between fat mass and BMD and total body bone mineral content (BMC) was found in human subjects (Goulding 2000, Blum 2003, Hsu 2006, Janicka 2007), especially when BMI is above 35 kg/m² (DeLaet 2005). Increased body weight with fat deposition is accompanied by a decline in relative bone strength in women (Xu 2010, Gnudi 2009, Rossen 2006) and decreased mechanical properties and performance of the cortical bone in obese mice (Ionova-Martin et al 2011). In addition, consistent with these findings in humans, HFD-induced obesity lowers bone quality measures in mice, and

also lowers BMD and BMC in total body bone, tibia, femur, and spine in the majority of rodent studies (Parhami 2001, Cao 2009, Cao 2010, Halade 2010, Yarrow 2016, Woo 2009, Chen 2010, Yan 2015, Núñez 2007, Lecka-Czernik 2015, Patsch 2011, Kyung 2009, Donner 2016). However in contrast, several studies showed an increased or null effect on BMD attributed to high fat feeding (Ionova-Martin 2010, Lv 2010, Ma 2010, Doucette 2015, Shen 2013, Gerbaix 2012). Importantly, most of the rodent studies were done in growing animals rather than after maturity, suggesting a detrimental effect of high fat feeding and obesity on bone development and maturation. Whether or not these adverse effects also occur in mature bone of older animals is less clear (Halade 2010, Gerbaix 2012).

2.2 Dietary fat and bone

Optimal nutrition is important to bone health via various mechanisms. The majority of research has focused on dietary Ca and vitamin D due to their crucial roles in bone metabolism and skeletal health (Cashman 2007). Other nutritional factors such as protein affect musculoskeletal function, and high protein intake may attenuate bone loss due to energy restriction and weight loss (Fournier 2016, Conigrave 2008, Shapses 2012). The effects of dietary fat on bone health have also been examined in clinical trials and animal studies (Shapses 2012, Reid 2008, Watkins 2006, Cao 2010). Dietary fat may benefit or harm bone health and appears to depend on the amount and the type of intake (Orchard 2010). Under a low or normal energy intake, dietary fat promotes bone mineralization possibly by increasing intestinal vitamin D and Ca absorption (Shapses 2012, Dawson-Hughes 2015). Excessive caloric intake from dietary fat leads to positive energy deposition and obesity that has been shown to adversely affect bone metabolism and compromise bone quality that increases fracture risk (Martínez-Ramírez 2007).

When comparing types of fatty acids, unsaturated fatty acids may function differently from saturated fatty acids in affecting bone cell fate and metabolism. For instance, in humans, intake or serum concentration of long chain polyunsaturated fatty acids (PUFA) is directly associated with BMD in older adults (Watkins 2006, Rousseau 2009, Farina 2007, Bhattacharya 2007) and thus PUFA may prevent bone loss. There is also evidence that dietary PUFA lowers fracture risk; however, observational reports are inconsistent and the association may vary among women and men and ethnicities (Chen 2010). The mechanism by which PUFA, especially ω -3 fatty acids, have positive effects on bone mass and quality is possibly by decreasing PGE₂ and inhibiting receptor activator of NF- κ B ligand-induced osteoclast formation and differentiation (Rahman 2008, Boeyens 2007). Dietary ω -3 PUFA also promotes bone formation by increasing osteoblast differentiation and survival. In

addition, to date there are no known negative effects of monounsaturated fatty acids (MUFA) on bone health, and MUFA attenuates BMD loss in older female mice (Wang 2016). Moreover, cross-sectional studies indicate that dietary MUFA is associated with reduced fracture risk in older adults (Trichopoulou 1997, Hayek 2012). However, the mechanism and effects of dietary MUFA on bone cell metabolism are unclear. In contrast, dietary saturated fatty acids (SFA) have been shown to have detrimental effects on bone health. High SFA intake impairs bone metabolism, reduces bone mass, and compromises bone quality and strength. Excess SFA induced obesity is associated with low BMD in elderly men and women, and accelerates the pathogenesis of osteoporosis and fracture risk (Corwin 2006, Shapses 2012, Parhami 2001). One mechanistic explanation for the effects of excess SFA intake on bone metabolism is prevention of osteoclastogenic bone formation (Parhami 2001, Chen 2015). Because osteoblasts and adipocytes are both derived from MSC, elevated bone-marrow adipogenesis further compromises aging-attenuated osteoblastogenesis (Kawai 2012, Moerman 2004). Additionally, high SFA feeding also impairs lipid metabolism that may indirectly stimulate osteoclast senescence and apoptosis (Chen 2015, Oh 2010, Manolagas 2007). Moreover, SFA mediates osteoclastogenesis and bone resorption possibly by increasing cellular oxidative stress (Yarrow 2016, Manolagas 2007) and the activity of inflammatory cytokines (IL1 β , IL-6, TNF α) (Nane 2003, Ragab 2002). There is also evidence showing that SFA accelerates bone turnover by preventing osteoclast apoptosis (Zhong 2011).

3. Calcium

3.1. Calcium and bone

Calcium is an essential mineral required for many physiological functions in the body. Only a small percentage (~1%) of total body Ca exists in the extracellular and intracellular fluid (Peng 2003, Peng 2003, Veldurthy 2016). The ionic form of Ca

functions as an intracellular messenger and is necessary in modulation of normal muscle contraction, cell signaling, blood clotting, neurotransmission, and enzyme secretions (Szent-Györgyi 1975, Ghosh 1995). About ninety-nine percent of total body Ca is in the form of crystal hydroxyapatite salt $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ and is stored in bones (Veldurthy 2016) (**Figure 5**). Adequate Ca status ensures the structural integrity of the bone (Cashman 2007). In the early stage of life, adequate Ca load is crucial for bone growth and development, so that the bones can reach to a high peak bone mass by early adulthood. After achieving peak bone mass, adequate Ca intake is necessary for maintaining normal bone mineralization and the prevention of osteoporosis (Welten DC 1995). Calcium deficiency, or hypocalcemia, caused by different mechanisms including low dietary intake, intestinal mal-absorption, hyperuricemia, thyroid surgery, or other pathological factors promotes bone turnover to maintain a normal serum Ca concentration (Fleet 2010, Fleet 2006, Boyle 2003, Wolf 2000). Subsequently, chronic loss of bone mass eventually leads to osteoporosis.

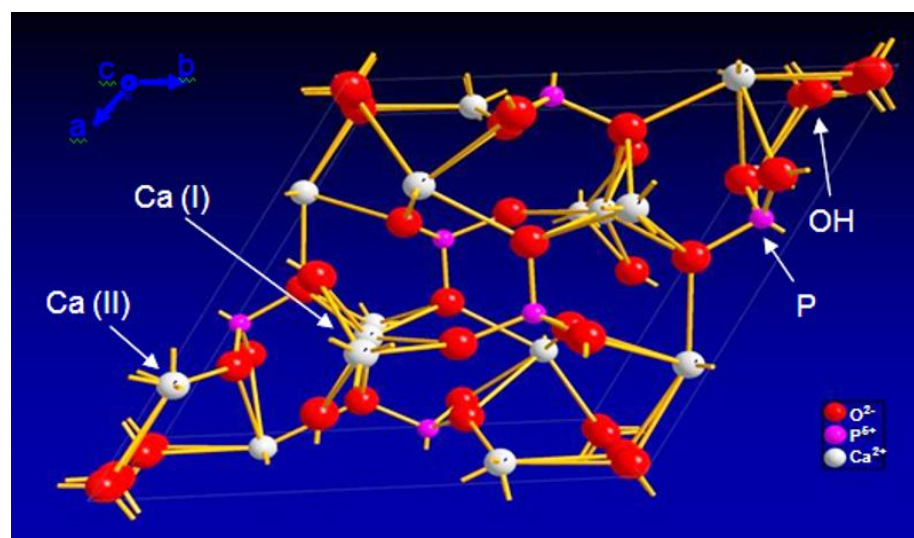


Figure 5. Hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$.

<http://www.medicalmcqs.com/wp-content/uploads/2013/12/Hydroxyapatite.png>

3.2. Intestinal Ca absorption

Dietary intake is the primary source of the body Ca pool--dairy products being one of the main ones (Recker 1985). Other food rich in Ca include: herring, tofu, sesame seeds, flax seeds, and green leafy vegetables (International Osteoporosis Foundation). Net intestinal Ca absorption is the difference between total dietary Ca load and fecal Ca loss (Christakos 2012). In humans, twenty to seventy percent of total dietary Ca can be absorbed from the small intestine (Fleet 2006). And the efficiency of intestinal Ca absorption is strongly related to the dietary Ca bioavailability, which can be influenced by the presence of other nutrients such as fat intake, fiber, cocoa, tea, nuts, the overall body Ca status, and luminal pH (Wolf 2000).

Importantly, there are two major pathways for dietary Ca entering blood circulation from the intestinal lumen: paracellular diffusion and transcellular movement (Fleet 2010, Peng 2003) (**Figure 6**). In particular, paracellular Ca movement occurs in all three segments of the small intestine; but it predominates in the ileum due to the long transition time (Christakos 2012). Under conditions of adequate dietary intake, paracellular absorption contributes the majority of external Ca to the body (Fleet 2012). Mechanistically, in the non-saturable, paracellular model of Ca absorption, Ca passively diffuses into blood circulation through tight junctions and intermediate structures between enterocytes. Paracellular diffusion is energy-independent, and the absorption rate depends on luminal Ca concentration (Kruger 1997, Bronner 1990, 2003). In addition, luminal pH, solubility or the form of Ca in the diet, and enterocyte permeability all affect the amount of Ca that can be passively absorbed (Bronner 1990).

In contrast, transcellular movement of Ca requires facilitative and active transporters. This mainly occurs at the proximal part of the small intestine (duodenum) and it involves three steps: 1. entry across the apical epithelial membrane of enterocytes, 2. diffusion through the cytoplasm, and 3. exit at the basolateral cell membrane (Bronner F 1990). Major Ca transporters include protein

channel Trpv6, Ca binding protein (CalbD_{9k}), and Ca ATPase. These Ca transporters act together and deliver Ca into blood circulation (Fleet 2010, Feher 1983, Benn 2008). In detail, known as gate-keeper, Trpv6 translocates Ca cross the epithelial membrane of enterocytes (Peng 1999, Peng 2003). Then, cytoplasmic CalbD_{9k} delivers Ca from the apical to the baso-lateral membrane of enterocytes (Walters 1989). There are studies reporting that intracellular Ca diffusion is the rate-limiting step of active Ca movement due to the low self-diffusion rate of Ca, and CalbD_{9k} also serves as intracellular Ca reservoir within cells (Bronner 2003). Finally, at the basolateral membrane, Ca ATPase utilizes ATP and actively pumps Ca into the circulation (Benn 2008).

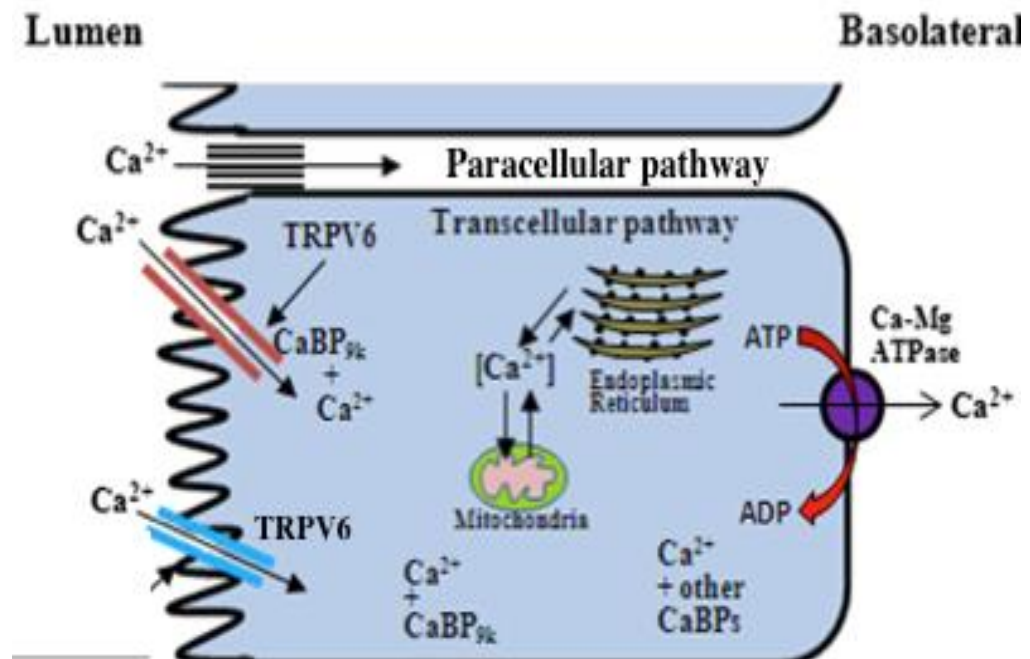


Figure 6. Intestinal calcium absorption¹.

¹ In the small intestine, paracellular and transcellular processes are the two major mechanisms by which dietary Ca is delivered to the blood circulation from the intestinal lumen. In paracellular movement, Ca passively diffuses into blood circulation through tight junctions and intermediate structures between enterocytes.

Transcellular transport of Ca requires facilitative and active transporters including TRPV6, CaBP9K, and Ca-Mg ATPase (Christakos 2012).

3.3. Factors influencing calcium status

The body's demand for Ca (and phosphate) fluctuates constantly as a result of environmental and physiological conditions. Factors such as dietary Ca loads in food, the efficiency of body Ca handling, growth, pregnancy, lactation, aging, and pathological conditions all affect the total body Ca pool (Fleet 2010). Maintaining optimal Ca metabolism is essential in protecting bone health as well as other non-calciotropic physiological functions (Deluca 2015, Veldurthy 2016). Hormonal regulation of Ca homeostasis via PTH, estradiol, and vitamin D is primarily achieved by affecting transcription of active Ca transporters involved in both intestinal Ca absorption and renal reabsorption (Favus 1981, Veldurthy 2016) (**Figure 7**). For instance, when serum Ca concentration is low, the vitamin D endocrine system directly up-regulates gene expression of TRPV6 and CalbD_{9k} in the small intestine and TRPV5 and CalbD_{28k} in the kidney to enhance the efficiency of dietary Ca absorption and renal Ca retention (Deluca 2015, Bouillon 2003). In addition, PTH and calcitriol also affect bone metabolism and release Ca to maintain serum concentration.

Dietary nutrients also influence Ca absorption. Protein, for example, may increase intestinal absorption. However, findings about the association between dietary protein intake and intestinal Ca absorption remain controversial (Dawson-Hughes 2015, Kerstetter 2003), and high protein intake results in increased urinary Ca loss that may lead to negative Ca balance and bone loss (Heaney 1982).

The effect of dietary fat intake on intestinal Ca absorption has been examined in both humans and rodents. However, similar to protein, the exact role of fat remains unclear, and fat intake could stimulate or inhibit intestinal Ca absorption depending

on the quantity and quality of the fatty acids. For example, there is evidence that high consumption of long-chain fatty acids interferes with Ca absorption by forming insoluble calcium-carboxylate (calcium-soap) (Gacs 1977). High fat feeding typically rich in SFA has been shown to greatly attenuate intestinal Ca absorption and increase fecal calcium loss (Agnew JE 1971, Tadayyon 1969, Cao 2011). In contrast, some studies showed that low amount of dietary fat in breast milk promotes Ca absorption in newborns.

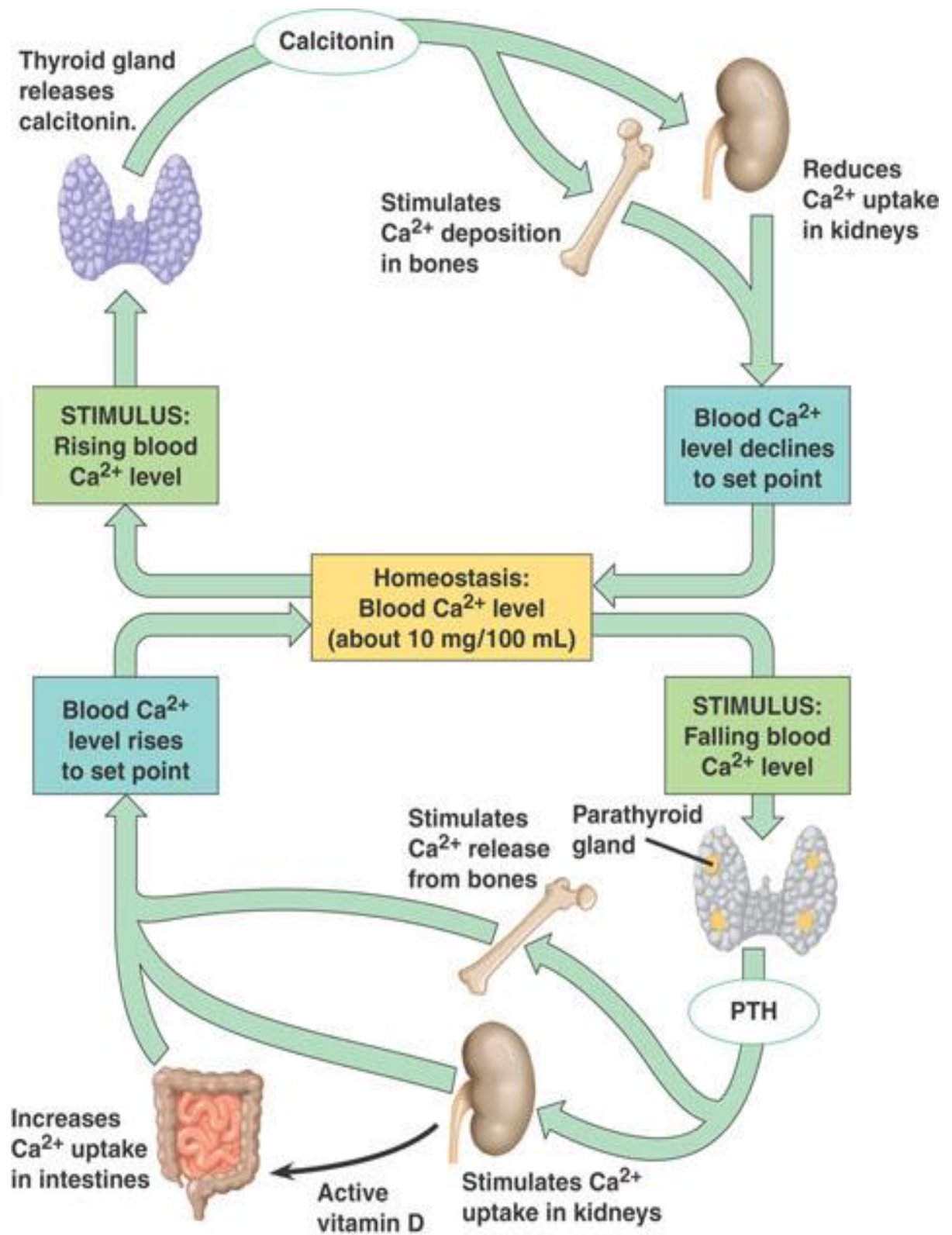


Figure 7. Calcium homeostasis¹.

http://hmsphysiology.pbworks.com/f/1267660258/45_11CalciumHomeostasis_L.jpg

¹When serum Ca concentration is low, parathyroid hormone (PTH) and calcitriol [1,25(OH)₂D] increase serum Ca concentration by directly up-regulating gene expression of Ca transporters in the small intestine and kidney to increase the efficiency of dietary Ca intake and urinary Ca reabsorption. In addition, PTH and vitamin D can also initiate bone resorption and mobilize calcified minerals in the bone to release Ca (Deluca HF 2012).

4. Vitamin D

Vitamin D is a lipid soluble vitamin that is an important calciotropic hormone involved in regulation of Ca and phosphorous homeostasis and bone metabolism. Due to ubiquitous expression of vitamin D binding receptor (VDR) in tissues, vitamin D also regulates many other physiological functions, such as immune functions, insulin sensitivity, cell differentiation and proliferation, and angiogenesis.

4.1 Vitamin D physiology and metabolism

Endogenous synthesis by skin under solar ultraviolet B radiation and dietary consumption (fish, liver, yolk, mushroom, and supplements) are the two sources of vitamin D (Olick 1981, Okano 1977, Brannon 2011, Tangpricha 2003). Vitamin D obtained from the diet can be either vitamin D₂ (ergocalciferol) in plants and fungi or vitamin D₃ (cholecalciferol) in animal source foods (**Figure 8**). For humans, the dietary reference intake (DRI) for vitamin D in the United State is 600 IU/d for adults and 800 IU/d for adults >70 years of age. In addition, for laboratory mice and rats, the recommended vitamin D level commonly added in chow or purified diet is 1000 IU/kg diet (National Research Council).

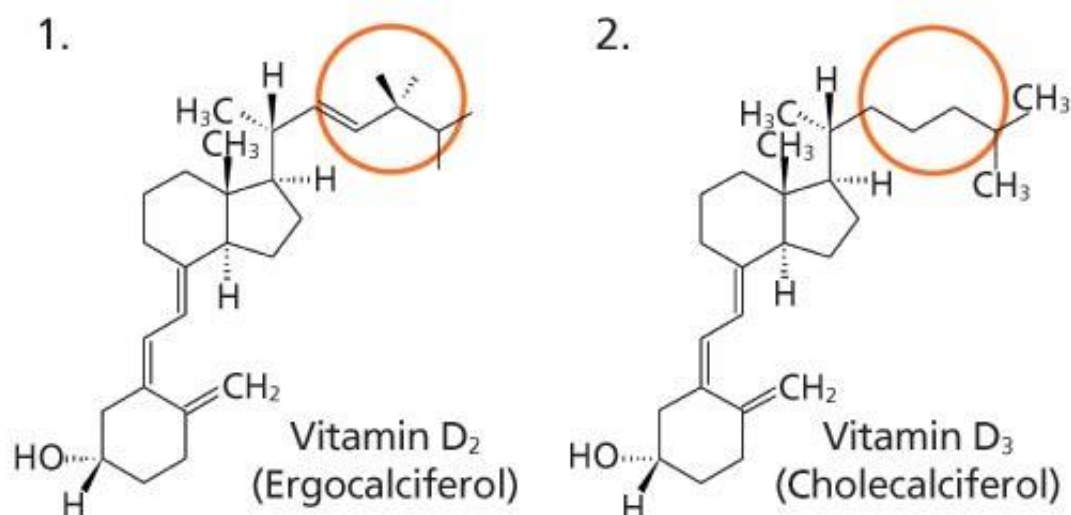


Figure 8: Structures of vitamin D₂ and D₃

<http://www.chromatographyonline.com/vitamin-d2-and-d3-separation-new-highly-hydrophobic-uhplcplc-phase>

Vitamin D has short half-life in circulation (about 1-2 days), and it is bound to vitamin D-binding protein (Mawer 1972). The vitamin D binding protein is synthesized in the liver and is evolutionarily related to albumin (Delanghe 2015). It has a high affinity for hydroxylated vitamin D metabolites including 25OHD (Herrmann 2017). Dietary or cutaneous synthesized vitamin D is biologically inert forms of the vitamin that can be stored in adipose tissue. However, the majority of vitamin D will be metabolized by two sequential hydroxylation reactions into the bioactive hormone in the liver and kidney (Mawer 1972, Deluca 2015, Holick 2002) (**Figure 9**). During the first step of bioactivation, which occurs in the liver, vitamin D 25-hydroxylase hydroxylates vitamin D at the 25-carbon position and forms 25 hydroxycholecalciferol (25OHD) (Bikle 2014, Christakos 2010). The 25OHD is the main circulating metabolite, and its serum concentration directly reflects the combination of dietary intake and sun-light exposure that determine the overall vitamin D status in the body (Holick 2002, Bikle 2014). The second activation step primarily occurs in the kidney in which 1 α -hydroxylase hydroxylates 25OHD at the 1 α -carbon position to form the

biologically active calcitropic hormone, 1,25-dihydroxycholecalciferol ($1,25(\text{OH})_2\text{D}$), also known as calcitriol (Bikle 2014, Christakos 2010). A second renal enzyme, 24-hydroxylase, initiates the degradation of excess vitamin D metabolites. 24-hydroxylase oxidizes excess $1,25(\text{OH})_2\text{D}$ and 25OHD at the C-24/C-23 positions to form calcitroic acids, which are water soluble and targeted for urinary excretion. (Bikle 2014, Jones 2012, Prosser 2004).

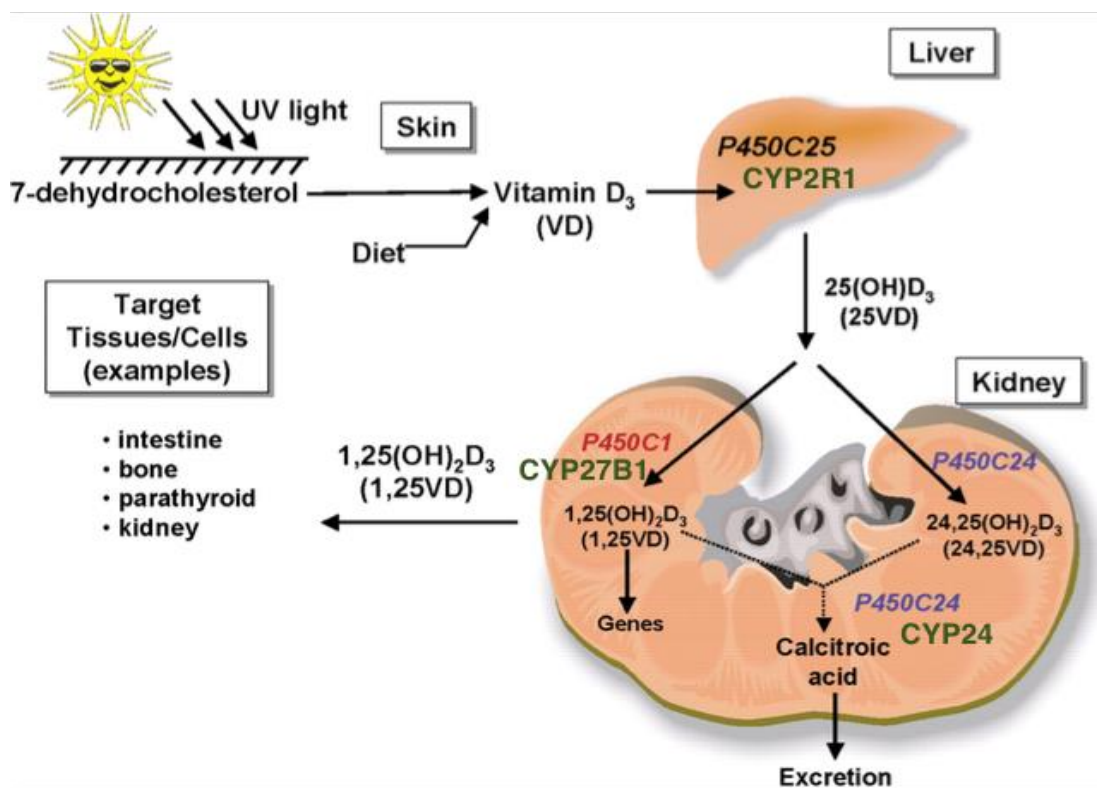


Figure 9. Vitamin D metabolism¹.

¹Dietary and endogenous synthesized vitamin D undergoes two sequential hydroxylation reactions in the liver and kidney that are catalyzed by 25-hydroxylase (CYP2R1) and 1 α -hydroxylase (CYP27B1) to be converted into the biologically active hormone $1,25(\text{OH})_2\text{D}$. Excess $1,25(\text{OH})_2\text{D}$ and 25OHD can be catabolized into water soluble calcitroic acids for urinary excretion by 24-hydroxylase (CYP24a1).

4.2 Vitamin D hydroxylases

Vitamin D 25-hydroxylase, 1 α -hydroxylase, and 24-hydroxylase are critically involved in vitamin D metabolic pathways as described above (Bikle 2014, Christakos 2010). Extensive effort has been put towards isolating and identifying the genes encoding these enzymes, as well as identification of regulating factors.

25-hydroxylase

In the liver, six enzymes are shown to have 25-hydroxylase activity (Takeyama 1997, Cheng 2003, Cheng 2004). They are: mitochondrial enzyme CYP27A1, and the microsomal enzymes CYP2C11, CYP2J2/3, CYP3A4, CYP2D25, and most importantly CYP2R1 (Zhu 2012, 2013). In detail, protein CYP27A1 is a multifunctional enzyme with broad substrate specificity, but is mainly involved in 26- or-27-hydroxylation of cholesterol and bile-acid synthesis (Usui 1990). CYP27A1 may not be important in vitamin D₃ activation in humans and rodents. Genetically modified CYP27A1 knockout (-/-) mice have normal or even elevated serum 25OHD concentration, when compared with wild type control mice (Rosen 1998, Aiba 2006, Zhu 2013). Protein CYP2C11 is both sex and species specific; to date, it has only been found in male rat liver (Zhu 2012, Hayashi 1988). Even though CYP2C11 has 25-hydroxylase activities, its primary function appears to be hydroxylation of testosterone (Rahmaniyan 2005). To date, enzyme CYP2D25 has only been isolated from experimental pig livers, and it is not found in humans or rodents (Achour 2011). CYP3A4 is primarily involved in drug metabolism, and shows low affinity toward vitamin D₃ (Gupta 2004).

In contrast, compared with other genes coding 25-hydroxylase, microsomal CYP2R1 is essential for hepatic vitamin D conversion in both humans and rodents (Cheng 2003, 2004, Roizen 2012). Enzyme CYP2R1 has high substrate affinity and specificity toward vitamin D (Shinkyo 2004), and it contributes a substantial amount

(~75%) of the hepatic 25OHD synthesis (Zhu 2013). In vivo studies show that serum 25OHD concentration is greatly attenuated in genetically modified CYP2R1^{-/-} mice when compared with wild type controls (Cheng 2003, Cheng 2004). Importantly, conversion of 25OHD is not hormonally regulated, though factors such as liver health and dietary nutrient intake may affect gene expression and activity of CYP2R1 (Takeyama 1997).

