# The Role of the Vitamin D Receptor in Aging Skeletal Muscle and Inflammatory Response

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## Abstract

Growing evidence suggests a strong and direct effect of vitamin D on skeletal muscle. Observational studies demonstrate a positive association between serum 25-hydroxyvitamin D (25OHD) concentration and muscle strength and function in older adults. In randomized controlled trials, vitamin D supplementation in older adults increased muscle strength and balance and reduced the risk of falls. The effects of vitamin D on aging muscle may be mediated by its receptor (VDR). VDR is expressed in human skeletal muscle and when bound to the active vitamin D metabolite, 1,25-dihydroxyvitamin-$D_3$ ($1,25(OH)_2D_3$), it regulates expression of genes that affect muscle cell differentiation and contractility. At present, data on the relationship between 25OHD and VDR expression in human skeletal muscle are limited.

In *Part 1* of this dissertation, we conducted three studies to examine the effect of various metabolites of vitamin D on human skeletal muscle. These studies examined 1) the effect of increasing concentrations of 1,25-dihydroxyvitamin $D_3$ ($1,25OH_2D_3$) on VDR gene expression in human primary myoblasts, 2) the association between serum 25-hydroxyvitamin D (25OHD) concentration and VDR protein concentration in muscle of older adults, and 3) the effect of 16-week supplementation with vitamin $D_3$ on VDR gene expression in muscle of older women. We found that increasing $1,25OH_2D_3$ concentration in human primary cell myoblasts significantly increased VDR mRNA expression compared to controls. In older mobility-limited adults, serum 25OHD was positively associated with VDR protein concentration. After 16 weeks of vitamin $D_3$ supplementation, VDR mRNA expression was significantly greater compared to placebo in muscle samples of older women.

In *Part 2* of this dissertation, we examined the role of vitamin D in skeletal muscle inflammation using two models. First, we assessed the cross sectional associations between 25OHD and/or VDR concentration with intramuscular inflammatory markers (IL-6 and TNFα) and related signaling pathways (NFkB and p38 MAPK phosphorylation). Second we investigated the longitudinal affects of 16 weeks of vitamin $D_3$ supplementation on alterations in skeletal muscle inflammatory markers (IL-6 and TNFα). We found that intramuscular VDR protein concentration was positively associated with intramuscular IL-6 gene expression but negatively associated with intramuscular IL-6 protein concentration in older mobility-limited adults. However, intramuscular IL-6 gene expression was not altered after 16 weeks of vitamin $D_3$ supplementation in older mobility-limited women. Neither intramuscular VDR nor serum 25OHD was associated with intramuscular TNFα.

In conclusion, these data suggest 1) a direct relationship between $1,25OH_2D_3$ and 25OHD concentrations with human intramuscular VDR mRNA and protein concentration and 2) a subsequent association with intramuscular IL-6. Our findings generate important hypotheses on the role of vitamin D in skeletal muscle; however, larger randomized trials are needed to confirm our findings.

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# INTRODUCTION

Growing evidence suggests that vitamin D affects skeletal muscle physiology and function and may play a particular role in aging skeletal muscle[1,2]. Numerous experiments in muscle cell culture and animal models demonstrate that this effect is mediated by the vitamin D receptor (VDR)[3,4]. Treatment with both 25-hydroxyvitamin-$D_3$ (25OHD) and 1,25-dihydroxyvitamin $D_3$ ($1,25OH_2D_3$) influence intramuscular VDR protein concentration and gene expression in rodents[5,6] and stimulate skeletal muscle signaling pathways involved in muscle proliferation, differentiation, and contractile function in muscle cell culture.[7-9] Yet, limited data are available on the VDR in aging human skeletal muscle. Thus far, VDR protein has been identified in human skeletal muscle of young and older women using a variety of VDR antibodies and techniques [2,10,11] and one randomized intervention study found that vitamin $D_3$ supplementation increased intramyonuclear VDR protein concentration.[12] However, there is a critical gap in the knowledge of specific mechanisms of vitamin D action in skeletal muscle. The proposed investigation aimed to expand our current understanding of the relationship between vitamin D status and human skeletal muscle VDR concentration. Further, we examined potential roles of VDR in skeletal muscle by determining whether VDR plays a role in intramuscular inflammation.

At present, research on the potential role of VDR on inflammatory pathways in skeletal muscle is scarce. Based upon studies in non-muscle tissues,[13-16] VDR influences markers of inflammation, possibly by interfering in pro-inflammatory signaling cascades. In rodent skeletal muscle, a recent study found that treatment with vitamin $D_3$ attenuated

exercise-induced muscle damage and inflammation through the modulation of MAPK and NF-κB, pathways possibly mediated by VDR, and resulted in significant reductions in intramuscular pro-inflammatory markers.[6] Further, vitamin $D_3$ supplementation in older healthy women decreased circulating markers of innate immunity.[17] Notably, these same pathways of inflammation have been implicated in sarcopenia as well as systemic inflammatory disorders such as obesity and diabetes.[18]

The proposed dissertation sought to fill these knowledge gaps by examining two major areas of investigation. The first area aimed to deepen current understanding between vitamin D status and VDR in human muscle by examining 1) the effect of increasing concentrations of 1,25-dihydroxyvitamin $D_3$ ($1,25OH_2D_3$) on VDR gene expression in human primary myoblasts, 2) the association between serum 25-hydroxyvitamin D (25OHD) concentration and VDR protein concentration in muscle of older adults, and 3) the effect of 16-week supplementation with vitamin $D_3$ on VDR gene expression in muscle of older women. The second area of investigation aimed to explore the role of VDR and 25OHD concentration in human intramuscular inflammatory markers. The central hypothesis of the proposed research was that the expression of VDR in the human skeletal muscle would increase with either parent or active vitamin D supplementation and would be associated with decreased markers of inflammation.

The research proposed in this project is significant because it elucidates an association between decreasing 25OHD, muscle weakness with aging, and potential whole-body effects linked to systemic inflammation such as insulin resistance, Type II diabetes, and cardiovascular disease. With increased knowledge of the role of VDR in skeletal muscle, possible therapeutic interventions may be designed to attenuate

deleterious functional consequences particularly with aging. More specifically, this project may further advance the field by providing insight into the effectiveness of vitamin D supplementation on outcomes of healthy aging.

# CHAPTER 1:

# BACKGROUND

## Introduction

Growing evidence suggests a strong and direct effect of vitamin D on skeletal muscle physiology and function, possibly mediated by the vitamin D receptor (VDR)[4]. This relationship may become increasingly important in aging skeletal muscle[1,2]. Several observational studies suggest that low vitamin D status, particularly in older adults, has been associated with reduced muscle mass, strength and performance, and an increased risk of falls.[1] In the National Health and Nutrition Examination Survey (NHANES) III in the United States, higher serum 25-hydroxyvitamin-D (25OHD) concentrations in older adults were associated with better lower extremity function[19] while the Longitudinal Aging Study in Amsterdam demonstrated that the lowest levels of 25OHD were associated with the greatest risk of falling.[20] In two large studies, the InCHIANTI and Malmo Osteoporosis Prospective Risk Assessment (OPRA), decreased handgrip strength was exhibited by those individuals with low vitamin D status.[21,22] A recent longitudinal study examining physical performance in the elderly (Lifestyle Interventions and Independence for Elders Pilot: LIFE-P), noted a clinically significant improvement in physical performance with increases in 25OHD.[22] Moreover, a number of intervention studies have shown that supplementation of vitamin $D_3$ increases appendicular muscle strength and performance and reduces the risk of falls mostly in older individuals with low baseline vitamin D status.[23-25] Lastly, a recent meta-analysis has demonstrated that muscle strength and balance are improved with parent vitamin D supplementation.[26]

The mechanism(s) by which vitamin D is exerting these beneficial effects in skeletal muscle has not been definitively established, but has been under intense investigation in the last several years. Some studies have concluded that the actions of the active form of vitamin D, $1,25(OH)_2D$, on skeletal muscle are *indirect* by way of calcium and/or phosphate homeostasis, possibly via mechanisms mediated by intestinal absorption. A study in vitamin D-deficient rats found muscle weakness only in those animals with concomitant hypophosphatemia and reported improvements in muscle strength only in the animals with resolution of hypophosphatemia with vitamin $D_3$ supplementation.[27] The actions of $1,25(OH)_2D$ on skeletal muscle may also occur via a *direc*t mechanism, namely through the VDR, which is present in the nucleus of skeletal muscle cells[10,12] and is known to regulate genomic transcription in vitro[28] (**Figure 1-1**). In muscle cell culture, when bound to the active vitamin D metabolite, $1,25(OH)_2D$, VDR will regulate expression of genes that affect muscle cell differentiation and contractility.[8] Research by Srikuea et al.[5] reported VDR and CYP27B1 (1-α-hydroxylase) gene expression in C2C12 myoblasts and adult mouse muscle cells using various techniques; and supplementation with vitamin $D_3$ has been shown to increase intramyonuclear VDR concentration and muscle fiber cross-sectional area in vastus lateralis muscle of older women.[12] However, little is known about the specific role of human skeletal muscle VDR *in vivo*. Thus, a key to identifying the mechanisms underlying the effect of vitamin D on human muscle may lie in a better understanding of the role of the intramuscular VDR.

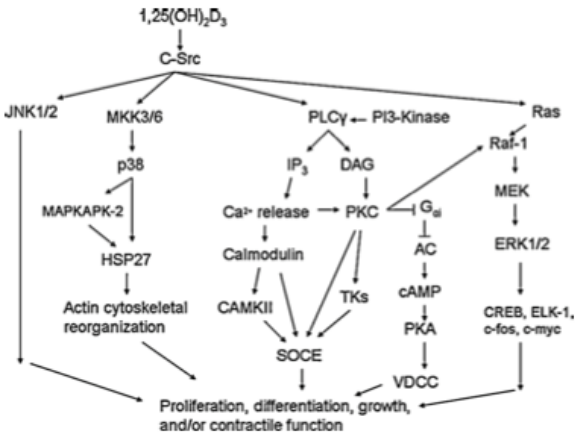


**Figure 1-1. Potential non-genomic signaling pathways of vitamin D[28]** (Reproduced from Dirks Naylor & Lennon-Edwards, 2010)

**Vitamin D Metabolism**

Vitamin D is a steroid hormone that is traditionally regarded as a regulator of bone metabolism through the homeostatic control of calcium and phosphate, although more recently it has been hypothesized to play a role in modulating multiple other cellular functions.[29-31] Vitamin $D_3$, the prohormone, is produced mostly in the epidermal layer of the skin via ultraviolet radiation of 7-dehydrocholesterol following exposure to sunlight. Vitamin $D_3$ can also be acquired through a few dietary sources including fish, dairy products, irradiated mushrooms and dietary supplements. Bound to a vitamin D binding protein, vitamin $D_3$ is transported to the liver where it is hydroxylated to $25OHD_3$, the major circulating form of vitamin D and a biomarker of vitamin D status. 25OHD is further hydroxylated to the active hormone, $1,25(OH)_2D_3$, primarily via activity of the mitochondrial cytochrome P450 enzyme, 1-α-hydroxylase, encoded by the gene *CYP27B1*. While this conversion has typically been thought to occur in the

kidney,[32] *CYP27B1* has also been shown to be present in several other tissues including the placenta, macrophages, and monocytes.[33] Most recently *CYP27B1* has been shown to be expressed in skeletal muscle. Srikuea and colleagues were able to clone the full length *CYP27B1* mRNA transcript from $C_2C_{12}$ myotubes and found evidence of constitutive expression of *CYP27B1* protein in both the cytoplasm and mitochondria of $C_2C_{12}$ myoblasts and the cytoplasm of $C_2C_{12}$ myotubes.[5] They also reported localized increases in *CYP27B1* protein expression in $BaCl_2$-induced regenerating muscle fibers of mouse tibialis anterior muscle. These data provide support for the concept that skeletal muscle is yet another target organ of vitamin D action (**Figure 1-2**).

