Effect of Early Life Vitamin D Supplementation on Bone Development

by

Kristina Anne Fielding

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Graduate Department of Nutritional Sciences
University of Toronto

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Abstract

Vitamin D is important for bone development with immunomodulatory effects. This study investigated whether feeding CD-1 and interleukin 10 (IL-10) knockout (KO) dams low (25 IU/kg diet) or high (5,000 IU/kg diet) vitamin D affected bone health of dams as well as their offspring. Offspring were weaned to 1 of the 2 diets and followed to young adulthood. Unlike CD-1 dams, IL-10 KO dams experienced greater femur strength with high vitamin D. CD-1 male offspring had reduced femur neck strength and female offspring had smaller, weaker femurs, and weaker lumbar vertebra 2 (LV2) with high maternal vitamin D. IL-10 KO male offspring had larger femurs and female offspring had stronger femurs when weaned to high vitamin D. Low vitamin D did not adversely impact bone health but the optimal level of dietary vitamin D seems to differ between healthy and inflammatory states.
Acknowledgements

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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25(OH)$_2$D</td>
<td>1,25-dihydroxycholecalciferol</td>
</tr>
<tr>
<td>7-DHC</td>
<td>7-dehydrocholesterol</td>
</tr>
<tr>
<td>25(OH)D</td>
<td>25-hydroxycholecalciferol</td>
</tr>
<tr>
<td>AI</td>
<td>adequate intake</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>anteroposterior</td>
</tr>
<tr>
<td>APase</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>BMC</td>
<td>bone mineral content</td>
</tr>
<tr>
<td>BMD</td>
<td>bone mineral density</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic proteins</td>
</tr>
<tr>
<td>CARD15</td>
<td>caspase recruitment domain family member 15</td>
</tr>
<tr>
<td>Cbfa1</td>
<td>core-binding factor gene</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>CYP24A1</td>
<td>24-hydroxylase enzyme</td>
</tr>
<tr>
<td>CYP27A1</td>
<td>25-hydroxylase enzyme</td>
</tr>
<tr>
<td>CYP27B1</td>
<td>1-hydroxylase enzyme</td>
</tr>
<tr>
<td>DAI</td>
<td>daidzein</td>
</tr>
<tr>
<td>DBP</td>
<td>vitamin D-binding protein</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DES</td>
<td>diethylstilbesterol</td>
</tr>
<tr>
<td>DPD</td>
<td>deoxypyridinoline</td>
</tr>
<tr>
<td>DRI</td>
<td>dietary reference intake</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>DSS</td>
<td>dextran sodium sulphate</td>
</tr>
<tr>
<td>E₂</td>
<td>estradiol</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>GEN</td>
<td>genistein</td>
</tr>
<tr>
<td>H⁺</td>
<td>hydrogen ions</td>
</tr>
<tr>
<td>hCG</td>
<td>human chorionic gonadotropin</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-γ</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IOM</td>
<td>Institute of Medicine</td>
</tr>
<tr>
<td>IR</td>
<td>insulin receptor</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LV</td>
<td>lumbar vertebra</td>
</tr>
<tr>
<td>MAP</td>
<td><em>Mycobacterium avium</em> subspecies <em>paratuberculosis</em></td>
</tr>
<tr>
<td>MDR1</td>
<td>multidrug resistant 1</td>
</tr>
<tr>
<td>ML</td>
<td>mediolateral</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>NCX1</td>
<td>Na⁺/Ca²⁺ exchanger 1</td>
</tr>
<tr>
<td>NLF</td>
<td>NOD-like receptor family</td>
</tr>
<tr>
<td>NOD2</td>
<td>nucleotide-binding oligomerization domain 2</td>
</tr>
<tr>
<td>NR1I2</td>
<td>nuclear receptor subfamily 1, group I, member 2</td>
</tr>
<tr>
<td>OCN</td>
<td>osteocalcin</td>
</tr>
<tr>
<td>OPG</td>
<td>osteoprotegerin</td>
</tr>
<tr>
<td>OPN</td>
<td>osteopontin</td>
</tr>
</tbody>
</table>
P1NP  procollagen type 1 amino-terminal propeptide
P_i  inorganic phosphate
PBM  peak bone mass
PBS  phosphate buffered saline
PICP  procollagen I carboxyterminal propeptide
PMCA  plasma membrane calcium ATPase
PND  postnatal day
PTH  parathyroid hormone
PXR  pregnane X receptor
RDA  recommended dietary allowance
RANK  receptor activator of nuclear factor kappa B
RANKL  RANK ligand
SD  standard deviation
SGA  small-for-gestational age
Th1 cell  type 1 helper T cell
Th2 cell  type 2 helper T cell
Th17 cell  type 17 helper T cell
TNF-α  tumor necrosis factor-α
T_reg  regulatory T cell
TRAP  tartrate resistant acid phosphatase
TRPV  transient receptor potential vaniloid
U-Dpyr  urinary deoxypyridinoline
U-Pyr  urinary pyridinoline
UC  ulcerative colitis
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>UVB</td>
<td>ultraviolet B</td>
</tr>
<tr>
<td>VDR</td>
<td>vitamin D receptor</td>
</tr>
<tr>
<td>WT</td>
<td>wildtype</td>
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Chapter One

INTRODUCTION
1.0 INTRODUCTION

Cholecalciferol (vitamin D₃), the “sunshine vitamin”, is a unique vitamin in that the major source for the body is the skin during exposure to ultraviolet B (UVB) irradiation¹-³. Both the main circulating metabolite, 25-hydroxycholecalciferol (25(OH)D), and active metabolite, 1,25-dihydroxycholecalciferol (1,25(OH)₂D), of vitamin D are important in regulating calcium homeostasis, bone growth and remodelling, body weight and the immune system⁴-⁶. Due to the higher prevalence of vitamin D deficiency and insufficiency of healthy individuals, and the awareness of the detrimental effects this may have on bone development, as well as overall health, it is prudent to better understand how vitamin D status may optimize bone health⁷-⁸. Of particular interest is the concept of nutritional programming, in which a nutritional intervention in utero through to 2 years of age can affect both the structural and functional development of the child⁹-¹⁰. By improving vitamin D status during critical periods of the life cycle such as intrauterine and early postnatal life, the prevalence of chronic bone diseases (ie: osteoporosis) during later life may be reduced. Moreover, there may be specific disease processes in which vitamin D may have a protective role. An example is inflammatory bowel disease (IBD).

IBD is a chronic, autoimmune disease characterized by a dysregulated immune response towards the normal bacteria flora of the intestine. This dysregulation leads to the enhanced production of proinflammatory cytokines which may play a critical role in the development of the bone abnormalities that present with this disease¹¹-¹³. Although the etiology is still unknown, a key aspect considered to contribute to the dysregulated immune response and subsequent bone abnormalities is vitamin D deficiency¹²,¹⁴. Similar to healthy individuals, poor vitamin D status is also prevalent in those affected with IBD¹²,¹⁵. Therefore, with the discovery of the immunomodulatory effects, in addition to the well-known effects of 25(OH)D and 1,25(OH)₂D on bone growth and development, improving vitamin D status may be an effective strategy to prevent or attenuate IBD. By improving vitamin D status during intrauterine and early postnatal life, the prevalence of autoimmune diseases (ie: IBD) and their associated abnormalities may be reduced.

In this research, the effects of low and supplemental levels of dietary vitamin D on health outcomes such as body weight and bone development were compared in dams and their offspring in both healthy and inflammatory mouse models. The CD-1 mouse model was used to represent a healthy state while the interleukin-10 (IL-10) knockout (KO) mouse model
represented a state of intestinal inflammation that is associated with inflammation-induced bone abnormalities. The first hypothesis of this study was that healthy and diseased dams receiving a supplemental level of vitamin D in the diet, beginning at 3-4 weeks of age and continuing throughout pregnancy and lactation, would have higher body weights and greater bone dimensions and bone strength at time of necropsy (end of lactation). The second hypothesis was that healthy and diseased offspring exposed to supplemental levels of vitamin D during intrauterine and early postnatal life would have higher body weights as well as greater bone dimensions and bone strength at young adulthood (12-14 weeks of age).
Chapter Two

LITERATURE REVIEW
2.0 LITERATURE REVIEW

2.1 Vitamin D

2.1.1 Vitamin D Sources

Vitamin D$_3$ is formed by UVB irradiation of its precursor 7-dehydrocholesterol (7-DHC), which is present in the skin of humans and animals whereas vitamin D$_2$ is formed by UVB irradiation of its precursor, ergosterol, which is present in plants, fungi and yeast$^{16}$. Although the principle source of vitamin D is endogenous production, with an increasing awareness of the association between higher UVB exposure and greater risk of skin cancer, it is strongly suggested to avoid the sunlight which limits this as a source of vitamin D$^{17}$. Therefore, it is prudent to use dietary sources and/or supplementation to meet the dietary reference intake (DRI) for vitamin D. Most diets are low in vitamin D as intake of the few sources that are vitamin D-rich or fortified occurs intermittently (Table 2.0). One of best vitamin D-rich sources is oily fish. The vitamin D content for 3.5 ounces (1 serving) of fish can range from 249 IU in farmed salmon to 981 IU in wild salmon$^{18-19}$. Therefore, depending on the type of fish, 1-3 servings would provide the recommended dietary allowance (RDA (1-70 years of age); 600 IU vitamin D/day)$^{20}$. Foods in Canada that are fortified with vitamin D include milk$^{19,21-22}$ and margarine$^{19,21-22}$. In the United States, yogurt$^{19,22}$, orange juice$^{19,22}$, and ready-to-eat breakfast cereals$^{19,22}$ are often fortified as well. Vitamin D fortification of foods can provide a range of 10-40 IU/serving from ready-to-eat breakfast cereals to 400 IU/serving from milk$^{22}$. Therefore, daily milk consumption could provide the RDA; however, milk consumption is variable. While 87% of Canadian children aged 1-3 years consume 1-2 cups of milk/day$^{23}$, 57% of adolescents aged 14-18 years consume only 1 cup of milk/day$^{23}$ and 59% of adults aged 19 years and older consume < 1 cup of milk/day$^{24}$. Therefore, with low consumption of vitamin D-rich and fortified foods it is imperative for many individuals to use vitamin D supplements to achieve the recommended level for vitamin D intake.

2.1.2 Vitamin D Metabolism

Vitamin D$_3$ is a steroid hormone that is unique as it is endogenously produced in the skin. Seven-DHC within the epidermis is converted to the thermally, unstable intermediate, previtamin D$_3$, by the action of UVB irradiation (wavelength 290-320 nm)$^{1-3}$. At body
Table 2.0 Vitamin D content of vitamin D-rich and fortified foods

<table>
<thead>
<tr>
<th>Vitamin D-rich foods&lt;sup&gt;1&lt;/sup&gt;</th>
<th>IU vitamin D/serving size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmon</td>
<td>249 ± 40</td>
</tr>
<tr>
<td>Farmed</td>
<td>981 ± 89</td>
</tr>
<tr>
<td>Wild</td>
<td>415 ± 112</td>
</tr>
<tr>
<td>Bluefish</td>
<td>342 ± 96</td>
</tr>
<tr>
<td>Swordfish</td>
<td>447 ± 126</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vitamin D-fortified foods</th>
<th>Fortification level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>400 IU/250 mL</td>
</tr>
<tr>
<td>Margarine</td>
<td>530 IU/100 g</td>
</tr>
<tr>
<td>Yogurt</td>
<td>89 IU/100 g</td>
</tr>
<tr>
<td>Orange juice</td>
<td>100 IU/1 cup</td>
</tr>
<tr>
<td>Ready-to-eat breakfast cereals</td>
<td>40-140 IU/100 g</td>
</tr>
</tbody>
</table>

<sup>1</sup>Serving size; 3.5 ounces
temperature, previtamin D$_3$ isomerizes to form the inactive metabolite, vitamin D$_3$$^{1,3}$. Once synthesized, vitamin D$_3$ is translocated from the dermis into the circulation by the vitamin D-binding protein (DBP)$^{25}$. Vitamin D$_3$ is transported to the liver by the DBP where it is hydroxylated by the enzyme, 25-hydroxylase (CYP27A1)$^{26-28}$, to form the main circulating vitamin D$_3$ metabolite, calcidiol (25(OH)D$_3$)$^{29-32}$. The half-life of 25(OH)D$_3$ is between 3-4 weeks$^{33}$. Once formed, 25(OH)D$_3$ re-enters the circulation bound to DBP and is transported to the kidneys$^{34-36}$ where it is hydroxylated by the enzyme, 1-hydroxylase (CYP27B1)$^{37}$, to form the active vitamin D$_3$ metabolite, calcitriol (1,25(OH)$_2$D$_3$)$^{34,38-40}$. The production of 1,25(OH)$_2$D$_3$ is highly regulated with parathyroid hormone (PTH)$^{41-43}$ and calcitonin$^{42,44}$ increasing CYP27B1 activity and 1,25(OH)$_2$D$_3$$^{41-43}$ and high levels of calcium$^{41}$ decreasing CYP27B1 activity. Extrarenal synthesis of 1,25(OH)$_2$D$_3$ has been documented in bone, in both osteoblasts$^{45-46}$ and osteoclasts$^{47-48}$, lymph nodes$^{49}$, macrophages$^{48}$, dendritic cells (DC)$^{50}$, placenta$^{49,51}$, keratinocytes of the dermis$^{49}$, adrenal gland$^{49}$, pancreas$^{49}$, brain$^{49}$, and colon$^{49}$. The half-life of 1,25(OH)$_2$D$_3$ is between 11-21 hours. Because 25(OH)D$_3$ has a longer half-life (3-4 weeks)$^{33}$, is present in higher concentrations and its synthesis is less tightly regulated compared to 1,25(OH)$_2$D$_3$, it is the more appropriate metabolite to measure for vitamin D status. Also of importance is the production of 24,25-dihydroxyvitamin D$_3$ and 1,24,25-trihydroxyvitamin D$_3$ via 24-hydroxylase (CYP24A1)$^{52-54}$, that is present in skin, intestine, liver, and kidney$^{55}$. This enzyme is responsible for the attenuation of the many effects of 1,25(OH)$_2$D$_3$ signalling such as maintaining calcium homeostasis as well as regulating bone growth and remodelling and the immune response$^{54-56}$.

2.2 Functions of 1,25(OH)$_2$D

2.2.1 Calcium Homeostasis

1,25(OH)$_2$D maintains serum calcium homeostasis through regulation of calcium at the intestine, bone and kidney. 1,25(OH)$_2$D stimulates intestinal calcium absorption by upregulating the expression of the calcium channel proteins, transient receptor potential vaniloid (TRPV5 and TRPV6) that mediate calcium uptake into the enterocyte$^{57-60}$. Also upregulated is the cellular calcium transfer protein, calbindin-D$_{9K}$ that allows intracellular diffusion of calcium through the enterocyte$^{57-58,60}$, and the calcium extrusion protein, plasma membrane calcium ATPase (PMCA$_{1b}$), which is responsible for calcium extrusion into the circulation$^{57-58}$.
Another main target of 1,25(OH)2D, to maintain calcium homeostasis, is bone. Vitamin D is needed for bone mineralization. During mineralization, hydroxyapatite (calcium ions and inorganic phosphate (Pi) groups) forms around osteoblasts on newly formed bone matrix causing osteoblasts to differentiate and mature into osteocytes. 1,25(OH)2D also acts on bone to mobilize calcium by increasing expression and activity of receptor activator of nuclear factor kappa B ligand (RANKL). Osteoclast precursor cells which express RANK, the receptor for RANKL, differentiate into mature, multinucleated resorptive osteoclasts in the presence of RANKL. Therefore, 1,25(OH)2D indirectly increases osteoclastogenesis in the presence of RANKL, and facilitates release of calcium to restore circulating levels of calcium.

The final major target for calcium homeostasis is the kidney. 1,25(OH)2D regulates calcium reabsorption by upregulating the expression of TRPV5 and TRPV6 in the apical membrane to increase calcium reabsorption. Calbindin-D9K and calbindin-D28K which facilitate the diffusion of calcium across the cell are also increased by 1,25(OH)2D. PMCA4, especially at the basolateral membrane, and Na+/Ca2+ exchanger 1 (NCX1) are also upregulated, resulting in a shift of the net flux of calcium to the basolateral side followed by the extrusion of calcium into the circulation. These actions correspond to an increase in calcium reabsorption from the urine into the circulation. Although a key factor in calcium homeostasis, vitamin D is imperative in maintaining optimal bone health.

### 2.2.2 Bone Growth and Remodelling

This thesis research focused on the role of vitamin D in bone growth and remodelling. The process of bone growth and remodelling is regulated by the 2 main bone cells osteoblasts and osteoclasts. The mesenchymal stem cell (MSC) lineage expresses the core-binding factor (Cbfa1) gene, which is an osteoblast differentiating factor. Osteogenic factors, such as bone morphogenetic proteins (BMP), enhance MSC expression of Cbfa1, thereby increasing the differentiation of MSC into osteoprogenitors, which express the mRNA of Cbfa1 and type I collagen. Osteoprogenitors are fully committed to the osteogenic lineage. Further differentiation of osteoprogenitors into mature osteoblasts is evident through the expression of different factors and activities. Alkaline phosphatase (APase) activity is an important factor as it is a direct indicator of osteoblast function. Osteoblasts also produce bone matrix proteins (ie: type I collagen, osteocalcin (OCN), and osteopontin (OPN)), which facilitate bone
mineralization\textsuperscript{61-63, 81-82, 84-87}. The initiation of mineralization includes the formation of hydroxyapatite, which arises from the crystallization of calcium ions and $\text{P}_i$ groups, on newly formed bone matrix (osteoid) by osteoblasts\textsuperscript{61-63}. During mineralization, the calcium and $\text{P}_i$ groups are deposited around osteoblasts, embedding them into the matrix and leading to their differentiation into mature osteocytes\textsuperscript{64-65}. The importance of osteocytes is in their maintenance of the structural integrity of bone\textsuperscript{88} as well as their actions on the other main bone cells, osteoblasts and osteoclasts. Osteocytes maintain the differentiation\textsuperscript{88-90} and mineralization activity\textsuperscript{88-89, 91}, and regulate RANKL expression\textsuperscript{88} of osteoblasts as well as regulate the formation and activation of osteoclasts\textsuperscript{88, 92-94}. One of the main functions of mature osteoblasts is to express RANKL\textsuperscript{45, 68-69, 71}, which is critical in osteoclastogenesis. Osteoblasts also secrete the RANKL decoy receptor, osteoprotegerin (OPG). OPG binds to RANKL to inhibit the cell to cell contact between osteoblasts and osteoclast precursors required for osteoclastogenesis\textsuperscript{68-71, 73, 95}. Therefore, OPG negatively regulates osteoclast formation, differentiation and resorptive activity\textsuperscript{68-71, 73, 95}.

Osteoclasts are derived from the hematopoietic stem cell lineage. Hematopoietic cells differentiate into monocytes and macrophage cells, which express the receptor for RANKL\textsuperscript{68, 71, 73, 96-99}. These cells then differentiate into osteoclast precursors in the presence of RANKL expressed by osteoblasts\textsuperscript{68, 71, 73, 96, 98, 100-101}. The survival and differentiation of osteoclast precursors, which express the receptor for RANKL, into mononuclear osteoclasts, also requires the presence of RANKL\textsuperscript{71, 73, 97-100}. The mononuclear osteoclasts then fuse to form mature, multinucleated osteoclasts, expressing the receptor for RANKL\textsuperscript{71, 73, 97-100}. The presence of RANKL is imperative for the activation and formation of the ruffled border, which provides the resorptive activity of osteoclasts\textsuperscript{69, 99}. Mature osteoclasts that have a developed ruffled border degrade both the inorganic (hydroxyapatite) and organic (matrix proteins) phases of bone\textsuperscript{85}. The secretion of hydrogen ions (H\textsuperscript{+}) by acid hydrolases results in the acidification of the inorganic matrix and the subsequent degradation of hydroxyapatite\textsuperscript{102-103}. Osteoclasts also secrete proteases to degrade the bone matrix proteins\textsuperscript{104-105}. In addition to osteoblasts and osteoclasts regulating bone growth and remodelling, vitamin D is also important due to its direct effects on these bone cells.
2.2.2.1 Effects of 25(OH)D on Bone Growth and Remodelling

In vitro studies have investigated the effects of both 25(OH)D and 1,25(OH)\(_2\)D on osteoblasts and osteoclasts and their respective functions of bone formation and resorption. It is well known that 1,25(OH)\(_2\)D is important in bone health and remodelling; however, studies have demonstrated the ability of bone cells to synthesize 1,25(OH)\(_2\)D from 25(OH)D\(_4\)-48. Studies show that osteoblasts express CYP27B1, and in turn, blockage of this enzyme reduces cellular responses to 25(OH)D\(_4\)-46. This demonstrates the autocrine effects of 1,25(OH)\(_2\)D\(_4\)-46 (Figure 2.0). In addition, it has been suggested that 25(OH)D may have direct actions due to its ability to bind to the vitamin D receptor (VDR)\(^{45}\). 25(OH)D is very important during bone formation as it is the main vitamin D metabolite present in bone matrix undergoing active mineralization\(^{106}\). Within osteoblasts, it has been demonstrated that physiological levels of 25(OH)D (100 nmol/L) upregulate the gene expression of bone matrix proteins, OCN\(^{45-46}\) and OPN\(^{45}\), as well as enhance the incorporation of calcium into the extracellular matrix (ECM), resulting in greater bone mineralization and strength, by way of local synthesis of 1,25(OH)\(_2\)D\(_{45-46}\). Interestingly, enhanced gene expression of bone matrix proteins by physiological levels of 25(OH)D was observed to be more effective than a pharmacological dose (1-10 nM) of 1,25(OH)\(_2\)D\(_{45-46}\), despite the fact that locally produced amounts of 1,25(OH)\(_2\)D (approximately 400-800 pM) were much lower\(^{46}\). This emphasizes the importance of 25(OH)D and its continuous metabolism to 1,25(OH)\(_2\)D by osteoblasts. In addition, physiological levels of 25(OH)D have been shown to enhance osteoblast differentiation\(^{45}\) by acting on MSC, osteoprogenitors, and osteoblast precursors\(^{107-108}\), and enhance osteoblast function\(^{45}\). Osteoblast metabolism of 1,25(OH)\(_2\)D from 25(OH)D by the CYP27B1 enzyme may also be a potential regulator of osteoblast differentiation and function, as there is a modest inhibitory effect on proliferation at physiological levels of 100 nmol/L\(^{45,108}\). Therefore, 25(OH)D has been shown to upregulate bone formation through its local metabolism to 1,25(OH)\(_2\)D in osteoblasts. However, in order to optimize bone growth and remodelling, bone resorption must be coupled with this process.

Physiological levels of 25(OH)D (100 nmol/L) have been shown to regulate the transcription of RANKL by way of osteoblast metabolism of 25(OH)D to 1,25(OH)\(_2\)D\(^{45}\) (Figure 2.0). In addition, 25(OH)D metabolism to 1,25(OH)\(_2\)D by CYP27B1 in both monocytes/macrophages and osteoclast precursors enhances their differentiation towards mature
Figure 2.0 Effects of 25(OH)D on osteoblasts
The actions of physiological levels of 25(OH)D (100 nmol/L) in vitro on bone formation cells via direct and indirect effects. The effects of 25(OH)D are important in maintaining a balance between bone formation and bone resorption to optimize bone health. 25(OH)D may potentially enhance bone formation by increasing the expression of OCN and OPN and potentially enhance bone resorption by increasing RANKL expression through direct action on osteoblasts. 25(OH)D is present during active mineralization to increase bone formation. Osteoblasts express CYP27B1 which metabolizes 25(OH)D to 1,25(OH)\textsubscript{2}D. Autocrine effects of 1,25(OH)\textsubscript{2}D include the upregulation of bone formation by increasing OCN expression and the upregulation of bone resorption by increasing RANKL expression. (? , may have potential effects;增多, increases)
osteoclasts47 (Figure 2.1). Specifically, monocytes and macrophages treated with RANKL had enhanced osteoclastogenesis in the presence of physiological levels of 25(OH)D (100 nmol/L) compared to RANKL alone47-48. Interestingly, the physiological levels of 25(OH)D (100 nmol/L), and subsequent osteoclast metabolism to 1,25(OH)₂D, increased osteoclastogenesis to a greater extent than the pharmacological levels of 1,25(OH)₂D that were added47. This may be due to the continuous local production of 1,25(OH)₂D, and its autocrine effects on osteoclast differentiation in the presence of CYP27B1. However, the osteoclast-mediated bone resorption was dose-dependently inhibited with increasing 25(OH)D concentrations, with a maximal inhibitory effect at 50 nmol/L48, which corresponds to the proposed physiological threshold for optimal bone health and protection from fracture by the Institute of Medicine (IOM)20, 109. These findings indicate the regulatory role of local osteoclast synthesis of 1,25(OH)₂D on both osteoclastogenesis and osteoclast activity. Specifically, this emphasizes the significance of sufficient serum 25(OH)D concentrations in optimizing bone resorption by increasing osteoclast differentiation while reducing resorptive activity. Therefore, at optimal serum 25(OH)D levels, local 25(OH)D metabolism to 1,25(OH)₂D may optimize communication and coupling between bone resorption and bone formation resulting in optimal bone health and remodelling.

2.2.2.2 Effects of 1,25(OH)₂D on Bone Growth and Remodelling

1,25(OH)₂D also affects both osteoblasts and osteoclasts and their respective functions of bone formation and resorption. With respect to osteoblasts, 1,25(OH)₂D stimulates their proliferation (the number of osteoblasts)77, differentiation72, 110, and mineralization (calcium deposition) of bone72, 77, 110-111 (Figure 2.2). During differentiation, 1,25(OH)₂D acts to increase all stages of development in the osteoblast lineage including MSC, osteoprogenitors, and osteoblast precursors107-108, 111. In addition, 1,25(OH)₂D upregulates APase activity, which is indicative of increased osteoblast function such as matrix and mineral production72, 77, 110-111, as well as upregulates the production of important bone matrix proteins, collagen67, 77, OCN67, 72, 84, 110-112 and OPN111-112 and their integration into the matrix77.

1,25(OH)₂D affects bone resorption as well. 1,25(OH)₂D upregulates the expression of RANKL by osteoblasts68-69, 72 (Figure 2.2). Another important aspect is the regulation of osteoblast expression of OPG. 1,25(OH)₂D has been shown to significantly reduce OPG expression, thereby permitting cell to cell contact between osteoblasts and osteoclast precursors, which enhances osteoclastogenesis70, 72, 95 (Figure 2.2). Finally, 1,25(OH)₂D reduces osteoclast
Figure 2.1 Effects of 25(OH)D on osteoclastogenesis and osteoclast activity
The actions of physiological levels of 25(OH)D (100 nmol/L) in vitro on osteoclastogenesis and osteoclast resorptive activity in the presence of RANKL via indirect effects. Osteoclasts and their precursors express CYP27B1 which metabolizes 25(OH)D to 1,25(OH)2D. The indirect effects of 25(OH)D are important in maintaining a balance between bone resorption and bone formation to optimize bone health. Osteoclasts descend from the hematopoietic stem cell lineage. Autocrine effects of 1,25(OH)2D include enhancing the synthesis of multinucleated osteoclasts at all stages of differentiation, while reducing the differentiation of activated osteoclasts and the formation of the ruffled bordered. 1,25(OH)2D also reduces the resorptive activity of activated osteoclasts through autocrine action to prevent the rate of bone resorption from exceeding that of bone formation, however there is a maximal inhibitory effect at 25(OH)D levels of 50 nmol/L. (●, enhances; ■, reduces)
Figure 2.2 Effects of 1,25(OH)2D on osteoblast differentiation and function
The actions of 1,25(OH)2D in vitro on osteoblast differentiation, bone formation activity and RANKL expression are important to maintain a balance between bone formation and resorption to optimize bone health. Osteoblasts descend from the mesenchymal stem cell lineage. 1,25(OH)2D enhances the production of mature osteoblasts by increasing differentiation at all stages of development. Increased osteoblast function of mineralization and matrix deposition by 1,25(OH)2D is evident via the increased expression of APase activity. 1,25(OH)2D also enhances bone formation by increasing osteoblast expression of bone matrix proteins (ie: OCN). Bone formation and resorption must be coupled during remodelling for optimal bone health. Therefore, 1,25(OH)2D also indirectly enhances osteoclastogenesis by acting on osteoblasts to increase the expression of RANKL while simultaneously reducing the expression of OPG. (+, increases; −, reduces)
resorptive activity to ensure bone resorption is coupled with bone formation\textsuperscript{48, 77} (Figure 2.3). While the regulatory effects of vitamin D on bone health have been well documented, studies show this vitamin is also involved in modulating the immune response.

### 2.2.3 Immunomodulatory Actions

In addition to the original actions of regulating calcium homeostasis and bone growth and remodelling, vitamin D has recently emerged as an important regulator of the immune system and therefore may be a key factor in autoimmune diseases. Research into the immunomodulatory effects of 25(OH)D is limited. However, it has been demonstrated that dendritic cells (DC) express CYP27B1, and therefore have the ability to metabolize 25(OH)D to 1,25(OH)$_2$D\textsuperscript{50}. In addition, DC have their development and function regulated by 25(OH)D\textsuperscript{50}. The presence of 25(OH)D has been shown to reduce DC maturation, tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and IL-12 cytokine production and DC-induced T cell proliferation; however, there was an increase in IL-1\(\beta\) and IL-6 production\textsuperscript{50}. The immunomodulatory effects of 1,25(OH)$_2$D have been well demonstrated. Primary targets include DC, type 1 helper T (Th1) cells (pro-inflammatory cytokines), monocytes, macrophages and type 2 helper T (Th2) cells (anti-inflammatory cytokines). 1,25(OH)$_2$D has been shown to inhibit DC formation\textsuperscript{113-114}, maturation\textsuperscript{113-116}, and differentiation\textsuperscript{114-115}, which indirectly reduces Th1 cell activation\textsuperscript{113, 116-117} and proliferation\textsuperscript{114}, as well as promote DC apoptosis\textsuperscript{113}. 1,25(OH)$_2$D has also been shown to decrease T cell activation and proliferation\textsuperscript{118-119}, resulting in reduced T cell RANKL production. In addition, 1,25(OH)$_2$D can decrease Th1 cell, macrophage and monocyte production of interferon-\(\gamma\) (IFN-\(\gamma\))\textsuperscript{113, 120-123}, TNF-\(\alpha\)\textsuperscript{121, 123-128}, IL-1\(\beta\)\textsuperscript{129} and IL-6\textsuperscript{124, 130}, leading to a reduction in T cell expression of TNF-\(\alpha\) and RANKL. 1,25(OH)$_2$D has also been shown to enhance the development of Th2 cells to shift the cytokine profile to an increase in IL-4\textsuperscript{128, 131} and IL-13\textsuperscript{132-133} expression and a decrease in IFN-\(\gamma\) expression\textsuperscript{131} as well as enhance the expression of regulatory T (T\textsubscript{reg}) cells\textsuperscript{134}. Therefore, research strongly illustrates the regulatory effect of 1,25(OH)$_2$D on the immune response, illustrating the involvement of vitamin D in many aspects of health.
Figure 2.3 Effects of 1,25(OH)_{2}D on osteoclastogenesis and osteoclast activity
The actions of 1,25(OH)_{2}D in vitro on osteoclast differentiation and resorptive activity are important to maintain a balance between bone resorption and formation to optimize bone health. Osteoclasts descend from the hematopoietic stem cell lineage. 1,25(OH)_{2}D enhances the production of multinucleated osteoclasts by increasing differentiation at all stages of development. Reduction in the formation of the ruffled border and osteoclast function of bone resorption by 1,25(OH)_{2}D is important to ensure the rate of bone resorption does not exceed that of formation. 1,25(OH)_{2}D also indirectly enhances osteoclastogenesis by stimulating osteoblast expression of RANKL, thereby increasing cell to cell communication between osteoblasts and the precursors of the osteoclast lineage. (↑, increases; ↓, reduces)
2.3 Vitamin D and Health Effects

2.3.1 Body Weight

Studies in healthy humans and animal models have found no association between vitamin D supplementation and body growth. Research in healthy pregnant women has shown that vitamin D supplementation of 400-4,000 IU/day does not decrease or increase the birth weight of the child\textsuperscript{135}. In agreement, vitamin D intervention in post-weaning rats and mice has also reported no change in body weight with vitamin D supplementation ranging from 400-20,000 IU vitamin D\textsubscript{3}/kg diet\textsuperscript{136}. However, vitamin D deficiency and insufficiency during the critical period of pregnancy is associated with reduced maternal weight gain\textsuperscript{137}. This is important as poor maternal weight gain during pregnancy can lead to poor growth and body size for the developing infant\textsuperscript{137-138}.

