The generation of C-3α epimer of 25-hydroxyvitamin D in adult Sprague Dawley rats and older adult humans

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DEDICATION

This thesis is dedicated to my beloved grandmother, Edda Bianchini.

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Manuscript 1

Manuscript 2

ABSTRACT

The C-3α epimer (C-3 epimer) of 25-hydroxyvitamin D_3 ($25(OH)D_3$) represents a high percentage of total $25(OH)D_3$ concentration, yet its origin and potential function(s) are unknown. *In vitro,* C-3 epimer is less potent than its $25(OH)D_3$ native form in exerting effects on calcium homeostasis. *Study 1*: The aim of this study was to determine C-3 epimer profiles in response to 2000 IU supplementation of vitamin D (VD) in an elderly population and to explore its relationship to calcium homeostasis. Elderly men (n=40) residing at a long-term care hospital in Quebec received a supplement of 2000 IU/day of VD_3 for 8 weeks. At beginning and end of study, dietary assessment was performed, anthropometric data was collected and blood sampled for measurement of iCa and VD metabolites: $25(OH)D_3$, 24,25 dihydroxyvitamin D ($24,25(OH)_2D_3$) and C-3 epimer were quantified using liquid chromatography tandem mass spectrometry (LC-MS/MS). Plasma total 25(OH)D, parathryoid hormone (PTH) and osteocalcin (OC) (Liaison, Diasorin Inc) were measured by immunoassays at beginning and end of study. Associations between these biochemical measures were assessed using Pearson correlation coefficients and multiple linear regression analysis was performed. Paired t-tests were used to detect differences of means before and after supplementation. Plasma 25(OH)D (LC-MS/MS) increased after 8 weeks of supplementation from 52.4 ± 2.4 nmol/L at baseline to 81.4 ± 2.3 nmol/L at week 8 ($p<0.05$). Despite the increase in 25(OH)D and intakes of VD over 2000 IU/d, C-3 epimer was not quantifiable in any of the participants and thus linkages to calcium homeostasis were not evident. These data suggest that C-3 epimer is not a major component of VD status in an elderly population with VD status above targets known to have benefits to bone health. To clarify, a younger mammalian system was studied in a dose-response manner. *Study 2*: The aim of this study was to establish if different doses of VD_3 result in a proportionate dose-response in C-3 epimer and determine the biological response of bone to C-3 epimer in a healthy adult rodent model. Sprague Dawley rats (12 weeks, n=36 female n=36 male) were randomized to 1 of 6 diets for 8 wk: control AIN93-M diet (1 IU VD_3/g diet) or experimental diets of 2 and 4 IU/g diet of cholecalciferol, 0.5 and 1 IU/g diet of C-3 epimer, and a 0.5 IU/g diet of 25(OH)D group. At wk 0, 4 and 8, blood samples were collected and whole body and regional bone mass and architecture examined. VD metabolites ($25(OH)D_3$, $24,25(OH)_2D_3$, C-3 epimer) were quantified using LC-MS/MS as well as bone biomarkers. MIXED model ANOVA for repeated measures was performed with Bonferroni adjustment. Exogenous cholecalciferol, 25(OH)D and

C-3 epimer diets were reflected in plasma concentrations in a dose-response manner. In females in the 4 IU cholecalciferol diet group, C-3 epimer concentration (mean 84.6 ± 25.5 nmol/L) was greater than control (mean 21.4 ± 7.6 nmol/L, $p<0.05$). In males, significant differences among diet groups for epimer concentrations were not present; the 4 IU cholecalciferol diet group had C-3 epimer concentrations of 32.1 ± 13.7 nmol/L compared to 9.2 ± 3.9 nmol/L on control diet. No significant differences were observed by 8 wk among diet groups for weight, dietary intake, iCa and bone biomarkers. These data suggest that circulating C-3 epimer concentrations are dependent on the amount of cholecalciferol intake and at moderate concentrations do not affect maintenance of bone mineral density in mature rodents. This thesis research showed that high C-3 epimer concentrations are not seen in very elderly men at ranges of VD status that are known (≥50 nmol/L) to have benefits for human health whereas in female rodents, C-3 epimer is generated in high concentrations in response to VD supplementation.

RÉSUMÉ

Le C-3α épimère (C-3 épimère) de l'hydroxyvitamine D_3 ($25(OH)D_3$) compte pour un pourcentage important de la concentration totale de $25(OH)D_3$ et pourtant, son origine et ses effets sont inconnus. *In vitro,* le C-3 épimère produit moins d'effet sur le métabolisme des cellules osseuses que sa forme originelle $25(OH)D_3$.

Étude 1 : L'objectif de cette étude était de déterminer la réponse en C-3 épimère à une supplémentation de 2000 IU de vitamine D (VD) chez une population âgée, et d'étudier ses effets sur le métabolisme des cellules osseuses. Pendant 8 semaines, 40 hommes âgés ont reçu 2000 IU/jour de VD_3. Au début de l'étude et après 8 semaines, les métabolites de VD présentes dans le plasma, dont $25(OH)D_3$, $24,25(OH)_2D_3$ et le C-3 épimère, ont été quantifiés, à l'aide de la chromatographique liquide et de la spectrométrie de masse (LC-MS/MS), et les différences ont été testées grâce à des tests pairés. Les associations entre ces marqueurs biochimiques ont été attestés au moyen de coefficients de corrélation de Pearson. Apres 8 semaines de supplémentation, le taux de 25(OH)D présent dans le plasma a augmenté (semaine 0: 52.4 ± 2.4 nmol/L; semaine 8: 81.4 ± 2,3, nmol/L $P<0.05$). tandis que le taux de C-3 épimère demeurait sous le seuil de détectabilité. Ces données suggèrent que chez les individus âgés dont les taux de VD sont supérieurs aux niveaux connus pour être bénéfiques à la santé osseuse, le C-3 épimère n'est pas un composant majeur de la VD. Afin de clarifier ce point, une seconde étude a été menée, visant à évaluer la réponse de l'organisme de plus jeunes mammifères. **Étude 2** : L'objet de cette étude est d'établir si différentes doses de VD_3 induisent une production proportionnelle de C-3 épimère, et de déterminer la réponse biologique osseuse au C-3 épimère chez un rongeur adulte en santé. Des rats Sprague Dawley (âgés de 12 semaines, n=36 femelles et n=36 mâles) ont été répartis de manière aléatoire selon 6 types de régimes pendant 8 semaines ; le régime de contrôle AIN93-M (1 IU VD_3/g de nourriture), 4 régimes expérimentaux avec respectivement 2 et 4 IU/g de nourriture de cholecalciferol, et 0.5 et 1 IU/g de nourriture de C-3 épimère, et un régime avec 0.5 IU/g de nourriture de 25(OH)D. Lors des semaines 0, 4 et 8, des échantillons sanguins ont été collectés, et la masse et l'architecture osseuse ont été examinées. Les métabolites de la VD ($25(OH)D_3$, $24,25(OH)_2D_3$, C-3 épimère) ont été quantifiés à l'aide de LC-MS/MS, de même que des biomarqueurs osseux. Une modélisation MIXTE ANOVA pour mesures répétées a été réalisée, avec un ajustement Bonferroni. Les taux de cholecalciferol

éxogène, 25(OH)D et de C-3 épimère mesurés étaient proportionnels aux taux de cholecalciferol, 25(OH)D et de C-3 épimère éxogènes des différents régimes. Chez les femelles du groupe associé au régime avec 4 IU de cholecalciferol, la concentration en C-3 épimère (84.6 ± 25.5 nmol/L) était supérieure à celle de l'échantillon de contrôle (21.4 ± 7.6 nmol/L, $p<0.05$). Chez les mâles, les concentrations d'épimères ne présentent pas des différences significatives : le taux de C-3 épimère a été mesuré à 32.1 ± 13.7 nmol/L pour le groupe recevant 4 IU de cholecalciferol et à 9.2 ± 3.9 nmol/L chez le groupe contrôle. Aucune difference significative n'a été détectée entre les différents groupes, en ce qui concerne le poids, la consommation alimentaire, le taux d'iCa, ou les biomarqueurs osseux. Ces données suggèrent que le C-3 épimère est sécrété de manière proportionnelle à la consommation de cholecalciferol, mais n'a pas d'effet sur le maintien de la densité minérale osseuse chez le rongeur adulte.Les recherches de cette thèse montrent que l'on n'observe pas chez l'homme très âgé de fortes concentrations de C-3 épimère, comme des niveaux de VD connus pour avoir des effets bénéfiques sur la santé chez l'être humain, tandis que chez la femelle rongeur, le C-3 épimère est sécrété en grande quantité suite à une supplémentation en VD.

LIST OF ABBREVIATIONS

$1,25(OH)_2D_3$: 1,25-dihydroxyvitamin D (Calcitriol)

$1,24,25(OH)_3D_3$: Calcitroic Acid

$25(OH)D_3$: 25-hydroxyvitamin D_3

$24,25(OH)_2D_3$: 24,25-dihydroxyvitamin D_3

3-epi-$25(OH)D_3$: C-3α epimer of 25-hydroxyvitamin D

3-epi-$1,25(OH)_2D_3$: C-3α epimer of 1,25-dihydroxyvitamin D

7-DHC: 7-Dehydrocholesterol

ANOVA: Analysis of Variance

BA: Bone Area

BGLAP: Bone Gamma-Carboxyglutamic Acid-containing Protein

BMD: Bone Mineral Density

BMC: Bone Mineral Content

BS/BV: Bone Surface to Bone Volume ratio (specific bone area)

BV/TV: Bone Volume to Tissue Volume ratio

vBMD: Volumetric Bone Mineral Density

aBMD: Areal Bone Mineral Density

C-3 epimer: C-3α epimer of 25-hydroxyvitamin D

Ct.Th: Average Cortical Thickness

Ct.Po: Cortical Porosity

CTX: Collagen C-telopeptde

CYP27A1: Cytochrome P450 27A1

CYP2R1: Cytochrome P450 2R1

CV: Coefficient of Variation

DBP: D-Binding Protein

DEQAS: Vitamin D Quality Assurance Scheme

DRI: Dietary Reference Intakes

DXA: Dual-energy X-ray Absorptiometry

EAR: Estimated Average Requirement

FGF23: Fibroblast Growth Factor 23

FGFR: Fibroblast Growth Factor Receptor

KO: Knock-Out

HPLC: High Pressure Liquid Chromatography

IOM: Institute of Medicine

LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry

M-CSF: Macrophage-Colony Stimulating Factor

MRI: Magnetic Resonance Imaging

nCT: Nanocomputed Tomography

NIST: US National Institute of Standards and Technology

OC: Osteocalcin

OPG: Osteoprotegerin

Po.V: Total Pore Volume

PTH: Parathyroid Hormone

pQCT: Peripheral Quantitative Computed Tomography

RANK: Receptor Activator of Nuclear Factor-κB

RANKL: Receptor Activator of Nuclear Factor-κB ligand

RDA: Recommended Dietary Allowance

RXR: Retinoid Acid Receptor

TRPV6: Transient Receptor Potential cation channel subfamily V member 6

Tb.N: Trabecular Number

Tb.Sp: Trabecular Separation

Tb.Th: Trabecular Thickness

UVB: Ultraviolet Beta

VD: Vitamin D

VDREs: Vitamin D Response Elements

μCT: Microcomputed Tomography

CONVERSION FACTORS

1 ng/mL of 25(OH)D = 2.5 nmol/L
1 μg of vitamin D = 40 IU

CONTRIBUTION OF AUTHORS

C. Bianchini is the primary author and candidate for this thesis. For manuscript 1, the candidate was responsible for measurement of 25(OH)D, PTH and OC with chemiluminescence assay and assisting dietary assessments as well as performing statistical analyses and data interpretation. For manuscript 2, the candidate was responsible for data collection, statistical analyses and data interpretation.

S. Agellon was the laboratory technician who helped with the blood sample processing, order management of all necessary assays and laboratory equipment required for both studies.

P. Lavery was the laboratory technician involved in animal welfare, blood sample collection, DXA and μCT analysis as well as animal sacrifice.

I. Germain was the PhD candidate who performed the Sainte Anne Hospital randomized controlled trial from which the blood samples were used for manuscript 1. She completed recruitment of participants as well as many dietary assessments and data entry.

H. Weiler is the principle investigator of these studies and was involved in the development and grant proposal stages. She supervised and ensured timely and efficient conduction and successful completion of both studies.

1.0 LITERATURE REVIEW

1.1 INTRODUCTION

The precursor of vitamin D_3, 7-dehydrocholesterol, is a prohormone that gives further rise to daughter hormones that exert their own biological effects. Of these metabolites 25-hydroxyvitamin D_3 ($25(OH)D_3$), is the major circulating form used for assessment of vitamin D status (1). The 25(OH)D metabolite was initially discovered to be effective in curing rickets and in the initiation of calcium transport (2). However as more metabolites were discovered, a more polar metabolite was identified that was formed in the proximal renal tubules of the kidney and initiated calcium transport in the duodenum of the intestine and mobilization of bone calcium more readily than $25(OH)D_3$. This metabolite is known as 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and has been recognized as the most biologically active metabolite (3). There is some evidence against and some evidence in favour of the 24-hydroxylated molecule, 24,25 dihydroxyvitamin D_3 ($24,25(OH)_2D_3$), as another indispensable and biologically important metabolite. The evidence regarding $24,25(OH)_2D_3$ as a biologically relevant molecule is mostly focused on fracture repair, however the most recent research supports the 24 hydroxylation as most important in generating $1,24,25(OH)_3D_3$ as a catabolite of $1,25(OH)_2D_3$ (4). Research on the biological significance of vitamin D metabolites has lead to further understanding of vitamin D and its physiological benefits, which have been shown to extend beyond skeletal health. These newly discovered health benefits along with vitamin D deficiency in many populations has substantially increased the demand for vitamin D assessments.

A number of analytical techniques exist for the quantitation of 25(OH)D, of which high pressure liquid chromatography (HPLC) and liquid chromatography tandem mass spectrometry (LC-MS/MS) are widely accepted. The latter has led to new discovery of a smaller peak that coelutes with 25(OH)D and is now known to be C-3α epimer of 25(OH)D (C-3 epimer). The conversion of the C3 ring of 25(OH)D from beta to alpha orientation results in the C-3 epimer (Figure 1). The interest in the C-3 epimer molecule can be mainly ascribed to evidence demonstrating that it can contribute significantly to total 25(OH)D measurements with highest concentrations found in infant populations (5). According to a recent review of the analytical measurement of C-3

epimer, approximately nine studies have quantified C-3 epimer in infant (<1 year of age), pediatric (1-18 years of age) and adult populations (> 18 years of age) where one was solely an adult investigation and two were unknown populations (6). The reported range of % C-3 epimer contribution of total 25(OH)D_3 concentrations across adult studies was rather wide (up to 47%) in comparison to the measured C-3 epimer mean of 4.3 nmol/L in these studies. In contrast, infant populations had higher mean C-3 epimer concentrations (18.2 nmol/L) better corresponding to the high range of % C-3 epimer contribution to total 25(OH)D (up to 61.1 %) (6). Therefore, further studies are required to clarify these discrepancies and verify the significance of C-3 epimer contribution in all populations.

All major vitamin D metabolites undergo this epimerization process, which has been shown to serve a regulatory function in other physiological pathways (7, 8). Both *in vitro* and *in vivo* studies suggest that C-3α epimer of 1,25$(OH)_2D_3$ (3-epi-1,25$(OH)_2D_3$) provides some of the physiological benefits of its native form (6). Compared to 1,25$(OH)_2D_3$, 3-epi-1,25$(OH)_2D_3$ binds to the vitamin D receptor (VDR) at approximately 2-3% (7). In contrast to its low affinity to the VDR, it is equally as effective in suppressing parathryoid hormone (PTH) relative to 1,25$(OH)_2D_3$ (9, 10). However, its capacity to induce certain genes such as *BGLAP* (osteocalcin), promote intestinal calcium reabsorption and its role in anti-proliferation and differentiation is reduced (6, 11). The higher metabolic stability of 3-epi-1,25$(OH)_2D_3$ over 1,25$(OH)_2D_3$ might compensate for its few equipotent physiological abilities (12). Indeed, in infants with variable amounts of C-3 epimer, bone health outcomes did not differ (13).

Owing to high amounts of the C-3 epimer in infants, sources were hypothesized to be from mothers milk and related microbiota, supplementation or dietary sources (5). The source of C-3 epimer in humans of all ages remains unknown, although it is now proposed to be either exogenously obtained or endogenously produced. Exogenous sources of C-3 epimer have been proposed such as vitamin D supplements, though a recent study reported that liquid vitamin D supplements contained no detectable amounts of C-3 epimer (14). Endogenous synthesis has been suggested as high doses of 25(OH)D (500 μg) given to adult rats resulted in rapid production of C-3 epimer at concentrations of 40.5 nmol/L after approximately 6 hours (7). It is

thus possible that both endogenous and exogenous sources contribute to generation of C-3 epimer, the extent of each source is unclear in humans.

In summary, there is a large need for standardization of $25(OH)D_3$ measurement as classification of vitamin D deficiency is being confounded by assay variability (15). The C-3 epimer is shown to be a contributor to this variability however the clinical consequences of reporting C-3 epimer concentrations are still unclear. The source and physiological role of C-3 epimer needs to be elucidated in order to understand the relevance of high or low values of C-3 epimer (6).

1.2 VITAMIN D

1.2.1 Sources

Ultraviolet beta (UVB) solar radiation (290-315 nm) is regarded as a major source of vitamin D as UVB elicits photochemical and thermal conversion of 7-dehydrocholesterol in the epidermis of the skin to vitamin D_3 (Figure 1) (16). The use of sunscreen and a darker skin pigmentation both absorb UVB rays and thus limit the amount of endogenous vitamin D_3 production. Along with these factors, latitude, altitude, air quality, time of day, month of year and cloud cover all affect the amount of UVB radiation reaching skin (17). MacLaughlin and Holick (18) demonstrated a twofold decrease in the capacity of the skin to produce vitamin D_3 in elderly people from 77 to 82 years of age when compared to younger adults of 8 to 18 years, confirming that the efficiency of cutaneous vitamin D_3 synthesis is partially dependent on age and ascribed mainly to a reduction in 7-dehydrocholesterol (18). As a basis for setting Dietary Reference Intakes (DRI), it is assumed that UVB exposure is minimal and that intakes are set at values that minimize the risk of dietary inadequacy (19). Considering Canada's high latitude (>42 °N), it can be assumed that endogenous synthesis of vitamin D is negligible during the winter for all Canadians and this therefore increases the reliance on dietary sources. In terms of dietary sources, vitamin D exists in 2 isoforms; vitamin D_2 or vitamin D_3 (17). Vitamin D_2, known as ergocalciferol, is a fungal secosteroid derived from photochemical conversion of ergosterol, whereas vitamin D_3 (cholecalciferol) is the animal form derived from cholesterol (4). Foods containing high amounts of vitamin D_3 are oily fish such as cod and tuna (4). Other dietary sources include supplements as well as fortified foods. Vitamin D_2 can be produced by

irradiation of ergosterol in shiitake mushrooms and yeast yet very little vitamin D_2 is obtained through the diet, and is mostly from supplementation (4). The Canadian Food and Drug Regulations require mandatory fortification of specific foods; margarine and milk (20). The fortification of foods in Canada has been proven to be beneficial in increasing concentrations of serum 25(OH)D through various randomized controlled trials (21). Another source of vitamin D is adipose stores. Brouwer et al (22) showed through an experiment in rats that adipose tissue acts as a safeguard to release vitamin D stores under fasting conditions. In humans, vitamin D storage can last from months up to years. Although adipose tissue is the main storage site for cholecalciferol, vitamin D is also stored widely in tissues such as muscle and liver (23).

1.2.2 Metabolism

In order to obtain vitamin D from the UVB radiation, 7-dehydrocholesterol must be photochemically converted into the secosteroid, previtamin D. This conversion occurs at the 5,7 diene bond in the B ring of the 7-dehydrocholesterol molecule (Figure 1) (3). Once the previtamin D isomerizes to cholecalciferol over a period of days, it must reach a tissue site expressing a 25-hydroxylase gene. It will then travel to tissues to be 1α-hydroxylated on C1 in order to gain access to target tissues expressing the VDR (24). An albumin-like protein known as the vitamin D binding protein (DBP), binds to vitamin D metabolites to escort them within the general circulation to their target tissues (3). Both endogenous and exogenous vitamin D_3 are transported via the DBP.

Vitamin D bound to DBP is transported to sites of storage or to the liver for conversion into 25(OH)D_3 (24). CYP27A1 and CYP2R1 are part of the cytochrome (CYP) p450 enzyme superfamily of monooxygenases, and are currently the most widely accepted enzymes responsible for hydroxylation at C-25. Found in the mitochondria and the microsome respectively, these enzymes both have high substrate affinity for vitamin D_3, however they are not very tightly regulated (25). Data on a mutation in CYP2R1 gives genetic evidence for the biological necessity of CYP2R1 in 25-hydroxylase activity yet CYP27B1, as well as other enzymes such as CYP2J2/3 and CYP3A4, remain implicated in catalyzing this hydroxylation (26-29). After the liver, 25(OH)D must be converted into 1,25$(OH)_2D_3$, the specific ligand for the VDR, to exert its biological effects. This hydroxylation occurs in the proximal renal tubules

or extrarenal tissues and is performed by the tightly regulated CYP27B1 enzyme, to produce the most biologically active metabolite, $1,25(OH)_2D_3$ (28, 30). Alternatively, $25(OH)D_3$ may undergo 24-hydroxylation in the liver to become the $24,25(OH)_2D_3$ when $25(OH)D_3$ is in excess (4). This hydroxylation occurs via CYP24A1 enzyme, which is also responsible for the formation of the $1,25(OH)_2D_3$ final product product known as calcitroic acid ($1,24,25(OH)_3D_3$) (31). CYP24A1 is found in highest concentrations in the mitochondria of the renal proximal tubules cells and together with $1,25(OH)_2D_3$, forms a physiological feedback loop in which it deactivates $1,25(OH)_2D_3$ and $1,25(OH)_2D_3$ in turn induces CYP24 transcription (32, 33). The CYP24A1 enzyme is highly regulated as it is responsible for vitamin D catabolism and the formation of the metabolites of the C23/C24 pathway (33-36).

1.2.3 $1,25(OH)_2D_3$ signaling

The intracellular mediator of $1,25(OH)_2D_3$ signaling is a ligand-induced transcription factor known as the vitamin D receptor (VDR). VDR is a nuclear receptor with high homology to other family members, such as receptors for steroid and thyroid hormones (24). Intracellular $1,25(OH)_2D_3$ stereospecifically binds to VDR, which functions as a heterodimer with related retinoid X receptors (RXRs). The heterodimeric complex translocates to the nucleus where it interacts with vitamin D response elements (VDREs) to activate or repress transcription of target genes. Some genes that are activated include those that encode the calcium binding protein, osteocalcin and CYP24A1.