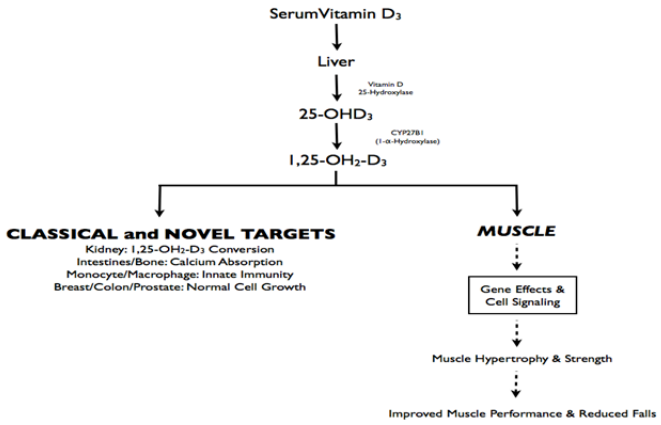


**Figure 1-2. Classical and novel targets for vitamin D.** Vitamin D has traditionally been accepted as a mediator of calcium absorption in the intestines, resulting in subsequent effects on bone. More recent actions have been well documented in a variety of other tissues, most recently in muscle.[34]

## Characterization of the Vitamin D Receptor

Once in the biologically active form, $1,25(OH)_2D$ binds to a classic steroid receptor, or VDR. The VDR is a molecule with a mass of approximately 50-60 kDa and

is widely distributed across several tissues.[29] Most effects of $1,25(OH)_2D$ appear to mediate the regulation of gene expression by activating transcription of genes whose promoters contain specific sequences known as vitamin D response elements (VDRE). Within the nucleus, the VDR heterodimerizes with the retinoid x receptor (RXR) and the VDR/RXR complex binds to the VDRE, resulting in the expression of genes involved in several components of cellular metabolism and function.[35] The most conserved domain in human VDR is the DNA-binding domain which comprises two zinc fingers. The proximal (N-terminal) zinc finger is specific for DNA binding to the VDRE while the second zinc finger provides at least one of the sites for the heterodimerization to RXR.[36]

As described with other steroid receptors, the VDR acts principally as a nuclear transcription factor; however, it has also been proposed to have a non-nuclear receptor mediating non-genomic actions.[37] The mechanisms of action of the non-nuclear VDR are significantly less well characterized. Thus far, reports have described that binding of $1,25(OH)_2D$ to a non-nuclear VDR initiates the formation of a second messenger or phosphorylation of intracellular proteins resulting in rapid cellular effects occurring within seconds to minutes. [3,4,38] Some have proposed it to be a novel membrane receptor,[37] while others have suggested a membrane-associated calcium-binding protein that functions as a calcium-specific ion channel.[39] More recent studies have suggested that the non-nuclear VDR is the nuclear VDR itself, translocated from the nucleus to the plasma membrane within cholesterol and sphingolipid-rich caveolae microdomains.[38,40] As VDR may also be affected by several coactivators, corepressors, and potentially

phosphorylation, the control of VDR activity may require distinct crosstalk involving signaling cascades between the nucleus and plasma membrane.[34]

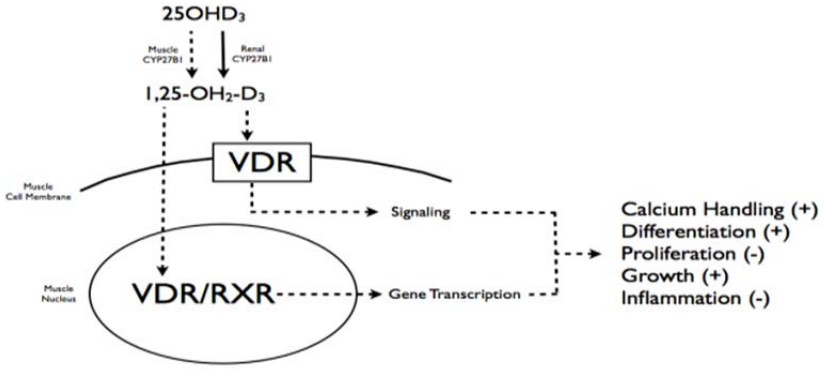


**Figure 1-3. Proposed hypothesis model for the direct effect of vitamin D on skeletal muscle.** Circulating and locally converted $1,25(OH)_2D_3$ have been proposed to act on skeletal muscle through the VDR (dotted line). The VDR is located both within the nucleus, which results in genomic actions, as well as outside of the nucleus, which may cause acute non-genomic signaling events. Current data suggest that the biological pathways affected by $1,25\text{-}OH_2\text{-}D_3$ in skeletal muscle include calcium handling/contraction, cellular differentiation and proliferation, growth pathways, and inflammation.[34]

## VDR in Skeletal Muscle

$1,25(OH)_2D$ exerts its effects primarily by binding to the VDR which has been identified in skeletal muscle.[5,10-12] VDR was initially identified in rodent skeletal myoblast cell lines,[41] subsequently in chick monolayers of myoblasts,[42] and in cloned human skeletal muscle cells.[43] Bischoff et al.[10] demonstrated the first *in situ* intramyonuclear staining for the VDR using VDR antibody 97A (Affinity BioReagents) in frozen cross-sections of human skeletal muscle and Ceglia et al.[12] corroborated this finding using a different monoclonal antibody to the VDR. Yet the isolation of the VDR in skeletal muscle tissue has not been consistent across all studies[44]. Many[2,10-12] but not

all studies[44] have demonstrated that VDR is expressed in skeletal muscle. A recent report questioned the selectivity of the VDR antibody 97A due to potential reaction with proteins on Western blot not related to the VDR, using a VDR knockout mouse model.[44] However, more recent studies using multiple techniques and alternate VDR antibodies lend support to the presence of VDR in skeletal myocytes.[5,11] Of note, a study in older women detected VDR in frozen muscle cross-sections by Western blot using multiple commercial antibodies to the VDR, including a monoclonal VDR antibody D-6 (Santa Cruz Biotechnology) which is reported to have the best specificity for VDR protein as it is not detected in muscle of VDR knockout mice by immunohistochemistry and Western blot.[11] A study by Srikuea et al.[5] combined the use of Western blot, immunocytochemistry, PCR cloning, and DNA sequencing to validate expression and concentration of the VDR in the $C_2C_{12}$ mouse cell line and adult mouse skeletal muscle cells. Although this last study did not use human muscle cells, the authors employed multiple analytic techniques to localize the presence of VDR in skeletal muscle cells. Most recently, Girgis and colleagues[45] demonstrated that $C_2C_{12}$ cells express VDR, CYP27B1 (1-α-hydroxylase), CYP24A1 - a classic VDR target gene, and vitamin D binding protein at the transcript level. The study also demonstrated increased expression of VDR mRNA following 48 hours of treatment with $1,25(OH)_2D_3$ with concomitant increases in CYP24A1. Together, these studies provide strong evidence for the presence of VDR in skeletal muscle cells.

**The Role of VDR in Skeletal Muscle**

Little is known about the regulation of the VDR in human skeletal muscle. Specifically, while there appears to be an interaction between $1,25(OH)_2D_3$ and skeletal muscle VDR in vitro,[3] it is unknown if or how the VDR is affected by circulating vitamin $D_3$, 25OHD, or $1,25(OH)_2D$ *in vivo*. Furthermore, studies that examine variations in VDR concentration in various populations are limited.

Experimental evidence based on data in the VDR-knockout mouse model, cell culture, and other rodent studies, demonstrate that effects of vitamin D on skeletal muscle may, at least in part, be mediated by the VDR. Notably, a targeted genetic deletion in VDR intestinal tissue results in a phenocopy of the VDR null mouse that presents with dramatic musculoskeletal and functional alterations.[46,47] The VDR knockout mouse model has muscle fibers that are approximately 20% smaller and more variable in size than wild type mice, and its muscle expresses increased levels of myogenic differentiation factors including Myf5, E2A and myogenin compared to the wild type.[46] In addition, the VDR null mutant mice have lower body size and weight and impaired motor coordination compared to wild type animals.[46,47] Interestingly, a knock-in of the VDR in the intestine of VDR knockout mice will rescue the skeletal phenotype, but whether it rescues the muscle phenotype is not known.[48]

Recent reports have identified alterations in expression and concentration of the VDR in skeletal muscle cells following administration of $1,25(OH)_2D_3$[5,6,8]. Prior studies in other tissues[49,50] have indicated that the content of VDR in target tissues is positively associated with the level of biological activity in response to vitamin D administration.

With regard to skeletal muscle, recent reports have emerged demonstrating that VDR mRNA expression increases in $C_2C_{12}$ myoblasts with $25OHD_3$ and $1,25(OH)_2D_3$ treatment.[5,8] Srikuea et al. noted increases in myoblast VDR expression following a single dose of $25OHD_3$[5] and both Srikuea and Garcia et al.[5,8] independently demonstrated a more than a fivefold increase in VDR mRNA expression following 4 days of $1,25(OH)_2D_3$ administration compared to control. The increased expression was further confirmed by Western blot analyses using whole-cell culture homogenates and immunofluorescence studies under similar conditions. Srikuea et al.[5] also examined regenerating mouse skeletal muscle in vivo and found that murine regenerating muscle fibers had greater expression of VDR compared to non-regenerating fibers. Therefore, an alteration in VDR content in skeletal muscle cells following $1,25(OH)_2D_3$ administration would lend support to the concept that there may be direct effects on muscle physiology and/or function.

Despite growing literature defining the role of VDR in cell culture and animal models, very little is known about the regulation or action of the VDR in human skeletal muscle. One study has suggested that aging may decrease overall expression of VDR in human skeletal muscle.[2] Much more recently, a randomized study in older vitamin D-insufficient women found that supplementation with vitamin $D_3$ increased intramyonuclear VDR concentration compared to placebo by 30% over a 4-month period.[12] Despite this recent work, however, studies that examine actions downstream of the VDR in human muscle are lacking.

**Vitamin D and Inflammation**

An inverse relationship between serum 25OHD concentration and circulating serum inflammatory markers has been reported,[51] and vitamin D supplementation results in a decreased innate immune response.[17] The precise mechanisms have not been well-characterized; however, several metabolites of vitamin D may play a role in mediating inflammation in non-muscular tissue (**Figure 1-4**). The active form of vitamin D, $1,25(OH)_2D_3$, is synthesized locally by macrophages and dendritic cells by the enzyme 1-alpha-hydroxylase (CYP27B1)[52] whose expression and action is modulated by inflammatory mediators.[53] In human prostate epithelial cells, $1,25(OH)_2D_3$ increases the expression of MKP5, a member of the MKP family of enzymes that dephosphorylates and inactivates MAPK's, thus decreasing intracellular IL-6. [54] Further, these authors demonstrate that up-regulation of MKP5 mRNA by $1,25(OH)_2D_3$ was dependent on the vitamin D receptor.[54] Similarly, Zhang and colleagues[55] have demonstrated that $1,25(OH)_2D_3$ attenuates monocyte and macrophage proinflammatory cytokine production, both IL-6 and TNFα, by upregulating intracellular MKP1 thereby decreasing p38 activation.