Research in humans investigating the effects of maternal vitamin D status on infant body weight has reported conflicting results depending on the stage of pregnancy that is investigated. Whereas one study found no association between early (approximately 11 weeks of gestation) maternal vitamin D deficiency (serum 25(OH)D < 28 nmol/L) and infant birth weight\textsuperscript{139}, many studies have reported a relationship between these 2 variables. One study reported that mothers who were vitamin D deficient (serum 25(OH)D < 37.5 nmol/L) or sufficient (serum 25(OH)D > 75 nmol/L) during early (< 22 weeks gestation) pregnancy were at an increased risk of their infant being small-for-gestational age (SGA) at birth\textsuperscript{140}. Another study reported a 140\% and 50\% increased risk of SGA at birth with maternal vitamin D deficiency (serum 25(OH)D levels ≤ 29.9 nmol/L) and insufficiency (serum 25(OH)D levels 30.0-49.9 nmol/L), respectively, during early (approximately 12 weeks of gestation) pregnancy\textsuperscript{141}. The same study found that maternal vitamin D deficiency and insufficiency resulted in significantly decreased infant weight at birth and 1 month of age compared to maternal vitamin D sufficiency (serum 25(OH)D levels > 50.0 nmol/L) during early pregnancy\textsuperscript{141}. However, studies investigating late (28 weeks of gestation and 1-4 hours postpartum) maternal vitamin D deficiency (serum 25(OH)D < 27.5 nmol/L) and infant birth weight found no association\textsuperscript{142-143}. Vitamin D status has also been associated with body weight in those affected with IBD. Reduced serum 25(OH)D levels were significantly associated with a lower body weight and body mass index (BMI)\textsuperscript{15}.
Animal studies of maternal deficiency have also implicated vitamin D as a factor in the regulation of offspring body growth in both healthy and diseased models. A study in pregnant guinea pigs investigating the effects of maternal vitamin D deficiency during pregnancy and lactation found that pups of sows consuming a diet devoid of vitamin D weighed significantly less at birth (7.5%) and 28 days of age (5.3%) than those pups of sows consuming a vitamin D sufficient diet (1,200 IU vitamin D3/kg diet)\textsuperscript{144}. Vitamin D deficiency also had a significant effect on body weight in the inflammatory mouse model of IBD, the IL-10 KO mouse. Although maternal and post-weaning vitamin D deficiency did not affect body size during early postnatal life, severe growth retardation presented at 7 weeks of age with mice deteriorating until necropsy at 9 weeks of age\textsuperscript{145}. However, littersmates who were weaned to a vitamin D sufficient diet (200 IU vitamin D3/day) were healthy and gained weight throughout development\textsuperscript{145}.

The role of vitamin D in the development of a healthy body size is supported by the many interactions of 1,25(OH)\textsubscript{2}D with factors involved in growth. 1,25(OH)\textsubscript{2}D has been shown to control (upregulate and inhibit) mRNA expression and secretion of human chorionic gonadotropin (hCG) (important in cellular differentiation and proliferation) from the multinucleated cells of the placenta, syncytiotrophoblasts\textsuperscript{146}. Progesterone and estradiol (E\textsubscript{2}) play an important role in fetal growth and development, and 1,25(OH)\textsubscript{2}D has been shown to increase their production from syncytiotrophoblasts\textsuperscript{147}. Other important factors involved in growth and development are insulin and glucose. 1,25(OH)\textsubscript{2}D has been shown to control the mRNA expression and protein level of the insulin receptor (IR), thereby increasing the responsiveness of cells to insulin\textsuperscript{148}. Therefore, 1,25(OH)\textsubscript{2}D is important in glucose transport, metabolism and homeostasis as it increases the production of insulin, while reducing the production of glucagon\textsuperscript{148-149}. As a result, vitamin D deficiency may impair these growth factors leading to restricted development and reduced body weight.

### 2.3.2 Bone Growth and Attainment of Peak Bone Mass

Bone is constantly undergoing changes in size, shape and structure throughout life\textsuperscript{150-152}. During puberty, a time of accelerated growth, there is a rapid accumulation in bone mineral content (BMC), bone mineral density (BMD) and bone area\textsuperscript{150-152} and 33-37% of peak bone mass (PBM) is achieved\textsuperscript{151,153}. Further acquisition of bone area and mineralization continues until early adulthood when maximal PBM is achieved\textsuperscript{151,153}. PBM is the maximum amount of
bone tissue attained at the end of skeletal maturation, and therefore is a key determinant of skeletal health throughout life\textsuperscript{151,153}. Therefore, optimizing PBM in early life may be a preventative strategy to attenuate the decline of bone tissue that occurs later in life, for example during chronic diseases such as osteoporosis and autoimmune diseases such as IBD.

Following the acquisition of PBM, there is a constant average annual loss of bone mass of approximately 0.25-0.70\%\textsuperscript{151,154}. However, small changes in PBM can have a profound effect on bone health later in life. Therefore, if a higher PBM is achieved during adolescence and early adulthood there will be more bone to lose during the aging process, times of stress such as pregnancy, or disease such as IBD. Subsequently, this may lower the risk of developing osteoporosis later in life. For instance, a 10\% increase in PBM can delay the onset of osteoporosis by 13 years\textsuperscript{154}. One aspect to consider when maximizing the attainment of PBM is the effect of nutrition and how this may influence bone development during early life. Nutritional programming is thought to occur during a sensitive period that exists from pre-pregnancy to 24 months of age during which nutrition can affect the structural and functional development of the child\textsuperscript{9-10, 155-156}. With the known effects of 25(OH)D and 1,25(OH)\textsubscript{2}D on the 2 main bone cells, osteoblasts and osteoclasts, their impact on bone development is an important area of research. Optimal vitamin D status during the critical period of intrauterine growth and early postnatal life may enhance the attainment of PBM in adolescence and early adulthood, resulting in a higher level of bone mass later in life when bone loss begins.

2.3.3 Vitamin D and Bone Development

Several longitudinal studies have illustrated the importance of vitamin D exposure in utero and during early life and its’ effects on bone health. Studies have investigated the relationship between maternal vitamin D status during pregnancy and the child’s bone health and development later in life. Newborns whose mothers had vitamin D status above the median (serum 25(OH)D levels > 42.6 nmol/L) had greater BMC at the tibia than newborns whose mothers were below the median\textsuperscript{157}. Fourteen months later the 2 groups of children had similar serum 25(OH)D concentrations and the children whose mothers had vitamin D status below the median had a greater gain in BMC, and therefore there was no difference between the 2 groups at study end\textsuperscript{158}. However, longer studies have shown vitamin D to have beneficial effects. A study investigating the effect of maternal vitamin D status on children’s bone development in early postnatal life found a significant effect on bone. Nine year old children whose mothers
were vitamin D replete (serum 25(OH)D concentrations > 50 nmol/L) during late pregnancy had higher whole body and lumbar spine BMC and BMD compared to those children whose mothers were vitamin D deficient (serum 25(OH)D concentrations < 27.5 nmol/L)\textsuperscript{143}. A similar study investigated the relationship between maternal UVB exposure during late pregnancy, which is our main source of vitamin D, and the child’s skeletal development at 9.9 years of age. Greater maternal UVB exposure was associated with higher whole body BMC and BMD in the child\textsuperscript{159}. This implies that vitamin D status during the critical period of intrauterine growth is significantly correlated with the child’s bone mineral accrual later in life.

In addition, the importance of vitamin D status during the early postnatal period on bone development has also been defined. A study investigating the effects of vitamin D status on bone health found that infants and toddlers (aged 8-24 months) who were vitamin D deficient (serum 25(OH)D levels < 50 nmol/L) had evidence of demineralization at the wrist and knee\textsuperscript{160}. A longitudinal study showed that 8 year old girls who were supplemented with 400 IU/day during the first year of life had greater BMC and BMD at the femur neck\textsuperscript{161}. Therefore, of importance is the possible beneficial impact of early vitamin D status on the acquisition of PBM, and as a result improvement in bone health and reduced risk of fracture in adulthood and in disease states in which bone development can be compromised.

### 2.4 Vitamin D Status in a Healthy Population

The importance of vitamin D for optimal bone development, as well as overall health, has increased the awareness of vitamin D status in healthy pregnant women and children. In 2010, the IOM released new DRI values for vitamin D based on achieving serum 25(OH)D levels (with minimal sun exposure) deemed adequate for optimal bone health\textsuperscript{20}. Although many health outcomes (ie: bone health, immune response, cancer, and cardiovascular disease) were evaluated with respect to determining the adequate vitamin D requirement, with the exception of measures for bone health, sufficient evidence was lacking for a cause and effect or dose-response relationship\textsuperscript{20}. Therefore based on studies reviewed, a serum 25(OH)D level between 40-50 nmol/L was proposed to benefit infants (< 1 year) and a serum 25(OH)D level > 50 nmol/L was proposed to benefit nearly all the population (\(\geq 1\) year)\textsuperscript{20}. Furthermore, serum 25(OH)D < 30 nmol/L are proposed to result in a risk of deficiency while insufficiency is categorized as serum 25(OH)D levels between 30-50 nmol/L for those 1 year and above\textsuperscript{20}. However, there are some researchers who advocate for higher cut-off values when defining
optimal serum 25(OH)D levels in order to benefit overall health (ie: immune response). Optimal serum 25(OH)D levels proposed for healthy pregnant women are ≥ 75 nmol/L and for children (newborn to 19 years of age) are ≥ 50 nmol/L. Tables 2.1 and 2.2 compare the serum 25(OH)D cut-off values for the new DRI for vitamin D with those proposed by some researchers for healthy pregnant women and children, respectively.

Therefore, evaluating the vitamin D status of a population is dependent upon the serum 25(OH)D cut-off values used to define deficiency, insufficiency and optimal status. Studies in healthy pregnant women have found that 44-82% are vitamin D deficient (serum 25(OH)D levels < 50 nmol/L) while 17-34% are insufficient (50 nmol/L ≤ serum 25(OH)D levels ≤ 74 nmol/L). The vitamin D status of children (newborn to 19 years of age) is also often less than optimal. As many as 4-49% of healthy children are vitamin D deficient (serum 25(OH)D levels < 37.5 nmol/L) while 21-38% are insufficient (37.5 nmol/L ≤ serum 25(OH)D levels < 50 nmol/L). The high occurrence of suboptimal vitamin D status is thought to be due to insufficient vitamin D intake, and therefore studies have investigated the effects of vitamin D supplementation.

2.5 Pregnancy and Lactation

Pregnancy and lactation are dynamic periods of bone health. Due to the high calcium demand for fetal skeletal development, pregnancy is a time of great maternal intestinal calcium absorption and calcium mobilization from bone tissue. During the second and third trimesters, fractional calcium absorption increased by as much as 20-57% and 25-72% above pre-pregnancy levels, respectively; however, serum calcium levels were significantly reduced. In addition, urine calcium excretion was increased throughout pregnancy, by as much as 46-125% during the third trimester compared to pre-pregnancy levels. Throughout lactation there was a 20% increase in fractional calcium absorption compared to pre-pregnancy levels, which significantly increased serum calcium levels during this time. The rate of calcium excretion returns to baseline during lactation.

Vitamin D metabolites also undergo change during pregnancy and lactation. Serum 25(OH)D levels are significantly increased in the third trimester. In addition, specifically at 12 weeks gestation maternal 1,25(OH)₂D levels have been shown to be triple that of normal, nonpregnant female subjects. There was also a significant increase in serum 1,25(OH)₂D
Table 2.1 Vitamin D status cut-off levels for healthy pregnant women

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<td>Insufficient</td>
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<td>Optimal</td>
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Table 2.2 Vitamin D status cut-off levels for children (newborn-19 years)

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<td>&gt; 50</td>
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</table>

N/A = not applicable
levels during the second and third trimesters compared to postpartum concentrations\textsuperscript{169-170, 173}, and this was positively associated with increased intestinal calcium absorption\textsuperscript{170}. The increase in serum 1,25(OH)\textsubscript{2}D concentrations during pregnancy is thought to be a result of the extrarenal production by the placenta\textsuperscript{49, 51}.

Studies during pregnancy and lactation have documented the occurrence of maternal bone changes during these periods. These changes may be a result of calcium mobilization from bone when maternal calcium absorption is not sufficient for the high calcium demand for fetal skeletal development\textsuperscript{170-171}. Compared to pre-pregnancy levels, there was a significant reduction in bone formation markers (ie: OCN\textsuperscript{169-170, 172}, procollagen I carboxypeptides (PICP)\textsuperscript{169}) during the second and third trimesters which returned to baseline during lactation\textsuperscript{169-170, 172}. However, this was accompanied by a significantly longer increase in bone resorption markers (ie: urinary collagen cross links\textsuperscript{169-172} and tartrate resistant acid phosphatase (TRAP)\textsuperscript{169}) throughout pregnancy and lactation that did not return to baseline until the resumption of menses\textsuperscript{169-170}. These results indicate the uncoupling of bone formation and resorption processes, which may precipitate bone loss following pregnancy and lactation.

In addition to the imbalance between bone remodelling processes, there was a significant reduction in lumbar spine, hip and whole body BMD at 2 weeks post-partum from pre-pregnancy levels compared to control, nonpregnant females\textsuperscript{174}. Although BMD at these sites began to increase around 4 months postpartum, BMD was still below baseline levels at 9 months postpartum in women who breastfed for longer than 4 months\textsuperscript{174}. A follow-up study indicated these reductions were temporary as BMD recovered to baseline levels at 19 months\textsuperscript{174}. These changes in BMD were not associated with changes in body weight or composition\textsuperscript{174}. Other studies have found significant reductions in lumbar vertebra (LV)\textsuperscript{170-172}, pelvis\textsuperscript{172} and total hip\textsuperscript{171} BMD during lactation, compared to pre-pregnancy levels, that were not associated with changes in body weight. However, the change in LV BMD was found to be temporary as it returned to baseline following the resumption of menses\textsuperscript{170}. Furthermore, following pregnancy and lactation there were reductions in arm and trunk BMD once menses had resumed\textsuperscript{170}.

Although bone formation markers return to pre-pregnancy levels during lactation, in many cases this has not compensated for the longer in duration increase in bone resorption markers resulting in a reduction in BMD. These findings are important to consider as studies have found a high prevalence of vitamin D deficiency and insufficiency in pregnant women.
Although these detrimental bone changes may be temporary, they may be more pronounced under deficient and insufficient conditions. As a result, some women may be at risk of developing osteoporosis due to the nature of pregnancy itself. A reduction in BMD and overall bone health can increase the risk of bone fragility and fracture, thereby potentially reducing one’s quality of life. Therefore, this emphasizes the need to investigate preventative measures to improve bone health prior to and during pregnancy and lactation to reduce or prevent the possible occurrence of osteoporosis later in life.

2.6 Osteoporosis

2.6.1 Characteristics of Osteoporosis

Osteoporosis is a chronic disease characterized by low bone mass and micro-architectural deterioration of bone tissue, with a subsequent increase in bone fragility and susceptibility to fracture risk\textsuperscript{175-176}. The mean age of diagnosis is often later in life between 45 and 65 years of age or at a time of first fragility fracture\textsuperscript{176-178}. The diagnosis of osteoporosis is based on the individuals’ T score, which is the deviation of the BMD measurement from the mean peak BMD of healthy, young adults\textsuperscript{175,179}. The diagnostic categories include normal BMD (T score $\leq 1$ standard deviation (SD) below the norm), low BMD (osteopenia) ($1 < T$ score $< 2.5$ SD below the norm) and osteoporosis (T score $> 2.5$ SD below the norm)\textsuperscript{175,179}. Common skeletal sites affected include the total hip, femur neck, mid-forearm and lumbar spine.

Quality of life is impaired in those diagnosed with osteoporosis. Women with osteoporosis report greater pain, impaired physical and social functioning, deteriorated mental health (ie: depression/anxiety) and vitality, poor emotional status and a reduced perception of overall health compared to nonosteoporotic women\textsuperscript{180}. Fragility fractures are the most severe symptom of osteoporosis. Following fracture, physicality, socialization, mental health, emotional status and overall well-being continued to deteriorate\textsuperscript{181}. Previous fracture is predictive of a higher risk of future fracture\textsuperscript{178}. Osteoporosis-related fractures are of major concern as they have been associated with increased risk of mortality. Canadians diagnosed with osteoporosis, over the age of 50 years, have a 2.53 increased risk of mortality in the first year and 2.27 increased risk in the second year following a vertebral fracture\textsuperscript{177}. In addition, there is a 4.19 increased risk of mortality in the first year following a hip fracture. Therefore,
due to the severe health risks associated with osteoporosis, it is important to develop preventative measures to reduce the occurrence of these detrimental end points.

2.6.2 Prevalence and Incidence of Osteoporosis

Osteoporosis is more prevalent in women than men regardless of skeletal site. Currently, Osteoporosis Canada estimates that 2 million Canadians suffer from this chronic disease. In Canada, 1 in 4 women and at least 1 in 8 men over the age of 50 years have osteoporosis. One study in Manitoba, Canada found that 30% of women over the age of 50 years presented with osteoporosis at 1 or more skeletal site. Specifically, 14% were osteoporotic at 1 site, 5% at 2 sites, and 11% at 3 or more sites. A prospective cohort study across Canada found that prevalence of osteoporosis differs by skeletal site. In women (50 years and older) there was a 9% prevalence at the total hip, 16% at the femur neck, and 12% at the lumbar spine. In men (50 years and older) osteoporosis prevalence was 0.9% at the total hip, 3% at the femur neck, and 3% at the lumbar spine. Prevalence also increases with age. At age 65 years and older, prevalence increases to 18% and 2% at the total hip, 30% and 5% at the femur neck, and 20% and 3% at the lumbar spine in women and men, respectively.

The prevalence of fractures in those diagnosed with osteoporosis is 19-80%. The incidence of fracture increases with an increasing number of osteoporotic sites. In women 50 years of age and older, the incidence of osteoporotic fracture was 132 persons per 1,000 person-years for 1 osteoporotic site, 72 persons per 1,000 person-years for 2 osteoporotic sites, and 237 persons per 1,000 person-years for 3 or more osteoporotic sites. In addition, fracture incidence varied with osteoporotic site. The incidence of osteoporotic fracture was 26 persons per 1,000 person-years at the lumbar spine, 41 persons per 1,000 person-years at the total hip, and 33 persons per 1,000 person-years at the femur neck. The high prevalence, and subsequent risk of fracture, of this chronic disease indicates the need to prevent its’ onset. Therefore, to reduce the prevalence of osteoporosis, the development of this disease and associated risk factors must be understood.
2.6.3 Etiology

2.6.3.1 Risk Factors for Osteoporosis and Related Fractures

In women, menopause is 1 of the most important determinants of bone loss. Osteoporosis is strongly associated with a longer period of menopause, a time in life when estrogen and progesterone production is significantly reduced and bone metabolism is imbalanced leading to greater bone loss\(^{185-186}\). As women approach menopause, 2-5% of bone mass is lost per year\(^{182}\). Pregnancy and lactation may also contribute to a loss of bone mass due to higher calcium needs for fetal skeletal development. The uncoupling of bone formation and resorption processes during pregnancy and lactation\(^{169-170,172}\), in addition to the significant reduction in lumbar spine, hip and whole body BMD at 9 months post-partum from pre-pregnancy levels in women who breastfed for longer than 4 months\(^{174}\), may increase the risk of developing osteoporosis later in life. Whereas another study found that women who had a history of pregnancy, but did not breast-feed, were at a 4.4-fold increased risk of developing osteoporosis later in life compared to those who did breast-feed for 1 month or longer\(^{187}\). Furthermore, women have a higher prevalence of osteoporosis-related fractures compared to men\(^{188}\). Low PBM is another important factor in the risk of osteoporosis as well as osteoporosis-related fractures. PBM is the maximum amount of bone tissue attained at the end of skeletal maturation\(^{151,153}\). Following acquisition there is a constant average loss of approximately 0.25-0.70% of bone mass per year\(^{151,154}\). Therefore the amount of bone mass achieved during growth and development can impact the development of this disease and subsequent fracture. This is further emphasized by the fact that a 10% increase in PBM during growth and development can delay the onset of osteoporosis by 13 years\(^{154}\) and reduce the risk of fracture by 50%\(^{189}\). Therefore, because bone is lost annually following the attainment of PBM, it is important to maximize the amount of bone mass achieved to slow or attenuate the detrimental effects of bone loss. Age is also associated with osteoporosis and osteoporosis-related fractures because once PBM is achieved there is a constant loss of bone each year\(^{151,154}\). As a result, as one ages there is a loss of bone mass and therefore an increased risk of osteoporosis\(^{186}\) and a significantly increased prevalence of osteoporosis-related fracture in older women and men\(^{176,188}\). Also associated with an increased risk of osteoporosis in women is body weight. It has been demonstrated that women over the age of 50 years are at an increased risk of developing osteoporosis when they weigh less than 127 pounds\(^{187}\). In women and men, weight
and height are associated with osteoporosis-related fractures, with thinner and shorter individuals having an increased risk of prevalence\textsuperscript{188}. Vitamin D may also be a critical factor in this disease. Sixty-four to 100\% of women with osteoporosis were documented to have serum 25(OH)D levels below optimal status (serum 25(OH)D levels < 75 nmol/L)\textsuperscript{190-191}, with 29\% having insufficient levels (50 < serum 25(OH)D levels < 75 nmol/L)\textsuperscript{190} and 71\% having deficient levels (serum 25(OH)D levels < 50 nmol/L)\textsuperscript{190}. Nutritional factors are important in determining the risk of developing osteoporosis-related fractures. Studies on calcium and vitamin D have demonstrated the beneficial effects on bone development, thereby highlighting the importance of these factors in reducing the risk of fracture associated with osteoporosis. The importance of vitamin D status on bone development during intrauterine growth has been demonstrated in 9 year old children. Those children whose mothers were vitamin D replete (serum 25(OH)D concentration > 50 nmol/L) during late pregnancy had higher whole body and lumbar spine BMC and BMD compared to children whose mothers were vitamin D deficient (serum 25(OH)D concentration < 27.5 nmol/L)\textsuperscript{143}. In addition, adequate calcium intake (1,200-1,500 mg/day) plus vitamin D supplementation (200-400 IU/day) of adolescent girls (average age 11-12 years) for 1 year increased BMC and BMD of the femur, LV, and trabecular bone of the tibia compared to controls\textsuperscript{192-193}. A prospective 3 year study emphasized the relationship between baseline vitamin D status and BMD. Adolescent girls (aged 9-15 years) who experienced menarche 2 years after the beginning of the study and had an average serum 25(OH)D level of 45 nmol/L had a 26\% and 27\% greater increase in femur neck and lumbar spine BMD respectively, compared to girls who had an average serum 25(OH)D level of 19 nmol/L\textsuperscript{194}. This study shows that poor vitamin D status during a period of growth and development could impact the acquirement of PBM. Therefore, optimizing calcium and vitamin D status during intrauterine and early postnatal life should maximize bone development and achievement of PBM subsequently resulting in a reduction in the risk of osteoporosis-related fractures later in life. There are many risk factors associated with the development of osteoporosis and related fractures and therefore therapeutic measures to prevent disease onset are an important area of research.

Vitamin D is involved in the development of bone, and with the increasing prevalence of vitamin D deficiency it is important to consider the impact poor status may have on health during critical periods such as pregnancy and lactation and intrauterine and early postnatal life. Furthermore, whether there are specific disease states in which less than optimal vitamin D
status may precipitate the development of the disease and associated abnormalities is an emerging area of study. With the recent discovery of the immunomodulatory effects of vitamin D there is interest in its’ possible role in chronic autoimmune diseases such as IBD. Compromised bone development has been documented in individuals with IBD and therefore it is important to investigate the possible factors involved. The many effects of vitamin D in the development of bone and the immune system may indicate a possible protective role against the development of IBD, and therefore supplementation with vitamin D should be investigated.

2.7 Inflammatory Bowel Disease

Vitamin D status may be important for preventing or attenuating inflammatory diseases (ie: IBD) because of its immunomodulatory effects. Of particular interest with those affected with IBD are the associated bone abnormalities and development as a result of the dysregulated immune response present in this disease195-205. Due to the severity of intestinal inflammation and deterioration in bone health with IBD, it is imperative to investigate strategies that may modulate disease development during intrauterine and early postnatal life.

2.7.1 Characteristics of Inflammatory Bowel Disease

IBD is a chronic autoimmune disorder in which the small and large intestines become red and swollen206-207. IBD includes 2 main intestinal disorders, ulcerative colitis (UC) and Crohn’s disease (CD)206, 208-209. The age of onset for IBD is most common during adolescence and young adulthood from age 15-29210-215. Symptoms of IBD include diarrhoea206, 208-209, abdominal pain206, 208-209, bleeding206, 208-209, 216, fatigue208-209, vomiting209, fever206, 209, weight loss206, 208, and anaemia217, which can all significantly deteriorate the quality of one’s life.

2.7.2 Prevalence and Incidence of Inflammatory Bowel Disease

The prevalence and incidence of IBD in developed countries are higher compared to those of developing countries, and continue to rise. This is particularly relevant as Canada has 1 of the highest estimated prevalence and incidence rates of IBD in the world214. In Canada, the estimated prevalence of IBD was 0.37% and the estimated incidence for IBD from 1984 to 1992 was 25 new cases per 100,000 person-years210. Then in 2000, the prevalence of IBD increased among the Canadian population to approximately 0.50%, with an incidence of approximately 45 new cases per 100,000 person-years during 1998 to 2000211. Specifically in
Ontario, Canada, the pediatric population has seen an increase in the prevalence of IBD from approximately 0.04% in 1994 to approximately 0.06% in 2005\textsuperscript{214}. In addition, the estimated incidence increased from 9.5 new cases\textsuperscript{214} per 100,000 children in 1994\textsuperscript{214} to 14.3 from 1997 to 2001\textsuperscript{218} to 11.9 from 2002 to 2006\textsuperscript{214,218}. In 2001, the estimated prevalence of IBD in the United States was 0.39%, with an annual estimated incidence of 17 cases per 100,000 persons from 1990 to 2000\textsuperscript{212}. The prevalence of IBD increased to approximately 0.51% from 2003 to 2005\textsuperscript{213}. Specifically, in the pediatric population of the United States, the estimated prevalence of IBD in 2006 was 0.02\textsuperscript{219}. Also, the annual incidence of IBD increased from approximately 1.1 cases per 100,000 children during 1991 to 1996\textsuperscript{220} to approximately 6.5 during 1996 to 2006\textsuperscript{219}. Populations outside of North America have also reported an increase in the incidence of IBD. The Finnish population reported an increase in the prevalence and incidence of IBD over the years. The prevalence of IBD in 1999 was approximately 0.44%, an increase from approximately 0.17% in 1986\textsuperscript{221}. The annual incidence rate of IBD increased from 19.5 to 29.2 per 100,000 persons from 1986 to 1999\textsuperscript{221}. In France, the pediatric incidence rate also significantly increased from 5.2 cases per 100,000 person-years from 1988 to 1990 to 6.7 from 2006 to 2007\textsuperscript{215}. With IBD prevalence on the rise, it is important to determine the factors involved with this increase. The etiology of IBD remains unknown; however, it is hypothesized to be a multifactorial disease and therefore both genetic and environmental factors are implicated in disease development.

2.7.3 Etiology of Inflammatory Bowel Disease

IBD predominantly occurs in the developed world, and although the reason is unclear, possible theories for lower incidence in the developing world include diet, lower fat and refined carbohydrate intake\textsuperscript{222-223}, and the hygiene hypothesis, which suggests those with a higher exposure to childhood infections or unsanitary conditions develop a more sufficient immune system and therefore experience less incidence of chronic diseases such as IBD\textsuperscript{224}. Other factors include low diagnostic awareness as well as the misdiagnosis as diarrhoea from infectious causes\textsuperscript{225}. Although the etiology is unknown, factors suspected to be involved in the development of IBD include genetic as well as environmental aspects (Figure 2.4).
Figure 2.4 Factors that may contribute to the development of IBD
2.7.3.1 Genetic Factors

The genetic aspect of IBD was illustrated with the demonstration of IBD-susceptible genes. Nucleotide-binding oligomerization domain 2 (NOD2) or caspase recruitment domain family member 15 (CARD15) is a member of the NOD-like receptor family (NLF) whose protein product may act as an integral component of the innate immune response to bacterial pathogens\textsuperscript{226-228}. Approximately 25-40\% of those affected with IBD have been identified as carriers of at least 1 of the 3 common variants associated with IBD susceptibility\textsuperscript{226-228}. In addition, carriers of this IBD-susceptible gene are 2.5 times more likely to develop IBD than non-carriers\textsuperscript{227}. Another important IBD-susceptible gene is the nuclear receptor subfamily 1, group I, member 2 (NR1I2) which codes for the pregnane X receptor (PXR)\textsuperscript{229}. PXR is important in regulating the metabolism of xenobiotics\textsuperscript{229}. Inheritance of polymorphisms of the NR1I2 gene may contribute to IBD susceptibility as 3 specific alleles are significantly more common in those affected with IBD compared to controls\textsuperscript{229}. The multidrug resistant 1 (MDR1) gene is also an IBD-susceptible gene as a specific risk allele has been associated with the development of CD\textsuperscript{230}. Furthermore, those with extensive UC have been shown to display the combined presence of risk alleles for IBD-susceptible genes NR1I2 and MDR1\textsuperscript{231}. The genetic predisposition of IBD has also been explored using both twin and family studies, but results are controversial. Concordance rates for IBD range from 25-35\%\textsuperscript{232-233} for monozygotic twins and 2-5\%\textsuperscript{232-233} for dizygotic twins. Studies among families have also implicated genetics as a factor as those who have 1 or more family members affected with IBD have a 2-13 times higher risk of developing the disease than the general population\textsuperscript{234-236}. However, while this indicates that genetics are an important component, it also emphasizes that genetics are not the sole determinant of IBD and that environmental factors are of importance in the development of this inflammatory disease.

