Vitamin D Insufficiency in Children with Duchenne Muscular Dystrophy

By

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Abstract

Duchenne muscular dystrophy (DMD) is an X-linked condition caused by mutations in the dystrophin gene, resulting in muscle function loss. Children with DMD are at risk for compromised bone health due to loss of ambulation and corticosteroid treatment. A retrospective study was conducted to determine if vitamin D levels were reduced in this population. The serum 25-OHD and 25-OHD standardized per unit of vitamin D supplementation in DMD patients were both lower than another cohort of patients with disability (Ostegenesis Imperfecta), and a cohort treated with glucocorticoids (Systemic Lupus Erythematosus). Controlling for the season, the significant determinants of serum 25-OHD as well as serum 25-OHD per unit of supplementation were the child's underlying condition, disease duration, and weight Z-scores. Our study suggests that children with DMD have on average lower serum 25-OHD despite high levels of supplementation in comparison to a glucocorticoid treated population, and a population with disability.

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List of Abbreviations

1,25-OHD	1,25-dihydroxyvitamin D
25-OHD	25-hydroxyvitamin D
7-DHC	7-dehydrocholesterol
AAP	American Academy of Pediatrics
ALP	Alkaline Phosphatase
ALTM	All-laboratory Trimmed Mean
ANOVA	Analysis of Variance
AP	Activating Protein
APC	Antigen Presenting Cells
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMI	Body:Mass Index
C-22	Carbon #22
CALIPER	Canadian Laboratory Initiative for Pediatric Reference Intervals
cAMP	Cyclic Adenosine Monophosphate
CHMS	Canadian Health Measures Survey
CPB	Competitive protein binding assay
CPS	Canadian Pediatric Society
CYP	Cytochrome P450
DBP	Vitamin D-Binding Protein
DMD	Duchenne Muscular Dystrophy
DNA	Deoxyribonucleic Acid
DXA	Dual Energy X-ray Absorptiometry
HPLC	High-performance liquid chromatography
IL	Interleukin
IOM	Institute of Medicine
IU	International Unit
KDOQI	Kidney Disease Outcomes Quality Initiative
MED	Minimal Erythemal Dose
mRNA	Messenger Ribonucleic Acid
NEJM	New England Journal of Medicine
NF-κB	Nuclear Factor Kappa-chain of B-cells
NHANES	National Health and Nutrition Examination Study
Nm	Nanometers
NS	Not Significant
OI	Osteogenesis Imperfecta
PMCA1b	ATP-dependent calcium pump type 1B
PPi	Inorganic Pyrophosphate
PTH	Parathyroid Hormone
RANK	Receptor Activator of Nuclear Factor Kappa-B
REDCap	Research Electronic Data Capture
RIA	Radioimmunoassay
RXR	Retinoid X Receptor
SD	Standard Deviation

SLE	Systemic Lupus Erythematosus
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
TGF	Transforming Growth Factor
T_H	T-Helper Cell
TNF	Tumor Necrosis Factor
TRPV6	Transient Receptor Potential channel type 6
UV	Ultraviolet
VDR	Vitamin D Receptor

1 Introduction

Duchenne muscular dystrophy (DMD) is a disease characterized by progressive loss of muscle

function. The absence of the dystrophin gene results in dystrophic muscle, resulting in

cardiovascular and pulmonary issues[1]. There has been an increasing awareness of the

importance of bone health in Duchenne muscular dystrophy (DMD). Patients with DMD

experience muscle weakness and inflammation. There is no current cure for this disease, only

treatments designed to slow the progression of symptoms. Glucocorticoids, such as deflazacort

and prednisone, are the standard treatment for patients with DMD to prolong ambulation, and

to maintain cardiovascular and respiratory function[2]. However, despite these beneficial effects,

the continual use of glucocorticoids has been shown to reduce bone mass, stunt growth, and

increase fractures in patients with DMD. [3] Glucocorticoids are often prescribed with calcium

and vitamin D supplementation to improve bone mass[4]. Despite the potential benefits of

vitamin D adequacy to improve bone health, high rates of vitamin D deficiency have been

described in cohorts of boys with DMD[3]. The cause of this deficiency and its contribution to

poor bone health is unclear and merits further investigation. Therefore, it is essential to

understand the vitamin D status in patients with DMD, as well as significant determinants to

serum vitamin D and bone health.

1.1 Vitamin D

Vitamin D is a fat-soluble secosteroid required for calcium homeostasis, and maintenance of

bone, cardiovascular, and immune health[5,6]. In recent years there has been increasing

awareness of the importance of vitamin D to prevent disease and disability. It has been

suggested that individuals with autoimmune, inflammatory, and bone-related diseases may

have low levels of serum vitamin D[7]. According to the Canadian Health Measures Survey in 2012, 35% of the Canadian population did not meet the standard required for optimal bone health[8]. Individuals may be at risk of vitamin D deficiency due to poor diet, low sunlight exposure, as well as underlying diseases and their respective treatments. Vitamin D deficient individuals are susceptible to osteomalacia, a condition of decreased mineralization that in children produces rickets, growth retardation, and skeletal deformities[9]. Despite the evident benefits of vitamin D adequacy, vitamin D deficiency is commonly reported in pediatric populations including Duchenne muscular dystrophy, Osteogenesis imperfecta, and systemic lupus erythematosus[10-12].

1.2 Vitamin D Synthesis and Metabolism

The active form of vitamin D, 1,25-dihydroxyvitamin D is derived from two major precursors: vitamin D_2 (ergocalciferol), and vitamin D_3 (cholecalciferol). Vitamin D_2 is obtained in the diet from some plants and fungi exposed to UV irradiation. Vitamin D_3 can be synthesized in the skin when exposed to UV irradiation and is also obtained in the diet from consumption of animal products such as eggs and liver[13].

1.2.1 Cutaneous Synthesis of Vitamin D

Synthesis of vitamin D_3 begins as acetyl-CoA is converted into lanosterol in the cholesterol synthesis pathway. Dehydrogenation of lanosterol converts it to 7-dehydrocholesterol (7-DHC) and can then be stored in the epidermis to be converted to vitamin D3, or to cholesterol[14]. Vitamin D_3 is synthesized from 7-dehydrocholesterol[14] in a 2-step process. UV-B light ranging in wavelength from 290-320nm first penetrates the layers of the epidermis and dermis to

convert 7-dehydrocholesterol to precalciferol[4]. Regions with the highest levels of precalciferol

formation are the stratum spinosum and stratum basale, both located in the epidermal layer of

the skin[15]. Heat in the form of body temperature (37°C) is then required to isomerize the

precalciferol molecule to cholecalciferol[15]. The synthesis of vitamin D_2 follows a similar

pathway in fungi and yeast. Ergocalciferol is formed through UVB irradiation of ergosterol[16].

UVB-exposure of ergosterol cleaves the B-ring forming pre-vitamin D_2, which isomerizes to

ergocalciferol in the presence of heat[16,17]. Structurally, ergosterol differs from 7-DHC in the

side chain, as it has an additional C22-C23 double bond as well as an additional methyl group

on C24[16].

Figure 1 - Chemical transformation of previtamin D to 25-hydroxyvitamin D. The reaction on

the top shows vitamin D2 synthesis and the one below shows vitamin D3 synthesis. (Taken

from: Bikle, 2014) [18]
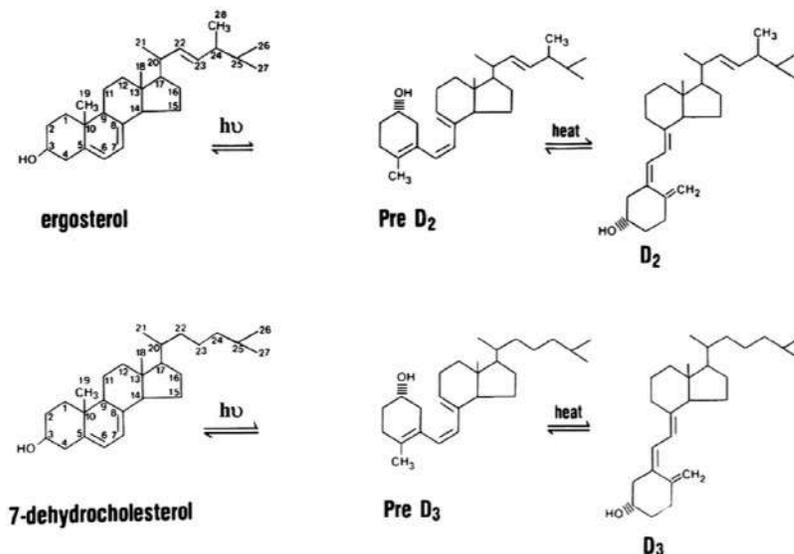
There are several advantages to synthesizing vitamin D_3 in the skin. First, the skin serves as a

reservoir for 7-DHC and precalciferol. Therefore, when the body requires additional vitamin D,

it is not limited by the amount of precursor in the skin. Second, the catalysis of precalciferol to

vitamin D_3 immediately occurs after the conversion of 7-DHC to precalciferol. This rapid reaction allows the skin to continually release vitamin D_3 into the bloodstream for up to 3 days upon exposure to UV-B light[15]. Lastly, excess exposure of the skin to sunlight will convert precalciferol to two biologically inactive isomers, lumisterol$_3$ and tachysterol$_3$ to prevent vitamin D toxicity[19,20].

Conversely, there are limitations in the process of synthesizing vitamin D_3 in the skin. The major limitations are skin pigmentation, habitat latitude, and the use of sunscreen[21]. Individuals with darker skin pigmentation have a greater concentration of melanin, a pigment in skin that absorbs light in the UV range[22]. Higher amounts of melanin in the skin will reduce the amount of UV-B light absorbed, and hence reduce the amount of vitamin D_3 that is synthesized[23]. Significantly higher levels of post-UV-B serum vitamin D_3 were measured in individuals with high skin reflectance (European ancestry) in comparison to those with low skin reflectance (African American ancestry)[24,25]. For this reason those with African American ancestry are suggested to take two times the recommended daily intake for vitamin D supplementation to achieve optimal vitamin D sufficiency[25]. The evidence regarding the effect of sunscreen use on vitamin D has been inconclusive. Regular sunscreen use has shown no significant change in serum vitamin D levels in two studies[26,27]. Conversely, another study has shown that sunscreen use was linked to significantly lower levels of circulating active vitamin D in comparison to non-users[28]. A review of this topic suggested that although doses of sunscreen used in studies reduced serum vitamin D, its daily regular use would have minimal effects on vitamin D levels[29]. A potential explanation for this discrepancy is that most individuals regularly use a lower concentration and a lower quantity of sunscreen in comparison to those who were tested in a long-term clinical study, and hence do not experience a significant decline in serum

vitamin D[30]. Other limitations of vitamin D_3 production include the concentration of 7-DHC in the skin, as the levels of 7-DHC decrease with increases in age, the energy of the UVB radiation penetrating the cutaneous layers of skin, the solar zenith angle (dependent on the season and latitude), and the temperature of the epidermis, which regulates the isomerization of precalciferol to vitamin D_3[21].

1.2.2   25-Hydroxylation

The liver acts as the main site to convert vitamin D_3 into 25-hydroxyvitamin D (25-OHD), the precursor to the active form of vitamin D. 25-hydroxylation of vitamin D_3 is catalyzed by cytochrome P450 (CYP) enzymes, whereby a hydroxyl group is added to the C-25 of vitamin D_3[31].

Figure 2 - 25-hydroxylation of vitamin D3 to become 25-hydroxyvitamin D3. (Taken from: ChemSpider Structure Search)

Cholecalciferol
(vitamin D3)

Calcidiol
(25-hydroxyvitamin D3)
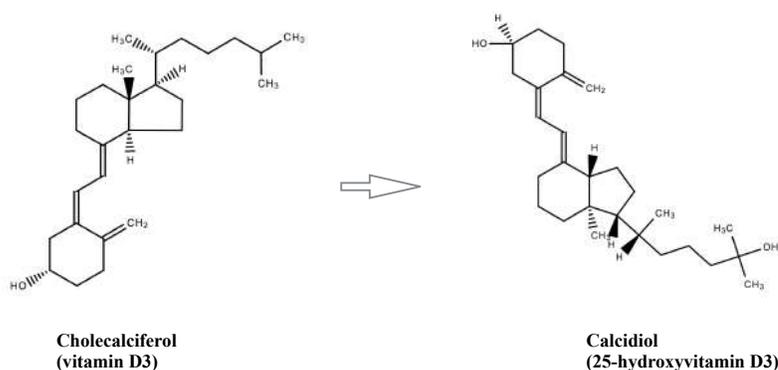
At least 6 CYP enzymes have been identified to hydroxylate vitamin D_3 *in vitro*, namely CYP3A4, CYP2J2, CYP2J3, CYP2C11, CYP27A1, and CYP2R1[32-34]. However, CYP2R1 has been proven to be the major enzyme involved in 25-hydroxylation of vitamin D_3 in the liver.

When CYP2R1 is deleted or mutated in mice, total circulating serum 25-OHD is dramatically reduced[35,36]. It has been shown that the remaining CYP enzymes impact 25-OHD production in tissues other than the liver, or contribute minimally to the circulating levels of 25-OHD[36]. Once 25-OHD is produced, it is transported to the kidney via vitamin D-binding protein (DBP) to be hydroxylated further to become the active metabolite. Regulation of CYP enzymes involved in hepatic 25-hydroxylation by parathyroid hormone (PTH), calcium, or phosphorus levels is still unclear. Liver CYP27A1 mRNA levels in rats are affected by factors including $1,25-OH_2D$, however regulation of CYP2R1 activity is uncertain.

1.2.3   1α-Hydroxylation

The complex of 25-OHD and DBP are filtered through the glomerulus into the nephron of the kidney, and reabsorbed in the proximal tubules by the endocytic receptor megalin[37]. Megalin is a 600-kDa transmembrane protein belonging to the low-density lipoprotein family[37]. Megalin is expressed on the apical side of epithelial cells in the proximal tubule and absorbs the complex of 25-OHD and DBP via receptor-mediated endocytosis[38]. Megalin knock-out mice have exhibited the inability to internalize 25-OHD and DBP, resulting in urinary excretion of 25-OHD and DBP, and ultimately vitamin D deficiency and bone disease[38]. 25-OHD is hydroxylated in the kidney by 25-hydroxyvitamin D_3-1α-hydroxylase, also known as the CYP27B1 enzyme[39]. Production of 1α-hydroxylase occurs in the epithelial cells of the proximal tubules in the kidney. 1α-hydroxylase hydroxylates 25-OHD at the C-1 position of the α-ring to generate 1,25-dihydroxyvitamin D_3 ($1,25-OH_2D$) the active form of vitamin D[21].

Figure 3 - 1α hydroxylation of 25-hydroxyvitamin D to become 1,25-dihydroxyvitamin D. The highlighted red circle indicates hydroxylation at the C-1 position of the α ring. (Taken from: ChemSpider Structure Search)

Calcidiol
(25-hydroxyvitamin D3)

Calcitriol
(1,25-dihydroxyvitamin D3)
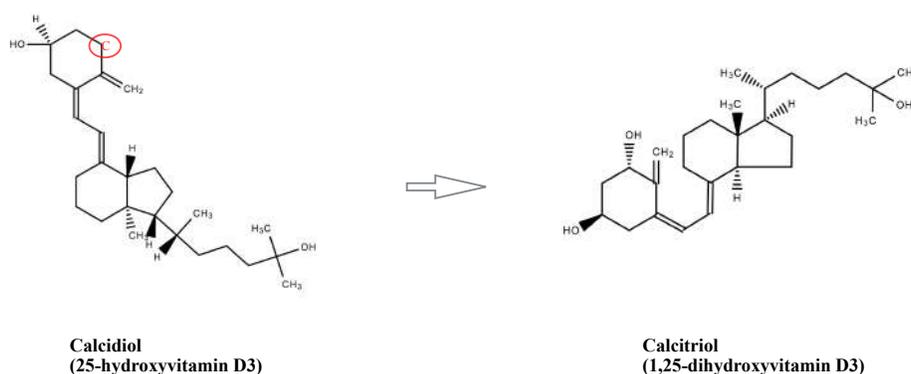
1α-hydroxylase activity is regulated via the cAMP and calcium/phospholipid pathways. Parathyroid hormone receptors are expressed in renal proximal tubule cells and an increase in parathyroid hormone levels stimulates these receptors to increase cAMP and stimulate an increase in 1α-hydroxylase mRNA, resulting in upregulation of 1,25-OH_2D production[40,41]. Activation of the calcium/phospholipid pathway results in downregulation of 1,25-OH_2D production in the epithelial cells of the proximal tubule[40]. The activated product 1,25-OH_2D will bind to DBP and will be transported to various tissues to exert its effect.