Besides MAPK activity, other signaling pathways have been implicated to increase production of cytokines due, in part, to activation of NFκB in in a cardiac myocyte model and dendtritic cells.[56,57] It has been shown in murine macrophage cells that $1,25(OH)_2D_3$ will directly modify NFκB transcriptional activation by upregulating IkB-α, an inhibitor of NFκB, and subsequently attenuating the nuclear translocation of p65, the pro-inflammatory transcription factor of the NFκB complex.[58]

Inflammatory cytokines within skeletal muscle contribute to accelerated muscle catabolism and dysfunction;[59] therefore, it would be of importance to identify whether vitamin D has a modulates inflammatory response at the level of skeletal muscle. Research thus far suggests that serum 25OHD is positively associated with skeletal muscle recovery after exercise and injury[6,60-62] and reduced circulating inflammatory markers and concomitant impairments in peak power in healthy adults.[61] Vitamin $D_3$ supplementation has resulted in an attenuation of exercise-induced muscle weakness in rat skeletal muscle.[6]

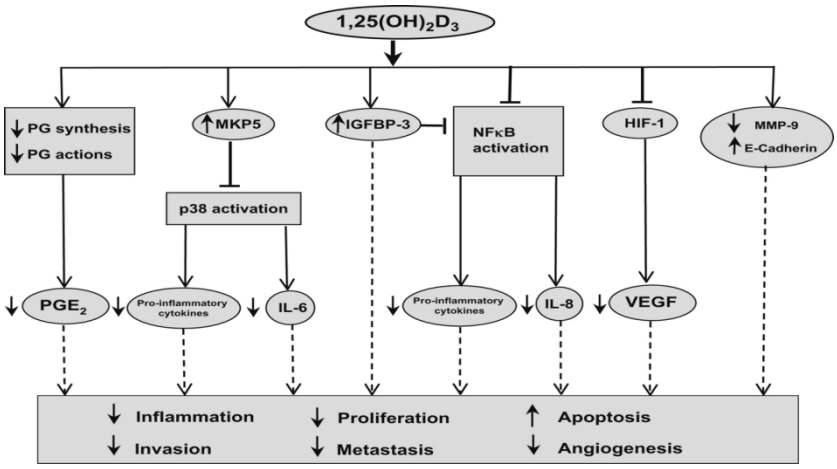


**Figure 1-4.** Overview of the molecular mechanisms underlying the anti-inflammatory actions of $1,25(OH)_2D_3$. (Reproduced from **Vanoirbeek et. al, 2011[63])**

**VDR and Inflammation**

VDR is expressed by tissues involved in the regulation of the inflammatory response[29] and studies suggest that VDR activation may, in fact, regulate intracellular inflammation.[13-15] Indeed, VDR null mutant mice have increased markers of intestinal epithelial cell inflammation[64] and demonstrate reduced levels of the NFκB inhibitory protein, IkB-α, resulting in increased NFκB activity in mouse embryonic fibroblasts.[13]

Further, in human osteoblasts, VDR will directly interact with p65, an NFκB subunit, when exposed to cytokine TNFα, decreasing the efficiency of VDR-mediated gene transcription.[65]

VDR may also have specific implications with regard to skeletal muscle-mediated inflammation, although the literature examining associations between intramuscular VDR and inflammation remain limited. In a randomized controlled study examining Sprague Dawley rats, $1,25(OH)D_3$ supplementation influenced exercise-induced muscle damage and inflammation through the modulation of MAPK and NFκB pathways possibly mediated by VDR.[6] Of note, this study found that skeletal muscle of vitamin D-treated rats exposed to high intensity exercise demonstrated an increase in skeletal muscle VDR with a concomitant reduction in the intramuscular mRNA expression of p38, ERK1/2, IKK and IkB, and subsequent reductions in intramuscular pro-inflammatory TNFα and IL-6 gene expression when compared to placebo[6].

The consequences of increased systemic inflammation may exhibit local effects on skeletal muscle as well as result in systemic biologic consequences. For example, in rats exposed to crush injury, vitamin D application resulted in significant increases in muscle cell regeneration and extracellular matrix proteins and decreases in muscle cell apoptosis[62]. This extra muscular inflammatory association may be important in humans due to a strong correlation between circulating cytokines and impaired muscle regeneration as seen in sarcopenia[66] and reports that higher plasma concentrations of IL-6 and TNFα are associated with lower muscle mass and lower muscle strength in aging populations.[67,68] It has yet to be determined if VDR plays a role in these associations.

In conclusion, preliminary evidence proposes that intramuscular VDR is affected by circulating 25OHD, and plays a role in human skeletal muscle-mediated inflammation. This project is significant because it will contribute valuable knowledge regarding the relationship between intramuscular VDR, 25OHD, aging muscle physiology, and potential effects linked to intramuscular inflammation. With increased understanding of the role of VDR in skeletal muscle, possible therapeutic interventions may be designed to address age-related functional consequences and may significantly advance the field by providing insight into the efficacy of clinically prescribed vitamin D supplements on vulnerable populations, namely the elderly.

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# CHAPTER II

## Skeletal muscle vitamin D receptor varies with vitamin D status

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RUNNING HEAD: Skeletal muscle VDR increases with vitamin D

Abbreviations: Vitamin D Receptor (VDR); 25-Hydroxyvitamin $D_3$ (25OHD); 1,25-Dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$); Short Physical Performance Battery (SPPB); mitogen activated protein kinase (MAPK); Body Mass Index (BMI ); Standard Deviation (SD)

Clinical Trial: NCT00986596 and NCT00635739 (www.clinicaltrials.gov)

## ABSTRACT

**Background:** Growing evidence suggests that vitamin D acts on skeletal muscle at least in part through the vitamin D receptor (VDR). **Objective:** Three studies were conducted to examine 1) the effect of increasing concentrations of 1,25-dihydroxyvitamin $D_3$ ($1,25OH_2D_3$) on VDR gene expression in human primary myoblasts, 2) the association between serum 25-hydroxyvitamin D (25OHD) concentration and VDR protein concentration in muscle of older adults, and 3) the effect of 16-week supplementation with vitamin $D_3$ on VDR gene expression in muscle of older women. **Design:** A human primary myoblast cell line was treated with increasing concentrations of $1,25OH_2D_3$. Muscle biopsies of older, mobility-limited adults were obtained at baseline and after 16 weeks of 4,000 IU/day vitamin $D_3$ supplementation. VDR gene expression and protein concentration were examined. **Results:** In human primary cell myoblasts, increasing $1,25OH_2D_3$ concentration augmented VDR mRNA expression and the fold change (mean ±SD) between control and 1nmol/L $1,25OH_2D_3$ was 1.36±0.33 ($p=0.05$). In older mobility-limited adults, serum 25OHD was positively associated with VDR protein concentration ($R=0.67$; $p=0.0028$) and VDR concentration differed significantly between those with vitamin D sufficiency (>20 ng/mL) and vitamin D insufficiency/deficiency (≤20 ng/mL; $p=0.02$). After 16 weeks of vitamin $D_3$ supplementation, VDR mRNA expression in muscle biopsies of older women increased (1.2 ±0.99 fold) versus a decrease (-3.2± 1.7 fold) in the placebo group ($p=0.04$). **Conclusions:** Our data show an increase in VDR mRNA expression in both treated human primary myoblasts and supplemented humans, suggesting a direct effect of 1,25-dihydroxyvitamin-$D_3$ on the

expression of VDR in muscle. Higher serum 25OHD was associated with greater VDR protein concentration in muscle of older adults.

# INTRODUCTION

Observational studies have suggested a positive association between serum 25-hydroxyvitamin D (25OHD) concentration and muscle strength and physical function in older adults (1-3), however, little is known regarding the specific mechanism of action that vitamin D exerts on skeletal muscle. It has been hypothesized that this effect is mediated by the vitamin D receptor (VDR). The VDR protein is present in human skeletal muscle cells at varying stages of development (4-6), although controversy exists regarding antibody specificity for the detection of VDR in skeletal muscle (7). Several groups have isolated VDR in human skeletal muscle tissue comparing antibodies, immunoblotting and gene expression analyses (4-6) and have demonstrated both gene and protein expression of VDR in skeletal muscle when utilizing established protocols (5). When bound to the active vitamin D metabolite, 1,25dihydroxyvitamin-$D_3$ ($1,25OH_2D_3$) in cell culture, VDR has been shown to regulate expression of genes that affect muscle cell differentiation (8) and proliferation (6). Additionally a non-nuclear skeletal muscle VDR has recently been demonstrated to mediate signal transduction resulting in non-genomic signaling events (9, 10).

Little is known about the factors that regulate expression of VDR in human skeletal muscle *in vivo*. As suggested by a prior report (11), age is inversely associated with VDR concentration. An age-associated reduction in VDR has been documented in older rat intestine and bone (12). It has also been shown that both 25OHD and $1,25OH_2D_3$ increase VDR protein content in $C_2C_{12}$ myoblasts (6, 13) and supplementation of vitamin $D_3$ in older adults with low baseline serum 25OHD

concentration increases nuclear VDR protein concentration by immunohistochemistry (14).

The aim of the present study was to examine the relationship between vitamin D and VDR gene expression and protein concentration in human skeletal muscle utilizing three complementary experimental models. The objective of the first study was to investigate the effect of different concentrations of $1,25OH_2D_3$ on VDR gene expression in human primary myoblast cell lines derived from three healthy individuals. The objective of the second study was to determine the relationship between VDR protein concentration in skeletal muscle and serum concentrations of 25OHD in older mobility-limited adults. The third study was to determine the effect of 16-weeks of vitamin $D_3$ supplementation on VDR gene expression in skeletal muscle in older women. Within study three, we also investigated if supplementation resulted in local alterations of VDR concentration within specific compartments of the skeletal muscle cell (nuclear vs. non-nuclear). We hypothesized that $1,25OH_2D_3$ would increase VDR mRNA expression of human primary cell myoblasts in a dose-dependent manner, that concentration of 25OHD in serum would be positively associated with VDR mRNA expression and protein concentration in the skeletal muscle of older adults and that supplementation with vitamin $D_3$ in older adults would lead to an increase in skeletal muscle VDR mRNA.

## SUBJECTS and METHODS

### Subjects

Cell Culture: Primary myoblast cell lines were established from three healthy young adults: age 19-30 years, BMI 20.3-23.4kg/$m^2$, 1 female and 2 males. The myoblast cell

culture portion of the study was approved by the Ethics Committee of the Karolinska Institutet (Stockholm, Sweden).

Human Subjects: Data for the human study were obtained from 20 older adults (male=8, female=12) who were mobility-limited as determined by the Short Physical Performance Battery (SPPB) (15). Participants had a mean age 77.9 ±4.05 years and mean BMI 27.06 ±2.94 kg/$m^2$, and a mean SPPB 8.65±1.23 (out of a possible 12). These subjects had been selected for a larger randomized study for which inclusion and exclusion criteria are presented elsewhere (16). Muscle biopsies were optional in this study and performed on a subset of randomized subjects. This study was approved by the Institutional Review Board of the Tufts University Health Sciences Campus (Boston, MA).