Renal $1,25(OH)_2D_3$ production is controlled by feedback and feed forward regulation directly through PTH and fibroblast growth factor 23 (FGF23) (37, 38). In addition, cellular $1,25(OH)_2D_3$ regulates its own action via interaction with the VDR to downregulate expression of the 1α hydroxylase gene (39). PTH is the main regulator of $1,25(OH)_2D_3$ production by the kidney and it does so mainly through transcriptional regulation of the CYP27B1 gene (40). An endocrinological feedback loop exists between PTH and $1,25(OH)_2D_3$ in that $1,25(OH)_2D_3$ decreases PTH gene transcription and PTH stimulates CYP27B1 and thereby $1,25(OH)_2D_3$ synthesis (41). When PTH is present, $1,25(OH)_2D_3$ acts on the distal nephron and the intestine to increase calcium reabsorption and on bone to increase bone resorption to help provide minerals for calcium homeostasis (38). Chapuy et al (42) have shown that a negative correlation exists

between PTH and 25(OH)D and that PTH levels plateau at 3.8 pmol/L when serum 25(OH)D concentrations reach the threshold of 78 nmol/L. Many others have observed this plateau as well, however the threshold for 25(OH)D has differed amongst several studies. Dawson Hughes et al (43) standardized the 25(OH)D data for different experiments and found a cluster range of 75 to 80 nmol/L. Contrary to PTH, FGF23 inhibits $1,25(OH)_2D_3$ production and $1,25(OH)_2D_3$ in turn stimulates FGF23 production (44). FGF23 is found in greatest expression in osteocytes and active osteoblasts and will bind with its receptor FGFR in the presence of the binding cofactor klotho to inhibit production of $1,25(OH)_2D_3$ through increasing expression of CYP24A1 (45, 46). PTH and FGF23 are also important regulators of the classical vitamin D minerals; calcium and phosphate.

Calcium and phosphate regulate CYP27B1 levels in an indirect manner. Calcium exerts its action on PTH which in turn stimulates CYP27B1 production. There is feedback regulation between PTH, calcium and phosphate in that PTH regulates serum concentrations of calcium and phosphate, which both, in turn, regulate the synthesis of PTH (4). When serum calcium concentrations are normal, calcium acts on the calcium receptor present in the parathyroid glands to suppress secretion (4). In terms of phosphate regulation of CYP27B1, FGF23 concentration was found to be elevated in X-linked hypophosphatemia and shown to have an impact on the regulation of decreasing levels of $1,25(OH)_2D_3$. Therefore FGF23 is accepted as a mediator of the effect of phospate levels on vitamin D metabolism (38).

The intestine, kidney and bone cells respond to $1,25(OH)_2D_3$ in order to maintain calcium as well as phosphate homeostasis (47). When circulating $1,25(OH)_2D_3$ reaches the enterocytes of the duodenum and jejunum, transport proteins such transient receptor potential cation channel subfamily V member 6 (TRPV6) and calcium binding protein calbindin- D_{9k} both act to stimulate calcium to be actively transported from the lumen into the blood (48). There is also a passive, paracellular mechanism by which $1,25(OH)_2D_3$ enhances intestinal calcium absorption through concentration-dependent diffusion (49). The way by which $1,25(OH)_2D_3$ enhances intestinal absorption of phosphate is thought to be through a saturable process, although the regulation of intestinal type IIb sodium phosphate co-transporter has been suggested as a plausible mechanism as well (50, 51).

The kidney is another target tissue of $1,25(OH)_2D_3$ regulation to maintain normal calcium concentrations. The distal nephron is the major site of calcium regulation and the site of vitamin D metabolite and PTH action (52, 53). The entry of calcium through the renal epithelial cell is facilitated by TRPV5 and calbindin helps to shuttle the calcium from the apical to the basolateral side of the epithelial cell (54, 55). The two calcium binding proteins calbindin- D_{28K} and calbindin-D_{9K} as well as TRPV5 channel translocation to the plasma membrane are induced in the kidney by $1,25(OH)_2D_3$. The effects of $1,25(OH)_2D_3$ in the kidney extend beyond enhancing tubular reabsorption of calcium in that it decreases its own production through regulation of hydroxylases CYP24A1 and CYP27B1.

1.2.4 CYP27B1 and CYP24A1

The CYP27B1 enzyme expression is not limited to the kidneys, and is infact expressed in a multitude of tissues. Some of the organs and tissues that contain CYP27B1 were shown to be lymph nodes, epithelial cells, skin, colon, pancreatic islets, brain sites, osteoblasts and cells of the placenta and the adrenal medulla (56, 57). The CYP27B1 gene has a region within its promoter making it sensitive to stimulation by PTH (58). Furthermore, the activity of the 1α-hydroxylase is inversely correlated to serum calcium levels and this has been shown to be mediated by PTH (59).

CYP24A1 is very important for regulating the action of $1,25(OH)_2D_3$ in target cells. Similar to CYP27B1, expression level is highest in the proximal tubules of the kidney, however most cells express the 24-hydroxylase activity *in vivo* (40). The mRNA levels can range from very small levels to hundred fold above basal levels in order to help catabolize $1,25(OH)_2D_3$ when needed (38). There is a strong double positive VDRE present in the CYP24A1 promoter causing the induction of CYP24A1 to be 10- to 100- fold higher in vitamin D sensitive cells. Together, $1,25(OH)_2D_3$, PTH and serum phosphorus help regulate CYP24A1 enzyme to protect against any hypercalcemia or hyperphosphatemia (60). When ionized calcium concentrations remain low, PTH will inhibit CYP24A1 to repress production of $1,24,25(OH)_3D$ as well as $24,25(OH)_2D_3$ and increase production of $1,25(OH)_2D_3$ (4). In terms of a role for CYP24A1 in $1,25(OH)_2D_3$ catabolism, studies have shown that CYP24A1 mRNA has widepread detection in most vitamin

D target cells (61). However, it is not detectable in vitamin D target cells that have not been exposed to $1,25(OH)_2D_3$ but levels of mRNA increase within hours of $1,25(OH)_2D_3$ exposure. These experiments led Holick (4) to propose 24-hydroxylation as a key player in the involvement of inactivating $1,25(OH)_2D_3$ in target cells and not solely important for the inactivation process of excess $25(OH)D_3$. Recently, St-Arnaud (62), developed a CYP24A1-deficient mouse strain and their results concluded that CYP24A1 deficiency delayed fracture repair. In summary, this enzyme is a major catabolic player in vitamin D metabolism. However, its anabolic role, which is associated with the potential for biological activity of $24,25(OH)_2D_3$ remains unclear.

1.2.5 $24,25(OH)_2D_3$

As previously mentioned, once $25(OH)D_3$ is brought to the kidney, it is further metabolized into either $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$. The interest in $24,25(OH)_2D_3$ has increased as a result of the findings that plasma levels are almost 30 times higher than $1,25(OH)_2D_3$ itself (3, 63). Recently, there is more focus on the metabolite's role in bone and cartilage and new activities of the metabolite such as the role in growth plate cartilage and the presence of a membrane receptor for $24,25(OH)_2D_3$ in fracture healing chicks (64). It has been demonstrated that a synergistic relationship exists between $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ (65). Norman et al (64), tested the hypothesis that $24,25(OH)_2D_3$ is required for healing fractures as well as developing normal bone integrity. This was studied in an *in vivo* chick model and the kidney enzyme activity of 24-hydroxylase was shown to increase threefold with fracture imposition (64). However, Holick et al (4) summarized the major lines of evidence implying the major role of $24,25(OH)_2D_3$ as an inactivation product. Notably, serum $24,25(OH)_2D_3$ concentration can reach very high levels (greater than 250 nmol/L (100 ng/mL)) in hypervitaminotic animals suggesting a lack of regulation (66). This lack of regulation and the absence of any evidence for a $24,25(OH)_2D_3$ receptor such as the VDR strongly suggest that $24,25(OH)_2D_3$ is a breakdown product of 25(OH)D, serving little biological function (66, 67).

In regard to supplementation, there are large inter-individual differences in terms of the response as measured using serum $25(OH)D_3$ concentration. Wagner et al (68) performed a randomized controlled trial with adult males and females (n=80) in Toronto, Canada to explore these differences. They received 28 000 IU of $25(OH)D_3$ weekly in the form of supplements or

fortified foods for a period of 8 weeks over winter months and the concentration of serum $24,25(OH)_2D_3$ and 25(OH)D were measured using LC-MS/MS. They found that serum $24,25(OH)_2D_3$ concentration was highly correlated with $25(OH)D_3$. At week 2, the ratio was smaller in the vitamin D supplementation group than the placebo group, which was proposed to result from a potential lag in the CYP24A1 enzyme. Thus a measurement of the ratio of $24,25(OH)_2D_3$:$25(OH)D_3$ in the early stage of supplementation may help one determine if larger supplementation is needed due to inter-individual differences in catabolism or to clarify if certain 25(OH)D concentration targets are feasible in all people.

1.2.6 VDR

The expression profiling of VDR in certain tissues has given insight on the regulation of gene transcription and novel function of vitamin D within these tissues (69, 70). As previously described, VDR heterodimerizes with RXRs to recognize VDREs in the presence of $1,25(OH)_2D_3$. This $1,25(OH)_2D_3$-VDR-RXR complex regulates expression of many genes that encode bone and mineral homeostasis effectors (71). In VDR knockout (KO) mice, intestinal transgenic expression of VDR results in normalization of serum calcium, bone density and bone volume (72). Physiological benefits of vitamin D have been shown to extend beyond the well-known benefits on bone health. It has been proposed that variant VDRE sequences induce conformational changes in the VDR-RXR complex to permit associations with differential comodulators in order to exert these actions in various tissues (73). These actions include vitamin D as regulator of innate and adaptive immunity, keratinocyte function in epidermis and hair follicles and musculoskeletal system function (24).

1.2.7 Vitamin D and bone

Before discussing the effects of vitamin D on bone, a brief overview of bone biology will be presented. Bone is a unique mass of connective tissue in that it is constantly regenerated throughout life by a process called bone turnover (74). The extracellular matrix of bone consists of collagen, water, mineral, noncollagenous proteins, and lipids. Hydroxylapatite, the inorganic component of bone, consists of carbonate, magnesium and acid phosphate as well inorganic phosphate and calcium, whose amounts depends on the diet and environment (74). Bone consists of dense, solid bone surrounding the marrow, known as cortical bone and an inner network of

rods and plates that fill up the marrow space, known as trabecular (cancellous) bone. The periosteum is a fibrous connective tissue sheath surrounding cortical bone that contains bone cells; osteoblasts and osteoclasts. In mature bone, these bone cells aid in the bone remodeling which occurs in part to help adapt to biomechanical forces and to remove old or damaged bone and replace with new, mechanically stronger bone (75).

Osteoblasts are bone forming cells that originate from mesenchymal stem cells. This commitment of mesenchymal cells to the osteoblast lineage requires the canonical Wnt pathway (76). These mononucleated cells deposit bone and some are trapped in their own bone matrix, evolving into osteocytes; terminally differentiated osteoblasts. Osteoblasts secrete the most abundant noncollagenous matrix protein of bone, osteocalcin (OC). They contain receptors for both estrogen and parathyroid hormone and are also responsible for regulation of osteoclast activity, the bone resorptive cell. The osteoclast precursor is generally agreed to be the bone marrow macrophage. The two essential cytokines involved in osteoclastogenesis are receptor activator of nuclear factor kB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) (77). RANKL interacts with its receptor, receptor activator of nuclear factors-κB (RANK), on the surface on osteoclast precursors, to induce differentiation (78). Osteoprotegerin (OPG) is produced by bone marrow stromal cells and is considered a decoy receptor for RANK. OPG competitively antagonizes RANKL by binding to RANK inhibiting RANKL from inducing osteoclast differentiation (79). Osteoclasts release hydrochloric acid, hydrolytic and proteolytic enzymes resulting is breakdown of bone and release of hydrolyzed collagen fragments such as N-terminal telopeptides and C-terminal peptides of type I collagen (CTX) from the bone matrix, which eventually enter into circulation and can serve as biomarkers for bone resorption (16). Both osteoclasts and osteoclasts together make up a local group of cells forming the bone remodeling unit.

The effect of $1,25(OH)_2D_3$ on bone is both direct and indirect through stimulation of intestinal calcium and phosphate absorption. VDR KO mice develop secondary hyperparathyroidism, hypocalcemia, and rickets after weaning, however when fed a calcium and phosphorus rescue diet, rickets and osteomalacia are prevented along with normalization of parathyroid levels. This suggests that $1,25(OH)_2D$ is exerting its major effect on bone indirectly via stimulation of

intestinal calcium and phosphorus absorption. *In vitro* studies have shown that $1,25(OH)_2D_3$ can increase the activity of osteoclasts either directly by promoting osteoclast formation or indirectly by acting on osteoblast to induce the secretion of vitamin D_3 responsive factors to promote osteoclast activity (80). Osteoblast lineage cells express the VDR and osteoblasts have the ability to synthesize $1,25(OH)_2D$ and express CYP27B1 and CYP24A1 (4). Based on murine studies, nuclear translocation of $1,25(OH)_2D_3$ increases RANKL through the VDRE in the RANKL gene promoter as well as increase expression of RANK receptor on osteoclasts and osteoclast precursors (81, 82). The presence of $1,25(OH)_2D_3$ down regulates OPG and thereby is permissive of osteoclast differentiation and subsequent bone resorption (83). The RANKL/OPG ratio is a good indicator of the potential for bone resorption and becomes particularly important to assess in an aging population as the ratio of resorption/formation increases with age, eventually leading to bone loss (84, 85).

The first 34 amino acids of 84 amino acid peptide of PTH effect bone and Ca++ metabolism (86). The mechanism by which PTH has this affect is through binding to cognate PTH receptors present on the surface of osteoblasts, resulting in an alteration of the actions of both osteoblasts and osteoclasts (87, 88). PTH has been shown to increase RANKL levels and decrease OPG levels (89). It has also been observed that PTH is capable of inhibiting an inhibitor of the Wnt pathway called sclerostin in osteocytes (90). The activation of B-catenin leads to activation of the Wnt pathway causing osteoprogenitor proliferation, thus the inhibition of sclerostin results in an increase in osteoblast number (4). Such increase would be consistent with higher bone mineralization.

1.2.8 Bone mass and microarchitecture assessment

For a very long time histological techniques have been used to provide relevant information on cellular and dynamic bone remodeling however they are limited in their capacity to assess structural parameters. As a result, imaging techniques are now widely used to directly measure structural parameters through obtaining a 2D or 3D image (91). Radiographs are produced by summation of attenuation along a single scan direction. They are helpful in assessment of fracture healing, used for evaluation skeletal morphology *in vitro* and *in vivo* and limited to 2D evaluations. Magnetic Resonance imaging (MRI) uses both a magnetic field and radio

frequencies to obtain images of the soft tissues. Since information is obtained regarding soft tissues that give a strong signal, the contrast between bone and soft tissue allows for assessment of bone architecture similar to histological analysis (92). MRI is not capable of obtaining information regarding mineral mass, unlike dual-energy X-ray absorptiometry (DXA). DXA makes use of two X-rays at different energy levels to provide the ratio of attenuation of high to low energy beams in order to differentiatiate between soft tissue from bone and lean tissue from fat. DXA provides measures of areal bone mineral density, bone mineral content as well as body composition and is therefore not useful in distinguishing bone architecture or estimates of volumetric density that requires 3-D analysis to qualitatively and quantitatively investigate trabecular and cortical bone compartments.

The gold standard for trabecular and cortical assessment in animal models is microcomputed tomography (μCT). This technique makes use of X-ray attentuation to reconstruct 3D images for assessment of volumetric bone mineral density and other measures of bone architecture and morphology (91). Both *ex vivo* and *in vivo* uCT can achieve a very small nominal voxel size of 5 μm (voxel represents a value on a regular grid in three dimensional space) while *in vivo* μCT scanning alone allows longitudinal measures of bone architecture. Peripheral quantitative computed tomography (pQCT) is also used for 3D assessment of bone and is available *in vivo* and *ex vivo*. However, it's accuracy for assessment of bone parameters in humans with thin cortices as occurs in aging or rodent models for experimental investiageion is debatable due to its image resolution and voxel size (approximately 70 μm) (91). Lastly, nanocomputed tomography (nCT) uses synchroton radiation to obtain very detailed imaging at extremely low resolutions (below 1 micron), yet this technique is costly and requires substantial technical expertise for standarized operation (91).

1.2.9 Vitamin D assessment

In order to assess sufficient vitamin D status for optimal bone health, the DRI values were updated in 2011 by the Institute of Medicine (IOM). In the update it was reaffirmed that serum 25(OH)D concentration is the best indicator of vitamin D status at this time. Plasma or serum 25(OH)D is a useful biomarker of vitamin D status as it has one of the longest half lives in the circulation relative to the other metabolites and it is free of tight homeostatic control. Adiposity,

liver and kidney function and effects of aging are amongst the many factors that must be taken into consideration when using 25(OH)D as a biomarker for vitamin D supply to target tissues (17). Plasma $1,25(OH)_2D$ is not a good biomarker of vitamin D status as it has been shown that $1,25(OH)_2D$ and 25(OH)D only correlate in severe vitamin D deficiency and since $1,25(OH)_2D$ has a short half-life owing to homeostatic control, it is not a suitable marker for status alone (17).

Serum 25(OH)D at a concentration of 50 nmol/L (20 ng/mL) was identified as that associated with achievement of bone health for 97% of individuals (19). In the case of persons older than 50 years of age, the intake distribution was the same for older and younger adults yet there was variability regarding benefits to bone health for older adults. In the Dietary Reference Intake for Calcium and Vitamin D manual, reasons for uncertainty regarding DRI values for vitamin D were listed. The lack of data on dose-response relationships examining responses and health outcomes from graded doses of vitamin D was a major factor. To summarize the Institute of Medicine's cutoff values, the committee suggested persons to be at risk for deficiency below 30 nmol/L (12 ng/mL) of serum 25(OH)D levels (19). Severe deficiency in most Canadian laboratories is < 25 nmol/L and risk of inadequacy has been suggested to be between 30 nmol/L and 50 nmol/L (12 and 20 ng/mL) (19). According to the IOM, evidence to support striving for concentrations beyond 75 nmol/L (30 ng/mL) was lacking and concentrations above 125 nmol/L might be of concern for toxicity (19). Lastly, it is important to consider that the IOM's dietary recommendations generated considerable controversy, as many believe that these recommendations were too conservative and ignored the well-supported evidence for vitamin D's health benefits on the immune system (93).

For measurement of serum 25(OH)D, the assays currently available are competitive protein binding assays, immunochemical assays, and chromatographic procedures such as high pressure liquid chromatography (HPLC) or the more recently developed liquid chromatography (LC) tandem-mass spectrometry (MS/MS) (4, 94). Immunoassays are currently the most popular method for $25(OH)D_3$ and $25(OH)D_2$ measurement, most using an automated platform (95). A measurement comparison was performed amongst LC-MS/MS, radioimmunoassay and automated immunoassay and the LC-MS/MS method resulted to be a sensitive and accurate alternative compared to DiaSorin RIA (94). In the NHANES 2009 discussion, the decision to

change from the DiaSorin RIA method of measurement of the samples of 1988-1994 to the LC-MS/MS was made (96). Some of the reasons leading to this decision were that RIA showed assay fluctuations for the NHANES samples and did not allow for separate quantification of $25(OH)D_3$ and $25(OH)D_2$. The assays based on vitamin D binding protein have difficulty with equal detection of $25(OH)D_2$ and $25(OH)D_3$ since binding proteins have been shown to have higher affinity for the $25(OH)D_3$ form compared to $25(OH)D_2$ (97).

Binkley et al (98) shed new light on the 25(OH)D measurement situation after discovering a two-fold difference in mean 25(OH)D values from 6 different methodologies. It was agreed that a standard material for both $25(OH)D_2$ and $25(OH)D_3$ was required to be used worldwide to improve comparability and consistency of measurement. The US National Institute of Standards and Technology (NIST) then developed four separate human serum-based reference material (SRM 972) for 25(OH)D measurement. This reference material has been successful in reducing inter-lab bias according to the most recent DEQAS report (99). The relatively new metabolite of interest, 3-epi-$25(OH)D_3$ (C-3 epimer) is not separated with many LC-MS/MS assays, thus there is detection of the epimer resulting in overestimation of total 25(OH)D serum concentrations as previously mentioned. In newer LC-MS/MS procedures, the C-3 epimer is usually separated and quantified along with 25(OH)D and this method is capable of providing $24,25(OH)_2D$ as well as $1,25(OH)_2D$ measurements. It is thus far considered as the best method for epimer detection (96). As this novel metabolite affects 25(OH)D assessment, DEQAS participants are encouraged to use NIST reference materials including those with known amounts of C-3 epimer.

Several analytical assays for C-3 epimer measurement exist. The most recent DEQAS (International Vitamin D External Quality Assessment Scheme) 2013 survey commented that none of the major immunoassays detected the epimer (99). Some of the HPLC and LC-MS/MS methods are capable of resolving the C-3 epimer as well as the Roche competitive protein binding method. This method demonstrated that the C-3 epimer metabolite contributes to the total 25(OH)D. It was suggested in the DEQAS report that the inclusion of C-3 epimer in the total 25(OH)D given by most HPLC and LC-MS/MS methods may contribute to their positive bias. As mentioned by Bailey et al (6), this might be due to the inability of many LC-MS/MS to differentiate co-eluting isomeric compounds with different structures, leading to the over-

estimation of 25(OH)D concentrations. Therefore, many new methods have been developed to improve sufficient chromatographic selectivity (100). However, the method of analysis for C-3 epimer detection must be carefully chosen as it has been speculated that C-3 epimer produces a greater signal than 25(OH)D in LC-MS/MS assays that do not have dedicated calibrators, leading to an overestimation of C-3 epimer concentrations. To overcome this problem, CN, PFP or chiral columns are being used to provide necessary selectivity for C-3 epimer separation (14) .

1.3 C-3 EPIMER

1.3.1 Source

The source of the epimer in humans remains unknown. Whether is if from fortified foods or dietary supplements versus endogenous production is of particular interest (96). It has been suggested that intestinal bacteria or the treatment of foods such as heating may be sources of the C-3 epimer (5). In infants, evidence suggests that bifidobacteria in breast milk might give rise to the C-3 epimer as Singh et al (5) discovered that the epimer decreases in the first year of life and thereafter, values were no longer detectable. This study was conducted with 183 infants (<1 yr, 116 males, 67 females) and C-3 epimer was detectable in 39 of the subjects. Recently, in agreement with these results, Yazdanpanah et al (14) observed a drop in epimer concentration after 12 months of age (n=63, ≤ 1 y) and a continual decrease throughout childhood.

If endogenously formed, the immaturity of tissues responsible for classical vitamin D metabolism, namely hepatic and renal tissue, could be responsible for the increase in 25(OH)D epimerization. In their study, Singh et al. tested whether deranged hepatic metabolism would reduce C-3 epimer formation and saw no difference between healthy adults and those with liver failure (5, 14). Yazdanpanah et al (14) assessed liquid vitamin D supplements for C-3 epimer concentration and ruled it out as a source. The peak identification in LC-MS/MS was less than 2% compared to that of cholecalciferol in the supplements and therefore could not explain elevated C-3 epimer concentrations in infants.