2.7.3.2 Environmental Factors

Environmental factors hypothesized to have an effect on the etiology of IBD include cigarette smoking, appendectomy surgery, oral contraceptive use, high sugar or fast food (modified fat) diets, breastfeeding, \textit{Mycobacterium avium} subspecies \textit{paratuberculosis} (MAP) infection, microbial dysbiosis and vitamin D status. Studies have shown that while current cigarette smoking almost doubles the risk of developing CD, it has a protective effect against the
development of UC, decreasing the risk by approximately 75%\textsuperscript{233, 236-238}. A strong positive association has been demonstrated between appendectomy and the incidence of CD\textsuperscript{236, 239}, whereas this surgery was protective against the development of UC\textsuperscript{236-237}. The current use of oral contraceptives increases the risk of developing CD and UC\textsuperscript{236, 240}. The impact of breastfeeding on the development of IBD has been controversial. Breastfeeding for a duration longer than 3 months has been shown to have a protective effect against the development of CD and UC\textsuperscript{236, 241}, whereas Thompson et al\textsuperscript{242} found breastfeeding to be protective against CD and associated with an increased risk of UC and Gilat et al\textsuperscript{239} found no association. The role of diet has also been implicated in IBD, with a 2.6 increased risk with the consumption of a high sugar diet (> 30 g/day) and a 3.6 increased risk with the consumption of a fast food meal 2 or more times per week\textsuperscript{243}. Another factor considered in the development of IBD is the infectious agent MAP. MAP has been detected in 35-45\% of those affected with IBD\textsuperscript{244-245}. The human gut microbiota is a highly diverse and abundant community of microbes that are beneficial to health however can also produce antigens which activate the immune response. Therefore, a balanced relationship must exist between the bacteria and human host. Microbial dysbiosis, characterized by a significantly lower bacterial load, altered bacterial composition, reduced bacterial diversity and reduced transcriptional activity, has been documented in those affected with IBD\textsuperscript{246-247}. Vitamin D status as a risk factor was the focus of this thesis research. With the knowledge of the prevalence of vitamin D deficiency and insufficiency in children and adults with IBD, the interest in the role of vitamin D in the development of this disease and its’ associated bone abnormalities has increased\textsuperscript{15, 248-252}. Many factors have been investigated with respect to the development of IBD, and although the importance of both genetic and environmental aspects has been identified, the etiology is still unknown.

2.7.4 Dysregulated Cytokine Production

Although the etiology of IBD is still unknown, the intestinal inflammation that presents during this disease is thought to be due to a loss of tolerance of the immune system against the normal bacterial flora of the intestine\textsuperscript{253-254}. During a regulated immune response, the innate and acquired immune systems provide protection against disease causing organisms by employing highly controlled effector mechanisms such as T cells. Following antigen presentation, these cells differentiate into Th1, Th2 and type 17 helper T (Th17) lymphocyte subsets that regulate one another, in order to provide the most useful and beneficial response for the host, as well as
regulate B cells, monocytes, macrophages and DC\textsuperscript{255-256}. These cells, in addition to their production of cytokines, are capable of destroying a broad range of foreign bodies while simultaneously recognizing and tolerating self-antigens\textsuperscript{255-256}. Therefore, by maintaining self-tolerance during the immunological response, the hosts’ defences are able to respond to, and eliminate, the threat with minimal damage\textsuperscript{256}. Following this process, apoptosis of the antigen-responsive cells is induced to ensure surrounding tissues and host functions are restored to their original state\textsuperscript{256}.

IBD is a specific autoimmune disease characterized by an inappropriate, sustained, and injurious response against self that is thought to emerge due to an unregulated immune response involving Th1 cells\textsuperscript{257-260}, macrophages\textsuperscript{260-265}, monocytes\textsuperscript{265-268}, and DC\textsuperscript{269-271}. These cells are involved in the perpetuation of the immune response and intestinal inflammation due to their proinflammatory cytokine production. Th1 cells produce IFN-\(\gamma\)\textsuperscript{257-259, 272}, TNF-\(\alpha\)\textsuperscript{258, 260, 263, 272-273}, IL-1\(\beta\)\textsuperscript{260, 263} and IL-6\textsuperscript{258, 260, 263, 272}, macrophages produce IFN-\(\gamma\)\textsuperscript{262, 265, 272}, TNF-\(\alpha\)\textsuperscript{260, 263, 272}, IL-1\(\beta\)\textsuperscript{260, 263, 274}, IL-6\textsuperscript{260, 263, 265, 272}, IL-8\textsuperscript{263-265} and IL-18\textsuperscript{272}, monocytes produce IFN-\(\gamma\)\textsuperscript{265}, TNF-\(\alpha\)\textsuperscript{266-268}, IL-1\(\beta\)\textsuperscript{266}, IL-6\textsuperscript{265, 267} and IL-8\textsuperscript{265}, and DC produce TNF-\(\alpha\)\textsuperscript{50, 270}, IL-1\(\beta\)\textsuperscript{50}, IL-6\textsuperscript{50, 269-271}, IL-8\textsuperscript{270} and IL-12\textsuperscript{50, 269, 271}.

\textbf{2.7.5 Inflammatory Bowel Disease-Associated Bone Abnormalities}

In addition to the dysregulated immune response and subsequent intestinal inflammation, those affected with IBD also present with bone abnormalities. Research has shown that animal models of IBD have significantly reduced bone mass, due to suppressed bone formation and increased resorption\textsuperscript{275-276}, as well as decreased BMC\textsuperscript{273, 277}, BMD\textsuperscript{273, 277}, and bone strength\textsuperscript{273, 275}. In addition, human studies have demonstrated that children\textsuperscript{195, 198, 200, 202-204} and adults\textsuperscript{196-197, 199, 201, 205} affected by IBD present with significantly reduced BMC\textsuperscript{203-205} and BMD\textsuperscript{195-202} compared to healthy controls. Osteopenia has been found in 24-38\% and osteoporosis in 13-32\% of those children affected with IBD\textsuperscript{278-279}. In adults with IBD, osteopenia has been found in 31-43\% and osteoporosis in 11-41\%\textsuperscript{196-197, 199, 201}. The seriousness of these bone abnormalities has been illustrated in studies investigating fracture risk in those affected with IBD. These studies have found there is a 40-63\% increased risk of vertebral, hip, rib and wrist/forearm fractures in adults with IBD\textsuperscript{280-282}. Although the etiology of the bone
abnormalities associated with IBD is unknown, it is hypothesized that the disease process itself may be a key factor.

One of the main factors hypothesized to be involved in the IBD-associated bone abnormalities is the elevated production of proinflammatory cytokines such as IFN-γ, TNF-α, IL-1β, IL-6, IL-8 and IL-12, as well as the increased T cell expression of RANKL and the detrimental effects this has on bone cells (Figure 2.5 and 2.6). Enhanced production of proinflammatory cytokines and RANKL has been associated with reduced BMD. Specifically, IFN-γ has been shown to induce osteoclast formation as well as increase T cell expression of TNF-α and RANKL. Proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 promote the differentiation of mature osteoclasts in the presence of RANKL, as well as increase their resorptive activity. Furthermore, TNF-α and IL-1β also induce osteoblast apoptosis and T cell RANKL expression. TNF-α has been found to inhibit the production of bone matrix proteins (ie: type I collagen and OCN). Earlier research has suggested that TNF-α has the ability to induce the differentiation of osteoclasts independent of RANKL; however, the number of mature, active osteoclasts is approximately half with TNF-α compared to those activated in the presence of RANKL. In addition, TNF-α is a predictor of the level of soluble RANKL because it induces osteoblast RANKL expression as well as inhibits the differentiation of osteoblasts by reducing preosteoblast expression of Cbfa1 by approximately 50%. TNF-α has also been shown to support the survival of osteoclasts. The increased production of TNF-α and IL-6 has been associated with reduced calcium integration into bone matrix as well as increased levels of IL-6 leads to misshapen and irregular osteoblasts. IL-8 increases the mRNA and protein expression of RANKL by osteoblasts, as well as stimulates osteoclast differentiation and resorption activity. IL-18 has been shown to increase T cell RANKL expression and osteoclast formation. In addition to their production of proinflammatory cytokines, DC also enhance the inflammatory process by inducing T cell proliferation. Therefore, it is postulated that the IBD-associated bone abnormalities are a consequence of the direct effects of the enhanced proinflammatory cytokine and RANKL production on the bone forming and resorbing cells.

The bone abnormalities associated with IBD are important to address as the onset of this chronic inflammatory disease is typically during adolescence and young adulthood, periods of rapid bone growth and development. Therefore, it is imperative to explore therapies...
Figure 2.5 Effects of proinflammatory cytokines on osteoclastogenesis and osteoclast activity during a state of inflammation

The dysregulated immune response of IBD presents with an enhanced production of proinflammatory cytokines. The inflammatory response is proposed to upregulate the stages of osteoclast differentiation leading to the enhanced production of activated osteoclasts. Development of the ruffled border and resorptive activity of mature, active osteoclasts is also enhanced. There is also indirect upregulation of osteoclastogenesis in the inflammatory state that occurs via the proinflammatory cytokine upregulation of RANKL expression by T cells. The overall increase in osteoclast resorptive activity is hypothesized to be a main contributor in the development of IBD associated bone abnormalities. (_ARROW, enhances)
Figure 2.6 Effects of proinflammatory cytokines on osteoblast differentiation and function during a state of inflammation

The dysregulated immune response of IBD presents with an enhanced production of proinflammatory cytokines. The inflammatory response is proposed to suppress the stages of osteoblast differentiation leading to the reduced synthesis of osteoblasts. Expression of bone matrix proteins (i.e., type I collagen and OCN) is reduced resulting in the inhibition of bone forming activity. Proinflammatory cytokines also act to impair bone formation by disrupting the process of mineralization and matrix deposition directly. There is an indirect upregulation of osteoclast synthesis as the proinflammatory cytokines enhance the expression of RANKL by osteoblasts which leads to cell to cell communication between osteoblasts and osteoclast precursors. Further suppression of bone formation occurs via the increased rate of osteoblast apoptosis. The overall decrease in osteoblast function and bone forming activity is hypothesized to be a main contributor in development of IBD associated bone abnormalities. (🔺, enhances; ❌, reduces)
that will maximize the attainment of PBM so there will be more bone mass to lose during both
the progression of the disease and the normal losses due to the effects of aging. One
preventative strategy of particular interest is vitamin D supplementation during intrauterine and
early life development. Based on the concept of nutritional programming, which is thought to
occur when nutrition affects the child’s structural and functional development during the
sensitive period of growth from gestation to 24 months of age\textsuperscript{9-10, 155-156}, vitamin D and its’
effects on bone and the immune system may play a critical role in the development of IBD. By
using nutritional intervention, there is the possibility to intervene and program early bone
growth and development prior to the onset of disease and associated abnormalities.

2.8 Vitamin D Status in those Affected with Inflammatory Bowel Disease

The immunomodulatory effects, in addition to its’ effect on bone development, has
increased the awareness of vitamin D status in those affected with IBD. Poor vitamin D status
has also been found in those diagnosed with IBD. Although no studies have reported the
prevalence of vitamin D deficiency and insufficiency during pregnancy and lactation in those
affected with IBD, research shows that 44-50\%\textsuperscript{250-252} of women of childbearing age (aged 15-40
years), affected with IBD, are vitamin D deficient (serum 25(OH)D levels < 50 nmol/L) while
26-35\%\textsuperscript{250-252} are insufficient (50 nmol/L ≤ serum 25(OH)D levels < 75 nmol/L). In addition,
studies in children (aged 8-22 years) affected with IBD have found that 19-46\%\textsuperscript{15, 248-249} are
deficient (serum 25(OH)D levels < 51 nmol/L) and 38-58\%\textsuperscript{248-249} are insufficient (51 nmol/L <
serum 25(OH)D levels < 75 nmol/L). The high occurrence of suboptimal vitamin D status is
thought to be due to insufficient vitamin D intake, and therefore studies have investigated the
effects of vitamin D supplementation.

2.9 Vitamin D Supplementation

The prevalence of vitamin D deficiency and insufficiency in healthy and diseased
populations has generated interest in studying the impact of vitamin D supplementation. With
the actions of vitamin D on bone and the immune system, it is important to also consider the
timing of vitamin D intervention to maximize the effects on development. With the recent
publication of the updated DRI’s for vitamin D based on serum 25(OH)D levels that optimize
bone health\textsuperscript{20}, it is of interest whether vitamin D intakes above the RDA will benefit skeletal as
well as non-skeletal conditions such as IBD.
2.9.1 Vitamin D Supplementation in the Healthy State

2.9.1.1 Human studies

2.9.1.1.1 Pregnancy and Lactation

The level of vitamin D supplementation required during pregnancy is important to determine as it must satisfy the needs of both mother and fetus. A recent randomized, double-blind, placebo-controlled trial investigated the level of vitamin D supplementation required by pregnant women to achieve serum 25(OH)D levels > 80 nmol/L\textsuperscript{135} (Table 2.3). Findings indicated that vitamin D supplementation of 4,000 IU/day (starting between 12-16 weeks of gestation) significantly increased serum 25(OH)D levels compared to 400 IU/day and 2,000 IU/day at 1 month prior to delivery and at delivery\textsuperscript{135}. Specifically, supplementation with 4,000 IU vitamin D/day significantly increased serum 25(OH)D levels above 80 nmol/L in more women (84\%) compared to 400 IU vitamin D/day (52\%)\textsuperscript{135}. There was no significant difference in the percentage of women with serum 25(OH)D > 80 nmol/L between 2,000 IU vitamin D/day (80\%) and 4,000 IU vitamin D/day\textsuperscript{135}. A daily supplement of 400 IU vitamin D/day provided a minimal increase (12.5 nmol/L) in serum 25(OH)D levels throughout the study. Furthermore, increased serum 25(OH)D levels in groups 2 and 3 led to a significant increase in serum 1,25(OH)\textsubscript{2}D levels\textsuperscript{135}. Neonatal serum 25(OH)D levels were significantly correlated with maternal serum 25(OH)D levels overall, 1 month prior to delivery and at delivery, and were significantly different among treatment groups\textsuperscript{135}. Average neonatal serum 25(OH)D levels were 45 nmol/L in group 1, 57 nmol/L in group 2, and 66 nmol/L in group 3\textsuperscript{135}. Therefore, this study illustrates the need for vitamin D intakes greater than 4,000 IU/day during pregnancy to achieve optimal serum 25(OH)D levels > 80 nmol/L\textsuperscript{135}.

The level of vitamin D supplementation required during lactation is also important to determine as it must satisfy the demands of both mother and developing child. One study investigated the level of vitamin D supplementation required by lactating mothers to prevent hypovitaminosis D in both themselves and their exclusively breastfed infant\textsuperscript{298} (Table 2.3). Hollis and Wagner\textsuperscript{298} found that the supplementation of lactating women with 2,000 or 4,000 IU vitamin D/day (including a 400 IU vitamin D\textsubscript{3} plus 1,600 or 3,600 IU vitamin D\textsubscript{2}) led to significant increases, in both mothers and their infants, in total serum 25(OH)D concentrations. Total maternal serum 25(OH)D levels increased to 90.3 nmol/L at 3 months with 2,000 IU
Table 2.3 Effect of vitamin D intervention on serum 25(OH)D levels in healthy pregnant and lactating women

<table>
<thead>
<tr>
<th>Study Design</th>
<th>Sample size, Gender Age at Intervention, Dose and Intervention Length</th>
<th>Outcomes</th>
</tr>
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</table>
| Randomized double-blind placebo-controlled study[^35] | • women (≥ 16 years of age), with confirmed singleton pregnancy < 16 completed weeks of gestation, were enrolled into the study.  
• randomization to 1 of 3 intervention groups (between 12-16 weeks of gestation) was based on baseline serum 25(OH)D levels:  
  1) ≤ 100 nmol/L were eligible for all 3 groups.  
  2) 100-150 nmol/L were eligible for groups 1 and 2.  
  3) > 150 nmol/L were eligible for group 1.  
• pregnant women were randomized into 1 of 3 groups; all of which received a standard daily prenatal vitamin containing 400 IU vit. D plus an additional daily supplement of vit. D₃ of:  
  1) 0 IU (placebo) (total of 400 IU). N=166.  
  2) 1,600 IU (total of 2,000 IU). N=167.  
  3) 3,600 IU (total of 4,000 IU). N=169.  
• subjects were followed monthly until delivery, and then 2 weeks postpartum.  
• maternal serum 25(OH)D and calcium levels were measured at baseline and monthly visits and cord blood was obtained at delivery. | • 1 month prior to delivery and at delivery, average serum 25(OH)D levels were significantly higher in group 3 (118 and 111 nmol/L, respectively) compared to groups 1 (79 and 79 nmol/L, respectively) and 2 (105 and 98 nmol/L, respectively) and higher in group 2 compared to group 1.  
• the primary goal of achieving serum 25(OH)D levels > 80 nmol/L around the time of delivery was achieved by approximately:  
  1) 52% in group 1.  
  2) 80% in group 2.  
  3) 84% in group 3.  
• significantly more women achieved serum 25(OH)D levels > 80 nmol/L in groups 2 and 3 compared to group 1.  
• 400 IU vit. D/day provided a minimal increase in serum 25(OH)D levels of 12.5 nmol/L throughout the duration of the study.  
• serum 25(OH)D levels directly influenced 1,25(OH)₂D levels, with serum 1,25(OH)₂D levels significantly increased in groups 2 and 3.  
• serum 25(OH)D levels > 100 nmol/L were required to support a maximal increase in serum 1,25(OH)₂D levels through renal and placental production.  
• there was no relationship between serum 25(OH)D and calcium levels throughout the study in all groups.  
• child birth weight did not differ between intervention groups.  
• neonatal serum 25(OH)D levels were significantly correlated with maternal serum 25(OH)D levels overall, at 1 month prior to delivery and at delivery, and were significantly different among treatment groups.  
• average neonatal serum 25(OH)D levels at birth for group 1 were 46 nmol/L, for group 2 were 57 nmol/L, and for group 3 were 66 nmol/L.  
• no adverse events (hypercalcemia and hypercalciuria) occurred as a result of vit. D supplementation or serum 25(OH)D levels. |
Randomized controlled trial

- 18 lactating mothers, who had given birth within the last month, were enrolled into the study (each subject acted as their own control).
  - women were randomized into 1 of 2 groups (n=9/group):
    1) receive a daily multivitamin (containing 400 IU vit. D₃) and 1,600 IU vit D₂/day.
    2) receive a daily multivitamin (containing 400 IU vit. D₃) and 3,600 IU vit. D₂/day.
  - vit. D status at baseline was compared with values at 3 other time points.
  - blood, urine, and milk samples were obtained from mothers at months 1, 2, 3, and 4 of lactation and infant blood was collected at months 1 and 4.
  - maternal and infant serum was monitored for calcium, vit. D₂, vit. D₃, 25(OH)D₂, and 25(OH)D₃ concentrations.
  - vit. D antirachitic activity in mother’s milk was assessed by measuring vit. D₂, vit. D₃, 25(OH)D₂ and 25(OH)D₃ concentrations in the milk.
  - study duration was 3 months.
  - mothers instructed to limit sun exposure as well as formula intake by child.

- dietary vit. D intakes were up to 10-fold higher than the 1997 DRI for lactating women for 3 months and no adverse events were reported.
  - serum calcium, vit. D, and 25(OH)D concentrations all remained in the normal range.
  - in group 1, total serum 25(OH)D concentrations significantly increased from 69.0 nmol/L at baseline to 90.3 nmol/L at 3 months and milk antirachitic activity significantly increased from 35.5 IU/L at baseline to 69.7 IU/L at 3 months.
  - in group 2, total serum 25(OH)D concentrations increased from 82.3 nmol/L at baseline to 111.3 nmol/L at 3 months and milk antirachitic activity significantly increased from 40.4 IU/L at baseline to 134.6 IU/L at 3 months.
  - in both groups, serum 25(OH)D₃ concentrations significantly decreased despite the daily 400 IU vit. D₃ supplement.
  - during the study, mothers receiving 4,000 IU vit. D/day had significantly higher serum 25(OH)D₃ levels compared to those receiving 2,000 IU vit. D/day.
  - infants of mothers receiving 2,000 IU vit. D/day had an increase in total serum 25(OH)D concentrations from 19.8 nmol/L at baseline to 69.5 nmol/L at 3 months.
  - infants of mothers receiving 4,000 IU vit. D/day had an increase in total serum 25(OH)D concentrations from 33.5 nmol/L at baseline to 77.0 nmol/L at 3 months.
  - infants of mothers receiving 4,000 IU vit. D/day had significantly higher serum 25(OH)D₂ levels compared to infants of mothers receiving 2,000 IU vit. D/day at 3 months.
vitamin D/day, whereas supplementation with 4,000 IU vitamin D/day increased levels to 111.3 nmol/L at 3 months\textsuperscript{298}. Supplementation with 4,000 IU vitamin D/day led to significantly higher 25(OH)D\textsubscript{2} levels in mothers and children compared to those supplemented with 2,000 IU vitamin D/day\textsuperscript{298}. Interestingly, both groups experienced a significant decrease in serum 25(OH)D\textsubscript{3} levels from baseline to 3 months\textsuperscript{298}. The vitamin D content of the mother’s milk was also investigated by measuring the antirachitic activity, which is the ability to prevent the development of rickets\textsuperscript{298}. For both groups, there was a significant increase in the antirachitic activity of milk following 3 months of vitamin D supplementation, directly attributable to the increase in serum 25(OH)\textsubscript{2} levels\textsuperscript{298}. Total infant serum 25(OH)D levels were also measured, and found to be significantly elevated in both groups, subsequently indicating the transfer of vitamin D from mother to child through mother’s milk\textsuperscript{298}. Infants whose mothers received 2,000 IU vitamin D/day achieved total serum 25(OH)D levels of 69.5 nmol/L at 3 months whereas those whose mothers received 4,000 IU vitamin D/day achieved a level of 77.0 nmol/L\textsuperscript{298}. Similar to their mothers, infants in the 4,000 IU vitamin D/day group had significantly higher 25(OH)D\textsubscript{2} concentrations\textsuperscript{298}. As previously mentioned, an important finding of this study was the decline in maternal serum 25(OH)D\textsubscript{3} levels despite the intake of 400 IU vitamin D\textsubscript{3}/day. This emphasized the importance of evaluating the ability of the 1997 DRI to achieve optimal vitamin D status for pregnant and lactating women, as well as their child, knowing the beneficial effects of vitamin D on bone growth and development, as well as possibly on overall health. It also indicated that maternal supplementation up to 10 times the 1997 DRI for vitamin D during lactation may be necessary to achieve optimal serum 25(OH)D levels in both mother and infant. However, despite these findings, as previously mentioned in 2010 the IOM released new DRI values for vitamin D based on achieving a serum 25(OH)D level adequate for optimal bone health\textsuperscript{20}. Based on available evidence, the serum 25(OH)D level deemed sufficient for optimal bone development was 50 nmol/L as levels > 75 nmol/L yielded inconsistent results\textsuperscript{20}. Therefore the RDA was set at 600 IU vitamin D/day (assuming minimal sun exposure) for pregnant and lactating women\textsuperscript{20}. However, because some researchers believe maternal and offspring bone development as well as overall health will benefit from higher serum 25(OH)D levels (> 75 nmol/L)\textsuperscript{143, 157, 162-164}, studies investigating bone outcomes in pregnant and lactating women and their infants following vitamin D supplementation at various levels above the RDA requires further exploration.
2.9.1.1.2 Childhood

A few studies have investigated the effect of vitamin D supplementation on vitamin D status and bone outcomes in healthy children (Table 2.4). A study in Danish girls (aged 11-12 years) investigated the effect of vitamin D₃ supplementation on bone mass and bone turnover for 1 year²⁹⁹. Vitamin D₃ supplementation with 200 IU/day and 400 IU/day significantly increased serum 25(OH)D levels above those in the placebo group, however the difference between vitamin D₃ supplement groups was not significant²⁹⁹. When defining optimal serum 25(OH)D levels as > 75 nmol/L, the level of vitamin D₃ supplementation in this study did not achieve this status. After 1 year, vitamin D₃ supplementation with 200 IU/day increased serum 25(OH)D levels to 53 nmol/L and 400 IU/day increased levels to 58 nmol/L²⁹⁹. Interestingly, supplementation with 400 IU vitamin D₃/day resulted in a significantly lower increase in LV₁-₄ bone mass compared to the other 2 groups²⁹⁹. There were no changes in whole-body or LV₁-₄ BMC or BMD, or OCN levels from baseline to 1 year for any of the 3 groups²⁹⁹. However, it is hypothesized that vitamin D₃ supplementation, and the resulting increase in serum 25(OH)D levels was not high enough to illicit a response²⁹⁹. Furthermore vitamin D intervention may have been too late after the proposed sensitive period of intrauterine growth as well as the duration of study may have been too short to observe beneficial effects on bone development. A similar study by Viljakainen et al.¹⁹³ randomized Finnish girls (average age 11 years) to receive a daily placebo, 200 IU vitamin D₃ or 400 IU vitamin D₃. Vitamin D₃ supplementation significantly increased serum 25(OH)D levels compared to the placebo after 1 year¹⁹³. Furthermore, 400 IU vitamin D₃/day resulted in significantly higher serum 25(OH)D levels compared to 200 IU vitamin D₃/day¹⁹³. Serum calcium levels did not differ among groups after 1 year¹⁹³. Vitamin D₃ supplementation significantly decreased urinary deoxypyridinoline (U-Dpyr) levels compared to the placebo, indicating the antiresorptive properties of vitamin D, however the effects of the higher dose of vitamin D₃ did not differ from the lower dose¹⁹³. The main bone outcome measures were LV₂-₄ and femur bone area, BMC and BMD. In contrast to the previous study, LV₂-₄ and femur BMD significantly increased with vitamin D₃ supplementation; however, there were no changes for the other bone outcomes¹⁹³. When considering only 80% compliance, the benefit of vitamin D₃ supplementation on bone mineral augmentation became significant. Bone mineral augmentation of LV₂-₄ was 12.5% higher in the group receiving 400 IU vitamin D₃/day and of the femur was 14.3% and 17.2% higher in groups
Table 2.4 Effect of vitamin D intervention on bone health in healthy children

<table>
<thead>
<tr>
<th>Study Design</th>
<th>Sample size, Gender</th>
<th>Outcomes</th>
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<tbody>
<tr>
<td>Randomized double-blind placebo-controlled intervention study&lt;sup&gt;299&lt;/sup&gt;</td>
<td>▪ 225 Danish girls (11-12 years of age) were randomized to 1 of 3 groups (N=75/group): 1) daily placebo. 2) 200 IU vit. D&lt;sub&gt;3&lt;/sub&gt;/day. 3) 400 IU vit. D&lt;sub&gt;3&lt;/sub&gt;/day. ▪ serum 25(OH)D, OCN, and calcium were measured. ▪ whole-body and LV&lt;sub&gt;1-4&lt;/sub&gt; BMC, BMD, bone mass and bone turnover were measured. ▪ the duration length was 1 year.</td>
<td>▪ calcium intake among all subjects at baseline: mean = 1,029 mg/day. ▪ after 1 year the only significant differences were in serum 25(OH)D levels and LV&lt;sub&gt;1-4&lt;/sub&gt; bone area. ▪ there were significant increases in serum 25(OH)D levels in groups supplemented with vit. D&lt;sub&gt;3&lt;/sub&gt; compared to placebo. 1) 200 IU vit. D&lt;sub&gt;3&lt;/sub&gt;/day increased serum 25(OH)D levels from 42 to 53 nmol/L at 1 year. 2) 400 IU vit. D&lt;sub&gt;3&lt;/sub&gt;/day increased serum 25(OH)D levels from 44 to 58 nmol/L at 1 year. ▪ the increase in LV&lt;sub&gt;1-4&lt;/sub&gt; bone area from baseline to 1 year was significantly less in the 400 IU vit. D&lt;sub&gt;3&lt;/sub&gt;/day group. ▪ there was no effect on whole-body and LV&lt;sub&gt;1-4&lt;/sub&gt; BMC and BMD, OCN, or calcium levels.</td>
</tr>
<tr>
<td>Randomized double-blind placebo-controlled intervention study&lt;sup&gt;193&lt;/sup&gt;</td>
<td>▪ 193 Finnish girls (average age 11 years) were randomized to 1 of 3 groups: 1) daily placebo (N=64). 2) 200 IU vit. D&lt;sub&gt;3&lt;/sub&gt;/day (N=58). 3) 400 IU vit. D&lt;sub&gt;3&lt;/sub&gt;/day (N=71). ▪ subjects were instructed to take 1 pill/day for 28 days of each month of the study. ▪ serum 25(OH)D, calcium, and OCN levels were measured. ▪ urinary bone resorption markers, pyridinoline (U-Pyr) and U-Dpyr were measured. ▪ LV&lt;sub&gt;2-4&lt;/sub&gt; and femur bone area, BMC and BMD were measured. ▪ the duration length was 1 year.</td>
<td>▪ at baseline, the average serum 25(OH)D level was 47 nmol/L, the average vitamin D intake was 195 IU/day and the average calcium intake was 1,198 mg/day. ▪ after 1 year, serum 25(OH)D levels were significantly increased in the 200 IU and 400 IU vit. D&lt;sub&gt;3&lt;/sub&gt; groups compared to the placebo group; however, levels did not reach optimal status (serum 25(OH)D levels &gt; 75 nmol/L). ▪ after 1 year, serum 25(OH)D levels were significantly higher in the 400 IU vit. D&lt;sub&gt;3&lt;/sub&gt; group compared to the 200 IU vit. D&lt;sub&gt;3&lt;/sub&gt; group. ▪ serum calcium, OCN and U-Pyr levels did not differ among groups after 1 year. ▪ U-Dpyr was significantly reduced in the 200 IU vit. D&lt;sub&gt;3&lt;/sub&gt; group compared to placebo at 1 year, however U-Dpyr levels in the 400 IU vit. D&lt;sub&gt;3&lt;/sub&gt; group did not differ from placebo or 200 IU vit. D&lt;sub&gt;3&lt;/sub&gt; groups. ▪ LV&lt;sub&gt;2-4&lt;/sub&gt; and femur BMD increased with vit. D&lt;sub&gt;3&lt;/sub&gt; supplementation compared to the placebo group. ▪ there were no changes in LV&lt;sub&gt;2-4&lt;/sub&gt; or femur bone area and BMC after 1 year. ▪ With 80% compliance, BMC: ▪ in LV&lt;sub&gt;2-4&lt;/sub&gt; was 12.5% higher in the group receiving 400 IU vit. D&lt;sub&gt;3&lt;/sub&gt;/day compared to the placebo group. ▪ in the femur was 14.3% and 17.2% higher in the groups receiving 200 IU vit. D&lt;sub&gt;3&lt;/sub&gt;/day and 400 IU vit. D&lt;sub&gt;3&lt;/sub&gt;/day, respectively, compared to the placebo group.</td>
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</table>
| Randomized double-blind placebo-controlled intervention study | 168 Lebanese girls (10-17 years of age) were randomized to 1 of 3 groups to receive a weekly:
1) placebo (N=55).
2) low vit. D dose of 1,400 IU (equivalent to 200 IU/day) (N=58).
3) high vit. D dose of 14,000 IU (equivalent to 2,000 IU/day) (N=55).
- serum 25(OH)D and 1,25(OH)2D levels and subtotal body (total body minus the head), LV, hip and forearm BMC and BMD were measured at baseline and 1 year.
- serum calcium and APase were measured at baseline, 6 months and 1 year.
- the duration length was 1 year. | at baseline, the mean serum 25(OH)D level for all subjects was 35 nmol/L.
at baseline there were significant positive associations between serum 25(OH)D levels and LV BMD, femur neck BMD and radius BMC and BMD.
- suboptimal calcium intake of 677 mg/day was documented at the start of the study.
average serum 25(OH)D and 1,25(OH)2D levels did not differ between placebo and low vit. D groups.
- high vit. D supplementation increased average serum 25(OH)D levels to 95 nmol/L; significantly higher than the placebo and low vit. D groups.
- high vit. D supplementation significantly increased average serum 1,25(OH)2D levels higher than the placebo and low vit. D groups.
- vit. D supplementation significantly increased hip BMC compared to placebo at 1 year.
- there were negative correlations for the high vit. D group between baseline serum 25(OH)D levels and % change in LV, femur neck and radius BMC and % change in subtotal and LV BMD.
- there was no significant effect on serum calcium levels except in 2 subjects in the placebo group who had levels above the upper limit for children (10.7 mg/dl). |
receiving 200 IU vitamin D₃/day and 400 IU vitamin D₃/day, respectively, compared to the placebo group¹⁹³. In another study of adolescent girls higher vitamin D supplements were used. Lebanese girls (aged 10 to 17 years) received a weekly placebo or were supplemented with 1,400 IU vitamin D/week (low vitamin D group) or 14,000 IU vitamin D/week (high vitamin D group)³⁰⁰. At baseline, serum 25(OH)D levels were significantly positively associated with LV BMD, femur neck BMD and radius BMC and BMD³⁰⁰. Serum 25(OH)D and 1,25(OH)₂D levels did not differ between placebo and low vitamin D supplementation after 1 year³⁰⁰. High vitamin D supplementation significantly increased average serum 25(OH)D levels from 35 nmol/L at baseline to 95 nmol/L after 1 year which is significantly higher than the other 2 groups³⁰⁰. High vitamin D supplementation also significantly increased 1,25(OH)₂D levels after 1 year compared to placebo and low vitamin D groups³⁰⁰. Vitamin D supplementation significantly increased total hip BMC compared to placebo at 1 year³⁰⁰. Lower baseline serum 25(OH)D levels were associated with greater percent changes in LV, femur neck and radius BMC and LV and subtotal body BMD for high vitamin D supplementation³⁰⁰. Therefore this study indicates that vitamin D supplementation above the 1997 adequate intake (AI) and 2010 RDA values during adolescence is beneficial to bone development after 1 year. However, with the short study duration whether benefits continue later in life was not investigated. Therefore, this thesis research focused on whether vitamin D supplementation during the critical periods of intrauterine and early postnatal life would benefit bone growth and development later in life at young adulthood.