Data for the supplementation study included 16 mobility-limited women aged 65 years and over with moderately low baseline 25OHD that were participating in a randomized controlled intervention study examining the effects of vitamin $D_3$ vs placebo on skeletal muscle morphology and VDR protein concentration (14). These participants had a mean age of 78.5±4.79 years, mean BMI of 27.0±5.5kg/$m^2$, a mean SPPB score of 7.9±1.55, and mean baseline 25OHD concentration of 18 ±3.86 ng/mL. Subjects were examined before and after 16 weeks of 4,000 IU per day of oral vitamin $D_3$ supplementation or placebo. This study protocol and inclusion/exclusion criteria have been described in detail elsewhere (14). This study was approved by the Institutional Review Board of the Tufts University Health Sciences Campus (Boston, MA).

Human Myoblast Cell Culture

Cell Line Preparation: Muscle biopsies were obtained from the vastus lateralis of three healthy young subjects via the percutaneous needle biopsy technique. Visible fat and connective tissue were removed, tissue was weighed, and then put in a 15 ml tube containing PBS + 1% AbAm (antibiotic/antimycotic, Sigma Aldrich) and was stored at 4°C overnight. The tissue was washed twice in serum free DMEM medium (Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (DMEM/F-12), Life Technologies) and transferred to a small sterile beaker with 0.25% trypsin, final volume 5 ml. The beaker was put on a magnetic stirrer in an incubator, 37 ° C, low speed for 20-30 minutes. The beaker stood for about 5 minutes inside the sterile hood, then supernatant was transferred to a sterile tube. Trypsin was stopped by adding DMEM medium with FCS and centrifuged at 300 x g for 10 minutes. The supernatant was removed and myoblasts were re-suspended in medium containing 20% FCS and 1% AbAm. Cell suspension was pre-plated for 20-30 minutes in a Petri dish. Cell solution was transferred to a 75mL culture flask. Isolated human myoblasts were cultured in DMEM-F12 with 20% FBS and 1% ABAM (proliferation media) at 37° C, 5% $CO_2$. Culture dishes were precoated with collagen I (Gibco® Collagen I, Bovine 5 mg/mL Invitrogen) and diluted to a final concentration of 50 µg/mL in 0.02 M acetic acid according to the manufacturers manual. Medium was switched every third day. Myoblasts were split into new flasks when reaching approximately 60-70% confluency. To control for cell culture population purity (i.e. whether or not the isolated population were predominantly of myogenic origin), cells

were immunocytochemically analyzed for the myogenic marker, desmin. A sample of cells was trypsinized in 0.25% Trypsin-EDTA for 5 min and spun down onto a cover glass for subsequent immunocytochemistry. The cells were then fixated in 4% formaldehyde for 8 minutes. Following fixation, cells were washed 3x5 minutes in 1X PBS (hereafter referred to simply as *washing step*) prior to a 30 min block in 4% bovine serum albumin ((BSA) Invitrogen). Mouse-derived monoclonal antibody against human desmin (DAKO D33) diluted 1:200 in 1X PBS containing 1% BSA and 0.1% Triton X was used for the 60 min primary antibody incubation. Following another washing step, the rat anti-mouse IgG monoclonal antibody (ALEXA Fluor 568) diluted 1:1000 in 1X PBS was used for the 30 min secondary antibody incubation. Following the subsequent washing step, the cells were mounted in Vectashield and 4',6-diamidino-2-phenylindole (DAPI) before quantification of the percentage of desmin positive cells. We calculated the percentage of desmin-positive nuclei by counting the total number of nuclei in three fields of 10X magnification and the number of desmin-positive nuclei in the same fields. A minimum of 100 cells was counted for each individual experimental set. All isolated cell populations with fewer then 85% desmin-positive cells were rejected for further analysis. Cells from passage two were utilized for analysis. Myoblasts were treated in triplicate for 18 hours with one of three doses of $1,25OH_2D_3$ (1pmol/L, 10pmol/L, and 1nmol/L).

mRNA Extraction: Total RNA was prepared by the Trizol method (Invitrogen) according to the manufacturer's protocol and quantified spectrophotometrically by

absorbance at 260 nm. One microgram of total RNA was reverse transcribed by Superscript reverse transcriptase (Life Technologies) using random hexamer primers (Roche Diagnostics) in a total volume of 20 µl.

Real Time qPCR: VDR: Quantitative real-time PCR was performed utilizing a commercially available reaction mixture (SsoAdvanced SYBR Green Supermix; Bio-Rad Laboratories, Hercules, CA) on a CFX96 Real Time System (Bio-Rad Laboratories, Hercules, CA). cDNA levels of VDR (QT01010170) were measured using commercially available primer mixtures (Quantitect Primer Assays: Qiagen). 5µL aliquots of cDNA together with 20µL iScript (12.5µL of iTaQ Supermix, 2.5µL of primer and 5µL of nuclease-free $H_2O$) were assayed in duplicate on a 96-well heat-sealed PCR plate (Bio-Rad Laboratories, Hercules, CA). Changes in target gene expression were calculated relative to values from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (QT00199633). Efficiencies of each primer set were assessed using a standard curve, and analyzed using 0.0025-25ng of control cDNA. CYP24A1: Detection of mRNA for CYP24A1 (Hs00167999_m1, Perkin-Elmer Applied Biosystems) was performed on an ABI-PRISM7700 Sequence Detector (Perkin-Elmer Applied Biosystems) with changes in target gene expression calculated relative to values of GAPDH (402869, Perkin-Elmer Applied Biosystems). Data are presented as fold change from control.

Biochemical Measures

25OHD Serum Assay: Fasting morning blood samples were drawn from human subjects at baseline and follow-up and archived. Serum 25OHD was analyzed utilizing a

commercially available assay kit (Diasorin, LIAISON® 25OH Vitamin D Total Assay). Vitamin D deficiency was defined as 25OHD serum concentrations below 12 ng/mL, insufficiency as 12-19 ng/mL and sufficiency ≥20 ng/mL (17).

Muscle biopsies: Baseline and 16-week follow-up human muscle biopsies were obtained from the vastus lateralis at the level of the mid-thigh under local anesthesia (1% Lidocaine). At baseline and following supplementation, the specimens were split and either flash frozen in liquid nitrogen or mounted in a vinyl cryomold (Tissue-Tek, USA) and secured using a viscous mounting medium (O.C.T., Tissue-Tek, USA) and then frozen in an isopentane/liquid nitrogen slurry. Samples were stored in liquid nitrogen until analysis.

Western Blotting Analysis: Immunoblotting was utilized to examine protein concentration of VDR in human skeletal muscle. Vastus lateralis muscle was prepared as previously described (18). Membranes were incubated overnight at 4°C with primary antibodies specific for VDR (1:1,000 in 5% bovine serum albumin and TBS-Tween; via R&D Systems, Minneapolis, MN and manufactured by Perseus Proteomics, Tokyo, Japan). Membranes were rinsed three times for 10 min in TBS-Tween and incubated at room temperature with secondary goat-anti mouse IgG2Aa HRP conjugate antibody (1:2000 in 5% nonfat dry milk and TBS-Tween; Invitrogen, Frederick, MD). Membranes were rinsed three times for 10 min in TBS-Tween and the immunoreactive proteins were detected with Supersignal Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) and quantified by optical density (Image Lab 3.0.1; Bio-Rad Laboratories, Hercules, CA). Changes in protein expression were calculated relative to values of constitutive

control GAPDH (14C10, Cell Signaling Technologies, Beverly, MA). Data are presented in arbitrary units and represent concentration as determined by optical density.

mRNA Preparation: Vastus lateralis muscle (~50mg) was homogenized by bead milling with zirconium oxide beads in 1mL PureZOL RNA Isolation Reagent (Bio-Rad Laboratories, Hercules, CA). RNA extraction was completed utilizing Aurum Total RNA Fatty and Fibrous Tissue RNA Extraction Kit (Bio-Rad Laboratories, Hercules, CA). cDNA conversion was performed utilizing a commercially available reaction mixture (iScript Reverse Transcription SuperMix for RT-qPCR Bio-Rad Laboratories, Hercules, CA) on a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). Real Time qPCR was performed as outlined above.

Immunohistochemistry: Immunohistochemistry and fluorescent microscopy methods were described in detail previously (5). Digital images of immunofluorescent marker staining of VDR, DAPI and laminin in a subgroup of the supplementation study (vitamin $D_3$ n=6; placebo n=5) were further analyzed by OracleBio (Glasgow, Scotland UK) to determine concentration of non-intramyonuclear-associated VDR protein signal. A software algorithm was utilized to detect total VDR signal area and VDR-positive myonuclei across individual field images of skeletal muscle. The VDR and DAPI layer images were overlaid for detection of positive nuclei. VDR signal was aggregated across the field, then VDR signal colocalized with myonuclei was subtracted from total VDR signal to determine the non-nuclear fraction. The non-nuclear signal was normalized to image area so that individual time points (pre- and post-) could be compared. The non-nuclear signal from three images was averaged for each time point per subject.

**Statistical Analysis**

All statistical analyses were performed using JMP statistical software (v. 10.0, SAS Institute Inc., Cary, NC) except for CYP24A1 data which were analyzed with STATISTICA statistical software (version 10 StatSoft Inc.) Statistically significant differences were evaluated between $1,25OH_2D_3$ concentrations by the use of one-way ANOVA. Tukey's post hoc test was used to locate differences in mean values. Associations between linear variables were examined with Pearson correlation analysis. Formal hypothesis testing within and across groups was completed with two-sided t-tests. All variables were examined for normal distribution, and were log-transformed before analysis as needed to better approximate a normal distribution. Cook's distance was applied to all data points to identify outliers and observations. A Cook's distance greater than three times the mean Cook's distance for that variable was determined as an outlier. Data are presented as means ±SD and were determined to be statistically significant at an alpha value of $\leq 0.05$.

As studies examining VDR protein in human skeletal muscle via Western Blot are lacking in the literature, we estimated statistical power a priori to detect between-group differences based on a 95% confidence interval with 80% power from previously available immunohistochemical studies in humans (4, 14). From these data, we estimated a between-group difference of 1.5 fold in VDR expression and our calculations required a within-group sample size of n=4 to give an 80% chance of rejecting the two-sided null hypothesis of no difference between the group means at the 0.05 level of significance.

## RESULTS

### Human Primary Myoblasts

VDR mRNA Fold Change: After being treated for 18 hours with three concentrations of $1,25OH_2D_3$ (1 pmol/L, 10 pmol/L,1 nmol/L), myoblast VDR mRNA increased in a dose-dependent manner (**Figure 1**). VDR was significantly increased (mean fold change ±SD) between control and 1 nmol/L $1,25OH_2D_3$ in human primary myoblasts (1.36 ±0.33; p=0.05).

CYP24A1 mRNA Fold Change: Gene expression of *CYP24A1* following stimulation with $1,25OH_2D_3$ demonstrated an induction of *CYP24A1* mRNA (mean fold change ±SD) following 18 hours of $1,25OH_2D_3$ stimulation in human primary cell myoblasts (1 pmol/L 203.6±349.5; 10 pmol/L 951.5 ±784.1; 1 nmol/L 8633.9 ±6957.5; $p<0.05$ between control and 10 pmol/L and 1 nmol/L, **Figure 2**). Gene expression of *CYP24A1* was expressed in very low to non-detectable concentration in myoblasts at the basal state, as made apparent by high CT-values (CT-value range 33-36). Thus, the fold change as presented in **Figure 2** may underestimate the true fold change of *CYP24A1* gene expression following $1,25OH_2D_3$ stimulation in myoblasts.

### Human Subjects

VDR Protein Concentration and Circulating 25OHD: Muscle cell VDR protein concentrations were significantly higher in vitamin D sufficient (n=12) versus vitamin D

insufficient/deficient older mobility-limited adults (n=8, p=0.02). Serum 25OHD (ng/mL) concentrations were positively associated with VDR protein concentration (R=0.67; p=0.0028; **Figure 3**). There was no significant correlation between serum 25OHD concentration and VDR mRNA expression nor a correlation between VDR mRNA expression and VDR protein concentration (data not shown).