1.3.2 C-3 epimer concentrations

Although the recent and past literature has mainly focused on C-3 epimer in infants and has found an inverse relationship between concentration of C-3 epimer and age, new research has put light on the possibility of detecting the epimer in adults (5, 101). Singh et al (5) detected the epimer in close to 23 percent of infants < 1 year of age (n=183, 116 males, 67 females) accounting for a relatively wide range of total serum 25(OH)D concentrations but none in adults. They also discovered an relationship between age and % C-3 epimer contribution of total 25(OH)D. However, at the NHANES roundtable discussion regarding the monitoring of serum 25(OH)D in late July 2009, one of the members had found the epimer in a range of 4-27% of the total serum 25(OH)D concentrations in adult patients however they found no correlation between age and epimer concentrations. Furthermore, Stepman et al (102) performed an ultra-performance LC/tandem MS (UPLC/MS/MS) on six infants to verify the results obtained from Singh et al (5) as well as on 32 adults to look at the possibility of finding the epimer in adult serum. The C-3 epimer accounted for 15-43 % of total 25(OH)D concentrations in infants whereas it ranged from 2.5-17% of total 25(OH)D concentrations in adults (102).

Recently, another study was performed using a highly sensitive method for C-3 epimer measurement looking at serum concentrations in 214 patients, ranging from neonates to 80 years of age and greater (101). The method used was HPLC-MS/MS and the results showed a small but detectable amount of C-3 epimer in 99% of the samples. In 92% of the samples, the concentration of the epimer was 3.0 ng/mL (7.5 nmol/L) or less. Unlike Singh et al (5), no relationship between age and epimer concentration was observed, yet the infant (less than 1 year) portion of the sample size was only two individuals. The explanation given by Lensmeyer et al (103) to account for this difference was assay sensitivity. The lower limit of detection for C-3 epimer was 0.1 ng/mL (0.25 nmol/L) for their study whereas Singh et al (5) did not report a lower limit of quantification for their modified LC-MS/MS method. However, Singh et al (5) did mention the concentrations of the epimer ranging from 5-92 ng/mL (12.5-230 nmol/L), which if 5 ng/mL (12.5 nmol/L) was actually applied as a lower limit for the data of Lensmeyer et al (103), the epimer would be detectable in very few samples.

It has been suggested that not accounting for the 25(OH)D_3 epimer can lead to an overestimation

of $25(OH)D_3$ concentrations which might lead to the misclassification of some people as vitamin D sufficient. Strathmann and colleagues (104) demonstrated this in their study of 626 healthy people aged 3 days to 94 years of age. They showed a misclassification of 9% of infants as sufficient with regard to $25(OH)D_3$ measurement and 15% of adults and children as sufficient; when considering the non-epimeric form of $25(OH)D_3$ only they were at risk of vitamin D deficiency (104). The reported findings from an epidemiological study of C-3 epimer showed that more sun exposure, higher intake of vitamin D supplements, higher alcohol intake and lower waist circumference all influence C-3 epimer concentrations in a non-hispanic white population (105). A review of C-3 epimer studies summarized the contribution of C-3 epimer to total 25(OH)D in different populations (6). The review showed 21.4 % in infant populations with data ranging from 0-61.1%, 5.9% in pediatric population with data ranging from 0-20.0% and 6.1% in adult population with data ranging from 0-47.0 % (Figure 2). At the NHANES rountable discussion, the importance of measuring C-3 epimer was considered (96). The panel identified a research need for additional studies that seek to determine the sources of C-3 epimer as well as the biological activity and consequences of elevated C-3 epimer concentrations.

1.3.3 Metabolism

In efforts to clarify if certain cell types are responsible for generation of C-3 epimer, Kamao et al (7) explored the metabolism of $25(OH)D_3$ among different cell lines; human osteosarcoma cell line (MG-63), rat osteosarcoma cell line (UMR 106), human colon adenocarcinoma cell line (Caco-2), porcine kidney cell line (LLC-PK1), and human hepatoblastoma cell line (Hep G2). In almost all cell lines, C-3 epimer was generated in greater amounts compared to $24,25(OH)_2D_3$. However, in agreement with the previous results demonstrating that the C-3 epimerization pathway does not occur in perfused rat kidney, in the LLC-PK_1 cell line, $24,25(OH)_2D_3$ was preferentially generated over C-3 epimer. The Hep G2 cells generated the most C-3 epimer compared to all other cell lines, suggesting the liver as a potential source. Their work demonstrated that the C-3 epimerization is tissue specific and substrate specificity for $25(OH)D_3$, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ epimerization also existed in a tissue-specific manner among the tested cell lines. In summary, the most plausible likely source is through endogenous metabolism, as rats given high doses of $25(OH)D_3$ readily produce the epimer (7, 9). Furthermore, C-3 epimer is generated in a dose-response manner with increasing dosages of

cholecalciferol in infants (13).

It has been demonstrated through several studies that all major vitamin D intermediate metabolites can undergo epimerization (7). Reddy et al (106) suggested bile acid hydroxysteroid dehydrogenases (HSDs) are responsible for the metabolism of 25(OH)D into its epimer form since epimerization is a common occurrence in bile acid metabolism. The HSD activities are widely found in the intestinal bacteria. The specific 3α and 3β HSDs have been shown to convert potent steroid hormones into less active forms (107). It has been suggested that the HSDs could act similarly in the epimerization of vitamin D, making it less active. Higashi et al (108) demonstrated that HSDs are part of the C-3 epimerization process, namely, 3αHSD or βHSD which have high substrate specificity for $24,25(OH)_2D_3$. Kamao and colleagues (7) examined the metabolism of C-3 epimer in recombinant E. coli cell culture systems and demonstrated that the epimer was metabolized by CYP27B1 at position C1 and by CYP24 at position C24 . However, a later experiment performed by Kamao et al (109) demonstrated that neither the typical vitamin D cytochrome P450 metabolizing enzymes nor hydroxysteroid epimerase were responsible for catalyzing the epimerization *in vitro* and the highest level of epimerization activity resided in microsomal fractions. Therefore, the epimerization most likely involves extra-renal tissues and enzymes not involved in classical vitamin D metabolism (6). The epimerization activity appears to be a unidirectional process. Reddy et al (106) noted that no reverse conversion of 3-epi-$1,25(OH)_2D_3$ into $1,25(OH)_2D_3$ was present in the HPLC as no peak for $1,25(OH)_2D_3$ was observed, congruent with work of Masuda and colleagues (110). Kamao et al (7) also did not detect any production of C-3β metabolites from C-3α substrates, concluding that the C-3α to β epimerization is unidirectional in biological systems.

1.3.4 Biological activity

In terms of biological activity, all of the C-3 epimer metabolites have been shown to have a lower binding affinity to the VDR compared to their native $25(OH)D_3$, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ forms (7). Using a calf thymus $1,25(OH)_2D_3$ receptor assay, 3-epi-$1,25(OH)_2D_3$ had a binding affinity of 2.1% of that of the native $1,25(OH)_2D_3$ form. Binding affinity of C-3 epimer for the DBP was also lower compared to the $25(OH)D_3$ at approximately 36-46% (6, 7). Further studies that demonstrated reduced affinity of 3-epi-$1,25(OH)_2D_3$ to the VDR relative to

the native form also showed that it has a decreased ability to stimulate gene expression and calcium transport in human colonic carcinoma cell line Caco-2 (11). Molnar et al (111) solved the crystal structure of 3-epi-1,25$(OH)_2D_3$ with human VDR-LBD and discovered that 3-epi-1,25$(OH)_2D_3$ does not interact with an important residue in ligand recognition by VDR; the Ser278 residue. Based on these findings, one can consider the 3-epi-1,25$(OH)_2D_3$ to be a partial agonist of VDR, however, all number of hydrogen bonds appear to be maintained in an alternative manner with the VDR complex (112).

Since the 3-epi-1α,25$(OH)D_3$ had been best described in osteoblast cell lines, Kadiyala et al (41) investigated the effect of the epimer on the *BGLAP* gene. They found that 3-epi-1α,25$(OH)D_3$ increased stabilization of the osteocalcin mRNA however the ability to increase *BGLAP* gene transcription and protein release was decreased 1000 fold compared to 1,25$(OH)_2D$. Furthermore, 3-epi-1α,25$(OH)D_3$ was demonstrated to induce CYP24 gene, however, to a much lesser degree compared to 1,25$(OH)_2D_3$ (7). Kamao et al (7) showed that the 3-epi-1α,25$(OH)D_3$ has the most biological activity out of all three epimers and was capable of inducing anti proliferation/differentiation activity. In MG-63 cells, it was shown that the transcriptional activity of human IC gene promoter containing VDRE was about 12% of 1,25$(OH)_2D_3$ for 3-epi-1,25$(OH)_2D_3$, whereas C-3 epimer and 3-epi-24,25$(OH)_2D_3$ had a very small effect even at high concentrations. Time courses changes in the amount of metabolites generated from the native C3β compounds and the C-3 epimers were examined and it was shown that the C-3 epimers had a slower metabolite rate, possibly compensating for their lower binding affinity for the VDR and thus contributing to their biological activity.

As mentioned, C-3 epimerization appears to be tissue specific and have tissue-specific functions. When explored in pulmonary alveolar type II cells, 3-epi-1,25$(OH)_2D_3$ stimulated surfactant synthesis in these cells as actively as 1α,25$(OH)_2D_3$, making it plausible that the epimer plays a role in lung development and function (113). Further evidence for equivalent biological activity of the epimer compared to its native form was found in bovine parathyroid cells. Brown et al (9) showed that 3-epi-1α,25$(OH)_2D_3$ was as effective as 1,25$(OH)_2D_3$ at suppressing PTH secretion in bovine parathyroid cells at diastereomer concentrations as high as 10^{-10} M. Furthermore, 3-epi-1α OHD_3 given to rats had very low calcemic effects but reduction of circulating PTH levels

was seen by 65 ± 7% (10). This parathyroid-suppressing action was proposed to be attributed to the high metabolic stability of 3-epi-1, $25(OH)_2D_3$. It has also been proposed that the epimer may be able to explain some of the variability found in previous studies in the PTH/$25(OH)D_3$ relationship considering its ability to suppress PTH secretion.

Since the apparent high metabolic stability of the epimer was suggested as an explanation for the observed C-3 epimer biological activity, Rhieu et al (12) performed experiments to further explore this stability. They were first to identify 3-epi-calcitroic acid as an inactive metabolite of 3-epi-1α,$25(OH)_2D_3$. Using both isolated rat kidney perfusion and purified rat CYP24A, they found that it was produced at concentrations threefold less compared to the inactive metabolite of non-epimeric $1,25(OH)_2D_3$, calcitroic acid. Based on molecular docking studies performed to explore these findings, Rhieu and colleagues concluded that the enhanced stability of 3-epi-$1,25(OH)_2D_3$ is achieved by binding to CYP24A1 in an alternative manner, slowing side-chain oxidation and thus inactivation.

In summary, most of the research on C-3 epimer physiological function have been performed in *in vitro* models. To date, *in vivo* studies regarding physiological function and studies that assess health risks associated with C-3 epimer concentrations independent of $25(OH)D_3$ and $1,25(OH)_2D_3$ are lacking (6). The detection in infant, child and adult samples has created debate about its clinical relevance and whether separation of the epimer is necessary for optimal vitamin D status measurement is truly dependent on its possible differential biological activity from the active $1,25(OH)_2D_3$ vitamin D metabolite.

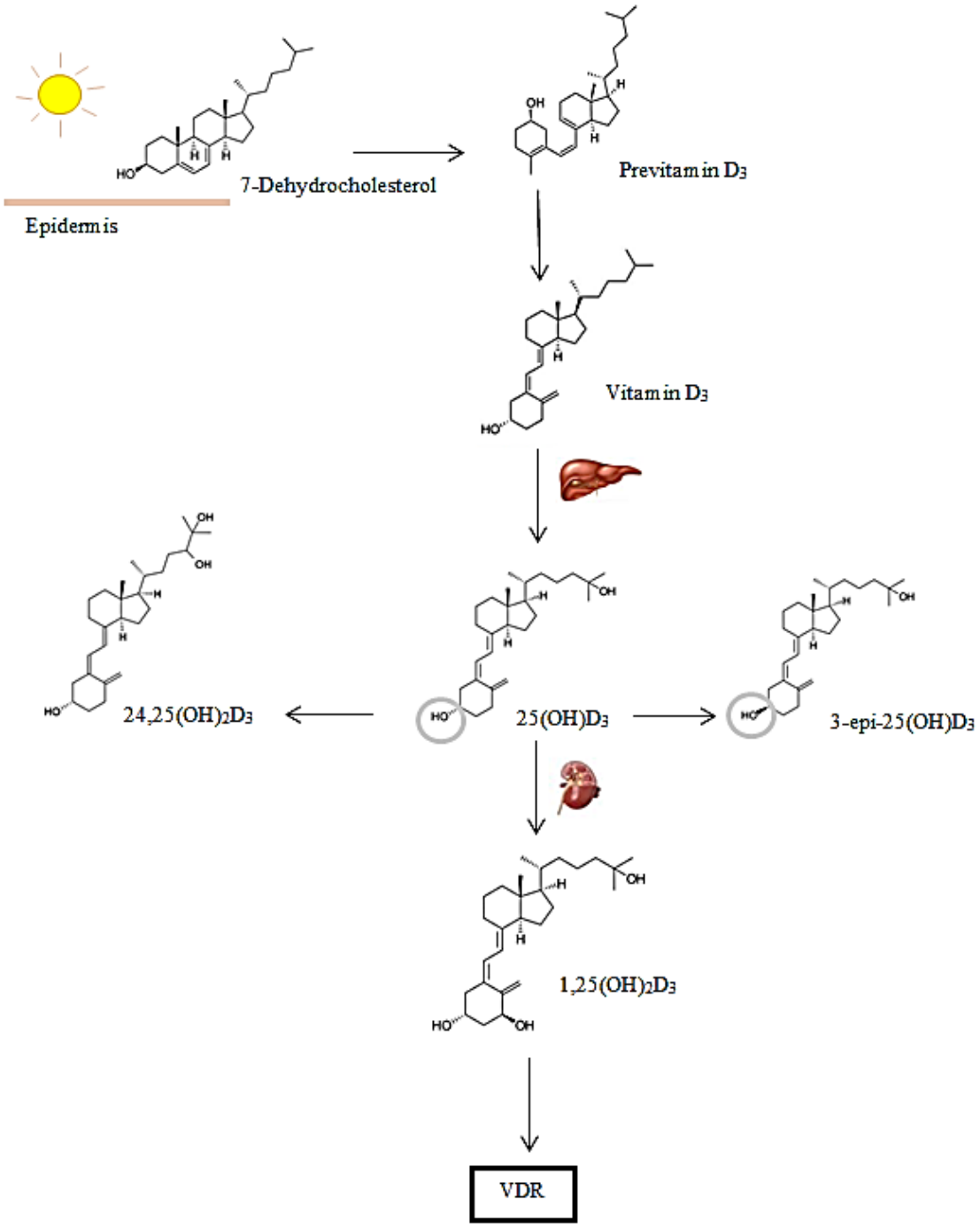

Figure 1: Vitamin D metabolism

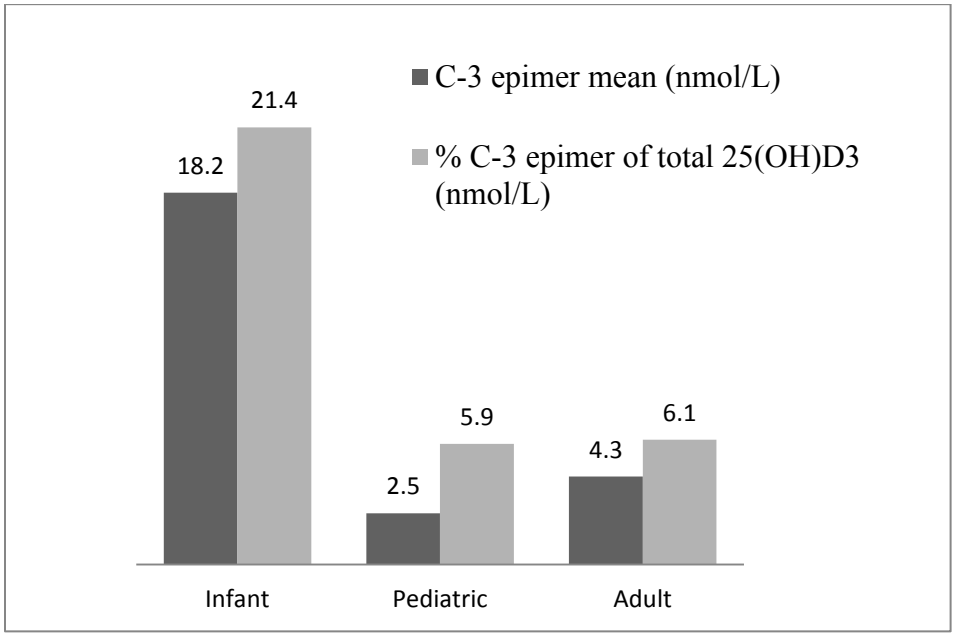

Figure 2: Quantification of C-3 epimer in infant, pediatric and adult populations. Data from Bailey et al. (6)

1.4 RATIONALE AND OBJECTIVES

The health benefits of vitamin D supplementation are widespread and are unequivocal in populations with specific risks for vitamin D deficiency. Therefore, the measurement, source and potential physiological function of C-3 epimer, which has been shown to confound total 25(OH)D measurement with certain analytical assays, has become increasingly important. From previous studies, endogenous production of C-3 epimer seems most plausible. Some studies show that C-3 epimer is equally potent as 25(OH)D in suppression of PTH and has lower calcemic effects. Therefore, C-3 epimer has a potential role in the treatment of hyperparathyroidism owing to protection against hypercalcemia and toxicity. C-3 epimer has been investigated in some infant, pediatric and adult populations, however, detection of C-3 epimer in an elderly (> 70 years of age) population in response to vitamin D supplementation has yet to be thoroughly explored. To date, *in vivo* studies of C-3 epimer on the important vitamin D functions such as maintenance of bone mineral density are lacking. Owing to the importance of maintaining bone mass in advanced aging such investigation requires animal model prior to clinical studies.

Therefore, the global objective of the studies in this thesis were:

Study 1: Investigate the C-3 epimer response to vitamin D supplementation in an elderly population.

Study 2: Expand knowledge of the role of C-3 epimer of 25(OH)D *in vivo* on maintenance of BMD in adult rodents and to obtain better knowledge of the C-3 epimer response to vitamin D intakes ranging from adequate to high, for a further understanding of the C-3 epimer source.

It was hypothesized that:

Study 1: Circulating C-3 epimer concentrations would increase in an elderly population in response to 2000 IU/day vitamin D supplementation; and

Study 2: Higher intakes of vitamin D would yield higher circulating concentrations of C-3 epimer in adult rats and the natural and preformed C-3 epimer would be equally as potent as 25(OH)D in establishing and maintaining bone mineral density.

2.0 MANUSCRIPT 1

C-3α epimer of 25-hydroxyvitamin D response to 2000 IU vitamin D supplementation in very elderly men

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2.1 ABSTRACT

A novel C-3α epimer of 25-hydroxyvitamin D_3 (C-3 epimer of 25(OH)D) is believed to be less biologically active than the native form; however, it may be equally potent in suppressing parathyroid hormone (PTH). PTH increases with age, suggesting that C-3 epimer may have applications in elderly populations. The aim of this study was to determine C-3 epimer profiles in response to 2000 IU supplementation of vitamin D (VD) in an elderly population and to explore its relationship to calcium homeostasis. Elderly men (n=40) residing at a long-term care hospital in Quebec received a supplement of 2000 IU/day of VD_3 for 8 weeks. At beginning and end of study, dietary assessment was performed, anthropometric data was collected and blood sampled for measurement of iCa and VD metabolites: $25(OH)D_3$, 24,25 dihydroxyvitamin D ($24,25(OH)_2D_3$) and C-3 epimer were quantified using liquid chromatography tandem mass spectrometry (LC-MS/MS). Plasma total 25(OH)D, PTH and osteocalcin (OC) (Liaison, Diasorin Inc) were measured by immunoassays at beginning and end of study. Pearson correlations were used to detect relationships between vitamin D metabolites and multiple linear regression analysis was performed. Paired t-tests were used to detect differences of means before and after supplementation. Mean age of participants was 89.8 ± 0.4 y, with a mean BMI of 26.3 ± 0.5 kg/m^2. Plasma 25(OH)D (LC-MS/MS) increased after 8 weeks of supplementation from 52.4 ± 2.4 nmol/L at baseline to 81.4 ± 2.3 nmol/L at week 8 ($p<0.05$). Despite the increase in 25(OH)D and intakes of vitamin D over 2000 IU/d, C-3 epimer was not quantifiable in any of the participants and thus linkages to calcium homeostasis were not evident. These data suggest that C-3 epimer is not a major component of vitamin D status in an elderly population with vitamin D status above targets known to have benefits to bone health.

2.2 **INTRODUCTION**

Recent discovery in the field of vitamin D has led to identification of a novel C-3 epimer of 25(OH)D (C-3 epimer) in serum samples from humans. The C-3 epimer has been previously detected in 99% of 214 measured adult serum samples with concentrations ranging from 0.25-59.25 nmol/L (0.1 to 23.7 ng/mL) (101). Although an age-related effect of C-3 epimer has been reported, this seems to be most evident in infant populations (5, 101). C-3 epimer is metabolized to a biologically active form, 3-epi-1,25$(OH)_2D_3$, which *in vitro* binds to the vitamin D receptor (VDR) at approximately 2-3% of the affinity of native 1,25-dihydroxyvitamin D (7). As expected with low binding to the VDR, active metabolites of C-3 epimer are less biologically active compared to those of 25(OH)D in the ability to stimulate intestinal calcium absorption and CYP24 and *BGLAP* gene (osteocalcin (OC) gene) induction (7, 11, 109). Conversely, the C-3 epimer has been shown to be equally effective in rats and bovine parathyroid cell lines as 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) in suppressing parathyroid hormone (PTH), which might be explained by the longer half-life of the epimer (9, 10, 112)**.** This is especially important in an aging population where concentrations of PTH have been shown to increase with age (109).

Fracture risk in the elderly is a growing health care problem as it increases exponentially with age. The increased fracture risk can be mostly explained by increased incidence of falls in aging, decreased bone mineral density and increased bone remodeling; a major determinant of which is changes in vitamin D metabolism (114, 115). According to the Dietary Reference Intake (DRI) for vitamin D, bone loss is used an indicator of vitamin D adequacy for those >70 years (116). If C-3 epimer represents a significant contribution to total 25(OH)D in an elderly population and if it is indeed able to equally reduce PTH compared to 25(OH)D, clinicians need to establish whether to use total 25(OH)D or 25(OH)D alone in evaluating vitamin D status. Furthermore, the changes in vitamin D metabolism that occur with age, such as reduced hydroxylation in the liver and kidney, might have a significant effect on C-3 epimer concentrations in this life stage group (117).