1.2.4 Vitamin D Transport

When vitamin D_3 is produced cutaneously, it quickly binds to the vitamin D-binding protein for its transport in the blood[42]. Vitamin D-binding protein is a 458-amino acid protein that is responsible for transporting vitamin D_3 and its downstream metabolites[42]. Vitamin D-binding protein has a role not only in transport of vitamin D, but also an active role in actin binding and the immune system, acting as a chemotaxis factor and macrophage-activating factor[43]. Among other serum proteins including albumin and high-density lipoprotein, vitamin D-binding protein has the highest affinity for unbound vitamin D_3 and acts as the major transport protein from the skin to the liver, where it is further processed to become 25-hydroxyvitamin D, the primary circulating form of vitamin D[44]. Of all vitamin D metabolites, vitamin D-binding protein has the highest affinity for 25-hydroxyvitamin D_3, followed by 1,25-dihydroxyvitamin D_3, and the least for vitamin D_3[44]. When comparing the transport of vitamin D_2 and vitamin D_3, the transport of vitamin D_3 appears to be more efficient as the presence of a methyl group at C-24 in vitamin D_2 lowers the affinity of DBP to vitamin D_2 in comparison to vitamin D_3[45].

1.2.5 24-Hydroxylation

Serum 25-OHD as well as active $1,25\text{-OH}_2D$ may be inactivated to prevent accumulation of either substance in cells. 25-hydroxyvitamin D_3-24-hydroxylase (CYP24A1) is a mitochondrial enzyme that catalyzes the conversion of both 25-OHD and $1,25\text{-OH}_2D$ to their 24-hydoxylated products in order for them to be excreted. CYP24A1 is expressed in cells where vitamin D exhibits physiological effects, namely in the kidney, bone, and intestines[46,47]. The presence of a vitamin D response element upstream of the promoter of CYP24A1 suggests that the rise in $1,25\text{-OH}_2D$ in cells will promote transcription of 24-hydroxylase. The inactivation pathway via 24-hydroxylase prefers $1,25\text{-OH}_2D$ over 25-OHD as the substrate, to create the biologically

inactive product calcitrolic acid[21]. The conversion of $1,25\text{-OH}_2\text{D}$ to calcitrolic acid prevents cell toxicity from accumulation of the active product[21].

1.3 Dietary Sources of Vitamin D

Despite the efficiency of cutaneous synthesis of vitamin D, UV light exposure may lead to DNA damage and cancer in humans. Within 290 -330 nm, the wavelength range required to convert 7-DHC to pre vitamin D_3, UV light can commonly dimerize pyrimidines, and lead to oxidative damage to DNA[48]. Oxidative damage to DNA can result in mutations which in turn may result in various types of skin cancer. Thus limiting exposure to UV light is recommended to reduce the risk of skin cancer.

Aside from cutaneous production, vitamin D_3 can be obtained via food sources, most of which contain small amounts of vitamin D unless they are fortified. In Canada, the average daily vitamin D intake amongst the population is 169 International Units (IU) [13]. The top food sources containing vitamin D include: milk, meat, fish, margarine, eggs, dairy and vegetables[13]. Meat and poultry contain traces of vitamin D_3 and 25-OHD and contribute little to dietary vitamin D intake, whereas milk, soy and nut beverages which are supplemented with vitamin D in Canada, act as the major source of vitamin D intake, representing 44% of Canadians' total intake of vitamin D[13].

Despite the poor vitamin D content in common food sources, individuals can alternatively obtain vitamin D via supplementation. The Endocrine Practice Guidelines Committee suggests children aged 1-18 to obtain 600 – 1000 IU of vitamin D to maintain vitamin D sufficiency[49]. To examine the effectiveness of supplementation compared to sunlight exposure on serum

vitamin D production, a study was performed to simulate atmospheric transmittances, suggesting the exposure times needed to achieve sufficient serum vitamin D[50]. Results showed that an individual with cream white skin would need to be exposed for 3-8 minutes with 25.5% of the body surface area exposed to the sun during the months of April to October in Boston, Massachusetts to receive an equivalent of 400 IU of vitamin D [50]. During the winter months, it is difficult for individuals in northern climates such as Canada to synthesize vitamin D via cutaneous mechanisms and therefore they must intake vitamin D from the diet and supplements.

1.4   Clinical Guidelines for Vitamin D Sufficiency

Serum vitamin D is typically measured using three main systems: Metric (ng/mL), International Units (IU), and Molar concentration (nmol/L). These three units are interconvertable, but the molar and metric systems are the most commonly used. 1 International Unit of serum vitamin D is equivalent to 25 ng/mL, or 62.5 nmol/L[51]. The cutoff for vitamin D sufficiency is unclear and varies between definitions obtained from multiple sources. The various definitions for vitamin D sufficiency are listed below in Table 1[52-55]. The American Academy of Pediatrics and the Kidney Disease Outcomes Quality

Table 1 – Definition of Vitamin D Status measuring 25-hydroxyvitamin D levels. AAP, American Academy of Pediatrics; IOM, Institute of Medicine; KDOQI, Kidney Disease Outcomes Quality Initiative; NEJM, New England Journal of Medicine; CPS, Canadian Pediatric Society

Vitamin D Status	25-Hydroxyvitamin D (nmol/L)				
	AAP 2008, IOM	Endocrine Society	KDOQI	Adult – NEJM 2007	CPS - 2007
Deficiency	< 37.5	< 50	< 37.5	< 50	< 25
Insufficiency	37.5 - 49	50-74	37.5 - 49	50-74	25-75
Sufficiency	> 50	> 75	> 50	> 75	> 75

Initiative define vitamin D deficiency to be lower than 37.5 nmol/L[52,53,56]. In contrast, the Endocrine Society and the New England Journal of Medicine define deficiency to be below 50 nmol/L[49,52]. The Canadian Pediatric Society has the lowest definition for serum vitamin D deficiency at 25 nmol/L[51]. In order to maintain sufficient serum vitamin D concentrations, the American Academy of Pediatrics suggests for children who are not ingesting a minimum of 1L of vitamin D-fortified milk, to ingest 400 IU of vitamin D per day to maintain a serum vitamin D concentration above 50 nmol/L[57]. Conversely, the Canadian Pediatric Society suggests that a dose of 400 IU per day of vitamin D supplementation may not be enough to reach the sufficiency concentration of 75 nmol/L defined by Canadian Pediatric Society standards[51]. The recommended amount of supplementation required to fulfill the Canadian Pediatric Society definition is still unclear and requires further studies to establish recommendations for dietary intakes.

1.5 25-Hydroxyvitamin D Measurement Assays

There are several assays that can be used to distinguish low and high serum levels of vitamin D. However, these measurement techniques have not been standardized, and they therefore may account for the discrepancies in measured serum vitamin D levels across different studies. Multiple studies have reported differences between assay measurements of 25-hydroxyvitamin D[58-60]. A study comparing 25-hydroxyvitamin D measurements between competitive protein binding assay (CPB), radioimmunoassay (RIA), and high-performance liquid chromatography (HPLC) showed the mean serum vitamin D measurements to be 80% higher when measured using the CPB assay than HPLC, followed by the RIA assay[61]. The international Vitamin D Quality Assessment Scheme has been monitoring the accuracy of serum vitamin D assays by comparing laboratory measures to the All-Laboratory Trimmed Mean (ALTM), a consensus mean indicative of a true measurement of serum vitamin D[62]. The average discrepancy between 6 serum assays and the ALTM was 7%, with the Nicols assay showing an approximate 30% positive bias[62]. Serum 25-OHD measured with DiaSorin Liason, a common immunoassay, was compared between two laboratories to Liquid Chromatography-Tandem Mass Spectrometry, the selected ideal method of serum 25-OHD measurement[63,64]. Results showed that between 1-in-5 and 1-in-3 patients were incorrectly identified as 'deficient' using Liason standards[64]. It is evident that without cross-calibration between collection sites, incorrect conclusions may be drawn from different serum 25-hydroxyvitamin D measurement assays.

1.6   Vitamin D Mechanism of Action and Physiological Effects

1.6.1   Receptor Mechanisms of Action

$1,25\text{-}OH_2D$ exerts its genomic effects by binding to the vitamin D receptor (VDR). The VDR is a member of the steroid nuclear receptor family. The receptor protein has three binding domains: an N-terminal domain, consisting of two zinc finger domains that bind to DNA at vitamin D response elements, a C-terminal domain, which binds to the ligand, and a hinge region linking the N- and C-terminal domains together[18]. The C-terminal domain consists of 12 helices, where the terminal helix acts as a gating system to encapsulate the ligand, forming a surface for coactivators to bind, as well as to increase the affinity of VDR with its binding partner, the retinoid X receptor[18]. Upon binding to the VDR, the receptor assembles as a heterodimer with the retinoid X receptor (RXR), and forms a complex with other co-activator proteins. This complex will subsequently bind the N-terminal domain of the VDR to DNA at vitamin D response element sites, where it will recruit additional co-regulatory proteins to activate its downstream genomic activity[18]. The VDR DNA binding upregulates transcription of downstream genes including osteopontin, 24-hydroxylase, and cyclin-dependent kinase inhibitor 1A[65]. Conversely, genes encoding for CYP27B1 and PTH are repressed via negative vitamin D response elements[65,66].

Alternatively, $1,25\text{-}OH_2D$ exerts non-genomic activity in intestinal calcium transport, chondrocyte growth, and keratinocyte activity in the skin. $1,25\text{-}OH_2D$ is able to bind to non-genomic membrane-associated rapid response steroid binding protein within caveolae/lipid rafts, to increase the activity of phosphatases, kinases, and ion channels[18,67].

1.6.2 Calcium Homeostasis

The classic effect of vitamin D is to regulate intestinal calcium transport and calcium homeostasis by interacting with its receptors in the intestine, bone, and kidney. The intestines play a major role in calcium absorption, where 70-80% of calcium absorption occurs in the ilium. Calcium absorption in the intestine begins on the apical side of enterocytes. $1,25\text{-}OH_2D$ promotes transcellular active calcium transport in the intestine via TRPV6, a transient receptor potential channel type located on the apical side of enterocytes. Vitamin D regulated apical transport of calcium via TRPV6 is the rate-limiting step in calcium absorption from the intestines when dietary calcium levels are low[68]. With normal/high dietary calcium intake, it is likely that the calcium traverses the intestine via the passive paracellular pathway[68,69]. Research has suggested that $1,25\text{-}OH_2D$ increases paracellular calcium transport by increasing the permeability of the tight junctions in enterocytes and is more important for increasing calcium uptake with low calcium intake[69-71]. Once calcium has been taken into the enterocytes the calcium binding protein calbindin-D_{9K} facilitates its movement to the basolateral side, where an ATP dependent calcium pump (PMCA1b) extrudes calcium out of the cell[72].

Calcium homeostasis is required for normal physiological activity and to prevent osteomalacia and rickets. Given the importance of vitamin D in maintaining serum calcium levels, vitamin D deficiency during development of bone in children will lead to bone deformation and rickets. In adults, low vitamin D levels may lead to hyperparathyroidism, leading to increased bone resorption, and osteoporosis. With bone mineralization defects, individuals with vitamin D deficiency will experience lowered structural support and an increased risk of bone fracture.

1.6.3 Parathyroid Glands

The parathyroid glands act as primary regulators of calcium in the bone, intestines and kidney. Parathyroid hormone (PTH) is synthesized in the chief cells of the parathyroid glands and is released in response to low serum calcium levels. PTH acts in bone to increase calcium and phosphate release from bone mineral via indirect activation of osteoclasts that resorb bone[73]. In the kidney, PTH inhibits reabsorption of phosphate in the proximal tubules, and enhances calcium reuptake in the distal tubule[73]. PTH also enhances the activity of CYP27B1 to hydroxylate 25-OHD to form the active product 1,25-OH2D. As outlined above the increase in vitamin D will increase calcium absorption in the intestines. Calcium then acts on a negative feedback system to inhibit the chief cells from secreting PTH[73]. Therefore, serum PTH is critical in the regulation of both calcium and vitamin D homeostasis.

Primary hyperparathyroidism occurs when adenomas appear on the parathyroid glands, which is then accompanied by hyperplasia, further growth of adenomas, and carcinoma[74]. These adenomas result in hypersecretion of PTH from the parathyroid glands, resulting in abnormally high serum levels of calcium, and eventually a reduction in bone mineral density[75]. Worse primary hyperparathyroidism symptoms including elevated PTH and serum calcium levels, are often associated with vitamin D deficient populations[76-79]. Secondary hyperparathyroidism occurs when there is an increase in PTH caused by external factors aside from damage to the parathyroid glands. The primary causes of secondary hyperparathyroidism are renal failure or vitamin D deficiency[80]. In individuals with chronic renal disease, 1α-hydroxylase is deficient or absent, and 1,25-OH$_2$D production is diminished. The decrease in serum 1,25-OH$_2$D results in low serum calcium, and subsequently elevates PTH production and secretion[81]. In vitamin D insufficiency an increase in PTH production enhances CYP27B1 levels and 25-OHD

hydroxylation, and therefore maintains a relatively normal level of 1,25-OH$_2$D with high levels of PTH.

1.6.4 Immunity and Inflammation

Aside from the classical effects on bone and calcium homeostasis, vitamin D has a strong role in strengthening innate and reducing adaptive immunity. Epithelial cells present in the skin, intestine, urogenital, and respiratory systems represent the first line of defense against invading pathogens. VDR and 1α-hydroxylase are expressed in these epithelial cells, and local production of 1,25-OH$_2$D increases the expression of tight junction and gap junction proteins between these epithelial cells[82-84]. Neutrophils, monocytes, and macrophages are important members of the innate immune system. The 1,25-OH$_2$D-VDR complex upregulates monocyte gene expression and the production of antibiotic proteins expressed on macrophages[85,86]. Chemotactic and phagocytic activity is also upregulated when the 1,25-OH$_2$D pathway is activated[87-90]. Dendritic cells are the major antigen-presenting cells (APCs), which activate the T-cell mediated response. VDR activation suppresses monocyte-derived dendritic cell maturation and thereby reduces total dendritic antigen presentation[91].

Vitamin D is a critical modulator of inflammatory cytokines. Vitamin D activity inhibits T-cell differentiation as it decreases the production of pro-inflammatory T$_h$1 cytokines including IL-2, IFNγ, and TNFα[92-95]. Conversely, vitamin D upregulates anti-inflammatory T$_h$2 differentiation by enhancing IL-4, IL-5, and IL-10 production[96,97]. Vitamin D further enhances anti-inflammatory effects via TNF-α suppression and upregulated NF-κB activity[98-100]. Vitamin D has also exhibited a large role in autoimmunity. It has been demonstrated that 1,25-OH$_2$D treatment of myeloid dendritic cells resulted in a decreased response from autoreactive T-cells,

the cells responsible for autoimmunity and autoimmune diseases[101]. Vitamin D has an inhibitory effect on the maturation of dendritic cells, which results in the destruction of tissues in autoimmune diseases[102].

1.7 Duchenne Muscular Dystrophy

1.7.1 Disease Pathology and Symptoms

Duchenne Muscular Dystrophy (DMD) is an X-linked disease with mutations in the dystrophin gene[1]. The dystrophin protein stabilizes the link between the extracellular sarcolemmal matrix and the actin cytoskeleton in muscle fibers[103]. Patients with DMD exhibit muscle weakness, muscle necrosis, and chronic inflammation in muscle fibers. Dystrophic muscle fibers have a large number of macrophages, T-cells, B-cells, and dendritic cells[104]. Pro-inflammatory chemokines are present in the muscle fibers before the disease onset, and initiate recruitment of T lymphocytes and macrophages[105-107]. TNF-α and TGFβ are known to induce muscle wasting in humans and muscle fibrosis in mouse models of DMD[108-110]. Chronic inflammation usually results in muscle damage and muscle function loss, where children with DMD exhibit progressive muscle weakness and typically lose ambulation by the age of 12[111]. Without medical or technological intervention, most people with DMD do not survive more than two decades. The standard treatment for DMD patients to prolong ambulation, increase muscle strength, and to maintain respiratory and cardiovascular function is to provide glucocorticoids. With glucocorticoid treatment and intervention to improve respiratory function DMD patients can live into their 30s but eventually succumb to cardiac or respiratory failure.

1.7.2 Glucocorticoids and Their Effects on Vitamin D

Glucocorticoids are the standard of treatment for patients with inflammatory diseases or autoimmune disorders[112,113]. Glucocorticoids are a class of steroid hormones naturally created in the adrenal cortex in humans[114]. Prednisone and deflazacort are the most common forms of synthetic glucocorticoid prescribed to patients with inflammatory diseases[115]. The anti-inflammatory and immunosuppressant effects of glucocorticoids are dependent on glucocorticoid receptor mediated transcriptional activation of genes in leukocytes[116,117]. Glucocorticoid receptor activation inhibits pro-inflammatory gene transcription including AP-1 and NF-κB, and upregulates anti-inflammatory immunomodulators including IL-10[118,119]. Glucocorticoids also have drastic effects on bone homeostasis. An increase in bone resorption is seen with glucocorticoid treatment, as it activates osteoclastogenesis by upregulating RANK ligand expression from osteoblasts and lowering expression of osteoprotegerin[120,121]. An increase in osteoblast apoptosis and a decrease in osteoblast differentiation and replication result in a decline in bone formation[122]. Together these increases in bone resorption and decreases in bone formation result in glucocorticoid-induced osteoporosis.