1 α -hydroxylase

The kidney is the primary tissue where circulating 1,25(OH)₂D is synthesized. Unlike 25-hydroxylase, thus far only one gene, CYP27B1, has been shown to code 1 α -hydroxylase (Bikle 2014). Mutation and deletion of gene CYP27B1 impairs 1 α -hydroxylase expression and activity, and leads to great reduction in circulating 1,25(OH)₂D with consequent impairment of bone metabolism and health in both humans and rodents (Fu 1997, Shinki 1997). Due to its potent endocrine role in Ca and bone metabolism, expression and activity of renal 1 α -hydroxylase is well regulated by three hormones that maintain serum concentration of 1,25(OH)₂ within a tight range. For example, under a normal physiological conditions, parathyroid hormone (PTH) increases circulating 1,25(OH)₂D, whereas fibroblast growth factor 23 (FGF23) lowers circulating concentration by transcriptionally up- or down-regulation of CYP27B1 gene expression (Bikle 1975, Urakawa I 2006). In addition, high serum 1,25(OH)₂D itself inhibits CYP27B1 in the kidney via the VDR and vitamin D inhibitory receptor pathway (Kim MS 2007). Furthermore, several other tissues, such as brain, intestine, adipose tissue, and bone, also express 1 α -hydroxylase, suggesting extrarenal synthesis of 1,25(OH)₂D is important for functions other than Ca absorption and bone (see below).

24-hydroxylase

Mitochondrial CYP24A1 is the only catabolic enzyme involved in vitamin D metabolism (Prosser 2004, Kundu 2014). In both humans and rodents, CYP24A1 has 24-hydroxylase activity, and it catalyzes the conversion of $1,25(\text{OH})_2\text{D}$ and 25OHD into 24-hydroxylated vitamin D metabolites including $1,25(\text{OH})_2\text{D}-26,23$ -lactone and calcitroic acid (Jones 2012, Bikle 2014). Because CYP24A1 mainly works to balance the effects of CYP27B1, the regulation of CYP24A1 is opposite that of renal CYP27B1 (Jones 2012, Bikle 2014); high serum concentration of $1,25(\text{OH})_2\text{D}$ and FGF23 up-regulate CYP24A1 gene expression, whereas elevated PTH attenuates CYP24A1 activity. Deletion or mutation of CYP24A1 causes toxic concentrations of $1,25(\text{OH})_2\text{D}$ and 25OHD to accumulate in the circulation, which leads to abnormal bone mineralization (Jones 2012).

4.3 Factors affecting vitamin D status

Vitamin D status can be affected by many factors. These include the amount of sun exposure, which can be affected by season and latitude, sunscreen usage, dietary vitamin D intake, supplementation, Ca and phosphate status, skin pigmentation, age, and sex (Matsuoka 1987) (**Figure 10**). Hormonal factors such as serum PTH and FGF23 regulate serum $1,25(\text{OH})_2\text{D}$ by directly affecting gene expression of 1α -hydroxylase in the kidney and by indirectly affecting serum Ca/P concentrations and bone turnover (Henry 2011, Quarles 2012). In addition, overall health status also has a role; chronic liver and kidney diseases may compromise vitamin D synthesis and bioavailability. Moreover, an increasing amount of effort has been put on focusing the effects of dietary nutrients, such as fat intake, on vitamin D regulation. For example, some studies show that dietary fat may promote intestinal absorption (Hollander D 1978, Dawson-Hughes 2014). In humans, clinical trials have shown that serum 25OHD concentration is higher in people who had fat-enriched

meals when compared with people who had low-fat meals after a single, high dose of oral vitamin D intake (Dawson-Hughes 2014, Raimundo 2011). However, other studies in rodents and humans have found that high fat intake has a null or negative impact on vitamin D absorption (Tangpricha 2003, Holvik 2007, Korkor 2009).

Importantly, there is an inverse association between body adiposity and serum 25OHD concentration in both human subjects and rodents across different age groups (Dyląg 2014, Gonzalez-Molero 2013). Obese individuals have a greater chance of developing vitamin D deficiency when compared with normal weight individuals. This may be due to poor dietary intake, or may be due to a sedentary lifestyle and low outdoor UV light exposure (Díez-Rodríguez 2014). Additionally, low serum 25OHD in overweight/obese populations may be attributed to a dilution effect by adipose tissue on fat-soluble vitamins (Wortsman 2000). Indeed, circulating 25OHD is elevated after weight reduction in obese people.

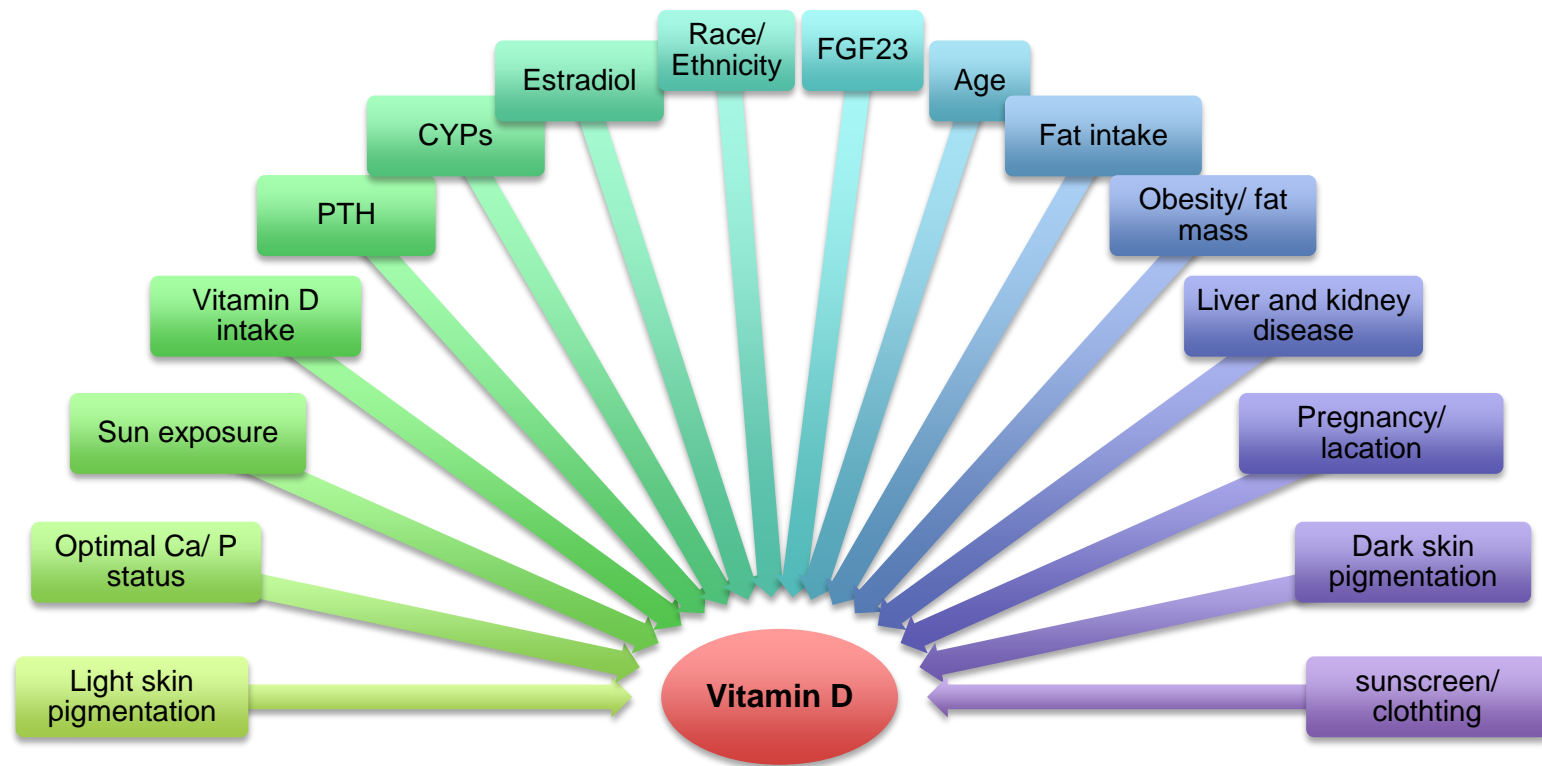


Figure 10. Factors affecting body vitamin D status¹.

¹Vitamin D status is influenced by age, skin pigmentation, and other factors affecting the amount of sun exposure. Dietary vitamin D, supplements, and fat intake also play a role. In addition, liver and kidney disease, Ca and phosphate status, hormonal influence of PTH, FGF23 and estradiol all affect vitamin D status (Matsuoka 1987).

4.4 Prevalence of vitamin D deficiency

Despite the fact that vitamin D fortification is common in foods such as milk, cheese, cereals, and orange juice, vitamin D deficiency is common in children and adults (Holick 2008). According to the NHANES data in year 2010, more than 25% of the US population was at risk of vitamin D inadequacy as indicated by serum 25OHD concentration between 21–29 ng/mL (52 to 72 nmol/L); and 8% of the population was at risk of vitamin D deficiency (serum 25OHD \leq 20 ng/mL [\leq 50 nmol/L]).

4.5 Physiological functions and consequences of deficiency

The vitamin D endocrine system is an essential regulator of Ca (and phosphate) homeostasis. Upon ligand-binding to its receptor VDR, 1,25(OH)₂D-VDR complex transcriptionally regulates genes involved in Ca transporters including TRPV6, CalbD_{9k}, and PMCA1B in the small intestine, and TRPV5, CalbD_{28k}, and PMCA1B in the kidney (Bikle 2012, 2014). Therefore, calcitriol directly regulates the body Ca pool by altering the efficiency of intestinal absorption and renal reabsorption. For example, under conditions of inadequate dietary intake and low circulating Ca concentration, 1,25(OH)₂D along with PTH up-regulate gene expression of Ca transporters that enhances intestinal absorption and decreases urinary Ca loss (Bikle 2014, Henry 2011, Quarles 2012). In addition, 1,25(OH)₂D may stimulate osteoclastogenesis and bone resorption to release Ca stored in bones. However, the specific role of 1,25(OH)₂D on bone metabolism and health remains elusive, and whether or not vitamin D prevents osteoporosis, falls, and fracture risk remains controversial (Bikle 2012). Even though there is a positive association between dietary vitamin D intake and bone mass (Bischoff-Ferrari 2006), it is unclear whether the beneficial effects of vitamin D on bone mass are directly attributed to its effects on the Ca and phosphate pools in the body, or are attributed to the direct effects of 1,25(OH)₂D on bone cells (osteoblasts vs. osteoclasts). Nevertheless, the

primary outcome of vitamin D deficiency in the body is the detrimental effect on Ca homeostasis and bone health. Only 10-15% of dietary Ca can be absorbed without vitamin D, while that percentage increases to 30-45% with adequate vitamin D intake (Holick 2007). Thus, chronic vitamin D deficiency results in classic Ca deficiency diseases, rickets in children and osteoporosis in adults (Holick 2007).

Besides the kidneys, many other tissues, such as adipocytes, brain, pancreas, and breast, express 1α -hydroxylase and VDR, indicating that these tissues can synthesis and utilize $1,25(\text{OH})_2\text{D}$. Therefore, vitamin D putatively has pleiotropic effects on a variety of physiological systems beyond bone and Ca metabolism (**Figure 11**). Locally synthesized $1,25(\text{OH})_2\text{D}$ functions more like paracrine or autocrine hormones, and approximately two hundred genes can be directly and indirectly regulated by the $1,25(\text{OH})_2\text{D}$ -VDR complex (Holick 2007). These genes are involved in regulation of inflammatory cytokines, oxidative stress responses, cellular proliferation, differentiation, apoptosis, and angiogenesis (Kong M 2014, Dusso 2005, Cui 2013, González-Molero 2014, Marcotrichino 2012). For example, in the pancreas, vitamin D enhances autophagy and reduces apoptosis of pancreatic β -cells, prevents insulinitis, and enhances insulin synthesis and sensitivity (Altieri 2016, Wang 2016). Vitamin D deficiency may increase the risk of developing insulin resistance and type II diabetes mellitus (Noyola-García 2016,). Moreover, vitamin D deficiency is closely associated with a variety of non-skeletal chronic diseases, including muscle weakness, type I diabetes, rheumatoid arthritis, hypertension, cardiovascular diseases, neurological disorders, and cancers (colon, breast and prostate) (Sergeev 2014, Littlejohns 2014, Bischoff-Ferrari 2006).

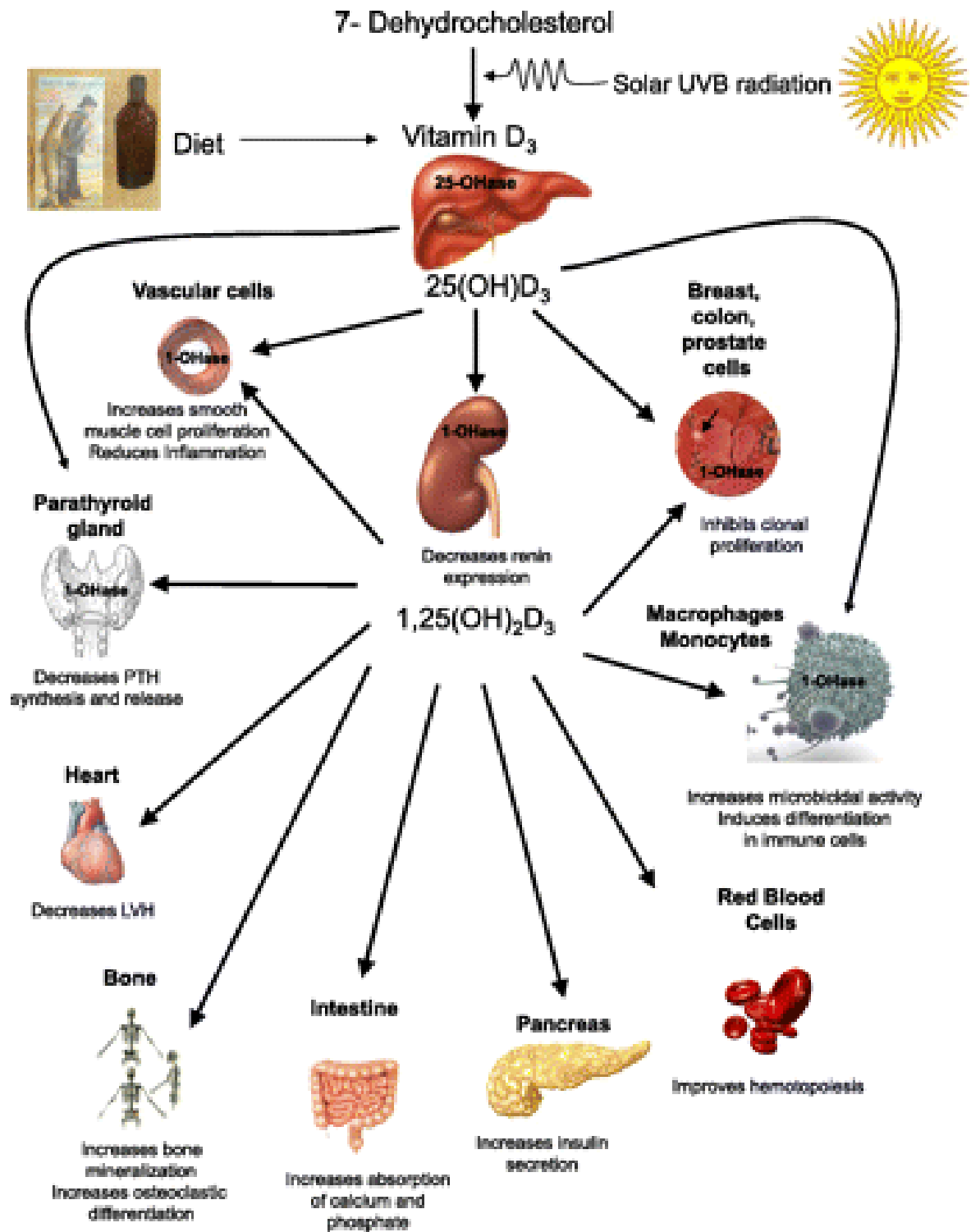


Figure 11. Physiological functions of Vitamin D¹.

<http://ajprenal.physiology.org/content/297/6/F1502>

¹ Beyond the calciotropic role in regulating Ca and bone metabolism, locally synthesized vitamin D modulates inflammatory cytokines, oxidative stress, cellular

proliferation, differentiation, apoptosis, and angiogenesis. Thus, vitamin D has pleiotropic effects on variety of physiological functions including insulin sensitivity, immunity, neurological function, and adipogenesis.

4.6 Vitamin D and obesity

Epidemiology studies report that obese individuals have lower circulating 25OHD concentration, and show greater risk to develop vitamin D deficiency, or hypovitaminosis D (Bell 1985, Liel 1988, Cheng 2010, Wortsman 2000). The inverse association between circulating 25OHD and obesity is found in association with all obesity related parameters, including BMI, fat mass, abdominal adiposity, and waist circumference (Liel 1988, Dyląg 2014, Lenders 2009, Gonzalez-Molero 2013, Tsiaras 2011, Tamer 2012). Nevertheless, the nature of the relationship between vitamin D deficiency and obesity remains elusive, and whether or not it is vitamin D deficiency that promotes adiposity or if it is obesity that causes vitamin D deficiency is unclear. It is possible that obesity and its related factors may directly cause vitamin D deficiency via various mechanisms. For example, poor dietary choices that are low in essential nutrients directly lead to low vitamin D consumption and deficiency. There is also evidence that high-fat meals interrupt intestinal vitamin D absorption, though low amounts of dietary fat facilitate vitamin D (fat-soluble) absorption (Dawson-Hughes 2013). Decreased outdoor physical activity and UVB radiation attenuate cutaneous vitamin D biosynthesis. Moreover, a greater amount of white adipose tissue may have dilution effects on lipid soluble vitamins that possibly decrease vitamin D bioavailability. Indeed, several clinical trials report that weight reduction without dietary supplementation increases circulating concentration of 25OHD in humans (Wortsman 2000, Gangloff 2016). Alternatively, vitamin D deficiency may contribute to obesity (Foss 2009). This theory is proposed based on an adaptive response for greater white adipose tissue accumulation in mammals to

maintain a normal physiological temperature and survival rate in cold winter time, and that circulating 25OHD fluctuates seasonally and falls in winter (Foss 2009). It is possible that increased appetite and food consumption and lowered energy expenditure are attributed to lowered vitamin D status in the body, suggesting a role of vitamin D on hypothalamic regulation of energy homeostasis (Foss 2009). Similar findings that vitamin D directly regulates thermogenesis in brown and beige adipose tissue and reduces obesity are also seen in rodent studies (Crane 2014). Moreover, VDR is widely distributed in the body and is found in bone marrow and adipocytes, and there has been evidence showing that $1,25(\text{OH})_2\text{D}$ affects bone marrow adipogenesis (Duque 2004). However, whether or not $1,25(\text{OH})_2\text{D}$ stimulates or suppresses adipocyte differentiation and increases fatty acid oxidation remains inconsistent among *in vivo* and *in vitro* studies (Ding 2012).

5. Serotonin, vitamin D and Obesity

5.1 Function and metabolism of serotonin in the CNS

Serotonin, also known as 5-hydroxytryptamine, is an important monoaminergic neurotransmitter that is involved in the regulation of neuroendocrine functions including energy balance, circadian rhythms, regulation of mood, stress response, and other behavior traits (Tecott 2007, Hale 2012, Voigt JP 2015). In particular, the serotonergic effectors projected to the arcuate nuclei in the brain is essential in hypothalamic regulation of feeding behavior. Extensive studies have demonstrated that serotonin is shown to suppress appetite, reduce food intake, and promote satiety (Tecott LH 2007, Voigt JP 2015). There is evidence that serotonin reduces dietary fat intake as well (Blundell JE 1995).

Within the neurons in the CNS, the essential amino acid tryptophan undergoes two sequential steps catalyzed by the enzymes tryptophan hydroxylase 2 (TPH2) and aromatic amino acid decarboxylase to be converted into serotonin

(**Figure 12**). The enzyme TPH2 is important (Champier 1997, Noguchi 1973) in regulating production of serotonin and is widely expressed in neuronal cells in the central nervous system (CNS) (Walther 2003, Gutknecht 2009). Studies from clinical trials and rodents have showed that central serotonin synthesis is greatly attenuated in rodents with dysfunctional TPH2 or genetically modified TPH2^{-/-} rodents (Brommage 2015, Kriegebaum 2010, Berger 2012). In addition, peripheral serotonin synthesized by tissues such as the gastrointestinal tract, lung, kidney, platelets, and brown adipose tissue is catalyzed by tryptophan hydroxylase 1 (TPH1) (Mohammad-Zadeh 2008). However, peripheral serotonin cannot travel cross the blood-brain-barrier into the brain, and central and peripheral serotonin are synthesized separately and function independently from each other.

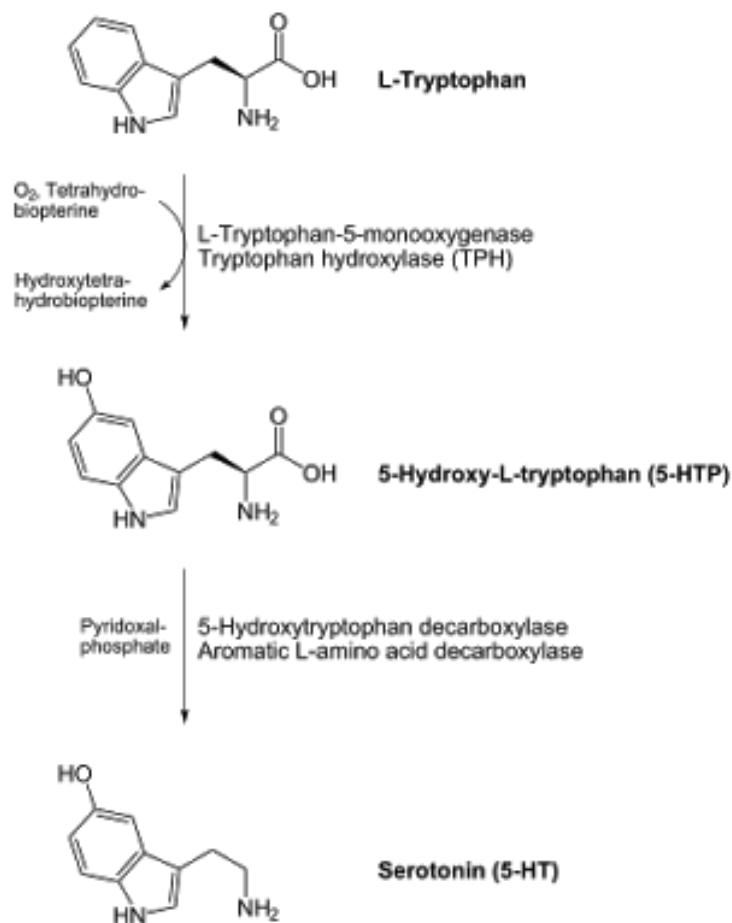


Figure 12. Conversion of tryptophan to serotonin.

<http://adhd-treatment-options.blogspot.com/2010/02/does-tyrosine-for-adhd-actually-work-a.html>

¹The synthesis of serotonin (5-hydroxytryptamine) initially involves the conversion of L-tryptophan to L-5-hydroxytryptophan by the enzyme tryptophan hydroxylase (TPH). There are two TPH enzymes: TPH1, which functions in peripheral tissues, and TPH2, which functions in the brain. Decarboxylation of L-5-hydroxytryptophan by the cytosolic enzyme, L-aromatic amino acid decarboxylase, then produces serotonin.

Newly synthesized serotonin in presynaptic neurons is temporally taken up and stored in synaptic vesicles via the action of vesicular monoamine transporter. Serotonin is eventually released into the synaptic cleft and binds to its receptors on the postsynaptic cell membrane to propagate serotonergic messaging between neurons (**Figure 13**). The diverse serotonergic effects are determined by the type of receptors that serotonin binds to (Mohammad-Zadeh 2008). In particular, when examining the role of serotonin in regulating energy homeostasis, serotonin receptors 5-HT1A and 5-HT2C are important in controlling feeding, including the amount and speed of food intake (Donovan 2013). Termination of the postsynaptic serotonergic effect is mediated by a transmembrane transporter: the serotonin reuptake transporter (SERT) (Mohammad-Zadeh 2008, Ni 2006). Located on the presynaptic cell membrane, SERT mediates reuptake of excess serotonin in the synaptic cleft and controls the abundance of serotonin; thus SERT regulates both the strength and duration of serotonergic effects.

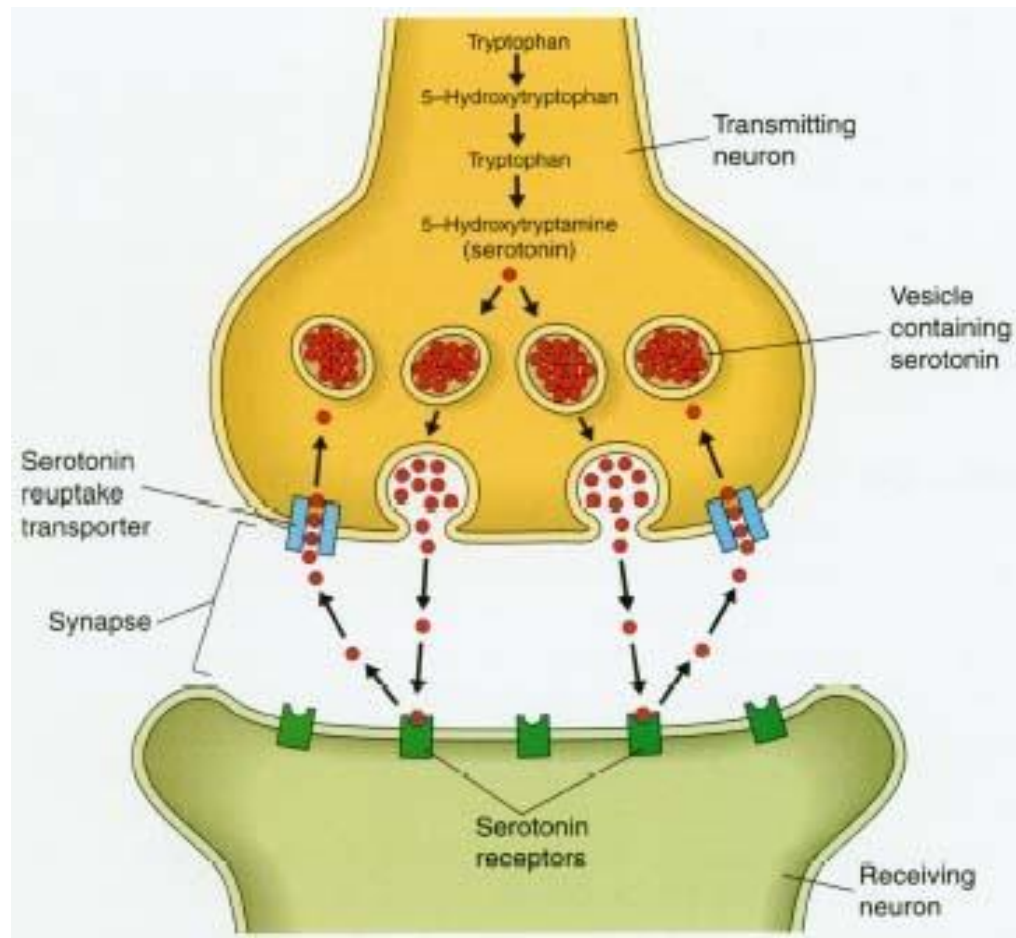


Figure 13. Serotonin pathway in the CNS

<https://github.com/research-team/NEUCOGAR/wiki/Serotonin-in-the-brain>

¹Serotonin in the presynaptic neurons is released into the synaptic cleft and binds to serotonin receptors on the postsynaptic cell membrane to propagate serotonergic messages between neurons. Termination of serotonergic signaling is mediated by the transmembrane serotonin reuptake transporter (SERT) (Mohammad-Zadeh 2008, Ni 2006).

5.2 Serotonin regulation, vitamin D, and obesity

Pharmaceutical methods can be used to alter serotonergic effects in the brain. Alternatively, non-pharmaceutical factors such as stress, aging, exposure to bright light, lack of physical activity and combinations of these factors have adverse

effects on serotonergic pathways (Young 2007). Dietary nutrients are also shown to affect serotonin synthesis, and high fat feeding, for instance, may compromise central serotonin synthesis. There also is evidence in rodents that serotonin concentration in the brain stem is greatly lowered in rats consuming a 20% high fat diet when compared with rats consuming a 5% low fat diet (Kimbrough 1984).

Recently, a putative activating vitamin D response element (VDRE) was identified in the promoter regions of the serotonin synthesizing enzymes, TPH1 and TPH2 (Patrick 2014, Patrick 2015), suggesting that vitamin D potentially affects central and peripheral serotonin synthesis by transcriptionally regulating the gene expression of TPH1/TPH2. Importantly, an *in vitro* study supports this assumption; gene expression of TPH2 was shown to have a dose-dependent response to 1,25(OH)₂D in rat brain cells (Kaneko 2016). Since both vitamin D and serotonin status are associated with eating disorders and obesity, it is possible that vitamin D regulates food intake and adiposity via the serotonergic pathway. However, to date, the relationship between vitamin D status and central serotonin status and pathways remains unclear.