VDR mRNA Expression in Human Skeletal Muscle Following Vitamin $D_3$ Supplementation: After 16 weeks of vitamin $D_3$ supplementation, we found an increase in muscle VDR mRNA expression (1.2 ±0.99 fold; n=9) versus a decrease (-3.2± 1.7 fold; n=11) in the placebo group (p=0.04; **Figure 4**). Independent of treatment group, the 16-week change in serum 25OHD concentration from baseline was not associated with a change in VDR mRNA expression in the muscle. Of note, one placebo subject whose sample demonstrated a VDR mRNA expression fold change of 69.55 (compared to an average of -3.2) was determined to be an outlier and was excluded from analysis.

Non-nuclear VDR Protein Concentration in Human Skeletal Muscle: There was no significant change in non-nuclear skeletal muscle VDR protein signal in human muscle cross-sections after supplementation with vitamin $D_3$ for 16 weeks. The mean percent change of total non-nuclear VDR area was 1.42 ± 1.21% for the vitamin $D_3$-supplemented group and -6.7 ± 5.26% for the placebo group (p=0.2, **Figure 5**).

## DISCUSSION

Our study demonstrated that VDR mRNA expression increased *in vitro* in human

primary cell myoblasts in a dose-dependent manner with increasing concentrations of $1,25OH_2D_3$ treatment. In human skeletal muscle tissue, VDR protein was strongly associated with circulating 25OHD concentration in older mobility-limited adults. Individuals with sufficient serum 25OHD (>20ng/mL) concentrations had significantly greater VDR protein concentrations than those with insufficient and deficient 25OHD (≤20ng/mL) concentrations. Sixteen weeks of vitamin $D_3$ supplementation in older mobility-limited women with low baseline 25OHD increased skeletal muscle VDR mRNA expression compared to placebo. Finally, data in this analysis and prior (14) suggest that the increase in skeletal muscle VDR protein following vitamin $D_3$ supplementation is localized within the nuclear compartment of the muscle cell.

The *in vitro* direct effect of $1,25OH_2D_3$ on myoblast VDR mRNA expression as well as the *in vivo* association between VDR protein with serum 25OHD, suggest that skeletal muscle VDR concentration may be dependent upon the availability of 25OHD and on local control of the active metabolite, $1,25OH_2D_3$. *In vitro*, myoblast VDR mRNA expression increased in a dose-dependent manner after treatment with $1,25OH_2D_3$, and the difference reached significance at the highest dose. The 36% increase at the highest dose in skeletal muscle VDR mRNA expression is similar to that found in vascular smooth muscle cells (19). The positive association between 25OHD concentration and protein concentration of VDR is also consistent with observations in other tissues (19-22), including $C_2C_{12}$ myoblasts in cell culture (6, 13). As studies in $C_2C_{12}$ muscle cells and adult mice have demonstrated a stimulatory effect of both 25OHD and $1,25OH_2D_3$ on VDR expression (6, 13), our results are consistent with previous findings. In addition, our ~20% increase in human skeletal muscle VDR mRNA

expression after vitamin $D_3$ supplementation versus placebo for 16 weeks provided additional evidence demonstrating a stimulatory effect of a parent vitamin D compound on VDR in muscle. As this percent increase appears modest *in vivo*, an explanation for the statistically significant between-group difference after supplementation is the 3-fold decrease of skeletal muscle VDR demonstrated by the placebo group. This result may have been due to the small sample size, however, we hypothesize that circulating 25OHD, perhaps through local activation to $1,25(OH)_2D_3$ at the tissue level, may be acting more to preserve skeletal muscle VDR protein rather than promoting de novo synthesis of new VDR protein in this aging population. A larger study sample is needed to further evaluate this finding. Furthermore, additional studies are needed to determine the metabolite most responsible for the increase in VDR expression in human skeletal muscle.

Much of the work to date examining the interaction between vitamin D and skeletal muscle has been conducted in animal cell culture and has revealed important functional interactions. Girgis and colleagues (13) recently demonstrated that $C_2C_{12}$ cells express VDR, *CYP27B1* (1-α-hydroxylase), *CYP24A1*, and vitamin D-binding protein at the transcript level. This study also demonstrated increased VDR mRNA following 48 hours of treatment with $1,25OH_2D_3$ with concomitant increases in *CYP24A1*, a classic VDR target gene responsible for local $1,25OH_2D_3$ degradation . Other researchers have also demonstrated that short treatment with $1,25OH_2D_3$ *in vitro* induces reverse translocation of the VDR from the nucleus to the plasma membrane (23, 24) and binding of $1,25OH_2D_3$ to non-nuclear VDR induces fast, non-transcriptional responses (9, 10). This non-genomic role of the VDR has specifically been implicated in stimulation of

transmembrane second messenger systems (10), tyrosine phosphorylation pathways (25) and mitogen-activated protein kinase (MAPK) pathways (i.e. ERK1/2, p38) (9).

Our data demonstrated a direct effect of the active metabolite $1,25OH_2D_3$ on human skeletal muscle, specifically a stimulatory effect of $1,25OH_2D_3$ on the expression of VDR mRNA as well as the known VDR target *CYP24A1* in primary myoblasts. Further, we demonstrated an increase in VDR mRNA expression after vitamin $D_3$ supplementation in humans, although determining whether the *in vivo* alteration in VDR mRNA was a result of increased 25OHD or $1,25(OH)_2D_3$ is outside the scope of this study. As there appears to be a direct relationship between human skeletal muscle and $1,25OH_2D_3$, with a potential interaction with 25OHD, these results lend support to hypotheses that in humans, VDR in human muscle may be involved in signaling events similar to those previously reported in animal and cell culture models and these pathways should be explored in future studies.

Our group previously demonstrated that after 16 weeks of vitamin $D_3$ supplementation, there was a significant increase only in intra-myonuclear VDR protein concentration (14). However, after 16 weeks of vitamin $D_3$ supplementation, we did not detect a significant change in VDR protein outside of the nucleus (non-nuclear). The possibility that there may be a more immediate non-nuclear VDR effect or acute translocation of the VDR needs further investigation. In fact, several groups (6, 23, 24) have reported a translocation in skeletal muscle within 24h after $1,25OH_2D_3$ treatment.

Our study had several strengths. We were able to examine a direct effect of the active form of $1,25OH_2D_3$ on human primary myoblasts in culture and then confirm our

findings with cross-sectional and longitudinal associations between human skeletal muscle VDR and serum 25OHD *in vivo*. The study demonstrated similar effects *in vitro*, in cross-sectional analysis as well as longitudinally following vitamin $D_3$ supplementation, which improves the validity of the conclusions. While this is not the first study to report a direct effect of varying vitamin D metabolites on skeletal muscle, it is the first study to demonstrate this relationship in human skeletal muscle tissue, both *in vitro* in human skeletal muscle primary myoblasts and *in vivo*. Limitations of the work in humans include a relatively small sample size and the fact that the work was conducted as a secondary analysis in the cross-sectional component of the study.

The results of this study lend further evidence to the effects of vitamin $D_3$ supplementation on the expression and concentration of VDR in human skeletal muscle. We found a positive association between circulating 25OHD and VDR concentration and an increase in VDR mRNA expression in human skeletal muscle after both 18 hours of $1,25OH_2D_3$ administration in human primary cells and after 16-weeks of vitamin $D_3$ supplementation *in vivo*. Finally, our results suggest that increasing concentrations of circulating 25OHD affect intra-myonuclear concentrations of VDR rather than non-nuclear VDR, at least over a 16-week period. Further translational studies are needed to examine both the acute and long-term effects of vitamin $D_3$ supplementation on key skeletal muscle biological pathways and how these effects influence muscle function.

**ACKNOWLEDGMENTS:** RMP, LC, TG, KO, BDH, RAF Designed Research; RMP, LC, TG, KO Conducted Research; LC, TG, KO, BDH, RAF Provided Essential Reagents and Materials; RMP, KO Analyzed Data; RMP, LC Wrote Paper; BDH, AHL, TG, RAF Had primary responsibility for final content. No authors declare a conflict of interest.

**LEGENDS FOR FIGURES**

**Figure 1. Human Primary Myoblast VDR mRNA Fold Change** After being treated for 18 hours with three concentrations of $1,25(OH)_2D_3$ (1pmol/L, 10pmol/L, 1nmol/L), fold change of VDR mRNA was significantly increased between control and 1nmol/L (1.36 ±0.33) in human primary myoblasts established from three healthy young adults ($p=0.05$). Data presented as (mean±SD) and analyzed via ANOVA and Tukey's post hoc test. ** denotes statistical significance versus control group, $p\leq0.05$

**Figure 2.** Gene expression of the 24-hydroxylase (CYP24A1) in human primary cell myoblasts stimulated with 1 pmol/L, 10 pmol/L and 1 nmol/L of $1,25(OH)_2D_3$ for 18h. Data presented as mean ± SD fold change relative to controls (1 pmol/L 203.6±349.5; 10 pmol/L 951.5 ±784.1; 1 nmol/L 8633.9 ±6957.5) * denotes statistical significance versus control group, $p\leq0.05$.

**Figure 3. Association between Serum Vitamin D and VDR** Serum 25OHD (ng/mL) strongly associated with VDR protein concentration ($R=0.67$; $p=0.0028$) in older mobility limited adults (n=20) as demonstrated by Pearson correlational analysis.

**Figure 4. Human VDR mRNA Fold Change** Mean (±SD) Fold Change of VDR mRNA was increased (1.2 ±2.6 times; n=9) in the vitamin $D_3$ supplementation group compared to a decrease in the placebo group (-3.2 ± 4.6 times; n=11) in older mobility limited

women ($p=0.04$) as analyzed by Student's t-test. * denotes statistical significance between groups $p \leq 0.05$

**Figure 5. Location of VDR in Human Skeletal Muscle after 12 Weeks of 4,000IU/day Vitamin $D_3$ Supplementation** The vitamin $D_3$ supplemented group (n=9) increased skeletal muscle plasma membrane VDR by 1.42% (±1.21) while the placebo group (n=11) decreased by -6.7% (±5.26) ($p=0.2$) as determined by Student's t-test.