2.9.1.1.3 Adulthood

Studies have been conducted in healthy adults to investigate vitamin D supplementation on serum 25(OH)D and bone health status (Table 2.5). Results from the Women’s Health Initiative (WHI) which provided 400 IU vitamin D₃/day plus 1,000 mg of calcium/day to a multi-ethnic postmenopausal group of women (aged 50-70 years) found greater preservation of hip BMD at the femur neck across 5 years compared to the placebo group (1,000 mg of calcium/day)³⁰¹. Furthermore, in the vitamin D₃ plus calcium group there was a significant 21% decrease in fracture risk in women over the age of 60 years³⁰¹. After 6 years, femur neck cross-sectional area which is consistent with greater bone strength had significantly increased compared to that of the placebo group³⁰¹. In addition to daily vitamin D supplementation, the effect of quarterly supplementation with high doses (100,000 IU/capsule) of vitamin D₃ has also
### Table 2.5 Effect of vitamin D intervention on bone health in healthy adults

<table>
<thead>
<tr>
<th>Study Design</th>
<th>Sample size, Gender Age at Intervention, Dose and Intervention Length</th>
<th>Outcomes</th>
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</table>
| Randomized double-blind placebo-controlled clinical trial (WHI)\(^{301}\) | • a subcohort of 1,970 multi-ethnic postmenopausal women (50-79 years of age) from the WHI were randomized to 1 of 2 groups: 1) receive 400 IU vit. D\(_3\)/day + 1,000 mg of calcium (N=991). 2) receive daily placebo + 1,000 mg of calcium (N=979). | • at baseline, whole-body, hip and LV BMD was significantly higher in the vit. D\(_3\) + calcium group.  
• greater preservation of hip BMD at the femur neck with vit. D\(_3\) + calcium compared to the placebo group over 5 years, with a difference observed as early as 2 years.  
• femur neck cross-sectional area (which is consistent with greater strength) significantly increased with vit. D\(_3\) + calcium compared to placebo at the 6\(^{th}\) annual visit.  
• in women > 60 years of age, there was a significant 21% decrease in the risk of hip fracture in the vit. D\(_3\) + calcium group. |
| Randomized double-blind trial\(^{302}\) | • 2,686 British men and women (65-85 years of age) were randomized to 1 of 2 groups: 1) doses of 100,000 IU vit. D\(_3\) every 4 months (equivalent to approximately 800 IU/day) (Men: N=1,019; Women: N=326). 2) placebo (Men: N=1,018; Women: N=323).  
• after 4 years, serum 25(OH)D levels in a subcohort of 235 subjects were measured approximately 3 weeks after a dose.  
• the incidence of fracture was recorded.  
• the trial duration was 5 years. | • subjects in the vit. D\(_3\) group had a 22% lower rate of first fracture at any site, and a 33% lower rate of fracture occurring at the hip, wrist or forearm, or LV.  
• in the subcohort, serum 25(OH)D levels were 40% higher in the vit. D\(_3\) group (74 nmol/L) compared to the placebo group (53 nmol/L). |
| Randomized double-blind placebo-controlled trial\(^{303}\) | • 2,578 men and women (70-97 years of age) from Amsterdam were randomized to 1 of 2 groups: 1) 400 IU vit. D\(_3\)/day (Men: N=333; Women: N=958). 2) daily placebo (Men: N=329; Women: N=958).  
• subjects were advised to consume at least 3 servings of dairy products/day and to ensure a calcium intake of at least 800-1,000 mg/day.  
• annual evaluations recorded the incidence of hip or other peripheral fractures.  
• a subcohort of 270 subjects was used to measure serum 25(OH)D levels.  
• the trial duration was 3-4 years. | • in the subcohort of 270 subjects, baseline average serum 25(OH)D levels were similar between the 2 groups (27 nmol/L in the vit. D\(_3\) group and 26 nmol/L in the placebo group).  
• after 1 year, the average serum 25(OH)D levels significantly differed between the 2 groups with 62 nmol/L in the vit. D\(_3\) group and 23 nmol/L in the placebo group.  
• after 3 years, a smaller sample of subjects from the subcohort were analyzed and serum 25(OH)D levels in the vit. D\(_3\) group (54 nmol/L) remained significantly higher than those of the placebo group (23 nmol/L).  
• the number of fractures between the 2 groups did not differ during the total follow-up period. |
### Randomized double-blind placebo-controlled trial

208 healthy postmenopausal African American women (50-75 years of age) were randomized to 1 of 2 groups:

1) receive 800 IU/day of oral vit. D₃ supplementation (after 2 years, the vit. D₃ supplementation dose was increased to 2,000 IU/day) + calcium supplement (1,200-1,500 mg).
2) receive daily placebo + calcium supplement (1,200-1,500 mg).

- BMD and serum calcium, PTH, 25(OH)D, 1,25(OH)₂D and OCN levels were measured.
- the trial duration was 3 years.

- at baseline, BMD at the total hip ranged from normal (65% of women) to osteopenic (33.6%) to osteoporotic (1.4%) and the mean serum 25(OH)D level was 47 nmol/L (range 12.5 to 99.7 nmol/L).
- no difference in rate of bone loss between the 2 groups.
- serum 25(OH)D levels were not associated with BMD changes in either of the 2 groups.
- BMD significantly declined at all sites, except for the LV, in both groups.
- mean serum 25(OH)D levels increased from 46.9 to 70.8 nmol/L within 3 months of 800 IU vit. D₃/day and to 86.9 nmol/L within 3 months of 2,000 IU vit. D₃/day.
- there was no significant change in serum 25(OH)D levels in the placebo group.
- 40% receiving 2,000 IU vit. D₃/day had serum 25(OH)D levels <80 nmol/L after 3 years.
- serum PTH levels declined in both groups at 3 months, but were not sustained; serum 1,25(OH)₂D levels declined in the placebo group; OCN levels were similar between groups.

### Randomized double-blind trial

63 hospitalized men and women (≥ 65 years of age) from Auckland, New Zealand were randomized into 1 of 3 treatment groups, with equal numbers of men and women in each group:

1) loading group: received ten 50,000 IU vit. D₃ tablets at entry followed by monthly placebo tablets for 8 months.
2) loading + monthly group: received ten 50,000 IU vit. D₃ tablets at study entry followed by monthly 50,000 IU vit. D₃ tablets for 8 months.
3) monthly group: received one 50,000 IU vit. D₃ + 9 placebo tablets at study entry followed by 50,000 IU vit. D₃ tablets for 8 months.

- serum 25(OH)D, calcium, PTH and P1NP levels were measured.

- mean baseline serum 25(OH)D level was 58 nmol/L.
- serum 25(OH)D levels were inversely related to PTH levels, however PTH declines very little after 50 nmol/L.
- loading and loading + monthly groups: rapid increases in 25(OH)D levels (mean increase was 58 nmol/L) in the first month with gradual declines to plateaus of 69 and 91 nmol/L, respectively.
- monthly group: gradual increase in serum 25(OH)D levels to a plateau of 80 nmol/L at 5 months.
- in deficient subjects, the loading dose was effective in rapidly correcting low serum 25(OH)D levels (< 50 nmol/L); increasing mean 25(OH)D from 31 nmol/L at baseline to 101 nmol/L 1 month later.
- vit. D₃ supplementation of non-deficient subjects led to a mean increase in serum 25(OH)D levels of 50 nmol/L and a mean peak of 130 nmol/L at 1 month.
- vit. D₃ supplementation did not significantly elevate serum calcium levels above the upper end of the reference range; serum PTH was significantly reduced at 1 month in the loading groups and at 3 months in the monthly group.
- P1NP did not change in subjects whose serum 25(OH)D was ≥30 nmol/L, however declined in those who had levels <30 nmol/L.
| Randomized double-blind placebo-controlled trial\(^{106}\) | • Australian women aged 70-80 years were randomized to 1 of 2 intervention groups:  
1) single oral dose of 500,000 IU vit. D\(_3\)/year (N=1,131).  
2) placebo/year (N=1,125).  
• participants were followed for 1 year after the last dose.  
• women were included if they had an increased risk of hip fracture as defined by maternal hip fracture, past fracture, or self-reported faller.  
• 150 substudy participants had serum 25(OH)D levels measured at baseline, 1, 3, and 12 months after dose.  
• the trial duration was 3-5 years. | • significantly more women (74%) in the vit.D\(_3\) group had ≥ 1 fall compared to the placebo group (68%).  
• the increased number of falls in the vit. D\(_3\) group was evident in all 3 classifications of falls:  
  → fall with fracture.  
  → fall without fracture.  
  → fall with soft tissue injury.  
• the incidence relative risk for falling was 1.15 in the vit. D\(_3\) group compared to the placebo group.  
• temporal effect of vit. D\(_3\) supplementation: the incidence relative risk for falling decreased from 1.31 in the first 3 months to 1.13 during the remaining 9 months.  
• the incidence relative risk for fracture was 1.26 compared to the placebo group.  
• there was no temporal effect of vit. D\(_3\) supplementation for fracture.  
• calcium intake did not differ between groups.  
• at baseline, the median serum 25(OH)D level was 53 nmol/L for the vit. D\(_3\) group and 45 nmol/L for the placebo group.  
• in the vit. D\(_3\) group, 1 month after the dose the median serum 25(OH)D level was > 120 nmol/L (82% were above 100 nmol/L and 24% were above 150 nmol/L) and 3 months after the dose the median was 90 nmol/L.  
• there was a marked increase in serum 25(OH)D levels for the vit. D\(_3\) group at 12 months (range 55-74 nmol/L). |
been investigated with respect to fracture risk. In a subcohort, serum 25(OH)D levels were significantly higher (40%) in the vitamin D₃ group compared to the placebo group. This study also found a protective effect of vitamin D₃ supplementation on fracture risk. There was a 22% risk reduction of first fracture at any site, and a 33% risk reduction of fracture at major osteoporotic sites (ie: hip, wrist or forearm, and LV) in the vitamin D₃ supplemented group compared to the placebo group. However other intervention studies have reported conflicting results. A lack of effect was observed by Lips et al. who evaluated the incidence of hip and other peripheral (ie: humerus, ankle, and leg) fractures in men and women randomized to receive either a 400 IU daily supplement of vitamin D₃ or placebo. At baseline, serum 25(OH)D levels did not differ between the 2 groups. After 1 year of intervention, the subcohort average serum 25(OH)D levels in the vitamin D₃ group (62 nmol/L) were significantly higher than in the placebo group (23 nmol/L). During the total follow-up period of the study, the number of fractures did not significantly differ between the 2 groups. In addition, a study that provided a vitamin D₃ supplement to postmenopausal African American women, aged 50-75 years, for 3 years found there were no differences in the rate of bone loss or reduction in BMD between the supplemented and placebo groups. Women were supplemented with 800 IU vitamin D₃/day for the first 2 years, however this was increased to 2,000 IU vitamin D₃/day for the third year over concern of not reaching optimal vitamin D status with the lower supplementation. Both the vitamin D₃ supplemented and placebo groups received 1,200-1,500 mg/day of calcium. Results demonstrated an increase in serum 25(OH)D levels from 46.9 nmol/L at baseline to 70.8 nmol/L after 3 months of 800 IU/day vitamin D₃ supplementation and to 86.9 nmol/L following 3 months of 2,000 IU vitamin D₃/day. However, even with vitamin D₃ supplementation, 40% of women still had serum 25(OH)D levels < 80 nmol/L at the end of 3 years. Moreover, there was no relationship between serum 25(OH)D levels and BMD as well as no difference in OCN levels between the groups. Another study investigated the effect of high dose vitamin D₃ supplementation (500,000 IU loading dose, 500,000 IU loading dose plus 8 monthly 50,000 IU doses, and 8 monthly 50,000 IU doses) administered in both vitamin D deficient (serum 25(OH)D levels < 50 nmol/L) and sufficient elderly subjects (≥65 years of age) on serum 25(OH)D levels and bone biomarker levels. As expected, this resulted in increased serum 25(OH)D concentrations. Procollagen type 1 amino-terminal propeptide (P1NP, a bone formation marker) levels remained unchanged in individuals with serum 25(OH)D ≥ 30 nmol/L; however, decreased in those with levels < 30 nmol/L. Serum PTH levels were reduced in all
3 groups. Importantly, in deficient subjects the loading dose rapidly corrected serum 25(OH)D levels from a mean of 31 nmol/L at baseline to 101 nmol/L 1 month later. In addition, the loading dose in sufficient subjects resulted in a slower increase in serum 25(OH)D levels, with peak values reaching approximately 130 nmol/L and no indications of hypercalcemia. Therefore, conflicting results as to the effect of vitamin D3 supplementation on structural and functional bone outcomes may indicate that nutritional intervention should occur earlier during rapid periods of development to exert a long-term beneficial response.

Sanders et al. found conflicting results with Australian women aged 70-80 years who were randomized to receive either a single annual oral dose of 500,000 IU vitamin D3 or placebo for 3 to 5 years (Table 2.5). Significantly more women (74%) experienced at least 1 fall compared to those women receiving a placebo (68%). Women in the vitamin D3 group experienced 15% more falls and 26% more fractures compared to the placebo group. In addition, there was a significant temporal effect of vitamin D3 supplementation for falls but not for fractures. In the vitamin D3 group, there was a 31% increased risk of falls in the first 3 months following vitamin D3 supplementation which decreased to a 13% increased risk during the remaining 9 months of the year. In the vitamin D3 group, the median serum 25(OH)D level increased from 53 nmol/L to > 120 nmol/L 1 month after supplementation, with 82% above 100 nmol/L and 24% above 150 nmol/L. At 3 months after supplementation the median serum 25(OH)D level had decreased to 90 nmol/L. At 12 months, serum 25(OH)D levels in the vitamin D3 group were on average 41% higher than that of the placebo group. Findings of this study indicate the importance of timing of vitamin D dosing, emphasizing that too much of a nutrient at one time may have detrimental effects.

### 2.9.1.2 Animal studies

Vitamin D supplementation of healthy rats and mice has shown to increase serum 25(OH)D levels and decrease serum 1,25(OH)2D and PTH levels (Table 2.6). In rats, by design, a higher vitamin D intake led to significantly higher serum 25(OH)D levels and lower serum 1,25(OH)2D levels. Increasing vitamin D supplementation (via dermal and oral methods) from 0.2 to 20 nmol/day led to an appropriate increase in serum 25(OH)D concentrations and decrease in serum 1,25(OH)2D concentrations. Also, dermal vitamin D supplementation (in
Table 2.6 Effect of vitamin D intervention on serum 25(OH)D levels in healthy animal models

<table>
<thead>
<tr>
<th>N and Animal Model</th>
<th>Gender, Age at Intervention, Dose and Intervention Length</th>
<th>Outcomes</th>
</tr>
</thead>
</table>
| Experiment 1 – 14 Wistar rats<sup>307</sup> | ▪ Exp. 1 – ♀ rats used, age not given. 50% consumed 7 nmol/day of vit. D from the diet and 50% consumed 7 nmol/day of vit. D from the diet plus received 18 nmol/day dermal vit.D supplementation in ethanol.  
▪ intervention length was 3 weeks. | ▪ Exp. 1 – rats given additional vit. D had higher serum 25(OH)D, lower serum 1,25(OH)2D, lower PTH, and higher kidney tissue VDR mRNA. |
| Experiment 2 – Wistar rats<sup>307</sup> | ▪ Exp. 2 – ♀ rats used, age not given.  
▪ 4 treatment groups: all on vit. D deficient diets supplemented with 0.5% elemental calcium.  
1) supplemented weekly with 1.5 nmol vit. D (0.2 nmol/day).  
2) supplemented with vit. D every second day to provide 2 nmol/day.  
3) supplemented with vit. D every second day to provide 20 nmol/day.  
Groups 1-3 received vit. D directly into the gut through a feeding tube.  
4) supplemented, via vit. D in ethanol applied to the skin, every second day to provide 20 nmol/day.  
▪ the preparation period lasted 3 weeks.  
▪ dietary calcium content was changed weekly between 0.01 and 1.5 g/100 g diet for another 3 weeks.  
▪ total intervention length was 6 weeks. | ▪ Exp. 2 – increasing vit. D supplementation led to increased levels of 25(OH)D and suppressed concentrations of 1,25(OH)2D.  
▪ dermal vit. D administration increases serum 25(OH)D levels to 85% of that obtained by oral dosing.  
▪ varying dietary calcium content had no effect on 1,25(OH)2D levels. |
| Experiment 1A – 51 Sprague Dawley rats<sup>136</sup> | ▪ Exp. 1A – 27 ♀ and 24 ♂ weanling rats fed AIN93G diet containing 400 IU vit. D<sub>3</sub>/kg diet until 10 weeks of age.  
▪ rats then randomized to modified AIN93M diets containing 400, 1,000, 5,000, 10,000 or 20,000 IU vit. D<sub>3</sub>/kg diet for 4 weeks. | ▪ Exp. 1 – feeding both rats and mice increasing amounts of vit. D<sub>3</sub> caused a linear increase in serum 25(OH)D<sub>3</sub> levels (92 nmol/L in the 400 IU vit. D<sub>3</sub>/kg diet and >800 nmol/L in the 20,000 IU vit. D<sub>3</sub>/kg diet).  
▪ serum 1,25(OH)2D<sub>3</sub> levels were suppressed with increasing vit. D<sub>3</sub> intake in both rats and mice.  
▪ dietary vit. D<sub>3</sub> had no effect on bone ash, bone calcium, or BMD. |
| Experiment 1B – 30 C57BL/6 mice<sup>136</sup> | ▪ Exp. 1B – ♀ mice fed AIN93G diets containing 400, 1,000, 5,000, 10,000 or 20,000 IU vit. D<sub>3</sub>/kg diet from weaning until 10 weeks of age. | ▪ Exp. 2 – feeding both rats and mice decreasing amounts of vit. D<sub>3</sub> caused serum 25(OH)D<sub>3</sub> concentrations to fall in a curvilinear relationship (with an inflection point at approx. 200 IU vit. D<sub>3</sub>/kg).  
▪ dietary vit. D<sub>3</sub> levels below 100 IU vit. D<sub>3</sub>/kg reduced serum 1,25(OH)2D<sub>3</sub> levels.  
▪ in mice, the lowest level of vit. D<sub>3</sub> intake (25 IU vit. D<sub>3</sub>/kg) led to a significant reduction in BMC and BMD. |
| Experiment 2A – 30 Sprague Dawley rats<sup>136</sup> | ▪ Exp. 2A – ♂ weanling rats fed AIN93G diets containing 50, 100, 200, 400 or 1,000 IU vit. D<sub>3</sub>/kg diet from weaning until 12 weeks of age. | |
| Experiment 2B – 36 C57BL/6 mice<sup>136</sup> | ▪ Exp. 2B – ♀ mice fed AIN93G diets containing 25, 50, 100, 200, 400, or 1,000 IU vit. D<sub>3</sub>/kg diet from weaning until 14 weeks of age. | |
| 40 female guinea pigs | at pregnancy, sows were randomized to 1 of 2 intervention diets throughout pregnancy and lactation:  
1) vit. D deficient diet (devoid of vitamin D).  
2) vit. D sufficient diet (1,200 IU vit. D₃/kg diet).  
  at birth, within litters, 2 pups were randomized to receive a daily:  
1) placebo supplement until 28 days of age.  
2) oral vit. D supplement (10 IU vit. D₃) until 28 days of age.  
sows were housed with their pups until 28 days of age.  
sow serum 25(OH)D levels were measured at conception, day 42 of pregnancy, and immediately postpartum.  
pup serum 25(OH)D, OCN, and DPD levels were measured within 48 hours after birth and at 28 days of age.  
in pups, bone area, BMC and BMD were measured at for the whole-body, LV, tibia and femur.  
in pups, tibia and femur bone size and strength were assessed.  
endogenous vit. D synthesis was prevented by placing UV filters on the lights. | serum 25(OH)D levels did not differ between groups prior to mating.  
at 42 days of pregnancy, sows consuming the vit. D deficient diet had significantly reduced serum 25(OH)D levels which remained decreased throughout pregnancy and lactation.  
at birth, serum 25(OH)D levels of pups from sows in group 1 were significantly lower than those from sows in group 2.  
pups of sows in group 2 had a significant increase in serum 25(OH)D levels from birth to 28 days of age.  
regardless of postnatal supplement group, pups of sows in group 1 had significantly lower serum 25(OH)D levels at 28 days of age compared to those pups (regardless of supplement group) from sows in group 2.  
pups of vit. D sufficient sows were significantly longer in length than those of vit. D deficient sows regardless of postnatal supplement.  
whole-body, LV, tibia and femur areas were significantly reduced in pups of vit. D deficient sows compared to pups of vit. D sufficient sows at birth.  
significant interaction between maternal diet and postnatal supplementation; pups of vit. D deficient sows who received a postnatal vit. D supplement did not differ from pups of vit. D sufficient sows at 28 days.  
pups of vit. D deficient sows had 7.1% lower whole-body and 7.2% lower tibia BMC compared to pups of vit. D sufficient sows at birth and 28 days of age.  
there was no effect on BMD at any skeletal site.  
at birth, serum OCN levels were significantly higher in pups of vit. D sufficient sows however at 28 days there was no difference among groups.  
there was no effect of maternal diet or postnatal supplementation on DPD levels. DPD levels decreased from birth except in placebo supplemented pups of vit. D deficient sows.  
no difference in tibia or femur size at birth or 28 days.  
tibia yield load was significantly decreased in pups of vit. D deficient sows at birth and 28 days; no difference in femur yield load at birth or 28 days. |
ethanol) proved to be an effective way to provide vitamin D as this led to an increase in serum 25(OH)D levels that was 85% of that for oral administration of the same dose. Another study investigated increasing vitamin D₃ diet supplementation of 10 week old rats for 4 weeks and weanling mice for 10 weeks and found similar results of a linear increase in serum 25(OH)D₃ concentrations. Increased dietary levels of vitamin D₃ ranging from 400 to 20,000 IU/kg diet led to serum 25(OH)D₃ levels from 92 nmol/L to greater than 800 nmol/L in rats and from approximately 95 nmol/L to greater than 500 nmol/L in mice, respectively. Results also showed a suppression of serum 1,25(OH)₂D₃ levels of approximately 80% in rats and 50% in mice. However, there was no effect of vitamin D₃ supplementation in mice on femur BMD. The response of serum 25(OH)D₃ concentrations to decreasing vitamin D₃ levels of 1,000 to 50 IU/kg diet in rats and 25 IU/kg diet in mice was also measured, and results demonstrated a curvilinear decrease. In mice, vitamin D₃ restriction below 100 IU/kg diet led to a significant reduction in serum 1,25(OH)₂D₃ whereas the 25 IU vitamin D₃/kg diet resulted in serum 25(OH)D₃ concentrations of approximately 35 nmol/L, which led to significantly reduced femur BMC and BMD. These studies indicate that serum 25(OH)D levels are influenced by dietary vitamin D supplementation. More importantly, findings by Fleet et al. show that vitamin D₃ supplementation up to 20,000 IU/kg diet of weanling mice did not enhance bone development as measured by BMD. However, this may be due to the age of mice at the time of vitamin D₃ supplementation. Because supplementation of mice began post-weaning, the proposed critical period wherein nutritional factors may influence structural and functional development would have been missed. More specifically, a lack of long-term effect on bone development would be understandable as the suggested periods of intrauterine and early postnatal life which are thought to be sensitive to nutritional intervention (ie: vitamin D supplementation) were not investigated. A novel feature of this thesis research was the study of the effect of maternal vitamin D supplementation during intrauterine and early postnatal life on bone outcomes later in life at young adulthood. Another unique aspect of this thesis was the functional measurement of bone strength at selected skeletal sites following vitamin D supplementation to mimic common fractures in humans.

The effect of postnatal vitamin D supplementation following maternal vitamin D deficiency has been investigated with respect to fetal bone mineralization at birth in guinea pigs (Table 2.6). Pregnant guinea pigs were randomized to a deficient (devoid of vitamin D) or
sufficient (1,200 IU vitamin D₃/kg diet) vitamin D diet throughout pregnancy and lactation. At birth, 2 pups were randomized to receive a daily placebo or vitamin D₃ supplement (10 IU). By design, sows consuming a vitamin D deficient diet had significantly reduced serum 25(OH)D levels compared to those on the sufficient diet at 42 days of gestation. At birth, pups from sows on a vitamin D deficient diet had significantly reduced serum 25(OH)D levels compared to those pups from vitamin D sufficient sows. In addition, supplementation did not affect pup serum 25(OH)D levels at 28 days of age as pups from sows on a vitamin D deficient had significantly lower levels. Whole-body, LV, tibia and femur areas were significantly reduced in pups of vitamin D deficient sows at birth. However, at 28 days of age pups of vitamin D deficient sows who received a postnatal vitamin D supplement no longer differed from pups of vitamin D sufficient sows in whole-body and femur areas. Maternal diet also had a significant effect on whole-body and tibia BMC. Pups of vitamin D deficient sows had 7.1% lower whole-body and 7.2% lower tibia BMC than pups of vitamin D sufficient sows at birth and 28 days of age. At birth, serum OCN levels were significantly higher in pups of vitamin D sufficient sows compared to vitamin D deficient sows. There was no effect of maternal diet or postnatal supplementation on deoxypyridinoline (DPD) concentration which measures osteoclast activity. However, DPD concentrations significantly decreased from birth to 28 days of age except in placebo supplemented pups from vitamin D deficient sows. There was no effect of maternal diet or postnatal supplementation on tibia or femur size. Tibia yield load was significantly decreased at birth and 28 days for pups of vitamin D deficient sows; however femur yield load was unaffected. These findings show that vitamin D deficiency during the critical period of intrauterine growth can detrimentally affect bone area and mineralization during early postnatal life. Furthermore, the importance of early vitamin D supplementation during early postnatal life is emphasized as it can rescue whole-body and femur area from maternal vitamin D deficiency. This thesis research focused on the effect of higher vitamin D supplementation during intrauterine and early postnatal life on bone outcomes later in life at young adulthood as well as the effect of vitamin D supplementation during pregnancy and lactation on maternal bone outcomes.
2.9.2 Vitamin D Supplementation in Inflammatory Bowel Disease

2.9.2.1 Human studies

The impact of vitamin D intervention on children affected with IBD has recently been investigated (Table 2.7). The effect of vitamin D supplementation on bone health in children (14 years of age) with IBD was studied by measuring BMD at LV2-4\textsuperscript{308}. Children with baseline LV2-4 BMD z scores greater than -1 were assigned to the control group (1,300 mg/day of calcium and 200 IU/day vitamin D), while those with scores less than -1 were assigned to 1 of 2 intervention groups (daily supplements of 1,000 mg of calcium for 12 months versus daily supplements of 1,000 mg of calcium for 12 months plus monthly supplements of 50,000 IU vitamin D\textsubscript{2} for 6 months)\textsuperscript{308}. There was no change in serum 25(OH)D levels for either intervention group from baseline to 12 months; however, there was a trend for increased serum 25(OH)D levels at 12 months in those who received monthly supplementation of 50,000 IU vitamin D\textsubscript{2} for the first 6 months\textsuperscript{308}. This study also showed that changes in BMD of LV2-4 were similar between the control and intervention groups as well as the calcium only and the calcium plus vitamin D\textsubscript{2} intervention groups. These findings indicate that calcium alone or in combination with vitamin D could not improve reduced BMD in those affected with IBD after 12 months of supplementation. However, this may further indicate the need for nutritional intervention beginning in utero and during early life development to have positive long-term effects on bone.