The source of C-3 epimer is still unknown making it difficult to proceed with clinical studies focused on improving vitamin D status. The proposed sources of C-3 epimer include endogenous production as well as bifidobacteria, fortification of foods and dietary supplements (5, 7).

Therefore, the aim of this study was to determine how C-3 epimer concentrations change in response to 2000 IU supplementation of vitamin D as a source of C-3 epimer and to associate the appearance of the epimer with changes in calcium homeostasis in the oldest of old men (118), a population in whom vitamin D studies are lacking. A secondary objective was to evaluate an immunoassay used in the routine assessment of vitamin D status to clarify whether such methodology is appropriate in view of possible C-3 epimer generation.

2.3 MATERIALS AND METHODS

Study protocol

This study was conducted over an 8 wk supplementation period during late fall to early winter (Nov-Dec) in which all participants received 2000 IU/d of vitamin D_3 with the aim of increasing serum 25(OH)D concentrations to >75 nmol/L since the epimer tends to be most commonly detected when vitamin D status is robust. The vitamin D_3 supplements were given in the form of tablets (Swiss Herbal Remedies Ltd, Richmond Hill for 4 wk followed by 4 wk of Webber Naturals, Coquitlam, BC due to change in hospital formulary). Potential participants were identified following a pre-screening of the Ste Anne Hospital population for entrance criteria and to be eligible for this study, men > 70 y were invited to participate and were included if the Mini-Mental State Examination score was at or above 18/30 and if baseline 25(OH)D concentration was <75 nmol/L. The exclusion criteria consisted of: palliative conditions (prognosis less than 4 months), untreated hyperparathyroid conditions, conditions that required parenteral or enteral feeding as a feeding method, metabolic bone diseases (excluding osteoporosis and osteomalacia) as well as end-stage renal disease due to altered vitamin D metabolism and use of vitamin D analogues. Glomerular filtration rate of the participants was estimated using the Cockgroft-Gault equation. Participants on medications were not excluded, all medications were recorded. Participants heights and weights were taken from their medical chart for calculation of body mass index (BMI: kg/m^2) at baseline and end of study. Vitamin D metabolites as well as biomarkers of bone metabolism, namely iCa, PTH and OC were measured at beginning and end of study.

Dietary assessment

Dietary assessment was performed for each participant at beginning and end of the study period for three consecutive days by nutrition researchers for main meals; night time snacks were recorded. Dietary vitamin D intake from all foods as well as the supplementation was assessed. The micronutrient and macronutrient composition of the intake was assessed using the hospital food composition database (Promenu version 4.75.40, CMR Progiciels Inc.) which incorporates the Canadian Nutrient File 2010 (119).

Biochemical assessments

Vitamin D supplementation began immediately after the baseline dietary assessment. Fasting blood samples were obtained between 6:30 and 8:00 am at the end of every three day dietary intake period at baseline and end of study. Serum albumin, phosphate, total and ionized calcium were measured by the Ste Anne Hospital laboratory using Vitros 250E (Ortho Clinical Diagnostics, Johnson & Johnson, version 250) and Symex XT-2000i (Sysmex, version XT-2000i/XT-1880i) auto analyzers. This laboratory meets the standards of International Organization for Standarization (ISO, Norm 15189 – Medical Laboratories) certification. Blood samples were measured the same morning of blood draw for measurement of 25(OH)D, osteocalcin and intact PTH using an autoanalyzer (Liaison, DiaSorin, Stillwater MN). The lower limit of quantification (LLOQ) for 25(OH)D using this assay is 10 nmol/L. The intra-assay CV values were 7.2%, 11.2%, 5.2% for 25(OH)D, PTH, OC respectively at week 0 and 6.3%, 5.3%, 5.0% for 25(OH)D, PTH, OC respectively at week 8. Aliquots of each sample were stored in a -80 freezer until further analysis for vitamin D metabolites. Plasma (20 μL) was measured for 3-epi-25(OH)D_3, 25(OH)D_3, 25(OH)D_2 and 24,25$(OH)_2D_3$ using liquid chromatography tandem mass spectrometry (LC-MS/MS) (Warnex Inc., Laval, QC: GLP laboratory). This laboratory is registered with the Vitamin D External Quality Assurance Scheme program. The analytical method included a Diels-Alder derivatization technique with substituted triazolinediones to increase MS response of the vitamin D metabolites and used the same methodology as Gallo et al (13). The LC-MS/MS technique used demonstrated high inter-assay precision and accuracy for calibration standards of 3-epi-25(OH)D_3 with a CV of $\leq$ 5%. Inter-Assay precision and accuracy for quality control samples for 3-epi-25(OH)D_3 in human plasma was $\leq$ 7.5%. Lower limits of quantification (LLOQ) were set based on the lowest standard for each metabolite; 12.5 nmol/L

(5.0 ng/mL) for 25(OH)D_2, 12.5 nmol/L (5.0 ng/mL) for 25(OH)D_3, 6.25 nmol/L (2.5 ng/mL) for 3-epi-25(OH)D_3 and 1 nmol/L (0.4 ng/mL) for 24,25$(OH)_2D_3$. Two NIST standards were assayed for measurement of 25(OH)D concentrations yielding errors in accuracy of 7% for NIST 968e level 2 (32.3 nmol/L; 12.9 ng/mL) and 2.5% for NIST 968e level 3 (57.7 nmol/L; 19.9 ng/mL) standard.

Ethics

The RCT from which samples were used for this study was reviewed and approved by the McGill Faculty of Medicine Institutional Review Board as well as Veteran Affairs Canada. All participants or their legal representative provided informed consent.

Statistical analysis

Paired t-tests were used to compare serum metabolite concentrations before and after the 8 week period. Normality and heteroscedasticity of the residuals were tested with the Shapiro-Wilk W and the White test, respectively. Pearson correlations were performed to test the association between vitamin D metabolites fufilling assumption of continuous, normally distributed data with a linear relationship. Spearman correlations were performed at endpoint to test for possible relationship between medication class use and 25(OH)D concentrations. Multiple linear regression was conducted to explain the variation in the concentrations of 24,25$(OH)_2D_3$ and 25(OH)D and Bland Altman (120) plot was used to examine the agreement between LC-MS/MS and chemiluminescence autoanalyzer for 25(OH)D. Models accounted for calcium intake, BMI, age and baseline vitamin D status. All data was analyzed with SAS 9.3 (SAS institute, Cary NC). Data are (mean ± SEM) unless otherwise stated.

2.4 RESULTS

Participants had a mean age of 89.8 ± 0.4 years and baseline BMI of 26.3 ± 0.5 kg/m^2. Serum concentrations of PTH (wk 0: 3.2 ± 0.3; wk 8: 3.1 ± 0.3, pmol/L) and OC (wk 0: 4.9 ± 0.6; wk 8: 4.9 ± 0.5, nmol/L) did not change over time. Vitamin D intakes, calcium intakes as well as ionized calcium did not significantly change over the 8 weeks (**Table 1**). At baseline, only 5.0 % of participants met the Recommended Dietary Allowance (RDA) of 800 IU for vitamin D whereas 12.5% met the Estimated Average Requirement (EAR) of 400 IU with diet alone. A

total of 27.5% of participants met the RDA of 1200 mg/d for calcium intakes and 40% met the EAR of 1000 mg/d. As expected, dietary intake did not change to a large degree after 8 weeks; a change in those meeting RDA for vitamin D changed to 7.5 % of participants and those meeting RDA for calcium decreased to 22.5% of participants. Those meeting EAR for calcium increased to 45% of participants. Medications commonly used are detailed in Table 1. Spearman correlations performed to study relationship between medication groups and 25(OH)D concentrations revealed no significant correlations ($p<0.05$).

Mean serum Total 25(OH)D concentration measured by chemiluminescence was 53.1 ± 2.3 nmol/L (21.2 ± 0.9 ng/mL) at week 0 and 70.6 ± 1.9 nmol/L (28.3 ± 0.7 ng/mL) at week 8. Based on LC-MS/MS analysis, 3-epi-25(OH)D_3 as well as 25(OH)D_2 were below the LLOQ in all participants (n=40). Paired t-tests revealed significantly increased plasma 25(OH)D after 8 weeks of supplementation, measured by LC-MS/MS; wk 0: 52.4 ± 2.4 nmol/L (21.0 ± 0.9 ng/mL) vs. wk 8: 81.4 ± 2.3 (32.5 ± 0.9 ng/mL) $p<0.001$) (Table 1). Pearson correlation between the LC-MS/MS and chemiluminescence methods was $r=0.871$ ($p<0.0001$) for baseline and $r=0.534$ ($p=0.0004$) for week 8 (**Figures 1a, 1b**). The mean difference (bias) between these methods for 25(OH)D concentrations was 0.69 nmol/L with a SD of the difference of 7.48 and a 95 confidence interval from –13.97 to 15.34 for baseline and bias of 10.74 ± 13.14 nmol/L with a 95 confidence interval from -36.48 to 15.01 for week 8 (**Figures 1c, 1d**).

Paired t-tests also revealed a significant increase in 24,25$(OH)_2D_3$ after 8 wk of supplementation; wk 0: 4.0 ± 0.3 nmol/L (1.6 ± 0.1 ng/mL) vs. wk 8: 6.8 ± 0.3 nmol/L (2.7 ± 0.1 ng/mL) $p<0.001$). A positive linear relationship was observed between 25(OH)D_3 and 24,25$(OH)_2D_3$ with a Pearson correlation of $r=0.76$ ($p<0.001$) at baseline (**Figure 2a**) and $r=0.34$ ($p=0.034$) at endpoint (**Figure 2b**). A multiple linear regression analysis for the relationship between 24,25$(OH)_2D_3$ and 25(OH)D_3, while accounting for calcium intake, BMI and age, indicated that greater age (y) was slightly related to lower concentrations of 24,25$(OH)_2D_3$ (nmol/L) with a coefficient of -0.072 ($p=0.01$).

Table 1: Characteristics and biochemistry of participants

	Week 0 (n=40)	Week 8 (n=40)
Age (years)	89.8 ± 0.4	n/a
BMI (kg/m^2)	26.3 ± 0.5	n/a
Total dietary vitamin D (IU/day)[1,2]	310.4 ± 25.2	302.9 ± 27.2
Dietary calcium (mg/day)[3]	1022.0 ± 75.0	983.2 ± 83.4
Total Calcium (nmol/L)	2.26 ± 0.01	2.28 ± 0.01
Plasma 25(OH)D (nmol/L)[4]	52.4 ± 2.4	81.4 ± 2.3*
Plasma $24,25(OH)_2D_3$ (nmol/L)	4.0 ± 0.3	6.8 ± 0.3*
$24,25(OH)_2D_3$: 25(OH)D (nmol/L)	0.07 ± 0.004	0.08 ± 0.004
Albumin (g/L)	35.17 ± 0.76	34.12 ± 0.76
Ionized calcium (mmol/L)	1.1 ± 0.01	1.1 ± 0.01
PTH (pmol/L)	3.2 ± 0.3	3.1 ± 0.2
OC (nmol/L)	4.9 ± 0.6	4.9 ± 0.5
Medication use n (%)		
Aspirin	13 (32.5)	11 (27.5)
Diuretics[5]	17 (42.5)	19 (47.5)
Beta Blockers	27 (67.5)	31 (77.5)
Calcium Channel Blocker	13 (32.5)	13 (32.5)
ACE inhibitors	9 (22.5)	8 (20.0)
ARB	5 (12.5)	5 (12.5)
Statins[5]	8 (42.5)	8 (42.5)
Oral Antidiabetics[5]	7 (17.5)	7 (17.5)
Anti-acids	23 (57.5)	24 (60.0)
Antihistamines	2 (5.0)	3 (7.5)
Antidepressants[5]	13 (32.5)	20 (50.0)

Data presented as mean ± SEM,
[1] excludes study supplement but may include meal replacement supplements
[2]week 0: 5% of participants met RDA, 12.5 % met EAR; week 8: 7.5 % of participants met RDA, 12.5 % met EAR
[3] week 0: 27.5 % of participants met RDA, 40% met EAR; week 8:22.5 % of participants met RDA, 45 % met EAR
[4]based on LC-MS/MS methodology
[5] known or suspected effect on vitamin D metabolism (121)
*significantly different from baseline ($p<0.05$)
Abbreviations: ACE: Angiotensin-convertin enzyme; ARB: Angiotensin receptor blocker

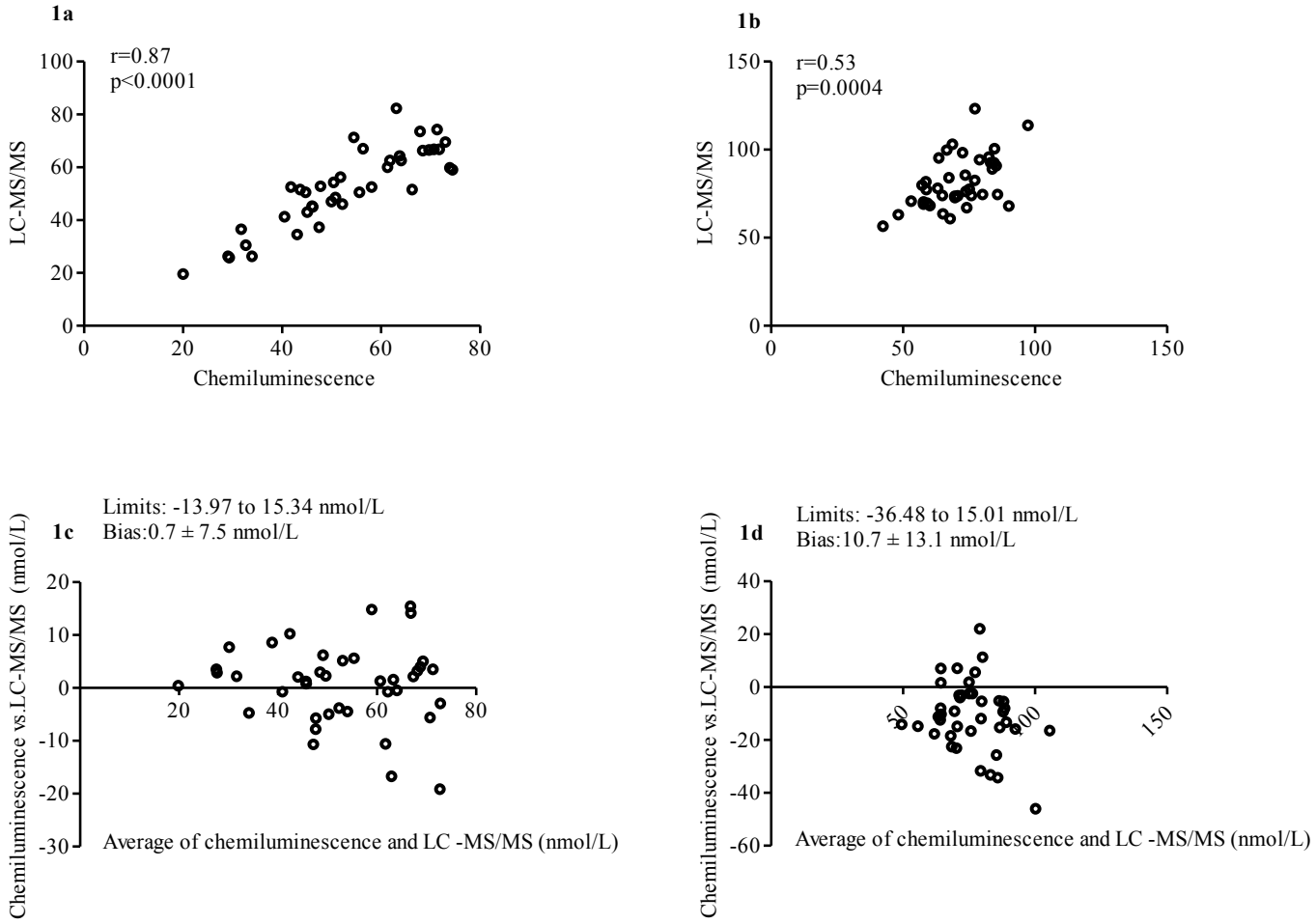

Figure 1: Relationship between LC-MS/MS and chemiluminescence for 25(OH)D_3 measurement. Correlation at baseline (1a) and week 8 (1b) with Bland Altman limits of agreement plots at baseline (1c) and wk 8 (1d). Data are n=40 per time-point

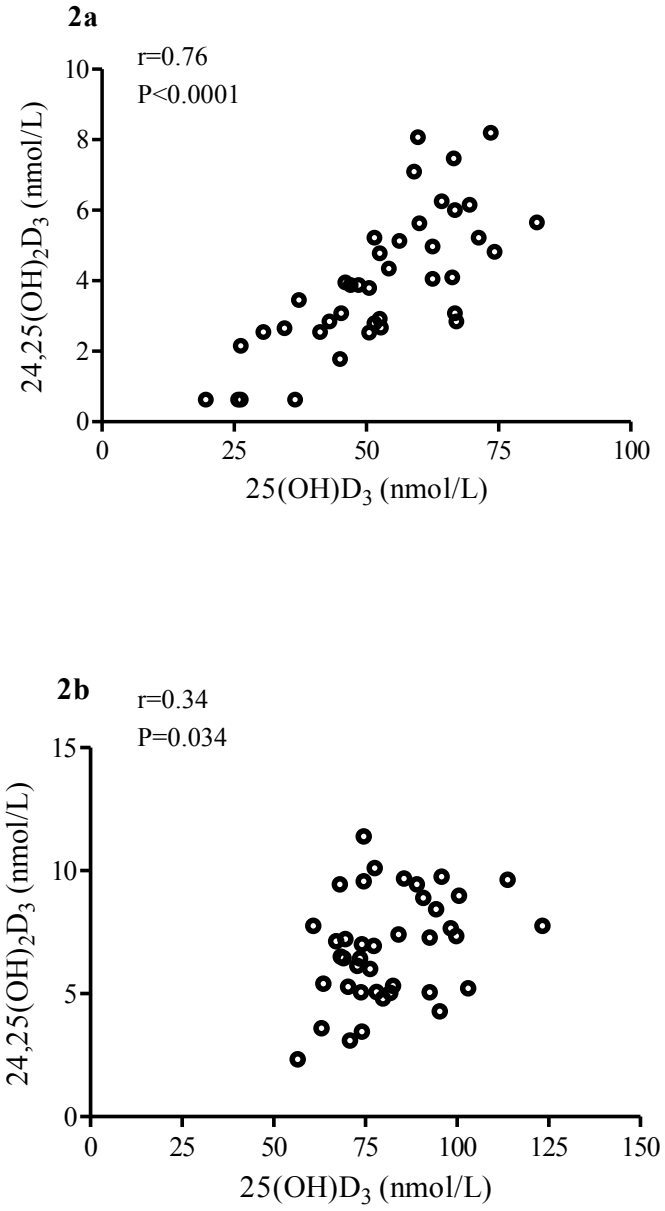

Figure 2: Relationship of plasma $25(OH)D_3$ and $24,25(OH)_2D_3$ measured with LC-MS/MS. Baseline values (2a) and wk 8 (2b). Data are n=40 per time-point.

2.5 DISCUSSION

This study was designed to examine whether vitamin D supplementation would result in generation of C-3 epimer in order to better understand its source and significance in the elderly which is an understudied population. Some studies suggest C-3 epimer is dependent on increasing 25(OH)D concentrations and thus we designed the study to elevate status within a robust range of over 75 nmol/L (101). We learned that in an elderly population, C-3 epimer is not generated in meaningful amounts in response to increasing concentrations of 25(OH)D well above the Institute of Medicine (IOM) 50 nmol/L cut-off for bone health using vitamin D intakes above 2000 IU/d. This suggests that C-3 epimer generation is not dependent on supplemental amounts exceeding the RDA or elevation of 25(OH)D beyond target IOM concentrations in this population.

The goal to elevate concentrations to above the 75 nmol/L value was based on the concentration of 25(OH)D required to observe a plateau in serum PTH values (43). Contrary to a previous 8 week supplementation trial of 800 IU/d (n=148 women, ~74 y) in which values of 25(OH)D significantly increased by 18.30 ± 20.94 nmol/mL (mean ± SD) from an initial value of 24.63 ± 12.14 nmol/mL and PTH decreased by 1.70 ± 1.87 pmol/L from 6.11 ± 2.34 pmol/L, PTH did not significantly change in these elderly male participants despite a clear elevation in 25(OH)D (122). In this population serum PTH was not elevated. This is consistent with the observation that our participants had adequate dietary Ca intake and serum 25(OH)D concentrations. It is thus possible that higher vitamin D status is required prior to generation of C-3 epimer and suppression of PTH. This is highly likely since Granado-Lorencio et al (123) evaluated C-3 epimer concentrations in patients (88% women, mean age 60.5 y) who displayed potential hypervitaminosis D (serum 25(OH)D concentrations above reference interval 15-64 ng/ml (37.5-162.5 nmol/L)) and found C-3 epimer concentrations ranging from 6 to 71.5 nmol/L (2–28.6 ng/mL). They also observed that participants with higher concentrations of C-3 epimer had lower PTH (<1.47 pmol/L).

It is important to note that the average age of participants for this study was 89 years and chronic diseases were not an exclusion criteria (unless prognosis was less than 4 months), thus this

sample is not necessarily representative of a healthy population, especially since these were institutionalized elderly men. Based on estimated glomerular filtration rate (48.3 ± 16.1 mL/min) kidney function in our participants was compromised. Indeed low $24,25(OH)_2D_3$ concentrations were observed, which might be indicative of a loss of renal function with advanced aging as the 24-hydroxylation occurs at the level of the kidney. However, $24,25(OH)_2D_3$ concentration did increase overall with higher concentrations of $25(OH)D_3$, suggesting that generalized impairment of vitamin D metabolism is not likely the reason we did not observe detectable levels of C-3 epimer. Use of medications known to affect vitamin D metabolism (121) could have been a factor in some, however none of our participants showed any significant relationship between medication class and 25(OH)D concentration using Spearman correlation.

The link between organ function and C-3 epimer has also been studied by Singh et al. whom investigated whether different health states effect C-3 epimer concentrations by observing adults with comprimised liver function (n=53; 25 males and 28 females, 20-87 y) and adults without known liver disease (n=147; 35 males and 112 females, 19-91 y) (5). In their study, they used a non-derivitized method of C-3 epimer separation which included the use of a 5-dinitrobenzoyl-(R)-phenylglycine column. In the first group, mean 25(OH)D concentrations were 42.5 nmol/L (range 0-142 nmol/L) and the second group 25(OH)D concentrations were 31.3 nmol/L (10-82.5 nmol/L). In agreement with our study, no C-3 epimer peaks were detected in either population. Thus, even with a very high range 25(OH)D concentrations (82.5 to 142 nmol/L), C-3 epimer is not readily detected in some populations.