6. Conclusion and rationale

High fat diets and obesity influence intestinal Ca absorption and bone health. However, the effects of dietary fat rather than diet induced obesity and type of dietary fatty acids (MUFA vs. SFA) on Ca and bone have not been addressed. In addition, although circulating vitamin D concentration is inversely associated with several parameters related to obesity (BMI and fat mass), the nature of the relationship between vitamin D deficiency and obesity remains unclear. This raises several questions of whether or not high fat feeding and obesity directly interfere with systemic vitamin D metabolism and lead to low vitamin D status that is beyond the simple explanation of the adipose dilution effect; and whether or not dietary vitamin D alters energy homeostasis and contributes to the pathogenesis of obesity. Therefore, the goal of this dissertation is to address these unanswered questions between high fat feeding and Ca and bone metabolism under different caloric intake and obesity conditions, and to explore the cause or effect relationship between vitamin D deficiency and obesity.

CHAPTER II: SPECIFIC AIMS AND HYPOTHESIS

Aim 1. To explore whether high fat feeding, in the absence of obesity or excess caloric intake, affects Ca and bone metabolism in an aging murine model. The secondary goal is to identify whether dietary MUFA or SFA has a differential effect on Ca and bone metabolism. We will test the hypothesis that HFD increases fractional Ca absorption and gene expression of Ca transporters in the small intestine. We will also test the hypothesis that dietary SFA have detrimental effects on bone, and dietary MUFA have a neutral or beneficial effect.

Aim 2. To determine whether HFD and obesity affects vitamin D metabolism and bone health in adult female mice; and to determine whether dietary MUFA and SFA have differential effects on vitamin D regulating enzymes. It is hypothesized that high fat feeding and obesity attenuate serum 25OHD concentration and impair vitamin D 25-hydroxylase in the liver. In addition, it is hypothesized that dietary MUFA and SFA differentially affect vitamin D regulating enzymes in the liver and kidney,

Aim 3. To determine if different levels of dietary vitamin D intake affect the pathogenesis of obesity under conditions of normal and excessive fat intake. The secondary objective is to explore whether vitamin D deficiency affects food intake and obesity by altering serotonin status in the CNS. It is hypothesized that supplemental vitamin D protects HFD induced obesity; whereas, low vitamin D feeding promotes food intake and adiposity, and compromises cerebral serotonin synthesis by down regulating gene expression of tryptophan hydroxylase 2 in the raphe and arcuate nuclei of mature female mice.

CHAPTER III: EXPERIMENTAL METHODS

Body composition. Total body composition including fat mass, lean mass, total body water, and free water were determined in live mice at the beginning or the end of each animal experiment via magnetic resonance imaging (EchoMRI Body Composition Analyzer EMR-129).

Bone densitometry. Total body BMD and BMC were determined at the end of each experiment using dual-energy X-ray absorptiometry after mice were killed and the blood and tissues were removed (DEXA) (GE-Lunar PIXImus mouse densitometer; software version 2.10.41). After total body BMD and BMC measurement, individual bones such as left femur, left tibia, left humerus, left radius, and lumbar spine (L1-5) were dissected, wrapped individually with 1x phosphate buffered saline (PBS) soaked gauze, and stored at -20°C. Regional BMD and BMC at specific anatomical bone sites were evaluated by placing the excised individual bone site on a Delrin block in the PIXImus. The mean and CV of three repeated scans of BMD of total tibia and total femur BMD were achieved in each study.

Bone micro-computed tomography. Left femurs were scanned using a microcomputerized tomography system (vivaCT 35, Scanco Medical AG, Brüttisellen, Switzerland) to analyze femoral geometric parameters. A scout view of the entire femur was first performed to measure the length of the femur from the upper extremity to the lower extremity. Then the proximal end of the femur corresponding to a 0.3-1.2 mm region below the lowest point of growth plate was scanned at 6µm isotropic voxel size to acquire a total of 220 µCT slides. All images were first smoothed by a Gaussian filter (sigma=1.2, support=2.0) and then a threshold was determined corresponding to 36% of the maximum available range of image gray scale values. The images of the secondary

spongiosa regions 0.3-0.9 mm below the lowest point of the growth plate were contoured to determine trabecular area. For cortical area, we analyzed the 0.3-mm region centered at the mid-point of each femur. Geometric trabecular volumetric bone mineral density (vBMD), bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), structure model index (SMI), cortical BMD, cortical bone area (Ct.Ar), cortical bone thickness (Ct.Th) and moment of inertia (J) were calculated by 3D standard microstructural analysis provided by the manufacturer (Boxian ML 2010).

Calcium absorption. Prior to any feeding intervention, eighteen mice (n=6 per dietary group) were randomly selected to assess Ca balance using a radioisotope method. Intestinal Ca absorption and metabolism were measured in the same mice at the beginning (wk0) and the end (wk8) of the study using a modified method as previously described [19]. Briefly, mice were individually kept in metabolic cages. After a 4d adaptation period (d 1-4), mice received an intramuscular injection of 2 MBq (0.054 mCi) of ^{45}Ca to trace the endogenous Ca secretion in the small intestine. Calcium balance was determined over a 5d period (d 5-9). Throughout the 5 days, urine samples were collected and the pH was adjusted to <2 with HCl to avoid Ca precipitation; fecal samples were collected and ashed at 600°C for 18h and the residue was dissolved in HCl (3 mol/L). Fecal and urinary ^{45}Ca were determined by liquid scintillation counting. Fecal and urinary total Ca concentration (Ca_{total}) was determined using atomic absorption spectrometry.

Calculations. Calcium balance, fractional calcium absorption (FCA), and endogenous fecal Ca were calculated using the following equations:

1. $\text{Ca consumption (mmol/d)} = \text{mean food consumption (g/d)} \times \text{food Ca (mmol/g)}$

2. $\text{Ca balance (mmol/d)} = \text{Ca consumption (mmol/d)} - (\text{fecal Ca}_{\text{total}} + \text{urine Ca}_{\text{total}})$
(mmol/d)
3. $\text{Endogenous fecal Ca (mmol/d)} = \text{urine Ca}_{\text{total}} \text{ (mmol/d)} \times (\text{fecal } ^{45}\text{Ca (mmol/d)} / \text{urine } ^{45}\text{Ca (mmol/d)})$
4. $\text{Unabsorbed dietary Ca (mmol/d)} = \text{fecal Ca}_{\text{total}} \text{ (mmol/d)} - \text{endogenous fecal Ca (mmol/d)}$
5. $\text{FCA} = (\text{Ca consumption (g/d)} - \text{unabsorbed dietary Ca (g/d)}) / \text{Ca consumption (g/d)}$
 $\text{Intestinal Ca secretion (mmol/d)} = \text{endogenous fecal Ca (mmol/d)} / (1 - \text{FCA})$

Lipid extraction. Approximately 100mg of liver tissue was homogenized with a tissue homogenizer in 700uL of 1xPBS. Protein concentration of homogenates was determined using BCA Protein Assay (Bio-Rad) with bovine serum albumin (BSA) as the standard. The rest of the liver homogenates were then diluted into 1 mg/mL with 1xPBS, and stored at -80°C. For hepatic lipid extraction, 20mL of 2:1 (v:v) chloroform-methanol (CHCl_3 -MeOH) was added to 1mL of the 1mg/mL liver homogenate, and the mixture was filtered through fat free #1 Whatman filter paper (150mm diameter). Then 4mL of 50mM KCL was added into the mixture, vortexed for 45sec, and centrifuged for 10 min at 1,200 x g, 4°C. The mixture was separated into 2 layers. The upper aqueous layer was discarded, and the lower lipid-containing layer was isolated and dried under N_2 . Total lipid percentage of each sample was calculated by dividing the isolated lipid weight by the tissue weight.

Hepatic triglyceride. For hepatic triglyceride (TG) analysis, isolated lipid samples were dissolved in 1:1 (v:v) CHCl_3 -MeOH and subjected to thin layer chromatograph (TLC) (Sigma-Aldrich). TLC plates were incubated in the nonpolar solvent 70:30:1 (v:v:v) of hexane:ethyl ether:acetic acid until separation of lipids was complete. The TLC plate

was then exposed in an iodine tank for approximate 5min. The TLC plates were then scanned and digitized signals were quantified using the picture analysis software (Image J, 1.48v). Hepatic TG concentration of each sample was calculated based on a lipid standard curve.

RNA isolation and real-time PCR. Total RNA from tissues, including liver, kidney, and intestinal mucosa cells, was extracted according to the TRIzol protocol (Ambion, Life Technologies). Total RNA of specific brain regions, including arcuate nuclei and raphe nuclei, was isolated using an RNA kit (RNAqueous, Ambion). The RNA concentration was quantified using a Nano-drop (ND-1000, V7.2.1). RNA was reverse transcribed into cDNA with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Primers of interested genes, including vitamin D 1 α -hydroxylase Cyp27b1, vitamin D 25-hydroxylase Cyp2e1, 24-hydroxylase Cyp24, VDR, TPH2, serotonin receptor 2C (Htr2c), serotonin transporter (SERT), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were purchased (Taqman, Applied Biosystems). Real-time PCR was conducted on samples using Step-One Plus real-time PCR system (Applied Biosystems). Expression of all genes was normalized to the expression of GAPDH as the endogenous control within each sample.

Western blotting. Approximately 200mg of wet weight of liver was homogenized with a tissuemizer (Fisher) in 1mL homogenizing buffer (0.33 mol/L sucrose, 5mmol/L HEPES, 1 mmol/L EDTA, 1mmol/L DTT, sterilized H₂O, pH7.4) containing 1 μ L protease inhibitor (Bio-Rad). Homogenates were centrifuged for 10 min at 12,000 x g, and the supernatant was collected. Protein concentration was determined using BCA Protein Assay (Bio-Rad) with BSA as the standard. Total protein (30 μ g) was separated by electrophoresis on gradient (4-12%) SDS-PAGE (Novex) gels followed by electro transfer to

nitrocellulose membranes (Bio-Rad). Membranes were blocked overnight at 4°C using 5% of BSA in 1xTBS-T (20 mmol/L Tris-HCl, 135 mmol/L NaCl, 0.1% Tween-20, pH 8.0). Membranes were probed with rabbit CYP2R1 primary anti-mouse antibodies (Abcam) and anti-rabbit secondary antibodies (GE Healthcare). Bound antibodies were detected using the ECL western blotting detection reagent (GE Healthcare) with X-OMAT LS film at room temperature. Films were scanned and digitized signals were quantified using gel analysis software (UN-SCAN-IT gel, version 6.1). Used membranes were stripped with stripping buffer (100mmol/L 2-mercaptoethanol, 2% SDS, 62.5 mmol/L Tris-HCl, pH6.7) at 40°C and re-probed with mouse β -actin (Cell Signaling) primary antibodies and anti-rabbit secondary antibodies to correct for unequal loading and transfer.

Serum biochemistry analysis. At the end of the study, approximately 500uL of trunk blood was collected from each mouse following decapitation. Blood was held at room temperature for at least 30min to complete coagulation. After being centrifuged for 10min at 1,200 x g, 4°C, the clear serum was collected. Serum biochemistry was determined using mouse/rat ELISAs: serum estradiol (Calbiotech, Cat# ES 180S-100), 25OHD (Egal Bioscience), FGF23 (Immunotopics), and PTH (Immunotopics).

Brain frontal cortex serotonin concentration. Brain cortex was homogenized with 1x PBS and serotonin concentration was determined with a mouse Elisa kit (Blue Genes).

CHAPTER IV: STATISTICAL ANALYSES

Data are presented in the text and in tables as means \pm SD and in figures as means \pm SEM. One-way ANOVA was used to evaluate the statistical differences among dietary groups for most of the experimental measurements in studies one and two. When the *F* ratio was significant, Tukey's HSD or Scheffe's post-hoc analysis was conducted. A *P* value <0.05 was considered significant, and a *P* value <0.1 was considered to be borderline significant. The effect of dietary fat on Ca metabolism in study two and most experimental outcomes from study three were evaluated using two-factor ANOVA with dietary treatment and time as the independent factors. Repeated measures ANOVA was used to compare the cumulative food intake and weekly body weights among groups in all three studies. The Pearson correlation coefficient was determined to assess the relation between body weight and serum 25(OH)D concentration. A power analysis for FCA and BMD in study one was done using a previous study examining energy-restricted rodents vs. controls with identical methods (^{45}Ca and DXA). It was found that with α set at 0.05 and 80% power, 6 or fewer mice were necessary for statistical significance. In addition, 4 mice were adequate (80% power with $\alpha = 0.05$) for BV/TV [19]. Grubb's test to identify outliers was used before statistical analysis. All analyses were conducted using the SAS statistical package (SAS Institute, Cary, NC, USA; v9.3).

CHAPTER V: EXPERIMENTS

**EXPERIMENT 1: HIGH FAT DIET ENRICHED WITH SATURATED, BUT NOT
MONOUNSATURATED FATTY ACIDS ADVERSELY AFFECTS FEMUR, AND BOTH
DIETS INCREASE CALCIUM ABSORPTION IN OLDER FEMALE MICE**

Abstract

Diet induced obesity has been shown to reduce bone mineral density (BMD) and Ca absorption. However, previous experiments have not examined the effect of high fat diet (HFD) in the absence of obesity or addressed the type of dietary fatty acids. The primary objective of this study was to determine the effects of different types of high fat feeding, without obesity, on fractional calcium absorption (FCA) and bone health. It was hypothesized that dietary fat would increase FCA and reduce BMD. Mature 8-month-old female C57BL/6J mice were fed one of three diets: a HFD (45% fat) enriched either with monounsaturated fatty acids (MUFAs) or with saturated fatty acids (SFAs), or a normal fat diet (NFD; 10% fat). Food consumption was controlled to achieve a similar body weight gain in all groups. After 8wk, total body bone mineral content and BMD as well as femur total and cortical volumetric BMD were lower in SFA compared with NFD groups ($P < 0.05$). In contrast, femoral trabecular bone was not affected by the SFAs, whereas MUFAs increased trabecular volume fraction and thickness. The rise over time in FCA was greater in mice fed HFD than NFD and final FCA was higher with HFD ($P < 0.05$). Intestinal calbindin- D_{9k} gene and hepatic cytochrome P450 2r1 protein levels were higher with the MUFA diet than the NFD diet ($P < 0.05$). In conclusion, HFDs elevated FCA overtime; however, an adverse effect of HFD on bone was only observed in the SFA group, while MUFAs show neutral or beneficial effects.

Keywords: Bone, calcium absorption, fatty acids, high-fat diet, mice.

Introduction

Total body Ca balance is determined by intestinal Ca absorption and urinary Ca excretion. Since plasma Ca concentration fluctuates constantly as a result of environmental and physiological impacts [Fleet 2007], maintaining Ca balance is important to ensure optimal metabolic function and structural integrity of bone [Fleet 2007, Cashman 2007]. Two major pathways of luminal Ca entering the circulation are paracellular and transcellular movement [Fleet 2007, Christakos 2012]. In particular, in the transcellular movement of Ca, crucial transporters including the apical epithelium protein channel transient receptor potential cation channel subfamily V member 5 and member 6 (TRPV5/TRPV6), intracellular calbindin-D9k (CalbD9k), CalbD28k, and the basolateral plasma membrane Ca ATPase act together and transport Ca into the blood [Fleet 2007]. The transcellular movement of Ca is principally regulated by the calciotropic hormone 1,25-dihydroxycholecalciferol, the bioactive metabolite of vitamin D that directly enhances Ca absorption in the small intestine and reabsorption in the kidney when serum Ca concentration is low. Other factors such as dietary fat also affect intestinal Ca absorption.

Dietary fat can stimulate or inhibit intestinal Ca absorption depending upon the type and amount of fat intake. High fat diets (HFD) may reduce Ca absorption by forming insoluble Ca soaps [Gacs 1997]. A moderately high fat ad libitum feeding also results in changes of the duodenal oxidation state that lowers Ca absorption in mice [Xiao 2010]. In addition, studies suggest that HFD that are typically rich in saturated fatty acids (SFAs), negatively affect bone mineral density (BMD) during growth in rat studies [Macri 2012]. This is supported by observational studies in adult humans showing that a high fat intake is inversely associated with BMD [Corwin 2006, Kwon 2015]. Conversely, we and others found that dietary fat intake was positively related to Ca absorption in both human and rodent studies [Shapses 2012, Wolf 2000, Suzuki 2010]. Since obesity is

associated with compromised bone quality both in clinical trials [Premaor 2010, Cao 2011] and rodent studies [Ionova-Martin 2011], it is possible that excessive fat in the diet is a contributing factor. Observational studies examining the effects on bone, however, suggest that dietary monounsaturated fatty acids (MUFAs) act differently than other fatty acids [Orchard 2010, Martínez-Ramírez 2007]. The effect of MUFAs on Ca absorption has not been examined previously but others have suggested that the type of fatty acids differentially influences intestinal Ca absorption [Haag 2003]. Fat intake or type may also affect liver function and influence the hydroxylation of vitamin D to 25-hydroxyvitamin D through the enzyme Cyp2r1.

In this study, we hypothesized that excess dietary fat, in the absence of diet-induced obesity, would increase fractional Ca absorption (FCA) in an older and estrogen insufficient mouse model. The primary objective was to examine whether high fat feeding affects Ca metabolism and its active transporters in the small intestine and bone in this older female model. In a secondary objective, we hypothesized that the type of dietary fatty acid (MUFAs or SFAs enriched) would differentially affect Ca metabolism and bone mass, mineral density, and quality.

Experimental Design

Eight-month-old female retired breeder C57BL/6J mice (n=29) weighing approximately 27g each were purchased from Jackson Laboratory. After arrival, mice had free access to a purified diet (slightly modified AIN93M formula) and tap water and were housed in groups of three or four in breeding cages in an environmentally controlled room (19–26°C; relative humidity 40–70%; 12 h light/dark cycle). All procedures were approved by the Rutgers University Institutional Animal Care and Use Committee. After one-week stabilization, weight-matched mice were randomly divided into three groups and provided with different diets for 8wk (**Table 1**). The normal fat diet (NFD) contained 15%

of calories from protein, 75% of calories from carbohydrate, and 10% of calories from fat. The 45% HFD was either enriched with MUFAs (15% protein, 39% carbohydrate, and 46% fat), or SFAs (19% protein, 37% carbohydrate, and 44% fat). All diets contained similar amounts of dietary Ca, and the actual measured values were 0.6%, 0.7%, and 0.7% of Ca in the in the NFD, MUFA, and SFA groups, respectively. Mice were pair-weighted fed during the feeding intervention, and the food intake of the HFD groups was controlled twice a week to maintain similar weight gain as the NFD control group [Devassy 2015]. One mouse from the SFA group was removed due to tooth abscess and weight loss. Food intake of a 7d period was measured twice during the study at the beginning (d 1-7) and the end (d 50-56) of the study. After 8wk, food was removed for approximately 16h before the mice were asphyxiated with CO₂. Blood samples were collected using cardiac puncture, and mice were killed by exsanguination. Liver, kidney, and uterus were quickly removed, weighed, and frozen in liquid N₂. The small intestine was removed and flushed with saline, and the mucosa was obtained by scraping on ice. The mucosa was immediately frozen on dry ice.

Results

Caloric intake and body weight. The daily caloric intake per mouse as calculated from the 7-day food intake at the end of the study was 12 ± 2 , 7 ± 1 , and 8 ± 1 kcal/d, in the NFD, MUFA, and SFA groups, respectively ($P < 0.05$). There was a modest gain in weekly body weights in all groups throughout the study ($P = 0.32$, **Figure 14**). Delta weight change from weeks 0 to 8 did not differ among groups, with values equal to 1.3 ± 1.9 , 1.2 ± 2.2 , and 1.4 ± 2.3 g/8wk in the NFD, MUFA, and SFA groups, respectively ($P = 0.96$).

Calcium metabolism. Dietary intake of Ca was provided at recommended levels (0.5% Ca) in all mice, and was 18 ± 3 , 14 ± 1 , and 16 ± 1 mg/day in the NFD, MUFA, and SFA

groups, respectively ($P < 0.05$). There was a trend for SFAs and MUFAs to have higher final FCA values than the NFD diet ($P = 0.06$), and the FCA rise over time (from week 0 to 8) did not differ between groups ($P = 0.12$; **Table 2**). However, when analyzing the combined HFD groups (SFA and MUFA) compared with the NFD, final FCA was significantly higher ($P < 0.05$), and the rise over time in FCA between the initial and final weeks was greater in the HFD than in the NFD group ($P < 0.05$, data not shown).

The high fat feeding tended to decrease fecal Ca excretion over the 8 weeks of food intervention when compared with the NFD group ($P = 0.06$, **Table 3**), but the greater rise in urinary Ca excretion and Ca balance in the HFD group compared with NFD was not statistically significant. None of the dietary treatments had a significant effect on final (wk8) Ca balance, endogenous fecal Ca loss, intestinal Ca secretion, or urinary Ca excretion.

Serum estradiol and uterine weight. Dietary fat did not change the final serum estradiol concentration, which was 6.6 ± 3.2 , 6.1 ± 3.3 , and 9.6 ± 3.9 pg/mL in the NFD, MUFA, and SFA groups, respectively ($P = 0.42$). In addition, functioning as an indicator of the estrogenic effect, the uterine weight was measured. There were no significant differences in uterine weight among groups, with the value equals to 87 ± 32 , 80 ± 36 , and 77 ± 25 mg in the NFD, MUFA, and SFA groups, respectively ($P = 0.61$).

Calcium transporter mRNA expression. Located at the apical epithelium, protein channel *Trpv6* transfers dietary Ca into the enterocytes cross the epithelial membrane. In this study, intestinal *Trpv6* mRNA abundance was similar among dietary groups (**Figure 15**) ($P = 0.49$). However, mRNA abundance of the intracellular Ca transporter *CalbD9k* in the small intestine was up regulated in the MUFA group when compared with the NFD group ($P < 0.0001$). Moreover, in the kidney, high fat feeding down regulated the mRNA abundance of the apical Ca channel *Trpv5* when compared with the NFD group ($P < 0.0001$) (**Figure 15**).

Bone densitometry and geometry. Total body and femur BMD was lower in SFA mice than in NFD mice ($P < 0.05$) (**Table 3**). Total body BMC was also lower in the SFA group than in the NFD group ($P < 0.05$), but no statistically significant effects of HFD on BMC or BMD were found at distal femur, tibia, humerus, radius, and lumbar spine (L1-5) (**Table 3**). Tibia lengths were similar in all groups with value equals to 17.5 ± 0.9 , 16.2 ± 0.7 , and 17.8 ± 0.6 mm in the NFD, MUFA, and SFA groups, respectively ($P = 0.75$). In addition, femur length did not significantly differ among groups, with lengths equal to 16.2 ± 0.7 , 16.5 ± 0.6 , 16.2 ± 0.7 mm in the NFD, MUFA, and SFA groups, respectively ($P = 0.71$).

Femoral geometric parameters were measured in a limited number of samples (**Table 4**). Trabecular BV/TV (%) was higher in the MUFA group than the NFD group ($P < 0.05$). Additionally, Tb.Th was higher in the MUFA group when compared with the NFD group ($P < 0.05$). There was a lower SMI with MUFA intake compared with the other diet groups ($P < 0.05$). No significant differences among diet groups were found for trabecular vBMD ($P = 0.23$), Tb.N ($P = 0.51$), or Tb.Sp. ($P = 0.61$). In the cortical region, the cortical vBMD was less in the SFA group when compared with the NFD group ($P < 0.05$). High fat feeding tended to be associated with a higher cortical porosity ($P < 0.07$), when compared the HFD group with the NFD group. Other cortical parameters including Ct.Ar ($P = 0.45$), Ct.Th ($P = 0.85$), and J ($P = 0.97$) were similar among diet groups.

Hepatic fat and Cyp2r1 protein level. The liver fat in this weight-controlled study did not significantly differ among groups, with 36 ± 14 , 31 ± 26 , and $32 \pm 13\%$ in the NFD, MUFA, and SFA groups, respectively ($P = 0.77$). Protein levels of 25-hydroxylase Cyp2r1 that plays an important role in the hepatic synthesis of 25-hydroxycholecalciferol (25OHD) was found to be higher in the MUFA than NFD group ($P < 0.05$) (**Figure 16**). Additionally, there was an insignificant inverse correlation between liver fat and hepatic Cyp2r1 protein levels ($r = -0.145$; $P = 0.48$).

Discussion

There is evidence that dietary fat affects bone metabolism, but previous studies have only examined a high fat intake when there were also excess caloric intake and greater body weight [Corwin 2006, Lorincz 2010]. In the current study, our findings indicate that controlled HFD feeding in mature mice increases intestinal FCA, whereas only the MUFA enriched diet increases the expression of intestinal CalbD_{9k} mRNA and hepatic Cyp2r1 protein. The lower areal BMD, cortical BMC and vBMD in the femur with the SFA-enriched diet, compared with a neutral or positive effect of the MUFA-enriched diet suggests that bone is differentially affected by the type of dietary fatty acids. Hence, these findings support our primary hypothesis that high fat feeding without excess caloric intake increases Ca absorption in older female mice. Additionally, compared with dietary MUFAs, high SFAs intake adversely affected total body and femoral cortical bone parameters, but showed no differential role on Ca metabolism.

Different types of dietary fatty acids have differential effects on osteoblastic activity [Watkins 2006, Bhattacharya 2007, Zhong 2011, Coetzee 2007]. In addition, there is a negative effect of HFD-induced obesity on the bones of young rodents [Cao 2010, Woo 2009]. Our findings indicate that there is also an adverse effect of HFD on mature bones even in the absence of obesity, but this only occurs in the presence of a high SFAs (not MUFAs) intake. In a cross-sectional study using the NHANES III dataset, dietary SFAs was associated with reduced femoral neck BMD in humans [Corwin 2006]. SFAs may adversely affect bones through several mechanisms. Rodent studies indicate that SFAs increase bone resorption by elevating the expression of inflammatory cytokines and the receptor activator of NF- κ B ligand [Oh 2010]. SFA mediates osteoclastogenesis and may decrease osteoclast apoptosis [Zhong 2011]. Additionally, SFAs interfere with osteoblastogenesis and bone formation [Parhami 2001].

There are no known mechanisms suggesting that MUFAs would have a negative effect on bone quality, and our findings indicate that BMD and cortical variables were similar in MUFA-enriched and NFD groups. Furthermore, we found that some femoral trabecular variables were higher in the mice fed the MUFAs-enriched diet, although the absolute values were low in all groups. The overall low values in these mice are likely attributed to their older age and history of repetitive pregnancies and lactations (retired breeders) [Griffin 1993]. It is possible that high dietary MUFAs have a protective effect on trabecular bone in general, but it is not clear if this would also occur under other conditions, such as younger, virgin or ad-libitum fed. Nevertheless, our findings are consistent with cross sectional reports in humans showing a positive effect of MUFA intake on bone [Orchard 2010, Trichopoulou 1997] and a reduction in fracture risk in an elderly population [Martínez-Ramírez 2007].

Intestinal Ca absorption varies with several factors including vitamin D concentration, estrogen status, and dietary nutrients [25]. In particular, fat consumption has been shown to have inhibit or have null effects on Ca absorption [Gacs 1977, Xiao 2010, Tadayyon 2969]. In contrast, we found that FCA tended to be higher with HFD feeding. The trend for higher Ca absorption in the HFD groups is consistent with findings that dietary fat is a significant positive predictor of FCA in clinical trials [Shapses 2012, Wolf 2010, Ramsubeik 2013]. However, the underlying mechanisms of how dietary fat affects Ca absorption remain unclear. A high fat intake increases circulating estrogen and vitamin D concentrations that would indirectly increase Ca absorption [Nagata 2014, Dawson-Hughes 2014]. However, we did not find that estrogen status differed between groups. It is possible that the higher FCA was due to a hyper-permeability of the intestinal membrane, which would increase passive leakage of dietary Ca [Brun 2007]. In fact, SFA overconsumption, and not obesity, has been shown to reduce the integrity of tight junctions and increase intestinal permeability [Suzuki 2010]. We suggest that a

high fat feeding affects both active and passive transport to raise intestinal Ca absorption and these findings occur without excess energy intake.

An indirect estimation of intestinal transcellular movement of Ca can be achieved by measuring the expression of Ca absorption-related genes such as *Trpv6*, *CalbD_{9k}*, and plasma membrane Ca ATPase. These Ca transporters transfer dietary Ca across the enterocytes from the intestinal lumen to the blood circulation [Fleet 2010, Christakos 2012]. The fat-induced greater *CalbD_{9k}* gene expression in this study is consistent with findings that short chain fatty acids elevated *CalbD_{9k}* expression *in vitro* [Fukushima 2012]. Nevertheless, it is unclear whether a higher FCA or transcellular Ca transport was activated by high fat diet since only *CalbD_{9k}* mRNA and not *Trpv6* was elevated. The down regulation of renal *Trpv5* gene expression by high fat intake may suggest an attenuated transcellular Ca movement and reabsorption by the kidney [Nijenhuis 2005]. However, urinary Ca excretion did not significantly differ among diet groups in this study. Also, SFA and MUFA diets induce a higher intestinal Ca absorption, suggesting that this is not contributing to differences in bone.

Hepatic Cyp2r1 is the major enzyme responsible for the first step of vitamin D hydroxylation to 25(OH)D [Zhu 2013, Cheng 2004]. Because fatty liver is associated with low serum 25(OH)D concentration [Barchetta 2012, Black 2014], we measured hepatic fat and found that liver fat did not differ among diet groups. However, there was an up-regulation of Cyp2r1 protein expression in the MUFA group despite an absence of fatty liver. This finding may offer a mechanism to explain the higher serum 25(OH)D found in elderly people consuming a MUFA-rich diet compared with diets rich in polyunsaturated fat or low in fat diets [Niramitmahapanya 2011]. Others have used intestinal Caco-2 cells to show that oleic acid compared with other fatty acids produces greater cholecalciferol basolateral efflux [Goncalves 2013]. It is possible that a greater intestinal vitamin D absorption or conversion in the liver contributes to a higher vitamin D status with MUFA.