**Figure 2-1**

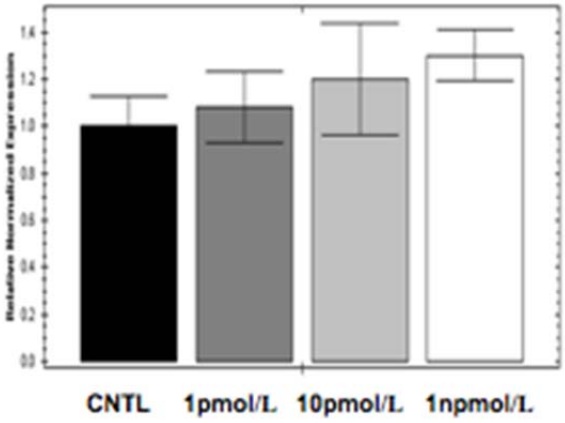


**Figure 2-2**

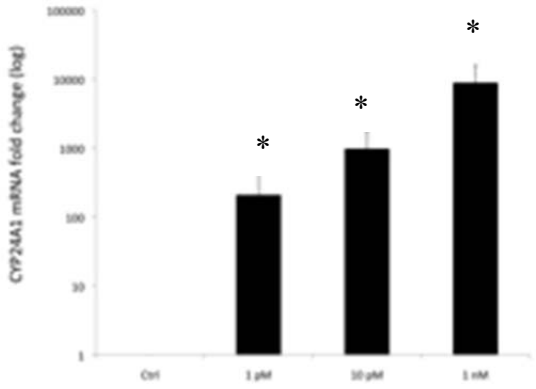

CYP24A1 mRNA fold change (log)
100000
10000
1000
100
10
1
*
*
*
Ctrl
1 pM
10 pM
1 nM

**Figure 2-3**

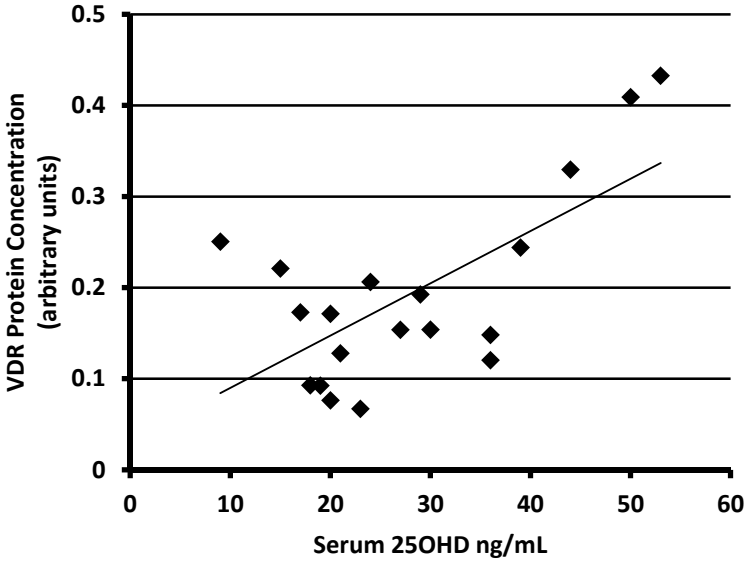

0.5
0.4
0.3
0.2
0.1
0
VDR Protein Concentration (arbitrary units)
0
10
20
30
40
50
60
Serum 25OHD ng/mL

**Figure 2-4**

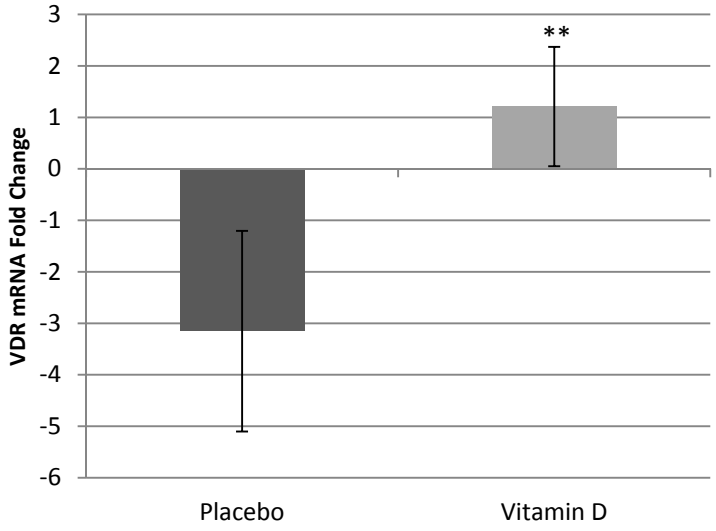
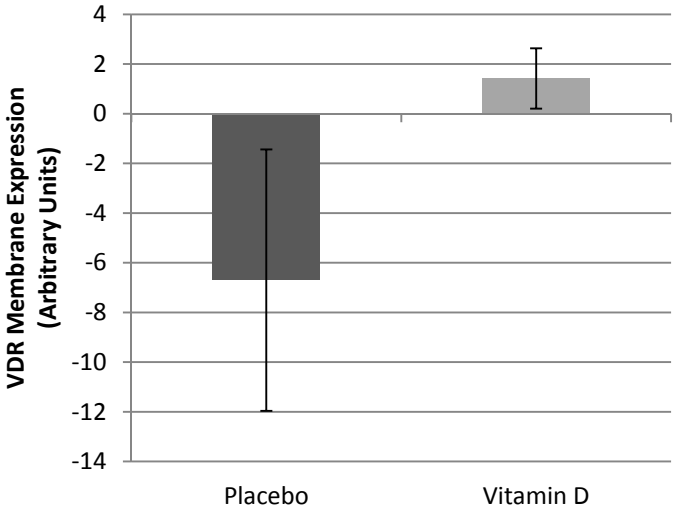


**Figure 2-5**


VDR Membrane Expression (Arbitrary Units)
4
2
0
-2
-4
-6
-8
-10
-12
-14
Placebo
Vitamin D

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# CHAPTER III

## Vitamin D Receptor Protein is Associated with Interleukin-6 in Human Skeletal Muscle

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**ABSTRACT**

**Introduction:** Vitamin D is associated with skeletal muscle physiology and function and may play a direct role in intramuscular inflammation, possibly via the vitamin D receptor (VDR). **Purpose:** We conducted two studies to examine 1) whether serum 25-hydroxyvitamin D (25OHD) and/or intramuscular VDR protein concentration are associated with intramuscular interleukin-6 (IL-6) and/or tumor necrosis factor-α (TNFα) gene expression and protein concentration and 2) whether 16-week supplementation with vitamin $D_3$ alters intramuscular IL-6 and/or TNFα gene expression. Potential signaling pathways were examined as a secondary analysis. **Methods:** Muscle biopsies of thirty older, mobility-limited adults were obtained at baseline. A subset of 12 women were supplemented with either 4,000 IU/day of vitamin $D_3$ (N=5) or placebo (N=7) and biopsies were repeated at 16 weeks. Serum 25OHD was measured and intramuscular VDR, IL-6 and TNFα gene expression and protein concentration were analyzed by PCR and Western blot, respectively. **Results:** Baseline serum 25OHD was not associated with intramuscular IL-6 or TNFα gene expression or protein concentration. Baseline intramuscular VDR protein concentration, adjusted for baseline serum 25OHD, was positively associated with intramuscular IL-6 gene expression (n=28; p=0.04), but negatively associated with intramuscular IL-6 protein (n=18; p=0.03). Neither intramuscular IL-6 nor TNFα gene expression was different between placebo (n=7) or vitamin $D_3$ supplementation groups (n=5) after 16 weeks (p=0.57, p=0.11, respectively). **Conclusion:** These data suggest that VDR is a better predictor than serum 25OHD concentration of intramuscular IL-6 gene and protein expression. A similar relationship

was not observed for TNFα expression. Further, supplementation with 4,000IU vitamin $D_3$ per day does not appear to affect intramuscular IL-6 or TNFα gene expression after 16 weeks.

## INTRODUCTION

Intramuscular inflammation has been demonstrated in a variety of skeletal muscle disorders[1,2] and contributes to muscle loss and dysfunction in systemic inflammatory disorders, such as insulin resistance, type 2 diabetes mellitus, and cardiovascular disease.[3]

An inverse relationship between serum 25-hydroxyvitamin-D (25OHD) and markers of inflammation has been shown in obesity[4], type 2 diabetes mellitus,[5] and cardiovascular disease.[6] Further, vitamin $D_3$ supplementation has been shown to result in a decreased innate immune response[7] although results from other studies have been mixed.[8,9]

Emerging evidence suggests that vitamin D may play a direct role in mediating inflammation within skeletal muscle.[10,11] Specifically, increased concentrations of circulating 25OHD aid muscular recovery from injury[12] and vitamin $D_3$ supplementation has been shown to reduce exercise-induced inflammation,[10] and may directly suppress intramuscular pathways of inflammation, particularly after exercise.[11] However, the mechanism by which vitamin D may directly impact muscle mediated inflammation is yet unclear.

Studies in other tissues such as fibroblasts and breast cancer cells, suggest that vitamin D receptor (VDR) activation may regulate intracellular inflammation.[13-15] VDR may also be implicated in skeletal muscle-mediated inflammation as has been noted in other tissues, such as vascular endothelial cells.[16] VDR is expressed in skeletal muscle tissue,[17,18] has been shown to decrease with age,[19] and increase with vitamin $D_3$ supplementation[11,18]. While it has been posited that chronically decreased expression of

VDR in aging skeletal muscle may play a role in decreased strength and functional ability, it may also have a role in intramuscular inflammation.

We hypothesized that serum 25OHD and intramuscular VDR gene expression and protein concentrations would be inversely related to inflammatory markers, IL-6 and TNFα. To investigate this hypothesis, we conducted a study to examine 1) the baseline association between serum 25OHD and intramuscular IL-6 or TNFα gene and protein concentrations, 2) the baseline association between intramuscular VDR gene expression or protein concentration with intramuscular IL-6 and TNFα gene and protein content in older mobility-limited adults. We also conducted a second study to assess changes in intramuscular IL-6 and TNFα after a 16-week supplementation of vitamin $D_3$ in older mobility-limited women. As a secondary analysis, we examined phosphorylation cascades that may be responsible for interactions between serum 25OHD, intramuscular VDR, and inflammatory markers IL-6 and TNFα.

## METHODS

### Subjects

Data for these studies were pooled from the baseline measurements of two larger randomized controlled studies for which inclusion and exclusion criteria are presented elsewhere.[18,20] Specific characteristics of study subjects are presented in Table 1. Only those subjects who provided a muscle biopsy (N=30; male=7, female=23) were included in the analysis. Participants were mobility-limited as determined by the Short Physical Performance Battery (SPPB<10)[21] and were not obese (BMI<30). In the longitudinal study, a subset of 12 women were either supplemented with 4,000 IU/day of vitamin $D_3$

(N=5) or placebo (N=7) and biopsies were repeated at 16 weeks. Both clinical studies were approved by the Institutional Review Board of the Tufts University Health Sciences Campus (Boston, MA).

Biochemical Measures

Archived fasting blood samples were assessed at baseline and 16-weeks. Serum 25OHD was analyzed utilizing Diasorin, LIAISON® 25 OH Vitamin D Total Assay. Vitamin D deficiency was defined as 25OHD serum concentrations below 12 ng/mL, insufficiency as 12-19ng/mL and sufficiency ≥20ng/mL.[22]

**Muscle biopsy**

Muscle biopsies were obtained from the vastus lateralis at the level of the mid-thigh under local anesthesia (1% lidocaine). The specimens were flash frozen in liquid nitrogen and stored in liquid nitrogen until analysis.

Western Blotting Analysis

Immunoblotting was utilized to examine intramuscular protein concentration of VDR, IL-6 and TNFα in the vastus lateralis muscle as previously reported (Pojednic et al, under review). Membranes were incubated overnight at 4°C with primary antibodies specific for VDR, IL-6 and TNFα (1:1,000 in 5% bovine serum albumin and TBS-Tween; VDR Perseus Proteomics via R&D Systems, Minneapolis, MN; IL6 AbCam ab6672, Cambridge, MA; TNFa D5G9 Cell Signaling, Danvers MA; phospho-p38 MAPK (Thr180/Tyr182) New England Biolabs Inc, Ipswich, MA; phospho-p65 (ser468) Cell Signaling, Danvers MA). Membranes were rinsed three times for 10 min in TBS-Tween

and incubated at room temperature for VDR with secondary goat-anti mouse IgG2Aa HRP conjugate antibody (1:2000 in 5% nonfat dry milk and TBS-Tween; Invitrogen, Frederick, MD) and for IL-6, TNFα, phospho-p38, phospho-p65 and GAPDH with anti-rabbit IgG AP-linked antibody (1:1000 in 5% nonfat dry milk and TBS-Tween; Cell Signaling, Danvers, MA). Membranes were again rinsed three times for 10 min in TBS-Tween and the immunoreactive proteins were detected with Supersignal Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) and quantified by optical density (Image Lab 3.0.1; Bio-Rad Laboratories, Hercules, CA). Changes in optical density were calculated relative to values from glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Cell Signaling, Danvers MA) and data are presented in arbitrary units.