The linear relationship between generation of increasing amounts $24,25(OH)_2D$ with increasing vitamin D status is in agreement with research by others that investigated the response of $24,25(OH)_2D_3$ and $25(OH)D_3$ to 28,000 IU/week supplementation (4000 IU/d) for 8 weeks (n=80, 40 males, 40 females, age not described) (68). Similar to our method, they used LC-MS/MS and compared 25(OH)D concentrations to an immunoassay (Diasorin, Liaison) and observed a positive bias as Liaison cross-reacts with $24,25(OH)_2D_3$ (124). We observed lower values by immunoassay with better agreement at baseline compared to week 8 when $24,25(OH)_2D_3$ concentrations were higher. Overall, the lack of C-3 epimer detection is not likely due to declining renal function based on ample generation of $24,25(OH)_2D_3$, but it may be

associated with older age or chronic diseases, as suggested in previous studies or function of other organs (5).

As 25(OH)D concentrations were elevated mainly through 2000 IU/d vitamin D supplementation in our study, the lack of C-3 epimer detection above the LLOQ of our methodology (6.25 nmol/L) suggests that supplements are not a significant source of C-3 epimer. This is in agreement with Yazdanpanah et al (14) that measured C-3 epimer concentrations in liquid vitamin D supplements and found that they did not contain any appreciable amounts of C-3 epimer and are thus unlikely to be a major source. A limitation of our study was the LC-MS/MS LLOQ for C-3 epimer (6.25 nmol/L). Although our ability to detect C-3 was previously demonstrated in other work (13), the assay was not sensitive enough to detect very low C-3 epimer concentrations that have been previously reported in adult samples (101). Furthermore, it is possible that we did not detect any C-3 epimer concentration in elderly men due to our small sample size (n=40), perhaps with a larger sample size or in a more healthy elderly population we would have detected C-3 epimer in some samples.

In summary, this report showed that high C-3 epimer concentrations are not seen in very elderly men at ranges of vitamin D status ($\geq$ 50 nmol/L) consistent with policy recommendations in support of bone health (125). Our study used LC-MS/MS for measurement of vitamin D metabolites in the oldest of old men (118) who resided in a hospital setting with minimal sun exposure. Importantly, dietary vitamin D and calcium was assessed along with other potential indicators of vitamin D status. Among other groups, the elderly are a particular population at risk for vitamin D deficiency owing to less than adequate intakes and limited opportunity for endogenous synthesis when residing in long-term care. Daily dosages of 2000 IU/d were effective in raising 25(OH)D concentrations with no apparent change in PTH and no detection of C-3 epimer concentrations. Therefore 2000 IU supplementation does not raise C-3 epimer concentrations in amounts that merit consideration in assessment of vitamin D status in elderly men. Additionally, PTH and OC were not changed with higher intakes suggesting 25(OH)D plasma values $\geq$ 50 nmol/L may be sufficient to assure good bone physiology. Future work regarding C-3 epimer in healthy versus diseased populations is required to further understand the relevance of C-3 epimer in all populations. Sex-specific effects should also be explored in C-3

epimer studies as it might give insight to the potential source. Furthermore, without more physiological data on the activity of C-3 epimer, careful consideration should be given to acquiring separate C-3 epimer measuerd values in populations where it represents a significant proprotion of total 25(OH)D.

2.6 ACKNOWLEDGEMENTS

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BRIDGE STATEMENT

The detection of C-3 epimer in adult populations is particulary important in regard to clarifying vitamin D status and may have a role in managing increasing parathryoid hormone (PTH) levels with age. Two studies have reported that 3-epi-1,25$(OH)_2D_3$ is equally potent in its ability to suppress PTH levels as the native 1,25$(OH)_2D_3$ (9, 10). In response to these results, and its decreased calcemic effects (10, 11), C-3 epimer has been proposed as potentially therapeutic for those with hyperparathyroidism (6). This relationship with PTH might also help to describe the inconsistencies in the PTH-25(OH)D dynamic. In the formerly reported results from our human study, C-3 epimer was assessed in elderly adults (n=40, 89 y) given one dosage of vitamin D over 8 weeks. As the participants were the oldest of old (118) men and most likely had declining renal function as well as other health disparities, we were unable to conclude if C-3 epimer was not detected in any of the samples due to older age or health states. Therefore, we conducted a study with different dosages of vitamin D in a healthy adult rodent model, and incoporated an examination of the possible effects of C-3 epimer on bone. An animal model was most appropriate for this study as we have yet to discover the biological function of C-3 epimer.

3.0 MANUSCRIPT 2

The generation of C-3α epimer of 25-hydroxyvitamin D and its biological effects on bone mineral density in adult rodents

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3.1 ABSTRACT

The source and biological function of C-3α epimer of 25(OH)D (C-3 epimer) is unknown. Elevated concentrations of the epimer in adult serum suggest it originates from supplemental vitamin D (VD). The objectives were to: (1) establish if increasing doses of cholecalciferol result in a proportionate dose-response in C-3 epimer; and (2) determine the biological response of bone to C-3 epimer treatment. Sprague Dawley rats (12 wk, n=36 female n=36 male) were randomized to either control AIN93-M diet (1 IU VD3/g diet) or one of five experimental diets for 8 wk: containing VD3 at 2 or 4 IU/g diet, C-3 epimer at 0.5 or 1 IU/g diet or 25(OH)D (0.5 IU/g diet). BW and food consumption were measured weekly. Blood was sampled at wk 0, 4 and 8 for assessment of VD metabolites and bone metabolism biomarkers. DXA (wk 0, 4 and 8) and *in vivo* micro CT (μCT) (wk 0 and 8) were performed followed by ex vivo μCT imaging and bone biomechanics. Differences were tested using mixed model ANOVA with Bonferroni adjustment. Weight, dietary intake and bone biomarkers, density and architecture did not differ among the VD diet or epimer diet groups throughout the study. The dose-response of VD generated significantly elevated C-3 epimer only in females with concentrations in the 4 IU VD diet (mean 84.6 (62.5) nmol/L) exceeding control (mean 21.4 (18.5) nmol/L, p=0.005). Male and female rats in the 25(OH)D group did not show a significant increase in C-3 epimer, whereas the 0.5 and 1 IU epimer groups exeeded 100 nmol/L of C-3 epimer by 8 wk in both sexes. These data suggest C-3 epimer is endogenously generated with higher intakes of VD. In conclusion, endogeous and exogenous C-3 epimer accumulates in serum, but does not alter bone health outcomes in an adult model over 8 wk.

3.2 INTRODUCTION

The appearance of C-3 epimer has caused many laboratories involved in vitamin D measurement to carefully consider C-3 epimer measurement and to exclude it from the assessment of vitamin D status as its source and function is unknown (6). Epimerization is an important chemical process for regulating certain steroid hormones in the body such as the hypothalamic gonadotropin-releasing hormone (8). Whether it has any *in vivo* biological significance in the vitamin D pathway has yet to be fully explored. Reddy et al (106) initially discovered the epimer of 1,25(OH)2D3 (3-epi-1,25(OH)2D3) in primary cultures of neonatal human keratinocytes. Although all major vitamin D metabolites are capable of being epimerized, 25(OH)D displays the highest kinetic parameters, making it the best substrate for epimerization (109).

Previously reported data has shown that *in vitro* 3-epi-1,25(OH)2D3 binds to the vitamin D receptor (VDR) with a lower affinity compared to the native 1,25(OH)2D3 form and is thus not as potent as 1,25(OH)2D3 in its calcemic abilities in a human colonic carcinoma cell line (Caco-2) (7, 11). However, it appears to be equally as potent in its ability to suppress PTH in bovine parathyroid cells and surfactant synthesis in alveolar cells (11, 113). This might be explained by the high metabolic stability of C-3 epimer, with its differential binding to CYP24A1 hydroxylase enzyme that reduces active site recognition (12). In human infants, concentrations as high as 60 nmol/L (24 ng/ml) do not appear to impact bone or PTH based on one dose-response trial using varied intakes of vitamin D (13). Although C-3 epimer concentrations are highest in infant populations, the range of C-3 epimer contribution of total 25(OH)D can reach up to ~50% in adult populations (6). Many studies have shown a relationship between absolute 25(OH)D and C-3 epimer concentrations, however further studies are required to understand if C-3 epimer quantification is required in all populations and if it does indeed have a biological effect that differs from 25(OH)D *in vivo*.

The aim of this study was to gain further knowledge of the source of C-3 epimer of 25(OH)D and its impact *in vivo* on maintenance of BMD in adulthood. We hypothesized that higher intakes of vitamin D3 would positively correlate with higher concentrations of C-3 epimer of 25(OH)D in adult rats and that the biological and preformed C-3 epimer would be equally as effective in maintaining BMD as 25(OH)D.

3.3 MATERIALS AND METHODS

Study protocol

Adult Sprague Dawley rats (36 male, 36 female, 12 wk old) were randomized to 1 of 6 diet groups following 1 wk of adaptation for a period of 8 wk: standard control AIN93-M diet (1 IU VD_3/g diet) or experimental diets of 2 and 4 IU/g diet of cholecalciferol, 0.5 and 1 IU/g diet of C-3 epimer, and a 0.5 IU/g diet of 25(OH)D reference group. The cholecalciferol diet groups along with control diet were chosen to examine if higher amounts of vitamin D stimulate endogenous synthesis of C-3 epimer. To fulfill the objective of examining biological activity *in vivo*, the two C-3 epimer diets were designed to test for biological equivalence to the reference group of native 25(OH)D. The span of 8 wk was chosen based on the half life of 25(OH)D (15-21 days), to make the study equivalent to three to four half lives. Furthermore, in adult rats, an 8 wk period is sufficient to observe differences in BMC in adult Sprague Dawley rats (126). Blood was drawn at beginning, middle and end of study for measurement of vitamin D metabolites as well as biomarkers of bone metabolism. Bone was assessed at all three time points using DXA and at wk 0 and wk 8 using *in vivo* μCT. Animals were exsanguinated under deep anaesthesia using cardiac puncture after final DXA and *in vivo* μCT scans. Right femur, tibia and lumbar spine 3 and 5 were wrapped in saline soaked gauze and stored in -20° C prior to *ex vivo* bone measurements.

Diets

The C-3 epimer and 25(OH)D_3 were supplied from Isosciences (Isosciences, LCC. Pennsylvania, USA) and supplied to Harlan (Harlan Laboratories, Madison, WI) for production of experimental diets. Experimental diets were made with modified AIN-93M (TD.94048) using an ethanol extracted version of casein called VFT & D deficient vitamin mix. Diet groups only differed by vitamin D, 25(OH)D or C-3 epimer content and were air dried during production to ensure no loss of vitamin D. Food was provided ad libitum and intake was monitored twice weekly by measure of disappearance. Food was withdrawn 24 hours prior to blood sampling at 0800 h with water provided *ad libitum.*

Biochemical assessment

At baseline, middle and end of study, approximately 800 µl of blood was sampled from the saphenous vein between 8:00 and 10:00 am to control for diurnal variation in the biomarkers. Blood was collected into heparinzed microtainers, centrifuged at 4 °C for 10 min at 2000 *g*. Blood was also collected in heparinzied capillary tubes for measurement of ionized calcium within 4 h of collection. Plasma was immediately aliquoted and stored at -80 °C for later analysis of bone biomarkers and vitamin D metabolites. CTx was measured at week 0, 4 and 8 with immunoassay (RatLap, Immunodiasnosticssytems, UK). RANKL and osteocalcin (OC) was measured with Rat Bone Panel Milliplex assays (Millipore and Luminex technology); OC at all timepoints and RANKL and endpoint only. PTH was measured at all timepoints using Rat Bioactive Intact PTH ELISA (Alpco, Salem, NH, USA). PTH, OC and CTx respective mean intra-assay CV's were 7.0 %, 6.2%, 3.5% and respective inter-assay CV's were 7.4 %, 14.1% and 6.2%. RANKL intra-assay CV was 7.9%. Plasma C-3 epimer, 25(OH)D and 24,25(OH)2D was measured in 20 µl of plasma using LC-MS/MS (Warnex Inc., Laval, QC: GLP laboratory). Interassay precision and accuracy for calibration standards for 25(OH)D3, 25(OH)D2 and C-3 epimer was ≤5%. Interassay precision for 24,25(OH)2D3 was 9.1%, 2.8% and 1.9% for calibrations at three different concentrations. Lower limits of quantification (LLOQ) were set at 12.5 nmol/L (5.0 ng/mL) for 25(OH)D2, 12.5 nmol/L (5.0 ng/mL) for 25(OH)D3, 6.25 nmol/L (2.5 ng/mL) for 3-epi-25(OH)D3 and 1 nmol/L (0.4 ng/mL) for 24,25(OH)2D3. Therefore we used half of the LLOQ (6.25 nmol/l) to assign a concentration of 3 nmol/L for those samples with no detectable C-3 epimer. The analytical LC-MS/MS used involved a Diels-Alder derivatization technique with substituted triazolinediones as previously described (13).

In vivo bone measurements

DXA regional and whole body scans (Hologic 4500, QDR Version 12,3, Hologic Incorporated Bedford., USA) were conducted at week 0, 4 and 8 of the study. To enable scanning, anesthesia was induced using 5 % isofluorane (Baxter, Illinois, USA) delivered using a chamber, followed by 2% maintenance using a cone mask. Rats were scanned in the dorsal position throughout the study period followed by measures of nose to tail length during anaesthesia. Regional scanning consisted of right and left femur, tibia and lumbar vertebrae (L1 to L4). Analysis was performed using DXA analysis software with a single operator performing the contouring to minimize bias

and inconsistencies. *In vivo* μCT (LaTheta LCT-200; Aloka, Tokyo, Japan) was performed at beginning and end of study period immediately following DXA measurements. Scan settings were 50 kV and 0.5 mA·s using a 120 mm specimen holder. Three separate scans were performed for each rat; whole body, lumbar vetebrae 1 to 5 and 1/3 distal femur and 1/3 proximal tibia. The three scans resulted in X-ray exposure of approximately forty minutes per animal at two time points. A previous study with similar exposure time showed that 8 weekly *in vivo* μCT scans (thirty-five minutes per scan) had no detrimental effects on bone (127). Whole body scanning was performed with in-plane pixel size of 120 x 120 μm^2 and pitch size of 1020 μm. Lumbar vertebrae as well as tibia/femur scans were performed with an isotropic resolution of 120 μm^3. Whole body was analyzed excluding skull with landmark at atlas vertebrae. Trabecular bone at distal tibia metaphysis and proximal femur metaphysis were assessed starting 10 slices from the beginning of the epiphyseal growth plate and spanned 12 slices, resulting in total volume of interest (VOI) of 1.440 mm^3.Vertebral analysis was performed on lumbar vertebra 3 using the entire vertebra from one intervertebral disc to the next.

Ex vivo bone density measurements

Long bones and vertebrae were excised for end-point measurement of bone architecture using high resolution μCT (model 1174, SkyScan, Antwerp, Belgium). Femur and tibia were stabilized using translucent dental wax during scanning. Lumbar vertebra 3 was placed in a tightly fitting rigid plastic tube. All bones were scanned at 50 kV and 800 μA with an exposure time of 6500 ms, 5500 ms and 8000 ms for tibia, femur and lumbar vertebra 3 respectively. Rotation for all bones was 0.9 degrees with 2 frame averages. A 0.4 mm aluminum filter was used for both femur and tibia and a 0.2 mm filter was used for the vertebra to reduce beam hardening artifacts (128). The tibia and femur were analyzed with isotropic resolution of 11.7 μm and the vertebrae with 14.2 μm. Mid-diaphyseal femur was scanned with isotropic resolution of 30.6 μm to enable cortical analysis. Three-dimensional (3D) reconstructions were obtained using two-dimensional cross-sectional images using manufacturer's software (NRecon v1.6.4.1; Skyscan). Acquired images were subsequently segmented into bone and marrow compartments (CT Analyser, v1.11.8.0; Skyscan). An adaptive threshold of 40-255 and a fixed global threshold of 80-255 Grey level value were applied for trabecular and cortical bone respectively. Trabecular tibia and trabecular femur analysis ROI started at a longitudinal distance of 1.17 mm and 1.75 mm from

the epiphysis respectively, and extended a further 2.34 mm and 1.75 mm in the distal direction for tibia and proximal direction for femur. To minimize bias and inconsistency, a single operator performed the contouring. The total ROI reached a total voxel size of 2.34 mm^3 for trabecular proximal tibia and 1.75 mm3 for trabecular distal femur analysis. Trabecular lumbar ROI was chosen 70 slices from epiphyseal growth plate structures and spanned the entire vertebra. Cortical femur ROI delineation was performed 200 slices from growth plate and extended over an area of 6.12 mm at mid-diaphysis.

Three-point flexure testing

Three point bending test was performed on the right femur at mid-diaphysis on an Instron 5544 (Norwood, MA, USA). Bones stored at -20 °C were completely thawed in a water bath at room temperature (23 °C) for at least 3 hours. Each femur was positioned anterior side up centered between the fulcrums with a constant span length of 16 mm for females and 19 mm for males. A 0.1 N load was applied at a preloading rate of 1 mm/sec until contact was made with the bone, followed by a rate of 0.1 mm/min until breakpoint. The femur length and anterior-posterior diameter at mid-diaphysis were measured before the test with a digital caliper (Fisher Scientific, St. Laurent, QC, Canada). Maximum flexure load (Fmax), maximum extension at maximum flexure load (dmax), and energy at break were derived from the load versus deformation curve generated with Bluehill version 2 software (Instron, Canton, MA). Stress (σmax) and strain (εmax) at maximum flexure load, and Young's modulus (stress/strain for linear portion of deformation curve) were derived using validated formulas (129).

$$\sigma = \frac{F_{max} L x/2}{4I}\ ,\ \varepsilon = \frac{12 x d_{max}}{L^2}$$

$$\text{Young's modulus} = \frac{KL^3}{48I}$$

where F_{max} = maximum flexure load; L = span length; x=anterior-posterior diameter); d_{max} = extension at maximum flexure load I = moment of inertia along the x-axis (MMIx obtained from Skyscan software)

Ethics

This study was reviewed and approved by the Macdonald Campus Facility Animal Care Committee and in accordance with the Canadian Council on Animal Care.

Statistical analysis

The sample size of n=12 (6 male, 6 female) was derived using femur BMD data for males and females combined. This data initially gave n=9 (α=0.05, β= 80) to detect 10% differences in a two sided t-test. The addition of three animals was to obtain an equal number of males and females and to account for the rare but possible loss of an animal or smaller effect size. Summary statistics were computed for all baseline characteristics, to ensure comparability between randomized treatment groups. Differences amongst groups were examined used MIXED model ANOVA for repeated measures with fixed effects of diet group, sex and time and random effects of study block and rat. Dietary intake was used as a random effect for biochemistry data. MIXED model ANOVA was performed on the cholecalciferol dose response (control diet, 2 IU vitamin D, 4 IU vitamin D) and another separate MIXED model ANOVA was performed on the C-3 epimer dose response (0.5 IU 25(OH)D, 0.5 IU C-3 epimer, 1 IU C-3 epimer), as these were separate questions and we did not intend to compare higher cholecalciferol groups to C-3 epimer groups. Bonferroni post-hoc testing analysis with adjustment for multiple comparisons was used to identify significant group differences. A total of 3 comparisons were made for each statistical model resulting in a significance level of 0.0167 (0.05/3). Pearson correlations were performed for relationship of vitamin D metabolites. Normality and homogenous variances were tested with Shapiro Wilk normality test and Levene's test respectively (outliers > 3 standard deviations from the mean were removed) with transformations (eg. logarithm) conducted where necessary to meet the assumptions of the post hoc testing. All testing was two-sided to enable acceptance or rejection of hypotheses. All data was analyzed with SAS 9.3 (SAS institute, Cary NC). Data is presented as (means (SD)) unless otherwise stated.

3.4 RESULTS

Dietary intake and anthropometry

There were no significant main effects of diet on weight gain, body length or dietary intake.

Weight of rodents in all 6 diet groups ranged from M: 427.5 - 437.2 g; F: 277.3 - 286.2 g at baseline and from M: 604.8 - 646.9 g; F: 339.5 - 368.3 g at end of study. For VD diets, a main effect of sex was present for intake, weight gain and body length ($p<0.0001$) and a main effect of time was present for weight gain and body length ($p<0.0001$) (**Appendix table 1**). There were no 2 way or 3-way interaction effects (diet, sex and time). For C-3 epimer and 25(OH)D diets, a main effect of sex was observed for intake, weight gain and body length ($p<0.0001$) and a main effect of time was present for intake ($p=0.0106$), body length ($p<0.0001$) and weight gain ($p<0.0001$). A 3-way interaction was found at baseline for dietary intake; females in the 0.5 25(OH)D group ate significantly less ($p<0.0048$) than females in the 0.5 C-3 epimer diet group.

LC-MS/MS

For all VD diet groups, there was a significant main effect of diet and time ($p<0.0001$) for plasma 25(OH)D (**Figure 1**), C-3 epimer (**Figure 2**) as well as $24,25(OH)_2D_3$ (**Figure 3**) concentrations whereas $25(OH)D_2$ was not detected in any of the samples. In both males and females consuming 4 IU vitamin D diet group, 25(OH)D (females $p=0.033$, males $p=0.008$) and $24,25(OH)_2D_3$ (females $p<0.0001$, males $p<0.0001$) concentrations were significantly higher than those in control group. Females demonstrated higher C-3 epimer concentrations with increasing concentrations of cholecalciferol in the diet (Figure 2b). In contrast, 25(OH)D and $24,25(OH)_2D_3$ concentrations significantly decreased with higher concentrations of C-3 epimer in the diet in both males and females (Figure 1c-d; Figure 3c-d). Exogenous C-3 epimer was successfully reflected in high endogenous plasma concentrations ranging from 268.8 (71.8) nmol/L in males to 291.3 (149.3) nmol/L in females after 8 weeks. Concentrations of C-3 epimer did not increase over time and in fact slightly decreased in males and females on the 0.5 IU 25(OH)D reference diet (Figure 2c-d). In males, the C-3 epimer % of total 25(OH)D was 12.5%, 23.7%, 30.2%, 39.6% for 0.5 IU 25(OH)D reference, control, 2 IU vitamin D, 4 IU vitamin D diets respectively. In females, the C-3 epimer % of total 25(OH)D was 27.8%, 39.3%, 53.4% and 63% for 0.5 IU reference, control, 2 IU vitamin D and 4 IU vitamin D diets respectively. A positive relationship was observed between 25(OH)D and $24,25(OH)_2D_3$ ($r=0.9$, $p<0.001$), 25(OH)D and C-3 epimer ($r=0.3$, $p=001$), C-3 epimer and $24,25(OH)_2D_3$ ($r=0.5$, $p<0.0001$) concentrations of rats on the vitamin D diets.

Bone biomarkers

For VD diets, baseline calcium concentrations were included in the statistical model as a covariate since small differences were seen at baseline. Significant main effects of time were observed for iCa ($p<0.0001$) as well as a diet by sex interaction ($p=0.0018$) where males on 2 IU VD diet differed from females on this diet ($p=0.0023$). No other interactions were significant. A significant main effect of PTH concentrations showed a trend for significant differences among diet groups ($p=0.0453$), however after Bonferroni corrections for control vs 4 IU VD diet ($p=0.0928$) as well for 2 IU vs 4 IU diet (0.0916) were not significant. A significant main effect for time was also observed for PTH ($p=0.0264$). No other significant main effects or interactions were present (**Table 1**).