Since the rise in Cyp2r1 was not significantly higher in the SFA enriched diet, differential vitamin D metabolism due to differences in dietary fatty acid type may be influencing bone.

One limitation of this study is that the protein intake was higher in the SFA than other diet groups (19% vs.15%). Nevertheless, because the protein intake was sufficient in all groups and within the normal range of intake (15-22%) [Yuen 1983, Nebot 2014], we would expect no differential effect on bone. For example, a previous study found that even at 40% (vs. 17%) protein intake had no effect on bone parameters in older mice [Mardon 2008]. Similarly, the vitamin/mineral content differed slightly between groups since intake was lower in the HFD group compared with the NFD group. However, in all groups there was sufficient intake of micronutrients, and therefore it is not expected to differentially affect bone or Ca metabolism. Specifically, all mice were consuming approximately 2000 IU/kg vitamin D and 0.6-0.7% Ca, intake levels which are both above the threshold to influence BMD and BMC in mice [Fleet 2008]. Serum 25(OH)D concentration was not measured in this study. It would be interesting to know if higher hepatic Cyp2r1 protein in the MUFA group is positively associated with higher serum 25(OH)D and if this affects Ca absorption or bone. In the same way, the proposed changes in Ca transporter function, based on mRNA abundance, would require additional measurements of protein levels and activity to confirm the biological relevance of these changes. Also, it is possible that a longer study [Cao 2011] would have resulted in greater differences in bone between treatment groups.

In conclusion, high compared to normal fat intake induced higher Ca absorption and this did not differ with the type of dietary fatty acids. In contrast, only high fat feeding with SFA adversely affected total and femoral areal bone mineral density under conditions of sufficient Ca intake and in the absence of excess caloric intake.

Table 1. Dietary composition and energy contents of experimental diets¹

Ingredient	NFD	MUFA	SFA
	g/kg diet		
Casein	146	183	242
L-cystine	3	4	3
Corn Starch	540	249	77
Maltodextrin	98	189	106
Sucrose	98	34	240
Cellulose	49	61	60
Soybean Oil	6	36	33
Coconut Oil	0	0	20
Lard	11	64	188
Olive Oil	26	153	0
Mineral Mix	10	12	14
Vitamin Mix V10001	10	12	12
Choline bitartrate	2	2	2
Calcium, mg/g diet	5.7	7.2	7.1
Energy, kcal/g diet	3.9	4.8	4.7
% Energy			
Carbohydrate	75	39	37
Protein	15	15	19
Fat	10	46	44
Saturated (%kcal of fat)	20	20	41
Monounsaturated (%kcal of fat)	60	60	41
Polyunsaturated (%kcal of fat)	20	20	18

¹ Prepared by Research Diets Inc., New Brunswick, NJ. MUFA, monounsaturated fatty acids; NFD, normal fat diet; SFA, saturated fatty acids.

²The mineral mix composition was as follows (amount in 10 g): 0.5 g Mg, 0.3 g S, 1.0 g Na, 1.6 g Cl, 6.0 mg Cu, 0.2 mg I, 45.0 mg Fe, 59 mg Mn, 0.2 mg Se, and 29 mg Zn.

³The vitamin mix composition was as follows (amount in 10 g): 4000 IU vitamin A palmitate, 1000 IU vitamin D₃, 50 IU vitamin E acetate, 0.5 mg menadione sodium bisulfite, 0.2 mg biotin (1.0%), 10 µg cyanocobalamin (0.1%), 2 mg folic acid, 30 mg nicotinic acid, 16 mg calcium pantothenate, 7 mg pyridoxine-HCl, 6 mg riboflavin, and 6 mg thiamin HC.

Table 2. Calcium metabolism in mice before and after 8wk feeding a normal fat diet or high fat diets enriched in MUFA or SFA¹

	Baseline (wk0)			Final (wk8)			<i>P</i>		
	NFD	MUFA	SFA	NFD	MUFA	SFA	Diet	Time	Diet x Time
Urinary Ca, $\mu\text{mol/d}$	1.80 \pm 1.3	1.87 \pm 1.0	1.48 \pm 1.0	2.24 \pm 1.5	3.36 \pm 3.3	3.17 \pm 1.7	0.64	0.08	0.67
Fecal Ca, mmol/d	0.30 \pm 0.06	0.27 \pm 0.05	0.33 \pm 0.05	0.31 \pm 0.07	0.18 \pm 0.05	0.24 \pm 0.04	0.01	0.01	0.06
Ca balance, mmol/d	0.10 \pm 0.03	0.12 \pm 0.03	0.09 \pm 0.02	0.14 \pm 0.05	0.16 \pm 0.04	0.15 \pm 0.03	0.25	0.001	0.61
Endogenous fecal Ca, mmol/d	0.11 \pm 0.05	0.08 \pm 0.05	0.07 \pm 0.05	0.13 \pm 0.06	0.24 \pm 0.03	0.19 \pm 0.08	0.96	0.01	0.25
Intestinal Ca secretion, mmol/d	0.11 \pm 0.09	0.13 \pm 0.11	0.10 \pm 0.06	0.23 \pm 0.13	0.39 \pm 0.20	0.38 \pm 0.22	0.23	0.001	0.45
FCA, %	31.9 \pm 14	33.6 \pm 10	24.9 \pm 8	38.5 \pm 9.8	57.7 \pm 11	56.7 \pm 20	0.10	0.001	0.12

¹ All values are means \pm SD (NFD, n=6; MUFA, n=6; SFA, n=6); means in a row with different superscripts differ, $P < 0.05$ by two-way ANOVA. FCA, fractional calcium absorption; MUFA, monounsaturated fatty acid; NFD, normal fat diet; SFA, saturated fatty acid.

Table 3. Bone densitometry in mice after 8wk of feeding normal or high fat diets enriched with MUFAs or SFAs¹

	Diet			
	NFD	MUFA	SFA	<i>P</i>
BMC, g				
Total body	0.41 ± 0.04 ^a	0.39 ± 0.03 ^{ab}	0.37 ± 0.04 ^b	0.02
Whole femur	0.026 ± 0.002	0.025 ± 0.002	0.025 ± 0.001	0.09
Distal femur	0.007 ± 0.001	0.007 ± 0.001	0.006 ± 0.002	0.12
Tibia	0.024 ± 0.002	0.024 ± 0.004	0.023 ± 0.002	0.69
Humerus	0.013 ± 0.001	0.013 ± 0.001	0.012 ± 0.001	0.85
Radius	0.009 ± 0.001	0.009 ± 0.001	0.008 ± 0.001	0.58
Lumbar spine	0.047 ± 0.004	0.045 ± 0.004	0.045 ± 0.002	0.62
BMD, g/cm ²				
Total body	0.046 ± 0.003 ^a	0.046 ± 0.002 ^a	0.044 ± 0.001 ^b	0.02
Whole femur	0.047 ± 0.002 ^a	0.047 ± 0.004 ^a	0.044 ± 0.002 ^b	0.03
Distal femur	0.062 ± 0.006	0.061 ± 0.013	0.053 ± 0.001	0.20
Tibia	0.045 ± 0.002	0.046 ± 0.004	0.043 ± 0.002	0.12
Humerus	0.040 ± 0.001	0.040 ± 0.001	0.038 ± 0.001	0.24
Radius	0.029 ± 0.001	0.029 ± 0.001	0.028 ± 0.004	0.12
Lumbar spine	0.047 ± 0.002	0.046 ± 0.001	0.046 ± 0.001	0.39

¹ All values are means ± SD (NFD: n=10; MUFA, n=10; SFA, n=8), Means in a row with different superscripts differ, *P* < 0.05 (one-way ANOVA followed by Tukey's post hoc test). BMC, bone mineral content; BMD, bone mineral density; MUFA, monounsaturated fatty acid; NFD, normal fat diet; SFA, saturated fatty acid.

Table 4. Femoral geometric structure in mice after 8wk of feeding normal or high fat diets enriched with MUFAs or SFAs¹

	Diet			
	NFD	MUFA	SFA	<i>P</i>
Trabecular				
BV/TV, %	0.55 ± 0.26 ^b	1.31 ± 0.54 ^a	0.66 ± 0.11 ^b	< 0.01
BV, mm ³	0.003 ± .001 ^b	0.009 ± .002 ^a	0.004 ± .001 ^b	< 0.01
TV, mm ³	0.644 ± 0.05	0.658 ± 0.04	0.675 ± 0.03	0.24
Tb.Th, mm	0.037 ± 0.01 ^b	0.048 ± 0.01 ^a	0.037 ± 0.01 ^b	0.01
Tb.N, 1/mm	7.814 ± 2.10	7.891 ± 1.72	6.435 ± 0.91	0.41
Tb.Sp, mm	0.186 ± 0.03	0.173 ± 0.05	0.158 ± 0.05	0.54
SMI	3.35 ± 0.45 ^a	2.49 ± 0.99 ^b	3.56 ± 0.30 ^a	0.05
vBMD, mg/cm ³	37.05 ± 5.4	33.58 ± 3.3	35.10 ± 1.9	0.68
Cortical				
Ct.Ar, mm ²	0.80 ± 0.06	0.84 ± 0.05	0.81 ± 0.04	0.41
Cr.Th, mm	0.16 ± 0.01	0.17 ± 0.01	0.16 ± 0.01	0.85
Ct.Po, %	2.40 ± 0.83	3.49 ± 1.20	3.54 ± 0.80	0.07
<i>J</i> , mm ⁴	0.43 ± 0.06	0.44 ± 0.03	0.43 ± 0.03	0.97
vBMD, mg/cm ³	1200 ± 19 ^a	1180 ± 20 ^{ab}	1170 ± 9.9 ^b	0.01

¹ All values are means ± SD (NFD: n=7; MUFA, n=5; SFA, n=4), Means in a row with different superscripts differ, *P* < 0.05 (one-way ANOVA followed by Tukey's post hoc test). BV, bone volume; Ct.Ar, cortical area; Ct.Po, cortical porosity; Ct.Th, cortical thickness; *J*, polar moment of inertia; MUFA, monounsaturated fatty acid; NFD, normal fat diet; SFA, saturated fatty acid; SMI, structure model index; Trabecular BV/TV, bone volume fraction; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; TV, total volume; vBMD, volumetric bone mineral density.

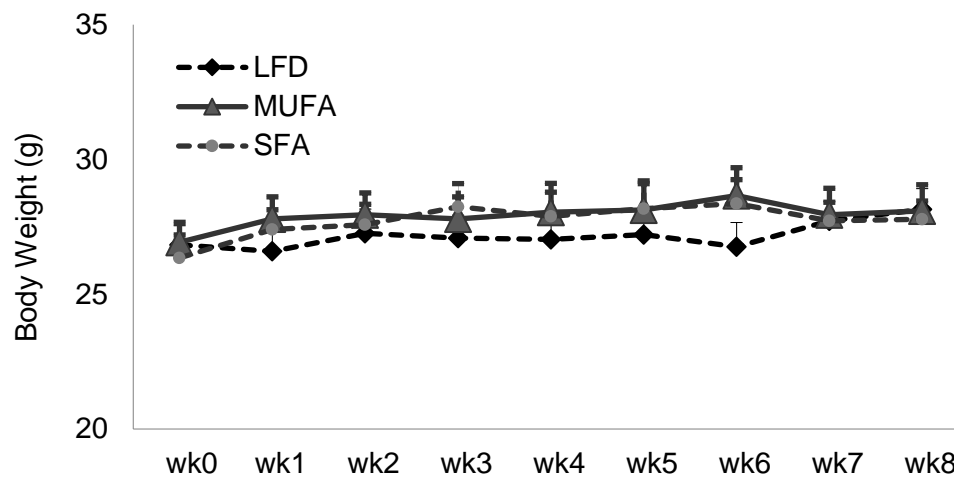


Figure 14. Weekly body weights in mice throughout the 8wk of feeding NFD or HFD enriched with MUFAs or SFAs.

Values are means \pm SEM, (NFD, $n=10$; MUFA, $n=10$; SFA, $n=8$). $P < 0.05$ by repeated measure ANOVA for the effect of diets on body weight over 8wk. MUFA, monounsaturated fatty acid; NFD, normal fat diet; SFA, saturated fatty acid.

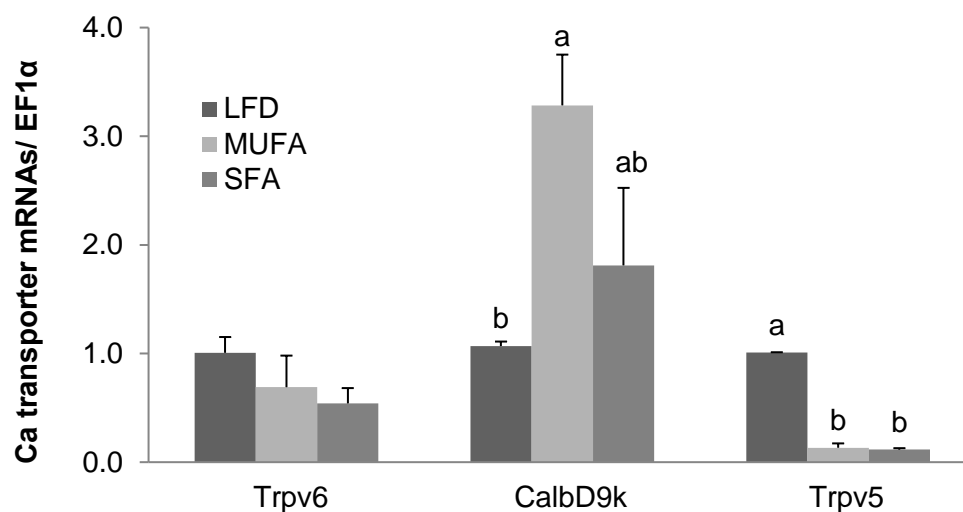


Figure 15. Relative mRNA abundance of intestinal Ca transporter *Trpv6* and *CalbD_{9k}* and renal *Trpv5* in mice after 8wk of feeding NFD or HFD enriched with MUFAs or SFAs.

All values are means \pm SEM, (n=6 for each group). $P < 0.05$ by one-way ANOVA followed by Tukey's post-hoc test. *CalbD_{9k}*, calbindin-D_{9k}; MUFA, monounsaturated fatty acid; NFD, normal fat diet; SFA, saturated fatty acid; *Trpv5*, transient receptor potential cation channel subfamily V member 5; *Trpv6*, transient receptor potential cation channel subfamily V member 6.

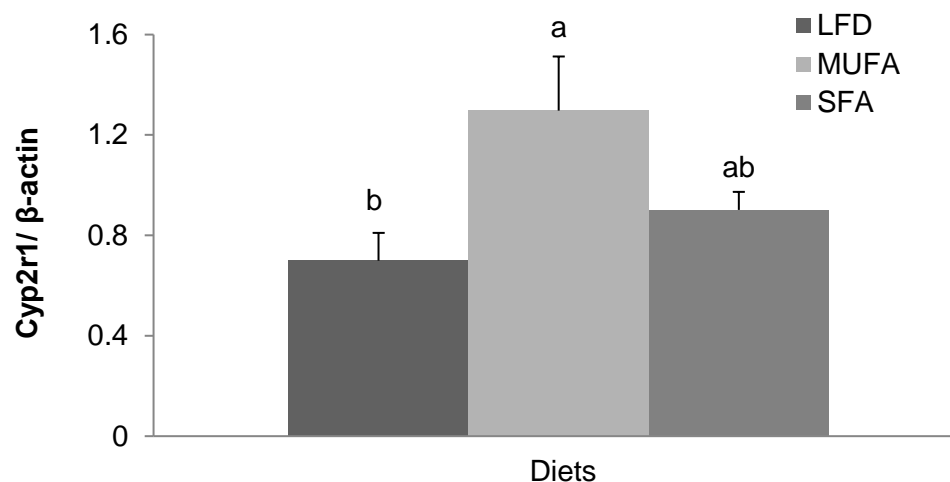


Figure 16. Hepatic vitamin D 25-hydroxylase Cyp2r1/β-actin protein level in mice after 8wk of feeding with normal fat diet and high fat diets enriched with MUFA or SFA.

All values are means \pm SEM, (NFD, n=10; MUFA, n=10; SFA, n=8). $P < 0.05$ by one-way ANOVA followed by Tukey's post-hoc test. Cyp2r1, cytochrome P450 2r1; MUFA, monounsaturated fatty acid; NFD, normal fat diet; SFA, saturated fatty acid.

**EXPERIMENT 2: EFFECTS OF DIETARY FAT LEVELS AND TYPES ON VITAMIN D
HYDROXYLASES AND BONE IN AGING FEMALE MICE**

Abstract

Obesity induced by high fat diets (HFDs) is inversely associated with vitamin D status and bone health in humans. Yet these associations and the direct effect of excessive fat intake on both hepatic and renal vitamin D metabolism have not been addressed. In this study, we sought to determine if excess energy intake from fat, or the type of fat, affects serum 25-hydroxycholecalciferol (25OHD) concentration and the enzymes involved in vitamin D metabolic pathways in an adult murine model. The second objective was a follow up of our recent findings that the type of fatty acids in HFDs differentially affects bone in non-obese mice. In this study, we overfed mice to achieve obesity with a HFD either rich in monounsaturated fatty acids (MUFAs) or saturated fatty acids (SFAs) and examined areal and volumetric bone mineral density (BMD) and quality. Twenty-one 8-month-old female C57BL/6J mice were fed *ad libitum* for 10wk with a normal fat diet (NFD) (10% fat as energy) or HFD (45% fat as energy) enriched with MUFA or SFA. We found that the HFD enriched with SFA, but not MUFA, resulted in greater energy intake, weight gain, total body fat, and liver fat compared with the NFD ($P < 0.05$). High SFA feeding resulted in a higher mRNA, but lower protein expression of hepatic Cyp2r1, and a lower renal Cyp24 mRNA abundance than in the NFD group ($P < 0.05$). Moreover, while BMD did not differ among groups, trabecular bone volume fraction was lower ($P < 0.05$) in the SFA than the NFD group (MUFA did not differ). In conclusion, high SFA and MUFA feeding differentially affected gene and protein expression of vitamin D hydroxylases that cannot be used to explain the lowered circulating 25OHD concentration. In addition, high SFA diet appears to negatively affect trabecular bone properties in older obese mice.

Key words. Bone mineral content, Cyp2r1, Cyp27b1, Cyp24a1, high fat diet, monounsaturated fat, saturated fat, and vitamin D hydroxylase.

Introduction

The vitamin D endocrine system is a well-known regulator that maintains skeleton integrity and calcium homeostasis. Upon ligand binding to its receptor, vitamin D transcriptionally regulates gene expression of calcium transporters and bone cells (osteoclasts vs. osteoblasts) (Fleet 2010, Christakos 2016). In addition to its calciotropic function, vitamin D affects inflammatory cytokines, fatty acid oxidation, and cellular proliferation, differentiation, and apoptosis (Dusso 2005, Omdahl 2002). Therefore, vitamin D status is related to several conditions including metabolic syndrome, type I and II diabetes, autoimmune disorders, and cancers (Fleet 2012, Bikle 2014).

Dietary intake and endogenous synthesis by skin are sources of vitamin D. Activation and deactivation of vitamin D involve several hydroxylation reactions that are catalyzed by cytochrome P450 enzymes (Bikle 2014, Deluca 2015). Mainly, if not solely, in the liver, 25-hydroxylase Cyp2r1 hydroxylates vitamin D at the C-25 position to produce 25-hydroxycholecalciferol (25OHD), the major circulating metabolite of vitamin D (Cheng 2003, Zhu JG 2013). In the kidney, renal 1 α -hydroxylase Cyp27b1 further hydroxylates 25OHD at the C-1 position and results in the formation of the metabolically active hormone 1,25-dihydroxycholecalciferol [1,25(OH)₂D] (Omdahl 2002). Enzyme Cyp27b1 is widely expressed in several other tissues, such as the brain and intestines; however, extra-renal synthesized 1,25(OH)₂D functions more like a paracrine or autocrine hormone (Bikle 2014). Furthermore, renal 24-hydroxylase, Cyp24a1, is the sole catabolic enzyme involved in the vitamin D metabolic pathways. It is shown to have 23- and 24-hydroxylase activity and is responsible for the degradation of excess circulating 25OHD and 1,25(OH)₂D (Henry 2011, Christakos 2016).

Vitamin D status can be affected by factors such as age, race/ethnicity, dietary intake, ultraviolet exposure, calcium status, and illness (Tsiaras 2011, Christakos 2016). Several studies have shown that obesity is inversely associated with serum 25OHD

concentration (Dyląg 2014, Gonzalez-Molero 2013, Tsiaras 2011). This is generally attributed to deposition into excess adipose tissue. However, the lowered 25OHD in obesity may also be due to lowered hepatic Cyp2r1 hydroxylase and synthesis of 25OHD. In addition, other mechanisms can also play a role; excess dietary fat, for example, may affect intestinal absorption or bioavailability of vitamin D. One clinical trial showed that the absorption of vitamin D supplements is higher in people consuming a normal fat meal than a high fat meal or no meal (Dawson-Hughes 2013), suggesting that both excess or the absence of dietary fat is detrimental to intestinal vitamin D absorption. Nevertheless, the impact of excessive fat intake and obesity on vitamin D metabolic pathways has not been examined. We hypothesized that the amount of fat would differentially affect vitamin D metabolism. In addition, we anticipated that the type of fatty acids would have a differential effect since evidence suggests they are differentially associated with serum 25OHD concentration in humans (Niramitmahapanya 2011). Specifically, the change in plasma 25(OH)D concentration after a vitamin D supplement is positively related to dietary monounsaturated fatty acids (MUFA) intake, but not saturated fatty acids (SFA) or polyunsaturated fatty acids (PUFA) intake (Niramitmahapanya 2011). Also, our previous findings indicate that hepatic Cyp2r1 protein abundance is higher with high dietary MUFA intake under conditions of normal energy intake in a non-obese mouse model (Wang Y 2016). In addition, in this previous study, we found that a high MUFA diet, but not high SFA, resulted in higher cortical bone mineral density (BMD) compared with the normal fat diet (NFD) in older female mice. These observations suggest that high dietary MUFA may uniquely affect vitamin D metabolism and bone properties.

In this study, we used an older female murine model to determine if high fat feeding (MUFA vs. SFA) induced obesity alters serum 25OHD concentration, and

whether this can be explained by changes in the regulating enzymes involved in vitamin D metabolism. The second objective was to identify whether excessive intake of fat enriched with MUFA has differential effects from SFA on vitamin D and bone in obese older mice.

Experimental Design. A total of twenty-one female retired breeder C57BL/6J mice about 8mo of age were purchased from Jackson Laboratory. After arrival, mice had free access to a purified diet (slightly modified AIN93M formula) and tap water and were housed in groups of three or four in breeding cages in an environmentally controlled room (19-26°C; relative humidity 40-70%; 12h light/dark cycle). All procedures were approved by the Rutgers University Institutional Animal Care and Use Committee. After a one-week acclimation period, weight-matched mice were divided into three groups and provided with one of the three diets for 10wk. The NFD contained 15% of calories from protein, 75% of calories from carbohydrate, and 10% of calories from fat. The 45% high fat diet (HFD) was either enriched with MUFA (15% protein, 39% carbohydrate, and 46% fat), or SFA (19% protein, 37% carbohydrate, and 44% fat). All diets contained adequate amounts of dietary Ca (5.5 g/kg diet) and vitamin D (1000 IU/kg diet). Details of the dietary composition are presented in **Supplemental Table 1**. Mice were fed *ad libitum* throughout the study, and weekly food intake and body weight were recorded twice a week. After 10wk, food was removed for approximately 16h before mice were killed by decapitation. Blood was collected, and liver and kidney were quickly removed, weighed, and frozen in liquid N₂.

Results

Energy and vitamin D intake. Throughout the study, weekly cumulative caloric intake was higher with HFD feeding than NFD ($P < 0.05$) (**Figure 17A**). All mice received an

adequate amount of vitamin D intake (0.025mg/kg diet or 1000IU/kg diet), as recommended by the National Research Council (1995) for maintaining normal health status. Actual daily intake from the final week was 2.57 ± 0.24 , 2.31 ± 0.34 , and 2.85 ± 0.17 IU/d, mouse in the NFD, MUFA, and SFA groups, respectively ($P = 0.26$).

Body weight and composition. High fat feeding led to a higher weight gain over 10wk than the NFD control ($P < 0.05$) (**Figure 17B**). Additionally, the absolute weight gain between the initial (wk0) and final weeks (wk10) was highest in the SFA group when compared with the NFD ($P < 0.05$) (**Table 5**). Mice from all diet groups had comparable amounts of total body water ($P = 0.49$) and lean mass ($P = 0.18$). However, fat mass and percent fat were higher in both MUFA and SFA groups, with the difference between the SFA and NFD groups significant ($P < 0.05$).

Hepatic total lipid and triglyceride. Hepatic lipid was higher in the MUFA and SFA groups than the NFD control, 1.46 ± 0.28 , 1.34 ± 0.14 , and 0.97 ± 0.18 mg/ mg of protein in the MUFA, SFA, and NFD groups, respectively ($P < 0.05$). High fat feeding did not alter hepatic triglyceride content as compared with the NFD group, 0.44 ± 0.2 , 0.49 ± 0.2 , and 0.43 ± 0.1 mg/ mg of protein in the MUFA, SFA and NFD groups, respectively ($P = 0.82$).

Serum Biochemistry. Ten weeks of high fat feeding (MUFA combined with SFA) lowered circulating 25OHD ($P < 0.05$). However, when examining the type of fatty acid, there was a trend for HFD-SFA to lower the serum 25(OH)D when compared with the NFD control ($P = 0.07$) (**Table 6**). No significant effects were observed of the amount (HFD vs. NFD) or the type (MUFA vs. SFA) of dietary fat on serum concentrations of 1,25(OH)₂D, PTH, FGF23, and estradiol after 10wk of feeding.

Vitamin D hydroxylase gene and protein expression. In the liver, vitamin D 25-hydroxylase Cyp2r1 mRNA expression was higher in the SFA than the NFD and MUFA groups ($P < 0.001$) (**Figure 18**). However, when further examining the protein abundance, there was a trend for Cyp2r1 protein being lower with SFA feeding than the MUFA ($P = 0.09$) (**Figure 19**).

In the kidney, the amount and the type of dietary fat showed no effect on gene expression ($P = 0.72$) or protein abundance ($P = 0.74$) of 1 α -hydroxylase Cyp27b1, when compared with the NFD group. On the other hand, HFD (MUFA combined with SFA) down-regulated renal 24-hydroxylase Cyp24a1 mRNA ($P < 0.05$) and tended to lower its protein abundance as well ($P = 0.07$) (**Figures 18 and 19**). However, It was the dietary SFA that showed a significant effect on down regulating Cyp24 mRNA ($P = 0.01$) (but not protein) when compared with the NFD control; dietary MUFA did not differ from the SFA or NFD groups.

Bone mass. After 10wk, total body and tibial BMC values were higher in the SFA than the MUFA and NFD groups ($P < 0.01$) (**Table 7**). There was a trend for dietary MUFA to have a higher aBMD at the radius ($P = 0.06$) (**Table 7**). Nevertheless, 10wk of HFD showed no significant effect on aBMD at any other anatomical bone sites in older female mice.

The effects of dietary fat on percent difference of aBMD after body weight adjustment were also compared with the weight-matched NFD controls (**Figure 19**). Non-significant lower BMD values in SFA-fed mice compared with MUFA-fed mice ($P < 0.1$) were found at the femur ($-28.4 \pm 9.3\%$ in SFA and $9.1 \pm 3.8\%$ in MUFA) and radius ($-18.1 \pm 5.8\%$ in SFA and $3.4 \pm 7.6\%$ in MUFA). Moreover, the absolute ratio of aBMD over body weight was lower for total body and all anatomical sites except tibia in the SFA group, but not

MUFA group, when compared with the NFD group ($P < 0.05$). Femoral BMC to BW ratio was also lower with high SFA feeding when compared with the NFD control ($P < 0.05$).

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Bone microarchitecture. Femoral length did not differ significantly among groups, with the actual lengths equal to 15.8 ± 0.5 , 15.7 ± 0.2 , and 15.9 ± 0.1 mm in the NFD, MUFA, and SFA groups, respectively ($P = 0.72$). When examining the geometric structure of femurs, BV/TV was lower in the SFA than the MUFA and NFD groups ($P < 0.05$) (**Table 9**). No other metaphyseal trabecular parameters including Tb.Th, Tb.N, Tb.Sp, and SMI differed among diet groups. In addition, dietary fat did not affect diaphysis cortical bone parameters including Tt.Ar, Ct.Ar, Ct.Th, J , and porosity when compared with the NFD control.

Discussion

Obesity is closely associated with vitamin D deficiency in humans across different age groups (Liel 1988, Dyląg H 2014, Lenders 2009, Gonzalez-Molero I 2013, Tsiaras 2011). Possible mechanisms include greater deposition of vitamin D (a fat soluble vitamin) into adipose tissue (Wortsman 2000) or altered intestinal absorption (Dawson-Hughes 2013). However, whether or not HFD and obesity directly affect overall vitamin D metabolism has not been fully addressed. Findings from the current study indicate that excess SFA intake leads to higher body weight and adiposity, whereas dietary MUFA only shows a moderate effect compared with NFD. Additionally, high intake of MUFA or SFA showed no adverse effect on the major vitamin D hydroxylases, suggesting that an alteration in vitamin D metabolism is not likely to explain lowered circulating 25OHD induced by HFD feeding in older female mice.