2.9.2.2 Animal studies

Research has examined the effect of vitamin D deficiency in a mouse model of IBD (Table 2.8). The effect of vitamin D deficiency (diet devoid of vitamin D), beginning in utero, on growth and development of the IL-10 KO mouse model was investigated. Vitamin D deficient IL-10 KO mice were severely growth retarded at 7 weeks of age compared to the vitamin D sufficient (200 IU vitamin D\textsubscript{3}/day) IL-10 KO mice and the vitamin D deficient wildtype (WT) mice\textsuperscript{145}. Also, vitamin D deficient IL-10 KO mice began to die as early as 7 weeks of age, as well as had a higher degree of wasting and mortality at 9 weeks of age compared to the vitamin D sufficient IL-10 KO and vitamin D deficient WT mice\textsuperscript{145}. Vitamin D deficiency exacerbated the development of IBD leading to diarrhoea and wasting at approximately 6 weeks of age in the vitamin D deficient IL-10 KO mice, whereas the vitamin D
### Table 2.7 Effect of vitamin D intervention on bone health in children with IBD

<table>
<thead>
<tr>
<th>Study Design</th>
<th>Sample size, Gender</th>
<th>Outcomes</th>
</tr>
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</table>
| Single-blind, prospective cohort study<sup>308</sup> | ▪ assigned to 1 of 2 groups based on baseline LV<sub>2-4</sub> BMD z score.  
▪ control group (N=33, 20 boys and 13 girls, mean age: 14 years): LV<sub>2-4</sub> BMD z score greater than -1. Follow standard dietary calcium (1,300 mg/day) and vit. D (200 IU/day) intake recommendations.  
▪ intervention group (N=39, 25 boys and 14 girls, mean age: 15 years): LV<sub>2-4</sub> BMD z score less than -1.  
▪ patients in the intervention group were randomized to 1 of 2 groups:  
1) receive a daily supplement of 1,000 mg of calcium for 12 months (N=19);  
2) receive a daily supplement of 1,000 mg of calcium for 12 months + monthly dose of 50,000 IU vit. D<sub>2</sub> (equivalent of 1,700 IU vit. D<sub>2</sub>/day) for the first 6 months of the study (N=20). | ▪ serum 25(OH)D levels at 12 months did not change for participants receiving calcium.  
▪ participants receiving calcium + vit. D<sub>2</sub> supplementation had a trend toward an increase in serum 25(OH)D concentrations at 12 months from baseline levels.  
▪ changes in z scores for height and weight were similar between the control and intervention groups after 12 months of supplementation.  
▪ changes in LV<sub>2-4</sub> BMD were similar between the control and intervention groups as well as between the calcium only and calcium + vit. D<sub>2</sub> intervention groups. |
Table 2.8 Effect of vitamin D intervention on overall health in an animal model of IBD

<table>
<thead>
<tr>
<th>Animal Model</th>
<th>Sample size, Gender</th>
<th>Age at Intervention, Dose and Intervention Length</th>
<th>Outcomes</th>
</tr>
</thead>
</table>
| C57BL/6 IL-10 KO mice and WT mice | IL-10 KO pregnant dams (n not given) were fed a vit. D deficient diet (devoid of vit. D), at 2 weeks of gestation, to ensure weanlings would be vit. D deficient.  
3-week-old IL-10 KO (gender not given) vit. D deficient pups were randomized to 1 of 2 groups:  
1) vit. D deficient diet (contained no vit. D)  
2) vit. D sufficient diet (200 IU vit. D/day).  
age- and sex-matched WT mice were used as controls.  
diets were high in calcium (1 g/100 g diet).  
mice were housed in yellow lighting to prevent endogenous synthesis of vit. D. | by 7 weeks of age, vit. D deficient IL-10 KO mice were significantly growth retarded compared with the vit. D sufficient IL-10 KO and vit. D deficient WT mice.  
vit. D deficient IL-10 KO began to die at 7 weeks of age, and by 9 weeks of age 58% were dead.  
vit. D sufficient IL-10 KO and vit. D deficient WT mice appeared healthy at 13 weeks of age.  
at 9 weeks of age, vit. D deficient IL-10 KO mice began to eat less and rapidly lost additional weight over the following 3 weeks.  
vit. D deficient IL-10 KO mice died after developing a wasting disease, which was preceded by diarrhoea.  
vit. D sufficient IL-10 KO mice did not develop diarrhoea. |
sufficient IL-10 KO mice and vitamin D deficient WT mice appeared healthy throughout the study\textsuperscript{145}. Although the bone abnormalities associated with IBD were not investigated, this demonstrates the importance of optimal vitamin D status on the development of this disease. Therefore, it is hypothesized that along with the exacerbated symptoms of diarrhoea and wasting, bone development may also be severely affected in vitamin D deficient IL-10 KO mice, with vitamin D supplementation possibly resolving the bone abnormalities.

The importance of investigating the effects of vitamin D supplementation are demonstrated in the detrimental health effects that occur as a result of deficiency. Poor vitamin D status can affect bone health in both healthy and IBD affected individuals. However, in addition to the deterioration of one’s health, there are also financial and psychosocial burdens that accompany these chronic diseases.

2.10 Costs of Chronic Diseases

2.10.1 Osteoporosis

Osteoporosis is a chronic disease that often results in 1 or more fragility fracture during one’s life. As a result, a majority of the cost associated with osteoporosis is in relation to these fragility fractures and the subsequent hospital visits following their occurrence. Medical costs can vary depending on fracture site. In Canada in 1996, annual osteoporotic hip fracture medical costs ranged from $21,385-44,156\textsuperscript{309}. Based on these values, the estimated annual burden of care associated with hip fracture in 1996 was $650 million\textsuperscript{309}. Then in 2006, the average annual cost of hospitalization following hip fracture was $21,550, whereas this cost was reduced to $12,220 for other skeletal sites (ie: mid-forearm or LV)\textsuperscript{310}. Hospital costs include physician care, hospital care and drug costs\textsuperscript{310}. In the United States in 2002, the average annual cost for medical expenses in those diagnosed with osteoporosis was $9,850\textsuperscript{311}. The national cost of osteoporosis in the elderly United States population was estimated at $2 billion\textsuperscript{311}. Also, from 2002 to 2006 the estimated annual cost following hip fracture ranged from $18,613-39,620 whereas the cost of fracture at other skeletal sites ranged from $15,890-24,660\textsuperscript{311-313}. These estimated costs include hospitalization, fracture care (inpatient and outpatient), medications, and long term healthcare\textsuperscript{311-312}. Based on these results, it was estimated that the national cost of fracture in the elderly United States population was $14 billion in 2002\textsuperscript{311}. The national cost
estimate for osteoporosis and related fractures was projected to be $22 billion in 2008, when considering the elderly population and medical inflation\textsuperscript{311}.

In addition to personal medical costs, fragility fractures also lead to a loss of productivity at work. Those who experience osteoporotic fractures are 3 times more likely to receive disability benefits, and their disability periods tend to last twice as long\textsuperscript{313}. Fracture patients also had significantly more days (up to 2 weeks) of medically related absenteeism, resulting in annual work loss costs of almost $5,000\textsuperscript{313}.

Although osteoporosis and related fractures lead to financial burden, one’s quality of life can also greatly suffer following fragility fracture. The most detrimental outcome associated with hip fracture is the increased risk of mortality. At 6 months following fracture, mortality was 14\% in women and 31\% in men, and at 12 months this increased to 22\% in women and 48\% in men\textsuperscript{314}. Furthermore, hip fractures often led to patients becoming bedridden and no longer being able to walk on their own\textsuperscript{314}. When assessing quality of life following an osteoporotic hip fracture, 75\% of women reported fair or poor general health status, whereas 42\% stated their health was worse or much worse compared to the year before\textsuperscript{314}. Spinal, hip and rib fractures were significantly correlated with an increase in pain\textsuperscript{315-316} and anxiety/depression\textsuperscript{316} as well as reductions in general health\textsuperscript{316}, mobility\textsuperscript{315-316}, self-care\textsuperscript{315-316}, happiness\textsuperscript{315}, vitality\textsuperscript{316} and an interest in life\textsuperscript{315} for up to 4 or 5 years following fracture.

\subsection*{2.10.2 Inflammatory Bowel Disease}

The severity of inflammation and symptoms that occur in IBD can be financially overwhelming to both the patient and society. IBD is a chronic autoimmune disease and therefore ongoing medical care is required to manage the disease and related symptoms. Physician visits, medications, laboratory tests, radiological tests, and surgeries are common for those affected with IBD\textsuperscript{317}. Approximately all those affected with IBD are on medication as well as annually undergo medical assessments such as chest and/or abdominal radiographs and serum chemistry and/or hematology tests\textsuperscript{317-318}, with an estimated 28-57\% requiring surgery\textsuperscript{206,317}. With annual medical tests and pharmaceutical costs accumulating to between $3,500-6,500\textsuperscript{317,319-320}, and hospitalization fees of approximately $12,500 per visit, the costs associated with IBD range between $1.4 and $1.6 billion\textsuperscript{318}. Costs have only further increased, with annual disease-attributable direct costs amounting to $6.3 billion in the United States in 2006\textsuperscript{320}. 
In addition to medical costs, there are also issues with respect to school or work performance and employment status. Severity of symptoms, age of onset and frequent medical assessments and/or hospital stays, which tend to occur during prime working years, led to 26-47%\textsuperscript{206,321} of individuals with IBD reporting a decrease in school or work productivity and/or employment\textsuperscript{206,321-323}, with as many as 80% who were hospitalized reporting less likely to be employed than those who were not\textsuperscript{321}. Children affected with IBD have also found the disease to affect their ability to sit through academic exams (17%) as well as attain the level of education they set out to achieve (14%)\textsuperscript{323}. Moreover, as many as 24% of those with IBD reported their disease had prevented them from seeking, or actually receiving, a promotion\textsuperscript{323}. Specifically, unemployed individuals affected by IBD also have an impact on society. In 1998, it was estimated that, in the United States, unemployment compensation as a result of IBD was approximately $1.04 billion, excluding other costs such as medical fees\textsuperscript{321}. Therefore, it is important to investigate strategies to reduce the occurrence of IBD in order to improve the health status of those afflicted with the disease, as well as decrease the financial burden it places on patients with IBD and society.

The financial deficit is not the only struggle those affected with IBD must face, there are also psychological and social costs that accompany this chronic disease. As many as 70% of IBD patients may be suffering from anxiety or depression\textsuperscript{324-327}. In addition, disease activity is positively associated with perceived stress and distress\textsuperscript{328}. A diminished quality of life has been related to a feeling of poor physical functionality, due to the illness and/or emotional problems associated with the illness, as well as poor social interaction\textsuperscript{324,326,328}. As reported by parents, children with IBD experience more social and competency problems compared to healthy controls\textsuperscript{325}.

Therefore, in addition to the detrimental physical health effects of chronic diseases such as osteoporosis and autoimmune diseases such as IBD, these diseases lead to great financial and psychosocial burdens as well. For these reasons it is imperative to investigate strategies to prevent the development of disease, thereby optimizing overall health and well-being. An important research tool to study specific interventions is the animal model. By using healthy and diseased mouse models that mimic the human situation, researchers can investigate possible nutritional strategies to improve health in the human population.
2.11 Animal Models for Nutritional Interventions

2.11.1 Healthy CD-1 Mouse Model

The CD-1 mouse is an important model for studying nutritional programming and its’ effect on bone as normal bone development during early life has been characterized in this mouse strain\textsuperscript{329}. It is critical to describe healthy bone development to quantify the changes of functional bone outcomes that occur with nutritional intervention. Ward et al\textsuperscript{329} characterized how bone mass (BMC and BMD) and biomechanical strength properties of healthy male and female CD-1 mice change at 1 month intervals during the first 4 months of life. Findings illustrated that 1 month old mice had the lowest whole femur BMC and BMD compared to all groups, and 3 and 4 month old mice had higher BMC and BMD compared to 2 month old mice\textsuperscript{329}. Also, males had higher femur BMC and BMD than females\textsuperscript{329}. By using strength properties as a surrogate measure of fracture risk, results found that 1 month old mice had the lowest yield load (a measure of the elastic limit of a bone; the contribution of mineral to bone strength), stiffness (a measure of the extrinsic rigidity of the bone tissue), and peak load (a measure of the maximum force the bone withstood before fracture; the contribution of matrix to bone strength) at the femur midpoint compared to all groups\textsuperscript{329}. At the femur neck, yield load was also lowest among 1 month old mice and 3 and 4 month old mice had higher peak load compared to 1 month old mice, whereas stiffness did not differ among age\textsuperscript{329}. Results for LV\textsubscript{1-4} showed that 1 month old mice had the lowest BMC and BMD compared to all groups, and 2 month old mice had lower BMC and BMD than 4 month old mice and lower BMD than 3 month old mice\textsuperscript{329}. BMC and BMD for LV\textsubscript{1-4} did not differ between genders\textsuperscript{329}. For biomechanical properties, 1 month old mice had the lowest peak load of LV\textsubscript{4} compared to all groups and 2 month old mice had lower peak load than 3 and 4 month old mice\textsuperscript{329}. Also, analyses of the relationship of whole femur BMC and femur biomechanical strength properties demonstrated that yield load, stiffness, and peak load at the femur midpoint and neck were each positively and significantly correlated with whole femur BMC\textsuperscript{329}. Peak load of LV\textsubscript{4} was also positively and significantly correlated with BMC of LV\textsubscript{1-4}\textsuperscript{329}. Therefore, findings indicated that the accumulation of bone mineral at the femur and LV occurs primarily during the first 3 months of life as there were no changes between 3 and 4 months. At 2 months of age the peak load at the femur midpoint, which is rich in cortical bone that is less metabolically active, was high and as a result did not significantly differ from 3 to 4 months of age\textsuperscript{329}. However, the
femur neck, which is rich trabecular bone that is more metabolically active, had a higher peak load later at 3 months of age. Peak load at LV4 was highest at 3 months of age with no change between 3 and 4 months. Therefore, CD-1 mice reach PBM between 3 and 4 months of age, and therefore this age represents young adulthood.

Another important aspect of the CD-1 mouse model is that it has been shown to respond to nutritional interventions. Kaludjerovic and Ward studied the effect of administering soy isoflavones, daidzein (DAI) and genistein (GEN), during the first 5 days of life, on bone mass and biomechanical strength at 4 months of age. Female mice treated with DAI, GEN, DAI+GEN or diethylstilbesterol (DES) had higher BMC and BMD of LV1-3 compared to the control group. Also, female mice treated with DAI or DES had a higher peak load at LV2 compared to the other treatment groups. DAI treatment led to a higher femur BMC compared to control and DES treatment as well as a higher femur BMD compared to all other groups. Females treated with DAI or DES had a greater femur midpoint peak load than those in the control group. Isoflavone and DES treatment resulted in a greater ultimate stiffness at the femur midpoint compared to the control treatment. Isoflavone treatment resulted in a greater yield load at the femur neck compared to the DES treatment and in the DAI treatment there was a higher femur neck peak load compared to the other groups. Therefore, this study found that early exposure of CD-1 mice to soy isoflavones resulted in improved bone development in female mice, which emphasizes the importance of nutritional programming, and the developmental effect it may have later in life.

### 2.11.2 Mouse Models of Inflammatory Bowel Disease

Animal models of IBD are essential in understanding the pathogenesis of the intestinal inflammation and resulting bone abnormalities that accompany this disease. Different IBD mouse models are created via chemical induction and genetic manipulation.

#### 2.11.2.1 Chemical Induction of Inflammatory Bowel Disease Mouse Model

The chemical induction of intestinal inflammation using dextran sodium sulphate (DSS) is a widely used IBD model. DSS is added to the drinking water, and upon ingestion the animal develops similar disease characteristics to those found in human IBD due to the toxic effect of interference with the barrier function of the intestinal epithelium. Disease
characteristics include reduced body weight\textsuperscript{276, 331-333}, diarrhoea\textsuperscript{322-333}, rectal bleeding\textsuperscript{322-333}, extensive intestinal inflammation\textsuperscript{277, 331-333}, intestinal infiltration of immune cells (ie: macrophages and lymphocytes)\textsuperscript{331-332}, and increased levels of proinflammatory cytokines IFN-\(\gamma\), TNF-\(\alpha\), IL-1\(\beta\), IL-6, and IL-12\textsuperscript{331, 333}. Similar to disease presentation in humans, research has also found the DSS experimental IBD model to develop bone abnormalities associated with the intestinal inflammation\textsuperscript{276}. With increased intestinal inflammation there was suppressed bone formation\textsuperscript{276-277}, increased bone resorption\textsuperscript{276}, low bone mass\textsuperscript{276}, and reduced BMC and BMD\textsuperscript{277}.

\textbf{2.11.2.2 Genetic Manipulation of Inflammatory Bowel Disease Mouse Model}

A common genetically manipulated IBD model is the IL-10 KO mouse. IL-10 is an anti-inflammatory cytokine that plays an essential role in regulating the intestinal Th1 immune response, while developing the Th2 response\textsuperscript{334}. IL-10 is important as it suppresses the synthesis of proinflammatory cytokines (ie: IFN-\(\gamma\), TNF-\(\alpha\), and IL-1\(\beta\))\textsuperscript{334-335}. The IL-10 KO mouse model spontaneously develops a mild form of IBD between 6-8 weeks of age when raised under conventional conditions with normal enteric bacterial flora\textsuperscript{253-254, 334, 336-337}. Disease development is proposed to result from the unregulated Th1 response against normal intestinal bacteria\textsuperscript{258, 336, 338}. This is emphasized by research demonstrating that IL-10 KO mice housed in germ-free conditions do not develop IBD\textsuperscript{253-254, 334}. The timing and presentation of disease in the inbred 129 Sv/Ev IL-10 KO mouse closely mimics that in humans\textsuperscript{334} as compared to outbred (129/Ola X C57BL/6) and inbred C57BL/6 mice, which develop a less severe disease later in life\textsuperscript{258, 338}. Therefore, the 129 Sv/Ev IL-10 KO IBD mouse model is important for investigating strategies to prevent the disease in humans. Disease characteristics include growth retardation, anaemia, diarrhoea, extensive intestinal inflammation, intestinal infiltration of immune cells, and increased production of proinflammatory cytokines (ie: IFN-\(\gamma\), TNF-\(\alpha\), IL-1\(\beta\), IL-6 and IL-12)\textsuperscript{253-254, 258, 334, 336, 338}.

As in humans affected with IBD, the IL-10 KO mouse model also develops bone abnormalities (ie: osteopenia and osteoporosis). Research indicates that the bone abnormalities are associated with the level of inflammation\textsuperscript{275}. Reduced bone development in the IL-10 KO mouse manifests as reduced trabecular and cortical bone mass\textsuperscript{275}, decreased bone formation\textsuperscript{275}, decreased femur length\textsuperscript{275}, reduced femur and LV\(_1\)-LV\(_3\) BMC and BMD\textsuperscript{273}, increased femur midpoint fragility and reduced strength at LV\(_3\)\textsuperscript{273, 275}.
There are several benefits to using genetically modified models of IBD versus chemically induced models to investigate preventative therapies. Firstly, IL-10 KO mice do not develop as severe clinical symptoms, such as diarrhoea, rectal bleeding and severe weight loss, as early (ie: 3-7 days after DSS administration) compared to chemically induced models of IBD (ie: DSS-induced mouse model)\(^{331-333}\). These symptoms are important to consider with chemically induced models of IBD as the toxicity to the intestinal epithelial cells and mucosal barrier can severely deteriorate the health of mice, resulting in premature death\(^ {332-333}\). Therefore, this is a major issue for study duration as it limits the length of time mice can be followed to investigate health and developmental outcomes, since mice are typically terminated between 8-10 days following chemical induction\(^ {276, 339}\). Another benefit of the IL-10 KO mouse is that it is a genetic model that spontaneously develops intestinal inflammation upon exposure to normal enteric bacterial flora\(^ {253-254, 334}\). Therefore, preventative interventions for the development of IBD can be investigated starting from pre-conception. This was critical for my research, which investigated the effect of early nutritional programming of vitamin D, beginning in utero and continuing throughout life, on the bone abnormalities associated with IBD.

2.12 Summary

Vitamin D is important during the growth and development of bone and the immune system, and may play a role in regulating body weight. However, following the attainment of PBM in adolescence and young adulthood, there is natural bone deterioration with the aging process, which may result in osteoporosis. Furthermore, those who suffer from IBD have associated bone abnormalities thought to be due to the inflammatory process. With the increasing prevalence of vitamin D deficiency, it is hypothesized that poor vitamin D status in healthy and diseased populations may play a role in the development of chronic disease. To investigate preventative nutritional interventions, healthy and inflammatory mouse models are used. The CD-1 mouse model is commonly used to investigate nutritional interventions and their possible effects in the healthy human population. Whereas the IL-10 KO mouse model mimics human IBD, and therefore can be used to investigate nutritional interventions that may attenuate the associated bone abnormalities. This thesis research investigated the effect of vitamin D supplementation on dams and their offspring. Studies have shown women to undergo major changes in bone health during pregnancy and lactation\(^ {169-171}\). Therefore, it was investigated whether vitamin D supplementation during these critical periods of growth and
development would lead to heavier body weights and larger and stronger bones in dams of a healthy and diseased mouse model. In addition, research has shown that intrauterine and early life vitamin D status is important for childhood bone development$^{143, 157, 159}$. Therefore, to determine if early exposure to vitamin D will improve offspring bone development, the study also investigated whether supplemental levels of vitamin D during intrauterine and early postnatal life would lead to heavier body weights and larger and stronger bones at young adulthood in a healthy and diseased mouse model.
Chapter Three

HYPOTHESES AND OBJECTIVES
3.0 HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

**CD-1 and IL-10 KO Dams:** It is hypothesized that a supplemental level of vitamin D during pregnancy and lactation will lead to heavier body weights and larger and stronger bones in a mouse model of health or inflammation.

**CD-1 Offspring:** In this mouse model of a healthy state, it is hypothesized that a supplemental level of vitamin D in utero and during suckling (postnatal day (PND) 1-21) will lead to heavier body weights and larger and stronger bones at young adulthood.

**IL-10 KO Offspring:** In this mouse model of inflammation, it is hypothesized that a supplemental level of vitamin D in utero and during suckling (PND 1-28) will lead to heavier body weights and larger and stronger bones at young adulthood.

3.2 Objectives

**CD-1 and IL-10 KO Dams:** To determine if a supplemental level of vitamin D during pregnancy and lactation results in heavier body weights and larger and stronger femurs and vertebra in a mouse model of health or inflammation.

**CD-1 Offspring:** To determine if a supplemental level of vitamin D in utero and during suckling (PND 1-21), in this mouse model of a healthy state, leads to heavier body weights and larger and stronger femurs and vertebra at young adulthood.

**IL-10 KO Offspring:** To determine if a supplemental level of vitamin D in utero and during suckling (PND 1-28), in this mouse model of inflammation, leads to heavier body weights and larger and stronger femurs and vertebra at young adulthood.
Chapter Four

MATERIALS AND METHODS
4.0 MATERIALS AND METHODS

4.1 Animals

All experimental procedures followed the policies outlined by the Canadian Council on Animal Care and were approved by the Animal Ethics Committee at the University of Toronto. Weanling (PND 22) CD-1 mice (n=11 males, n=29 females) were obtained from Charles River Laboratories Canada (St. Constant, QC, Canada) and fed a standard chow diet for 1 day. CD-1 mice were housed in the intervention study room under standard environmental conditions (12-h-light, 12-h-dark cycle; 23°C) with incandescent lighting free of UVB irradiation. On PND 23 CD-1 females were randomized to 1 of 2 intervention diets containing either low (modified AIN93G, 25 IU vitamin D₃/kg diet; Diet# 119289, Dyets Inc. Bethlehem, PA, USA) or supplemental (modified AIN93G, 5,000 IU vitamin D₃/kg diet; Diet# 119290, Dyets Inc. Bethlehem, PA, USA) levels of vitamin D₃. They were acclimatized to the incandescent lighting and intervention diets for approximately 4 weeks, at which time they were bred harem-style (3 females, 1 male) at 7 weeks of age (sexually mature). CD-1 females remained on their respective diets throughout breeding, pregnancy and lactation. CD-1 males were placed on a control diet (AIN93G, Dyets Inc. Bethlehem, PA, USA) at PND 23 under incandescent lighting which continued until breeding, at which time they consumed the same diet as the females. Pregnant CD-1 females were housed individually and checked daily for litters. All litters had 8-14 pups. CD-1 offspring were sexed by measuring the anogenital distance, and weaned at PND 21 and housed according to gender. At weaning, CD-1 dams were sacrificed and offspring were randomized to 1 of the 2 vitamin D intervention diets. Offspring diet groups differed based on postnatal weaning diet as well as vitamin D exposure in utero and during suckling. Offspring exposed to low vitamin D in utero and during suckling who were weaned to a low vitamin D diet were designated low, low (LL), whereas those weaned to a high vitamin D diet were designated low, high (LH). Similarly, those offspring exposed to high vitamin D in utero and during suckling who were weaned to a low vitamin D diet were designated high, low (HL), whereas those weaned to a high vitamin D diet were designated high, high (HH). CD-1 offspring were studied until 14 weeks of age (young adulthood) at which time they were sacrificed. For all CD-1 mice, food intake was measured every 2-3 days, fresh water was provided ad libitum every 2-3 days and body weight was recorded weekly.
Male and female 129 Sv/Ev IL-10 KO mice (n=4 males, n=4 females) were generously provided by Dr. Fedorak, University of Alberta. Upon arrival, IL-10 KO mice were housed in quarantine, according to gender, and fed a Fenbendazole diet for 4.5 weeks to ensure the mice had no gastrointestinal parasites. After 2 weeks in quarantine the IL-10 KO mice were set up in breeding pairs. Upon release from quarantine, breeding pairs were housed in the breeding room under standard environmental conditions (12-h-light, 12-h-dark cycle; 23°C) with normal lighting and placed on the control diet (AIN93G, Dyets Inc. Bethlehem, PA, USA). IL-10 KO males remained with females throughout pregnancy to take advantage of the fertile postpartum estrus that occurs following parturition. Weaning occurred at PND 21 when breeding females were pregnant, and therefore male and female offspring were housed together for 1 week due to their small size, and at PND 28 when breeding females were not pregnant. In-house breeding continued for 4.5 months to build a breeding colony. Once the colony was generated, IL-10 KO female offspring were randomly designated for the vitamin D intervention study where they were eventually bred and offspring were studied. IL-10 KO female offspring were transferred to the intervention study room at PND 28 where they were housed under standard environmental conditions (12-h-light, 12-h-dark cycle; 23°C) with incandescent lighting free of UVB irradiation and randomized to 1 of 2 intervention diets containing either low (modified AIN93G, 25 IU vitamin D₃/kg diet; Diet# 119289, Dyets Inc. Bethlehem, PA, USA) or supplemental (modified AIN93G, 5,000 IU vitamin D₃/kg diet; Diet# 119290, Dyets Inc. Bethlehem, PA, USA) levels of vitamin D₃. IL-10 KO females were acclimatized to the incandescent lighting and intervention diets for approximately 3-4 weeks, at which time they were bred harem-style (3 females; 1 male) between 7-8 weeks of age (sexually mature). IL-10 KO females were bred later than CD-1 mice, which were bred at 7 weeks of age, due to their small size. IL-10 KO females remained on their respective diets throughout breeding, pregnancy and lactation. Sexually mature IL-10 KO males, of comparable age and size to the females, were transferred from the breeding room to the intervention room for mating. Pregnant IL-10 KO females were housed individually and checked daily for litters. All litters had 3-8 pups. IL-10 KO offspring were sexed by measuring the anogenital distance, and weaned at PND 28 due to their small size. At weaning, IL-10 KO dams were sacrificed and offspring were randomized to 1 of the 2 vitamin D intervention diets and housed according to gender. Offspring diet groups differed based on postnatal weaning diet as well as vitamin D exposure in utero and during suckling. Offspring exposed to low vitamin D in utero and during suckling...
who were weaned to a low vitamin D diet were designated low, low (LL), whereas those weaned to a high vitamin D diet were designated low, high (LH). Similarly, those offspring exposed to high vitamin D in utero and during suckling who were weaned to a low vitamin D diet were designated high, low (HL), whereas those weaned to a high vitamin D diet were designated high, high (HH). IL-10 KO offspring were studied until 12 weeks of age (young adulthood) at which time they were sacrificed. Food intake was measured every 2-3 days, fresh water was provided ad libitum every 2-3 days and body weight was recorded weekly. In-house breeding was continuous until at least 15 mice/gender/group were acquired in the intervention room.

At necropsy, dams and offspring were asphyxiated with carbon dioxide, blood was collected via cardiac puncture and mice were killed via cervical dislocation. The right femur and LV1-3 were cleaned of soft tissue and stored at -80°C until analyses were performed.

4.2 Diet

Low and supplemental vitamin D levels in the diet were designed such that mice serum 25(OH)D levels would resemble those of individuals who were vitamin D deficient and sufficient, respectively. Based on the study by Fleet et al.136, a low diet containing 25 IU vitamin D₃/kg diet (Diet# 119289, Dyets Inc. Bethlehem, PA, USA) should result in a serum 25(OH)D level of approximately 35 nmol/L (representing vitamin D deficiency) and a supplemental diet containing 5,000 IU vitamin D₃/kg diet (Diet# 119290, Dyets Inc. Bethlehem, PA, USA) should result in a serum 25(OH)D level of approximately 200 mol/L.

4.2.1 Diet Analysis

The levels of vitamin D in the low and supplemental intervention diets were specifically analyzed (Maxxam Analytics, Mississauga, ON, Canada). The 2 diets were packaged separately in ziplock bags and transported to Maxxam Analytics. Analysis was blinded as the diets were designated “low vitamin D diet” and “high vitamin D diet”. The vitamin D level of the low diet was not detected as the analysis had a reportable detection limit of 200 IU vitamin D/kg diet. The vitamin D level of the supplemental diet was 4,730 IU vitamin D/kg diet. These vitamin D levels were satisfactory to proceed with the intervention study.
4.3 Serum 25(OH)D Levels

Blood was collected via cardiac puncture and centrifuged at 2,500 rpm for 25 minutes. Serum was collected and stored at -80°C. Serum 25(OH)D levels were measured for a subset of CD-1 mice (n=6-7 females/group, n=3 males/group). Serum 25(OH)D levels were measured by Dr. Vieth’s lab (University of Toronto and Mount Sinai Hospital, Toronto, Canada) using the automated IDS-iSYS 25OHD chemiluminescence immunoassay (Immunodiagnostic Systems Inc, Fountain Hills, AZ, USA). The reportable range of the assay was 12.5-350 nmol/L.

4.4 Bone Dimensions

4.4.1 Right Femur

CD-1 (n=14-15 mice/gender/group; 1 mouse/gender/dam/group) and IL-10 KO (n=15-26 mice/gender/group) femurs were removed from storage at -80°C and rehydrated in phosphate buffered saline (PBS) for 1.5 hours at room temperature. Femurs were weighed using a digital scale accurate to 4 decimal places. Femur length, anteroposterior (AP; depth) width and mediolateral (ML; width) width were measured using electronic precision calipers accurate to 0.01 mm (Lee Valley, Burlington, ON, Canada). Length was measured from the proximal tip of the femur head to the distal tip of the medial condyle. The femur midpoint was determined based on the length of the femur. AP width and ML width were measured at the femur midpoint as femurs are not cylindrical at the midpoint.