For the epimer and 25(OH)D diet groups, a significant main effect of time ($p=0.012$) was observed where males overall increased iCa concentrations over time and interaction of sex*time was present ($p=0.007$) (Table 1). A main effect of time (0.0054) for PTH concentrations was present as concentrations decreased overall for the 3 diet groups and a significant interaction effect of diet by sex by time was present ($p=0.0453$) (Table 1) where male rats in the 1 IU epimer group had transiently lower PTH compared to the 25(OH)D reference group.

For VD diet groups, a main effect of time and sex ($p<0.0001$) and an interaction of diet by sex ($p=0.0062$) was present for CTx, this interaction of diet*sex was due to baseline differences of higher CTx concentrations in 4 IU VD diet groups vs control ($p=0.0012$) (**Appendix table 2**). No main effects or interactions were observed for OC (Appendix table 2).

For C-3 epimer diets and 25(OH)D diet groups, a main effect of time ($p=0.0387$) only was present for OC as concentrations overall decreased across all three diets for both males (wk 0: 12.5(4.6) nmol/L; wk 8: 12.2(6.1) nmol/L) and females (17.4 (12.4) nmol/L; wk 8:16.6 (21.1) nmol/L) (Appendix table 2). A main effect of time ($p<0.0001$) and sex ($p=0.022$) was present for CTx concentrations and no interaction effects were observed. Circulating concentration of RANKL was only measured at endpoint of study as other biomarkers did not show significant differences among diet groups; no differences were observed among diet groups and no main effects or interaction effects were observed.

Body composition

For animals on VD diets, a main effect of time and sex was present for lean body mass, whole body BMD and BMC ($p<0.0001$). There was an interaction effect of sex by time ($p<0.0001$) for all parameters and no main effect of diet nor other interaction effects (**Appendix table 3**). For C-3 epimer and 25(OH)D diets, there was a main effect of time for lean body mass, whole body BMD and BMC ($p<0.0001$) and a main effect of sex for lean body mass and BMD ($p<0.0001$).

Bone density and architecture

DXA derived measures of aBMD and BMC of right and left femur, right and left tibia, lumbar vertebrae (L1-L4), and whole body showed main effects of increasing values over time ($p<0.0001$), but no differences among diets groups were observed at baseline or by end of study (**Appendix table 4**). Further analysis with *in vivo* μCT also showed no significant differences among diet groups in whole body vBMD, trabecular BMD and cortical BMD as well as trabecular vBMD of lumbar vertebra 3, right femur and right tibia, however there was a main effect of increasing values over time ($p<.0001$)

Ex vivo μCT analysis revealed small differences in trabecular lumbar bone with significant differences in BS/BV (specific bone area) which is a measure for the bone surface per given bone volume. In females, the 1 IU C-3 epimer diet group had significantly lower BS/BV (32.26 (4.66) mm^2/mm^3, $p=0.014$) compared to the 0.5 IU 25(OH)D reference group (37.32 (2.40) mm^2/mm^3) (**Figure 4**). In addition, trabecular separation of tibia was higher in males fed the 2 vs 4 IU diet (**Appendix table 5**). There was also a trend for significantly larger BV/TV in 4 IU VD diet compared to 2 IU VD diet. No other significant differences among diet groups for trabecular femur, tibia or vertebral analyses were present.

Cortical analysis of right femur at mid-diaphysis showed no significant differences among diet groups. However, three-point bending test performed at mid-diaphysis on right femur showed a trend of significantly higher flexure extension (mm) and strain (%) in males on the 0.5 C-3 epimer diet compared to the 1 IU C-3 epimer diet (**Table 2**).

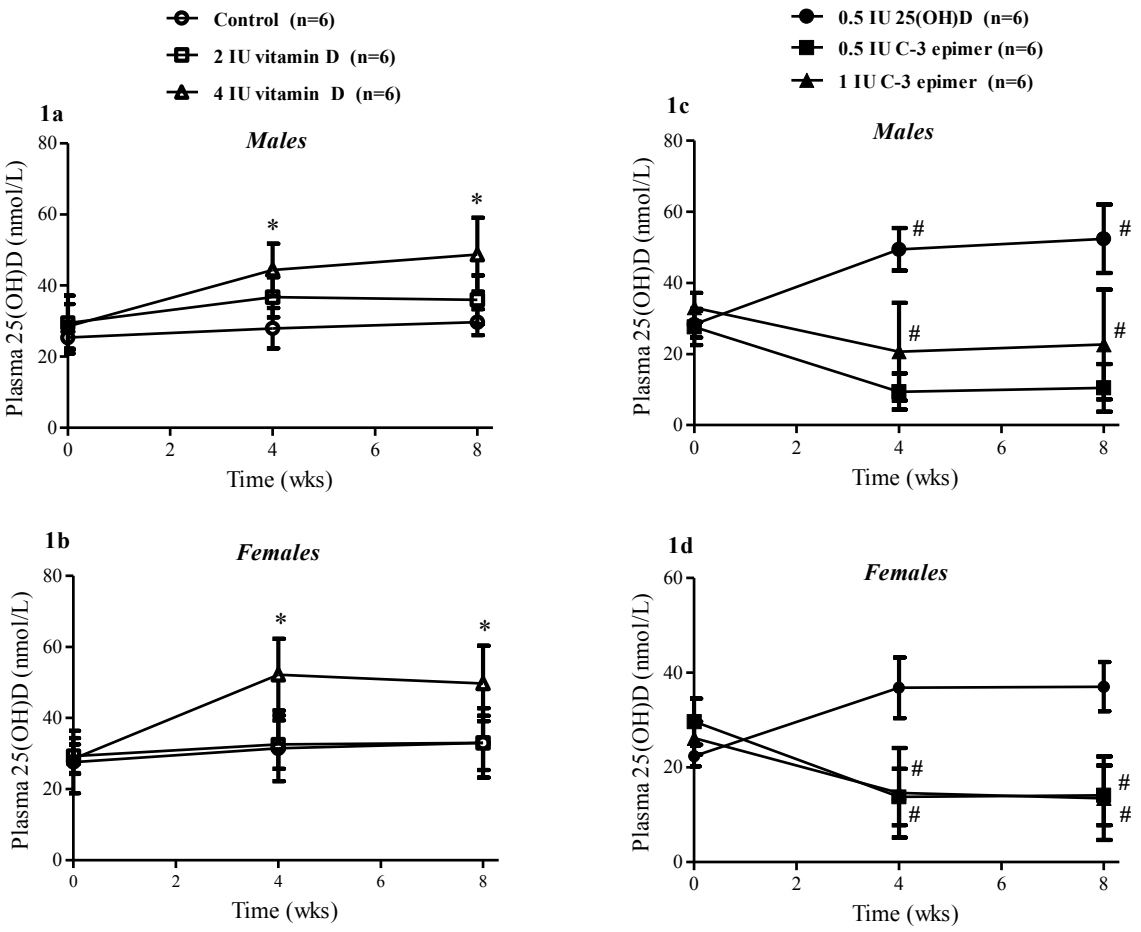

Figure 1: Plasma 25(OH)D_3 concentrations in adult Sprague Dawley rats over 8 weeks of consuming vitamin D dose-response diets and C-3 epimer dose response diets. Significant differences identified with MIXED model ANOVA with Bonferroni post-hoc adjustment ($p<0.05$). Points represent mean ± SD, n=6 per sex/diet. *significantly different from control AIN93-M diet
significantly different from 0.5 IU 25(OH)D reference diet.

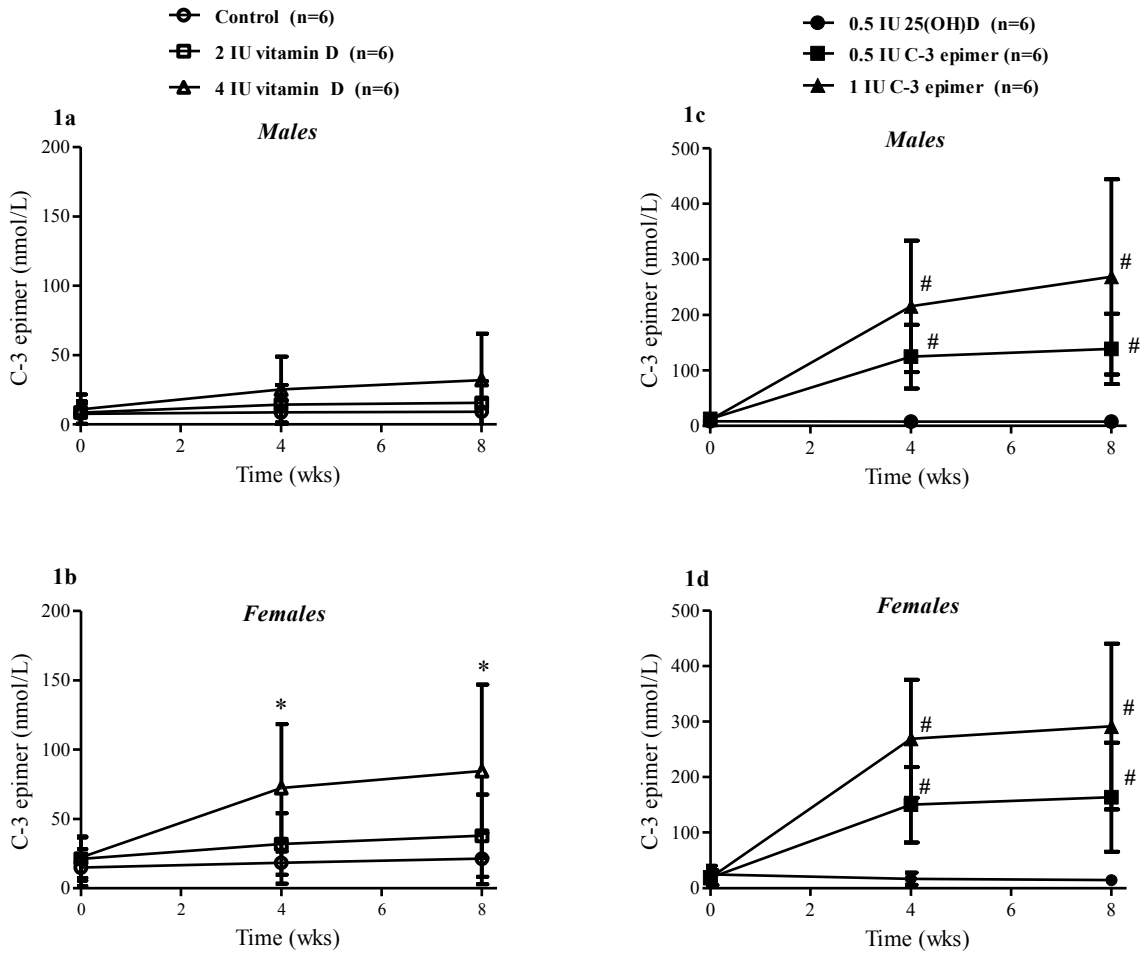

Figure 2: Plasma C-3 epimer concentrations in adult Sprague Dawley rats over 8 weeks of consuming vitamin D dose-response diets and C-3 epimer dose response diets. Significant differences identified with MIXED model ANOVA with Bonferroni post-hoc adjustment ($p<0.05$). Points represent mean ± SD, n=6 per sex/diet. *significantly different from control AIN93-M diet
[#] significantly different from 0.5 IU 25(OH)D reference diet.

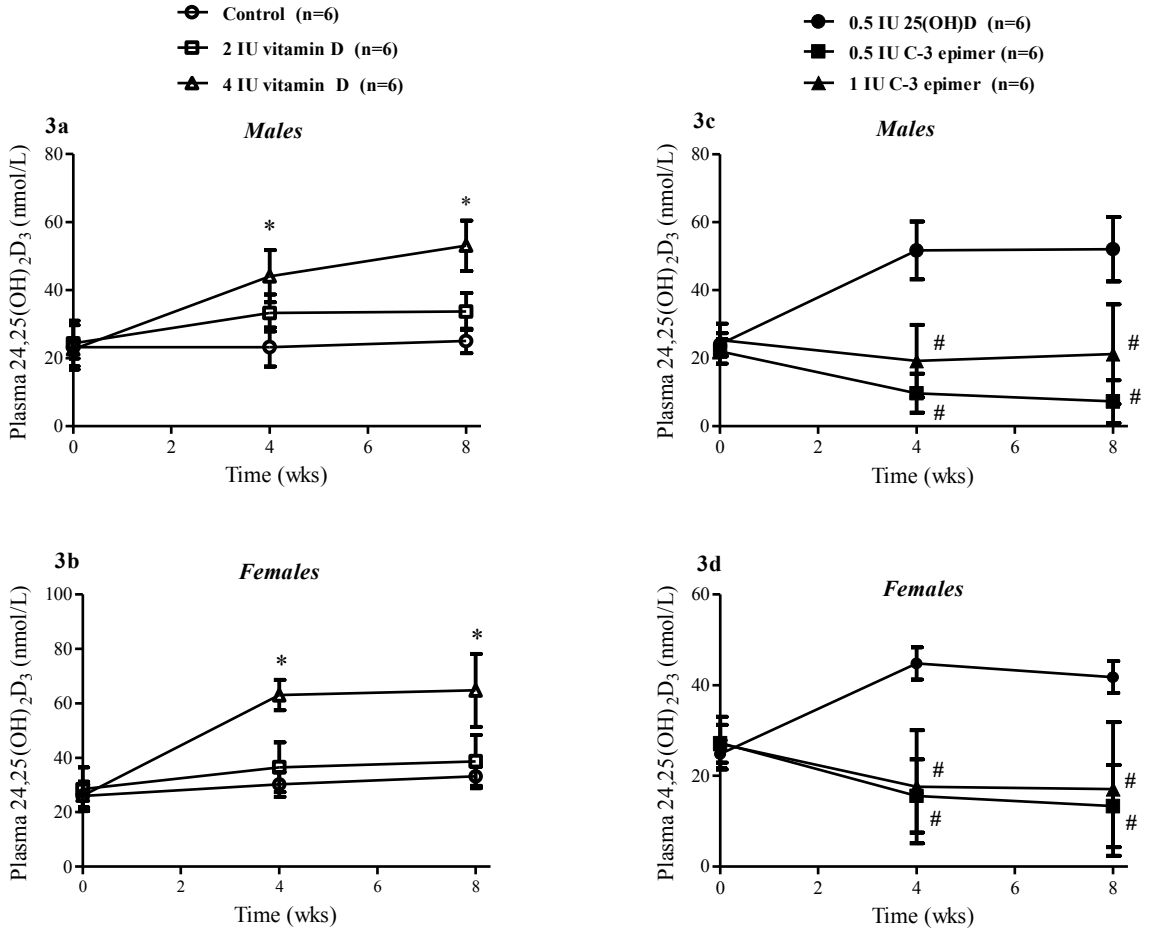

Figure 3: Plasma $24,25(OH)_2D_3$ concentrations in adult Sprague Dawley rats over 8 weeks of consuming vitamin D dose-response diets and C-3 epimer dose response diets.Significant differences identified with MIXED model ANOVA with Bonferroni post-hoc adjustment ($p<0.05$). Points represent mean ± SD, n=6 per sex/diet. *significantly different from control AIN93-M diet
significantly different from 0.5 IU 25(OH)D reference diet.

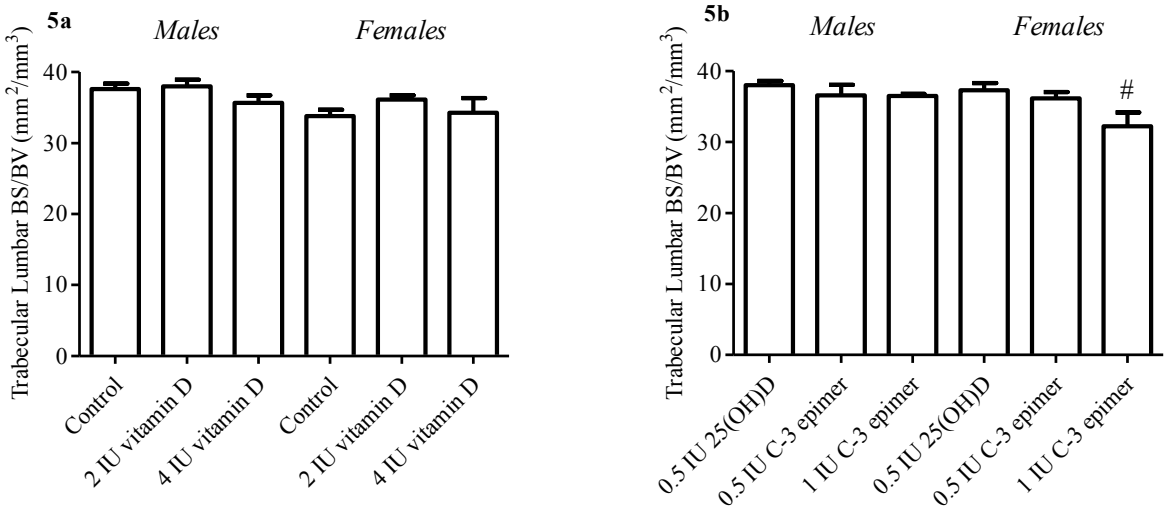

Figure 4: μCT analysis of lumbar vertebra (L3) in 20 week old Sprague Dawley rats. Significant differences identified with MIXED model ANOVA with Bonferroni post-hoc adjustment ($p<0.05$). Bars represent mean ± SD, n=6 per sex/diet $^{\#}$ significantly different from 0.5 IU 25(OH)D reference diet.

Table 1: iCa and PTH concentrations of Sprague Dawley rats on vitamin D and C-3 epimer diets over 8 weeks

			Diets				**Interaction**	**Diets**				**Interaction**
	Sex	Time	Control (n=6)	2 IU VD3 (n=6)	4 IU VD3 (n=6)	All (n=18)	diet*sex*time p value	0.5 IU 25(OH)D (n=6)	0.5 IU C-3 epimer (n=6)	1 IU C-3 epimer (n=6)	All (n=18)	diet*sex*time p value
iCa	M	0	1.32 (0.04)	1.36 (0.02)	1.31 (0.03)	1.33 (0.04)^{A}	0.248	1.34 (0.02)	1.33 (0.02)	1.33 (0.01)	1.33 (0.02)^{A}	0.531
(mmol/L)		4	1.31 (0.03)	1.32 (0.05)	1.27 (0.03)	1.30 (0.04)^{B}		1.32 (0.01)	1.32 (0.03)	1.31 (0.04)	1.31 (0.03)^{A}	
		8	1.32 (0.03)	1.36 (0.04)	1.35 (0.03)	1.35 (0.04)^{A}		1.36 (0.03)	1.36 (0.03)	1.34 (0.04)	1.35 (0.03)^{B}	
	F	0	1.33 (0.03)	1.34 (0.01)	1.34 (0.02)	1.34 (0.02)^{A}		1.32 (0.03)	1.32 (0.05)	1.35 (0.04)	1.33 (0.04)^{AB}	
		4	1.31 (0.02)	1.29 (0.05)	1.32 (0.03)	1.31 (0.03)^{B}		1.33 (0.02)	1.33 (0.02)	1.34 (0.03)	1.33 (0.02)^{AB}	
		8	1.33 (0.02)	1.31 (0.02)	1.33 (0.03)	1.33 (0.02)^{AB}		1.34 (0.03)	1.31 (0.03)	1.34 (0.04)	1.33 (0.03)^{AB}	
PTH	M	0	16.5 (5.5)	18.9 (8.0)	28.1 (15.7)	21.2 (11.3)^{AB}	0.859	18.5 (6.3)^{ab}	35.3 (25.8)^{ab}	31.7 (15.2)^{ab}	28.1 (17.5)^{AB}	0.0450
(pmol/L)		4	18.4 (11.2)	11.5 (2.9)	30.8 (24.8)	20.3 (17.0)^{AB}		37.5 (33.7)^{a}	20.9 (13.1)^{ab}	14.8 (9.9)^{b}	24.4 (22.6)^{AB}	
		8	10.3 (5.8)	13.9 (4.2)	19.3 (17.7)	14.5 (8.3)^{AB}		11.8 (5.6)^{ab}	14.3 (6.0)^{ab}	19.7 (12.9)^{ab}	15.5 (9.1)^{AB}	
	F	0	14.4 (15.6)	20.5 (13.9)	15.7 (7.9)	16.8 (12.4)^{AB}		17.3 (11.7)^{ab}	15.3 (11.4)^{ab}	15.2 (6.3)^{ab}	15.9 (9.4)^{A}	
		4	23.3 (29.2)	11.5 (2.9)	30.8 (24.8)	26.4 (22.6)^{A}		27.9 (21.6)^{ab}	27.0 (10.3)^{ab}	45.2 (37.6)^{ab}	32.6 (24.6)^{B}	
		8	14.6 (8.2)	11.3 (3.8)	18.9 (10.9)	14.9 (8.3)^{B}		12.8 (7.6)^{ab}	15.3 (8.4)^{ab}	11.9 (2.8)^{ab}	13.4 (6.4)^{A}	

Data presented as mean (SD). Main and interaction effects tested with MIXED model ANOVA with Bonferroni post-hoc adjustment ($p<0.05$). Differences among time*sex are demarked by different upper case superscripts in the same column. Interaction effects and differences are demarked by no common lowercase superscripts.