Glucocorticoid doses within normal physiological ranges are not likely to change VDR mRNA levels, but large doses of glucocorticoids used in studies have been found to lower VDR mRNA, and thus lower vitamin D activity via the vitamin D receptor[123]. Glucocorticoid users are shown to have a 2-fold increase in Vitamin D deficiency in comparison to untreated individuals[123].

1.7.3 Vitamin D Role in DMD

There are few bone health studies of children with DMD. However, it is evident from these few studies that many children with DMD are either 25-OHD deficient or insufficient. Low serum 25-OHD levels were first documented in a study analyzing serum 24,25-dihydroxyvitamin D, where DMD patients had on average lower serum 25-OHD than the controls[124]. Glucocorticoid treated DMD patients have shown a lower average 25-OHD serum level in comparison to non-treated groups[125]. Studies have also shown that without vitamin D supplementation, patients had insufficient serum levels of vitamin D in comparison to those with some form of supplementation[125].

Currently, there are no studies analyzing the effect of vitamin D on inflammation in dystrophic muscle. However, based on the role of vitamin D in immunomodulation and in NF-κB suppression, it is possible that vitamin D has a positive restorative effect to reduce muscle inflammation. While the low levels of serum 25-OHD in DMD patients have been found in these studies, the causes of this vitamin D insufficiency in patients are unclear and merit further investigation.

1.8 Systemic Lupus Erythematosus

1.8.1 Disease Pathology and Symptoms

Systemic Lupus Erythematosus (SLE) is a chronic inflammatory autoimmune disease. The pathogenesis of SLE is relatively unclear to date, however the disease presents apoptosis and tissue damage[126]. The major contribution to the symptoms seen in SLE is from autoantibody

production. Autoantibodies target self-molecules found in the nucleus, cytoplasm, and surface of cells. Antinuclear antibodies are prevalent in over 95% of all SLE patients[127,128]. Anti-DNA antibodies can bind to DNA located at the basement membrane of glomeruli, causing nephritis and activation of the complement system in the kidneys[129]. These symptoms of SLE are characteristic of the disruption of not only B-cell activation, but also T-cell differentiation, and abnormal dendritic cell activity[130]. The up regulation of pro-inflammatory cytokine production in SLE leads to an increase in disease severity and inflammation[131]. This stimulates B-cells to release auto-reactive antibodies, and shifts T-cell differentiation towards T_H1, leading to hyperactivity of T_H1 cells[132]. Abnormal T-cell immunity responses result in chronic tissue damage and inflammation[133,134]. Established treatments for individuals with mild SLE symptoms include non-steroidal anti-inflammatory drugs, the antimalarial drug hydroxychloroquine, and glucocorticoids[135]. Individuals with moderate to severe SLE showing lupus nephritis symptoms are typically treated with azathioprine, a purine given to inhibit lymphocyte growth, and to lower production of antibodies and natural killer cell activity[136]. SLE is a relapsing disease with phenotypes varying from individual to individual. Childhood-onset SLE has a prevalence of 3.3-8.8 per 100 children, where most children reveal symptoms between the ages of 11-12, and rarely under the age of 5[137].

1.8.2   Vitamin D Role in SLE

The effects of 1,25-OH$_2$D are beneficial for inflammation and autoimmune diseases by reducing T_H1 and T_H17 cells, while shifting T-helper cell differentiation towards T_H2[132]. Regulatory T-cells are increased with an increase in 1,25-OH$_2$D, which reduces B-cell activation and suppresses auto-reactive T-cell activity, thereby increasing tolerance of self-

antigens[138-141]. Ex-vivo experiments show that 1,25-OH$_2$D reduces serum antibody production, and IFN-α, a prominent cytokine involved in natural killer and macrophage activation[138,142].

Numerous clinical studies have correlated low levels of serum 25-OHD to an increase in SLE disease activity. However, little research has been performed to look at this relationship in pediatric populations. Average serum 25-OHD measured in Saudi children with SLE was 51 nmol/L, deficient by the American Academy of Pediatric standards, yet insufficient according to the Canadian Pediatric Society definitions[11]. Disease activity is normally assessed using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI), a qualitative system associating a higher score with worse symptoms[143]. Using this index, studies have shown an inverse correlation between 25-OHD and overall disease activity[11,144].Furthermore, a significant improvement in serum 25-OHD levels[11] and SLEDAI scores[144] were seen following vitamin D supplementation.

Therefore, vitamin D has shown positive restorative effects on the management of symptoms of SLE and improvement in SLE disease activity is associated with vitamin D supplementation.

1.9   Osteogenesis Imperfecta

1.9.1   Disease Pathology and Symptoms

Osteogenesis Imperfecta (OI) is an inherited systemic disorder characterized by bone and connective tissue deformities. OI is divided into four major types, where in the mildest form, Type 1, individuals show blue sclerae and deafness, and the moderate (type IV) to severe (II and III) types patients exhibit bone fractures, and short stature[145]. Over 90% of individuals

affected with this disorder exhibit mutations in type 1-collagen genes COL1A1 or COL1A2, which encode for pro-α1 and pro-α2 chains of type 1 procollagen respectively[146,147]. Type 1 collagen normally forms into larger procollagens, which are modified post-translationally via hydroxylations and glycosolyations to form mature fibrils[145]. These mature fibrils are the basic units contributing to the structural properties of tendons, ligaments and bones[148]. Bone mechanical properties depend on the interactions between these mature collagen fibrils and mineral hydroxyapatite[145]. Mutations in COL1A1/COL1A2 greatly reduce the production of type 1 procollagen, which then reduces the total amount of collagen introduced into the fibrils, ultimately reducing the strength of the bone. Bisphosophonates are antiresorptive agents that inhibit osteoclast activity and are the main method of treatment for children with OI to improve bone mineral density and reduce the risk of fracture[146,149]. Children with OI are mainly divided into four major categories: type I to type IV. Groups I and IV are associated with an autosomal dominant form of inheritance, and types II and III showed autosomal recessive inheritance[150]. The four types of OI were used in clinical practice to represent varying degrees of severity: mild (OI type I), lethal (OI type II), severely debilitating (OI type III), and moderately debilitating (OI type IV).

1.9.2   Bisphosphonates and Their Role on Vitamin D

Bisphosphonate treatment is used to treat multiple skeletal disorders in children, as well as postmenopausal osteoporosis, and glucocorticoid-induced osteoporosis in adults[151-153]. Bisphosphonates are synthetic derivatives of inorganic pyrophosphate (PPi), a natural compound consisting of two phosphate groups linked by an ester group[154]. PPi prevents mineralization of bone by binding to inorganic hydroxyapatite crystals[155]. Bisphosphonates function in a similar manner when administered, as their high affinity for inorganic crystals

prevents bone calcification. The modern bisphosphonates prescribed to individuals (alendronate, pamidronate, zoledronic acid) contain a nitrogen-side chain. These nitrogen-containing bisphosphonates inhibit the intracellular mevalonate pathway in osteoclasts, the pathway required for bone resorption and survival[154,156].

The effect of bisphosphonates on serum 25-OHD is rather unclear. The interaction between vitamin D levels and the effect of bisphosphonates on lumbar spine and hip bone mineral density was reported to be insignificant when comparing postmenopausal patients with vitamin D deficiency, insufficiency, and sufficiency[157]. Yet, spine and hip bone mineral density were reported in a different study to be significantly higher in vitamin D replete post-menopausal patients treated with bisphosphonates (defined as > 50 nmol/L) in comparison to vitamin D depleted patients (defined as < 50 nmol/L) [158]. Vitamin D insufficiency was the major identified cause of lowered bone mineral density in all bisphosphonate-treated patients[159]. While these studies suggest that vitamin D sufficiency is important for good outcomes of bisphosphonate therapy there is no evidence that bisphosphonates affect vitamin D status. Nevertheless, the impact of bisphosphonates on vitamin D status merits further research.

1.9.3   Vitamin D Role in OI

Very little research has been done looking at the effect of vitamin D insufficiency in children with OI. Vitamin D has a crucial role in calcium homeostasis and the maintenance of bone. Therefore, one should expect vitamin D deficient children with OI to have lower bone mineral density measures. In studies analyzing vitamin D status in patients with OI, approximately 50% of the patient populations or greater were deemed insufficient or deficient[12,160]. When adjusting for age, OI severity, and gender, serum 25-OHD was positively correlated to lumbar

spine bone mineral density in children and adolescents with OI[161]. Therefore, in order to improve bone health in children with OI, serum vitamin D should be elevated through means of supplementation or dietary intake of vitamin D.

2   Research Objectives and Hypotheses

2.1.1   Primary Objective

The primary objective of this present study was to investigate the vitamin D status and vitamin D standardized for vitamin D supplementation in a pediatric Duchenne muscular dystrophy population. Anticipating that patients with DMD will be vitamin D deficient, we then wished to determine if glucocorticoid treatment or disability in this population might contribute to vitamin D deficiency. In order to do this the results from the DMD population will then be compared to another pediatric population treated with glucocorticoids but not disabled (systemic lupus erythematosus patients), as well as another disabled pediatric population not treated with glucocorticoids (Osteogenesis imperfecta patients). Vitamin D was measured as serum 25-OH D levels. To control for the level of vitamin D supplementation in the three groups of patients a standardized vitamin D level was calculated by dividing serum 25-OHD by the international units of vitamin D supplementation to obtain a ratio for each patient.

2.1.2   Secondary Objectives

The secondary objectives of this present study were to compare the vitamin D status to secondary parameters to identify determinants of serum vitamin D in the three populations. The secondary parameters include: age, gender, height, weight, body-mass-index, ambulatory status, glucocorticoid dose per weight, parathyroid hormone, lumbar spine bone mineral density, total body bone mineral density, fat mass, and total body fat %.

2.2   Hypotheses

With our three patient populations, we set out to address the following hypotheses regarding their vitamin D statuses:

- The Duchenne muscular dystrophy population will have an insufficient average serum vitamin D level, below the Canadian Pediatric Society standard of 75 nmol/L[51].

- The Duchenne muscular dystrophy population will have lower levels of serum 25-hydroxyvitamin D in comparison to the CALIPER group, between the ages of 3 to 9 years

- The Duchenne muscular dystrophy population will have significantly lower serum vitamin D levels than the Osteogenesis imperfecta and systemic lupus erythematosus populations despite similar or higher levels of vitamin D supplementation

- Possible determinants of low serum vitamin D and standardized vitamin D for vitamin D supplementation include glucocorticoid dose and ambulatory status

- Poor bone health in Duchenne muscular dystrophy patients will be associated with poor ambulatory status

3 Materials and Methods

3.1 Study design, sample size, and populations

Upon receiving research ethics approval from the Holland-Bloorview Kids Rehabilitation

Hospital Research Ethics Board and the Hospital for Sick Children Research Ethics Board in

Toronto (Appendix Section 9.1 and 9.2), a retrospective study was conducted to assess the

vitamin D status in children with DMD, SLE, and OI. The estimated sample size for each

group was 70, given a one-way ANOVA measurement with a power of 90% at a significance

of 0.05 to detect a difference of 10 nmol/L of 25-hydroxyvitamin D between three groups.

DMD Population

Children with DMD at the Holland-Bloorview Rehabilitations Children Hospital were included

in this study. The medical charts of children with DMD that were followed at Holland-

Bloorview from January 1st 2008 to December 31st 2014 were reviewed. Patients from our

primary DMD population had their disease diagnosis confirmed via gene analysis or muscle

biopsy. Only male DMD subjects between the ages of 5 to 18 were included in this study. To

be eligible for this study, children with DMD needed a minimum of one serum 25-OHD

measurement between the dates of January 1st 2008 to December 31st 2014. In total, 83 out of

90 patients met these criteria and were included in the study.

SLE and OI Population

The medical charts of children with either SLE or OI who were followed at the Hospital for

Sick Children from January 1st 2008 to December 31st 2014 were reviewed. The children must

have had either a genetic or a clinical diagnosis of the disease to be included in the study. Each

patient in the study must have had a minimum of one serum 25-OHD measurement within the

aforementioned dates. In total, 170 of 194 SLE patients and 90 out of 114 OI patients met these criteria and were included in the study. Of the 90 OI patients, there were 44 type I (mild), 24 type IV (moderate), and 21 type III (severe) patients included in this study.

Normative Population

The normative comparative population data used in this study was extracted from the Canadian Laboratory Initiative for Pediatric Reference Intervals (CALIPER) study. These values are normal reference endocrine biomarkers obtained from otherwise healthy children of different ages in the Greater Toronto community[162]. The advantages of comparing our study population data to the CALIPER data is that the reference data comes from healthy children, and that the values are obtained from the same geographical area as the DMD, SLE, and OI populations.

3.2 Clinical, laboratory, and radiological data collected

Medical records were reviewed and data was extracted and recorded onto the RedCap database hosted at the Hospital for Sick Children. REDCap (Research electronic data capture) is an online secure application used for data capture in research[163]. Patients with one or more serum 25-OHD measurements were included in this study. Out of all possible serum 25-OHD measurements, the biochemistry data point closest to December 31st 2014 was included as the sole 25-OHD measurement for this study. For the DMD, SLE, and OI cohorts, the following general data were collected: Month and year of birth, date of diagnosis, age at diagnosis, type of glucocorticoid taken, date of glucocorticoid therapy initiation , date of visit, season of visit, age, height, weight, ambulatory status (Vignos scale for DMD patients), calcium supplementation dose, vitamin D supplementation dose, glucocorticoid dose, additional therapeutics. The Vignos scale is a quantitative scale measuring mobility ranging from fully

ambulatory patients with a score of 0, to wheelchair-bound individuals with a score of 9. The

following laboratory biochemistry data values were collected: date of blood collection, total

calcium, phosphate, serum creatinine, 25-hydroxyvitamin D, PTH, alkaline phosphatase, urine

calcium:creatinine ratio, albumin, blood nitrogen urea, creatinine clearance. Bone mineral

density (BMD) data values collected include: date of dual x-ray absorptiometry (DXA) scan,

lumbar spine BMD, lumbar spine BMD Z-score, total body BMD, total body BMD Z-score,

total bone mineral content (BMC), fat mass, lean mass, and total body fat %. The lumbar and

total BMD Z-scores were standardized at the hospital for Sick Children, and used in this study.

For more specific data collection tables, refer to sample data collection tables in the Appendix

to this thesis.

3.3 Statistical analysis

The correlation analyses were performed using RStudio version 0.99.485 (RStudio, Boston,

MA). The descriptive characteristics and linear regression analyses were performed using

SPSS version 22 (SPSS, Chicago, IL). According to the definitions of vitamin D status

established by the Canadian Pediatric Society, we divided our cohorts into three groups each

based upon their serum 25-OHD levels: deficient (< 25 nmol/L), insufficient ($25 - 75$ nmol/L),

and sufficient (> 75 nmol/L). Patient BMI values were calculated and together with the height

and weight, were standardized to Z-scores using the World Health Organization Growth charts

for Canada version 2014[164].

The study characteristics were recorded in frequencies, percentages, or means with standard

deviations. The Shapiro-Wilk test was performed on each study cohort data value set to

determine normality. One-way ANOVA followed by Bonferroni post-hoc analysis was

performed to detect statistical significance between the means of the descriptive data. Chi-squared test was used to detect significant differences between proportions measured in the descriptive characteristics. Spearman's rank correlation was used to determine correlation between serum 25-OHD, serum 25-OHD per IU of vitamin D supplementation, and BMD Z-score to the secondary parameters measured in each group. Simple linear regression was used to identify predictors of serum 25-OHD, serum 25-OHD per IU of vitamin D supplementation, lumbar spine BMD Z-score, and total body BMD Z-score in the DMD cohort.

The data from all three cohorts were combined into one unified cohort, and multiple linear regression analysis was performed to determine biological, pharmacological, and radiological determinants of serum 25-OHD and serum 25-OHD per IU of vitamin D supplementation in this unified cohort. The β values used in the multiple linear regression model were not standardized to a linear value between 0 to 1. Variables with a p-value on simple linear regression of <0.1 were entered into the linear regression analysis. A backwards selection method was utilized.

4 Results

4.1 DMD Vitamin D Status

To determine if DMD was associated with vitamin D insufficiency, we first determined the vitamin D status of the DMD population in our study. The distribution of serum vitamin D in the DMD population is shown in Fig. 4.