4.4.2 LV2

LV1-3 from CD-1 and IL-10 KO mice with corresponding right femurs were removed from storage at -80°C. LV2 was dissected from the intact spine (LV1-3) and soft tissue and spiny processes were removed. LV2 was then rehydrated in PBS for 1 hour at room temperature. LV2 was weighed using a digital scale accurate to 4 decimal places. The height, AP width (depth) and ML width (width) of LV2 were measured using electronic precision calipers accurate to 0.01 mm (Lee Valley, Burlington, ON, Canada). Height was measured along the longitudinal midline of the vertebra, while AP width and ML width were measured at the midpoint.
4.5 Biomechanical Strength Testing

The biomechanical strength properties of the right femur (femur midpoint fracture and femur neck fracture) and LV2 (compression fracture) were measured by 3 point bending and compression testing, respectively. Because the proportion of cortical and trabecular bone varies depending on the region of the femur, the femur midpoint, which contains mostly cortical bone, and the femur neck, which contains mostly trabecular bone, were selected for testing the functional outcome measure of bone, fracture resistance. Testing was completed using the materials testing system and specialized software (Model 4442, Instron Corp., Norwood, MA, USA; Bluehill 2). The femur neck (hip) and lumbar spine are common fracture sites in humans, and therefore measuring the strength of these regions is relevant to the human situation.

4.5.1 Three-Point Bending Test

Three-point bending was performed at the femur midpoint to determine the yield load (a measure of the elastic limit of the bone; measures the contribution of mineral to bone strength) and peak load (a measure of the fracture threshold of the bone; measures the contribution of matrix to bone strength) at a femoral site rich in cortical bone. Right femurs were removed from storage at -80°C and rehydrated in PBS for 3 hours. The structural properties of the femur were tested by placing the femur midpoint directly under the crosshead with the posterior surface of the femur placed on 2 supports of the bending jig. For the CD-1 mice the supports were set 6 mm apart, whereas they were set 5 mm apart for the IL-10 KO mice due to the difference in femur length. The crosshead, which was rounded to reduce shear force, was lowered at a constant speed of 2 mm/min until bending ultimately resulted in fracture.

4.5.2 Femur Neck Fracture Test

Following the three-point bending test, the femur neck compression test was performed to determine the peak load at a femoral site rich in trabecular bone. The test was conducted by placing the proximal half of the right femur in a customized jig and the flat-edged crosshead was lowered at a constant speed of 2 mm/min on the proximal femur head until fracture occurred.
4.5.3 LV2 Compression Test

LV compression testing was performed on individual vertebra (LV2) to determine the peak load at a vertebral site rich in trabecular bone. LV2 were removed from storage at -80°C and rehydrated in PBS for 3 hours. The structural properties of LV2 were tested by placing the distal side flat in the middle of the stainless steel plate, while a second suspended steel plate descended and applied a vertical force at a constant rate of 2 mm/min until LV2 was compressed. Peak load was determined as the first peak of the load-deformation curve.

4.6 Statistical Analyses

Statistical analyses were performed using Sigma-Stat (Version 3.5, Jandel Scientific, Chicago, Illinois, USA). Results are expressed as mean ± SEM. T-tests were performed to determine differences in dams (body weight and bone dimensions and strength outcomes), litter characteristics (litter size, number of males and females per litter, and litter weight), offspring post-weaning food intake and gender post-weaning food intake and bone strength outcomes. Two-way analysis of variance (ANOVA), with diet of dams and offspring as factors, was performed to determine differences in offspring body weight, serum 25(OH)D levels, bone dimensions and strength outcomes. Student-Newman-Keuls test was used for comparison of multiple means when statistical differences were detected. Linear regression was performed to determine the correlation between peak load at multiple skeletal sites. Statistical significance was defined as p < 0.05.
Chapter Five

RESULTS
5.0 RESULTS

5.1 Dams

5.1.1 CD-1 Study

**Body weight:** Diet did not result in differences in body weight at the end of lactation.

**Litter characteristics:** Pup litter size and the number of males and females per litter did not differ between dams fed a low versus high vitamin D diet (Table 5.0). At PND 1 and 14, average pup litter weights did not differ between dams fed a low versus high vitamin D diet (Table 5.0).

**Femur outcomes:** Femur weight, length, AP width and ML width did not differ between dams fed a low versus high vitamin D diet (Table 5.1). There were no differences between dams fed a low versus high vitamin D diet for yield load or peak load at the femur midpoint, or peak load at the femur neck (Table 5.1).

**LV₂ outcomes:** LV₂ weight, height, AP width, ML width and peak load did not differ between dams fed a low versus high vitamin D diet (Table 5.2).

5.1.2 IL-10 KO Study

**Body weight:** Diet did not result in differences in body weight at the end of lactation.

**Litter characteristics:** Pup litter size did not differ between dams fed a low versus high vitamin D diet (Table 5.3). Dams fed a low vitamin D diet had fewer (p=0.035) males than dams fed a high vitamin D diet (Table 5.3). Diet did not affect the number of females in a litter (Table 5.3). At PND 14, average pup litter weight did not differ between dams fed a low versus high vitamin D diet (Table 5.3).

**Femur outcomes:** Femur weight, length, AP width and ML width did not differ between dams fed a low versus high vitamin D diet (Table 5.4). There was no difference between dams for yield load at the femur midpoint; however dams who consumed a high vitamin D diet throughout pregnancy and lactation had a higher (p=0.006) peak load at the femur midpoint compared to those who consumed a low vitamin D diet during the same time period (Table 5.4). There was no difference between dams for peak load at the femur neck (Table 5.4).
Table 5.0 Litter size, gender per litter and body weight for CD-1 pups whose dams were fed low or high vitamin D diets

<table>
<thead>
<tr>
<th></th>
<th>Dam Diet²</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Litter size</td>
<td>11.0 ± 0.4</td>
<td>12.0 ± 0.4</td>
</tr>
<tr>
<td>Gender per litter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>6.0 ± 0.5</td>
<td>6.0 ± 0.4</td>
</tr>
<tr>
<td>Females</td>
<td>5.0 ± 0.3</td>
<td>6.0 ± 0.3</td>
</tr>
<tr>
<td>PND body weight (g)³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PND 1</td>
<td>1.96 ± 0.04</td>
<td>1.88 ± 0.03</td>
</tr>
<tr>
<td>PND 14</td>
<td>9.43 ± 0.19</td>
<td>9.06 ± 0.19</td>
</tr>
<tr>
<td>PND 21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>15.79 ± 0.32</td>
<td>14.79 ± 0.29</td>
</tr>
<tr>
<td>Females</td>
<td>15.25 ± 0.23</td>
<td>14.20 ± 0.25</td>
</tr>
</tbody>
</table>

1 Values are expressed as mean ± SEM.
2 Vitamin D Levels: 25 IU/kg diet (Low; n=14); 5,000 IU/kg diet (High; n=15).
3 PND: postnatal day. At PND 1 and 14 genders were combined; gender was determined at PND 21.
NS, non-significant, p > 0.05
Table 5.1 Weight, dimensions and biomechanical strength properties of the right femur from CD-1 dams that received low or high levels of vitamin D in the diet during pregnancy and lactation\(^1\)

<table>
<thead>
<tr>
<th>Dam Diet(^2)</th>
<th>Low</th>
<th>High</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight (mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>98.3 ± 1.9</td>
<td>102.2 ± 3.4</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Average length (mm)</td>
<td>16.10 ± 0.11</td>
<td>16.28 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>Average AP width (mm)(^3)</td>
<td>1.42 ± 0.02</td>
<td>1.42 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Average ML width (mm)(^4)</td>
<td>1.81 ± 0.02</td>
<td>1.84 ± 0.02</td>
</tr>
<tr>
<td>Femur midpoint</td>
<td>Yield load (N)</td>
<td>9.28 ± 0.41</td>
<td>9.87 ± 0.71</td>
</tr>
<tr>
<td></td>
<td>Peak load (N)</td>
<td>18.88 ± 0.81</td>
<td>19.11 ± 0.64</td>
</tr>
<tr>
<td>Femur neck</td>
<td>Peak load (N)</td>
<td>12.70 ± 0.83</td>
<td>12.62 ± 0.92</td>
</tr>
</tbody>
</table>

\(^1\) Values are expressed as mean ± SEM.
\(^2\) Vitamin D Levels: 25 IU/kg diet (Low; n=14); 5,000 IU/kg diet (High; n=14).
\(^3\) AP width: anteroposterior width at the femur midpoint.
\(^4\) ML width: mediolateral width at the femur midpoint.

NS, non-significant, p > 0.05
Table 5.2 Weight, dimensions and biomechanical strength properties of LV2 from CD-1 dams that received low or high levels of vitamin D in the diet during pregnancy and lactation

<table>
<thead>
<tr>
<th>LV2 dimensions</th>
<th>Dam Diet</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (mg)</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>30.5 ± 1.0</td>
<td>32.5 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>Average height (mm)</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>3.56 ± 0.06</td>
<td>3.61 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Average AP width (mm)(^3)</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>3.65 ± 0.05</td>
<td>3.72 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Average ML width (mm)(^4)</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>3.40 ± 0.02</td>
<td>3.42 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Peak load (N)</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>56.97 ± 2.77</td>
<td>56.82 ± 2.31</td>
<td></td>
</tr>
</tbody>
</table>

1 Values are expressed as mean ± SEM.
2 Vitamin D Levels: 25 IU/kg diet (Low; n=14); 5,000 IU/kg diet (High; n=14).
3 AP width: anteroposterior width.
4 ML width: mediolateral width.
NS, non-significant, p > 0.05
Table 5.3 Litter size, gender per litter and body weight for IL-10 KO pups whose dams were fed low or high vitamin D diets

<table>
<thead>
<tr>
<th></th>
<th>Dam Diet²</th>
<th></th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>Litter size</td>
<td>5.0 ± 0.4</td>
<td>6.0 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Gender per litter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>2.0 ± 0.3</td>
<td>3.0 ± 0.2</td>
<td>0.035</td>
</tr>
<tr>
<td>Females</td>
<td>3.0 ± 0.3</td>
<td>2.0 ± 0.3</td>
<td>NS</td>
</tr>
<tr>
<td>PND body weight (g)³</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PND 14</td>
<td>6.72 ± 0.23</td>
<td>6.78 ± 0.23</td>
<td>NS</td>
</tr>
<tr>
<td>PND 21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>8.21 ± 0.30</td>
<td>8.78 ± 0.20</td>
<td>NS</td>
</tr>
<tr>
<td>Females</td>
<td>8.72 ± 0.25</td>
<td>8.12 ± 0.23</td>
<td>NS</td>
</tr>
<tr>
<td>PND 28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>13.55 ± 0.33</td>
<td>13.70 ± 0.42</td>
<td>NS</td>
</tr>
<tr>
<td>Females</td>
<td>12.62 ± 0.21</td>
<td>11.83 ± 0.45</td>
<td>NS</td>
</tr>
</tbody>
</table>

¹ Values are expressed as mean ± SEM.
² Vitamin D Levels: 25 IU/kg diet (Low; n=15); 5,000 IU/kg diet (High; n=16).
³ PND: postnatal day. At PND 1 and 14 genders were combined; gender was determined at PND 21.

NS, non-significant, p > 0.05
Table 5.4 Weight, dimensions and biomechanical strength properties of the right femur from IL-10 KO dams that received low or high levels of vitamin D in the diet during pregnancy and lactation

<table>
<thead>
<tr>
<th>Dam Diet</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
</tr>
<tr>
<td>Femur dimensions</td>
<td></td>
</tr>
<tr>
<td>Weight (mg)</td>
<td>65.0 ± 1.0</td>
</tr>
<tr>
<td>Average length (mm)</td>
<td>14.90 ± 0.10</td>
</tr>
<tr>
<td>Average AP width (mm)</td>
<td>1.31 ± 0.01</td>
</tr>
<tr>
<td>Average ML width (mm)</td>
<td>1.47 ± 0.01</td>
</tr>
<tr>
<td>Femur midpoint</td>
<td></td>
</tr>
<tr>
<td>Yield load (N)</td>
<td>9.84 ± 0.53</td>
</tr>
<tr>
<td>Peak load (N)</td>
<td>18.30 ± 0.57</td>
</tr>
<tr>
<td>Femur neck</td>
<td></td>
</tr>
<tr>
<td>Peak load (N)</td>
<td>10.36 ± 0.38</td>
</tr>
</tbody>
</table>

1 Values are expressed as mean ± SEM.
2 Vitamin D Levels: 25 IU/kg diet (Low; n=14); 5,000 IU/kg diet (High; n=15).
3 AP width: anteroposterior width at the femur midpoint.
4 ML width: mediolateral width at the femur midpoint.

NS, non-significant, p > 0.05
LV2 outcomes: There were no differences between dams for LV2 weight, height, AP width, ML width or peak load (Table 5.5).

5.2 Male Offspring

5.2.1 CD-1 Study

Body weight: At PND 21, pups who were exposed to high vitamin D in utero and throughout suckling weighed less (p=0.023) than those exposed to low vitamin D (Table 5.0). Throughout development, from 4 to 14 weeks of age, neither dam nor pup diet had an effect on body weight (Figure 5.0).

Post-weaning food intake: There was no difference in average daily food intake between males consuming a low versus high vitamin D diet (Table 5.6). By design, males consuming a high vitamin D diet had a higher (p<0.001) vitamin D intake than those consuming a low vitamin D diet (Table 5.6). There was no difference in calcium intake (Table 5.6).

Serum 25(OH)D levels: Dam diet had no effect on serum 25(OH)D levels; however, male mice who consumed a high vitamin D diet post-weaning had higher (p<0.001) serum 25(OH)D levels than those who consumed a low vitamin D diet (Figure 5.1).

Femur outcomes: Femur weight, length, AP width and ML width did not differ among groups for male mice (Table 5.7). Yield load and peak load at the femur midpoint did not differ among groups (Table 5.7). Males exposed to high vitamin D in utero and throughout suckling had a lower (p=0.039) peak load at the femur neck compared to those exposed to low vitamin D during the same time period (Table 5.7).

LV2 outcomes: Pup diet had a significant effect on the weight of LV2. Males who consumed a high vitamin D diet post-weaning had heavier (p=0.043) LV2 compared to those who consumed a low vitamin D diet (Table 5.8). There were no differences among males for LV2 height, AP width, ML width or peak load (Table 5.8).

Correlation between peak load at multiple skeletal sites: There was a relationship between femur midpoint and LV2 (p=0.016) peak load. The peak load of LV2 can positively predict (r=0.323) the peak load of the femur midpoint (Table 5.9). However, there were no relationships between the femur neck and femur midpoint peak load, and femur neck and LV2.
Table 5.5 Weight, dimensions and biomechanical strength properties of LV2 from IL-10 KO dams that received low or high levels of vitamin D in the diet during pregnancy and lactation

<table>
<thead>
<tr>
<th>Dam Diet</th>
<th></th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>LV2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (mg)</td>
<td>19.8 ± 0.4</td>
<td>20.5 ± 0.6</td>
</tr>
<tr>
<td>Average height (mm)</td>
<td>3.37 ± 0.05</td>
<td>3.39 ± 0.05</td>
</tr>
<tr>
<td>Average AP width (mm)</td>
<td>3.43 ± 0.03</td>
<td>3.37 ± 0.04</td>
</tr>
<tr>
<td>Average ML width (mm)</td>
<td>2.79 ± 0.02</td>
<td>2.78 ± 0.01</td>
</tr>
<tr>
<td>LV2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak load (N)</td>
<td>44.55 ± 1.22</td>
<td>47.25 ± 1.67</td>
</tr>
</tbody>
</table>

1 Values are expressed as mean ± SEM.
2 Vitamin D Levels: 25 IU/kg diet (Low; n=14); 5,000 IU/kg diet (High; n=15).
3 AP width: anteroposterior width.
4 ML width: mediolateral width.
NS, non-significant, p > 0.05
Figure 5.0 Body weight for male CD-1 mice

Body weights of male mice that were exposed to low or high levels of vitamin D in utero and during suckling and then received a low or high vitamin D diet post-weaning and throughout adulthood. Values are expressed as means ± SEM (Low Low, n=17; Low High, n=16; High Low, n=17; High High, n=18). The closed circles represent males exposed to low vitamin D in utero and during suckling, and low vitamin D post-weaning and the open circles represent males exposed to low vitamin D in utero and during suckling, and high vitamin D post-weaning. The closed triangles represent males exposed to high vitamin D in utero and during suckling, and low vitamin D post-weaning and the open triangles represent males exposed to high vitamin D in utero and during suckling, and high vitamin D post-weaning. There were no significant differences in body weight among the 4 groups from 4 to 14 weeks of development.
Table 5.6 Post-weaning average daily food intake, vitamin D intake and calcium intake for male and female CD-1 mice

<table>
<thead>
<tr>
<th></th>
<th>Diet²</th>
<th>P-value</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male³</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food consumed (g/day)</td>
<td>4.03 ± 0.04</td>
<td>4.09 ± 0.03</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Vitamin D intake (IU/day)</td>
<td>0.10 ± 0.001</td>
<td>20.44 ± 0.16</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Calcium intake (mg/day)</td>
<td>20.15 ± 0.19</td>
<td>20.44 ± 0.16</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Female⁴</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food consumed (g/day)</td>
<td>3.88 ± 0.28</td>
<td>3.48 ± 0.06</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Vitamin D intake (IU/day)</td>
<td>0.10 ± 0.007</td>
<td>17.40 ± 0.28</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Calcium intake (mg/day)</td>
<td>19.38 ± 1.41</td>
<td>17.40 ± 0.28</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male³ vs. Female⁴</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food consumed (g/day)</td>
<td>4.06 ± 0.03</td>
<td>3.69 ± 0.16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin D intake (IU/day)</td>
<td>10.27 ± 1.67</td>
<td>8.12 ± 1.37</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Calcium intake (mg/day)</td>
<td>20.29 ± 0.13</td>
<td>18.46 ± 0.78</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

¹ Values are expressed as mean ± SEM.
² Vitamin D Level: 25 IU/kg diet (Low); 5,000 IU/kg diet (High).
³ Male: Low n=19; High n=19.
⁴ Female: Low n=19; High n=22.
NS, non-significant, p > 0.05
Figure 5.1 Serum 25(OH)D levels for male CD-1 mice

Serum 25(OH)D levels for male mice that were exposed to low or high levels of vitamin D in utero and during suckling and received a low or high vitamin D diet post-weaning and throughout adulthood. Values are expressed as means ± SEM (Low Low, n=2; Low High, n=3; High Low, n=3; High High, n=3). Different letters indicate there was a significant difference among groups. Dam diet did not affect serum 25(OH)D levels; however, pup diet had an effect. Male mice that consumed a high vitamin D diet post-weaning had significantly higher (p<0.001) serum 25(OH)D levels than male mice that consumed a low vitamin D diet post-weaning.
Table 5.7 Weight, dimensions and biomechanical strength properties of the right femur from male CD-1 mice exposed to low or high vitamin D in utero through to young adulthood

<table>
<thead>
<tr>
<th></th>
<th>Dam Diet²</th>
<th>Pup Diet²</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td>Dam Diet</td>
</tr>
<tr>
<td>Femur dimensions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (mg)</td>
<td>Low 121.9 ± 3.9</td>
<td>High 120.0 ± 2.2</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Low 115.5 ± 2.9</td>
<td>High 118.2 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>Average length (mm)</td>
<td>Low 16.15 ± 0.11</td>
<td>High 16.13 ± 0.08</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Low 16.10 ± 0.09</td>
<td>High 16.32 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Average AP width (mm)³</td>
<td>Low 1.55 ± 0.03</td>
<td>High 1.53 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Low 1.54 ± 0.02</td>
<td>High 1.54 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Average ML width (mm)⁴</td>
<td>Low 2.01 ± 0.03</td>
<td>High 2.06 ± 0.04</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Low 2.05 ± 0.03</td>
<td>High 2.01 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Femur midpoint</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yield load (N)</td>
<td>Low 15.83 ± 0.76</td>
<td>High 16.80 ± 0.93</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Low 16.64 ± 0.67</td>
<td>High 16.23 ± 0.79</td>
<td></td>
</tr>
<tr>
<td>Peak load (N)</td>
<td>Low 37.48 ± 1.39</td>
<td>High 36.84 ± 1.92</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Low 36.29 ± 1.45</td>
<td>High 34.59 ± 1.64</td>
<td></td>
</tr>
<tr>
<td>Femur neck</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak load (N)</td>
<td>Low 23.29 ± 1.16</td>
<td>High 21.44 ± 0.89</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td>Low 20.21 ± 0.90</td>
<td>High 20.47 ± 0.78</td>
<td></td>
</tr>
</tbody>
</table>

¹ Values are expressed as mean ± SEM.
² Vitamin D Levels: 25 IU/kg diet (Low); 5,000 IU/kg diet (High).
³ AP width: anteroposterior width at the femur midpoint.
⁴ ML width: mediolateral width at the femur midpoint.

Dam diet, Pup diet: Low, Low n=14; Low, High n=14; High, Low n=14; High, High n=15
NS, non-significant, p > 0.05
Table 5.8 Weight, dimensions and biomechanical strength properties of LV₂ from male CD-1 mice exposed to low or high vitamin D in utero through to young adulthood

<table>
<thead>
<tr>
<th>LV₂ dimensions</th>
<th>Dam Diet¹</th>
<th>Pup Diet²</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td>P-value</td>
</tr>
<tr>
<td>Weight (mg)</td>
<td>Low</td>
<td>36.8 ± 0.6</td>
<td>39.9 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>37.8 ± 1.2</td>
<td>38.9 ± 0.8</td>
</tr>
<tr>
<td>Average height (mm)</td>
<td>Low</td>
<td>3.46 ± 0.03</td>
<td>3.51 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>3.55 ± 0.04</td>
<td>3.50 ± 0.03</td>
</tr>
<tr>
<td>Average AP width (mm)³</td>
<td>Low</td>
<td>3.77 ± 0.06</td>
<td>3.80 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>3.87 ± 0.05</td>
<td>3.91 ± 0.05</td>
</tr>
<tr>
<td>Average ML width (mm)⁴</td>
<td>Low</td>
<td>3.53 ± 0.03</td>
<td>3.49 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>3.48 ± 0.03</td>
<td>3.50 ± 0.03</td>
</tr>
<tr>
<td>Peak load (N)</td>
<td>Low</td>
<td>76.95 ± 4.21</td>
<td>76.14 ± 2.55</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>74.93 ± 3.05</td>
<td>69.76 ± 4.31</td>
</tr>
</tbody>
</table>

¹ Values are expressed as mean ± SEM.
² Vitamin D Levels: 25 IU/kg diet (Low); 5,000 IU/kg diet (High).
³ AP width: anteroposterior width.
⁴ ML width: mediolateral width.

Dam diet, Pup diet: Low, Low n=14; Low, High n=14; High, Low n=14; High, High n=15
NS, non-significant, p > 0.05
Table 5.9 Correlation between peak load at multiple skeletal sites for male and female CD-1 mice

<table>
<thead>
<tr>
<th>Peak Load (N)</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r-value</td>
<td>P-value</td>
</tr>
<tr>
<td>Femur midpoint vs. LV₂</td>
<td>0.323</td>
<td>0.016</td>
</tr>
<tr>
<td>Femur neck vs. femur midpoint</td>
<td>0.165</td>
<td>NS</td>
</tr>
<tr>
<td>Femur neck vs. LV₂</td>
<td>0.092</td>
<td>NS</td>
</tr>
</tbody>
</table>

\(^1\) n=54  
NS, non-significant, \( p > 0.05 \)
peak load (Table 5.9).

### 5.2.2 IL-10 KO Study

**Body weight:** At PND 21 and 28 males did not differ in body weight whether exposed to low or high vitamin D in utero and throughout suckling (Table 5.3). Throughout development, from 5 to 12 weeks of age, neither dam nor pup diet had an effect on body weight (Figure 5.2).

**Post-weaning food intake:** There was no difference in average daily food intake between males consuming a low versus high vitamin D diet (Table 5.10). By design, males consuming a high vitamin D diet had a higher ($p<0.001$) vitamin D intake than males consuming a low vitamin D diet (Table 5.10). There was no difference in calcium intake (Table 5.10).

**Femur outcomes:** There were no differences in femur weight, length or AP width (Table 5.11). Males who consumed a high vitamin D diet post-weaning had larger ($p=0.033$) ML widths than those who consumed a low vitamin D diet (Table 5.11). Yield load and peak load at the femur midpoint, as well as peak load at the femur neck, did not differ among males (Table 5.11).

**LV2 outcomes:** LV2 weight, height, AP width, ML width and peak load did not differ among males (Table 5.12).

**Correlation between peak load at multiple skeletal sites:** There were no significant relationships between the femur midpoint and LV2 peak load, femur neck and femur midpoint peak load, and femur neck and LV2 peak load (Table 5.13).

### 5.3 Female Offspring

#### 5.3.1 CD-1 Study

**Body weight:** At PND 21, pups who were exposed to high vitamin D in utero and throughout suckling weighed less ($p=0.003$) than those exposed to low vitamin D (Table 5.0). Throughout development, from 4 to 14 weeks of age, neither dam nor pup diet had an effect on body weight (Figure 5.3).

**Post-weaning food intake:** There was no difference in average daily food intake between females consuming a low versus high vitamin D diet (Table 5.6). By design, females consuming a high vitamin D diet had a higher ($p<0.001$) vitamin D intake than those consuming
Figure 5.2 Body weight for male IL-10 KO mice
Body weights of male mice that were exposed to low or high levels of vitamin D in utero and during suckling and then received a low or high vitamin D diet post-weaning and throughout adulthood. Values are expressed as means ± SEM (Low Low, n=15; Low High, n=15; High Low, n=26; High High, n=23). The closed circles represent males exposed to low vitamin D in utero and during suckling, and low vitamin D post-weaning and the open circles represent males exposed to low vitamin D in utero and during suckling, and high vitamin D post-weaning. The closed triangles represent males exposed to high vitamin D in utero and during suckling, and low vitamin D post-weaning and the open triangles represent males exposed to high vitamin D in utero and during suckling, and high vitamin D post-weaning. There were no significant differences in body weight between the 4 groups from 5 to 12 weeks of development.
Table 5.10 Post-weaning average daily food intake, vitamin D intake and calcium intake for male and female IL-10 KO mice¹

<table>
<thead>
<tr>
<th></th>
<th>Diet²</th>
<th></th>
<th>P-value</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male³</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food consumed (g/day)</td>
<td>2.61 ± 0.08</td>
<td>2.54 ± 0.03</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Vitamin D intake (IU/day)</td>
<td>0.07 ± 0.002</td>
<td>12.71 ± 0.14</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Calcium intake (mg/day)</td>
<td>13.07 ± 0.38</td>
<td>12.71 ± 0.14</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Female⁴</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food consumed (g/day)</td>
<td>2.26 ± 0.09</td>
<td>2.28 ± 0.07</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Vitamin D intake (IU/day)</td>
<td>0.06 ± 0.002</td>
<td>11.40 ± 0.37</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Calcium intake (mg/day)</td>
<td>11.28 ± 0.45</td>
<td>11.40 ± 0.37</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
<th>P-value</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Male³ vs. Female⁴</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food consumed (g/day)</td>
<td>2.58 ± 0.04</td>
<td>2.27 ± 0.06</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Vitamin D intake (IU/day)</td>
<td>5.90 ± 0.89</td>
<td>5.73 ± 0.76</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>Calcium intake (mg/day)</td>
<td>12.91 ± 0.22</td>
<td>11.34 ± 0.29</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

¹ Values are expressed as mean ± SEM.
² Vitamin D Level: 25 IU/kg diet (Low); 5,000 IU/kg diet (High).
³ Male: Low n=28; High n=24.
⁴ Female: Low n=30; High n=30.
NS, non-significant, p > 0.05
Table 5.11 Weight, dimensions and biomechanical strength properties of the right femur from male IL-10 KO mice exposed to low and high vitamin D in utero through young adulthood

<table>
<thead>
<tr>
<th>Dam Diet 2</th>
<th>Pup Diet 2</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Femur dimensions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (mg)</td>
<td>Low 59.2 ± 1.1</td>
<td>61.7 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>High 58.8 ± 1.1</td>
<td>60.7 ± 1.2</td>
</tr>
<tr>
<td>Average length (mm)</td>
<td>Low 14.28 ± 0.05</td>
<td>14.50 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>High 14.50 ± 0.07</td>
<td>14.57 ± 0.06</td>
</tr>
<tr>
<td>Average AP width (mm)</td>
<td>Low 1.23 ± 0.01</td>
<td>1.24 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>High 1.23 ± 0.01</td>
<td>1.25 ± 0.01</td>
</tr>
<tr>
<td>Average ML width (mm)</td>
<td>Low 1.47 ± 0.01</td>
<td>1.50 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>High 1.46 ± 0.01</td>
<td>1.49 ± 0.01</td>
</tr>
<tr>
<td>Femur midpoint</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yield load (N)</td>
<td>Low 10.36 ± 0.36</td>
<td>10.83 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>High 9.95 ± 0.28</td>
<td>10.49 ± 0.26</td>
</tr>
<tr>
<td>Peak load (N)</td>
<td>Low 21.41 ± 0.76</td>
<td>21.03 ± 0.72</td>
</tr>
<tr>
<td></td>
<td>High 20.45 ± 0.50</td>
<td>21.84 ± 0.60</td>
</tr>
<tr>
<td>Femur neck</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak load (N)</td>
<td>Low 10.08 ± 0.58</td>
<td>10.82 ± 0.54</td>
</tr>
<tr>
<td></td>
<td>High 9.30 ± 0.43</td>
<td>9.82 ± 0.38</td>
</tr>
</tbody>
</table>

1 Values are expressed as mean ± SEM.
2 Vitamin D Levels: 25 IU/kg diet (Low); 5,000 IU/kg diet (High).
3 AP width: anteroposterior width at the femur midpoint.
4 ML width: mediolateral width at the femur midpoint.

Dam diet, Pup diet: Low, Low n=15; Low, High n=15; High, Low n=26; High, High n=23
NS, non-significant, p > 0.05
**Table 5.12** Weight, dimensions and biomechanical strength properties of LV2 from male IL-10 KO mice exposed to low or high vitamin D in utero through young adulthood¹

<table>
<thead>
<tr>
<th>LV2 dimensions</th>
<th>Dam Diet²</th>
<th>Pup Diet²</th>
<th>P-value</th>
<th>Dam Diet</th>
<th>Pup Diet</th>
<th>Dam Diet X Pup Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (mg)</td>
<td>Low 16.6 ± 0.4</td>
<td>17.2 ± 0.4</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>High 17.8 ± 0.4</td>
<td>17.3 ± 0.3</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Average height (mm)</td>
<td>Low 3.08 ± 0.05</td>
<td>3.08 ± 0.04</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>High 3.15 ± 0.04</td>
<td>3.09 ± 0.04</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Average AP width (mm)³</td>
<td>Low 3.31 ± 0.02</td>
<td>3.40 ± 0.04</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>High 3.37 ± 0.03</td>
<td>3.40 ± 0.03</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Average ML width (mm)⁴</td>
<td>Low 2.71 ± 0.01</td>
<td>2.71 ± 0.02</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>High 2.70 ± 0.01</td>
<td>2.71 ± 0.02</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Peak load (N)</td>
<td>Low 49.92 ± 2.23</td>
<td>51.66 ± 1.51</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>High 47.41 ± 1.34</td>
<td>51.64 ± 1.27</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

¹ Values are expressed as mean ± SEM.
² Vitamin D Levels: 25 IU/kg diet (Low); 5,000 IU/kg diet (High).
³ AP width: anteroposterior width.
⁴ ML width: mediolateral width.