Table 2: Bone architecture of right femur mid-diaphysis of 20 week old Sprague Dawley rats on vitamin D and C-3 epimer diets

		Diets			Main effect	Diets			Main effect
Parameters	Sex	Control (n=6)	2 IU VD3 (n=6)	4 IU VD3 (n=6)	Diet p value	0.5 IU 25(OH)D (n=6)	0.5 IU C-3 epimer (n=6)	1 IU C-3 epimer (n=6)	Diet p value
Tt.Ar (mm^2)	M	13.75 (1.22)	13.75 (1.50)	13.46 (0.28)	0.352	14.05 (0.73)	14.64 (1.34)	14.04 (0.79)	0.576
	F	9.72 (0.70)	10.0 (0.70)	9.35 (0.52)		9.84 (1.37)	10.01 (0.97)	10.30 (1.00)	
Ct.Ar (mm^2)	M	9.50 (1.04)	9.22 (1.08)	9.34 (0.24)	0.592	9.34 (0.69)	9.76 (0.66)	9.53 (0.70)	0.624
	F	6.37 (0.35)	6.83 (0.64)	6.19 (0.29)		6.51 (0.68)	6.46 (0.42)	6.72 (0.43)	
Ct.Ar/Tt.Ar (%)	M	0.690 (0.022)	0.670 (0.035)	0.694 (0.013)	0.888	0.664 (0.027)	0.669 (0.040)	0.678 (0.025)	0.793
	F	0.657 (0.017)	0.681 (0.041)	0.670 (0.035)		0.664 (0.022)	0.648 (0.040)	0.655 (0.038)	
Ct.Th (mm)	M	0.768 (0.071)	0.781 (0.062)	0.809 (0.024)	0.324	0.788 (0.051)	0.802 (0.047)	0.801 (0.036)	0.798
	F	0.644 (0.011)	0.671 (0.022)	0.642 (0.024)		0.660 (0.026)	0.645 (0.037)	0.655 (0.035)	
vBMD	M	1.78 (0.27)	1.86 (0.24)	1.87 (0.27)	0.253	1.86 (0.24)	1.85 (0.25)	1.86 (0.23)	0.841
	F	1.92 (0.31)	1.93 (0.30)	1.74 (0.29)		1.91 (0.33)	1.83 (0.30)	1.92 (0.30)	
Maximum Load (N)	M	167.9 (9.4)	155.8 (15.5)	177.2 (21.3)	0.369	176.1 (17.3)	160.8 (38.3)	164.7 (7.9)	0.762
	F	126.9 (18.3)	129.4 (12.2)	125.4 (12.4)		130.3 (17.0)	132.6 (17.3)	129.9 (21.1)	
Extension (mm)	M	1.11 (0.16)	1.09 (0.21)	1.14 (0.08)	0.890	1.26 (0.23)	1.29 (0.09)	1.08 (0.18)	0.036[a]
	F	0.87 (0.08)	0.92 (0.07)	0.90 (0.12)		0.96 (0.14)	0.92 (0.17)	0.83 (0.12)	
Energy at break (mJ)	M	87.1 (14.5)	78.2 (19.5)	89.8 (20.7)	0.603	108.4 (28.2)	104.8 (27.8)	87.1 (15.4)	0.241
	F	42.5 (6.8)	49.4 (14.1)	49.8 (7.1)		48.9 (18.4)	54.8 (22.7)	43.0 (19.1)	
Stress (MPa)	M	99.7 (14.2)	84.6 (10.9)	95.8 (14.7)	0.963	101.4 (13.3)	83.4 (23.3)	116.6 (15.1)	0.267
	F	117.5 (24.6)	122.8 (24.0)	121.7 (37.2)		125.3 (34.6)	116.5 (17.4)	95.0 (22.0)	
Strain (%)	M	6.96 (0.87)	6.91 (1.69)	7.12 (0.64)	0.924	8.22 (1.28)	8.56 (0.71)	7.01 (1.30)	0.064
	F	6.67 (0.41)	6.91 (0.48)	6.81 (1.12)		7.33 (1.13)	7.09 (1.44)	6.54 (0.91)	
Elastic Modulus (MPa)	M	19.5 (6.5)	13.7 (5.9)	18.4 (4.7)	0.852	15.8 (3.3)	11.2 (5.1)	19.3 (7.4)	0.908
	F	42.1 (5.8)	45.9 (11.5)	45.7 (12.6)		35.7 (23.2)	40.3 (10.0)	29.9 (7.0)	

Data presented as mean (SD). Main and interaction effects tested with MIXED model ANOVA with Bonferroni post-hoc adjustment ($p<0.05$).

Diet*sex interaction differences demarked by no common lower case superscripts ($p<0.05$). [a]No significant differences among groups after Bonferroni correction.

3.5 DISCUSSION

To our knowledge, this is the first study to investigate a dose reponse relationship of endogenous and exogenous C-3 epimer on maintenance of calcium homeostasis and bone mineral density. Our dose-response of cholecalciferol and C-3 epimer in rodents showed detectable levels of C-3 epimer in 77% of 12-20 wk old Sprague Dawley rat plasma samples (n=216) with a lower limit of quantification of 6.25 nmol/L (2.50 ng/mL). The range of plasma C-3 epimer concentrations of rats fed non-epimeric diets (control, 2 IU vitamin D, 4 IU vitamin D, 0.5 IU 25(OH)D) was from 6.3-84.6 nmol/L. These highly detectable levels and wide C-3 epimer range are in agreement with research in adult human populations where 99% had detectable levels of C-3 epimer and a range of 0.2-59.3 nmol/L (101).

In our adult rodent model, a C-3 epimer dose response relationship was observed with higher intakes of cholecalciferol, significantly apparent in females. This data provides evidence for the endogenous synthesis of C-3 epimer and agrees with previous human studies demonstrating a significant positive correlation between the epimer and 25(OH)D concentrations in adult populations (130). This effect has also been seen in an infant population where increasing dosage of vitamin D supplementation (400, 800, 1200, 1600 IU) results in higher plasma C-3 epimer concentrations (13). To date, the research shows high absolute C-3 epimer concentrations in infants that decrease to adult concentrations at ~one year of age and generally lower C-3 epimer concentrations in adults that remain relatively constant over time (6). In agreement with this research, our study showed that C-3 epimer concentrations remained stable in adult rodents on control AIN93-M diet (1 IU vitamin D/g) over an 8 week period.

The vitamin D requirement for rodents has not been officially defined (131, 132). However, the AIN recommended control diet of 1000 IU vitamin D3/kg diet was used and contrary to previous findings that showed high serum 25(OH)D concentrations (>130 nmol/L) in Sprague Dawley rats fed AIN93-M diet, we did not see such high values (131). The concentrations of 25(OH)D in both male and female control rats over this 8 week study were below the recommendation for bone health (50 nmol/L) in humans (19). Only with the 4 IU vitamin D diet did 25(OH)D concentrations reach 50 nmol/L in both sexes. This might be due to assay differences as Fleet et

al (131) performed enzyme immunoassays (Immunodiagnostic Systems) for 25(OH)D detection that may overestimate the values due to cross reactivity with other vitamin D metabolites such as $24,25(OH)_2D_3$. Indeed in our study, $24,25(OH)_2D_3$ concentrations >20 nmol/L were generated in rodents from control, 2 IU vitamin D, 4 IU vitamin D and 0.5 IU 25(OH)D diet groups at all time points. Nonetheless, our values for bone outcomes were similar to previous data on adult rodent models (133) and no differences in any bone outcomes between the control and higher vitamin D intake diet groups were observed, suggesting that vitamin D status was sufficient to support calcium homeostasis and bone metabolism in the adult rat.

As expected, higher catabolism of 25(OH)D was observed with increasing 25(OH)D concentrations as seen by the positive correlation between 25(OH)D and $24,25(OH)_2D_3$ for the vitamin D dose response diets (control, 2 IU, 4 IU vitamin D/g diet). Interestingly, higher amounts of C-3 epimer were also associated with higher concentrations of $24,25(OH)_2D_3$. Based on previous studies demonstrating the involvement of CYP24 enzyme in the C-3 epimerization pathway, these results indicate that higher epimerization might be seen with higher CYP24 enzyme activity (7). As we discovered that rats consuming 0.5 IU 25(OH)D reference diet group did not have increased C-3 epimer concentrations over time and in fact had slightly decreased values (Figure 2c-d), it seems more plausible that epimerization occurs before the 25 hydroxylation step and could possibly be at the level of cholecalciferol metabolism in the gastrointestinal tract or liver.

Exogenous C-3 epimer was readily reflected in plasma C-3 epimer concentrations up to 291.3 ± 61.0 nmol/L. The fact that 25(OH)D and $24,25(OH)_2D_3$ concentrations significantly decreased with higher concentrations of C-3 epimer in the diet gives evidence that exogenous C-3 epimer is not readily converted back into its native 25(OH)D form, congruent with previous studies (7, 110). Rodents with these very high C-3 epimer concentrations had no significant negative effects on whole body and regional areal and volumetric BMD, BMC as well as other important bone parameters. Interestingly, a significant transient reduction of PTH concetrations at week 4 was observed in the 1 IU C-3 epimer diet group compared to 0.5 25(OH)D diet group. Whether C-3 epimer is as effective as vitamin D in suppressing PTH requires longer term *in vivo* studies, however, it is important to note that high concentrations of C-3 epimer had no overall negative

effect on PTH concentration. Furthermore, ionized calcium concentrations were not altered in any of the C-3 epimer diet groups vs 25(OH)D reference group. This suggests that C-3 epimer may in fact be equally potent as native 25(OH)D in its ultimate calcemic effects in a healthy state where bone mass is relatively stable.

In terms of bone microarchitecture, the 2 IU/kg VD diet consumed over 8 weeks resulted in higher trabecular separation in tibia compared to 4 IU VD diet. These differences are most likely not due to higher vitamin D intakes as trabecular separation values for rats on 4 IU diets were fairly similar to rats on control diet. The significant trend for lower entension at breakpoint shown from bone biomechanics may reflect minor changes in bone with high C-3 epimer concentrations (> 200 nmol/L). These small changes were apparent in lumbar vertebra (L3) showing lower BS/BV in higher C-3 epimer group compared to 1 IU C-3 epimer group and 25(OH)D reference group. However, these small significant changes did not result in altered bone strength or BMD of the C-3 epimer diet group compared to other diet groups.

To our knowledge this is the first study to administer exogenous C-3 epimer by the dietary route to measure the outcome of C-3 epimer concentrations. Exogenous C-3 epimer was mixed in the diet formula without any additional vitamin D and thus we were able to uniquely observe any potential negative or positive outcomes related to C-3 epimer concentrations independent of 25(OH)D and $1,25(OH)_2D_3$. In terms of limitations, although our sample size (n=6) was robust enough to detect differences in vitamin D metabolite biochemistry, it was not sufficient to detect significant differences in bone biomarkers OC, CTx and RANKL as well as some bone parameters. Furthermore our study period of 8 weeks was not long enough to predict if increasing or decreasing vitamin D metabolite concentrations would eventually plateau. Our values appeared to continue to change over the full 8 weeks with some evidence of the beginnings of a plateau.

In summary, endogenous synthesis of C-3 epimer follows a dose-response to cholecalciferol intake in adult rodents. The response was more prominent in females compared to males. Additionally, consumption of C-3 epimer in place of cholecalciferol displaces 25(OH)D concentrations. Whether C-3 epimer was endogenously generated or consumed, high plasma

concentrations of C-3 epimer do not significantly alter major bone biomarkers and have no negative effects on overall bone density and architecture in an adult rodent model over the short term. However, small effects were seen in PTH and trabecular architecture with the 1 IU C-3 epimer diet and thus longer term larger studies are warranted to ensure C-3 epimer has no consequences to bone. These studies along with additional scientific research on the metabolism, physiological function and source as well as epidemiological studies are required to further understand the physiological effects and relevance of C-3 epimer.

3.6 ACKNOWLEDGEMENTS

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4.0 GENERAL DISCUSSION

4.1 FINDINGS

The overall aim of this work was the expand the knowledge of C-3 epimer, a relatively novel metabolite of vitamin D with unknown source and biological function. Many researchers have emphasized the importance C-3 epimer resolution in clinical routine vitamin D testing as the lack of C-3 epimer concentration quantification could be leading to misdiagnosis of vitamin D sufficiency, which may be problematic for those at risk of bone diseases (6, 101). However, until we obtain further understanding of the physiological function(s) of C-3 epimer and its impact on bone health relative to 25(OH)D, the overall importance of its measurement remains questionable.

The work of Singh et al (5) showing that C-3 epimer can account for a significant proportion of total 25(OH)D in infants drew a lot of scientific interest in C-3 epimer measurement, particulary in infants populations. To date, much of the work agrees with these findings, including work performed in our laboratory by Gallo et al. demonstrating C-3 epimer concentrations reaching up to on average 60 nmol/L in infants given liquid supplements (13). Thus far, data on adults suggests low total contribution of C-3 epimer to total 25(OH)D however ranges within and across studies were wide and methods of C-3 epimer detection varied (6). Therefore, C-3 epimer detection in adults requires further clarification. Additionally, the potent ability of the biologically active metabolite of C-3 epimer to suppress PTH makes it particularly interesting to study aging populations as PTH concentrations increase with age (9, 10). Thus, we decided to focus on an elderly adult population to obtain a clearer understanding of the prevalence of detectable C-3 epimer with aging and went forth with a healthy adult rodent model as C-3 epimer was not detected in the elderly.

The goal of the first study was to determine how C-3 epimer concentrations change in response to 2000 IU supplementation of vitamin D (VD) in the oldest of old men (118). Based on a previous study in elderly veterans (n=34, 33 male, 1 female, > 70 y), 2000 IU was sufficient to raise 25(OH)D concentrations and correct hypovitaminosis D in those with fairly low 25(OH)D levels (134). Therefore, we used 2000 IU/d to raise plasma 25(OH)D concentrations to above a

75 nmol/L range to ensure that C-3 epimer detection would not be affected by low 25(OH)D concentrations. Vitamin D intakes were below the RDA for almost all participants and calcium intakes were below the RDA for approximately half of participants. Our supplementation of 2000 IU/day successfully raised 25(OH)D levels (n=40) by 30 nmol/L within an 8 week period. This information is important to note as vitamin D deficiency, especially among those in institutions, can be rather high and is concerning for health implications (135). C-3 epimer was not detected in elderly males even with significantly increased 25(OH)D concentrations (> 75 nmol/L) in response to 8 weeks of vitamin D supplementation.

Overall, our results show that C-3 epimer concentrations are not evident at 25(OH)D concentrations > 75 nmol/L in advanced aging. However, it should be appreciated that elderly persons (>70 y) have declined organ function and thus differential vitamin D metabolism (117). Although C-3 epimer concentrations are not correlated with age, the very old age of our participants could have affected C-3 epimer detection (101, 104). The differences between adult and infant C-3 epimer concentrations are likely reflective of the plausible source of C-3 epimer. The dramatic changes in the intestinal microbiome with age, that evolves with the shift from infant to adult diets, may be associated with these differential C-3 epimer profiles since intestinal bacteria is a potential source (5, 136). It also remains possible that contrary to infants, the supplementation of 2000 IU/day was not sufficient to raise C-3 epimer concentrations to a detectable concentration with our limit of detection (6.25 nmol/L) (13).

The first branch of the second study was therefore designed to explore different dosages of vitamin D on C-3 epimer concentrations in a healthy rodent adult model. We used the AIN93-M control diet containing 1000 IU vitamin D/kg diet, an experimental diet of 2000 IU vitamin D/kg diet and an experimental diet containing double the amount used in the vitamin D supplementation in the elderly; 4000 IU vitamin D/kg diet. Contrary to the findings in elderly men, C-3 epimer was generated in a dose response, particulary in female rodents. Although C-3 epimer was generated in both sexes with all diets, only the 4000 IU diet was significantly different from the control diet in females after 8 weeks ($p<0.05$). This therefore suggests that the differences seen between adult rodents (male and female) and elderly males could be due to sex-effects. Infact, most of bone parameters, bone biomarkers and vitamin D metabolite

concentrations were significantly different between sexes, most likely due to faster growing males and/or sex hormone differences. The possibility that the higher dosage of vitamin D caused the observed increase in C-3 epimer generation was ruled out as 25(OH)D concentrations of male and female adult rodents were not higher compared to 25(OH)D concentrations in elderly men after 8 weeks.

The second branch of the rodent study was to gain a better understanding of the physiological function of C-3 epimer and its effects on bone outcomes. Since the biological function of C-3 epimer is still unknown we used half of the NRC recommendation for vitamin D (500 IU vitamin D/kg diet) for our 25(OH)D reference group and two experimental C-3 epimer diet groups (500 IU and 1000 IU vitamin D/kg diet). Blood C-3 epimer concentrations were fairly elevated with exogenous C-3 epimer though there were no significant differences between diet groups specifically for body composition, anthropometry, diet intake, iCa, OC as well as bone parameters. Whether C-3 epimer is as effective as the active $1,25(OH)_2D_3$ metabolite in suppressing PTH requires longer term *in vivo* studies since the significant reduction of PTH concentrations observed in the 1 IU C-3 epimer diet group compared to 0.5 IU 25(OH)D diet group was transient and only observed at week 4.

Although rodents on either C-3 epimer diet showed no significant differences compared to the 25(OH)D reference diet for most bone mass indices, we cannot reject or accept our hypothesis that exogenous epimer is equally potent as 25(OH)D in maintenance of bone mineral density since differences were also not observed between these diets. The small significant decrease in proximal tibia BS/BV of C-3 epimer diet groups compared to reference 25(OH)D diet at week 8 was most likely due to the minor differences seen in bone parameters in this group compared to other diet groups at baseline. Overall, it is most important to mention that high concentrations of C-3 epimer (up to 291.3 ± 60.9 nmol/L) had no negative effects on bone. It still remains possible that as suggested, C-3 epimerization is an alternative pathway for breakdown of 25(OH)D (137).

4.2 STRENGTHS AND LIMITATIONS

The methodology used for C-3 epimer detection in both studies is a unique proprietary Diels-Alder procedure involving several purification steps. Whereas underivatized $25(OH)D_3$ and its 3-epimer can be successfully resolved chromatographically on CN or, better, on PFP columns, this Diels-Alder procedure was used to get sufficient sensitivity using the available mass spectrometry equipment with 20 μL of sample. Thus a strength of this work is the unique and precise measurement of C-3 epimer with high capabilities for C-3 resolvement which has been questionable in other studies. Additionally, in both studies, resolvement of C-3 epimer was performed as well as measurement of $24,25(OH)_2D_3$ which assists in better understanding of 25(OH)D concentrations (6).

A specific strength of *study 1* was that it was the first supplementation trial in elderly men with the objective of measuring C-3 epimer concentrations. Accuracy of methodology for 25(OH)D measurement was ensured by analyzing and comparing both plasma and serum 25(OH)D concentrations with LC-MS/MS and chemiluminescence respectively. Furthermore, none of the anthropometric data and dietary intake data were self-reported. All anthropometric measures were made by health care professionals or researchers creating very little patient bias in the data and dietary intake was assessed by a certified dietitian or dietetic students over a 3 day period.

The major strength of *study 2* was that it was the first *in vivo* dose-response investigation of C-3 epimer. To our knowledge this was the first study to administer exogenous C-3 epimer by the dietary route to measure the outcome of C-3 epimer concentrations. Exogenous C-3 epimer was mixed in the diet formula without any additional vitamin D and thus we were able to uniquely observe any potential negative or positive outcomes related to C-3 epimer concentrations independent of 25(OH)D and $1,25(OH)_2D_3$. We also demonstrated that 500 IU and 1000 IU C-3 epimer/kg diet resulted in very high C-3 epimer concentrations. Our analysis of bone parameters included both DXA and *in vivo* μCT which allowed us to compare aBMD to vBMD and measure any longitudinal changes in bone parameters. Changes over time were evident, but no differences among the diets were noted.

Both of the studies addressed in this thesis had their limitations. Exclusively affecting *study 1*, the LLOQ for C-3 epimer (6.25 nmol/L) was too high to allow detection of possibly low C-3 epimer concentrations in elderly men. This was considered a limitation since a previous study in adults using LLOQ of 0.25 nmol/L detected C-3 epimer concentrations in 99% of samples (n=214) (101). However, the lack of C-3 epimer detection in *study 1* could also be attributed to the small sample size which might have been insufficient to observe detectable C-3 epimer concentrations in elderly males. A small sample size was also a limitation of *study 2.* This limitation along with a short 8 week study duration most likely affected our ability to see significant changes in bone parameters and microarchitecture as well as further changes in PTH, however this could also be due to a mature and fully grown adult model.

Lastly, a limitation to both studies was not measuring the vitamin D sources for C-3 epimer content. In *study 1*, although C-3 epimer was not generated, measurement of C-3 epimer content in supplements would allow us to conclude instead of speculate that the supplements were infact not a significant source of C-3 epimer. In *study 2*, the measurement of C-3 epimer content in the vitamin D diets would help us make conclusions regarding endogenous generation as the major source of epimerization in female rats. However, we did assess whether heating would destroy the epimer and therefore had the diets produced with airdrying instead of heating.

4.3 CONCLUSIONS

C-3 epimer is not generated in response to 2000 IU/d vitamin D supplementation in advanced aging in humans but is generated in a dose-response manner with higher intakes of cholecalciferol in adult females rodents with no overall consequences to bone in both rodent sexes. *In study 1*, high concentrations of 25(OH)D (above 50 nmol/L) did not result in C-3 epimer generation above 6.25 nmol/L, suggesting C-3 epimer concentrations are not significant in this population, even in response to supplementation. *In study 2*, C-3 epimer concentrations were higher with increased cholecalciferol intakes indicating endogenous generation in a rodent model. Furthermore, we discovered that high plasma C-3 epimer concentrations did not result in any significant effects on bone health compared to the all other diets over 8 weeks. This work has thus shown that high concentrations of C-3 epimer is not detrimental to bone in an adult

rodent model and the source of C-3 epimer may be mostly through endogenous production with increasing intakes of cholecalciferol. Currently, the clinical evidence is not strong enough for the decision of whether or not quantification of C-3 epimer is necessary in vitamin D testing. The results of this thesis can help fill this research gap and contribute to the knowledge in the scope of vitamin D today.

4.4 FUTURE DIRECTIONS

Future studies should include longer study duration periods as well as larger samples sizes in both human and rodent studies. A recent epidemiological study showed that higher intake of vitamin D from supplements is associated with both higher C-3 epimer concentrations as well as a higher percent of C-3 epimer relative to total 25(OH)D (105). Therefore, in order to discover the potential source of C-3 epimer, vitamin D supplements and all other sources of vitamin D should be assessed for C-3 epimer content. Additionally, younger models and models of bone loss, such as the ovariectomized rat, should be explored to further confirm that C-3 epimer has no consequences to bone. These studies along with additional scientific research on the metabolism, physiological function and source as well as epidemiological studies are required to further understand the physiological effects and relevance of C-3 epimer.