Figure 4: Distribution of serum 25-OHD levels in the DMD population. Deficiency: < 25nmol/L; Insufficiency:25 – 75 nmol/L; Sufficiency: > 75 nmol/L; the red dotted line indicates 75nmol/L, the cutoff for serum 25-OHD sufficiency by CPS standards.
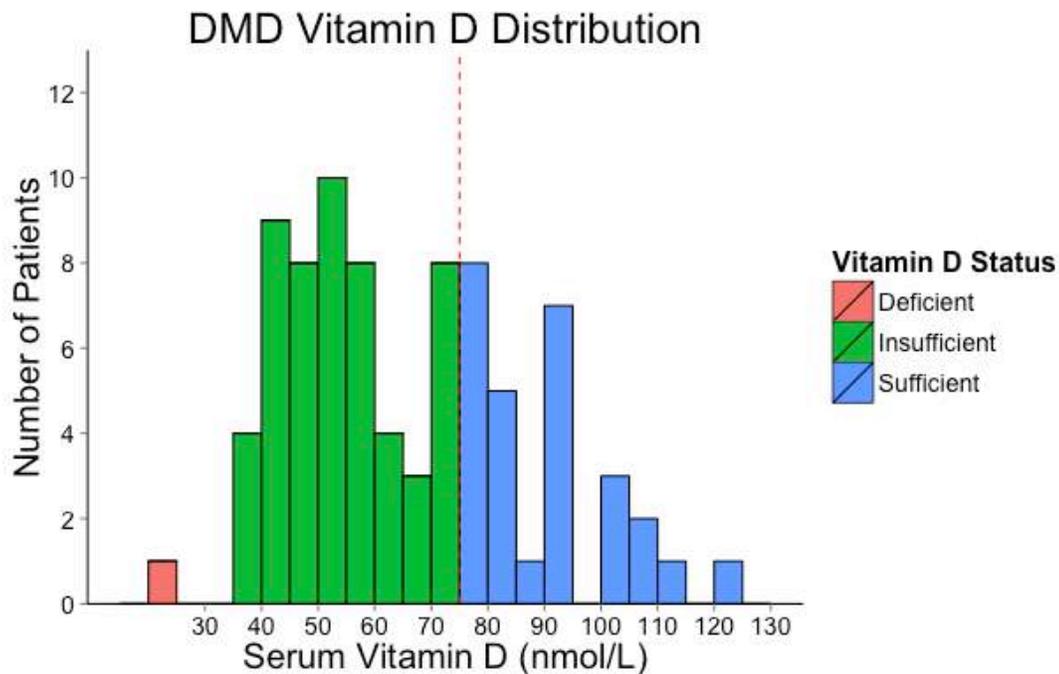
Our study examined serum 25-OH vitamin D levels in 83 patients with DMD. Of these patients one patient was vitamin D deficient, 57 patients (68.7%) were vitamin D insufficient, and 25 patients (30.1%) were vitamin D sufficient. On average, patients had a serum vitamin D level of 65.1 ± 21.4 nmol/L. Using the Wilcoxin signed rank test, the average serum vitamin D level

of DMD patients was significantly lower than the Canadian Pediatric Society standard of 75 nmol/L (p < 0.001) supporting the hypothesis that DMD is associated with lower than desirable vitamin D levels. To compare these results to the healthy pediatric population, the vitamin D levels of healthy children from the Canadian Laboratory Initiative for Pediatric Reference Intervals (CALIPER) [162] study were extracted and compared to the vitamin D levels of the DMD cohort. As shown in Figure 5 no significant difference was observed when comparing the average 25-OHD levels between the CALIPER and DMD population. However, when this data was plotted over the range of ages (Figure 6A) DMD patients seemed to have lower average levels of vitamin D at younger ages. To test this further we divided the patients into three age categories and compared the average 25-OH vitamin D levels in DMD patients with healthy controls in each category, Figure 6B. The 29 DMD patients between 3 to 8.9 years of age, had an average serum vitamin D level that was significantly lower than the CALIPER study population (p = 0.01 Bonferonni post-hoc analysis).

Figure 5: Mean serum 25-OHD comparisons between patients from CALIPER and DMD cohorts.
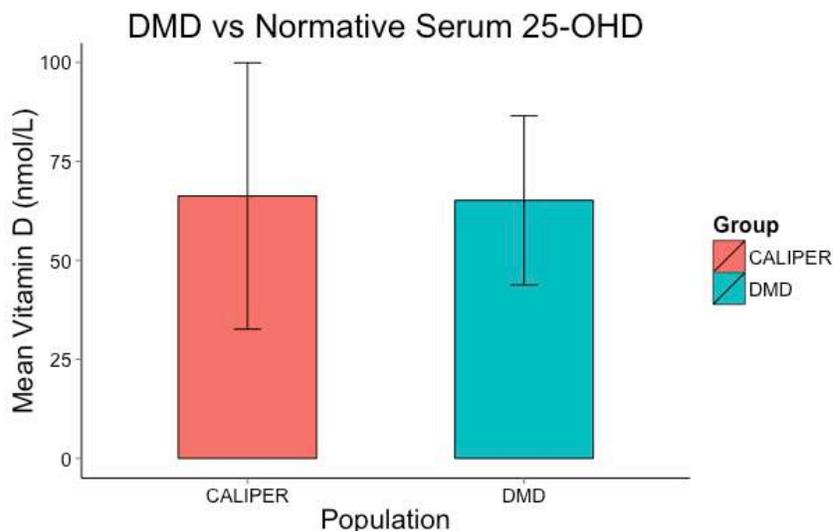
Figure 6A: Scatterplot showing serum 25-OHD of DMD and CALIPER patients across ages.
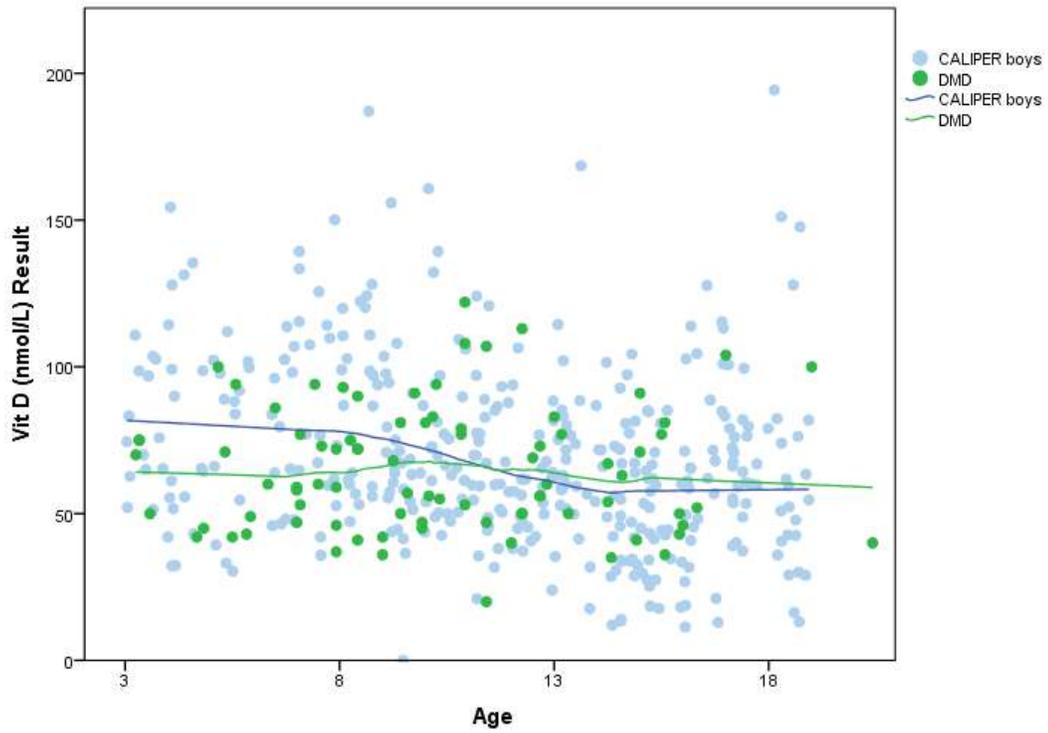
Figure 6B: Bar graph showing the average serum 25-OHD in the CALIPER, DMD, SLE, and OI populations across 3 age groups.
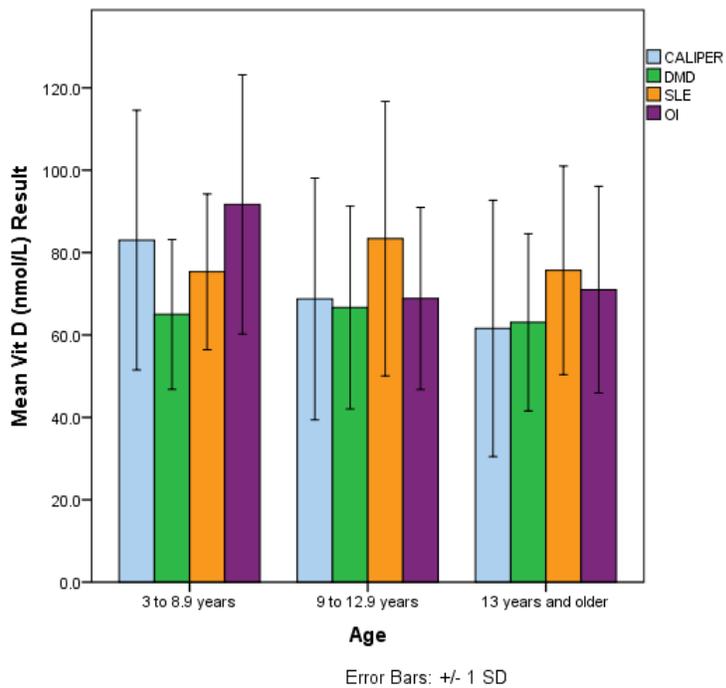
These results indicate that the average serum vitamin D level in children with DMD was below the sufficiency standard of 75 nmol/L, set by the Canadian Pediatric Society and that younger children with DMD are at the greatest risk for vitamin D insufficiency.

4.2   Comparison of Vitamin D Status of DMD, SLE, and OI Populations

Having established that the DMD cohort had on average an insufficient level of serum 25-OHD, we sought to determine factors that may have contributed to these results. Children with DMD have reduced ambulation that may have limited their exposure to sunlight and therefore we compared their vitamin D levels to those in another pediatric population with disability, patients with Osteogenesis Imperfecta (OI). Another factor that may have contributed to their vitamin D insufficiency was the glucocorticoid medications that were taken by DMD patients to decrease the rate of muscle loss. To determine if glucocorticoids might have increased vitamin D insufficiency we also examined the vitamin D status in a group of patients with systemic lupus erythematosus (SLE) as these patients also take glucocorticoids but are not disabled. The distributions of serum vitamin D levels for the OI and SLE populations are shown below in Figure 7 and Figure 8 respectively.

Figure 7: Serum 25-OHD distribution in the OI population. Deficiency: < 25nmol/L;

Insufficiency:25 – 75 nmol/L; Sufficiency: > 75 nmol/L; the red dotted line indicates 75nmol/L,

the cutoff for serum 25-OHD sufficiency by CPS standards.
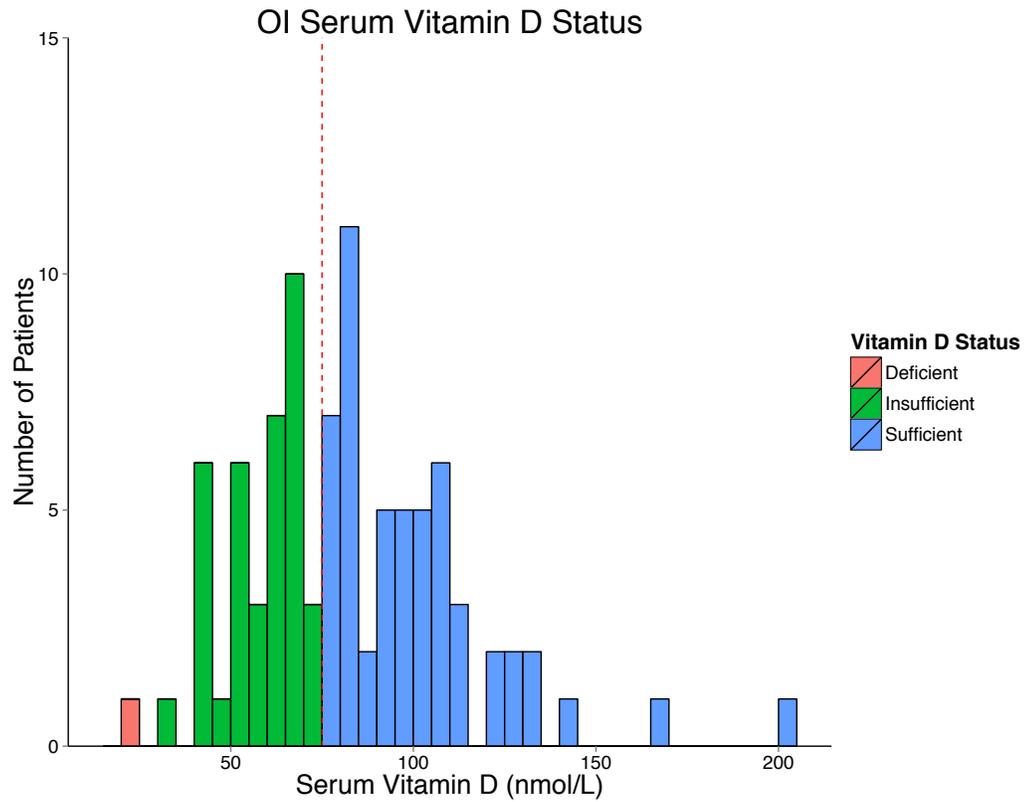
Figure 8: Serum 25-OHD distribution in the SLE population. Deficiency: < 25nmol/L; Insufficiency: 25 – 75 nmol/L; Sufficiency: > 75 nmol/L; the red dotted line indicates 75nmol/L, the cutoff for serum 25-OHD sufficiency by CPS standards.
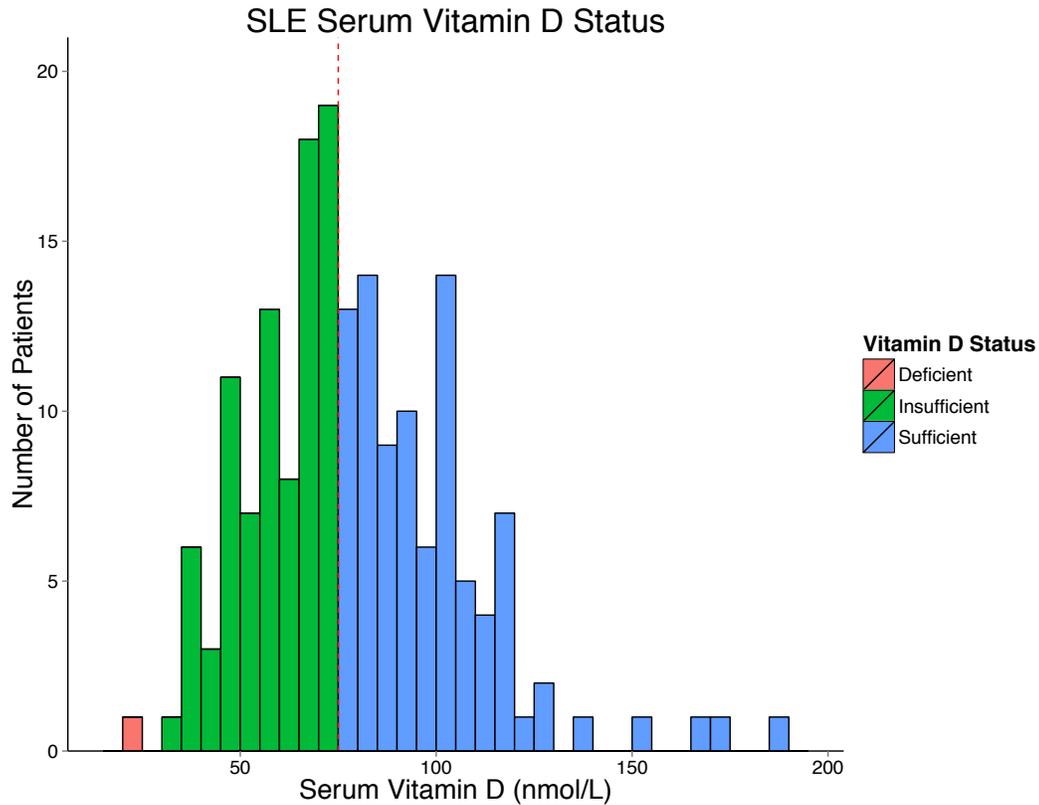
As shown in Figure 7, of the 90 OI patients with a serum 25-OHD measurement, one was deficient, 36 were insufficient (39.6%), and 53 were sufficient (58.2%). In contrast, out of a total of 176 SLE patients with a serum 25-OHD measurement, one was deficient, 86 were insufficient (48.6%), and 90 were vitamin D sufficient (50.8%).