There have been studies showing that the risk of developing non-alcoholic fatty liver and other chronic liver diseases is inversely associated with vitamin D status in human subjects and rodents (Eliades 2013, Dasarathy 2014, Roth 2011, Han 2015). However, whether HFD induced fatty liver compromises hepatic vitamin D hydroxylation and directly lowers circulating 25OHD is unknown. In the current study, HFD enriched with SFA and MUFA expectedly resulted in a fatty liver that was significantly correlated with final body weight. Nevertheless, though a lower serum concentration of 25OHD was related to body adiposity and liver fat, these inverse relationships did not reach statistical significant. Since the majority of circulating 25OHD (~75%) is attributed to the hepatic conversion catalyzed by 25-hydroxylase coding by Cyp2r1 (Cheng 2003, Zhu 2013), we examined both the gene and protein abundance of hepatic Cyp2r1. It was found that hepatic Cyp2r1 gene expression, but not protein expression, was up-regulated due to excessive HFD-SFA intake when compared with the NFD and HFD-MUFA intake. Perhaps, this may be explained by a compensatory mechanism of increase in gene

expression in response to the lowered Cyp2r1 protein level due to high SFA intake. In addition, Park et al (2015) also found a lower Cyp2r1 mRNA due to HFD-SFA feeding in young male mice. However, the protein level of Cyp2r1 in their study needs to be confirmed in the future, because there is no defined correlation from gene to protein translation (Nedergaard 2013, Wang 2006). Plus, HFD-SFA feeding did not alter serum 25OHD concentration in the previous study (Park 2015). Furthermore, in the current study, we found a greater protein level of hepatic Cyp2r1 with high HFD-MUFA intake, when compared with the SFA intake. This is consistent with our previous finding of an up-regulation of hepatic Cyp2r1 protein level due to high MUFA intake in older female mice (Wang 2016). Yet, the positive effect of MUFA on hepatic Cyp2r1 protein expression occurred under conditions of normal energy intake and in the absence of diet induced obesity (Wang 2016). Therefore, overall, these findings suggest a differential effect of SFA and MUFA on hepatic 25-hydroxylase and conversion to 25OHD. This might mechanistically explain a greater increase of circulating 25(OH)D in response to vitamin D supplementation when healthy older individuals consume high dietary MUFA rather than high PUFA or SFA (Niramitmahapanya 2011).

Due to the potent calciotropic effect of endocrine hormone $1,25(\text{OH})_2\text{D}$ on calcium and bone metabolism, the expression and activity of renal 1α -hydroxylase Cyp27b1 is tightly regulated by hormonal factors such as PTH and FGF23 to maintain a stable serum concentration of $1,25(\text{OH})_2\text{D}$ (Bikle 2014, Henry 2011, Quarles 2012). Nutritional factors such as dietary fat may also affect Cyp27b1 expression in the kidney. In the current study, high SFA and MUFA feeding did not alter gene or protein expression of Cyp27b1, and the circulating $1,25(\text{OH})_2\text{D}$ concentration did not differ among diet groups. This is expected given dietary calcium and vitamin D were consumed at adequate levels, and we used adult mice. Indeed, serum concentrations of PTH and FGF23 also did not differ among diet groups; though the absolute

concentrations were generally high in all groups, this may be typical in older rodents (van der Meijden 2015, Douard 2012). However, a HFD-SFA diet up-regulated renal Cyp27b1 mRNA, and higher serum concentrations of PTH and 1,25(OH)₂D were observed in obese young male mice when compared with the lean controls (Park 2015). These findings suggest that sex and age could play a role in the regulation of renal Cyp27b1 expression.

Protein Cyp24a1 is crucially involved in the vitamin D catabolic pathways and is responsible for the deactivation of excess 25(OH)D and 1,25(OH)₂D (Deluca 2015, Dusso 2015, Veldurthy 2016). In the current study, we found down-regulation of gene expression of 24-hydroxylase with excessive SFA feeding, and the protein level of Cyp24a1 was also lower in the HFD groups compared with the NFD control. We suggest that the down-regulated Cyp24a1 protein in response to HFD feeding may prevent further oxidation of vitamin D metabolites in order to maintain serum 25(OH)D and 1,25(OH)₂D concentrations.

In the current study, excessive intake of dietary SFA or MUFA did not adversely affect BMC of the total body or individual bone sites, as compared with normal fat intake. Nevertheless, despite a greater BMC in the SFA group that might be attributed to greater mechanical loading as a result of excess body weight (Shapses 2012, Zillikens 2010), aBMD was not significantly affected. Indeed, after adjusting bone parameters for body weight, we found that BMD and BMC were lower with HFD-SFA intake. Therefore, obesity may initially benefit bone, whereas detrimental effects may be expected to be seen over the long term (Lecka-Czernik 2015). Moreover, it is also possible that adipocyte derived hormones, such as leptin or estradiol, could benefit bone and calcium metabolism (Reid 2002). However, it is unlikely that estradiol played a role in the current study since serum estradiol concentration was not altered by high fat feeding in these older female mice. In addition, in a previous *ad libitum* HFD study that induced both

higher weight and circulating estradiol, there was no improvement in bone structure in ovariectomized mice (Cao 2016). Furthermore, our femoral microarchitecture results revealed that high SFA feeding adversely affects trabecular BV/TV values, whereas the MUFA-rich diet showed a neutral effect. Despite a negative effect on trabecular bone, 10wk of high SFA feeding did not alter femoral cortical parameters. This could be explained by a positive effect of body mass attributed to diet induced obesity. Similar findings that intragastric high fat feeding has less effect on tibial cortical BMD than on trabecular parameters were observed in young female rats (Chen 2015). Overall, our and other findings support the conclusion that dietary SFA, but not MUFA, adversely affects bone mass and quality in rodents and humans (Trichopoulou 1997, Martínez-Ramírez 2007, Hayek 2012, Wang 2016), suggesting that the type of dietary fat differentially affects bone health. In addition, extensive studies have shown that high SFA feeding and obesity promote oxidative stress, increased inflammatory cytokines, and bone resorption, and impair bone formation (Chen 2015, Pacifici 1996, Yarrow 2016). Thus, the deleterious effects of excess SFA consumption on bone could be exacerbated during prolonged feeding or under a more severe (morbid) obese condition (Lecka-Czernik 2015, Shen 2013).

The current study addressed the effects of HFD on vitamin D regulating enzymes and bone health in older female mice under conditions of obesity and normal vitamin D and calcium intake. One possible limitation is that while the major hydroxylases involved in vitamin D metabolic pathways are shared between rodents and human subjects (Christakos 2016, Zhu 2013), it is likely that differences exist since even among mice there are distinct genetic variations in calcium and bone metabolism within different murine strains (Replogle 2014). In addition, while the study was powered to test differences in bone and Cyp2r1 protein expression (Wang 2016), it may not have been

adequately powered to detect differences in other vitamin D regulating hormones or enzymes.

In conclusion, the inconsistent findings of high fat feeding (MUFA vs. SFA) on gene or protein abundance of vitamin D hydroxylases cannot be used to explain the lower circulating 25OHD concentration in mature mice. In addition, high SFA intake, but not MUFA, adversely affected trabecular bone parameters, though no detrimental effects of SFA on bone mass were seen in mature mice under the condition of excess caloric intake and obesity.

Table 5. Body weight and composition in mice after 10wk of *ad libitum* feeding with normal or high fat diets enriched with MUFA or SFA¹

	Diet			<i>P</i>
	NFD	MUFA	SFA	
Delta weight change, g	3.2 ± 1.7 ^b	5.1 ± 3.5 ^{ab}	8.2 ± 3.3 ^a	0.02
Total water, g	18.3 ± 4.6	17.0 ± 4.0	16.0 ± 0.7	0.49
Lean mass, g	19.6 ± 1.1	19.8 ± 1.0	20.5 ± 0.6	0.17
Fat mass, g	5.8 ± 1.3 ^b	9.3 ± 2.9 ^{ab}	12.8 ± 4.9 ^a	0.005
Percent fat, %	21.1 ± 3.4 ^b	29.7 ± 6.7 ^{ab}	35.1 ± 3.8 ^a	0.004

¹ All values are means ± SD (n=7 per group). Labeled means in a row with different superscripts differ, *P* < 0.05 by one-way ANOVA, followed by Tukeys's post hoc test. MUFA, monounsaturated fatty acid; NFD, normal fat diet; SFA, saturated fatty acid

Table 6. Serum biochemistry in mice after 10wk of *ad libitum* feeding with normal or high fat diets enriched with MUFA or SFA¹

	Diet			<i>P</i>
	NFD	MUFA	SFA	
25(OH)D, nmol/L	48.7 ± 6.1	42.0 ± 7.2	40.3 ± 7.2	0.19
1,25(OH) ₂ D, pmol/L	251 ± 165	249 ± 109	261 ± 116	0.99
PTH, pmol/L	51.9 ± 32	40.3 ± 25	42.6 ± 41	0.79
FGF23, pg/mL	426 ± 82	578 ± 52	531 ± 60	0.20
Estradiol, pg/mL	13.6 ± 2.6	13.6 ± 2.3	12.4 ± 2.8	0.64

¹All values are means ± SD (n=7 per group). Labeled means in a row with different superscripts differ, *P* < 0.05 by one-way ANOVA, followed by Tukeys's post hoc test. 1,25(OH)₂D, 1,25-dihydroxy-cholecalciferol; 25(OH)D, 25-hydroxy-cholecalciferol; FGF23, fibroblast growth factor 23; MUFA, monounsaturated fatty acids; NFD, normal fat diet; PTH, parathyroid hormone; SFA, saturated fatty acids.

Table 7. Bone densitometry in mice after 10wk of *ad libitum* feeding with normal or high fat diets enriched with MUFA or SFA¹

	Diet			<i>P</i>
	NFD	MUFA	SFA	
BMC, g				
Total body, g	0.486 ± 0.030 ^b	0.512 ± 0.043 ^b	0.604 ± 0.103 ^a	0.01
Femur	0.007 ± 0.001	0.007± 0.001	0.006 ± 0.001	0.36
Tibia	0.020 ± 0.001 ^b	0.020 ± 0.001 ^b	0.022 ± 0.001 ^a	0.01
Lumbar Spine	0.048 ± 0.004	0.048 ± 0.005	0.053 ± 0.006	0.08
Humerus	0.013 ± 0.001	0.012 ± 0.001	0.013 ± 0.001	0.57
Radius	0.009 ± 0.001	0.010 ± 0.002	0.010 ± 0.001	0.21
BMD, g/cm ²				
Total body	0.052 ± 0.002	0.054 ± 0.003	0.056 ± 0.005	0.16
Femur	0.060 ± 0.003	0.061 ± 0.003	0.055 ± 0.01	0.40
Tibia	0.046 ± 0.002	0.047 ± 0.002	0.048 ± 0.003	0.13
Lumbar Spine	0.050 ± 0.004	0.051 ± 0.002	0.053 ± 0.005	0.23
Humerus	0.045 ± 0.002	0.044 ± 0.003	0.044 ± 0.003	0.86
Radius	0.033 ± 0.001	0.037 ± 0.004	0.034 ± 0.002	0.06

¹ All values are means ± SD (n=7 for each dietary group). Labeled means in a row with different superscripts differ, *P* < 0.05 by one-way ANOVA, followed by Tukey's post hoc test. BMC, bone mineral density; BMD, bone mineral density; MUFA, monounsaturated fatty acids; NFD, normal fat diet; SFA, saturated fatty acids.

Table 8. Femoral geometric parameters in mice after 10wk of *ad libitum* feeding with normal or high fat diets enriched with MUFA or SFA¹

	Diet			<i>P</i>
	NFD	MUFA	SFA	
Metaphysis				
Trabecular				
BV/TV, %	3.29 ± 0.49 ^a	2.74 ± 0.37 ^{ab}	2.35 ± 0.57 ^b	0.02
BS/TV, mm ² /mm ³	6.64 ± 1.40	5.81 ± 0.77	5.64 ± 2.24	0.79
BS/BV, mm ² /mm ³	207 ± 61.9	214 ± 34.3	229 ± 61.4	0.49
Tb.N, 1/mm	8.85 ± 2.89	6.89 ± 0.97	6.65 ± 2.37	0.19
Tb.Th, mm	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.91
Tb.Sp, mm	0.17 ± 0.14	0.26 ± 0.25	0.22 ± 0.25	0.75
SMI	4.90 ± 1.15	4.65 ± 0.75	4.50 ± 0.27	0.72
Diaphysis Cortical				
Tt.Ar, mm ²	0.86 ± 0.04	0.86 ± 0.06	0.88 ± 0.08	0.77
Ct.Ar, mm ²	0.79 ± 0.04	0.79 ± 0.06	0.81 ± 0.08	0.78
Ct.Ar/Tt.Ar, %	0.92 ± 0.01	0.92 ± 0.01	0.92 ± 0.01	0.96
Ct.Th, mm ²	0.18 ± 0.01	0.18 ± 0.01	0.19 ± 0.02	0.70
<i>J</i> , mm ⁴	0.42 ± 0.05	0.40 ± 0.06	0.42 ± 0.05	0.85
Ct.Porosity, %	7.70 ± 0.48	7.71 ± 0.65	7.59 ± 1.07	0.96

¹All values are means ± SD (n=7/ diet group), Means in a row with different superscripts differ, *P* < 0.05 by one-way ANOVA, followed by Tukeys's post hoc test. BV/TV, bone volume fraction; BS/TV, bone surface density; BS/BV, specific bone surface, Ct.Ar, cortical bone area; Ct.Ar/Tt.Ar, cortical area fraction; Ct.Th, cortical thickness; *J*, polar moment of inertia; MUFA, monounsaturated fatty acid; NFD, normal fat diet; SFA, saturated fatty acid; SMI, structure model index; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; Tt.Ar, total cross-sectional area; vBMD, volumetric bone mineral density.

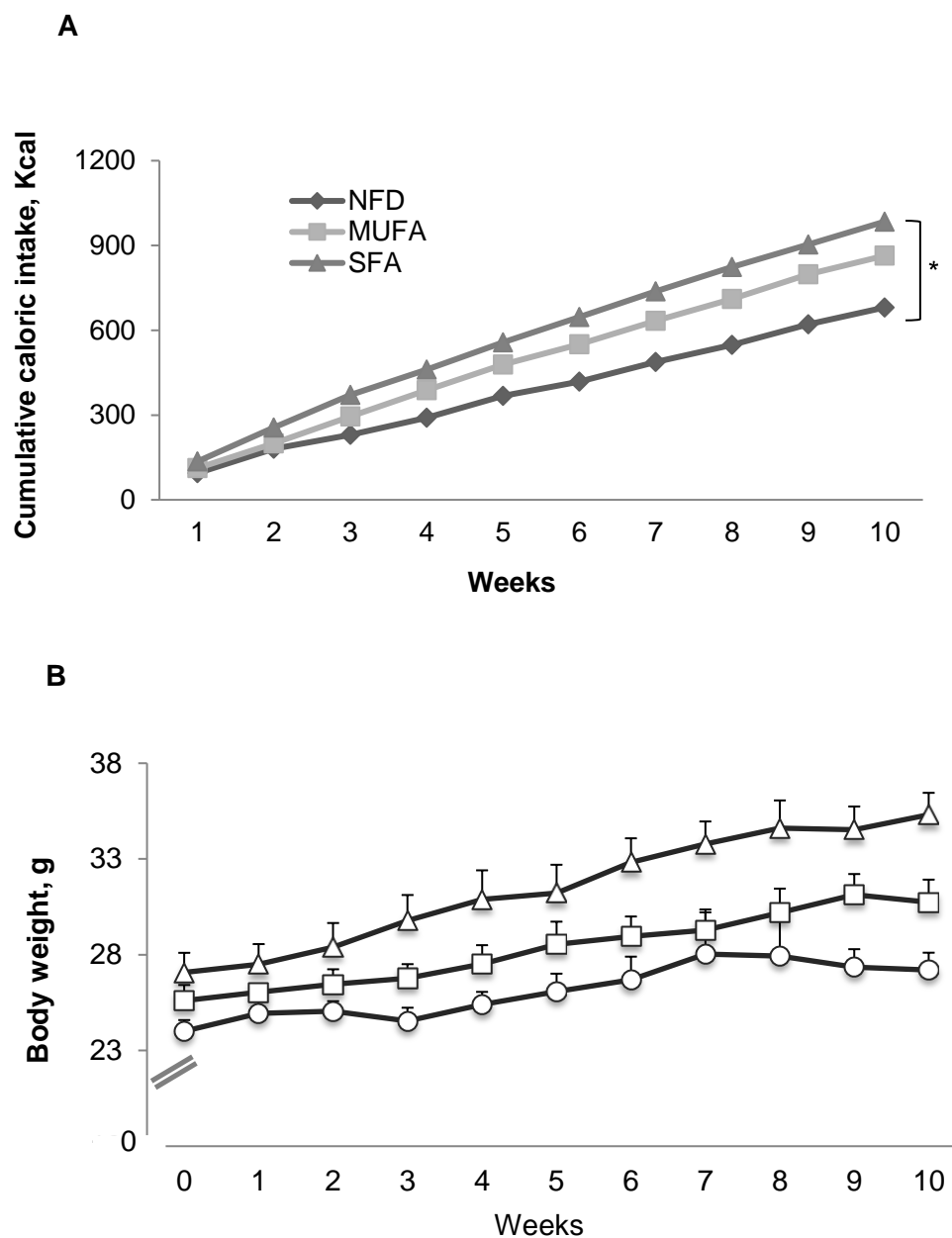
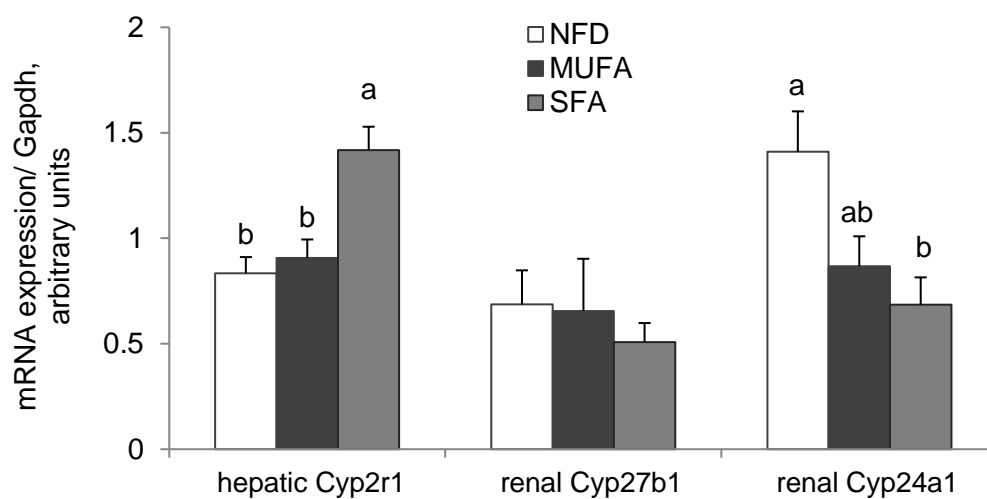


Figure 17. Weekly cumulative caloric intake (A) and body weight (B) in mice throughout the 10wk of *ad libitum* feeding with NFD or HFD enriched with MUFA or SFA.

Values are means \pm SEM, (n=7 per diet group) $P < 0.05$ by one-way ANOVA, followed with Tukey's post hoc test. Error bars in Figure A were very small and cannot be seen. MUFA, monounsaturated fatty acids; NFD, normal fat diet; SFA, saturated fatty acids.



Values are means \pm SEM, (n=7 per group). $P < 0.05$ by one-way ANOVA using Tukey's post hoc test. Cyp2r1, cytochrome P450 2r1; Cyp27b1, cytochrome P450 27b1; Cyp24a1, cytochrome P450 24; MUFA, monounsaturated fatty acids; NFD, normal fat diet; SFA, saturated fatty acids.

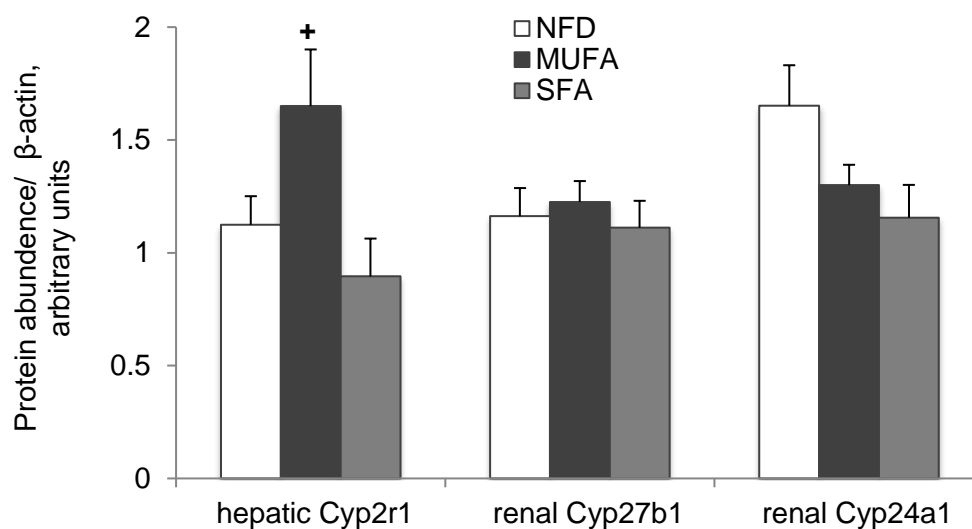


Figure 19. Vitamin D hydroxylases protein abundance in mice after 10wk of *ad libitum* feeding with NFD or HFD enriched with MUFA or SFA.

Values are means \pm SEM, (n=7 per group). ⁺*P* < 0.1 by one-way ANOVA. Cyp2r1, cytochrome P450 2r1; Cyp27b1, cytochrome P450 27b1; Cyp24a1, cytochrome P450 24; MUFA, monounsaturated fatty acids; NFD, normal fat diet; SFA, saturated fatty acids.

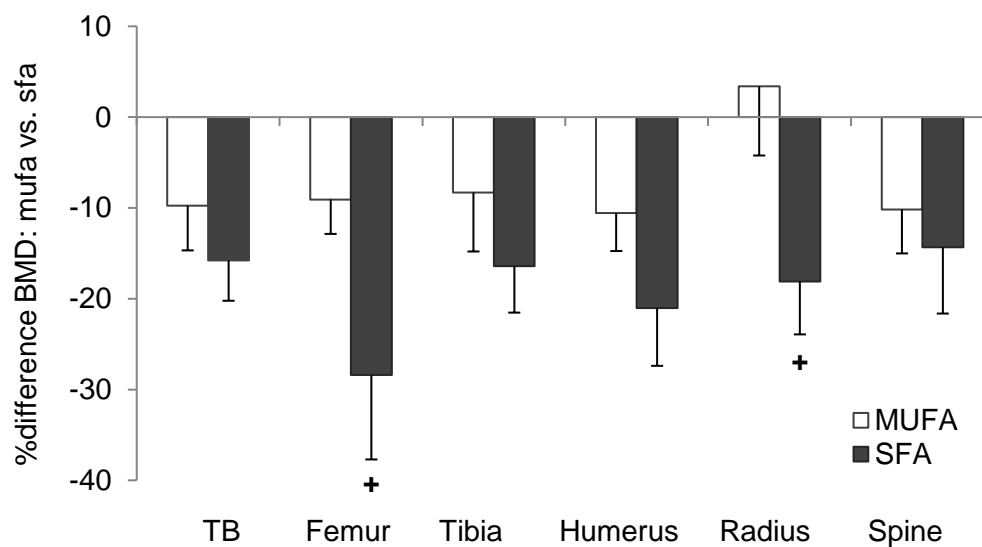


Figure 20. Percent difference for BMD values adjusted for body weight for HFD fed mice compared with their weight-matched NFD fed controls.

Values are means \pm SEM, (n = 7 per group). *Different from MUFA, $P < 0.1$ by one-way ANOVA. BMD, bone mineral density; LS, lumbar spine (L1-4); MUFA, monounsaturated fatty acids; NFD, normal fat diet; SFA, saturated fatty acids; TB, total body.

**EXPERIMENT 3: EFFECTS OF DIETARY VITAMIN D DEFICIENCY AND HIGH FAT
FEEDING ON ADIPOSITY, FOOD INTAKE, AND CORTICAL SEROTONIN IN
MATURE MICE**

Abstract

Low circulating hydroxycholecalciferol (25OHD) is commonly found in obese individuals, due at least in part to volume dilution of vitamin D in adipose tissue. However, whether vitamin D deficiency contributes to obesity and exacerbates related metabolic outcomes is unclear. To address this question, female retired breeder C57BL/6J mice (~8mo old) were *ad libitum* fed a high-fat diet (HFD – 45% of energy as fat) containing low (100 IU/kg), normal (1,000 IU/kg), or high (10,000 IU/kg) vitamin D content. After 10wk, mice fed the HFD with low D diet had greater energy intake, weight gain, fat mass, and hepatic fat than the HFD normal D and high D groups ($P < 0.05$). To follow-up and to assess a potential mechanism underlying our findings, we designed a second experiment to examine whether serotonin indices in the frontal cortex were affected by low D intake. Mice were fed either a normal fat diet (NFD – 10% of energy as fat) or the HFD, with each diet containing low or normal vitamin D. By the end of the experiment, both HFD and low D attenuated serum 25OHD ($P < 0.05$). While the HFD-low D intake appeared to increase weight and adiposity compared to the HFD-normal D diet, this was not significant in this second experiment. However, low D intake, regardless of the presence of HFD, lowered gene expression of 1 α -hydroxylase *Cyp27b1* and frontal cortex serotonin concentrations in the brain ($P < 0.05$). There was no effect of the diets on expression of genes involved in central serotonergic pathways including tryptophan hydroxylase 2, serotonin receptor 2C, and serotonin transporter. Taken together, these findings suggest that both HFD and low D intake lower circulating 25OHD, while supplemental vitamin D has no effect on preventing HFD-induced obesity. While we found that vitamin D deficiency had inconsistent effects on food intake and weight gain, our findings suggest that low D also lowers cerebral serotonin concentration. Whether serotonin regulates appetite, depression, and other cognitive outcomes associated with low vitamin D status should be explored further.

Key words. Vitamin D deficiency, saturated fatty acids, vitamin D hydroxylase, tryptophan hydroxylase 2, serotonin.

Introduction.

Vitamin D inadequacy is commonly found in children and adults (Holick 2007). According to the recent NHANES data reported in year 2010, more than 30% of the population in the United States has vitamin D inadequacy or deficiency indicated by serum concentration of 25-hydroxycholecalciferol (25OHD) less than 30 ng/mL. Importantly, vitamin D deficiency is closely related to many chronic diseases such as type II diabetes, autoimmune disease, and cancers (Cui 2013, González-Molero 2014). Circulating 25OHD status is inversely associated with obesity related parameters such as adiposity, body mass index (BMI), and waist circumference (González-Molero 2014, Lagunova 2009, Tamer 2012, Cheng 2010, Oliai 2015). In addition, obese individuals are at greater risk of developing vitamin D deficiency than lean people. Proposed causes by which obesity may affect vitamin D status include insufficient dietary intake, low sunlight exposure, and sequestration of vitamin D in adipose tissue (Tsiaras 2014, Christakos 2016, Drincic 2012). Alternatively, vitamin D deficiency may contribute to obesity. Besides its classic function as a calciotropic hormone in regulating calcium and bone metabolism (Christakos 2016), vitamin D also affects proliferation and differentiation of various genes involved in many physiological activities. Thus, it is possible that vitamin D may have a direct effect on regulating energy homeostasis, though the underlying mechanisms remain unclear.

The neurotransmitter serotonin is involved in several neurophysiological functions including control of energy balance, appetite, regulation of mood, and other behavioral traits (Tecott LH 2007, Hale 2012, Voigt JP 2015). Local synthesis of serotonin from tryptophan in the central nervous system (CNS) is catalyzed by tryptophan hydroxylase-2 (TPH2) (Walther 2003, Gutknecht 2009). TPH2 is essential in serotonin synthesis, and its expression and activity are closely associated with central serotonin concentration in humans and rodents (Walther 2003). Synthesis of serotonin in

the CNS is completely inhibited in Tph2 knockout mice (Brommage 2015, Kriegebaum 2010), and in clinical trials, people with dysfunctional TPH2 attributed to genetic polymorphisms have compromised serotonergic neurotransmission and suffer anxiety and depressive disorders (Berger 2012).

Recently, a vitamin D response element has been identified in the promoter region of TPH2 (Patrick 2014, Patrick 2015, Kaneco 2015), suggesting vitamin D may have a direct effect on serotonin metabolism in the CNS. Indeed, one *in vitro* study demonstrated that there is a dose dependent increment of Tph2 gene expression in both human brain glioblastoma/astrocytoma U87-MG cells and rat embryonic medullary raphe RN46A-B14 cells responding to treatment with 1,25-dihydroxycholecalciferol [1,25(OH)₂D] (Kaneco 2015). Since extensive studies have examined the effects of vitamin D and serotonin in regulating neurological health, eating behavior, and energy balance (Zhang 2005, Nash 2005, Alfawaz 2014), it is possible that vitamin D deficiency related to obesity is mechanistically mediated via a vitamin D regulated serotonin system. However, the relationship between vitamin D, energy balance, and central serotonin synthesis has not been investigated.

In the current study, the primary goal was to determine the effect of vitamin D status on weight gain and adiposity under conditions of normal and high fat diet intake in aging female mice. The secondary goal was to investigate whether low vitamin D feeding affects obesity via alteration of serotonergic pathways in the CNS. We hypothesized that low dietary vitamin D intake would promote energy intake, attenuate central serotonin production, and promote obesity, and these effects would be exacerbated by excessive dietary saturated fatty acids (SFA) intake.