Dam diet, Pup diet: Low, Low n=15; Low, High n=15; High, Low n=26; High, High n=23
NS, non-significant, p > 0.05
Table 5.13 Correlation between peak load at multiple skeletal sites for male and female IL-10 KO mice

<table>
<thead>
<tr>
<th></th>
<th>Male&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Female&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r-value</td>
<td>P-value</td>
</tr>
<tr>
<td>Peak Load (N)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femur midpoint vs. LV&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.226</td>
<td>NS</td>
</tr>
<tr>
<td>Femur neck vs. femur midpoint</td>
<td>0.055</td>
<td>NS</td>
</tr>
<tr>
<td>Femur neck vs. LV&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.119</td>
<td>NS</td>
</tr>
</tbody>
</table>

<sup>1</sup> n=75  
<sup>2</sup> n=80  
NS, non-significant, p > 0.05
Figure 5.3 Body weight for female CD-1 mice

Body weights of female mice that were exposed to low or high levels of vitamin D in utero and during suckling and received a low or high vitamin D diet post-weaning and throughout adulthood. Values are expressed as means ± SEM (Low Low, n=16; Low High, n=17; High Low, n=20; High High, n=17). The closed circles represent females exposed to low vitamin D in utero and during suckling, and low vitamin D post-weaning and the open circles represent females exposed to low vitamin D in utero and during suckling, and high vitamin D post-weaning. The closed triangles represent females exposed to high vitamin D in utero and during suckling, and low vitamin D post-weaning and the open triangles represent females exposed to high vitamin D in utero and during suckling, and high vitamin D post-weaning. There were no significant differences in body weight among the 4 groups from 4 to 14 weeks of development.
a low vitamin D diet. There was no difference in calcium intake (Table 5.6).

**Serum 25(OH) levels:** Dam diet had no effect on serum 25(OH)D levels; while consumption of a high vitamin D diet post-weaning resulted in higher (p<0.001) serum 25(OH)D levels than pups who consumed a low vitamin D diet (Figure 5.4).

**Femur outcomes:** There was a significant interaction between dam diet and pup diet for femur weight. Females fed a high vitamin D diet post-weaning and who were exposed to high vitamin D in utero and throughout suckling had lighter (p=0.044) femurs compared to those exposed to low vitamin D in utero and throughout suckling (Table 5.14). Femur length and AP width did not differ among diet groups; but females exposed to high vitamin D in utero and during suckling had smaller (p=0.010) ML widths than those exposed to low vitamin D (Table 5.14). Females exposed to high vitamin D in utero and throughout suckling had a lower (p=0.014) yield load at the femur midpoint than those exposed to low vitamin D (Table 5.14). Peak load at the femur midpoint did not differ among diet groups for females (Table 5.14). Females exposed to high vitamin D in utero and throughout suckling had a lower (p=0.039) peak load at the femur neck compared to those exposed to low vitamin D (Table 5.14).

**LV₂ outcomes:** LV₂ weight, height, AP width or ML width did not differ among groups (Table 5.15). Females who were exposed to high vitamin D in utero and throughout suckling had lower (p=0.041) peak load compared to those who were exposed to low vitamin D (Table 5.15).

**Correlation between peak load at multiple skeletal sites:** There were no significant relationships between the femur midpoint and LV₂ peak load, and femur neck and femur midpoint peak load (Table 5.9). There was a relationship between the femur neck and LV₂ (p=0.008) peak load. The peak load of LV₂ can positively predict (r=0.349) the peak load of the femur neck (Table 5.9).

### 5.3.2 IL-10 KO Study

**Body weight:** At PND 21 and 28 females did not differ in body weight whether exposed to low or high vitamin D in utero and throughout suckling (Table 5.3). Throughout development, from 5 to 12 weeks of age, neither dam nor pup diet had an effect on body weight (Figure 5.5).

**Post-weaning food intake:** There was no difference in average daily food intake between females consuming a low versus high vitamin D diet (Table 5.10). By design, females
Figure 5.4 Serum 25(OH)D levels for female CD-1 mice
Serum 25(OH)D levels for female mice that were exposed to low or high levels of vitamin D in utero and during suckling and received a low or high vitamin D diet post-weaning and throughout adulthood. Values are expressed as means ± SEM (Low Low, n=4; Low High, n=6; High Low, n=4; High High, n=6). Different letters indicate there was a significant difference among groups. Dam diet did not affect serum 25(OH)D levels; however, pup diet had an effect. Female mice that consumed a high vitamin D diet post-weaning had significantly higher (p<0.001) serum 25(OH)D levels than female mice that consumed a low vitamin D diet post-weaning.
Table 5.14 Weight, dimensions and biomechanical strength properties of the right femur from female CD-1 mice exposed to low or high vitamin D in utero through to young adulthood

<table>
<thead>
<tr>
<th></th>
<th>Dam Diet&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Pup Diet&lt;sup&gt;2&lt;/sup&gt;</th>
<th>P-value</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td></td>
<td>Dam Diet</td>
<td>Pup Diet</td>
<td>Dam Diet X Pup Diet</td>
</tr>
<tr>
<td>Femur dimensions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (mg)</td>
<td>Low</td>
<td>103.8 ± 2.8&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>107.6 ± 2.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NS</td>
<td>NS</td>
<td>0.044</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>104.9 ± 1.7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>99.1 ± 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Average length (mm)</td>
<td>Low</td>
<td>16.02 ± 0.08</td>
<td>16.29 ± 0.09</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>16.28 ± 0.08</td>
<td>16.27 ± 0.11</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Average AP width (mm)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Low</td>
<td>1.47 ± 0.03</td>
<td>1.50 ± 0.01</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>1.47 ± 0.01</td>
<td>1.44 ± 0.02</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Average ML width (mm)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Low</td>
<td>1.82 ± 0.02</td>
<td>1.83 ± 0.03</td>
<td>0.010</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>1.77 ± 0.02</td>
<td>1.75 ± 0.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femur midpoint</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yield load (N)</td>
<td>Low</td>
<td>18.91 ± 1.23</td>
<td>18.40 ± 0.80</td>
<td>0.014</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>16.46 ± 0.83</td>
<td>16.32 ± 0.67</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Peak load (N)</td>
<td>Low</td>
<td>32.75 ± 1.23</td>
<td>33.36 ± 1.31</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>31.86 ± 1.40</td>
<td>31.08 ± 0.94</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Femur neck</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak load (N)</td>
<td>Low</td>
<td>18.62 ± 0.77</td>
<td>19.55 ± 1.20</td>
<td>0.039</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>17.61 ± 1.02</td>
<td>16.58 ± 0.67</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Values are expressed as mean ± SEM. Values with different letters are significantly different, p < 0.05
<sup>2</sup> Vitamin D Levels: 25 IU/kg diet (Low); 5,000 IU/kg diet (High).
<sup>3</sup> AP width: anteroposterior width at the femur midpoint.
<sup>4</sup> ML width: mediolateral width at the femur midpoint.

Dam diet, Pup diet: Low, Low n=14; Low, High n=14; High, Low n=15; High, High n=15
NS, non-significant, p > 0.05
Table 5.15 Weight, dimensions and biomechanical strength properties of LV$_2$ from female CD-1 mice exposed to low or high vitamin D in utero through to young adulthood$^1$

<table>
<thead>
<tr>
<th>LV$_2$ dimensions</th>
<th>Dam Diet$^2$</th>
<th>Pup Diet$^2$</th>
<th>P-value</th>
<th>Dam Diet</th>
<th>Pup Diet</th>
<th>Dam Diet X Pup Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>52.3 ± 2.5</td>
<td>53.0 ± 2.4</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>49.7 ± 2.6</td>
<td>54.2 ± 3.4</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Average height (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>3.80 ± 0.08</td>
<td>3.84 ± 0.07</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>3.72 ± 0.05</td>
<td>3.89 ± 0.10</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Average AP width (mm)$^3$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>3.47 ± 0.05</td>
<td>3.58 ± 0.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>3.63 ± 0.05</td>
<td>3.63 ± 0.04</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Average ML width (mm)$^4$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>3.45 ± 0.04</td>
<td>3.39 ± 0.03</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>3.41 ± 0.03</td>
<td>3.42 ± 0.02</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Peak load (N)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>87.87 ± 4.05</td>
<td>88.55 ± 7.23</td>
<td>0.041</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>73.57 ± 5.11</td>
<td>80.95 ± 3.85</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Values are expressed as mean ± SEM.
2 Vitamin D Levels: 25 IU/kg diet (Low); 5,000 IU/kg diet (High).
3 AP width: anteroposterior width.
4 ML width: mediolateral width.

Dam diet, Pup diet: Low, Low n=14; Low, High n=14; High, Low n=15; High, High n=15
NS, non-significant, p > 0.05
Figure 5.5 Body weight for female IL-10 KO mice

Body weights of female mice that were exposed to low or high levels of vitamin D in utero and during suckling and received a low or high vitamin D diet post-weaning and throughout adulthood. Values are expressed as means ± SEM (Low Low, n=24; Low High, n=22; High Low, n=19; High High, n=20). The closed circles represent females exposed to low vitamin D in utero and during suckling, and low vitamin D post-weaning and the open circles represent females exposed to low vitamin D in utero and during suckling, and high vitamin D post-weaning. The closed triangles represent females exposed to high vitamin D in utero and during suckling, and low vitamin D post-weaning and the open triangles represent females exposed to high vitamin D in utero and during suckling, and high vitamin D post-weaning. There were no significant differences in body weight between the 4 groups from 5 to 12 weeks of development.
consuming a high vitamin D diet had a higher (p<0.001) vitamin D intake than females consuming a low vitamin D diet (Table 5.10). There was no difference in calcium intake (Table 5.10).

Femur outcomes: There were no differences in femur weight, length, AP width or ML width (Table 5.16). Females who consumed a high vitamin D diet post-weaning had a higher (p=0.005) yield load compared to those fed a low vitamin D diet (Table 5.16). Peak load at both the femur midpoint and femur neck did not differ among females (Table 5.16).

LV2 outcomes: LV2 weight, height, AP width, ML width and peak load did not differ among females (Table 5.17).

Correlation between peak load at multiple skeletal sites: There were no significant relationships between the femur midpoint and LV2 peak load, femur neck and femur midpoint peak load, and femur neck and LV2 peak load (Table 5.13).

5.4 Comparison of Male Versus Female Offspring

5.4.1 CD-1 Study

Post-weaning food intake: Males consumed more (p<0.001) food and had higher vitamin D (p<0.001) and calcium (p<0.001) intakes compared to females (Table 5.6).

Femur outcomes: At the femur midpoint, yield load did not differ between males and females (Figure 5.6); however, males had a higher (p<0.001) peak load compared to females (Figure 5.7). At the femur neck, males had a higher (p<0.001) peak load compared to females (Figure 5.8).

LV2 outcomes: Females had a higher (p=0.023) peak load at LV2 compared to males (Figure 5.9).

5.4.2 IL-10 KO Study

Post-weaning food intake: Males consumed more (p<0.001) food and had higher vitamin D (p=0.025) and calcium (p<0.001) intakes compared to females (Table 5.10).
Table 5.16 Weight, dimensions and biomechanical strength properties of the right femur from female IL-10 KO mice exposed to low and high vitamin D in utero through young adulthood

<table>
<thead>
<tr>
<th></th>
<th>Dam Diet&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Pup Diet&lt;sup&gt;2&lt;/sup&gt;</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td>Dam Diet</td>
</tr>
<tr>
<td>Femur dimensions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (mg)</td>
<td>Low 54.8 ± 1.2</td>
<td>High 55.8 ± 1.3</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>High 55.2 ± 1.1</td>
<td>56.5 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Average length (mm)</td>
<td>Low 14.03 ± 0.09</td>
<td>High 14.01 ± 0.11</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>High 14.07 ± 0.09</td>
<td>14.19 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Average AP width (mm)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Low 1.23 ± 0.01</td>
<td>High 1.22 ± 0.01</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>High 1.22 ± 0.01</td>
<td>1.24 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Average ML width (mm)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Low 1.39 ± 0.02</td>
<td>High 1.42 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>High 1.39 ± 0.01</td>
<td>1.41 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Femur midpoint</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yield load (N)</td>
<td>Low 9.05 ± 0.36</td>
<td>High 9.43 ± 0.25</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>High 8.68 ± 0.35</td>
<td>10.07 ± 0.22</td>
<td></td>
</tr>
<tr>
<td>Peak load (N)</td>
<td>Low 18.22 ± 0.41</td>
<td>High 18.62 ± 0.59</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>High 18.44 ± 0.43</td>
<td>19.83 ± 0.60</td>
<td></td>
</tr>
<tr>
<td>Femur neck</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak load (N)</td>
<td>Low 9.80 ± 0.27</td>
<td>High 9.59 ± 0.43</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>10.10 ± 0.41</td>
<td>10.03 ± 0.37</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Values are expressed as mean ± SEM.
<sup>2</sup> Vitamin D Levels: 25 IU/kg diet (Low); 5,000 IU/kg diet (High).
<sup>3</sup> AP width: anteroposterior width at the femur midpoint.
<sup>4</sup> ML width: mediolateral width at the femur midpoint.

Dam diet, Pup diet: Low, Low n=24; Low, High n=22; High, Low n=19; High, High n=20
NS, non-significant, p > 0.05
Table 5.17 Weight, dimensions and biomechanical strength properties of LV$_2$ from female IL-10 KO mice exposed to low or high vitamin D in utero through young adulthood$^1$

<table>
<thead>
<tr>
<th>LV$_2$ dimensions</th>
<th>Dam Diet$^2$</th>
<th>Pup Diet$^2$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td>Dam Diet</td>
</tr>
<tr>
<td>Weight (mg)</td>
<td>Low</td>
<td>18.1 ± 0.3</td>
<td>17.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>17.8 ± 0.4</td>
<td>18.4 ± 0.4</td>
</tr>
<tr>
<td>Average height (mm)</td>
<td>Low</td>
<td>3.14 ± 0.03</td>
<td>3.06 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>3.11 ± 0.03</td>
<td>3.15 ± 0.03</td>
</tr>
<tr>
<td>Average AP width (mm)$^3$</td>
<td>Low</td>
<td>3.26 ± 0.03</td>
<td>3.30 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>3.25 ± 0.04</td>
<td>3.29 ± 0.03</td>
</tr>
<tr>
<td>Average ML width (mm)$^4$</td>
<td>Low</td>
<td>2.66 ± 0.01</td>
<td>2.66 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>2.67 ± 0.01</td>
<td>2.69 ± 0.01</td>
</tr>
<tr>
<td>LV$_2$ Peak load (N)</td>
<td>Low</td>
<td>46.19 ± 1.37</td>
<td>47.31 ± 1.52</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>44.05 ± 1.54</td>
<td>45.35 ± 1.98</td>
</tr>
</tbody>
</table>

1 Values are expressed as mean ± SEM.
2 Vitamin D Levels: 25 IU/kg diet (Low); 5,000 IU/kg diet (High).
3 AP width: anteroposterior width.
4 ML width: mediolateral width.

Dam diet, Pup diet: Low, Low n=24; Low, High n=22; High, Low n=19; High, High n=20
NS, non-significant, p > 0.05
Figure 5.6 Yield load at the femur midpoint for CD-1 males and females
Comparing yield load at the femur midpoint between CD-1 males and females. Values are expressed as means ± SEM (Males, n=57; Females, n=55). Gender did not affect yield load at the femur midpoint.
Figure 5.7 Peak load at the femur midpoint for CD-1 males and females
Comparing peak load at the femur midpoint between CD-1 males and females. Values are expressed as means ± SEM (Males, n=56; Females, n=54). Different letters indicate there was a significant difference between males and females. Gender had a significant effect on peak load at the femur midpoint. Males had a higher (p<0.001) peak load compared to females.
Figure 5.8 Peak load at the femur neck for CD-1 males and females
Comparing peak load at the femur neck between CD-1 males and females. Values are expressed as means ± SEM (Males, n=54; Females, n=58). Different letters indicate there was a significant difference between males and females. Gender had a significant effect on peak load at the femur neck. Males had a higher (p<0.001) peak load compared to females.
Figure 5.9 Peak load at LV2 for CD-1 males and females
Comparing peak load at LV2 between CD-1 males and females. Values are expressed as means ± SEM (Males, n=55; Females, n=57). Different letters indicate there was a significant difference between males and females. Gender had a significant effect on LV2 peak load. Females had a higher (p<0.001) peak load compared to males.
**Femur outcomes:** At the femur midpoint, gender had a significant effect on both the yield load and peak load. Males had greater yield load (p<0.001) and peak load (p<0.001) compared to females (Figure 5.10 and Figure 5.11, respectively). Gender did not affect peak load at the femur neck (Figure 5.12).

**LV2 outcomes:** Males had a higher (p<0.001) peak load at LV2 compared to females (Figure 5.13).
Figure 5.10 Yield load at the femur midpoint for IL-10 KO males and females
Comparing yield load at the femur midpoint between IL-10 KO males and females. Values are expressed as means ± SEM (Males, n=75; Females, n=81). Different letters indicate there was a significant difference between males and females. Gender had a significant effect on yield load at the femur midpoint. Males had a higher (p<0.001) yield load than females.
Figure 5.11 Peak load at the femur midpoint for IL-10 KO males and females
Comparing peak load at the femur midpoint between IL-10 KO males and females. Values are expressed as means ± SEM (Males, n=75; Females, n=81). Different letters indicate there was a significant difference between males and females. Gender had a significant effect on peak load at the femur midpoint. Males had a higher (p<0.001) peak load compared to females.
Figure 5.12 Peak load at the femur neck for IL-10 KO males and females
Comparing peak load at the femur neck between IL-10 KO males and females. Values are expressed as means ± SEM (Males, n=75; Females, n=80). Gender did not have a significant effect on peak load at the femur neck.
Figure 5.13 Peak load at LV2 for IL-10 KO males and females
Comparing peak load at LV2 between IL-10 KO males and females. Values are expressed as means ± SEM (Males, n=76; Females, n=82). Different letters indicate there was a significant difference between males and females. Gender had a significant effect on LV2 peak load. Males had a higher (p<0.001) peak load compared to females.
Chapter Six

DISCUSSION AND CONCLUSIONS
6.0 DISCUSSION AND CONCLUSIONS

6.1 Discussion

This study was the first to investigate the effects of vitamin D supplementation on body weight and bone dimensions and strength in a mouse model of health, as well as a mouse model of inflammation with associated bone abnormalities. We found that the response to vitamin D differed depending on the model system. Overall, the IL-10 KO mice responded more positively to supplemental levels of vitamin D than the CD-1 mouse model.

Dams: The effects of vitamin D intervention on dams were studied due to the major changes in maternal bone tissue that have been documented during pregnancy and lactation\textsuperscript{169-171}. A similarity between dams in the healthy and inflammatory mouse model was a lack of effect of vitamin D supplementation on body weight, femur and LV\textsubscript{2} dimensions, and LV\textsubscript{2} peak load. However, IL-10 KO dams consuming a high vitamin D diet had a significantly higher peak load at the femur midpoint compared to IL-10 KO dams consuming a low vitamin D diet. Therefore, in the inflammatory mouse model, vitamin D supplementation appears to confer protection in cortical bone. Findings of this thesis research also show no effect of low or supplemental levels of vitamin D on body weight at the end of lactation in CD-1 or IL-10 KO dams. In addition, PND 1 and 14 litter weight for CD-1 offspring and PND 14 litter weight for IL-10 KO offspring, which may act as a surrogate of maternal weight gain, did not differ throughout suckling. This is interesting as research in humans suggests there is an association between vitamin D deficiency and insufficiency during pregnancy and lower maternal weight gain\textsuperscript{137}. One possible explanation for these conflicting results is the lack of definition for low vitamin D status in animal models. Because vitamin D status, as characterized by serum 25(OH)D levels, has not been defined for mice, serum 25(OH)D levels outlined for humans are extrapolated and used in animal studies. Therefore, it is unknown if the diet containing 25 IU vitamin D/kg diet resulted in a low vitamin D status in these dams. As a result, it is unclear if no effect is due to a lack of association between the 2 variables or if low vitamin D status was not attained. More research is needed to explore the relationship between low vitamin D status and body weight, as well as to determine the serum 25(OH)D levels of wild, free-living mice.

During pregnancy and lactation, major changes in maternal bone tissue have been documented. Research in healthy pregnant females suggests despite increased calcium
absorption in the second and third trimesters\textsuperscript{169-170}, calcium mobilization of maternal bone tissue may be necessary to meet the high calcium demand for fetal skeletal development\textsuperscript{170-171}. Calcium mobilization is supported by studies demonstrating an increase in bone resorption during the second and third trimester\textsuperscript{169-172} as well as a reduction in maternal BMD up to 9 months post-partum\textsuperscript{170-172, 174}. These detrimental changes in bone health are important to investigate as increased bone resorption and reduced BMD may result in an increased risk of fracture or developing osteoporosis later in life. Our findings show neither vitamin D supplementation nor lower levels affected bone growth or strength of CD-1 dams. There were no observed differences between CD-1 dam groups for femur dimensions, including weight, length, AP width and ML width, or strength properties, including yield load and peak load at the femur midpoint and peak load at the femur neck. Similarly, there were no differences between diet groups for LV\textsubscript{2} dimensions, including weight, height, AP width and ML width, or peak load of LV\textsubscript{2}. The results are interesting as the high vitamin D diet was expected to place CD-1 dams in a state of vitamin D sufficiency (serum 25(OH)D > 75 nmol/L), similar to that of measured serum levels in female CD-1 offspring, and therefore lead to significantly higher bone size and strength. Vitamin D supplementation was expected to lead to significantly higher serum 25(OH)D and 1,25(OH)\textsubscript{2}D levels\textsuperscript{169-170, 173} resulting in subsequently higher intestinal calcium absorption as previously confirmed in humans\textsuperscript{170}, thereby reducing the need for calcium mobilization from bone. In addition, optimal vitamin D status was expected to enhance bone formation and inhibit bone resorption, thereby rescuing bone from the imbalance in the remodelling process observed during pregnancy\textsuperscript{169-172}. However, results indicated no benefit to bone strength with the supplemental level of vitamin D. These findings may be due to delayed dietary intervention; CD-1 dams were randomized to the vitamin D intervention diets after 3 weeks of age, and therefore the proposed beneficial effects of high vitamin D exposure in utero and during early life were not achieved. This indicates that supplemental vitamin D exposure following 3 weeks of life was unable to optimize bone size and strength during pregnancy and lactation or perhaps there is simply no effect. Both of these aspects are possibilities. Also unexpected was the lack of lower bone size and strength in those CD-1 dams consuming the low vitamin D diet. By design, the low vitamin D diet was expected to result in low vitamin D status, thereby reducing intestinal calcium absorption and further exacerbating calcium mobilization from bone due to a lack of protection against the imbalanced bone remodelling that occurs during pregnancy. Based on these findings, it is hypothesized that although dams were
assumed to have a lower vitamin D status according to human vitamin D status, these levels may not represent a deficient state in mice. Further investigation with lower levels of dietary vitamin D may elucidate the serum levels at which mice become deficient, and subsequent bone abnormalities present. As previously mentioned, the serum 25(OH)D levels of wild, free-living mice are not known but would reveal whether habitual levels are the same as human levels.

The only difference between IL-10 KO dams was a significantly greater resistance to fracture at the femur midpoint in those fed a high vitamin D diet compared to those fed a low vitamin D diet. This was interesting as cortical bone, which is present in high amounts at the femur midpoint, is less metabolically active than trabecular bone, which is present in low amounts at the femur midpoint\textsuperscript{329-330, 342}. Therefore, because trabecular bone is more metabolically active it has shown to be more vulnerable to bone abnormalities in the IL-10 KO mouse model\textsuperscript{273, 275} as well as patients with IBD\textsuperscript{201, 343-345}. However, in concurrence with animal studies\textsuperscript{273, 275}, our findings show that cortical bone was also affected during this chronic inflammatory disease, and that it was responsive to a supplemental level of vitamin D. Bone quality and strength are greatly impaired in IBD as demonstrated by human studies; lower BMC and BMD as well as an increased risk of osteopenia, osteoporosis and fracture are reported in individuals with IBD\textsuperscript{195-201, 203-205, 280-282}. Therefore, preventing, rather than treating, these deleterious bone outcomes is important. Peak load measures the contribution of matrix proteins to the strength of bone. Therefore, greater strength at the femur midpoint with supplemental levels of vitamin D in IL-10 KO dams fed the high vitamin D diet suggests a beneficial effect of higher serum 25(OH)D and 1,25(OH)\textsubscript{2}D levels on the bone matrix. Osteoblasts, which express the enzyme CYP27B1, are affected by 25(OH)D via local metabolism to 1,25(OH)\textsubscript{2}D\textsuperscript{45-48}. As found with in vitro studies\textsuperscript{45-46}, our findings suggest that higher serum 25(OH)D levels upregulated the expression of OCN and OPN, which indicates enhanced bone formation. Therefore, the enhanced incorporation of matrix proteins into bone tissue may have attributed to the higher bone strength at the femur midpoint. Furthermore, physiological levels of 25(OH)D (100 nmol/L) have been shown to enhance osteoblast differentiation\textsuperscript{45} by acting on MSC, osteoprogenitors, and osteoblast precursors\textsuperscript{45, 107-108}. Our higher serum 25(OH)D level may have resulted in a higher number of mature, active osteoblasts ultimately producing a stronger femur midpoint. In addition, serum 25(OH)D levels of 50 nmol/L exert a maximal inhibitory effect on osteoclast-mediated bone resorption\textsuperscript{48}. Therefore, the higher bone strength suggests that the supplemental levels of vitamin D reduced bone resorption.
The direct effects of 1,25(OH)₂D on osteoblasts are similar to those observed with the local metabolism of 25(OH)D. 1,25(OH)₂D stimulates osteoblast proliferation (the number of osteoblasts)⁷⁷ and differentiation⁷²,¹¹⁰. During differentiation, 1,25(OH)₂D acts to increase all stages of development in the osteoblast lineage including MSC, osteoprogenitors, and osteoblast precursors¹⁰⁷-¹⁰⁸,¹¹¹. Therefore, the higher strength at the femur midpoint may be attributed to an enhanced state of bone formation due to the increase in mature, active osteoblasts.

Furthermore, higher bone strength following vitamin D supplementation may be the result of a stronger bone matrix by higher levels of 1,25(OH)₂D. 1,25(OH)₂D optimizes bone matrix by increasing osteoblast production of bone matrix proteins (ie: collagen⁶⁷,⁷⁷, OCN⁶⁷,⁷²,⁸⁴,¹¹⁰-¹¹² and OPN¹¹¹-¹¹²) and enhancing their integration into the matrix⁷⁷. Greater bone strength may also be due to the 1,25(OH)₂D reduction of osteoclast resorptive activity. In addition to the many direct effects of 25(OH)D and 1,25(OH)₂D on bone development, these vitamin D metabolites also have important immunomodulatory actions.

Previous studies have investigated the poor bone health associated with IBD and 1 major factor thought to contribute to the bone abnormalities is the disease process itself, the dysregulated immune response and over-expression of IFN-γ⁶⁵,²⁶²,²⁶⁵,²⁷², TNF-α⁶⁰,²⁶³,²⁷⁴,²⁸³,²⁴⁶, IL-1β²⁶⁰,²⁶³,²⁶⁵,²⁶⁶-²⁶⁸, IL-6²⁰²,²⁶⁰,²⁶³,²⁷²,²⁸³,²⁹⁶, IL-8²⁶³-²⁶⁴, IL-12²⁷²,²³⁴ and RANKL²⁸³,²⁴⁹. Based on our findings of higher bone strength at the femur midpoint with vitamin D supplementation, the increased production of proinflammatory cytokines during IBD may be critical in the pathogenesis of these bone abnormalities. It has been demonstrated that DC express CYP27B1, and therefore locally metabolize 25(OH)D to 1,25(OH)₂D⁵⁰. 25(OH)D regulates DC development and function by reducing their maturation, cytokine production (TNF-α and IL-12) and induction of T cell proliferation⁵⁰. Furthermore, the effects of 1,25(OH)₂D are also important as this metabolite decreases the proinflammatory cytokine production of DC (ie: TNF-α⁵⁰,²⁷⁰, IL-1β⁵⁰, IL-6⁵⁰,²⁶⁹-²⁷¹, IL-8²⁷⁰ and IL-12⁵⁰,²⁶⁹,²⁷¹), Th1 cells (ie: IFN-γ²⁵⁷-²⁵⁹,²⁷², TNF-α²⁵⁸,²⁶⁰,²⁶³,²⁷²-²⁷³, IL-1β²⁶⁰,²⁶³ and IL-6²⁵⁸,²⁶⁰,²⁶³,²⁷²), monocytes (ie: IFN-γ²⁶⁵, TNF-α²⁶⁶-²⁶⁸, IL-1β²⁶⁰, IL-6²⁶⁵,²⁶⁷ and IL-8²⁶⁵), and macrophages (ie: IFN-γ²⁶²,²⁶⁵,²⁷², TNF-α²⁶⁰,²⁶³,²⁷²,²⁷⁴, IL-1β²⁶⁰,²⁶³,²⁷⁴, IL-6²⁶⁰,²⁶³,²⁶⁵,²⁷², IL-8²⁶³-²⁶⁵ and IL-18²⁷²), while increasing the anti-inflammatory cytokine production of Th2 cells (ie: IL-4¹²⁸,¹³¹ and IL-13¹³²-¹³³). Therefore, the higher strength at the femur midpoint may be an indirect result of higher levels of 25(OH)D and 1,25(OH)₂D inhibiting the proinflammatory cytokine production of immune cells. By inhibiting the production of these cytokines, 25(OH)D and 1,25(OH)₂D indirectly reduce
the differentiation of mature osteoclasts as well as decrease their resorptive activity, reduce osteoblast and T cell expression of RANKL, and attenuate osteoblast apoptosis. In addition, these metabolites will also indirectly promote proper osteoblast differentiation, maturation and production of bone matrix proteins. Therefore, findings suggest that the supplemental level of vitamin D attenuated the proinflammatory cytokine induction of bone resorption, in addition to driving bone towards a heightened state of formation and matrix integration. This protective effect of 25(OH)D and 1,25(OH)2D ultimately led to a higher peak load at the femur midpoint.