The average serum 25-OHD levels for all three patient groups are shown in Table 2 and Figure 9. It was noted that the average vitamin D level in the DMD population was in the insufficient range whereas the average levels for both the OI and SLE patients were in the vitamin D

sufficient range. One-way ANOVA identified a significant difference between the average

serum 25-OHD of DMD patients with both the OI and SLE patient cohorts (Figure 9, p <

0.001) suggesting that DMD patients have significantly lower vitamin D levels than two other

pediatric patient groups with diseases that affect bone health.

Figure 9: Average serum 25-OHD measured in the DMD, OI and SLE populations.
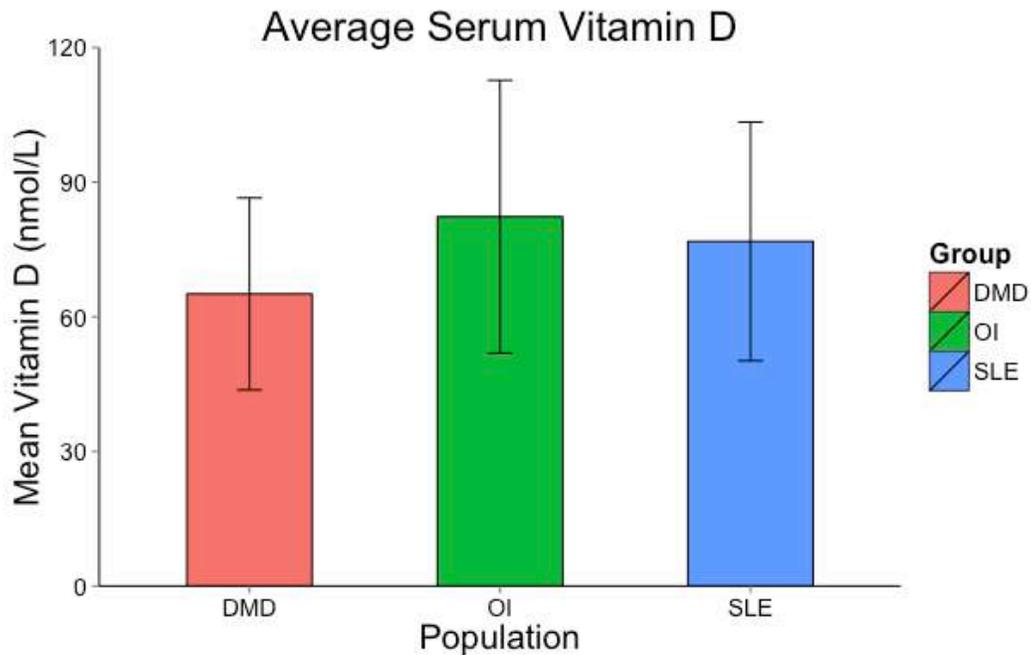
Post-hoc Bonferroni analysis showed that the DMD population had a significantly lower

average 25-OHD when compared to either the SLE (p = 0.002) or the OI cohorts (p < 0.001).

Table 2: Vitamin D characteristics for DMD, SLE, and OI

Vitamin D Characteristics	DMD	OI	SLE	ANOVA p-value
25-OHD (nmol/L)	65.1 (21.4)	82.3 (30.4) [a]	76.8 (26.6) [b]	<0.001
Vitamin D supplementation (IU)	1627 (1011)	705 (443) [a]	1006 (339) [b]	<0.001
Vitamin D supplementation per weight (IU/kg)	63.3 (53.0)	28.5 (24.3) [a]	18.9 (9.0) [b]	<0.001
25-OHD per IU of vitamin D supplementation	0.05 (0.02)	1.5 (0.1) [a]	0.09 (0.07) [b]	<0.001

Results are shown in mean (SD). [a] *p < 0.05 when comparing DMD to OI;* [b] *p < 0.05 when comparing DMD to SLE using Bonferroni post-hoc analysis.*

One potential reason why the DMD patients might have lower vitamin D levels is the level of vitamin D intake. While we did not have information about the dietary intake from any of the three patient groups we did have access to information about the prescribed levels of vitamin D supplementation. All three of these patient populations were seen by physicians who are attentive to their patients' bone health and typically prescribe vitamin D supplementation to maintain optimal bone health. To take into account the different levels of supplementation in each population and their effect on serum vitamin D levels, vitamin D supplementation and 25-OHD per IU of supplementation was compared between the DMD, OI and SLE populations. As shown in Table 2, the average vitamin D supplementation prescribed for DMD patients was actually higher than that for OI ($p < 0.001$) or SLE ($p < 0.001$) populations. To account for the dose of supplementation given to each patient, the serum 25-OHD was standardized per IU of vitamin D prescribed. Post-hoc analysis showed that the DMD population had on average lower serum 25-OHD per IU of supplementation in comparison to the OI and SLE populations (Figure 10, $p < 0.001$).

Figure 10: Vitamin D (left) and vitamin D per IU of supplementation comparison between DMD, SLE, and OI populations.
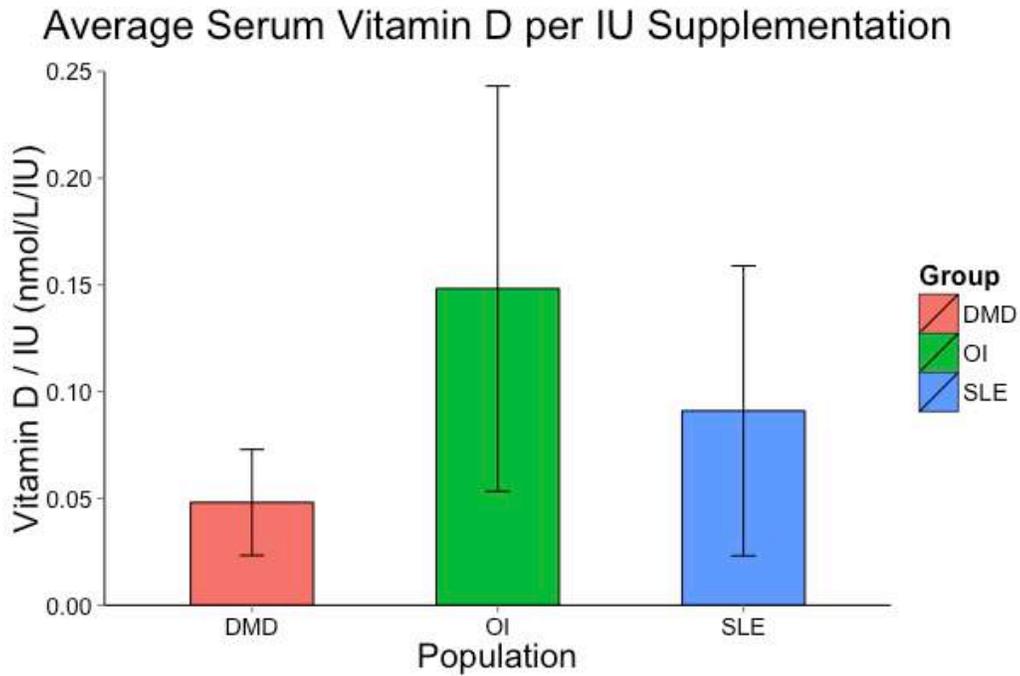
These findings suggest that children with DMD may have a relative resistance to vitamin D supplementation, as the DMD population has on average lower 25-OHD serum concentrations despite higher supplementation doses, and hence have a lower serum 25-OHD per IU of vitamin D supplementation.

To further compare the DMD patients with the OI and SLE patients the general anthropometric, pharmacological, biochemistry, and bone mineral density data was collected for each of the three patient cohorts. The results are listed in Table 3.

Table 3: Cohort characteristics for DMD, OI, and SLE populations.

	DMD (n=83)	OI (n=90)	SLE (n=194)	P value
Cohort characteristics				
Age (years)	10.3 (3.8)	8.7 (4.8)	15.6 (2.6)	<0.001[b]
Disease duration (years)	10.3 (3.8)	8.7 (4.8)	3.5 (2.5)	<0.001 [b]
% Males	100	50	22	<0.05[C]
Season of Visit (% summer: fall: winter: spring)	37:16:25:22	21:41:18:20	29:22:26:23	<0.05[C]
Height (Z-score)	-1.6 (1.9)	-1.5 (2.0)	-0.4 (1.3)	<0.001 [b]
Weight (Z-score)	-0.6 (1.5)	-0.5 (1.5)	0.3 (1.3)	<0.001 [b]
BMI (Z-score)	0.4 (1.6)	0.5 (1.4)	0.7 (1.2)	N.S
Glucocorticoid dose (mg/kg of Deflazacort equivalent)	0.58 (0.32)	0	0.16 (0.22)	<0.001 [a b]
Serum Biochemistry				
Total calcium (mmol/L) (normal: 2.25 – 2.63)	2.40 (0.1)	2.51 (0.1)	2.38 (0.1)	<0.001[a]
Phosphate (mmol/L)	1.53 (0.2)	1.58 (0.2)	1.36 (0.2)	<0.001 [b]
PTH (pg/mL) (normal: 10-55)	20.2 (11.0)	31.0 (19.1)	N/A	0.05
ALP (IU/L)	87 (52)	218 (79)	112 (74)	<0.001[a]
Bone Mineral Density				
Total Body BMD (Z score)	-4.28 (2.0)	-1.0 (1.4)	-0.44 (1.2)	<0.001 [a b]
Body Fat (% of total weight)	36.1 (14.4)	27.4 (10.7)	33.4 (10.1)	0.001 [a]

Results are recorded as mean (SD). [a] p < 0.05 when comparing DMD to OI; [b] p < 0.05 when comparing DMD to SLE using ANOVA and Bonferroni post-hoc analysis; [c] p < 0.05 when comparing between all groups using Chi-squared tests. N.S: not significant, p > 0.05.

As shown in Table 3 the number of patients that were included in the study for the SLE cohort was much greater than either the DMD or OI, these numbers reflect the relative prevalence of the three diseases and the number of patient records available to study. The average patient age

in the SLE cohort was also significantly higher than the DMD and OI cohorts reflecting the later onset of SLE compared to DMD and OI. BMI Z-scores were not significantly different amongst all three groups, despite DMD patients having a greater height and weight Z-score in comparison to the SLE patients. The patients with SLE were treated with prednisone, a similar glucocorticoid to the deflazacort treatment given to patients with DMD. To compare glucocorticoid doses between groups, the prednisone dose for the SLE patients was converted into deflazacort equivalents based on the relative potencies of the two drugs where 1 mg prednisone = 1.2 mg of deflazacort. Adjusted glucocorticoid dose per weight was higher in the DMD cohort than the SLE cohort. Although differences were seen in total calcium, phosphate, PTH, and ALP between the groups, the serum biochemistry values were all within normal ranges and are therefore not likely to be of clinical significance. Total body fat % was significantly higher in the DMD cohort in comparison to the OI cohort ($p < 0.05$) but not different from the SLE cohort. Total body BMD in the SLE population was within the normal range, while the OI population had lower BMD levels and the DMD cohort had the worst bone health with significantly lower total body BMD than the other two patient cohorts ($p < 0.05$).

4.3   Correlates of Low Serum 25-OHD in DMD

As noted above children with DMD had a lower average serum 25-OHD in comparison to an ambulatory glucocorticoid treated SLE population, and a disabled OI population not treated with glucocorticoids. To determine correlates of low serum 25-OHD in children with DMD, the standardized measurement of 25-OHD per IU of supplementation was compared to age, bone mineral content, BMI Z-score, fat mass, body fat %, glucocorticoid dose, lumbar BMD Z-score, serum PTH, vitamin D supplementation, total body BMD Z-score, and ambulatory status. When comparing these secondary parameters, Spearman's rank correlation showed a

41

significant negative correlation between serum 25-OHD per IU supplementation and serum

PTH (Figure 11, ρ = -0.304, p = 0.04). Ambulatory status was evaluated in children with DMD

using the Vignos scale, a qualitative scale where a value of '0' represents complete ambulation,

and '9' representing wheelchair bound[165]. A positive correlation was observed between serum

25-OHD per IU supplementation and the Vignos functional scale (Figure 12, ρ = 0.292, p =

0.019). No significant correlations were observed when comparing serum 25-OHD per IU

supplementation and the other secondary parameters.

Figure 11: Serum 25-OHD per IU of supplementation correlation with serum PTH.
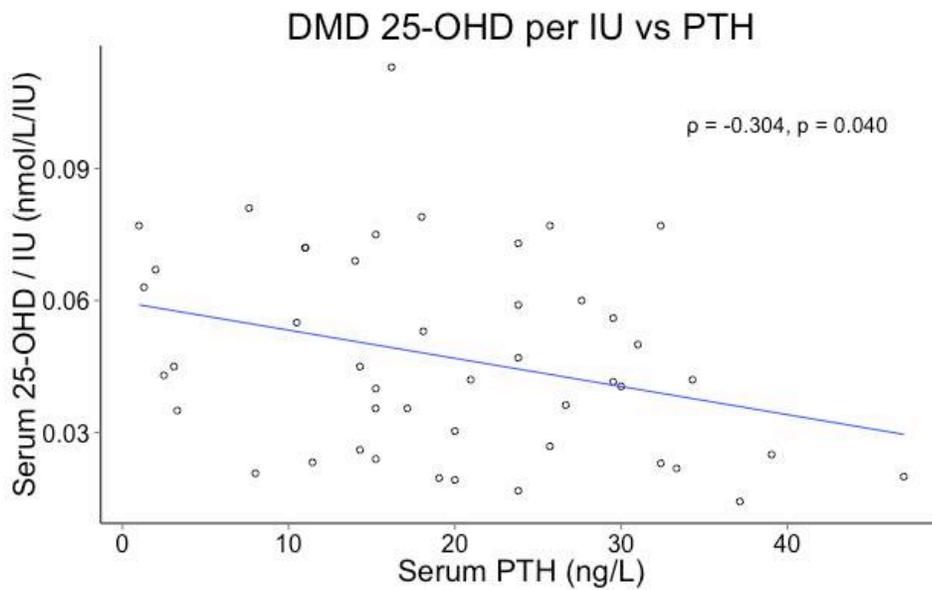
Figure 12: Serum 25-OHD per IU of supplementation correlation with Vignos functional scale.

0 on the Vignos scale indicates full ambulation and 9 indicates wheelchair bound.
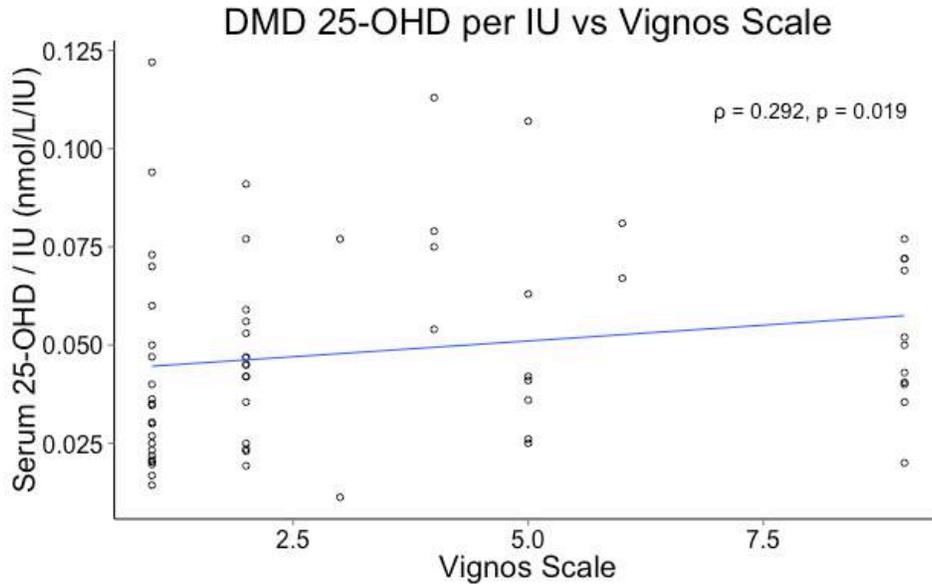
Serum 25-OHD itself was also compared to the following secondary parameters to identify significant associations: age, bone mineral content, BMI Z-score, fat mass, body fat %, glucocorticoid dose, lumbar BMD Z-score, serum PTH, vitamin D supplementation, total body BMD Z-score, and ambulatory status. Spearman's rank correlation identified a significant positive correlation between serum vitamin D and supplementation in DMD patients (Figure 13, $\rho = 0.281$, $p = 0.020$). No other significant correlations were identified with the other secondary parameters.