Experiment Design.

Experiment 1: Effect of dietary vitamin D on adiposity in diet-induced obese mice.

Twenty-one mice (n=7 per group) were assigned to receive a diet high in SFA (45% energy as fat, 20% energy as protein, and 35% energy as carbohydrate) containing 1 of 3 dietary vitamin D levels for 10wk: 100 (low), 1000 (control), or 10,000 (high) IU vitamin D/Kg diet (**Supplemental Table 1**). After 10wk, mice were killed by decapitation, serum was collected and stored at -80 °C, and the liver was obtained and immediately frozen in liquid N₂.

Experiment 2: Effect of low vitamin D intake on serotonin pathways in lean and obese mice. Forty mice (n=10 per group) were assigned in a 2 x 2 factorial design to receive a NFD (10% energy as fat, 20% energy as protein, and 70% energy as carbohydrate) or a high fat diet (SFA – macronutrient content same as in Experiment 1), and a normal (ND – 1000 IU/Kg diet) or low (LD – 100 IU/Kg diet) level of vitamin D. During the feeding period, four mice and their data from the NFD-ND (n=1), NFD-LD (n=1), and SFA-LD (n=2) groups were removed due to severe dermatitis attributed to aging. After 10wk, food was removed for approximately 16h before mice were killed by decapitation. Blood was collected, and cerebral cortex, liver and kidney were quickly removed, weighed, and frozen in liquid N₂. The midbrain was sectioned and preserved in RNA ladder for further dissections of the raphe nucleus and arcuate nucleus.

Results

1. Experiment 1: Effect of dietary vitamin D on adiposity in diet-induced obese mice.

Caloric intake and body weight. In mice receiving the SFA diet with low vitamin D (SFA-LD), feeding resulted in greater cumulative caloric intake over time compared with mice fed the SFA diet with normal (SFA) or high vitamin D (SFA-HD) (**Figure 21A**) ($P < 0.001$). Low dietary vitamin D intake did not affect body weight over the 10wk feeding

period (**Figure 21B**); however, one-way ANOVA indicated that final body weight was significantly higher in the SFA-LD group than the SFA and SFA-HD groups ($P = 0.04$) (**Table 9**). The delta weight change between baseline and wk10 was also higher in the SFA-LD group compared with the SFA and SFA-HD groups ($P = 0.003$).

Body composition and bone mineral density. Body fat mass ($P = 0.03$) and percent fat ($P = 0.05$) were greater in the SFA-LD group than in the SFA and SFA-HD groups (**Table 9**). However, there were no significant differences in final lean mass and body water among the diet groups. Total body BMC and BMD values were also comparable among the groups.

Hepatic lipid and triglyceride levels. Wet liver weight was lower in the SFA-HD group when compared with the SFA and SFA-LD groups, with 1.28 ± 0.20 , 1.09 ± 0.11 , and 1.30 ± 0.16 g in the SFA, SFA-HD, and SFA-LD groups, respectively ($P < 0.05$). Hepatic total lipid level was higher in the SFA-LD group, when compared with the SFA group ($P = 0.04$) (**Figure 22A**). In addition, hepatic triglyceride content was lower in the SFA-HD than the SFA group ($P = 0.03$) (**Figure 22B**).

Serum biochemistry. Serum 25OHD concentration directly reflected dietary intake, with low and high vitamin D intake leading to lower and higher serum 25OHD when compared with the SFA control group ($P < 0.001$) (**Table 10**). Serum concentrations of PTH, FGF23, and estradiol did not differ among the diet groups.

2. Experiment 2: Effect of low vitamin D intake on serotonin pathways in lean and obese mice.

Energy intake and body weight. Cumulative caloric intake and weekly body weight of mice during the feeding period are presented in **Figure 23**. Consumption of the SFA diet resulted in higher caloric intake and total body weight over time ($P < 0.001$) compared with the NFD diet irrespective of vitamin D content. No effect of low dietary vitamin D or

an interaction effect between dietary fat and vitamin D was observed on food intake and body weight over time.

Body composition and bone mineral density. Prior to being assigned to their respective dietary groups, mice had similar amounts of body fat and fat free mass (data not shown). Final body composition is presented in **Table 11**. After 10wk of feeding, mice from both SFA groups had greater amounts of body fat, lean mass, and total water when compared with the NFD controls irrespective of vitamin D status ($P < 0.05$). There was no significant effect of the vitamin D deficient diet and no interaction effect between dietary fat and vitamin D on any of the body compartments. Moreover, all mice had comparable amounts of total body BMD and BMC, and neither dietary SFA or vitamin D showed an effect (**Table 11**).

Hepatic lipid. No significant differences in total liver weights were detected among the mice fed the different dietary groups [liver tissue weights: 1.2 ± 0.2 , 1.1 ± 0.2 , 1.3 ± 0.2 , and 1.3 ± 0.2 g in the NFD, NFD-LD, SFA, and SFA-LD groups, respectively ($P > 0.05$)]. However, hepatic total lipid level was higher in the mice fed the SFA diet compared with those fed the NFD diet [lipid levels: 128 ± 32 , 111 ± 16 , 145 ± 18 , and 166 ± 19 ug/ug protein in the NFD, NFD-LD, SFA, and SFA-LD groups, respectively ($P < 0.05$)]. There was no significant effect of low dietary vitamin D and no interaction effect between dietary fat and vitamin D on tissue weight and total lipid level of the liver.

Serum biochemistry. Both low vitamin D and high SFA feeding were associated with lower serum 25OHD concentration compared with normal vitamin D and NFD controls, respectively ($P < 0.001$). However, no interaction effect between dietary fat and vitamin D on serum 25OHD was observed (**Figure 24**). Serum PTH concentration was higher in those fed the SFA diet [PTH concentrations: 117 ± 33 , 73 ± 17 , 235 ± 37 , and 165 ± 36 ng/mL in the NFD, NFD-LD, SFA, and SFA-LD groups, respectively ($P < 0.01$)]. Low vitamin D did not alter serum PTH, and no interaction effect between dietary fat and

vitamin D was found.

High SFA feeding led to higher fasting serum glucose levels [glucose concentrations: 128 ± 32 , 111 ± 16 , 166 ± 19 , 145 ± 18 mg/dL in the NFD, NFD-LD, SFA, and SFA-LD groups, respectively ($P < 0.001$)]. In addition, when examining the effect of vitamin D on fasting glucose, it was found that serum concentration was lower due to low dietary vitamin D feeding under condition of the same amount of fat intake ($P < 0.05$). However, there was no interaction effect between dietary fat and vitamin D on fasting serum glucose level.

Vitamin D hydroxylase mRNA expression. In the liver, the relative mRNA abundance of Cyp2r1 was higher in those mice fed the SFA diet compared with those fed the NFD diet ($P < 0.001$). There was no significant effect of dietary vitamin D and no dietary fat-vitamin D interaction effect on hepatic Cyp2r1 mRNA expression (**Figure 25**). In the kidney, high SFA did not alter Cyp27b1 mRNA. In contrast, low dietary vitamin D up-regulated Cyp27b1 mRNA, ($P < 0.01$). No interaction effect between dietary fat and vitamin D on Cyp27b1 expression was observed. Renal Cyp24 mRNA was similar among the diet groups, and neither dietary fat or vitamin D level altered its expression.

Vitamin D and serotonin metabolizing enzyme mRNA expression in the brain. In the arcuate nucleus, high SFA and low vitamin D feeding down-regulated expression of Cyp27b1 ($P < 0.05$ and $P < 0.01$, respectively). A weak dietary fat-vitamin D interaction effect on Cyp27b1 mRNA in the arcuate nucleus was observed, but this did not reach statistical significance ($P = 0.09$) (**Table 13**). In addition, relative mRNA abundance of Vdr was lower in mice fed the SFA diet compared with those fed the NFD diet ($P < 0.05$). No significant effects of dietary fat, vitamin D, or the interaction of dietary fat and vitamin D were found on the expression levels of TPH2, SERT, or Htr2c in the arcuate nucleus.

In the dorsal raphe nucleus, low vitamin D feeding down-regulated Cyp27b1 mRNA expression ($P < 0.01$) (**Table 12**). The relative expression of Htr2c also was lower

with low vitamin D intake, but did not reach statistical significance ($P = 0.07$). No effect of dietary fat and no interaction effect between dietary fat and vitamin D on Cyp27b1 and Htr2c was observed. Expression of Vdr, TPH2, and Sert were not different among the diet groups.

Cerebral cortex serotonin concentration. Serotonin concentration in the cerebral cortex was lower in those mice fed the low vitamin D diets ($P < 0.05$). There was no significant effect of dietary SFA and no dietary fat-vitamin D interaction effect on serotonin concentration in the cortex (**Figure 26**).

Correlation test. Serum 25OHD concentration was inversely associated with body weight after adjusting for dietary vitamin D intake ($r = -0.35$, $P < 0.05$), and was directly associated with serotonin concentration in the cerebral cortex after adjustment for final body weight ($r = 0.35$, $P = 0.04$) (**Figure 27**).

Discussion

Lowered circulating 25OHD is commonly found in obese individuals (Dylag 2014, Gonzalez-Molero 2013, Cheng 2010, Oliai 2015). However, it is unclear whether excess saturated fat intake and obesity directly cause reduced vitamin D status, or if vitamin D deficiency contributes to body adiposity. The primary objective of the current study was to investigate the role of dietary vitamin D in the pathogenesis of diet-induced obesity. In our first experiment, we found that a high saturated fat diet that was also low in vitamin D increased food intake, weight gain, adiposity, and fatty liver compared with the same high fat diet with normal level of vitamin D. Supplemental vitamin D 10x greater than that typically supplied in a mouse diet showed no effect on high fat diet-induced obesity. In our second experiment, we sought to follow up the findings regarding the effect of vitamin D deficiency on energy balance under conditions of normal and high fat intake, and to further explore whether this is mechanistically mediated through

serotonergic pathways.

In the second experiment, in contrast to experiment 1, although the average values of body weight and fat mass were higher in mice fed a high fat diet with low vitamin D compared with those fed a high fat diet with normal vitamin D, the differences did not reach statistical significance. In addition, low vitamin D feeding showed a null or even a mild negative effect on weight gain in older female mice fed a normal fat diet. The reason for the discrepant findings between experiments 1 and 2 is unclear, but may be explained by the fact that the cumulative food intake was lower in all mice from the first experiment compared with all mice in the second experiment. The higher overall food intake in the second experiment may have attenuated the influence of low vitamin D on body weight and adiposity. However, the reason for the different overall food intakes between the two experiments is unclear because all mice were fed, handled, and housed similarly in both experiments. Effects of vitamin D on body weight have been observed in other rodent studies. For example, Brouwer-Brolasma et al (2015) found excess food intake and weight gain due to low vitamin D feeding when older male mice were fed a 20% moderate fat diet, while Domingues-Faria et al (2014) made a similar observation in older rats fed a normal fat diet. In young male mice, Trinco et al (2016) found greater food intake and weight gain attributed to vitamin D deficiency combined with a 35% high fat diet. Notably, in this latter study, total body weight did not start to differ from the control animals fed the high fat diet with normal vitamin D until 12wk of feeding (Trinco et al 2016). Moreover, null and preventive effects of vitamin D deficiency on weight gain and adiposity have been seen in young mice as well (Liu 2015, Borges 2016, Kong 2014, Bhat 2014). The reasons for the different findings among the studies in young animals are unclear.

Similar to body weight and fat mass, we also found discrepant effects of vitamin D deficiency on hepatic fat accumulation in the mice fed the high fat diet

between experiments 1 and 2; a significant effect was observed in experiment 1, but not in experiment 2. This difference may also be related to the overall food intake differences between the two experiments. In humans, circulating 25OHD is closely correlated with the pathogenesis and progression of several chronic liver diseases (Barchetta 2011, Eliades 2013, Dasarathy 2014, KO 2016). In rodent studies, effects of vitamin D deficiency on fatty liver diseases have been inconsistent (Liu 2015, Kong 2014). Taken together, our findings and those of other research groups suggest that vitamin D deficiency may promote obesity and fatty liver, but the magnitude of the effects may be influenced by other factors such as dietary fat intake, period of deficiency, gender, age, and genetic variation.

Diet is an important contributor to vitamin D status (Tsiaras 2014, Domingues-Faria). As expected, we found that dietary vitamin D level was directly correlated with circulating 25OHD, and there was an inverse correlation between body weight and 25OHD. These findings are consistent with observations in human subjects that circulating vitamin D is lower in obese individuals (Gonzalez-Molero I 2014, Cheng 2010). Potential mechanisms that contribute to vitamin D deficiency in obesity include poor eating habits, sedentary lifestyle, reduced exposure to sunlight, and an adipose tissue dilution effect (Tsiaras 2014, Christakos 2016, Drincic 2012). In addition, there have been some studies showing that high fat feeding and obesity may affect vitamin D regulating enzymes (Park 2015, Wang 2016, Wamberg 2013). Thus, it is possible that low circulating 25OHD attributed to high fat feeding and obesity is due to a compromised hepatic activation of vitamin D. When examining gene expression of the major vitamin D 25-hydroxylase, Cyp2r1, in the liver, we found an up-regulation of Cyp2r1 message due to high fat feeding, suggesting a possible compensatory mechanism in response to reduced serum 25OHD concentration. However, using a young murine model, Park et al (2015) showed a decrease of gene expression of Cyp2r1 in high fat diet-induced obese

male mice when compared with low fat diet controls. Notably, they did not see serum changes of 25OHD concentration in the young mice (Park 2015).

Expression and activity of vitamin D 1 α -hydroxylase, Cyp27b1, in kidney is also important in maintaining circulating 25OHD concentrations. Environmental and hormonal factors, including low dietary vitamin D, low calcium intake, and high PTH, have been shown to up-regulate Cyp27b1 (Fleet 2016 Deluca 2016). The role of dietary fat on Cyp27b1 is less studied. In the current study, up-regulation of renal Cyp27b1 message was expectedly seen due to low vitamin D intake in the second experiment. However, we did not observe an effect of dietary fat on Cyp27b1 expression. Additionally, neither the high fat diet nor low vitamin D feeding altered renal vitamin D 24-hydroxylase, Cyp24, the catabolic enzyme responsible for degradation of excess vitamin D. These findings are in contrast to previous observations of higher and lower gene expression of Cyp27b1 and Cyp24 due to high fat feeding in young male mice (Park 2015).

The vitamin D endocrine system is essential for regulating calcium homeostasis and maintaining optimal skeletal health. Vitamin D deficiency attributed to low intake, high fat feeding, and obesity may negatively affect bone mass and quality (Fleet 2016, Yarrow 2016, Wang 2016, Shapses). Interestingly, our current data from both experiments showed that total body BMD and BMC were not altered by vitamin D deficiency in lean and high fat diet-induced obese mice. This may be partially explained by adequate amounts of calcium and phosphate content in the diet that help to prevent bone loss (Cifuentes 2016). Additionally, we used mature mice. Previous studies have shown detrimental effects of vitamin D deficiency on bone mineralization and development in young animals. The response of bone mineralization and structures to vitamin D deficiency may vary greatly in older people and mice (Deluca 1984, Meijden 2015). Furthermore, a mechanical loading effect attributed to heavier body weight with

excess caloric intake from fat may benefit bone mass initially (Bonewald 2008). It is also possible that fat-derived bone-favoring hormones promote bone health; however, serum estradiol did not differ among the dietary groups when examined in our first experiment. Therefore, it is possible dietary vitamin D deficiency and obesity may not initially translate into low bone mass. Over longer periods, more adverse effects on bone may occur due to high fat intake, obesity, vitamin D deficiency, or a combination of these factors.

Studies report that vitamin D influences obesity, metabolic syndrome, and insulin resistance. Some studies report that vitamin D supplementation protects against obesity and its morbidities by promoting glucose uptake, insulin sensitivity, leptin secretion, and adipose tissue apoptosis (Sergeev 2014, Marcotorchino 2012, Kong 2013). In addition, vitamin D deficiency may contribute to the pathogenesis of obesity, and indeed low intake and low serum 25OHD concentration are directly associated with all morbidities associated with obesity (Lagunova 2009, Tamer 2012, Cheng 2010). Mechanistically, cell studies have demonstrated that vitamin D appears to affect lipid metabolism and adipogenesis. Depending on the stage of adipocyte differentiation, the active form of vitamin D, 1,25(OH)₂D, can promote or inhibit adipogenesis *in vitro* (Mutt 2014, Blumberg 2006, Kong 2006, Nimitphong 2012, Narvaez 2013). However, whether vitamin D deficiency regulates energy balance in humans and experimental animals is unclear. Recently, a vitamin D responding element has been identified in the enzyme, TPH2, suggesting that vitamin D may affect local synthesis of serotonin in the CNS. Central serotonin is important in regulating food intake and appetite, and its concentration is inversely associated with weight gain (Tecott 2007, Hale 2012, Voigt JP 2015). Though studies have demonstrated independent roles of vitamin D and serotonin in regulating energy balance, the relationship between vitamin D and serotonin remains unclear. Therefore, in our second experiment, we sought to explore the hypothesis that

vitamin D deficiency promotes weight gain by affecting serotonergic pathways in the CNS. Interestingly, our data indicated that the serotonin concentration in the frontal cortex was lower in mice fed the low vitamin D deficient. In addition, there was a positive association between brain serotonin and serum 25OHD concentration. These are consistent with findings from a cell study of dose dependent changes in TPH2 expression in response to 1,25OHD treatment (Kaneco 2015). Our data suggest extends this *in vitro* observation by demonstrating that systemic vitamin D deficiency compromises central serotonin synthesis *in vivo*.

We also examined gene expression of markers involved in vitamin D and serotonergic pathways. In the arcuate nucleus, an essential brain region in regulating appetite and energy consumption, we found a lower gene expression of vitamin D 1 α -hydroxylase due to low dietary vitamin D intake. However, vitamin D deficiency showed no effect on gene expression of TPH2, the serotonin reuptake transporter, SERT, and the serotonin receptor, Htr2c. Moreover, since serotonin is exclusively synthesized in discrete collections of brainstem neurons known as the raphe nuclei in mammals (Hornung 2003), we also examined gene expression of these genes in raphe nuclei. Consistently, only a lower vitamin D 1 α -hydroxylase was observed due to vitamin D deficiency in the raphe nucleus; expression of none of the other vitamin D and serotonergic-related genes were affected by vitamin D deficiency. Thus, our data suggest that peripheral vitamin D deficiency leads to lower vitamin D in the brain, which may impair serotonin production in the CNS. However, due to the complexity of the serotonergic system, and that the overall serotonergic effect depends on other factors such as serotonin synthesis, neuronal release, re-uptake, and circulating concentration, the exact meaning of the observed association between vitamin D deficiency and lowered serotonin in the cerebral cortex remains unclear. It is possible that lowered serotonin concentration may or may not directly translate into compromised serotonergic

effect and physiological changes systemically. Thus, in-depth studies and behavioral tests such as swimming test and marble burying tests should be conducted in the future to investigate the role of vitamin D on the serotonin axis.

One limitation of the current study is that gene expression of Cyp27b1 and TPH2 were not examined in the cerebral frontal cortex, although this is the area of the brain in which lowered serotonin concentration was observed. Similarly, serotonin concentrations within the arcuate and raphe nuclei were not measured due to the limited tissue size. However, the change of serotonin concentration in the frontal cortex is believed to be a good indicator of overall concentration in the brain. In addition, though Htr2c is particularly important in regulation of food intake, we only examined the effect of vitamin D deficiency on gene expression of Htr2c, while other receptors, such as Htr1c, might have been affected.

In conclusion, both high fat feeding and low vitamin D intake are related to lower serum 25OHD concentration, and supplemental vitamin D has no beneficial effects on preventing high fat-induced obesity. Vitamin D deficiency combined with high fat feeding may contribute to greater increases in total body adiposity and fatty liver, and this may be related to reduction of serotonin concentration in the brain.

Table 9. Body composition and bone density in mice after 10wk of *ad libitum* feeding with SFA diet containing normal, high, and low amount of vitamin D content¹

	Diet			<i>P</i> value
	SFA	SFA-HD	SFA-LD	
Final weight, g	35.3 ± 5.6 ^b	34.2 ± 3.0 ^b	40.2 ± 3.8 ^a	0.04
Weight change, g/10wk	8.2 ± 3.3 ^b	9.1 ± 3.0 ^b	13.6 ± 1.4 ^a	0.003
Fat mass, g	12.8 ± 4.9 ^b	12.3 ± 2.2 ^b	17.1 ± 1.9 ^a	0.03
Percent fat, %	35.1 ± 8.9	35.7 ± 3.8	42.5 ± 2.9	0.05
Lean mass, g	20.5 ± 0.6	19.9 ± 1.3	20.3 ± 1.2	0.64
Body water, g	16.0 ± 0.7	15.7 ± 1.0	15.4 ± 1.1	0.52
Total body BMC, g	0.60 ± 0.1	0.59 ± 0.1	0.56 ± 0.1	0.61
Total body BMD, mg/cm ²	55.8 ± 4.6	56.4 ± 3.0	55.8 ± 4.1	0.96

¹All values are means ± SD (n=7 per group). Labeled means in a row with different superscripts differ, *P* < 0.05 by one-way ANOVA, following with Newman-Keuls post hoc test. BMC, bone mineral content; BMD, bone mineral density; SFA, saturated fatty acids; SFA-HD, saturated fatty acids with high vitamin D content; SFA-LD, saturated fatty acids with low vitamin D content.

Table 10. Serum biochemistry in mice after 10wk of *ad libitum* feeding with SFA diet containing a normal, high, or low amount of vitamin D content¹

	Diet			<i>P</i> value
	SFA	SFA-HD	SFA-LD	
25OHD, ng/mL	17.5 ± 2.9 ^b	35.9 ± 3.6 ^a	4.49 ± 7.1 ^c	<0.001
PTH, pg/mL	406 ± 389	529 ± 411	560 ± 350	0.76
FGF23, pg/mL	531 ± 60	599 ± 134	515 ± 91	0.32
Estradiol, pg/mL	12.4 ± 2.8	13.1 ± 10	10.8 ± 0.7	0.07

¹All values are means ± SD (n=7 per group). Labeled means in a row with different superscripts differ, *P* < 0.05 by one-way ANOVA, followed by Newman-Keuls post hoc test. 25OHD, 25-hydroxycholecalciferol; FGF23, fibroblast growth factor 23; PTH, parathyroid hormone; SFA, saturated fatty acids; SFA-HD, saturated fatty acids with high vitamin D content; SFA-LD, saturated fatty acids with low vitamin D content.

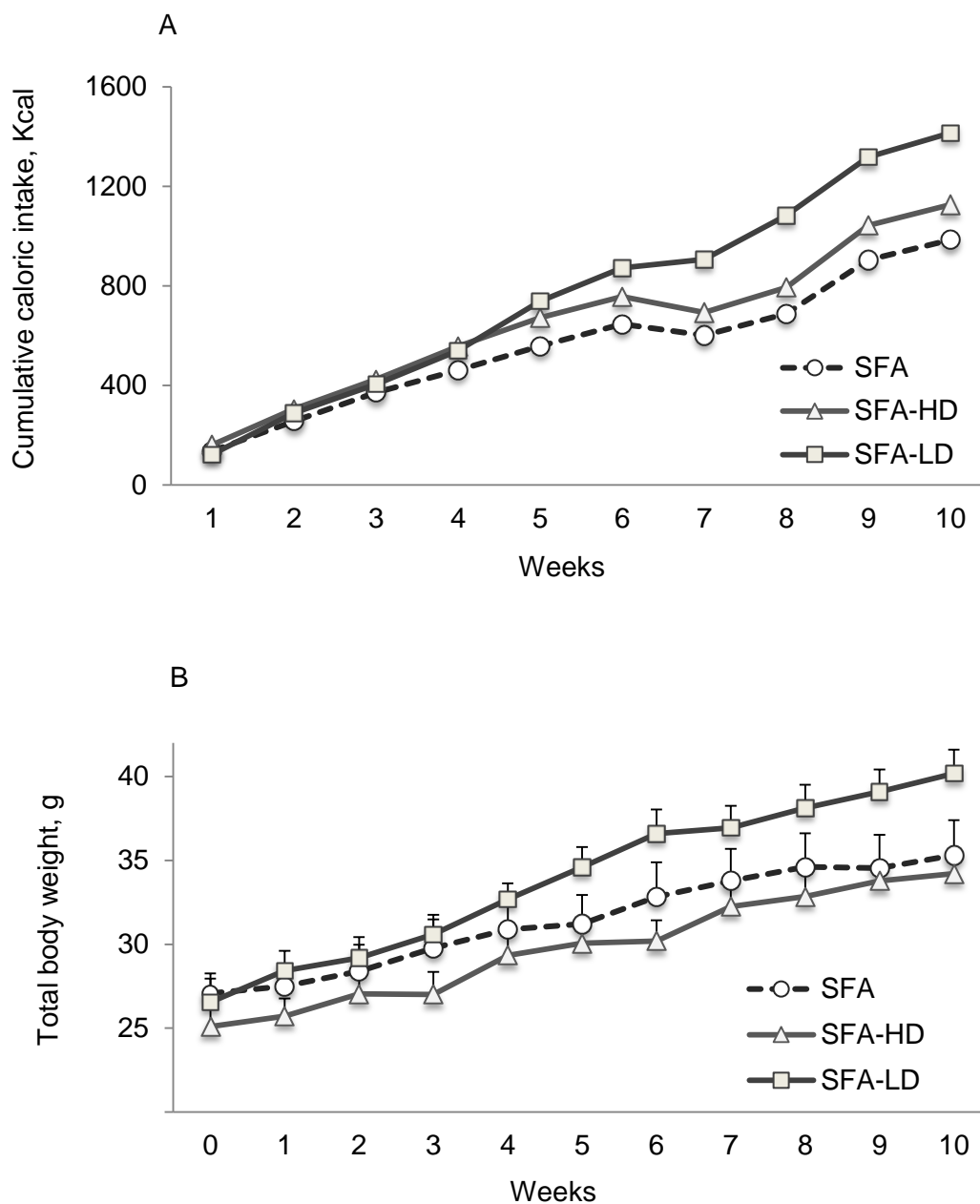


Figure 21. Weekly cumulative caloric intake (A) and body weight ($P=0.82$) (B) in mice during the 10wk of *ad libitum* feeding with SFA diet containing a normal, high, and low amount of vitamin D content¹.

Values are means \pm SEM ($n=7$ per diet group). $P < 0.05$ by one-way ANOVA, followed by Newman-Keuls post hoc test. Error bars in Figure A are very small and cannot be seen. SFA, saturate fatty acids; SFA-HD, saturated fatty acids with high vitamin D content; SFA-LD, saturated fatty acids with low vitamin D content.

Figure 1.2

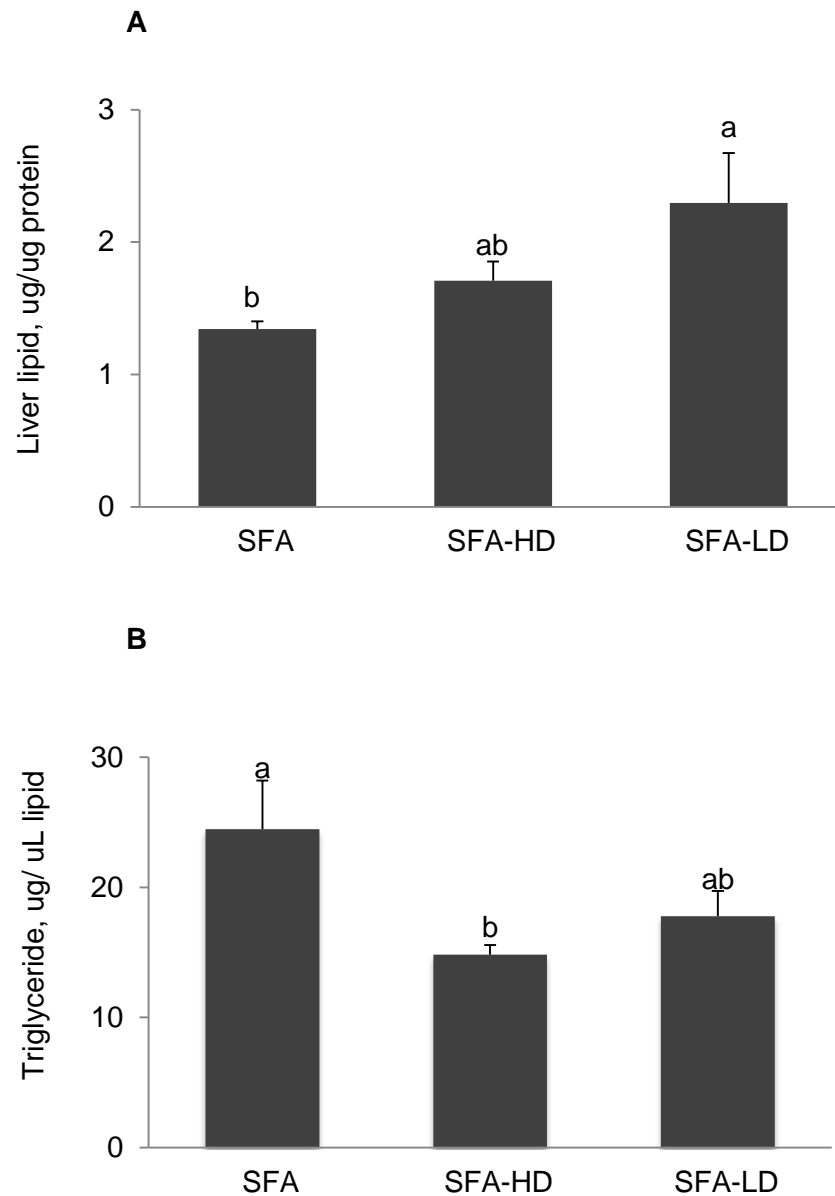


Figure 22. Liver lipid (A) and triglyceride (B) levels in mice after 10wk of *ad libitum* feeding with SFA diet containing a normal, high, and low amount of vitamin D content¹.