**mRNA Preparation**

Vastus lateralis muscle was prepared for mRNA analysis as reported previously (Pojednic et al, 2014, under review). mRNA extraction was completed utilizing Aurum Total RNA Fatty and Fibrous Tissue RNA Extraction Kit (Bio-Rad Laboratories, Hercules, CA). cDNA conversion was performed utilizing a commercially available reaction mixture (iScript Reverse Transcription SuperMix for RT-qPCR Bio-Rad Laboratories, Hercules, CA) on a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA).

**Real Time qPCR**

Quantitative real-time PCR was performed utilizing a commercially available reaction mixture (SsoAdvanced SYBR Green Supermix; Bio-Rad Laboratories, Hercules, CA) on a CFX96 Real Time System (Bio-Rad Laboratories, Hercules, CA). cDNA levels of VDR (QT01010170), IL-6 (QT00083720), and TNFα (QT01079561) were measured using commercially available primer mixtures (Quantitect Primer Assays: Qiagen) as previously reported (Pojednic et al, under review). Changes in target gene expression were calculated relative to values from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (QT00199633). Efficiencies of each primer set were assessed using a standard curve, and analyzed using 0.0025-25ng of control cDNA. Data are presented as fold change from baseline.

**Statistical Analysis**

Statistical analysis was completed using SAS JMP software (v. 10.0, SAS Institute Inc, Cary, NC). Correlational analyses were utilized to determine associations between serum 25OHD and intramuscular IL-6 and TNFα gene expression and protein concentration. Multivariable linear regression was used to determine whether VDR, adjusted for serum 25OHD, accounts for the variability of IL-6 and TNFα gene or protein content in the cross-sectional analyses. The proportion of variation in IL-6 and TNFα accounted for by the main variables (intramuscular VDR and serum 25OHD) was calculated as partial $R^2$ values. Body Mass Index (BMI) was originally included in each model, but determined to be non-significant ($p>0.05$), so this variable was removed. In the longitudinal analysis,

student's t-test was used to analyze differences in intramuscular IL-6 and TNFα mRNA fold change between placebo and supplemented groups. Secondary correlational analyses were used to assess associations between VDR or 25OHD with intramuscular inflammatory markers and signaling molecules in the cross-sectional study. Statistical significance was set at $p \leq 0.05$ for all analyses. Variables were each examined for normal distribution, and were log-transformed if necessary to better approximate normal distribution.

## RESULTS

**Subject Characteristics:** Subjects from the two studies were pooled for the baseline cross-sectional analysis. Mean age (±SD) of the baseline sample (N=30) was 78.2±4.4 years, mean BMI was 27.0±3.5 kg/m$^2$, and mean serum 25OHD was 22.5±10.6 ng/mL (range 9 to 53 ng/mL). A comparison between subjects from the two studies is presented in **Table 1**.

### Skeletal Muscle IL-6 and TNFα Gene Expression

*Cross-sectional Analysis*

In the combined group at baseline, it should be noted that within subset 1, two subjects did not have VDR protein concentration measured and were, thus, excluded from the cross sectional analysis. Neither baseline 25OHD nor VDR gene expression (adjusted for baseline 25OHD) was associated with IL-6 gene expression (n=28; p=0.92 and p=0.21, respectively). Intramuscular VDR protein concentration was positively associated with

intramuscular IL-6 gene expression (Total Model $R^2$ = 0.15; VDR p=0.04; **Figure 1**). Baseline 25OHD, VDR gene expression or protein concentration (adjusted for baseline 25OHD) were not associated TNFα gene expression (n=28; p>0.18).

*Longitudinal Analysis*

At baseline, older mobility-limited women (subset 1, N=12) were classified as either vitamin D insufficient or deficient. After 16 weeks, all vitamin D-supplemented subjects were classified as vitamin D sufficient while placebo subjects did not reach sufficient 25OHD levels.[18]

There was no significant difference between the fold change of intramuscular IL-6 mRNA expression between the placebo (mean 0.45, 95% CI -2.53, 3.43) and supplemented groups (mean 3.02, 95%CI -7.21, 13.26; p=0.57, **Figure 2A**) after 16 weeks of vitamin $D_3$ supplementation. With regard to intramuscular TNFα, after 16 weeks of vitamin $D_3$ supplementation, there was no significant difference in the fold change between the placebo (Mean:-2.46, 95% CI: -5.37-0.45), and supplemented groups (Mean:-6.83, 95% CI: -5.68-19.38; p = 0.11, **Figure 2B**).

**IL-6 and TNFa Protein Concentration and Signaling Pathways**

*Cross-Sectional Analysis*

In subset 2 (Table 1; N=18) for which intramuscular IL-6 and TNFα protein was available for analysis by Western Blot, baseline serum 25OHD was not associated with

intramuscular IL-6 protein concentration (p=0.13; **Figure 3A**). Baseline VDR protein concentration (adjusted for serum 25OHD) was negatively associated with intramuscular IL-6 protein concentration (Total Model $R^2$ = 0.35; p=0.03; **Figure 3B**). Neither serum 25OHD (p=0.93) nor VDR protein (adjusted for serum 25OHD) was associated with intramuscular TNFα protein (Total Model $R^2$=0.11; p=0.40).

Further analysis was undertaken to determine potential signaling pathways responsible for the correlation between intramuscular VDR and IL-6 protein. Both intramuscular phosphorylated NFkB and p38 were examined. Intramuscular phosphorylated NFkB was not found to be significantly associated with serum 25OHD (p=0.70), IL-6 protein (p=0.99) or TNFα protein (p=0.73) in this subset. NFkB was positively associated with intramuscular VDR protein concentration, but it was not statistically significant below the 0.05 level (p=0.06). Intramuscular phosphorylated p38 was not significantly associated with serum 25OHD (p=0.22), VDR (p=0.41), or TNFα protein (p=0.53). However, intramuscular phosphorylated p38 was positively associated with IL-6 protein (p= 0.002).

## DISCUSSION

This study found that intramuscular IL-6, a marker of inflammation, was associated with intramuscular VDR in older mobility-limited adults. Intramuscular VDR protein concentration was positively associated with intramuscular IL-6 gene expression, but negatively associated with intramuscular IL-6 protein concentration. There was no

significant change in intramuscular IL-6 gene expression after 16 weeks of vitamin $D_3$ supplementation. Because the sample size was small, it is premature to conclusively rule out possible contributions of serum 25OHD to intramuscular inflammation; however, we were able show noteworthy evidence that VDR may be associated with intramuscular IL-6 in human skeletal muscle.

The different relationship between VDR protein concentration and IL-6 gene expression versus protein concentration is unclear. It may be due to chance or to the small sample analyzed. Another potential hypothesis may be a trigger or signaling effect through which VDR protein may attenuate translation of IL-6 protein since VDR has been shown to have rapid non-genomic effects in skeletal muscle.[23,24] Lastly, as IL-6 has several proposed roles in skeletal muscle, including participation in inflammatory pathways,[25,26] it is also possible that VDR protein decreases this particular inflammatory response through a signaling process as noted in animal models.[11]

We did not observe a relationship between intramuscular TNFα, intramuscular VDR, or serum 25OHD. Our results are consistent with those reported for vascular endothelial cells in middle aged and older adults.[16] These authors concluded there was no involvement in vitamin D-related effects on endothelial cell inflammation with regard to TNFα.

In order to assess possible signaling relationships between VDR and IL-6 gene expression and protein concentration, we examined two key signaling pathways, p38 MAPK and NFκB. We did not find an association between serum 25OHD or intramuscular VDR with intramuscular NFκB or MAPK p38, although associations

between NFκB and VDR did trend toward significance. While our sample size prohibits us from ruling out these pathways as a potential link between intramuscular inflammation and VDR, our results may suggest that any relationship may be outside this particular p38 MAPK pathway in human skeletal muscle. It is recommended that future studies with larger sample sizes re-examine these as well as alternative signaling pathways to determine the mechanistic relationship between intramuscular VDR and intramuscular IL-6. This is of interest due to the proposed non-inflammatory properties of IL-6 in skeletal muscle[25,26] and the noted lack of association between serum 25OHD and IL-6 in our results.

Our study had several strengths including our ability to examine both a cross-sectional sample of human biopsied muscle and a longitudinal model following vitamin $D_3$ supplementation versus placebo. Limitations of this study were that it had a small sample size and was a secondary analysis of data pooled from two studies.

The results of our study lend evidence to the potential relationship between VDR and IL-6 in human skeletal muscle. We found that intramuscular VDR protein concentration is positively correlated with intramuscular IL-6 gene expression but negatively associated with intramuscular IL-6 protein concentration in older mobility limited adults. Neither intramuscular VDR nor serum 25OHD appear to be associated with intramuscular TNFα in humans. These findings should be considered hypothesis generating and need to be confirmed in larger randomized trials specifically designed to examine intramuscular inflammation. Future studies are needed to examine the

relationship between protein concentrations of VDR and IL-6 in skeletal muscle after supplementation, IL-6-related signaling pathways and implications on muscle function.

**Table 1 Participant Descriptive Statistics** (mean ± SD)

| | **Subset 1** | **Subset 2** | **p value** |
|---|---|---|---|
| Age (years) | 78.5 ± 4.79 | 77.9 ± 4.05 | 0.71 |
| Weight (kg) | 67.2 ± 14.2 | 73.97 ± 9.11 | 0.08 |
| Height (cm) | 157.5 ± 9.43 | 165.28 ± 7.82 | 0.007* |
| BMI | 27.0 ± 5.5 | 27.06 ± 2.94 | 0.88 |
| SPPB | 7.9 ± 1.55 | 8.65 ± 1.23 | 0.09 |
| Gender (f) | 100% | 63% | |
| **N** | **12** | **18** | |

## LEGENDS FOR FIGURES

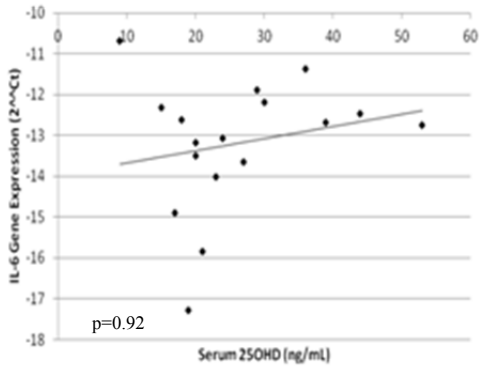
**FIGURE 1. Cross Sectional Association between IL-6 Gene Expression vs VDR protein and Serum** 25OHD In a sample of older mobility limited adults (n=28), 25OHD was not associated with intramuscular IL-6 gene expression (p=0.92; Figure 1A). However, in a multilinear regression model adjusted for serum 25OHD, intramuscular VDR protein concentration (log VDR) was positively associated with intramuscular IL-6 gene expression (Total Model $R^2 = 0.15$; p=0.04*, Figure 1B). 25OHD was determined by serum assay and RT-PCR analyses were carried out using VDR and anti-IL6 primers. Changes in Ct values were calculated relative to values from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and data is presented in arbitrary units (*$p<0.05$)

**FIGURE 2. Vitamin D Supplementation Does not Significantly Alter Intramuscular IL-6 or TNFα gene expression** In a cohort of older mobility women (N=12) supplemented with 4,000IU Vitamin $D_3$ for 16 weeks, there was no significant difference between the fold change of intramuscular IL-6 mRNA between the placebo (N=7) and Vitamin $D_3$ supplemented groups (N=5, p=0.57, Figure 2A) nor was there a significant difference in TNFα (p=0.11, Figure 2B) as assessed by student's t-test (p*$<0.05$).