Figure 13: Correlation between serum 25-OHD and vitamin D supplementation in DMD cohort.
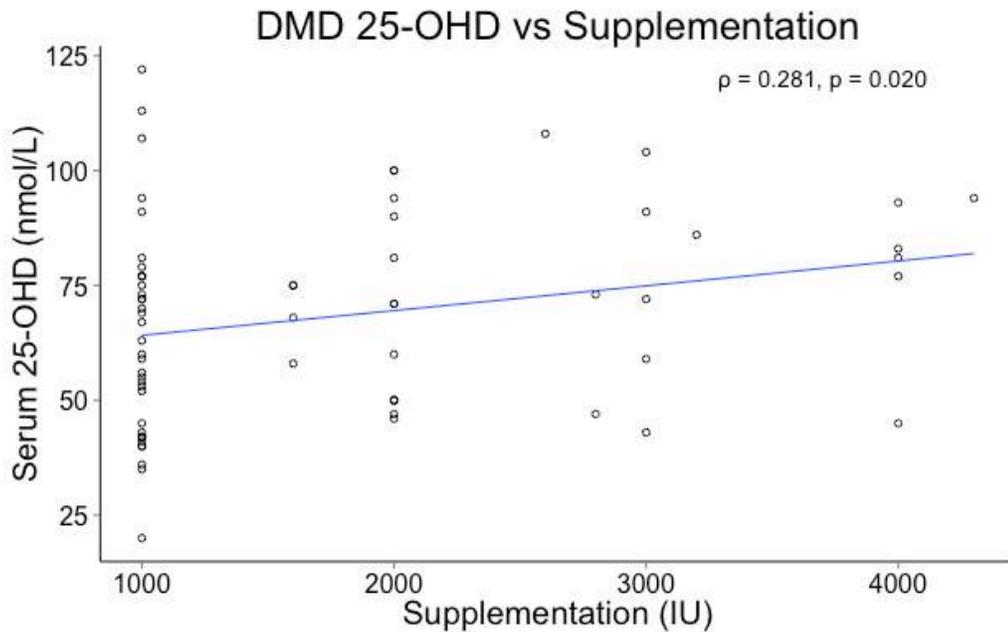
These correlation findings suggest that vitamin D levels are feeding back at the level of the parathyroid gland to decrease PTH release. The increase in vitamin D levels with a decrease in ambulatory status may reflect the longer duration of vitamin D supplementation for DMD patients as they age rather than any negative effect of vitamin D on muscle function. An increase in supplementation was also correlated with an increase in serum 25-OHD suggesting that DMD patients do absorb vitamin D taken orally. Importantly, no correlation was seen between glucocorticoid dose and serum 25-OHD suggesting that glucocorticoids may not be regulating vitamin D absorption or metabolism.

4.4 Determinants of Vitamin D in a Unified Cohort

As the patient numbers studied here are relatively small, the data from DMD, SLE and OI

patients were combined in a multiple linear regression to identify predictors of serum 25-OHD

and serum 25-OHD per IU of supplementation in the entire study cohort. Multiple linear

regression analysis identified having DMD, season of visit, disease duration, and weight Z-

score to be significant determinants of 25-OHD in the entire study population (Table 4).

Glucocorticoid dose was not found to be a significant determinant of serum 25-OHD.

Table 4: Linear regression to identify significant determinants of serum 25-OHD in the entire

cohort including DMD, OI, and SLE patients.

Model parameter	ß	P value
Constant	83.0	<0.0001
Having DMD	-12.2	0.001
Serum taken in the fall	7.60	0.03
Disease duration (years)	-0.8	0.02
Weight (Z score)	-3.4	0.001
$R^2 = 0.12$		

Analysis performed using backwards selection technique. R^2 = Expected variation from the

model / total variation in the data

This linear regression model confirmed that having DMD contributed to vitamin D

insufficiency and specifically accounted for a 12.2 nmol/L decrease in 25-OHD. As anticipated

serum 25-OHD levels were higher in the fall after summer exposure to greater levels of UV

irradiation and blood serum 25-OHD collection in the fall contributed to a 7.6 nmol/L increase

in 25-OHD. Disease duration was negatively correlated with serum 25-OHD, and every year

of disease duration decreased 25-OHD by 0.8 nmol/L. Every 1 unit increase in weight Z-score

resulted in a 3.4 nmol/L decrease in serum 25-OHD. However, this model only accounted for 12% of the variance seen in the total serum 25-OHD in the patients, suggesting that there were other determinants unaccounted for in this study that were important determinants of serum 25-OHD. Interestingly in this analysis vitamin D supplementation dose was not a significant determinant of 25 OHD values.

Following this analysis the predictors of 25OHD in the total cohort, a linear regression analysis of the determents of 25OHD concentration was conducted in the children with DMD.

Table 5: Linear regression to identify significant determinants of serum 25-OHD in the DMD cohort.

Model parameter	ß	P value
Constant	55.4	<0.0001
Vitamin D supplementation dose (IU)	0.006	0.009
$R^2 = 0.08$		

Unlike the results from the combined cohort, vitamin D supplementation dose was the only significant determinant for serum 25-OHD concentrations in the boys with DMD. Of note this again only accounted for 8% of the variance in serum 25 OHD values explained by this model.

The standardized value of serum 25-OHD per IU of supplementation was also analyzed for significant determinants. In the entire cohort, having DMD, having OI, disease duration and weight Z-scores were significant determinants of serum 25-OHD per IU of supplementation. Glucocorticoid dose was again found not to be a significant determinant of serum 25-OHD per IU of supplementation.

Table 6: Linear regression to identify significant determinants of 25-OHD per IU of

supplementation in the entire cohort.

Model parameter	ß	P value
Constant	0.101	< 0.001
Having DMD	-0.024	0.04
Having OI	0.069	< 0.001
Disease duration (years)	-0.004	0.002
Weight (Z score)	-0.007	0.01
$R^2 = 0.27$		

Linear regression was performed using the backwards selection technique.

These linear regression results suggested that the underlying condition, disease duration and

weight Z-scores were significant determinants of 25-OHD and 25-OHD per IU of

supplementation. However, the secondary parameters tested against the model only explained

part of the variation seen in the data. Therefore, there were factors not recorded in this

retrospective study that determined the majority of the variation seen in both 25-OHD and 25-

OHD/IU of vitamin D supplementation.

4.5   Bone Health in the Unified Cohort

Vitamin D plays a critical role in bone homeostasis. Although bone mineral density is one of

multiple contributors to bone strength and bone health, BMD is a widely used non-invasive

predictor of osteoporosis and risk of fracture. Total Body BMD was measured in each cohort

and correlated to secondary parameters to identify determinants of bone health. Lumbar BMD

was only recorded in the DMD cohort. The bone mineral density results are shown in Table 7.

Table 7: Bone mineral density measurements in the DMD, OI, and SLE cohorts.

Bone Mineral Density	DMD	OI	SLE	P-value
Total Body BMD (Z score)	-4.28 (2.0)	-1.0 (1.4)	-0.44 (1.2)	<0.001 [a b]
Lumbar BMD (Z-score)	-2.48 (1.2)	N/A	N/A	N/A

Results are recorded as mean (SD). [a] p < 0.05 when comparing DMD to OI; [b] p < 0.05 when comparing DMD to SLE using ANOVA and Bonferroni post-hoc analysis;

In the DMD cohort, the average patient total body BMD Z-score was measured to be -4.28 ± 2.0, indicating significant bone fragility in these patients. The average lumbar spine BMD Z-score was -2.48 ± 1.16 in the DMD cohort. Patients with DMD had slightly lower total body BMD Z scores than the OI and SLE cohorts. No significant correlations were identified between total body BMD and secondary parameters. In the OI population, average total BMD Z-score was -0.85 ± 1.46. A significant positive correlation was seen between BMD Z-score and BMI Z-score of OI patients (Figure 14, $\rho = 0.301$, p-value = 0.027). No significant correlations were identified between BMD Z-score and other secondary parameters in the OI population.

Figure 14: Correlation between Total body BMD Z-score and BMI Z-Score in the OI population.
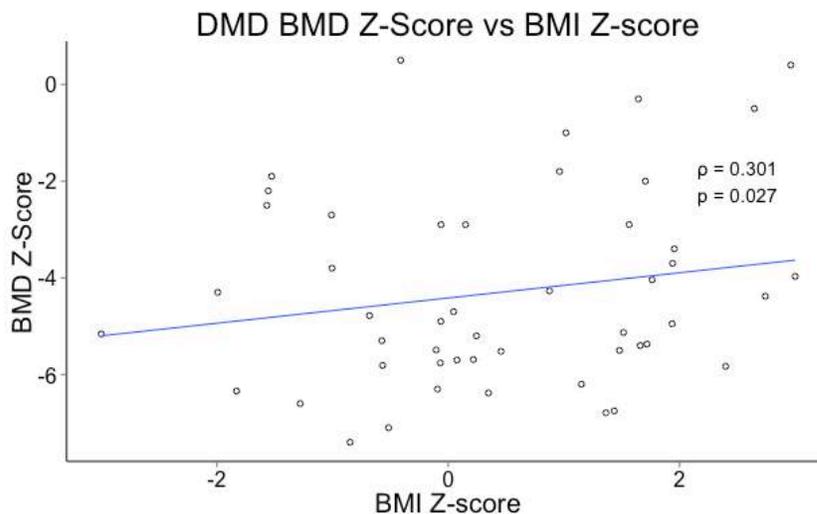
In the SLE population, the average total body BMD Z-score was -0.441 ± 1.179. Spearman's

rank correlation identified a significant correlation between BMD Z-score and total body fat%

(Figure 15, ρ = 0.220 p-value = 0.011), as well as BMI Z-score (Figure 16, ρ = 0.509, p-value

= 1.16E-9). No other significant correlations were identified in the SLE cohort.

Figure 15: Correlation between Total body BMD Z-score and fat % in the SLE population.
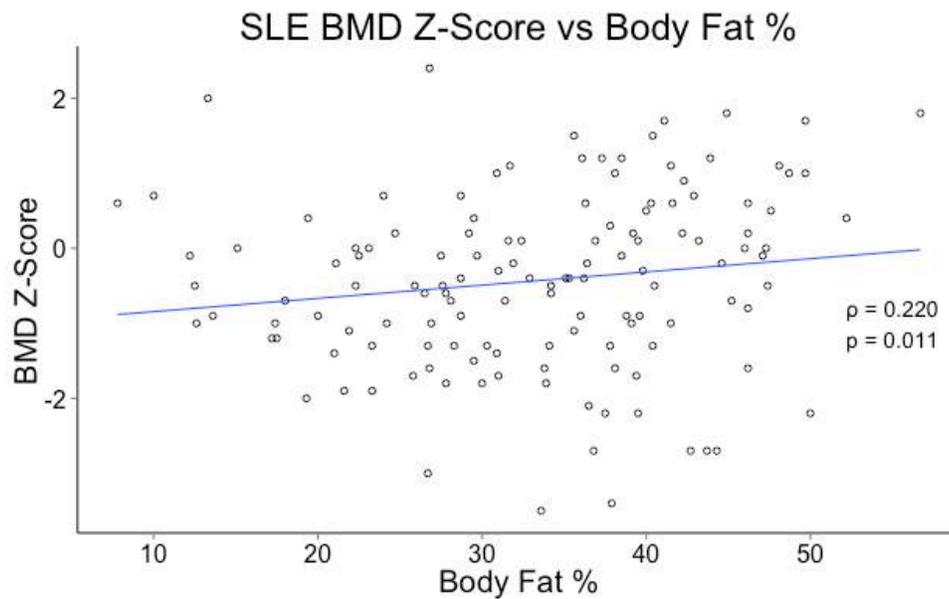
Figure 16: Correlation between Total body BMD Z-score and BMI Z-score in the SLE

population.
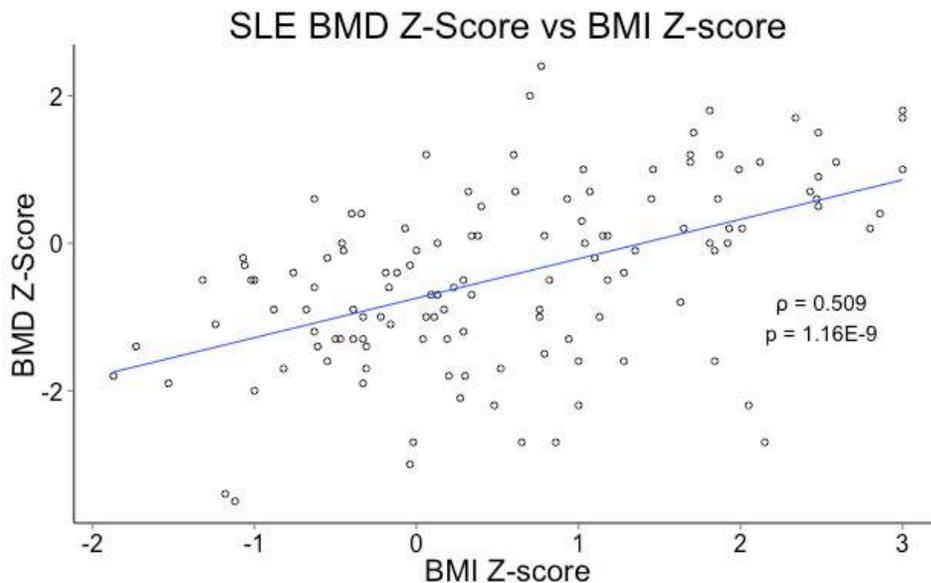
With a significantly lower average total body BMD Z-score recorded in the DMD population,

multi-linear regression analysis was then performed to identify the secondary parameters that

were predictors of low BMD in this population. In the DMD cohort, the ambulatory status of

the patients was a significant determinant of total body BMD Z-score (Table 8, p = 0.019).

Table 8: Multilinear regression model showing significant determinants of Total body BMD Z-

Score in the DMD population.

Model parameter	ß	P value
Constant	-3.61	<0.0001
Vignos	-0.23	0.019
$R^2 = 0.10$		

These results suggest that every unit of increase in the Vignos functional scale is associated

with a 0.23 decrease in total body BMD Z-score. Furthermore, significant determinants of

lumbar spine BMD Z-score include the age and glucocorticoid dose adjusted for the patients'
weight (Table 9).

Table 9: Multilinear regression showing significant determinants of lumbar spine BMD Z-score in the DMD population.

Model parameter	ß	P value
Constant	0.2	0.67
Age (years)	-0.191	<0.001
Glucocorticoid dose (mg/kg)	-1.061	0.010
$R^2 = 0.44$		

These results suggest for every unit increase in age or glucocorticoid dose adjusted for weight, there is a 0.191 and 1.061 decrease in lumbar spine BMD Z-score respectively.

Overall, the DMD population had on average significantly lower total body BMD Z-score in comparison to the SLE and OI populations. Ambulatory status was determined to be the sole predictor of total body BMD. Age and glucocorticoid dose were accurate predictors for lumbar spine BMD, accounting for 44% of the variation in the lumbar spine BMD data. However, there was a large amount of variation in the total body bone mineral density data that could not be explained by the linear regression models. There were other factors not accounted for in our study parameters that may have contributed to the poor bone health in these DMD children.

4.6 Summary of Findings

The objective of this study was to analyze the vitamin D status, identify possible determinants of 25-OHD and 25-OHD standardized per IU of supplementation, and to identify determinants

of bone health in the DMD population. To summarize the significant findings from this analysis:

1. Children with DMD had on average an insufficient level of serum 25-OHD, below the Canadian Pediatric Society standard of 75 nmol/L.

2. Children with DMD had relatively lower serum 25-OHD between the ages of 3 to 8.9 years compared to the healthy pediatric population data.

3. Compared to children with OI and SLE, children with DMD had lower serum 25-OHD, despite higher vitamin D supplementation.

4. In the total cohort, having DMD, the season and weight Z-score were significant determinants of serum 25-OHD. Glucocorticoid dose was not significantly associated with serum 25-OHD or 25-OHD per IU of supplementation.

5. In the DMD cohort alone, vitamin D supplementation was the only significant determinant of serum 25-OHD. However, given only 8% of the variance in the 25-OHD values were explained by the linear regression model, other non-measured variables may have a significant role in determining serum 25-OHD.

6. Boys with DMD had on average lower total body BMD Z-score compared to children with OI and SLE.

7. Neither serum 25-OHD nor serum 25-OHD per IU of supplementation was a significant predictor for both total body and lumbar spine BMD in the DMD cohort.

5 Discussion

The primary aim of this study was to evaluate the vitamin D status in children with DMD and to compare their average vitamin D level to an ambulatory population treated with glucocorticoids, and another non-ambulatory population not on glucocorticoid treatment. We were able to demonstrate that despite being on larger vitamin D supplementation, boys with DMD have lower serum 25-OHD concentrations compares to children with SLE and OI.