Values are means \pm SEM (n=7 per diet group). $P < 0.05$ by one-way ANOVA, followed by Newman-Keuls post hoc test. SFA, saturated fatty acids; SFA-HD, saturated fatty acids with high vitamin D content; SFA-LD, saturated fatty acids with low vitamin D content.

Table 11. Body composition and bone density in mice after 10wk of *ad libitum* feeding with normal or high saturated fat diets containing either normal or low amount of vitamin D content¹

	NFD		SFA		<i>P</i> value		
	Normal vitD	Low vitD	Normal vitD	Low vitD	Fat	Vitamin D	Fat x VitD
Weight change, g/10wk	-0.87 ± 3.0 ^b	-2.93 ± 2.3 ^b	5.96 ± 5.1 ^a	6.90 ± 4.2 ^a	<0.001	0.66	0.25
Fat mass, g	4.6 ± 2.2 ^b	3.7 ± 0.9 ^b	10.2 ± 4.6 ^a	11.6 ± 3.0 ^a	<0.001	0.83	0.31
Fat change, g/10wk	-1.50 ± 2.0 ^b	-2.45 ± 1.4 ^b	4.06 ± 3.8 ^a	5.45 ± 3.9 ^a	<0.001	0.83	0.25
Lean mass, g	21.6 ± 1.0 ^b	21.3 ± 0.9 ^b	22.6 ± 1.6 ^a	22.5 ± 1.1 ^a	0.01	0.60	0.82
Free water, g	0.21 ± 0.2	0.14 ± 0.1	0.27 ± 0.4	0.19 ± 0.3	0.51	0.39	0.95
Total body water, g	20.0 ± 1.0 ^b	19.8 ± 0.7 ^b	21.8 ± 2.3 ^a	20.5 ± 0.9 ^a	0.03	0.17	0.29
Total body BMC, g	0.51 ± 0.08	0.48 ± 0.05	0.52 ± 0.08	0.52 ± 0.12	0.13	0.98	0.55
Total body BMD, mg/cm ²	54.9 ± 2.8	53.6 ± 1.7	54.3 ± 2.2	54.2 ± 3.4	0.43	0.76	0.55

¹All values are means ± SD (n=8-10 for dietary group). Labeled means in a row with different superscripts differ, *P* < 0.05 by two-factor ANOVA, followed by Newman-Keuls post hoc test. BMC, bone mineral content; BMD, bone mineral density; NFD, normal fat diet; NFD-LD, normal fat diet with low vitamin D content; SFA, saturated fatty acids, SFA-LD, saturated fatty acids with low vitamin D content; VitD, vitamin D.

Table 12. Relative gene expression of markers in vitamin D and serotonin system of mice after 10wk of *ad libitum* feeding with normal or high saturated fat diets containing either normal or low amount of vitamin D content¹

	NFD		SFA		P value		
	Normal vitD	Low vitD	Normal vitD	Low vitD	Fat	Vitamin D	Fat x VitD
Accurate Nucleus							
Cyp27b1	1.00 ± 0.42	0.56 ± 0.21	0.63 ± 0.14	0.42 ± 0.21	0.01	0.001	0.09
Vdr	1.00 ± 0.30	1.11 ± 0.27	0.84 ± 0.14	0.62 ± 0.44	0.01	0.95	0.22
Tph2	1.00 ± 0.26	0.81 ± 0.39	1.43 ± 0.88	0.99 ± 0.58	0.08	0.14	0.60
Htr2c	1.00 ± 0.30	1.63 ± 1.03	1.16 ± 0.37	1.05 ± 0.60	0.59	0.08	0.23
Sert	1.00 ± 0.37	1.61 ± 0.73	1.71 ± 0.47	1.35 ± 0.82	0.09	0.21	0.08
Raphe Nucleus							
Cyp27b1	1.00 ± 0.54	0.47 ± 0.18	0.89 ± 0.48	0.54 ± 0.26	0.90	0.005	0.52
Vdr	1.00 ± 0.55	1.25 ± 0.98	0.75 ± 0.93	1.31 ± 0.84	0.76	0.18	0.60
Tph2	1.00 ± 0.99	0.18 ± 0.42	0.88 ± 1.09	0.69 ± 1.12	0.55	0.14	0.35
Htr2c	1.00 ± 0.17	0.9 ± 0.26	1.09 ± 0.39	0.82 ± 0.12	0.88	0.07	0.27
Sert	1.00 ± 0.30	0.25 ± 0.44	0.97 ± 1.07	0.8 ± 1.18	0.44	0.18	0.40

¹All values are means ± SD (n=8-10 per dietary group). Labeled means in a row with different superscripts differ, $P < 0.05$ by two factor ANOVA. Cyp27b1, cytochrome P450 family 27 subfamily B member 1 (vitamin D 1 α -hydroxylase); Htr2c, 5-hydroxytryptamine (serotonin) receptor 2C; NFD, normal fat diet; Sert, serotonin transporter; SFA, saturated fatty acids; Tph2, tryptophan hydroxylase-2; Vdr, vitamin D receptor; VitD, vitamin D.

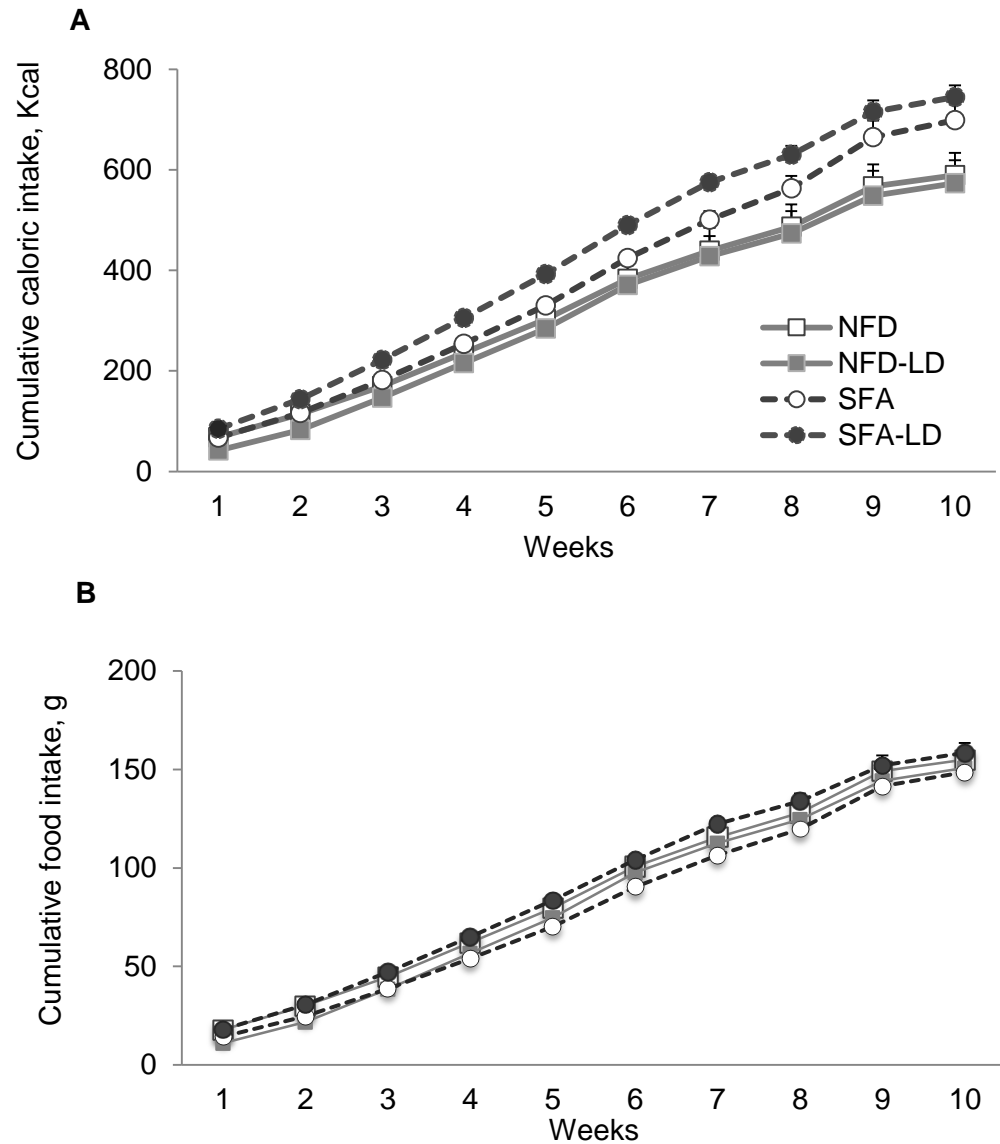


Figure 23. Weekly cumulative caloric intake (A), weekly cumulative food intake (B) in mice during the 10wk of *ad libitum* feeding with normal or high saturated fat diets containing either normal or low amount of vitamin D content.

Values are means \pm SEM (n=8-10 per diet group). * $P < 0.05$ by repeated measure of ANOVA. NFD, normal fat diet; NFD-LD, normal fat diet with low vitamin D content. Error bars are very small and cannot be seen in the figures. SFA, saturated fatty acid, SFA-LD, saturated fat with low vitamin D content.

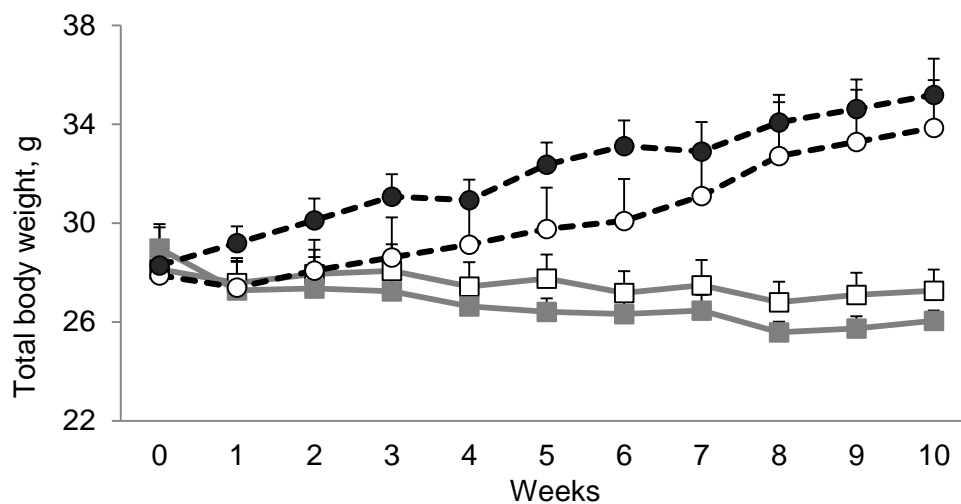


Figure 24. Weekly body weight in mice during the 10wk of *ad libitum* feeding with normal or high saturated fat diets containing either normal or low amount of vitamin D content:

Values are means \pm SEM (n=8-10 per diet group). * $P < 0.05$ by repeated measure of ANOVA. NFD, normal fat diet; NFD-LD, normal fat diet with low vitamin D content. SFA, saturated fatty acid, SFA-LD, saturated fat with low vitamin D content.

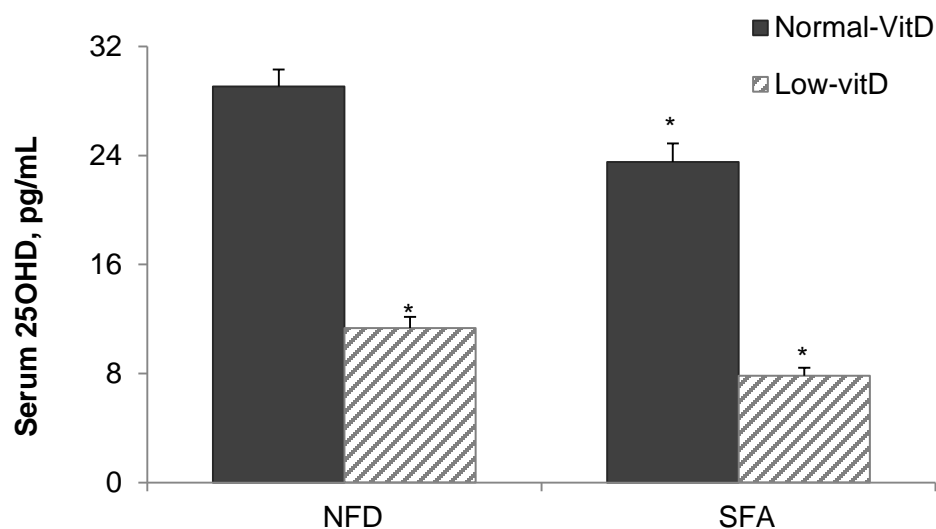


Figure 25. Serum 25OHD concentration in mice after 10wk of *ad libitum* feeding with normal or high saturated fat diets containing either normal or low amount of vitamin D content.

Values are means \pm SEM. * $P < 0.05$ by two-factor ANOVA followed with Newman Keuls post hoc test. 25OHD, 25-hydroxycholecalciferol; NFD, normal fat diet; SFA, saturated fatty acid.

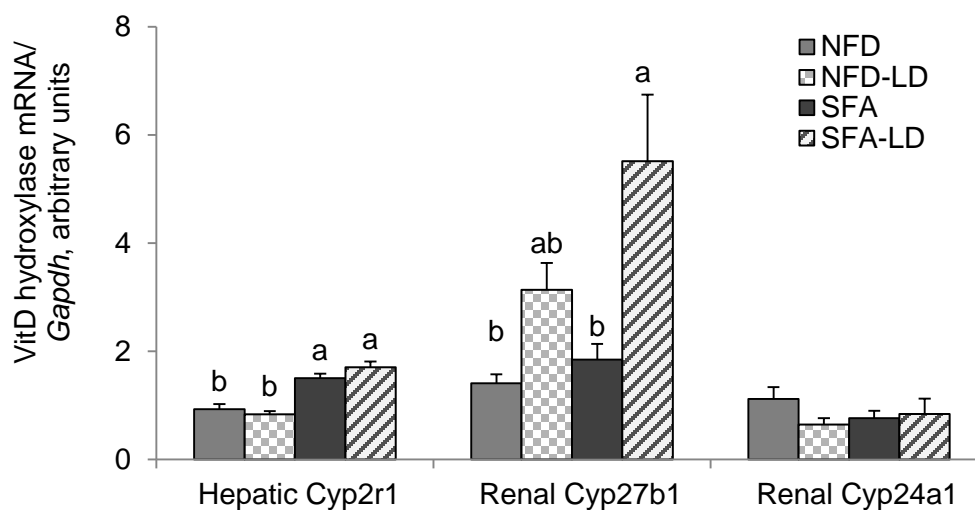


Figure 26. Vitamin D hydroxylases mRNA expression in mice after 10wk of *ad libitum* feeding with normal or high saturated fat diets containing either normal or low amount of vitamin D content.

Values are means \pm SEM. $P < 0.05$ by two-factor ANOVA. Cyp2r1, cytochrome P450 family 2 subfamily R member 1; Cyp24a1, cytochrome P450 family 24 subfamily A member 1; Cyp27b1, cytochrome P450 family 27 subfamily B member 1; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; NFD, normal fat diet; NFD-LD, normal fat diet with low vitamin D content; SFA, saturated fatty acids; SFA-LD, saturated fatty acids with low vitamin D content; vitD, vitamin D.

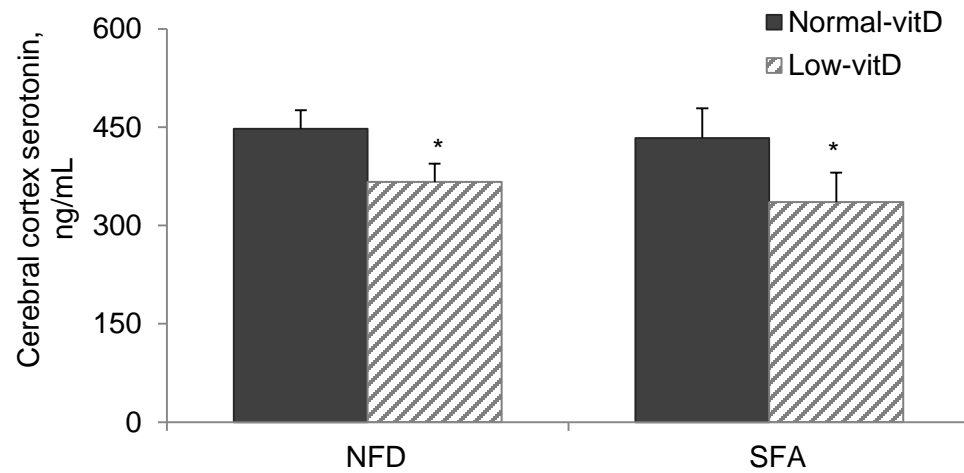


Figure 27. Cerebral cortex serotonin concentration in mice after 10wk of *ad libitum* feeding with normal or high saturated fat diets containing either normal or low amount of vitamin D content.

Values are means \pm SEM (n=8-10 per diet group). * $P < 0.05$ by two-factor ANOVA. NFD, normal fat diet; SFA, saturated fatty acids; vitD, vitamin D.

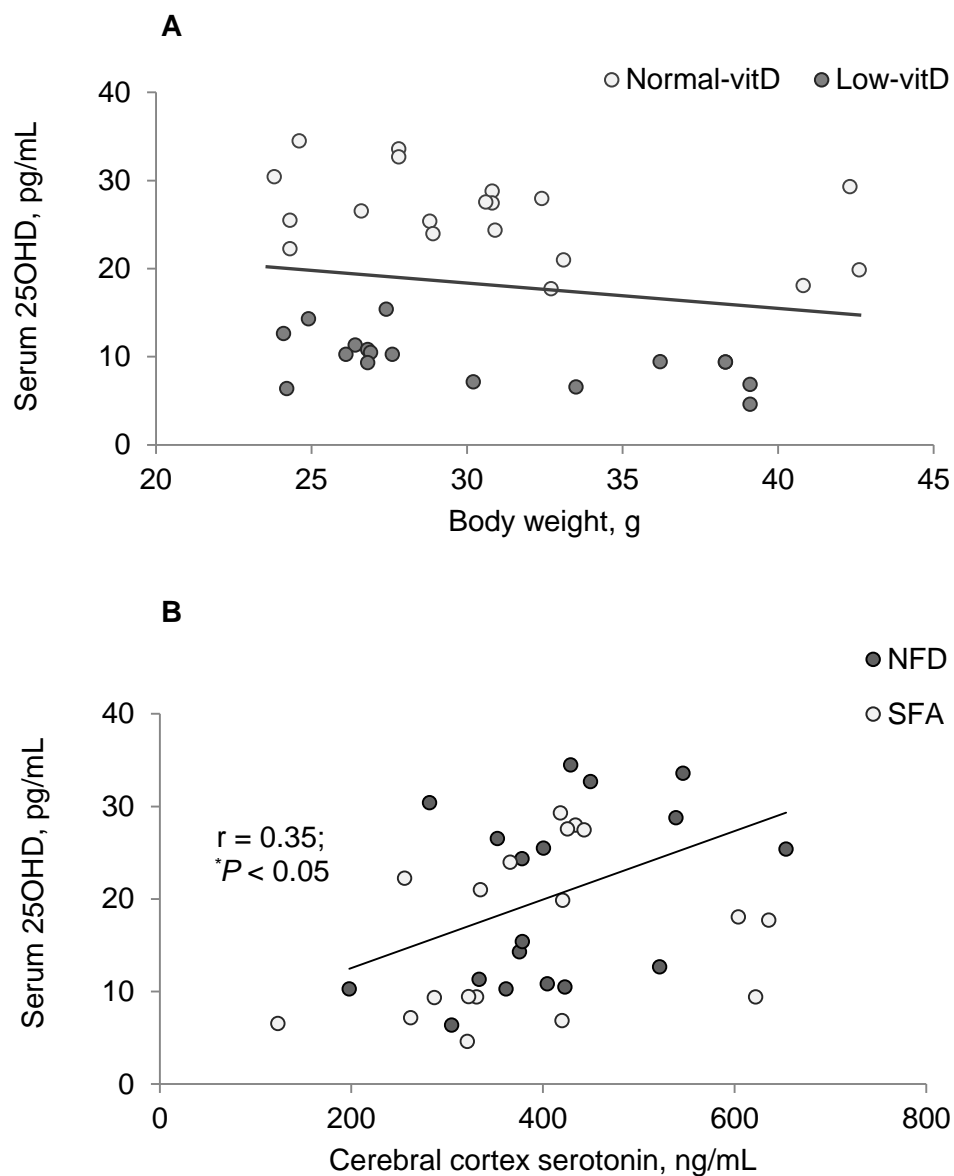


Figure 28. Relationship between serum 25OHD and body weight (A) and cerebral serotonin concentration (B) in mice after 10wk of *ad libitum* feeding with normal or high saturated fat diets containing either normal or low amount of vitamin D content.

* $P < 0.05$ by Pearson correlation. NFD, normal fat diet; SFA, saturated fatty acids; vitD, vitamin D.

CHAPTER VI: CONCLUSION AND FUTURE DIRECTIONS

Dietary fat is known to promote greater food intake and weight gain that eventually leads to positive energy deposition and obesity. High fat intake also affects Ca and vitamin D metabolism as well as bone health, while different types of dietary fatty acids may have differential effects (e.g. MUFA vs. SFA). In this dissertation, the first study addressed the role of high fat feeding on intestinal Ca absorption and bone health under conditions of normal energy intake and the absence of obesity using an aging female murine model. The second study examined the effect of different levels and types of fatty acid intake on vitamin D metabolism and bone in obese adult mice. The third study explored the role of dietary vitamin D (deficiency and excess) on food intake, body composition, and central serotonin status.

1. Effects of dietary fat on calcium absorption and bone

Role of dietary fat on calcium absorption and its intestinal transporters

Dietary fat influences intestinal Ca absorption, but the effects may vary depending on the amount and the type of fatty acids that are consumed (Gacs 1997, Xiao 2010). Previous findings from our laboratory and others found that dietary fat can be a positive predictor of intestinal fractional calcium absorption (FCA) in humans (Shapses 2012, Wolf 2000). Following this observation, the first aim of this dissertation was to investigate the role of high dietary MUFA and SFA intake on FCA under conditions of adequate Ca and vitamin D intake in normal weight adult mice. It was found that FCA was higher with HFD intake when compared with normal fat intake, and the effect of HFD on intestinal FCA was not influenced by the type of dietary fat (i.e. MUFA vs, SFA). The expression of Ca absorption-related genes, including the gate-keeper *Trpv6* and intracellular transporter *CalbD_{9k}*, was assessed to give an indirect estimation of transcellular movement of Ca in enterocytes. Interestingly, greater

intracellular Ca transporter *CalbD_{9k}* gene expression attributed to HFD was found, and this is consistent with *in vitro* findings that short chain fatty acids elevate *CalbD_{9k}* expression (Fukushima 2012). Nevertheless, it is unclear whether a higher FCA or transcellular Ca transport was activated by HFD since only gene expression of *CalbD_{9k}*, but not *Trpv6*, was elevated. Also, protein expression and activity of Ca transporters will need to be examined to determine the exact effects of HFD on FCA. In addition, paracellular movement contributes more than half of Ca absorption when dietary intake is adequate (Fleet 2010). Plus, there is evidence that HFD may change the permeability of the intestinal membrane. It is possible that increased FCA in HFD-fed mice is due to an elevated passive diffusion rate that is attributed to induced hyper-permeability of the intestinal membrane (Suzuki 2010, Brun 2007). Therefore, whether or not high fat feeding increases Ca absorption via enhancing passive leakage of dietary Ca into the circulation should be investigated. Thus, future studies should include histological analysis of structural changes and membrane proteins of enterocytes after different amounts and types of fat intake.

Role of high fat feeding on bone mass and quality

There are studies reporting that high fat intake and diabetic obesity compromise bone health and increase fracture risk in humans (Crown 2006, Cao 2010); however whether or not dietary fat, rather than obesity, has a direct effect on bone health has not been addressed. The results presented in this dissertation show that bone density, including total body BMC and BMD, as well as femur total and cortical vBMD, were lower due to high SFA intake when compared with normal fat intake in normal weight mice. In contrast, high MUFA feeding showed no negative effect on bone mass, and it increased trabecular volume fraction and thickness. Moreover, in the presence of obesity, although a higher total body BMC was associated with HFD intake, body BMD values were not

altered. However, femoral bone quality was compromised due to HFD-SFA indicated by a lower trabecular BV/TV value as compared with high MUFA and normal fat intake. It is possible that HFD induced greater body weight and mechanical loading, which initially benefits bone mass (Felson 1993); nonetheless, dietary fatty acids also affect bone cell fate and metabolism. Thus, high SFA intake may adversely affect bone health by enhancing osteoclastogenesis and preventing osteoclast apoptosis (Oh 2010), and by interfering with osteoblastogenesis and bone formation (Parhami 2010). In contrast, there is no known mechanism suggesting that MUFA would have a negative effect on bone quality. Thus, future studies should aim at examining the effects of MUFA on bone cells. Additionally, it is possible that high dietary MUFA has a protective effect on trabecular bone in general, but whether or not this would occur under other conditions such as in younger, virgin mice or in pregnancy, are unclear and should also be addressed.

2. Effects of high fat feeding on vitamin D metabolism

Role of high fat feeding and obesity on serum 25OHD

There are reports in humans showing that circulating vitamin D status is inversely associated with BMI (body weight and fat), and obese individuals have a greater risk to develop vitamin D deficiency (Dyląg H 2014, Gonzalez-Molero I 2013, Cheng 2010, Oliai 2015). However, whether or not high fat feeding and obesity directly lower vitamin D status, and whether or not different types of dietary fat will have differential effects on vitamin D have not been addressed in laboratory animals. In this dissertation, the role of dietary fat on vitamin D metabolism was examined systematically in adult mice. It was found that total serum 25OHD was consistently lower due to fatty liver and obesity induced by a HFD under conditions of both adequate and low vitamin D intake, and the effects of HFD were not significantly affected by the type of dietary fat (i.e. MUFA vs.

SFA). In addition, there is evidence showing that protein binding to free 25OHD might change the bioavailability of circulating vitamin D (Powe 2011). Thus, it will be interesting to see if HFD feeding has an effect on free and bioavailable 25OHD, albumin-bound, and vitamin D binding protein-bound 25OHD, and how the different forms are related to obesity, bone, and other biological functions.

Role of dietary fat on 25-hydroxylase

The majority of dietary and skin synthesized pre-vitamin D is delivered to the liver to be metabolized into 25OHD, the major circulating metabolite (Cheng 2003, Zhu JG 2013). Thus, liver health should be significant in 25-hydroxylation of vitamin D (Okano 2015), and compromised liver health may contribute to reduced serum 25OHD concentration observed in obese individuals. Nevertheless, to date only a few studies have focused on the role of dietary fat intake on hepatic 25-hydroxylase Cyp2r1, and most used HFDs that are typically rich in SFA (Park 2015). The novelty of the current dissertation is that we addressed the role of both different amounts (normal vs. high) and types (MUFA vs. SFA) of dietary fatty acids on expression of Cyp2r1 in lean and obese adult mice. Under conditions of normal energy intake and absence of fatty liver and obesity, an up-regulation of Cyp2r1 protein level due to high MUFA intake was found when compared with high SFA and normal fat intake. In addition, under conditions of excess energy intake, fatty liver and obesity, the Cyp2r1 message level was higher due to high SFA than high MUFA and normal fat intake. However, a lower protein level of Cyp2r1 was surprisingly found due to high SFA but not MUFA intake. It is possible that the up-regulated gene expression of Cyp2r1 with high SFA was a compensatory mechanism in response to the lowered Cyp2r1 protein level, which may help to explain the lower 25OHD in the serum. Furthermore, dietary MUFA played a different role than SFA in altering hepatic 25-hydroxylase regardless of the presence of fatty liver and

obesity. Mechanistically this may explain a greater increase of circulating 25(OH)D in response to vitamin D supplementation when healthy older individuals consume a diet high in MUFA rather than high in PUFA or SFA (Niramitmahapanya 2011). Furthermore, even though Cyp2r1 is the main 25-hydroxylase and is responsible for more than 75% of vitamin D synthesis in the liver, other genes such as Cyp2j1 also have 25-hydroxylase activity and contribute the rest of 25% hepatic production of 25OHD (Zhu 2013, 2014, Cheng 2003). The primary goal of the current dissertation focused on Cyp1, but it is also important to know whether or not dietary fatty acids and fatty liver would affect expression and activity of other genes coding 25-hydroxylase beside Cyp2r1. Taken together, these data suggest that dietary MUFA and SFA have distinct effects on altering Cyp2r1 expression that cannot completely explain the lowered 25OHD observed in adult mice.