**Offspring:** The role of vitamin D during growth and development differs between the healthy and inflammatory mouse models. Although exposure to high levels of vitamin D in utero and during suckling in the CD-1 offspring resulted in lower body weights at PND 21, IL-10 KO offspring body weights were not affected. The bone of CD-1 and IL-10 KO mice responded differently to the vitamin D interventions as well. Supplemental vitamin D exposure during intrauterine and early postnatal life resulted in a lower femur neck peak load in CD-1 male offspring and a smaller femur ML width and lower femur midpoint yield load, femur neck peak load and LV2 peak load in CD-1 female offspring whereas there was a protective effect of the supplemental pup diet in the IL-10 KO male and female offspring. The unexpected effects observed in the healthy offspring may result from maternal vitamin D supplementation further increasing serum 25(OH)D and 1,25(OH)2D levels in both the dam and offspring, as 25(OH)D and 1,25(OH)2D levels are already increased during pregnancy. Therefore, in the developing offspring the supplemental levels of vitamin D may result in serum 25(OH)D and 1,25(OH)2D levels that lead to poor bone development. Furthermore, following the sensitive periods of intrauterine and early postnatal life vitamin D does not appear to have an impact on bone strength. In contrast to the CD-1 offspring, IL-10 KO male offspring had significantly larger ML widths and female offspring had a significantly higher femur midpoint yield load when consuming a high vitamin D diet post-weaning. Therefore, in the inflammatory mouse model the main factor affecting bone outcomes appears to be pup diet. The severe intestinal inflammation and injury in the IL-10 KO mouse may have resulted in malabsorption of vitamin D which has been documented in humans with IBD. Therefore, in this model it is hypothesized that maternal serum 25(OH)D and 1,25(OH)2D levels were not elevated enough to cross the placenta or raise the antirachitic activity of milk to levels that would benefit the developing offspring. Another explanation as to the lack of effect of maternal
vitamin D supplementation on offspring bone outcomes is that vitamin D may not have an effect during the developmental periods of intrauterine and early postnatal growth. Therefore, studies investigating higher maternal vitamin D supplementation in this inflammatory mouse model may provide insight as to the lack of effect observed in this study. Findings also suggest that bone development in IL-10 KO mice post-weaning is responsive to vitamin D intervention, whereas the healthy model was more responsive to maternal vitamin D.

The effects of vitamin D appeared to differ between genders in the CD-1 mouse model. Strength outcomes were detrimentally affected more so in CD-1 female offspring exposed to high vitamin D, suggesting an interaction between vitamin D and estrogen. Human studies have demonstrated that during intrauterine growth, human amniotic fluid of female fetuses had significantly higher E\textsubscript{2} levels than males\textsuperscript{351-352}. Furthermore, in vitro studies have shown that in human osteoblasts\textsuperscript{353} and rat colonic tissue\textsuperscript{354}, VDR expression is significantly increased in the presence of 17β-E\textsubscript{2}. This suggests that higher levels of estrogen may result in an increased sensitivity to vitamin D. Therefore, if serum 25(OH)D and 1,25(OH)\textsubscript{2}D levels were too high, leading towards enhanced resorptive activity following maternal vitamin D supplementation, exposure to higher estrogen levels in females may have further increased vitamin D activity towards a state of resorption significantly reducing the strength of bone. The difference in response to vitamin D supplementation was also apparent between genders of the IL-10 KO mouse model. Similar to the CD-1 mouse model, this may be due to higher estrogen levels in females and therefore a higher sensitivity to vitamin D. However, in contrast to the CD-1 mouse model, estrogen may have had a positive effect. It is hypothesized that because the IL-10 KO mouse model may have reduced vitamin D absorption, the supplemental diet results in serum 25(OH)D and 1,25(OH)\textsubscript{2}D levels that positively interact with estrogen and its’ ability to enhance vitamin D sensitivity resulting in enhanced bone formation in the IL-10 KO female offspring. Both CD-1 male and female offspring appeared to be more responsive to vitamin D supplementation than IL-10 KO male and female offspring. A main factor possibly leading to this disparity is malabsorption of vitamin D due to the intestinal inflammation and injury in the IL-10 KO model\textsuperscript{273}.

High vitamin D exposure in utero and throughout suckling resulted in significantly lower body weights at PND 21 in CD-1 male and female offspring. These results were unexpected as many studies have reported an association between maternal vitamin D deficiency during early
pregnancy and the infant being SGA at birth\textsuperscript{139-141}. One study had similar findings where maternal vitamin D deficiency (serum 25(OH)D < 37.5 nmol/L) as well as sufficiency (serum 25(OH)D > 75 nmol/L) during early pregnancy resulted in an increased risk of the infant being SGA at birth\textsuperscript{140}. During pregnancy, serum 25(OH)D and 1,25(OH)\textsubscript{2}D levels are increased\textsuperscript{135, 169-170, 173} and although 1,25(OH)\textsubscript{2}D does not readily cross the placenta, it is hypothesized that supplemental levels of vitamin D raise 1,25(OH)\textsubscript{2}D levels such that there is transfer to the developing offspring\textsuperscript{56}. Developing offspring may be exposed to levels of 25(OH)D and 1,25(OH)\textsubscript{2}D that impair development. 1,25(OH)\textsubscript{2}D is involved in many aspects of fetal growth and development. 1,25(OH)\textsubscript{2}D may inhibit the expression and secretion of hCG, progesterone and estradiol, as well as decrease IR expression resulting in a reduced response to insulin\textsuperscript{146-148}. These changes can result in restricted growth and development.

By design, the 2 vitamin D diets led to significantly different serum 25(OH)D levels in CD-1 offspring at young adulthood, with the low vitamin D diet resulting in deficient concentrations (serum 25(OH)D levels < 50 nmol/L) and the high vitamin D diet resulting in optimal concentrations (serum 25(OH)D levels > 75 nmol/L); serum 25(OH)D levels as defined by human studies\textsuperscript{143, 157, 160, 162-164}. Unexpectedly, high vitamin D exposure in utero and during early postnatal life resulted in a significantly higher fracture risk at the femur neck later in life in CD-1 male offspring. This result was surprising as human studies have shown positive associations between maternal vitamin D status and bone outcomes in children later in life\textsuperscript{143, 157, 159}. Therefore, it is postulated that the supplemental level of vitamin D in the CD-1 dam diet resulted in the exposure of the developing offspring to serum levels of 25(OH)D and 1,25(OH)\textsubscript{2}D that were too high. This finding emphasizes the importance of the concept of nutritional programming\textsuperscript{9-10, 155-156}, as it indicates how influential the external environment (ie: nutritional factors) can be on the development of offspring during the sensitive periods of intrauterine and early postnatal life. LV\textsubscript{2} were significantly heavier in CD-1 males consuming a high vitamin D diet post-weaning but strength of LV\textsubscript{2} did not differ among CD-1 males.

Findings indicate maternal vitamin D supplementation may have been too high, ultimately leading to the high vitamin D exposure of the developing male offspring in utero and during suckling. Studies investigating vitamin D supplementation in animals\textsuperscript{136, 355-356} and humans\textsuperscript{357-358} have documented that serum 25(OH)D levels increase\textsuperscript{136, 355-358} whereas 1,25(OH)\textsubscript{2}D levels decrease\textsuperscript{136, 355}, increase\textsuperscript{300} or remain unchanged\textsuperscript{355-358}. However, vitamin D
supplementation during pregnancy results in a significant increase in 1,25(OH)\(_2\)D levels, above the already increased levels observed during this time\(^{135}\). Furthermore, several studies have indicated that the detrimental effects of toxic levels of vitamin D may not be a product of 1,25(OH)\(_2\)D concentrations, and therefore it is hypothesized that 25(OH)D may be the main metabolite responsible for the toxicity symptoms\(^{356,359}\). In humans, severe toxicity was apparent with serum 25(OH)D levels > 1,600 nmol/L, and hypercalcemia appeared at levels just above 1,250 nmol/L\(^{356,359}\). Although serum 25(OH)D levels of male adult offspring on the supplemental diet did not reach levels of toxicity as defined for humans, neither the true definition of toxicity for mice nor the levels reached in the developing offspring of dams fed a supplemental diet are known. Therefore, further investigation of these 2 aspects may explain the detrimental effects observed with supplemental levels of vitamin D during intrauterine and early postnatal life.

Cells of the osteoclast lineage express the enzyme CYP27B1, and therefore locally synthesize 1,25(OH)\(_2\)D from 25(OH)D\(^{45-48}\). The autocrine effects of 1,25(OH)\(_2\)D\(^{45-46}\) on monocytes/macrophages and osteoclast precursors increases their differentiation (in the presence of RANKL) towards mature osteoclasts\(^{47}\). Therefore, it is hypothesized that continuous stimulation by locally synthesized 1,25(OH)\(_2\)D drives osteoclasts into a higher state of resorptive activity. Because this is occurring during a time that is proposed to be particularly sensitive to influential factors, supplemental exposure of vitamin D at this level may over-stimulate osteoclast development, subsequently programming an exaggerated resorptive response throughout development. With enhanced bone resorption, a loss of bone matrix\(^{85,104-105}\) may have resulted in the lower bone strength at the femur neck. Furthermore, osteoblasts also possess the enzyme CYP27B1 and therefore are affected by the autocrine effects of 1,25(OH)\(_2\)D. Local production of 1,25(OH)\(_2\)D, from serum 25(OH)D levels of 100 nmol/L, enhances RANKL expression of osteoblasts\(^{45}\), which further increases osteoclastogenesis and resorptive activity. In addition to the upregulation of osteoclastogenesis and osteoclast resorptive activity, there is a modest inhibitory effect on osteoblast proliferation at serum 25(OH)D levels of 100 nmol/L\(^{45,108}\), which further causes an imbalance in the opposing processes of bone formation and resorption. Therefore, the high exposure of vitamin D during the sensitive periods of intrauterine and early postnatal growth may have a greater inhibitory effect on the developing osteoblasts, subsequently reducing their proliferation and further driving bone towards a state of resorption.
Serum 25(OH)D and 1,25(OH)₂D levels are both increased during pregnancy. Therefore, with maternal vitamin D supplementation the paracrine effects of 1,25(OH)₂D may have also influenced the lower peak load at the femur neck in CD-1 male offspring. 1,25(OH)₂D acts on osteoblasts to upregulate the expression of RANKL while simultaneously reducing the expression of OPG. With enhanced RANKL expression, the cell to cell communication between osteoblasts and osteoclast precursors may be increased, thereby exaggerating the rate of osteoclast differentiation and activation. This effect is further amplified by the reduced expression of OPG. By decreasing the expression of OPG, the regulation over RANKL and subsequent osteoclast differentiation and activation is lost, thereby further reducing the quality of bone matrix and bone strength.

Skeletal sites of CD-1 female offspring were more influenced by high vitamin D exposure during intrauterine and early life growth compared to males as both cortical and trabecular bone were affected. CD-1 female mice who consumed a high vitamin D diet post-weaning, who were also exposed to high vitamin D in utero and during suckling had significantly lighter femurs compared to those who consumed a high vitamin D diet post-weaning but were exposed to low levels of vitamin D in utero and during suckling. Our study also found that those who were exposed to high levels of vitamin D in utero and during suckling had a significantly smaller femur ML width and lower femur midpoint yield load, femur neck peak load and LV₂ peak load. A lower yield load and peak load suggests a loss in both bone mineral and matrix, respectively, suggesting that maternal supplementation may have been too high. These findings are in contrast to human studies, which have shown positive associations between maternal vitamin D status and bone outcomes in children later in life. The importance of the concept of nutritional programming is emphasized in our findings as they indicate the strong influence that early exposure to nutritional factors can have on the development of offspring later in life. The supplemental level of vitamin D may have led to serum 25(OH)D and 1,25(OH)₂D levels that resulted in a state of bone resorption, reducing both the mineral and matrix components of bone.

The regulation of serum 25(OH)D levels is important as this is the primary metabolite present in bone matrix undergoing active mineralization. Therefore, the smaller femur size and reduced yield load may be a result of dysregulated mineralization due to too high of serum 25(OH)D levels during critical periods of development. Similar to CD-1 male offspring, the
lower femur neck peak load and LV2 peak load, in addition to the lower femur midpoint yield load, in the female offspring may also be due to the autocrine effects of 1,25(OH)2D on cells of the osteoclast lineage. With high serum 25(OH)D levels and the resulting continuous production of 1,25(OH)2D, there may be a shift towards increased bone resorption that is programmed into development during intrauterine and early postnatal growth. Lower bone strength from enhanced osteoclast differentiation and activity may also result from the indirect autocrine and paracrine effects of 1,25(OH)2D on osteoblasts leading to the upregulated expression of RANKL and simultaneous inhibition of OPG expression. Furthermore, osteoblast metabolism of 1,25(OH)2D modestly inhibits osteoblast differentiation and bone formation at physiological levels of 25(OH)D (100 nmol/L). Therefore, with the supplemental levels achieved in the CD-1 female offspring a suppression of osteoblast differentiation would reduce the number of osteocytes and therefore impair bone integrity and mineralization, subsequently lowering bone strength.

Similar to our findings, a study on 24-hydroxylase KO dams also found bone abnormalities in offspring as a result from the disruption of 1,25(OH)2D catabolism. High levels of maternal 1,25(OH)2D led to increased fetal levels, which ultimately resulted in severe impairment of bone mineralization. Specifically, 5-day-old pups presented with reduced amounts of mineralized tissue at the mandible and clavicle. Double KO mice, for 24-hydroxylase and VDR, showed normal bone formation at all skeletal sites, further indicating the detrimental role of elevated 1,25(OH)2D levels on mineralization. Therefore, these findings show that although 1,25(OH)2D is not readily transferred across the placenta, when maternal levels are high there is a corresponding increase in fetal levels. This study also indicates the detrimental effects of high levels of 1,25(OH)2D on developing bone.

Studies investigating vitamin D supplementation have also found detrimental outcomes with relatively high vitamin D. An animal study investigating the impact of 1 subcutaneous dose of 2,000 IU vitamin D less than 24 hours after birth found a significant decrease in whole body BMC and BMD in male rats at 5 months of age. However, there was no effect in females. This is important as it suggests there may be a sensitive period following birth where high exposure to nutritional substances (ie: vitamin D) can detrimentally influence bone quality and development later in life. Furthermore, in a randomized, double-blind, placebo-controlled study the effect of an annual oral dose of 500,000 IU vitamin D on fall and fracture
risk in elderly women (aged 70 years and older) for 5 years was investigated. Results indicated that women receiving an annual high-dose of vitamin D experienced 15% more falls and 26% more fractures than the placebo group, with the number of falls exacerbated in the 3 months following the vitamin D dose. Baseline measurements of serum 25(OH)D and calcium levels did not differ between the vitamin D and placebo groups; however, 1 month after dosing in the vitamin D group the median serum 25(OH)D level was slightly greater than 120 nmol/L, with 82% higher than 100 nmol/L and 24% higher than 150 nmol/L. At 3 months, the median serum 25(OH)D level was approximately 90 nmol/L and 12 months later was on average 41% higher than the placebo group. Conversely, a similar 5 year study with an annual dose of 400,000 IU vitamin D divided into 1 dose every 3 months found that those men and women (aged 65-85 years) in the vitamin D group had a 22% reduction in the rate of first fracture at any site and a 33% reduction in the rate of fracture at major osteoporotic sites (hip, wrist or forearm, or vertebrae). Therefore, considering the findings of these 2 human studies this suggests that using lower and more frequent doses of vitamin D are more beneficial for bone health. Although these studies were focused on adults over the age of 65 years, the results relate to our findings which suggest that high doses of vitamin D may be harmful to bone. This may be especially true during intrauterine and early life development, a period characterized by differentiation and growth which is proposed to be sensitive to environmental influences such as nutritional factors.

In contrast to our findings in the CD-1 mouse model, IL-10 KO mice responded favourably to supplemental vitamin D. This may be because intestinal malabsorption resulted in higher vitamin D needs. Neither low nor supplemental levels of vitamin D affected IL-10 KO male or female offspring body weight throughout development. This was interesting as research in healthy humans has suggested an association between maternal vitamin D deficiency during early pregnancy and SGA infants at birth. Vitamin D status has also been associated with body weight in those affected with IBD; however the relationship between maternal vitamin D status and infant weight at birth still needs to be studied. Reduced serum 25(OH)D levels in children and adolescents (aged 8-22 years) affected with IBD were significantly associated with a lower body weight and BMI. Furthermore, a study involving C57BL/6 IL-10 KO mice demonstrated an association between vitamin D status and body weight. Offspring whose dams were vitamin D deficient throughout pregnancy and lactation and who were weaned to a vitamin D deficient diet (diet devoid of vitamin D) were severely growth retarded beginning at 7 weeks.
of age compared to littermates weaned to a vitamin D sufficient diet\textsuperscript{145}. The conflicting results of this thesis research may be due to the uncertainty as to whether mice were vitamin D deficient at serum 25(OH)D levels defined as deficient in humans. Therefore, future studies are required to elucidate the true serum 25(OH)D levels associated with vitamin D deficiency in this inflammatory mouse model.

Bone strength outcomes of IL-10 KO male offspring were unresponsive to the 2 levels of vitamin D. Although the ML width of the femur was significantly larger in those consuming a high vitamin D diet post-weaning, this structural change was not functionally relevant as there was no difference in strength at this skeletal site among groups. These results were surprising as previous research has indicated the bone abnormalities of this mouse model to be responsive to nutritional intervention\textsuperscript{361}. In contrast to the CD-1 mouse model, the lack of response to supplementation may be due to poor vitamin D intestinal absorption. However, another explanation may be that IL-10 KO male offspring are not responsive to vitamin D intervention. Therefore, further investigation with higher levels of vitamin D is required to determine if the issue is malabsorption. The lack of response to the low vitamin D diet was also interesting. Cantorna et al.\textsuperscript{145} observed severe wasting and presentation of disease symptoms (ie: diarrhea) in C57BL/6 IL-10 KO mice who were truly devoid of vitamin D beginning in utero and continuing throughout postnatal life. However, our 129 Sv/Ev IL-10 KO mice consuming the diet containing 25 IU vitamin D/kg diet appeared healthy and continued to grow until 12 weeks of age.

There were no differences in femur (weight, length, AP width or ML width) or LV\textsubscript{2} (weight, height, AP width or ML width) dimensions, or peak load at the femur midpoint, femur neck or LV\textsubscript{2} among IL-10 KO female offspring groups. However, females consuming a high vitamin D diet post-weaning had a significantly higher yield load at the femur midpoint compared to those consuming a low vitamin D diet. This was interesting as the femur midpoint is mainly composed of cortical bone. Cortical bone is less metabolically active and therefore, thought to be less affected by therapeutic intervention\textsuperscript{329-330,342}. However, as previous studies have shown, skeletal sites rich in cortical bone are compromised in IL-10 KO mice\textsuperscript{273,275} but also improved using dietary intervention\textsuperscript{361}. Similar to the male offspring, IL-10 KO female offspring experienced a beneficial effect of vitamin D supplementation which was in contrast to the CD-1 female offspring.
A higher yield load, which measures the contribution of mineral to bone strength, suggests that exposure to a high vitamin D diet during growth and development results in greater elasticity and mineral content at the femur midpoint at young adulthood in IL-10 KO female offspring. Similar to IL-10 KO dams, the mechanism by which supplementation leads to a higher yield load may be through higher serum 25(OH)D and 1,25(OH)2D levels, and the effect these metabolites have on bone cells. 25(OH)D is important in bone mineralization as it is the main vitamin D metabolite present in bone matrix undergoing active mineralization\cite{106}, and therefore supplemental levels of 25(OH)D may optimize the integration of calcium into bone matrix\cite{61-63} resulting in a greater mineral content. The effects of 25(OH)D on osteoblasts are also critical in bone mineralization. Physiological levels of 25(OH)D (100 nmol/L), through the metabolism to 1,25(OH)2D, enhance the incorporation of calcium into the ECM, and therefore increase bone mineralization and strength\cite{45-48}. Furthermore, physiological levels of 25(OH)D enhance osteoblast differentiation and function leading to the increased differentiation of osteocytes\cite{45} and greater structural integrity\cite{88}. In addition to enhancing bone formation, 25(OH)D also reduces bone resorption to improve bone development. Increasing 25(OH)D concentrations dose-dependently inhibit osteoclast-mediated bone resorption, with a maximal inhibitory effect at 50 nmol/L\cite{48}. The paracrine effects of 1,25(OH)2D are also important as this metabolite stimulates the differentiation of osteoblasts\cite{72,110}, thereby increasing the production of osteocytes and enhancing the mineralization of bone\cite{72,77,110-111} as well as reduces the resorptive activity of osteoclasts\cite{48,77}. Therefore, it is hypothesized that the supplemental level of vitamin D enhanced bone mineralization\cite{45-48,61-63,72,77,110-111} while simultaneously reducing hydroxyapatite degradation\cite{48,77,85,102-103} resulting in a higher yield load at the femur midpoint. However, vitamin D supplementation may also affect the bone abnormalities by regulating the enhanced immune response that presents with IBD.

Studies on adults\cite{196-197,199,201,205} and children\cite{195,198,200,202-204} affected with IBD have demonstrated the severe bone abnormalities (ie: reduced BMC and BMD, and increased risk of osteopenia, osteoporosis and fracture) that present with this chronic disease. With studies implicating the enhanced production of proinflammatory cytokines and RANKL as a main factor involved in the development of these abnormalities\cite{202,283,294,296}, preventative interventions that may reduce the dysregulated immune response are important. Recently, immunomodulatory actions of vitamin D have been described and therefore, the higher yield load at the femur midpoint of IL-10 KO female offspring following post-weaning vitamin D
supplementation may be due to a diminished immune response as previously described for IL-10 KO dams receiving the supplemental vitamin D diet. Autocrine and paracrine effects of 1,25(OH)₂D have been described to reduce the proinflammatory cytokine production of immune cells (ie: DC, Th1 cells, monocytes, and macrophages)⁵⁰, ²⁵⁸, ²⁶⁰, ²⁶³, ²⁷²-²⁷³ while increasing the anti-inflammatory cytokine production of Th2 cells¹²⁸, ¹³¹, ¹³²-¹³³. By diminishing the dysregulated immune response, the vitamin D metabolites indirectly reduce the heightened state of bone resorption²⁸⁴, ²⁸⁶-²⁹², ²⁹⁷, attenuate osteoblast apoptosis²⁹³ and promote proper osteoblast differentiation and maturation²⁹⁴, ²⁹⁶. As a result, the increased degradation of hydroxyapatite from bone tissue is attenuated⁸⁵, ¹⁰²-¹⁰³ and osteoblast function is enhanced, thereby leading to overall mineralization of bone tissue⁶¹-⁶⁵. Therefore, the higher yield load at the femur midpoint of IL-10 KO female offspring implicates the disease process in the development of IBD associated bone abnormalities as well as demonstrates the ability of vitamin D supplementation to protect bone mineral composition from this chronic, autoimmune disease.

**Correlation between peak load at multiple skeletal sites:** The relationship between peak load at various skeletal sites differs by gender in the CD-1 mouse model. In CD-1 male offspring, there was a significantly modest positive relationship between the peak load at the femur midpoint and LV₂. This was interesting as these 2 sites differ in their bone composition, with the femur midpoint rich in cortical bone and LV₂ rich in trabecular bone³²⁹-³³⁰, ³⁴². However, this relationship makes biological sense as cortical and trabecular bone are interdependent in that they are anatomically and functionally distinct, yet are both regulated by osteoblasts and osteoclasts³⁶². In CD-1 female offspring, there was a significantly modest positive relationship between the peak load at the femur neck and LV₂. These sites are rich in trabecular bone and therefore an association is expected. In contrast to our findings in the CD-1 mouse model, there were no significant relationships between the peak load of various skeletal sites in IL-10 KO male and female offspring.

**Offspring gender comparison:** In each mouse model we statistically examined the effect of gender on post-weaning food intake and bone strength outcomes. CD-1 and IL-10 KO male offspring consumed more food, and therefore had a higher vitamin D and calcium intake, compared to CD-1 and IL-10 KO female offspring, respectively. CD-1 male offspring had a higher peak load at both the femur midpoint and femur neck and IL-10 KO male offspring had a higher yield load and peak load at the femur midpoint, as well as a higher peak load at LV₂.
compared to CD-1 and IL-10 KO female offspring, respectively. This makes biological sense as male mice are naturally larger than female mice. However, yield load at the femur midpoint did not differ between CD-1 genders and this suggests that for healthy CD-1 offspring bone mineralization that occurs during growth and development at this skeletal site does not differ between males and females. Gender also did not affect peak load at the femur neck in IL-10 KO offspring. This finding suggests that for IL-10 KO offspring the accumulation of bone matrix at this skeletal site is not affected by gender. Peak load at LV2 was higher in CD-1 female offspring compared to male offspring suggesting that the accumulation of bone matrix at this skeletal site was greater in females.

6.2 Conclusions

**CD-1 and IL-10 KO Dams:** Supplemental levels of vitamin D during pregnancy and lactation did not affect body weight, or dimensions or strength of femurs and LV2 at the end of lactation in CD-1 dams. In IL-10 KO dams, supplemental levels of vitamin D during pregnancy and lactation did not affect body weight, femur and LV2 dimensions, or femur neck and LV2 strength but did result in higher peak load at the femur midpoint at the end of lactation.

**CD-1 Offspring:** In males, supplemental exposure to vitamin D in utero and during suckling (PND 1-21) resulted in a lower body weight at PND 21 and a lower femur neck peak load at 14 weeks of age. Femur dimensions, midpoint yield load and peak load, and LV2 dimensions and peak load were not affected. In females, supplemental exposure to vitamin D in utero and during suckling resulted in a lower body weight at PND 21, and lower femur ML width, femur midpoint yield load, and femur neck and LV2 peak load at 14 weeks of age. Femur length, AP width, femur midpoint peak load or LV2 dimensions were not affected.

**IL-10 KO Offspring:** In males and females, supplemental exposure to vitamin D in utero and during suckling (PND 1-28) did not affect body weight, or dimensions or strength of femurs and LV2.
Chapter Seven

FUTURE DIRECTIONS
7.0 FUTURE DIRECTIONS

A serum 25(OH)D level of 50 nmol/L has been deemed necessary for optimal bone development by the IOM. However, some researchers suggest this cut-off value is too low, especially to benefit overall health, and therefore urge for serum 25(OH)D levels > 75 nmol/L to be considered optimal. This thesis research was conducted in a healthy and inflammatory animal model to determine the impact of intrauterine and early postnatal life vitamin D supplementation on bone health at young adulthood. A benefit of using a mouse model to investigate the effects of nutritional intervention is the useful structural and functional information (ie: bone size and strength) collected that otherwise is not available in human studies. However, because these studies were conducted in an animal model it is unclear how the findings may relate to humans. Therefore, human studies are required to determine the true impact of higher vitamin D supplementation during pregnancy and lactation in healthy and inflammatory states of health on child bone development and overall health later in life. Only one study has investigated the impact of vitamin D supplementation during healthy pregnancy on maternal and neonatal serum 25(OH)D levels but bone outcomes were not investigated. In addition, vitamin D intervention did not begin until 12-16 weeks of gestation. Vitamin D supplementation trials in adults with varying levels of supplementation have yielded conflicting results. Table 7.0 outlines aspects of bone development that may be useful to measure in states of health and/or inflammation in future human studies. Findings may provide a more comprehensive understanding of the effects of vitamin D supplementation throughout sensitive periods of growth on bone development and overall health.

7.1 Future Direction of Human Studies

Longitudinal intervention trials are required to determine the effects of maternal vitamin D supplementation during pregnancy and lactation on offspring bone development later in life. Findings from this thesis research in the healthy mouse model emphasize the importance of maternal vitamin D supplementation with levels that will optimize serum 25(OH)D and 1,25(OH)2D levels in the growing fetus without reaching levels that are too high for skeletal development. Although one intervention trial investigated the effect of maternal vitamin D supplementation during pregnancy on infant serum 25(OH)D levels at birth, assessment of bone development must also be investigated. Bone outcomes such as BMC and BMD could be
### Table 7.0 Future directions for human studies

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<td><strong>All studies:</strong></td>
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measured at various timepoints throughout development into young adulthood to assess the impact of vitamin D supplementation on achieving PBM. In addition, measurement of bone biomarkers for formation and resorption could indicate the influence of vitamin D supplementation on the coupling of these 2 processes and the effect on bone health. One study in young girls indicated that the benefit of vitamin D supplementation on whole-body BMD may be dependent upon VDR genotype\textsuperscript{299}. Therefore, further investigation of susceptible genotypes may allow researchers to target vitamin D intervention to those who are more responsive.

Future studies must also focus on the effect of vitamin D intervention on overall non-skeletal health, and the level of supplementation required to achieve potential benefit. Studies could also investigate further vitamin D fortification of foods commonly consumed during childhood and adolescence to increase vitamin D intake as there are concerns with respect to compliance when relying on children to consume vitamin D supplements\textsuperscript{193}.

In humans affected with IBD issues of nutrient malabsorption\textsuperscript{361-362} have been documented, with 1 study focusing specifically on vitamin D malabsorption\textsuperscript{350, 363-364}. Future studies could further investigate these findings to establish the true effect of intestinal inflammation on the absorption of vitamin D, and whether this is a major factor in determining vitamin D status.

One limitation of maternal vitamin D supplementation trials is the confounding fetal and postnatal exposure to factors that may impede the characterization of the relationship between vitamin D and bone. One example is the concurrent supplementation with calcium which is a confounder in many human intervention trials. Due to the complex interaction between these 2 nutrients, supplementation with both impedes the ability of researchers to determine the effects of each nutrient individually. Therefore future studies, especially in children, could focus more on vitamin D supplementation alone.

7.2 Future Direction of Animal Studies

This thesis research focused on the functional outcome of bone size and strength in a healthy and inflammatory mouse model. One limitation of the mouse model as a research tool, especially for this thesis research, is the lack of definition for characterizing vitamin D status in either mouse model. Because serum 25(OH)D levels representing deficiency, insufficiency and sufficiency have not been defined for mice, studies investigating vitamin D intervention must
use human levels, which may be too high. Therefore, prior to further investigation of vitamin D intervention on bone outcomes in a mouse model of health or inflammation, future research should investigate the vitamin D status of mice living in the natural environment. Mice are commonly nocturnal and typically hide among brush and debris thereby limiting their exposure to UVB irradiation and subsequent endogenous synthesis of vitamin D$_3$. In addition, it is assumed that ingestion of vitamin D would be minimal to nonexistent. Therefore it is hypothesized that serum 25(OH)D levels of free living mice would represent a state of deficiency. The deficient status could then be replicated in a laboratory setting and the impact on bone outcomes such as size and strength could be quantified.

Furthermore, it may be prudent to investigate the impact of vitamin D intervention on alternative animal models such as the guinea pig. Similar to human studies$^{143, 157, 170-172, 174}$ the guinea pig model has reported that vitamin D deficiency (no vitamin D in the diet) during pregnancy results in reduced femur BMD in dams and reduced whole body BMC in fetuses at day 57 of gestation$^{365}$. Therefore, future studies investigating the structural and functional impact of vitamin D supplementation on strength outcomes and overall health may be an area of interest for this animal model.
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8.0 REFERENCES


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