5.1 Definition of Vitamin D Sufficiency

Extensive studies have established the role of vitamin D for bone health[18,19,166]. However, the optimal level of vitamin D for better health outcomes is still open for debate[167-170]. The definition of vitamin D sufficiency vary from 50 nmol/L defined by the American Academy of Pediatrics, to 75 nmol/L defined by the Canadian Pediatric Society. The discrepancy between the American and Canadian definitions for vitamin D sufficiency is primarily based on results from adult bone health and vitamin D literature. Only a few pediatric studies have analyzed the effect of raising serum 25-OHD above 75 nmol/L on bone health[171-173]. The results on bone health has been inconsistent, therefore the AAP has not adopted the 75 nmol/L definition for vitamin D sufficiency. Conversely, the CPS defines the optimal level of 25-OHD as the level at which serum parathyroid hormone levels and intestinal calcium absorption are stabilized[51,174,175]. For our purposes, we determined serum 25-OHD sufficiency to be greater than 75 nmol/L in accordance with the CPS definitions[51]. Serum 25-OHD levels between 25 and 75 nmol/L were determined to be insufficient, and levels below 25 nmol/L were deficient.

5.2 Vitamin D status of DMD Patients

Our findings show that out of 83 patients with DMD, one patient was vitamin D deficient, 57 patients were vitamin D insufficient, and only 25 patients were vitamin D sufficient. The

average serum 25-OHD amongst these patients was 65.1 nmol/L, below the sufficiency standard of 75 nmol/L. There have been a couple of other studies that have reported low serum 25-OHD concentrations in patients with DMD [3,10,125,176]. Out of 157 boys with DMD, Munot et al. identified that 78% of these boys had inadequate vitamin D levels, below 50 nmol/L[10]. Bianchi et al. identified the average serum 25-OHD to be 14 ± 6 ng/mL (35 ± 15 nmo/L) in the non-corticosteroid treated boys with DMD[125]. After one year of observation prior to 25-OHD supplementation, 60.6% of DMD patients had a serum 25-OHD below 50 nmol/L[3]. Thus our findings align with the results from previous reports, indicating vitamin D insufficiency in various DMD populations.

5.3   Comparison of Vitamin D between DMD and Healthy Populations

In comparison to the healthy local pediatric data obtained from the Canadian Laboratory Initiative for Pediatric Reference Intervals (CALIPER) study, the average serum 25-OHD in our DMD cohort was not significantly different. However, between the ages of 3 to 8.9 years, average DMD serum 25-OHD was significantly lower than the CALIPER study. To this date, no known study has compared the serum 25-OHD of a healthy local control population and patients with DMD, especially for boys in the age range of DMD patients. In general, data regarding serum 25-OHD in healthy children is lacking. A study in Edmonton surveying individuals between the ages of 2 to 16 identified the average serum 25-OHD in these participants to be 47.2 nmol/L, where 34% of individuals had a serum 25-OHD below 40 nmol/L, and 6% below 25 nmol/L[177]. Based on the Canadian Health Measures Survey obtained from 2012 to 2013, 78% of Canadians between the ages of 3 to 11 years had a serum 25-OHD greater than 50 nmol/L[8]. Data from the National Health and Nutrition Examination Study (NHANES) in 2003 and 2004 in the U.S. shows that average serum 25-OHD measurements for children aged 1-5 years was greater than 55 nmol/L, and the percentage of children aged 1-5

years with 25-OHD concentrations below 27.5 nmol/L was minimal[178]. In another U.S. study surveying serum 25-OHD in healthy children ranging from 6 to 23 months of age in Alaska, 31 % of 133 children had a serum 25-OHD below 62.5 nmol/L[179]. Therefore, vitamin D insufficiency is common amongst these pediatric populations as well as our DMD cohort. However additional studies directly comparing serum 25-OHD of a healthy control population to a DMD population need to be performed to identify the prevalence of vitamin D insufficiency in the DMD cohort in comparison to healthy pediatric individuals. The variation in the healthy population cohort in comparison to the DMD cohort may be explained by differences in the measurement assays. The DMD vitamin D was measured using high-tandem liquid chromatography, and the CALIPER study used radioimmunoassay. On average, there is a 7% discrepancy amongst assays, which may explain the difference between DMD and CALIPER cohort between the ages of 3 to 8.9 years[62].

5.4 Sunlight Influence on Vitamin D

There are multiple potential contributing factors to low serum 25-OHD levels, one of which is sunlight exposure. Vitamin D intake from sunlight exposure depends on the latitude, skin pigmentation, sunscreen usage, and the season[19]. Holick estimates that with 1 minimal erythemal dose (MED, the equivalent of the amount of sunlight required to create minimal pinkness in the skin after a full day of exposure), the body creates approximately 20,000 IU of vitamin D[53]. Therefore according to the Institute of Medicine[180], which requires children to receive 600 IU of vitamin D per day, children only require 0.033 of an MED to receive sufficient cutaneous synthesis of vitamin D per day. Children, especially infants, may require less sunlight exposure to cutaneously synthesize optimal amounts of serum 25-OHD due to a greater surface area to body volume ratio, and a greater capacity to synthesize cutaneous 25-OHD than adults[181]. In our study, we were unable to measure sunlight exposure in our unified

cohort. However, when combining the DMD, SLE, and OI cohorts, multiple linear regression analysis identified that having serum 25-OHD taken in the fall was a significant determinant of higher serum 25-OHD. Our findings were in line with the findings of Godzik et al., who identified that the overall serum 25-OHD of young adults in Toronto with an average age of 21 years had a serum 25-OHD of 54.4 nmol/L in the fall, which was significantly higher than the 38.4 nmol/L measured in the winter[182]. A United States study has identified the contribution of seasonal variability to serum 25-OHD, confirming that serum 25-OHD are highest in the late summer months, and lowest in the winter months[183]. Similarly, Gill and Kalia have shown that an average individual with type II skin (white skin) and with ¼ of total skin exposed were able to produce 1000 IU of vitamin D in Toronto with 14 to 30 minutes of exposure during the spring, summer, and autumn months[184]. However, individuals with type II skin with ¼ of total skin exposed to the sun in the winter months were unable to produce 1000 IU of vitamin D in Toronto[184]. Our findings agree with these previous findings in that greater serum 25-OHD was measured in the fall months in our unified cohort. However, the absence of a relationship between winter month of collection and serum 25-OHD in our study cannot be explained by exposure to UV light and may indicate the effects of vitamin D supplementation. The direct effect of sunlight exposure on serum 25-OHD in children is still unclear and merits further investigation. Furthermore, data recording the level of sunlight exposure in DMD children will be important for further understanding of the low serum vitamin D levels found in these patients.

5.5 Dietary Influence on Vitamin D

Foods including fish such as sardines, mackerel, salmon, and cod liver oil, and egg yolks are high in vitamin D content[57]. However, minimal amounts of these dietary sources of vitamin D are ingested daily by children[57]. In Canada, vitamin D is added to cow's milk and margarine to

prevent osteoporosis, osteomalacia, and rickets[185]. According to the data collected from the Canadian Health Measures Survey from 2009 to 2012, children between the ages of 3 to 5 years who drank milk more than once a day had on average a serum 25-OHD level of 75 nmol/L[186]. In contrast, children between the ages of 3-5 years who drank milk less than once a day had an average serum 25-OHD of 60 nmol/L[186]. It has been concluded by others that children are spending more time indoors[49], and are drinking less vitamin D fortified milk, which is leading to low serum 25-OHD[187].

Dietary vitamin D data was not collected in any of the patient charts for our study. From our study poor serum 25-OHD levels were found in DMD patients, however we are unable to determine if dietary vitamin D intake was a factor in this finding.

5.6 Vitamin D Supplementation Effect on Serum Vitamin D

Vitamin D supplementation is often one of multiple treatments for patients with poor bone health. To prevent vitamin D deficiency, the Institute of Medicine recommended children from the ages of one to eighteen years of age to take 600 IU of vitamin D supplementation per day[180]. However, Roth et al[177] found that few children who were treated with a dose of 1.3μg/kg/day (800 IU per day in a 2 year old weighing 15 kg) reached a serum 25-OHD level of 75 nmol/L or greater. Supplementation with 400 IU of vitamin D per day raised serum 25-OHD concentrations by 7-12 nmol/L in adults[188]. Yet, to increase serum 25-OHD from 50 to 80 nmol/L, an approximate intake of 1700 IU of vitamin D per day was required[189]. In our study, the DMD cohort was supplemented with an average dose of 1627 IU per day, or 63.3 IU/day/kg when adjusted for weight (1.57 μg/kg/day), well above the recommended daily intake for healthy children. Yet despite this, only 30.1% of the DMD cohort achieved a serum 25-OHD in the sufficient range. Bianchi et al.[3] performed a prospective study of 33 boys with

DMD with a mean age of 8.4 years. They showed that two years of treatment with 0.8 µg/kg/day of calcifediol was sufficient to correct serum 25-OHD in patients with DMD to a 25-OHD level greater than 50 nmol/L[3]. In our DMD cohort, vitamin D supplementation was identified as a significant positive determinant of serum 25-OHD, showing that increased vitamin D intake would increase serum vitamin D levels in these patients. However, despite the significantly high level of supplementation in comparison to the SLE and OI populations, the DMD cohort had a significantly lower serum 25-OHD compared to the other cohorts, and the serum 25-OHD in these patients was not restored to a level of 75 nmol/L. Our results suggest the high level of supplementation were still not sufficient to raise serum 25-OHD levels to sufficiency in approximately 70% of the DMD population. Poor vitamin D supplementation adherence may be a possible explanation for the low 25-OHD levels seen in DMD patients. No studies have looked at the medical adherence of DMD patients prescribed to vitamin D supplements, however there is evidence of poor adherence amongst individuals with chronic illnesses. Between 30 to 70% of patients with chronic diseases had poor medical compliance due to prolonged treatment, large number of treatments, and periods of illness remission[190-192]. In particular in Bianchi's study, the DMD children were specifically asked about compliance and were asked to bring back the calcifedol bottles. They reported a high compliance rate (as defined by taking at least 80% of the doses) in 84% of their cohort[3]. In another study analyzing medical adherence in children with asthma, only 58.4% of 24 children with asthma used their prescribed corticosteroids[193]. In addition, Mackner and Crandall identified that 48% of 50 children with inflammatory bowel disease from ages 11 to 17 were adherent to all prescribed medications[194]. Children with DMD are typically prescribed daily glucocorticoids along with vitamin D supplements to improve overall mobility and muscle function[195]. Given the retrospective nature of our study we were unable to collect compliance data but postulate they

may have been much lower in our cohort. Poor medication compliance could therefore explain the low 25-OHD seen in children with DMD despite high vitamin D supplement doses prescribed by pediatricians; however, further studies would be required to determine if this was the case.

5.7   Comparison Between DMD and OI Vitamin D Levels

The DMD population is both a glucocorticoid-treated, and low ambulatory population. To determine the influence of glucocorticoids on serum vitamin D, we compared our DMD cohort to a control cohort with decreased ambulation (OI). Populations with poor mobility are often associated with vitamin D insufficiency[196-198]. Our DMD cohort had on average lower serum 25-OHD levels than the OI cohort suggesting that disability may not contribute to the deceased 25-OHD levels seen in DMD patients. Greenway and Zacharin studied the vitamin D status of disabled children in Victoria, Australia, and determined the average serum 25-OHD in wheelchair-bound children to be 56.4 nmol/L[197]. In a study by Finbraten et al., serum 25-OHD was measured to be 53 nmol/L in non-ambulatory children with cerebral palsy, a disease that is commonly associated with poor ambulation[199]. Additionally, Thouvenot et al. identified that serum 25-OHD was negatively correlated to the degree of disability in patients with multiple sclerosis[200]. The low 25-OHD levels found in our DMD population coincide with these previous findings, indicating that vitamin D insufficiency is common in disabled populations. In contrast however, our decreased ambulatory control cohort (the children with OI) had a mean 25-OHD concentration in the sufficient range (greater than 75 nmol/L). Our findings conflict with the findings of Wilsford et al., where the average 25-OHD in 80 patients with OI was measured to be 23 ng/mL (57.4 nmol/L), well below the sufficiency range[160]. Of the 80 patients with OI in their study, 35 individuals had serum 25-OHD levels below 80 nmol/L[160]. In addition, Kadhim et al. identified vitamin D insufficiency (< 80 nmol/L) in 31 out of 60

patients with OI. Despite lower vitamin D supplementation than the DMD cohort, the patients

with OI in our study had on average a greater 25-OHD, above the 75 nmol/L sufficiency

cutoff. With all of the possible factors contributing to a greater 25-OHD in the OI population,

we were unable to identify the determinants of sufficient 25-OHD in the OI cohort. However,

since the average 25-OHD in the DMD population was significantly lower than our control

disabled population, it led us to believe that the glucocorticoid treatment rather than disability

in the DMD population may have contributed to low serum 25-OHD.

5.8   Glucocorticoid Influence on DMD Serum Vitamin D

Interestingly, results of our analyses did not identify a significant correlation between

glucocorticoid use and serum 25-OHD in the DMD population. Vitamin D deficiency had been

previously identified in glucocorticoid treated DMD studies[3,125,201]. Bianchi et al. identified that

corticosteroid-treated patients with DMD had an average 25-OHD of 13.5 nmol/L, in

comparison to corticosteroid-naïve patients with an average of 36.5 nmol/L[125]. In a later study,

Bianchi et al. identified average baseline 25-OHD measurements of 20 out of 33 DMD patients

with glucocorticoid treatment to be below 50 nmol/L[3]. Furthermore, Skversky et al.

[123]identified in a general cross-sectional analysis that the odds of vitamin D deficiency in

individuals was 2-fold greater in those treated with steroids in comparison to those free of

steroid use. However, in our DMD cohort, glucocorticoid usage neither correlated with nor

significantly determined serum 25-OHD, despite these patients having a significantly lower

average serum 25-OHD in comparison to the control glucocorticoid-free cohort (OI).

5.9   Comparison Between DMD and SLE Vitamin D

Glucocorticoid-treated populations are at risk of vitamin D deficiency[123,202]. In a study

determining the 25-OHD in children with asthma, Searing et al. identified 47% of 100 children

with asthma to have a serum 25-OHD level below 75 nmol/L[202]. The mechanisms by which glucocorticoids affect serum vitamin D have not been clearly established. Nevertheless, it has been established that glucocorticoid use improved the overall motor capabilities, yet increased the risk of fracture in DMD patients[203,204]. We compared our DMD population to a control corticosteroid treated population (patients with SLE) to identify the influence of disability on serum 25-OHD. As we hypothesized, our results showed a significantly lower average 25-OHD in the DMD population in comparison to the control glucocorticoid population (patients with SLE). However, the average serum 25-OHD of SLE patients was greater than the 75 nmol/L sufficiency range. This opposes the findings of AlSaleem et al., where the average 25-OHD of 24 patients with SLE was found to be 51.1 nmol/L, much lower than the sufficiency standard[11]. Most of these patients were given 800 IU of vitamin D supplementation prior to the study, which is a dose lower than the average of 1000 IU in our retrospective SLE cohort. We are unable to explain the high measured 25-OHD levels in our SLE cohort. Still, these results suggest that potentially lower ambulation in combination with glucocorticoids may contribute to low serum 25-OHD levels in DMD patients.

5.10   Disability Influence on DMD Serum Vitamin D

We hypothesized that ambulatory status may be a key contributor to low 25-OHD found in DMD patients. In our DMD cohort, the Vignos functional scale was found to be a significant positive correlate to serum 25-OHD when standardized per IU of supplementation. A decrease in overall ambulatory status in our DMD cohort was correlated to an increase in serum 25-OHD per IU of vitamin D supplementation. These results may appear to be contradictory to previous studies, as low sun exposure and thus low cutaneous synthesis of vitamin D has been commonly linked to decreased ambulation in disabled populations[205,206]. A study in Australia

determined that increasing disability status in patients with multiple sclerosis was strongly associated with lower 25-OHD levels and reduced sunlight exposure[207]. While the findings in our DMD population seems counter intuitive, we must consider the possible relationship between mobility and potential for sunlight exposure in the DMD patient cohort. A Vignos rank of 7 is defined as a patient with leg braces, whereas a Vignos rank of 9 means that a patient is wheelchair-bound[165]. A systematic review analyzing the developmental and social benefits of wheelchair intervention revealed that wheelchair-bound individuals have a reduced need for a personal caregiver, and an increase in mobility[208]. With an increase in overall mobility, wheelchair-bound individuals may experience more time outdoors, resulting in greater sunlight exposure. This may explain the higher level of 25-OHD per IU of supplementation for individuals with a higher Vignos functional scale score. Further information about sunlight exposure in the DMD population will be required to determine the role of increased mobility with wheelchair use on cutaneous vitamin D.

5.11   Weight Influence on DMD Serum Vitamin D

Weight Z-score was a significant negative determinant of serum 25-OHD and serum 25-OHD per IU of supplementation in our unified study population. Obesity has been identified as a significant issue in patients with DMD[209]. Generally, an increase in fat mass and weight gain has been seen in patients with neuromuscular disease in comparison to healthy controls[210]. Furthermore, steroid treatment with either prednisone or deflazacort has been shown to further exacerbate weight gain in patients with DMD[211,212]. Our DMD cohort had significantly higher percent body fat than the OI cohort, despite the absence of significant difference in BMI Z-scores. However, these patients are losing significant muscle mass with disease progression and therefore the percentage body fat is a more useful measure of obesity than BMI in these patients. Obesity in individuals has been previously linked to low serum 25-OHD levels[213-216].