Role of dietary fat on renal 1 α -hydroxylase and 24-hydroxylase

1 α -hydroxylase (Cyp27b1) converts 25OHD to the active metabolite 1,25(OH)₂D in the kidney (Omdahl 2002). Due to the potent hormonal effect of 1,25(OH)₂D on Ca and bone metabolism, expression and activity of Cyp27b1 is well regulated by serum concentration of 1,25(OH)₂D and the hormone PTH and FGF23 to maintain a tight serum concentration 1,25(OH)₂D. There is one rodent study showing that a HFD rich in SFA up-regulated renal Cyp27b1 gene expression and increased serum 1,25(OH)₂D in young male animals (Park 2015). However, whether or not dietary fat has a similar effect on Cyp27b1 expression in adult rodents has not been addressed. Interestingly, in this dissertation, gene and protein expression of Cyp27b1 along with the serum concentration of PTH, FGF23, and 1,25(OH)₂D were not altered due to HFD, suggesting a null effect of dietary fat on renal Cyp27b1 in adult mice. This could be due to less vitamin D demand for maintaining bone turnover in adult mice compared with young

mice. Nonetheless, the exact underlying mechanism remains unknown and should be examined in future studies. Furthermore, 24-hydroxylase (Cyp24a1) is a catabolic enzyme responsible for inactivation of vitamin D metabolites, including 1,25(OH)₂D and 25OHD (Henry 2011, Christakos 2016, Pike 2012). Interestingly, we found that HFD feeding down-regulated Cyp24a1 expression, with high SFA intake having a greater effect than high MUFA and normal fat intake. It is possible that lowered Cyp24a1 gene and protein expression is a compensatory mechanism in response to lowered serum 25OHD concentration. Thus, future studies should aim at analyzing the serum and urinary concentration of inactive vitamin D metabolites including 24,25(OH)₂D and calcitric acid (1 α -hydroxy-23-carboxy-24,25,26,27-tetranorvitamin D₃) to investigate this hypothesis.

3. Effects of dietary vitamin D on adiposity and central serotonin status

Role of dietary vitamin D on obesity

Whether or not vitamin D intake directly affects food intake and weight gain in aging mice was investigated in this dissertation using two parallel studies. In the first experiment, low vitamin D intake combined with a HFD led to greater food intake and weight gain overtime that resulted in higher adiposity and a fatty liver compared with the same HFD with normal level of dietary vitamin D. Supplemental vitamin D did not prevent HFD-induced obesity. In the second experiment, although the average values of body weight, fat mass, and liver fat were also higher in mice fed with low vitamin D combined with HFD compared with those fed a HFD with normal vitamin D, the differences among groups did not reach statistical significance. Interestingly, low vitamin D feeding showed a null or even a mild negative effect on weight gain and liver fat in aging mice fed a normal fat diet. The role of low vitamin D intake on promoting obesity and hepatic fat accumulation have also been seen in some other studies using growing

and adult animals (Brouwer-Brolasma 2015, Domingues-Faria 2014, Trinco 2016, Liu 2015, Borges 2016, Kong 2014, Bhat 2014, Barchetta 2011, Eliades 2013, Dasarathy 2014). Nevertheless, the results remain inconsistent, and the differences among studies and the biochemical explanation are unclear. Taken together, results from our work and others indicate that supplemental vitamin D may have no beneficial effect on preventing HFD induced obesity, whereas dietary vitamin D deficiency may promote obesity and fatty liver. However, the magnitude of the effects may be influenced by other factors such as dietary fat intake, period of deficiency, gender, age, and genetic variation.

Role of low vitamin D intake on central serotonin status

Vitamin D deficiency and low central serotonin status are both associated with eating and mood related disorders (Tecott LH 2007, Hale 2012, Voigt JP 2015). In addition, a recent study identified a vitamin D response element in the enzyme, tryptophan hydroxylase (TPH), which is essential for serotonin synthesis. Thus, it is possible that vitamin D regulates energy homeostasis via altering serotonin metabolism. This dissertation was the first to explore whether or not low dietary intake induced vitamin D deficiency contributes to obesity by compromising central serotonin status *in vivo* using an adult murine model. It was found that low dietary vitamin D intake down-regulated vitamin D 1 α -hydroxylase (Cyp27b1) gene expression in the brain (arcuate nucleus and raphe nucleus), indicating an attenuated local synthesis of the active vitamin D hormone, 1,25(OH)₂D. Importantly, lower serotonin concentration in the cerebral cortex was also found due to vitamin D deficiency. In addition, there was a direct association between central serotonin and serum total 25OHD concentrations. These are consistent with findings from a cell study that showed a dose-dependent change in TPH2 expression in response to 1,25(OH)₂D treatment (Kaneco 2015). Therefore, overall these data indicate that vitamin D status affects serotonin status in the brain, suggesting an underlying

mechanism by which vitamin D deficiency may affect energy homeostasis. However, in this study local concentrations of $1,25(\text{OH})_2\text{D}$ and expression and activity of markers involved in serotonergic pathways, including TPH2, Sert, and several serotonin receptors, were not analyzed in the cerebral cortex due to limited tissue size. Thus, it will be interesting to further examine the effects of vitamin D deficiency on serotonergic metabolism in the CNS. In addition, whether or not lowered serotonin concentration due to vitamin D deficiency would directly translate into compromised serotonergic effects and physiological changes systemically remains unknown. In-depth studies and behavior tests should be conducted in the future to investigate the role of vitamin D on the serotonin axis and energy homeostasis.

4. Summary

This dissertation addressed the role of different amounts and types of dietary fat intake on Ca metabolism and bone health in adult female mice. Overall, HFD increases intestinal Ca absorption over time even under conditions of normal energy and adequate Ca intake, and this did not differ due to the type of dietary fatty acids (MUFA vs. SFA). In contrast, dietary fat rich in SFA has detrimental effects on bone quality regardless of the presence of obesity, whereas high MUFA intake shows a neutral or beneficial effect. Moreover, the nature between body vitamin D status and obesity was addressed. It was found that HFD and obesity contribute to attenuated circulating 25OHD. However, this cannot be explained due to the differential effects of high MUFA and SFA intake on expression of major vitamin D regulating enzymes. Moreover, low dietary vitamin D combined with HFD could promote food intake and weight gain and lower cerebral serotonin concentration, but supplemental vitamin D may not prevent HFD induced obesity in aging mice.

APPENDIX: ADDITIONAL AND PRELIMINARY EXPERIMENTS

Supplemental Table 1. Dietary composition

	NFD	NFD-LD	SFA	SFA-LD	SFA-HD
Ingredient	g/kg Die				
Casein, 80 Mesh	200	200	200	200	200
L-Cystine	3	3	3	3	3
Corn Starch	452.2	452.2	72.8	72.8	72.8
Maltodextrin 10	75	75	100	100	100
Sucrose	172.8	172.8	172.8	172.8	172.8
Cellulose, BW200	50	50	50	50	50
Butter	23	23	98	98	98
Coconut Oil	0	0	52	52	52
Flaxseed Oil	0	0	52.5	52.5	52.5
Olive Oil	2	2	0	0	0
Soybean Oil	20	20	0	0	0
Mineral Mix S10026	10	10	10	10	10
DiCalcium Phosphate	13	13	13	13	13
Calcium Carbonate	5.5	5.5	5.5	5.5	5.5
Potassium Citrate, 1 H ₂ O	16.5	16.5	16.5	16.5	16.5

Vitamin Mix V10001	10	10	10	0	0
Vitamin Mix V13201 (No D3)	0	0	0	10	10
Choline Bitartrate	2	2	2	2	2
Vitamin D3 (100,000 IU/gm)	0	0	0	0.00086	0.086
Energy, kcal/g diet	3.85	3.85	4.73	4.73	4.73
% Energy					
Protein	19	19	24	24	24
Carbohydrate	67	67	41	41	41
Fat	4	4	24	24	24
Saturated (%kcal of fat)	40	40	60	60	60
Monounsaturated (%kcal of fat)	30	30	20	20	20
Polyunsaturated (%kcal of fat)	30	30	20	20	20

¹Prepared by Research Diets, Inc., New Brunswick, NJ. LD, low vitamin D content; HD, high vitamin D content; NFD, normal fat diet; SFA: saturated fatty acid.

²The mineral mix composition was as follows (amount in 10 g): 0.5 g Mg, 0.3 g S, 1.0 g Na, 1.6 g Cl, 6.0 mg Cu, 0.2 mg I, 45.0 mg Fe, 59 mg Mn, 0.2 mg Se, and 29 mg Zn.

³The vitamin mix composition was as follows (amount in 10 g): 4000 IU vitamin A palmitate, 1000 IU vitamin D₃, 50 IU vitamin E acetate, 0.5 mg menadione sodium bisulfite, 0.2 mg biotin (1.0%), 10 µg cyanocobalamin (0.1%), 2 mg folic acid, 30 mg nicotinic acid, 16 mg calcium pantothenate, 7 mg pyridoxine-HCl, 6 mg riboflavin, and 6 mg thiamin HC.

Supplemental Table 2. Fatty Acid Profiles

	NFD	MUFA	SFA
	40:30:30 S:M:P	20:60:20 S:M:P	60:20:20 S:M:P
	gm	gm	Gm
Butter	23	20	98
Coconut Oil	0	0	52
Flaxseed Oil	0	0	52.5
Olive Oil	2	144.5	0
Soybean Oil	20	38	0
Total fat added:	45	202.5	202.5
C2, Acetic	0	0	0
C4, Butyric	0.74	0.64	3.14
C6, Caproic	0.44	0.38	2.17
C8, Caprylic	0.25	0.22	5.08
C10, Capric	0.58	0.50	5.52
C12, Lauric	0.64	0.56	27.50
C14, Myristic	2.3	2	19.16
C14:1, Myristoleic	0.35	0.3	1.47
C15:0	0	0	0
C16, Palmitic	8.36	27.40	32.98
C16:1, Palmitoleic	0.55	1.905	2.25
C16:2	0	0	0
C16:3	0	0	0

C16:4	0	0	0
C17:0	0	0	0
C18, Stearic	3.59	7.33	19.52
C18:1, Oleic	12.06	117.57	35.62
C18:2, Linoleic	11.44	35.67	8.9371
C18:3, Linolenic	1.90	4.40	29.35
C18:4	0	0	0
C20, Arachidic	0.22	0.19	0.93
C20:1	0.01	0.43	0
C20:2	0	0	0
C20:3,	0	0	0
C20:4, Arachidonic	0	0	0
C20:5,	0	0	0
C21:5	0	0	0
C22, Behenic	0.01	0.87	0
C22:1, Erucic	0	0	0
C22:4, Clupanodonic	0	0	0
C22:5	0	0	0
C22:6	0	0	0
C24, Lignoceric	0.01	0.72	0
C24:1	0	0	0
T otal	43.4	201.1	193.6
Saturated (g)	17.1	40.8	116.0

Monounsaturated (g)	13.0	120.2	39.3
Polyunsaturated (g)	13.3	40.1	38.3
Saturated (%)	39.5	20.3	59.9
Monounsaturated (%)	29.8	59.8	20.3
Polyunsaturated (%)	30.7	19.9	19.8

5. A-1 Effects of dietary fat on Ca absorption and bone in ovariectomized mice

Hypothesis and Objective. To determine the effects of dietary fat on Ca absorption and bone mineral density under conditions of normal energy and Ca intake in ovariectomized (OVX) female mice while aging.

Experimental Design. Total twenty-eight female retired breeder C57B/6J mice were ovariectomized to induce estradiol deficiency before feeding. After surgery and recovery, weight-matched mice were divided into three groups, and weight-control fed with three types of diet for 8wk: 10% NFD, 45% SFA and 45% MUFA diet. All procedures were similar as previously described in main experiment 1.

Results and Discussion. After 8wk, body weight did not differ among groups. The OVX mice from different diet groups had comparable amount of body lean mass and fat mass (**Figure A1-1b**). As an indication of estradiol status in the body, uterus weight did not differ among groups, with actual weights equal to 87.8 ± 3.3 , 78.4 ± 5.0 , and 85.7 ± 9.7 g in the NFD, MUFA, and SFA groups, respectively. Moreover, high fat feeding without excess caloric intake showed no effect on body BMC and BMD values at total body level or individual anatomical bone sites in OVX mice (**Table A1-1**). When comparing BMD and BMC values to sham mice from main experiment 1, all BMD and BMC values were remarkably lower with OVX mice than the sham mice. This indicates the significant effect of estradiol in bone metabolism and health (Rodan GA 2000), while HFD only showed a minor or null effect on bone compared with estradiol. Furthermore, fractional Ca absorption, Ca balance, urinary and fecal Ca were comparable among dietary groups, and 8wk of HFD feeding did not alter Ca metabolism in OVX mice (**Table A1-2**). In addition, the delta change of FCA between initial (wk0) and final week (wk8) did not differ due to HFD feeding (**Figure A1-2**).

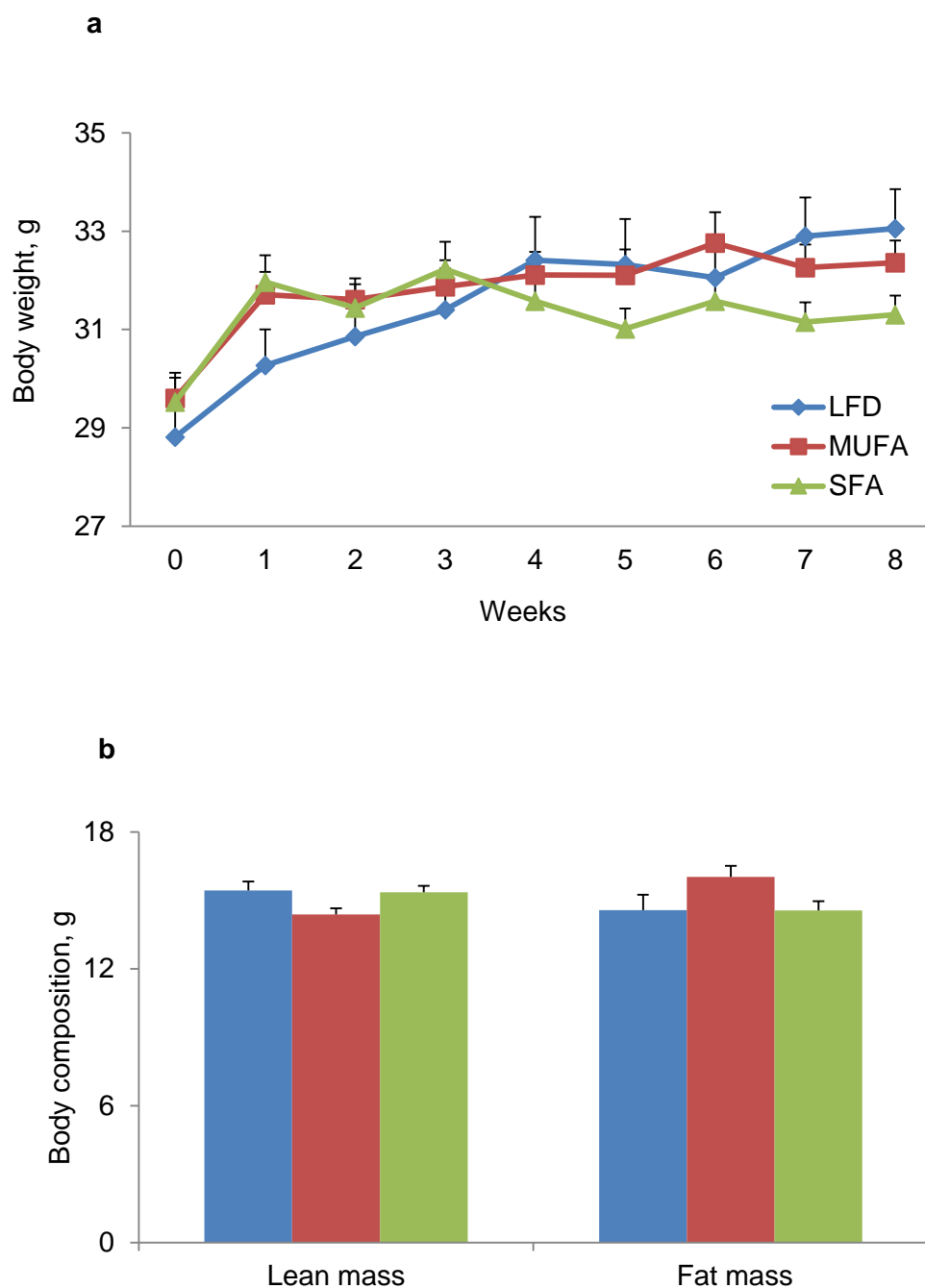


Figure A1-1. Weekly body weight (a) and body composition (b) in mice after 8wk of *ad libitum* feeding with NFD or HFD enriched with MUFA or SFA (n=6/ diet group). Values are means \pm SEM. $P < 0.05$ by one-way ANOVA using Scheffe's post hoc test.

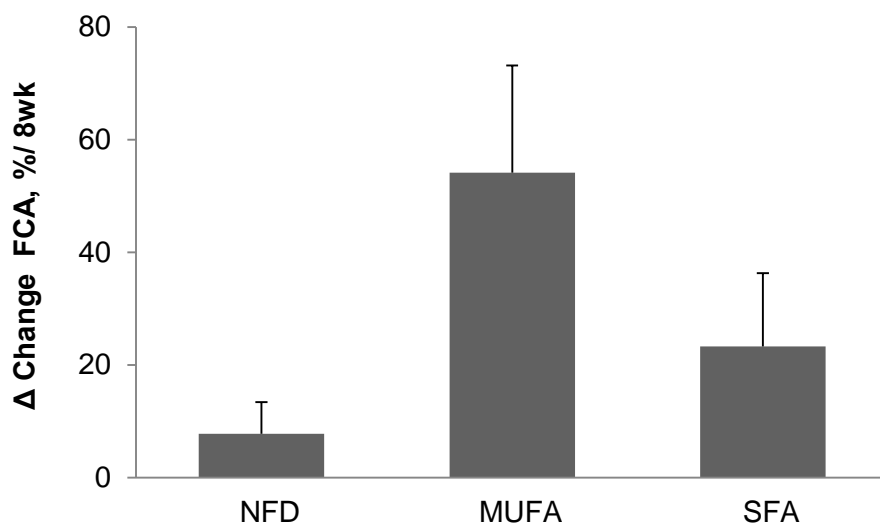


Figure A1-2. Delta change of fractional Ca absorption in mice between and after 8wk of *ad libitum* feeding with NFD or HFD enriched with MUFA or SFA (n=6/ diet group). Values are means \pm SEM.

Table A1-1 Bone densitometry in OVX mice after 8wk of feeding with normal or high fat diets enriched with MUFAs or SFAs¹.

	Diet			<i>P</i>
	NFD	MUFA	SFA	
BMC, mg				
Total Body	36.9 ± 12.1	35.3 ± 14.0	34.9 ± 9.3	0.45
Whole Femur	24.4 ± 0.4	22.1 ± 1.1	24.1 ± 0.4	0.07
Distal Femur	3.3 ± 0.3	3.5 ± 0.2	4.0 ± 0.1	0.71
Tibia	38.0 ± 0.5	38.0 ± 0.5	41.3 ± 0.5	0.87
Humerus	11.8 ± 0.2	11.8 ± 0.2	12.0 ± 0.1	0.62
Radius	8.67 ± 0.2	8.83 ± 0.2	8.67 ± 0.3	0.85
Lumbar Spine	39.0 ± 0.8	40.1 ± 0.9	39.8 ± 1.8	0.80
BMD, mg/cm ²				
Total body	43.4 ± 0.4	42.6 ± 0.5	43.2 ± 0.3	0.41
Whole femur	43.3 ± 0.7	41.9 ± 0.5	42.7 ± 0.7	0.27
Distal femur	48.9 ± 4.7	40.7 ± 4.5	47.3 ± 1.9	0.38
Tibia	51.3 ± 1.8	49.4 ± 1.9	49.7 ± 1.3	0.71
Humerus	35.3 ± 0.8	36.3 ± 0.4	35.6 ± 0.3	0.44
Radius	28.2 ± 0.4	28.0 ± 0.3	27.7 ± 0.3	0.56
Lumbar spine	42.2 ± 0.9	43.4 ± 0.4	42.1 ± 0.6	0.35

¹ All values are means ± SD (n=6/ diet group), Means in a row with different superscripts differ, *P* < 0.05 (one-way ANOVA followed by Tukey's post hoc test). BMC, bone mineral content; BMD, bone mineral density; MUFA, monounsaturated fatty acid; NFD, normal fat diet; SFA, saturated fatty acid.

Table A1-2 Calcium metabolism in OVX mice after 8wk of feeding with normal fat diet or with high fat diets enriched in MUFAs or SFAs¹

	Diet			<i>P</i>
	LFD	MUFA	SFA	
Urinary Ca, $\mu\text{mol/d}$	2.41 ± 1.25	4.57 ± 3.54	4.76 ± 4.66	0.44
Fecal Ca, mmol/d	0.27 ± 0.04	0.22 ± 0.08	0.23 ± 0.06	0.30
Ca balance, mmol/d	0.10 ± 0.03	0.14 ± 0.04	0.16 ± 0.04	0.05
Endogenous fecal Ca, mmol/d	0.12 ± 0.07	0.27 ± 0.18	0.16 ± 0.15	0.14
FCA, %	38.6 ± 12.7	50.4 ± 23.2	55.8 ± 31.0	0.15

¹All values are means \pm SD (n=6/ diet group); means in a row with different superscripts differ, $P < 0.05$ by two-way ANOVA. Ca, calcium; FCA, fractional calcium absorption; MUFA, monounsaturated fatty acids; NFD, normal fat diet; SFA, saturated fatty acids.

6. A-2: The effects high fat feeding on intestinal Ca transporters in older mice.

Objective and Hypothesis. In the previous study (Chapter 6), a weight-controlled feeding experiment has already been performed in older female mice to examine the effects of dietary fatty acids (MUFA vs. SFA) on Ca metabolism and transporters without excessive energy intake and obesity. In the preliminary study, we used the similar diets and *ad libitum* fed mice to understand if diet induced obesity would have additional effects on gene expression of Ca transporters in the small intestine and kidneys. The secondary goal was to investigate whether dietary MUFA and SFA would differentially affect Ca transporters. It was hypothesized that high fat feeding would up-regulate Ca transporters, and dietary MUFA would be associated with higher gene expressions.

Experimental Design. A total of eleven female retired breeder mice were group-housed and *ad libitum* fed with one of three diets for 8wk: 10% NFD, 45% SFA and 45% MUFA (as described in chapter 6). Body weights were

Results and Discussion. After 10wk, mice from both HFD groups had greater body weight and fat mass, with the average value of the MUFA group significantly different from the NFD group ($P < 0.05$) (Figure A2-1). In the small intestine, HFD feeding (MUFA and SFA combined) up regulated gene expression of Trpv6 ($P = 0.004$). When examining the difference between types of dietary fat, Trpv6 mRNA was significantly higher with MUFA feeding than the NFD ($P = 0.02$), whereas, the SFA group did not differ from either MUFA or LFD (Figure A2-2a). Since expression and activity of the Ca transporter is closely associated with active Ca absorption, the higher Trpv6 mRNA suggests a greater intestinal absorption. However, both HFD combined or individual

MUFA or SFA did not affect gene expression of other Ca transporters in the small intestine including *CalbD_{9k}*, NCX1, and PMCA1b.

In the kidney, HFD (MUFA and SFA combined) greatly up regulated mRNA expression of intracellular Ca transporter *CalbD_{9k}*. In addition, gene expression of basolateral Ca pump PMCA1b was lower with HFD feeding (Figure A2-2b), indicating an attenuated Ca reabsorption in the kidney. There was no significant difference of mRNA expression in *Trpv5*, *CalbD*, and NCX1 among diet groups. **Because** *CalbD₉* also functions as an intracellular reservoir that may temporally store excess Ca in cells to prevent intracellular Ca toxification, it is possible that down-regulated PMCA1b plus up-regulated *CalbD₉* led to attenuated renal Ca reabsorption in the kidney, which could be a compensatory mechanism responding to elevated intestinal Ca absorption with high HFD feeding under conditions of excess caloric intake and obesity.

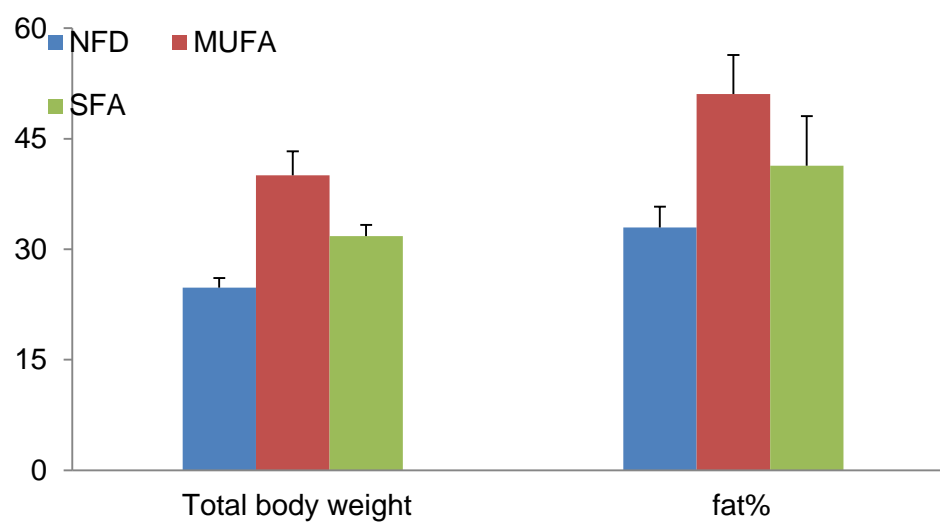


Figure A2-1. Total body weight and percent fat in mice after 8wk of *ad libitum* feeding with NFD or HFD enriched with MUFA or SFA (NFD, n=4; MUFA, n=4; SFA n=3). Values are means \pm SEM. $P < 0.05$ by one-way ANOVA using Tukey's post hoc test.

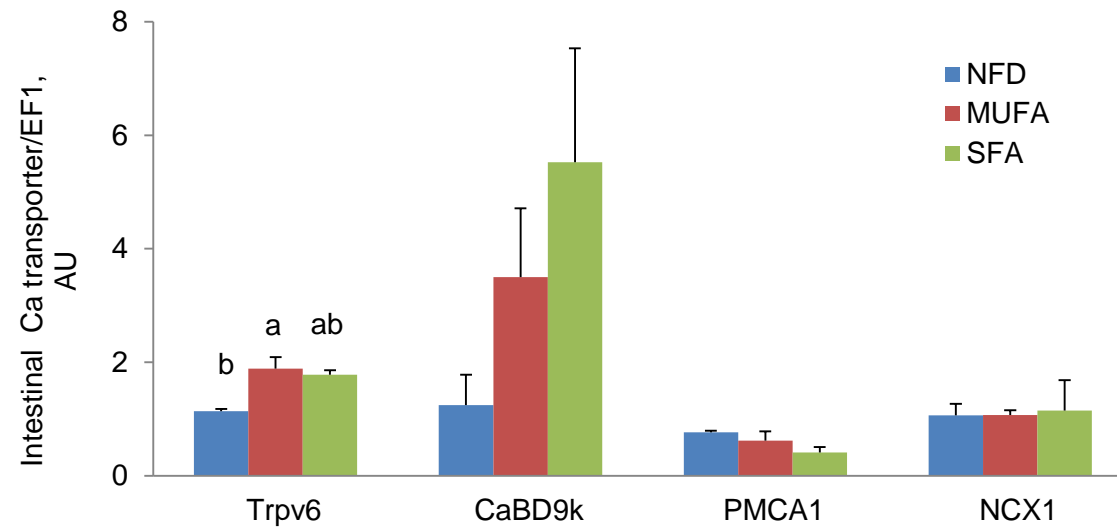


Figure A2-2. Calcium transporters in the small intestine in mice after 8wk of *ad libitum* feeding with NFD or HFD enriched with MUFA or SFA.

Values are means \pm SEM (NFD, n=4; MUFA, n=4; SFA n=3). $P < 0.05$ by one-way ANOVA using Tukey's post hoc test.

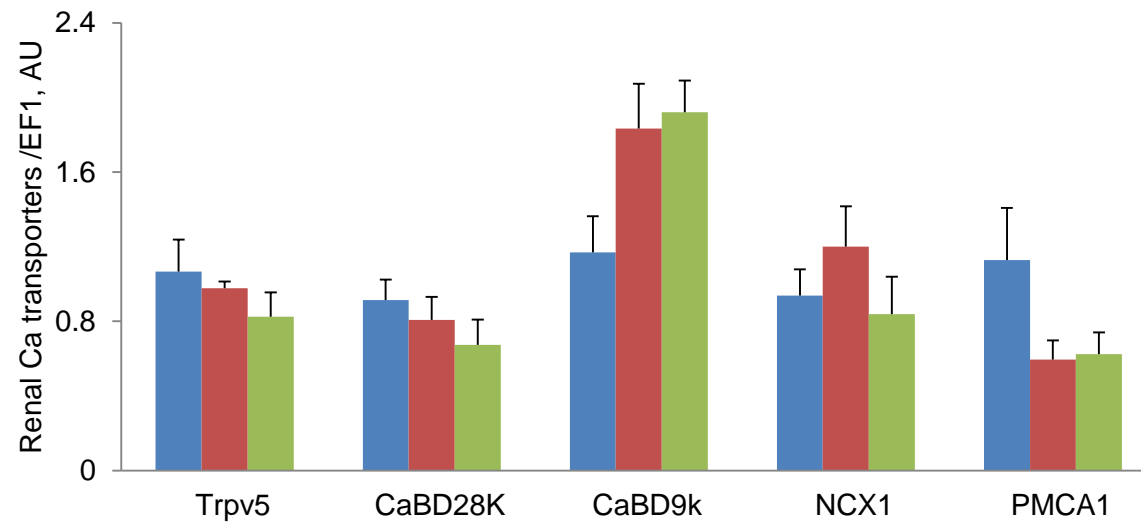


Figure A2-3. Calcium transporters in the kidney in mice after 8wk of *ad libitum* feeding with NFD or HFD enriched with MUFA or SFA.

Values are means \pm SEM (NFD, n=4; MUFA, n=4; SFA n=3). $P < 0.05$ by one-way ANOVA using Tukey's post hoc test.

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