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6.0 APPENDIX

Appendix table 1: Dietary intake and anthropometry of adult Sprague Dawley rats on vitamin D and epimer diets over 8 weeks

			Diets				Interaction	Diets				Interaction
			Control	2 IU VD3	4 IU VD3	All	diet*sex*time	0.5 IU 25(OH)D	0.5 IU C-3 epimer	1 IU C-3 epimer	All	diet*sex*time
	Sex	Time	(n=6)	(n=6)	(n=6)	(n=18)	p value	(n=6)	(n=6)	(n=6)	(n=18)	p value
Diet Intake (g/day)	M	0	25.9 (2.4)	24.5 (1.9)	24.7 (2.8)	25.0 (2.3)^{A}	0.335	25.3 (3.6)^{ab}	24.4 (1.0)^{ab}	23.6 (0.4)^{ab}	24.4 (2.2)^{A}	0.005
		4	27.9 (3.0)	25.4 (2.4)	26.4 (2.6)	26.6 (2.7)^{B}		26.4 (3.4)^{ab}	25.3 (0.9)^{ab}	26.2 (0.6)^{ab}	26.0 (2.0)^{B}	
		8	27.5 (3.4)	26.5 (1.8)	26.7 (2.6)	26.9 (2.6)^{B}		26.6 (2.8)^{ab}	26.6 (26.6)^{ab}	25.6 (0.6)^{ab}	26.2 (1.8)^{A}	
	F	0	18.4 (1.1)	18.5 (0.5)	19.7 (0.7)	18.8 (1.0)^{C}		17.5 (0.9)^{a}	19.8 (2.0)^{b}	18.6 (0.7)^{ab}	18.7 (1.6)^{C}	
		4	17.4 (2.3)	18.4 (1.0)	18.3 (2.1)	18.0 (1.8)^{C}		17.8 (1.6)^{ab}	17.5 (1.5)^{ab}	17.4 (0.8)^{ab}	17.5 (1.3)^{D}	
		8	17.5 (1.1)	18.3 (0.8)	18.5 (1.6)	18.1 (1.2)^{C}		17.9 (0.3)^{ab}	19.5 (0.9)^{ab}	17.0 (0.6)^{ab}	18.1 (1.2)^{CD}	
Body Weight (kg)	M	0	437.2 (20.2)	434.7 (23.7)	428.4 (24.2)	433.4 (21.7)^{A}	0.868	430.1 (31.0)	427.5 (21.2)	429.9 (32.2)	429.2 (26.8)^{A}	0.995
		4	558.8 (45.8)	550.0 (50.9)	559.2 (38.1)	556.0 (42.7)^{B}		548.8 (50.9)	549.0 (32.1)	546.7 (42.3)	548.2 (39.9)^{B}	
		8	646.9 (46.6)	607.3 (59.4)	621.9 (51.6)	625.4 (52.4)^{C}		611.6 (64.2)	611.7 (41.9)	604.8 (64.2)	609.4 (54.4)^{C}	
	F	0	277.6 (35.7)	286.2 (17.9)	281.5 (25.5)	281.8 (25.9)^{D}		277.3 (16.1)	280.5 (26.6)	277.4 (29.6)	278.4 (23.3)^{D}	
		4	318.6 (318.6)	331.8 (33.1)	337.5 (30.2)	329.3 (30.9)^{E}		323.4 (19.4)	315.4 (20.3)	319.5 (35.4)	319.4 (24.7)^{E}	
		8	349.8 ± 13.1	368.3 ± 15.9	353.2 ± 12.6	357.1 (33.2)^{F}		367.0 (31.9)	351.8 (13.4)	339.5 (38.1)	352.8 (30.3)^{F}	
Body Length (cm)	M	0	42.8 (1.0)	43.5 (0.3)	43.5 (1.4)	43.3 (1.0)^{A}	0.376	43.7 (1.45)	42.3 (1.7)	43.1 (1.1)	43.1 (1.5)^{A}	0.332
		4	45.4 (1.3)	45.6 (1.0)	46.1 (1.2)	45.7 (1.1)^{B}		46.5 (1.1)	45.2 (1.8)	45.9 (1.2)	45.9 (1.4)^{B}	
		8	47.0 (1.9)	46.9 (1.0)	48.0 (1.0)	47.3 (1.4)^{C}		47.8 (1.8)	46.7 (2.4)	46.8 (0.9)	47.1 (1.8)^{C}	
	F	0	38.8 (1.5)	39.1 (0.8)	38.8 (0.5)	38.9 (1.0)^{D}		38.6 (0.7)	38.9 (0.6)	38.4 (1.0)	38.6 (0.8)^{D}	
		4	40.3 (1.5)	40.9 (1.0)	40.9 (0.7)	40.7 (1.1)^{E}		41.0 (1.1)	41.2 (0.8)	40.7 (1.2)	40.9 (1.0)^{E}	
		8	41.5 (1.8)	41.8 (1.0)	41.3 (0.9)	41.5 (1.2)^{F}		41.5 (1.1)	41.2 (1.3)	41.6 (1.7)	41.4 (1.3)^{E}	

Data presented as mean (SD). Main and interaction effects tested with MIXED model ANOVA with Bonferroni post-hoc adjustment ($p<0.05$). Differences amongt diet*sex*time groups are demarked by different lower case superscripts. Time*sex effects and differences denoted by different upper case superscripts in the same column.

Appendix table 2:OC, Ctx and RANKL concentrations of Sprague Dawley rats on vitamin D diets over 8 weeks

			Diets				**Interaction**	**Diets**				**Interaction**
	Sex	Time	Control (n=6)	2 IU VD3 (n=6)	4 IU VD3 (n=6)	All (n=18)	p value Diet*Sex*Time	0.5 IU 25(OH)D (n=6)	0.5 IU C-3 epimer (n=6)	1 IU C-3 epimer (n=6)	All (n=18)	p value Diet*Sex*Time
OC	M	0	14.7 (1.7)	12.3 (4.7)	10.5 (6.0)	12.5 (4.6)	0.120	13.2 (2.9)	18.0 (13.5)	14.2 (5.7)	15.1 (8.4)	0.383
(nmol/L)		4	12.9 (4.8)	13.0 (3.8)	12.3 (1.7)	12.7 (3.4)		16.5 (7.8)	14.2 (8.9)	17.4 (11.6)	16.0 (9.1)	
		8	12.6 (6.5)	13.7 (8.0)	10.4 (3.7)	12.2 (6.1)		12.6 (4.4)	9.6 (2.5)	11.0 (3.2)	11.1 (3.5)	
	F	0	14.2 (5.4)	14.2 (7.17)	23.9 (19.2)	17.4 (12.4)		16.3 (3.7)	15.0 (3.6)	12.3 (5.4)	14.6 (4.4)	
		4	16.4 (4.1)	21.7 (24.3)	13.0 (5.0)	17.0 (14.1)		14.6 (7.8)	20.9 (14.5)	12.8 (4.2)	16.1 (10.3)	
		8	28.9 (34.4)	10.1 (4.3)	10.7 (5.4)	16.6 (21.1)		18.2 (11.1)	13.2 (6.8)	7.6 (2.7)	13.0 (8.5)	
CTX	M	0	67.6 (14.8)	65.2 (14.5)	100.4 (54.4)	77.7 (35.6)^{A}	0.472	91.5 (25.1)	67.3 (16.3)	74.8 (16.8)	77.9 (21.4)^{A}	0.386
(ng/mL)		4	48.5 (12.2)	57.9 (22.3)	67.3 (19.7)	57.9 (19.1)^{B}		48.9 (15.2)	50.9 (31.6)	47.4 (14.6)	49.1 (20.6)^{B}	
		8	37.6 (9.4)	40.6 (13.5)	52.7 (9.5)	43.6 (12.3)^{B}		45.8 (16.1)	43.9 (8.4)	41.9 (7.5)	43.9 (10.8)^{B}	
	F	0	41.9 (12.9)	40.4 (7.7)	31.9 (6.0)	38.1 (9.9)^{D}		37.3 (12.1)	45.5 (8.9)	39.3 (11.6)	40.7 (10.8)^{C}	
		4	23.8 (6.1)	20.6 (3.5)	16.1 (4.0)	20.2 (5.5)^{E}		19.3 (7.8)	21.7 (5.8)	26.1 (11.6)	22.3 (8.7)^{D}	
		8	18.7 (3.9)	19.0 (3.9)	13.6 (1.6)	17.1 (4.0)^{E}		14.4 (4.0)	19.1 (7.6)	17.7 (11.4)	17.1 (8.0)^{D}	
							Diet * Sex					Diet * Sex
RANKL	M	8	0.61 (0.11)	0.72 (2.89)	0.87 (0.21)	0.73 (0.23)	0.177	0.58 (0.13)	0.64 (0.09)	0.68 (0.16)	0.63 (0.25)	0.831
(pmol/L)	F	8	0.62 (0.09)	0.59 (0.15)	0.58 (0.21)	0.60 (0.15)		0.50 (0.06)	0.65 (0.14)	0.72 (0.40)	0.62 (0.13)	

Data presented as mean (SD). Main and interaction effects tested with MIXED model ANOVA with manual Bonferroni post-hoc adjustment ($p<0.05$). Differences among time *sex are demarked by different upper case superscripts in the same column. Diet*sex interaction differences demarked by no common lower case superscripts.

Appendix table 3: Body composition of adult Sprague Dawley rats on vitamin D and epimer diets over 8 weeks

			Diets				Interaction	Diets				Interaction
			Control	2 IU VD3	4 IU VD3	All	Diet*Sex*Time	0.5 IU 25(OH)D	0.5 IU C-3 epimer	1 IU C-3 epimer	All	Diet*Sex*Time
	Sex	Time	(n=6)	(n=6)	(n=6)	(n=18)	p value	(n=6)	(n=6)	(n=6)	(n=18)	p value
Lean mass (g)	M	0	338.9 (31.8)	342.4 (34.7)	344.4 (18.8)	341.9 (27.6)^{A}	0.722	347.1 (24.7)	341.2 (30.1)	340.8 (26.9)	343.0 (25.8)^{A}	0.304
		4	401.1 (40.7)	402.4 (33.8)	422.5 (37.5)	408.6 (36.6)^{B}		424.8 (15.6)	413.4 (64.9)	405.0 (16.4)	414.4 (38.2)^{B}	
		8	438.3 (43.8)	434.4 (54.4)	453.8 (26.8)	442.2 (41.5)^{C}		442.7 (17.1)	455.3 (73.0)	420.9 (17.5)	439.6 (44.3)^{C}	
	F	0	226.5 (31.6)	227.1 (26.5)	225.8 (29.8)	226.5 (27.6)^{D}		231.3 (18.3)	231.0 (30.5)	225.0 (23.6)	229.1 (23.3)^{D}	
		4	233.0 (30.7)	241.7 (16.9)	231.3 (27.4)	235.4 (24.6)^{D}		240.4 (24.6)	234.4 (9.8)	231.6 (19.7)	235.5 (18.3)^{D}	
		8	258.8 (23.3)	257.2 (16.1)	247.2 (16.3)	254.4 (18.5)^{E}		270.1 (19.8)	254.5 (7.2)	252.5 (27.8)	259.0 (20.5)^{E}	
aBMD (g/cm^2)	M	0	0.152 (0.006)	0.151 (0.005)	0.151(0.003)	0.151 (0.004)^{A}	0.104	0.152 (0.007)	0.154 (0.006)	0.154 (0.003)	0.153 (0.00526)^{A}	0.883
		4	0.168 (0.007)	0.171 (0.006)	0.171 (0.003)	0.170 (0.005)^{B}		0.170 (0.007)	0.176 (0.004)	0.175 (0.003)	0.174 (0.00543)^{B}	
		8	0.180 (0.008)	0.179 (0.005)	0.182 (0.004)	0.180 (0.006)^{C}		0.179 (0.009)	0.186 (0.005)	0.185 (0.005)	0.183 (0.00697)^{C}	
	F	0	0.145 (0.004)	0.146 (0.005)	0.146 (0.008)	0.146 (0.006)^{D}		0.144 (0.005)	0.147 (0.005)	0.151 (0.006)	0.147 (0.00549)^{D}	
		4	0.165 (0.003)	0.162 (0.005)	0.162 (0.006)	0.163 (0.005)^{E}		0.162 (0.008)	0.164 (0.004)	0.167 (0.004)	0.164 (0.00559)^{E}	
		8	0.170 (0.005)	0.166 (0.004)	0.167 (0.008)	0.168 (0.006)^{F}		0.166 (0.010)	0.169 (0.006)	0.173 (0.005)	0.169 (0.00752)^{F}	
BMC (g)	M	0	10.635 (0.471)	10.788 (0.286)	10.567 (0.482)	10.663 (0.408)^{A}	0.849	10.747 (0.645)	10.920 (0.420)	10.762 (0.603)	10.809 (0.536)^{A}	0.891
		4	14.372 (0.903)	14.385 (0.779)	14.642 (1.037)	14.466 (0.866)^{B}		14.065 (1.729)	14.722 (0.703)	14.580 (1.038)	14.456 (1.194)^{B}	
		8	16.715 (1.309)	16.813 (1.149)	16.748 (1.120)	16.759 (1.124)^{C}		16.578 (1.660)	17.087 (0.844)	16.905 (1.445)	16.857 (1.297)^{C}	
	F	0	7.840 (0.829)	8.018 (0.793)	7.847 (0.525)	7.902 (0.690)^{D}		7.740 (0.382)	7.845 (0.242)	8.122 (0.407)	7.902 (0.369)^{D}	
		4	9.903 (0.863)	9.985 (0.823)	9.940 (0.213)	9.942 (0.658)^{E}		9.878 (0.532)	9.995 (0.465)	10.073 (0.724)	9.982 (0.555)^{E}	
		8	10.920 (1.052)	11.383 (1.323)	10.943 (0.387)	11.082 (0.966)^{F}		11.017 (0.881)	11.390 (0.860)	11.233 (0.901)	11.213 (0.842)^{F}	

Data presented as mean (SD). Main and interaction effects tested with MIXED model ANOVA with Bonferroni post-hoc adjustment ($p<0.05$). Differences among time *sex are demarked by different upper case superscripts in the same column

Appendix table 4: Regional analysis of femur, tibia and lumbar spine of 20 week old Sprague Dawley rats on vitamin D and C-3 epimer diets

		Diets			Main Effect	Diets			Main effect
Femur	Sex	Control (n=6)	2 IU VD3 (n=6)	4 IU VD3 (n=6)	Diet p value	0.5 IU 25(OH)D (n=6)	0.5 IU C-3 epimer (n=6)	1 IU C-3 epimer (n=6)	Diet p value
BA (cm^2)	M	1.7 (0.1)	1.7 (0.2)	1.7 (0.1)	0.628	1.6 (0.1)	1.7 (0.1)	1.7 (0.1)	0.811
	F	1.4 (0.1)	1.4 (0.1)	1.4 (0.1)		1.5 (0.1)	1.4 (0.1)	1.5 (0.1)	
BMC (g)	M	0.755 (0.090)	0.737 (0.067)	0.732 (0.058)	0.392	0.725 (0.062)	0.750 (0.066)	0.778 (0.068)	0.673
	F	0.508 (0.039)	0.532 (0.064)	0.513 (0.026)		0.510 (0.072)	0.535 (0.049)	0.523 (0.058)	
aBMD (g/cm^2)	M	0.452 (0.043)	0.432 (0.047)	0.440 (0.042)	0.275	0.454 (0.039)	0.450 (0.018)	0.467 (0.032)	0.704
	F	0.361 (0.016)	0.373 (0.039)	0.360 (0.014)		0.349 (0.028)	0.389 (0.030)	0.354 (0.027)	
vBMD (g/cm^3)	M	0.497 (0.023)	0.485 (0.031)	0.525 (0.042)	0.323	0.500 (0.040)	0.516 (0.051)	0.521 (0.029)	0.748
	F	0.646 (0.039)	0.618 (0.064)	0.652 (0.071)		0.624 (0.030)	0.623 (0.041)	0.637 (0.059)	
trab vBMD (g/cm^3)	M	0.369 (0.008)	0.396 (0.044)	0.377 (0.012)	0.855	0.391 (0.050)	0.375 (0.015)	0.373 (0.062)	0.583
	F	0.580 (0.050)	0.571 (0.064)	0.585 (0.084)		0.563 (0.045)	0.554 (0.047)	0.577 (0.054)	
Tibia									
BA (cm^2)	M	2.0 (0.2)	2.0 (0.1)	2.0 (0.1)	0.400	2.1 (0.1)	2.0 (0.1)	2.0 (0.1)	0.435
	F	1.6 (0.03)	1.6 (0.1)	1.6 (0.1)		1.6 (0.1)	1.7 (0.1)	1.6 (0.1)	
BMC (g)	M	0.507 (0.058)	0.488 (0.049)	0.495 (0.048)	0.644	0.520 (0.059)	0.517 (0.030)	0.532 (0.040)	0.904
	F	0.353 (0.021)	0.363 (0.044)	0.357 (0.029)		0.350 (0.050)	0.370 (0.046)	0.348 (0.033)	
aBMD (g/cm^2)	M	0.255 (0.014)	0.242 (0.016)	0.253 (0.027)	0.859	0.250 (0.020)	0.257 (0.010)	0.267 (0.024)	0.347
	F	0.225 (0.010)	0.221 (0.023)	0.224 (0.012)		0.220 (0.020)	0.223 (0.021)	0.217 (0.014)	
vBMD (g/cm^3)	M	0.429 (0.023)	0.415 (0.038)	0.445 (0.030)	0.199	0.415 (0.038)	0.437 (0.051)	0.449 (0.036)	0.948
	F	0.634 (0.050)	0.581 (0.077)	0.651 (0.090)		0.607 (0.036)	0.606 (0.035)	0.601 (0.082)	
trab vBMD (g/cm^3)	M	0.333 (0.015)	0.329 (0.016)	0.341 (0.022)	0.525	0.331 (0.017)	0.346 (0.028)	0.341 (0.016)	0.797
	F	0.416 (0.013)	0.401 (0.040)	0.415 (0.026)		0.418 (0.008)	0.423 (0.008)	0.413 (0.022)	
Lumbar Spine									
BA (cm^2) (L1-L4)	M	2.1 (0.2)	2.1 (0.1)	2.1 (0.1)	0.166	2.2 (0.1)	2.1 (0.2)	2.2 (0.1)	0.466
	F	1.6 (0.1)	1.6 (0.1)	1.6 (0.1)		1.6 (0.2)	1.6 (0.1)	1.6 (0.1)	
BMC (g) (L1-L4)	M	0.665 (0.079)	0.678 (0.060)	0.673 (0.052)	0.439	0.688 (0.082)	0.720 (0.108)	0.718 (0.063)	0.487
	F	0.492 (0.042)	0.488 (0.043)	0.497 (0.022)		0.487 (0.078)	0.490 (0.051)	0.507 (0.045)	
aBMD (g/cm^2) (L1-L4)	M	0.321 (0.024)	0.322 (0.024)	0.323 (0.011)	0.440	0.348 (0.093)	0.338 (0.031)	0.329 (0.018)	0.862
	F	0.311 (0.018)	0.312 (0.015)	0.307 (0.019)		0.307 (0.012)	0.299 (0.019)	0.312 (0.010)	
vBMD (g/cm^3) (L3)	M	0.483 (0.026)	0.500 (0.033)	0.492 (0.019)	0.864	0.488 (0.039)	0.508 (0.037)	0.488 (0.017)	0.518
	F	0.560 (0.017)	0.551 (0.032)	0.561 (0.008)		0.546 (0.027)	0.541 (0.017)	0.567 (0.027)	
trab vBMD (g/cm^3) (L3)	M	0.357 (0.005)	0.359 (0.004)	0.357 (0.002)	0.713	0.356 (0.006)	0.354 (0.008)	0.358 (0.004)	0.156
	F	0.375 (0.003)	0.372 (0.006)	0.379 (0.016)		0.366 (0.008)	0.370 (0.004)	0.374 (0.011)	

Data presented as mean (SD). Main and interaction effects tested with MIXED model ANOVA with Bonferroni post-hoc adjustment.

Appendix Table 5 : Trabecular analysis of femur and tibia metaphysis and lumbar spine (L3) of 20 week old Sprague Dawley rats on vitamin D and C-3 epimer diets

		Diets			Main effect	Diets			Main Effect
		Control	2 IU VD3	4 IU D3	Diet	0.5 IU 25(OH)D	0.5 IU C-3 epimer	1 IU C-3 epimer	Diet
Femur	Sex	(n=6)	(n=6)	(n=6)	p value	(n=6)	(n=6)	(n=6)	p value
BV/TV (%)	M	18.93 (5.67)	17.70 (3.70)	23.44 (5.93)	0.529	17.66 (3.52)	19.89 (6.35)	22.95 (2.17)	0.768
	F	30.29 (3.66)	32.54 (10.89)	31.93 (9.12)		30.96 (8.82)	31.29 (4.83)	29.25 (5.10)	
Tb.Th (mm)	M	0.093 (0.010)	0.092 (0.005)	0.098 (0.008)	0.859	0.090 (0.005)	0.093 (0.007)	0.094 (0.002)	0.985
	F	0.089 (0.003)	0.091 (0.008)	0.087 (0.009)		0.090 (0.009)	0.087 (0.004)	0.087 (0.004)	
Tb.Sp (mm)	M	0.273 (0.056)[ab]	0.331 (0.100)[a]	0.234 (0.048)[b]	0.027	0.293 (0.118)	0.251 (0.048)	0.233 (0.041)	0.474
	F	0.167 (0.016)[c]	0.198 (0.093)[c]	0.158 (0.025)[c]		0.172 (0.024)	0.171 (0.027)	0.181 (0.062)	
Tb.N (1/mm)	M	2.02 (0.49)	1.92 (0.35)	2.38 (0.45)	0.420	1.96 (0.36)	2.12 (0.56)	2.45 (0.16)	0.540
	F	3.39 (0.33)	3.53 (0.93)	3.60 (0.70)		3.39 (0.74)	3.57 (0.47)	3.38 (0.56)	
Tibia									
BV/TV (%)	M	14.93 (3.36)	13.17 (2.27)	18.44 (4.12)	0.046[#]	16.45 (5.23)	17.01 (6.83)	18.67 (3.06)	0.428
	F	45.59 (5.07)	39.63 (11.13)	46.88 (7.45)		42.06 (5.99)	45.20 (4.84)	46.56 (7.72)	
Tb.Th (mm)	M	0.088 (0.008)	0.088 (0.005)	0.087 (0.006)	0.272	0.086 (0.003)	0.087 (0.007)	0.083 (0.008)	0.230
	F	0.076 (0.011)	0.066 (0.012)	0.075 (0.011)		0.065 (0.014)	0.071 (0.012)	0.079 (0.005)	
Tb.Sp (mm)	M	0.25 (0.04)[ab]	0.32 (0.09)[a]	0.20 (0.02)[b]	0.005	0.251 (0.071)	0.264 (0.133)	0.222 (0.024)	0.838
	F	0.09 (0.01)[c]	0.12 (0.09)[c]	0.09 (0.01)[c]		0.088 (0.011)	0.086 (0.006)	0.095 (0.023)	
Tb.N (1/mm)	M	1.69 (0.41)	1.50 (0.26)	2.10 (0.41)	0.286	1.92 (0.59)	1.95 (0.75)	2.29 (0.60)	0.720
	F	6.03 (0.81)	5.94 (1.47)	6.28 (0.55)		6.63 (0.84)	6.43 (0.63)	5.82 (0.74)	
Lumbar Spine (L3)									
BV/TV (%)	M	31.50 (4.63)	31.56 (2.24)	34.01 (2.28)	0.506	32.32 (3.63)	34.17 (6.51)	33.22 (2.52)	0.237
	F	38.81 (2.86)	35.72 (3.22)	34.31 (3.29)		33.91 (3.84)	36.32 (2.69)	38.45 (4.18)	
Tb.Th (mm)	M	0.094 (0.039)	0.093 (0.003)	0.098 (0.007)	0.290	0.093 (0.003)	0.096 (0.006)	0.094 (0.002)	0.107
	F	0.098 (0.004)	0.093 (0.002)	0.102 (0.024)		0.091 (0.003)	0.092 (0.005)	0.106 (0.002)	
Tb.Sp (mm)	M	0.199 (0.032)	0.197 (0.014)	0.196 (0.013)	0.482	0.194 (0.024)	0.189 (0.018)	0.197 (0.017)	0.492
	F	0.188 (0.012)	0.193 (0.010)	0.248 (0.150)		0.195 (0.018)	0.183 (0.009)	0.214 (0.086)	
Tb.N (1/mm)	M	3.34 (0.48)	3.39 (0.19)	3.48 (0.25)	0.629	3.47 (0.369)	3.54 (0.473)	3.54 (0.268)	0.766
	F	3.98 (0.20)	3.84 (0.31)	3.50 (0.80)		3.73 (0.356)	3.93 (0.166)	3.77 (0.805)	

Data presented as mean (SD). Main and interaction effects tested with MIXED model ANOVA with Bonferroni post-hoc adjustment ($p<0.05$).

Diet*sex interaction differences demarked by no common lower case superscripts.

[#] Interaction not significant after Bonferroni post-hoc adjustment.