Wortsman et al. determined that average serum 25-OHD in 19 obese individuals was 50.0 nmol/L, which was significantly lower when compared with 84.4 nmol/L measured in 19 non-obese individuals[213]. It has been postulated that the low serum 25-OHD in obese patients may be due to avoidance of sunlight exposure[217], or increased 25-OHD deposition in fat compartments[213]. In our DMD cohort, disease duration and glucocorticoid treatment may have caused the increase in total fat mass and thus total weight. This total increase in fat mass may explain the decreased serum levels of 25-OHD in the DMD cohort.

5.12 Bone Mineral Density in the DMD Population

Low BMD has been identified in numerous studies with DMD patients[3,125,176,218]. Decreased BMD Z-score in children has been associated with lower ambulatory status. In our study, children with DMD had an average total body BMD Z-score of -4.28 ± 2.0 and lumbar spine BMD Z-score of -2.48 ± 1.2, clearly outlining the poor bone status of our DMD population. These findings are similar to those of Bianchi et al., where the average total body BMD Z-score and lumbar spine BMD Z-score were of approximately -3 and -4 respectively (estimated from graphical representation in reference 117) in corticosteroid treated patients with DMD[125]. Our results also identified that the ambulatory status of DMD patients was a significant predictor of total body BMD Z-score. This contrasts with the results of Soderpalm et al., where a significant correlation between the heel BMD Z-score and the Vignos functional scale was identified in DMD patients, but no correlation was found between the Vignos scale and total body BMD[201]. However, the correlation between ambulatory status and total body BMD had been established in other populations with a similar disability. Fibraten et al. determined that non-walking children with cerebral palsy had significantly lower total body BMD Z-score (ranging from -1.7 to -5.4) in comparison to walking children (ranging from -0.8 to -1.5)[219]. Despite no known study identifying a correlation between total body BMD and ambulatory

status in children with DMD, our findings suggest that low ambulatory status is a significant determinant of decreased total body BMD Z-score in DMD patients.

5.13 Lumbar Spine BMD in the DMD Population

Lower lumbar BMD Z-scores are typically found in corticosteroid-treated DMD patients. Bianchi et al. [125] determined that the average lumbar spine BMD Z-score was low in DMD patients, but much lower in glucocorticoid treated DMD patients. Trabecular bone mass found in the lumbar spine was significantly reduced in long-term glucocorticoid therapy studies[220-222]. In accordance with the results of these previous studies, the glucocorticoid dose in our DMD cohort was found to be a significant negative determinant of lumbar spine BMD Z-score. In addition, our results identified a significant association between age and lumbar BMD Z-score in the DMD cohort. In healthy children, studies have identified a gradual increase in lumbar spine BMD Z-score with age. In DMD patients after one observational year, BMD Z-score progressively decreased[3]. However, no studies to our knowledge have analyzed long-term lumbar spine BMD Z-score change with age in DMD patients. In a recent prospective study, Bianchi et al. showed that total body and lumbar spine BMD increased in patients treated with calcifediol over two years[3].

5.14 Vitamin D Influence on Bone Mineral Density in the DMD Population

Interestingly, neither serum 25-OHD nor serum 25-OHD per IU of supplementation was found to be a significant determinant of total body or lumbar spine BMD Z-score in children with DMD. Numerous studies show a significant effect of vitamin D status on BMD in children[223-226]. Farrar et al. identified that adolescents with seasonal vitamin D deficiency had low BMD

measures[223]. Furthermore, Karalus et al. determined that with vitamin D supplementation to improve serum 25-OHD, significant improvements have been seen in the lumbar spine BMD. However in our study, serum 25-OHD was not seen as a significant determinant for either lumbar spine BMD or total body BMD Z-score. This finding suggests that factors other than vitamin D status determined BMD in the DMD patients and from our analysis these appeared to be disability for total body BMD and glucocorticoid dose and patient age for lumbar spine BMD.

6 Strengths and Limitations

The major strength of this study is that this study provides insight on vitamin D supplementation standards and bone health for children with DMD, SLE, and OI. Overall, given the importance of vitamin D in bone health, very little research has been performed to identify causes of low serum 25-OHD in the DMD population. Recently, a study was performed to identify the dose of supplementation that is required for optimal serum 25-OHD levels in children with DMD[227]. Alshaikh et al. concluded that a 2-month regimen of 6000 IU followed by a daily maintenance doses of 1000-1500 IU per day were associated with serum 25-OHD levels greater than 75 nmol/L[227]. Considering the high intake toxicity of vitamin D to be 240,000 to 4,500,000 IU in children and adolescents[228], our study suggests that children with DMD could and should be supplemented with more vitamin D to normalize their serum 25-OHD levels.

There were several interesting findings in our study that merit further exploration. First, both our SLE and OI populations had average serum 25-OHD levels above the 75 nmol/L vitamin D sufficiency standard. Previous studies have identified vitamin D insufficiencies in these two populations, but we were unable to identify determinants of the high serum 25-OHD in these populations. In addition, DMD boys with a greater Vignos scale score (lower ambulatory status) had on average higher serum 25-OHD per IU of supplementation. We postulated that as children progressed from moving with a leg brace (Vignos scale of 7) to being wheelchair-bound (Vignos scale of 9), their overall mobility increased, which led to an increase in sunlight exposure and cutaneous synthesis of vitamin D. However, this does not explain a lower serum 25-OHD seen in children with a Vignos scale of 1.

There are several limitations in our study. With a retrospective study, there was no knowledge of supplementation compliance amongst all patients included in this study. Since there is no DMD patient group that is not disabled, or free of glucocorticoid treatment, we chose the SLE and OI populations as comparator groups for our study. We did not match our SLE and OI cohorts to our primary DMD study group via their age or sex. If the groups were split to only include male patients, or compared to the average age, we would not have had enough patients to reach a significant power for our study. When the patient charts were reviewed, we had no knowledge of any dietary vitamin D intake, and therefore could not have standardized the level of serum 25-OHD according to the diet. Lastly, there was not a baseline measurement of serum 25-OHD for each patient before vitamin D supplementation, and therefore the change of serum 25-OHD and length of supplementation in each patient was unknown. Future studies regarding serum 25-OHD deficiency in DMD patients would require a prospective study. Such a study should record serum 25-OHD changes over time, measure compliance in taking supplements and record dietary information to standardize the serum 25-OHD to the total vitamin D intake. Sunlight exposure would also need to be tracked in order to gain some understanding of cutaneous vitamin D production in the patient population. This future study would also require a multicenter approach in order to capture a larger patient population using unified methodology across centres.

7 Conclusion

Overall, vitamin D insufficiency is prevalent amongst children with Duchenne muscular dystrophy. Despite high levels of vitamin D supplementation, serum 25-OHD were still below the sufficiency range. This discrepancy may be explained by other unaccounted factors including dietary intake of vitamin D, sunlight exposure, and vitamin D supplementation compliance. To identify significant determinants of 25-OHD in children with DMD, we analyzed the influence of glucocorticoid dose and ambulatory status. Glucocorticoid dose was not significantly correlated to serum 25-OHD in children with DMD. Ambulatory status was negatively correlated to serum 25-OHD per IU of supplementation and was a significant determinant for total body BMD. Age and glucocorticoid dosage were significant determinants of lumbar spine BMD. Our findings have important implications for vitamin D dosage regiments for children with DMD. We suggest increasing the dosage of vitamin D supplementation to improve serum 25-OHD in children with DMD and overall bone health.

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9 Appendices

9.1 REB Approval from Holland-Bloorview for DMD Population

Holland Bloorview
Kids Rehabilitation Hospital

Holland Bloorview
Kids Rehabilitation Hospital
150 Kilgour Road
Toronto ON Canada M4G 1R8
T 416 425 6220
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A teaching hospital affiliated
with the University of Toronto

Holland Bloorview Research Ethics Board
Ethics Approval Notification

The Holland Bloorview Research Ethics Board operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans, the Ontario Personal Health Information Protection Act, 2004, ICH Good Clinical Practice Consolidated Guideline E6, and Health Canada Part C Division 5 of the Food and Drug Regulations

Study Title: Children with DMD require greater supplementation with vitamin D to normalize serum 25-hydroxyvitamin D levels than another pediatric corticosteroid treated population and another population with a physical disability
File Number: 15-619
Principal Investigator: Laura McAdam
Co-Investigators: Jennifer Harrington, Etienne Sochett, Earl Silverman, Derek Stevens
Original Approval Date: January 26, 2016
Expiry Date: January 26, 2017
Review Type: Delegated

January 26, 2016

Dear Dr. McAdam,

The Holland Bloorview Research Ethics Board (REB) has reviewed the above named study. This was a delegated review. The board is granting ethics approval for a period of one year. The approval of this study includes the following documents:

- Protocol (version dated June 16r, 2015)
- TAHSN form received November 24, 2015
- Data Collection forms – DMD (version received November 24, 2015)
- Data Collection forms – OI (version received November 24, 2015)
- Data Collection forms – SLE (version received November 24, 2015)

This study must be conducted in accordance with the description in the application and any supplementary documents for which ethics approval has been granted. The REB needs to be notified of any unanticipated or unintentional divergence or departures from the protocol through a "Protocol Deviation Form". Any intentional changes to the protocol need be submitted through an "Amendment Form" to the REB for approval *before* the changes are implemented, except where necessary to eliminate immediate hazards to the participants.

Any adverse events that occur as a result of your study must be reported to the REB by submitting an "Adverse Event/Unanticipated Problem Form".

If the study is expected to continue beyond the new expiry date, you must request another renewal, at least thirty days prior to the expiry date, by submitting an "Annual Renewal Form". When the study is completed or terminated, you need to submit a "Study Closure Form" to the REB.
Best wishes for the successful completion of your project.

Sincerely,

Stephen Ryan, PhD, PEng
Chair, Research Ethics Board
P: 416 425 6220 x3526
sryan@hollandbloorview.ca

A world of possibility

9.2 REB Approval from the Hospital for Sick Children for OI and SLE Populations

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SickKids

RESEARCH ETHICS BOARD APPLICATION FORM

Form Version Date: October 1, 2012

REB RECEIVED

MAR 17 2015

REB APPLICATION # (for REB use only)

`1 0 0 0 4 9 2 1 4`

REB protocol number (REB use only)

`1 0 0 0 3 6 3 0 7`

PART 1: STUDY INFORMATION

1. PROJECT

Complete project title

Children with Duchenne Muscular Dystrophy require greater supplementation with 25-hydroxyvitamin D than other pediatric corticosteroid populations and populations with a physical disability.

Short title (max 50 characters)

Vitamin D deficiency and insufficiency in DMD

Lay summary (max 750 characters)

There has been increasing awareness of the importance of bone health in Duchenne muscular dystrophy (DMD). Boys with DMD usually present symptoms beginning at ages 3 to 5, with inability to walk occurring between the ages of 7 and 12. There is no current cure for this disease, only treatments designed to slow the progression of symptoms. Despite the potential benefits of vitamin D to improve bone health, high rates of vitamin D deficiency have been described in cohorts of boys with DMD. To look at the the differential effects of glucocorticoid treatment and chronic illness on the ability to achieve vitamin D sufficiency we plan to compare the results of our cohort of boys with DMD with three control groups.

List in point form the objectives of your study

- To determine if children with DMD require greater supplementation with vitamin D to normalize serum 25-hydroxyvitamin D levels than another pediatric coritcosteroid treated population and another population with a physical disability
- To determine if daily steroid dose or cumulative steroid dose has a greater association to serum 25-hydroxyvitamin D levels
- To determine if there is a difference between steroid treatment (Deflazacort vs. prednisone/prednisolone) on serum 25-hydroxyvitamin D levels
- To determine if there is a relationship between serum 25-hydroxyvitamin D levels and bone mineral density
- To determine if there is a relationship between of fractures and serum 25-hydroxyvitamin D levels
- To determine if fat mass is associated serum 25-hydroxyvitamin D levels
- To determine if there is a relationship between serum 25-hydroxyvitamin D and parathyroid hormone levels
- To determine if there is a relationship between muscle function and serum 25-hydroxyvitamin D levels
- To determine if there is an association between ambulatory status and serum 25-hydroxyvitamin D levels

Duration of study

Anticipated study start date: Feb 17, 2015

Anticipated study completion date: Apr 1, 2016

2. RESEARCH TEAM

All SickKids Staff, trainees, or volunteers must complete the mandatory online ethics training course (TCPS 2).

PI: Dr. Etienne Sochett

Research Ethics Board Application Form

Short Title: Vitamin D deficiency and insufficiency in DMD

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Form Version Date: October 1, 2012

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9.3 Sample Collection Data Sheet for DMD Population

Year 1 Data

Record ID   _____

Date of visit   _____

Season of visit   ◯ Summer: June to August
                                                            ◯ Fall: Sept to Nov
                                                            ◯ Winter: Dec to March
                                                            ◯ Spring: April to May

Age at visit   _____

Anthropometric data

Height (cm)   _____

Weight (kg)   _____

Body mass index (kg/m2)   _____

Pubertal status   ◯ Pre-pubertal
                                                            ◯ Mid-pubertal
                                                            ◯ Post-pubertal

Muscle Function (Brooke Scale Grade)   ◯ 1. Starting with arms at the sides, the patient
                                                            can abduct the arms in a full circle until they
                                                            touch above the head
                                                            ◯ 2. Can raise arms above head only by flexing the
                                                            elbow (shortening the circumference of the
                                                            movement) or using accessory muscles
                                                            ◯ 3. Cannot raise hands above head, but can raise an
                                                            8-oz glass of water to the mouth
                                                            ◯ 4. Can raise hands to the mouth, but cannot raise
                                                            an 8-oz glass of water to the mouth
                                                            ◯ 5. Cannot raise hands to the mouth, but can use
                                                            hands to hold a pen or pick up pennies from the
                                                            table
                                                            ◯ 6. Cannot raise hands to the mouth and has no
                                                            useful function of hands

Muscle Function (Vignos Function Scale)   ◯ 1. Walks and climbs stairs without assistance
                                                            ◯ 2. Walks and climbs stair with aid of railing
                                                            ◯ 3. Walks and climbs stairs slowly with aid of
                                                            railing (over 25 seconds for eight standard steps)
                                                            ◯ 4. Walks unassisted and rises from chair but
                                                            cannot climb stairs
                                                            ◯ 5. Walks unassisted but cannot rise from chair or
                                                            climb stairs
                                                            ◯ 6. Walks only with assistance or walks
                                                            independently with long leg braces
                                                            ◯ 7. Walks in long leg braces but requires
                                                            assistance for balance
                                                            ◯ 8. Stands in long leg braces but unable to walk
                                                            even with assistance
                                                            ◯ 9. Is in a wheelchair
                                                            ◯ 10. Is confined to a bed

Ambulatory status   ◯ Walking independently
                                                            ◯ Walking with assistance
                                                            ◯ Non-ambulatory

Pharmacological data

Mean elemental calcium supplementation over previous year (mg/day)
_____

Mean vitamin D supplementation over previous year (IU/day)
_____

Duration of glucocorticoid treatment
_____

Mean dose of glucocorticoid over previous year (mg/day)
_____

Type of bisphosphonate taken (if applicable)
_____

Mean dose of bisphosphonate over previous year (mg/day)
_____

Type of anti-seizure medication taken (if applicable)
_____

Mean dose of anti-seizure medication over previous year (mg/day)
_____

Additional therapeutics
_____

Mean dose of additional therapeutics (mg/day)
_____

Dietary intake of calcium
_____

Biochemistry

Ionised calcium (mmol/L)
_____

Total calcium (mmol/L)
_____

Phosphate (mmol/L)
_____

Serum creatinine (micromol/L)
_____

25-Hydroxyvitamin D
_____

Parathyroid Hormone (pg/ml)
_____

Alkaline Phosphatase (IU/L)
_____

Urine calcium:osmolarity ratio
_____

Albumin (g/dL)
_____

Urea Nitrogen Blood (BUN) (mg/dL)
_____

Creatinine Clearance (mL/minute)
_____

Fracture History

Number of previous peripheral fractures

- ○ 1
- ○ 2
- ○ 3
- ○ 4
- ○ 5
- ○ 6
- ○ 7
- ○ 8
- ○ 9

(Does not include fractures of nose, fingers, toes)

History of vertebral compression fractures

- ○ Yes
- ○ No

Bone mineral density

Lumbar spine BMD (g/m2)   _____

Lumbar spine BMD Z score   _____

Total body BMD (g/m2)   _____

Total body BMD Z score   _____

Total body BMC (g)   _____

Fat mass (g)   _____

Lean mass (g)   _____