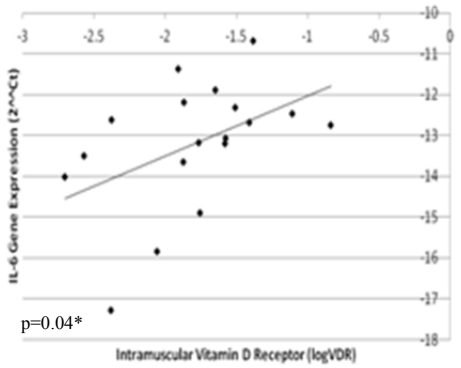
**FIGURE 3. Cross Sectional Association between Intramuscular IL-6 Protein Expression vs Serum 25OHD and Intramuscular VDR Protein** In a sample of older mobility limited adults (n=18), 25OHD was not associated with intramuscular IL-6 protein expression (p=0.13; Figure 3A). However, in a multilinear regression model adjusted for serum 25OHD, intramuscular VDR protein concentration (log VDR)

negatively predicted intramuscular IL-6 protein concentration (Total Model $R^2 = 0.35$; $p=0.03$, Figure 3B). 25OHD was determined by serum assay and western blot analyses were carried out using anti-VDR and anti-IL6 antibodies. Changes in optical density were calculated relative to values from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and data is presented in arbitrary units (*$p<0.05$)

**FIGURE 3-1**

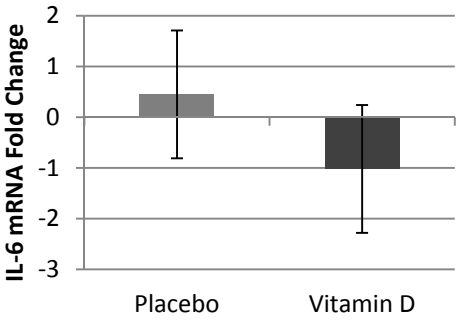

IL-6 Gene Expression (2^^Ct)
Serum 25OHD (ng/mL)
p=0.92

A.

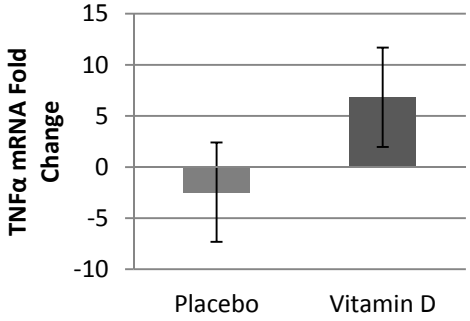

IL-6 Gene Expression (2^^Ct)
Intramuscular Vitamin D Receptor (logVDR)
p=0.04*

B.

**FIGURE 3-2**

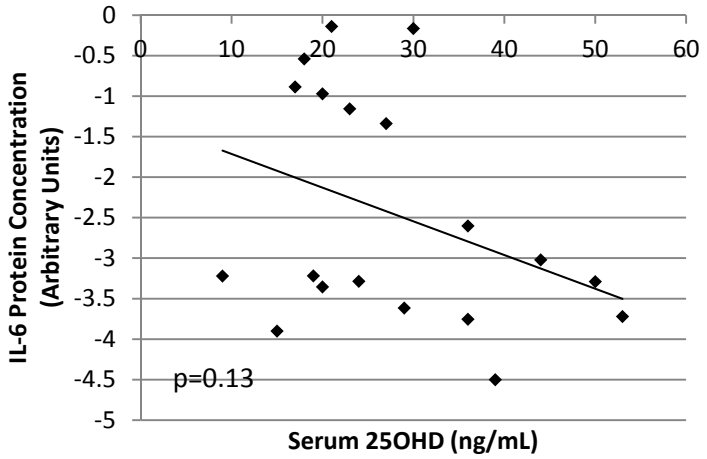

IL-6 mRNA Fold Change
2
1
0
-1
-2
-3
Placebo
Vitamin D

A.

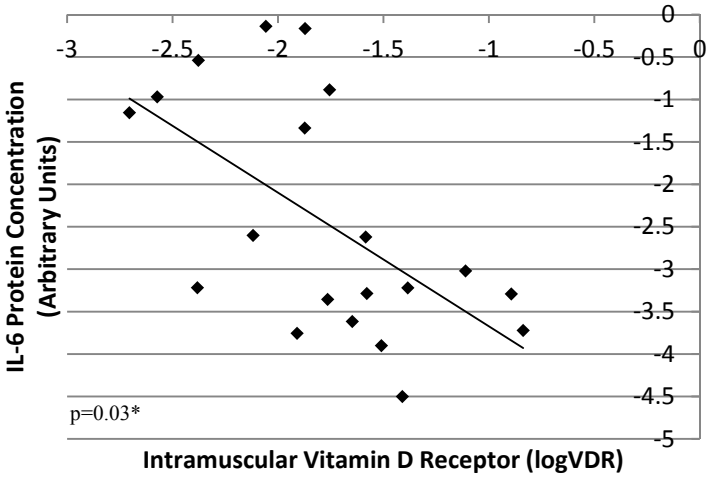

TNFα mRNA Fold Change
15
10
5
0
-5
-10
Placebo
Vitamin D

B.

**FIGURE 3- 3**


0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
-4.5
-5
0
10
20
30
40
50
60
IL-6 Protein Concentration (Arbitrary Units)
p=0.13
Serum 25OHD (ng/mL)

A.


-3
-2.5
-2
-1.5
-1
-0.5
0
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
-4.5
-5
IL-6 Protein Concentration (Arbitrary Units)
p=0.03*
Intramuscular Vitamin D Receptor (logVDR)

B.

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# CHAPTER IV

## Summary and Discussion

A growing number of studies have indicated a beneficial effect of vitamin D on skeletal muscle strength and physical performance, particularly with aging. Emerging evidence from both preclinical and clinical studies has begun to elucidate major molecular mechanisms by which vitamin D acts on skeletal muscle cells. Recent data localizing CYP27B1 and VDR protein expression in skeletal muscle cells provides support for a direct effect of vitamin D on muscle physiology. As in classic vitamin D target tissues, administration of parent and active forms of vitamin D increases VDR gene and protein expression in muscle indicating increased biological activity. Although VDR downstream signaling cascades have not been well-characterized, administration of $1,25(OH)_2D$ in skeletal muscle cells appears to influence biological pathways of muscle contraction, cell proliferation, cell differentiation, growth, and inflammation. This work has further characterized the relationship between vitamin D and skeletal muscle and has established a potential role of VDR with regard to intramuscular inflammation.

The first part of this project demonstrated that vitamin D participates in a direct interaction with human skeletal muscle. Specifically, we were able 1) to demonstrate that treatment with $1,25OH_2D_3$ increases skeletal muscle VDR gene expression in human primary myoblasts, 2) to show a positive association between serum 25OHD and VDR protein concentration in the skeletal muscle of older mobility-limited adults, and lastly, show an increase in VDR protein expression in vivo after 16 weeks of supplementation with vitamin $D_3$ in older mobility limited women. Immunoblotting,

immunohistochemistry, and real time polymerase chain reaction (RT-PCR) has demonstrated this effect in *in vitro* and *in vivo* models of human skeletal muscle. The second component of this project has demonstrated a potential interaction between intramuscular VDR and interleukin-6 (IL-6) in skeletal muscle. Moreover, this relationship may be unique to skeletal muscle in that the relationship appears to affect IL-6, but not known intramuscular MAPK p38 regulatory cascades or TNFα. This is of note due to implications that could potentially signify a role for VDR in human skeletal muscle outside of traditional inflammatory pathways.

We have shown that vitamin D, both $1,25OH_2D_3$ and serum 25OHD, appears to affect intramuscular VDR gene expression, protein concentration, and IL6, however there are unknown signals, triggers, and pathways that are potentially involved in the regulation of these outcomes. While we have demonstrated a direct effect of vitamin D on skeletal muscle, specific roles in skeletal muscle physiology and function have not yet been determined.

**Vitamin D and Skeletal Muscle Interaction**

Recently there have been important advances in our understanding of the interaction between vitamin D and skeletal muscle (reviewed in Pojednic & Ceglia[1]). Specifically, VDR is emerging as an important intermediary of critical physiological pathways in skeletal muscle. It has been reported by several groups that VDR mRNA expression increases in $C_2C_{12}$ myoblasts with vitamin D treatment[2-4]. Garcia et al.[4] and Srikuea et al.[3] demonstrated a more than fivefold increase of VDR following 4 days of

$1,25(OH)_2D_3$ administration that was further confirmed by Western blot analyses. Two of the three studies noted similar effects on VDR expression following a treatment with $25OHD_3$, particularly in differentiating cells, suggesting a common mechanism.[3,4] Our results demonstrated similar findings in human primary myoblasts and biopsied vastus lateralis muscle from older mobility limited adults. Namely, we were able to show an increase in VDR protein concentration and attenuated decreases in VDR gene expression with $1,25(OH)_2D_3$ and vitamin D supplementation, respectively.

While the relationship between vitamin D treatment and VDR expression is becoming more established in the literature, the potential downstream effects of this interaction are yet unclear. Many projects are focused on the role of vitamin D and VDR in skeletal muscle proliferation and differentiation. Srikuea et al.[3] recently examined regenerating mouse skeletal muscle in vivo and found that murine regenerating muscle fibers had greater expression of VDR compared to non-regenerating fibers suggesting a link between muscle cell regeneration and activation of VDR. Further, a recent study by Tanaka and colleagues[5] demonstrated that silencing VDR in $C_2C_{12}$ and G8 murine cells via siRNA resulted in decreased myosin heavy chain mRNA as well as decreased expression of mRNA and protein levels of a variety of myogenic factors including MyoD, myogenin, MRF4, and Myf5. Morphological changes in the myotube formation were also noted. These authors suggest skeletal muscle may indeed require VDR mediated signaling for successful myoblast differentiation into myocytes. However, as these results are only yet emerging, further translational experiments using both in vitro and in vivo skeletal muscle samples need to be performed.

With regard to a potential role for vitamin D and VDR in skeletal muscle mediated inflammation, very few studies have yet examined this relationship. Outside of our results demonstrating an association with intramuscular IL6 gene expression and intramuscular VDR protein concentration, only two other studies have demonstrated a direct link between vitamin D, VDR and inflammatory pathways in skeletal muscle. In rats exposed to crush injury, vitamin D supplementation resulted in significant increases in muscle cell regeneration and extracellular matrix proteins and decreases in muscle cell apoptosis[6]. In a randomized controlled study in rats, vitamin D influenced exercise-induced muscle damage and inflammation through the modulation of MAPK and NFκB pathways possibly mediated by VDR[7]. Specifically, this latter study found that skeletal muscle of vitamin D-treated rats exposed to high intensity exercise demonstrated an increase in skeletal muscle VDR with reduced expression of p38, ERK1/2, IKK and IkB, and subsequent reductions in pro-inflammatory TNFα and IL-6[7].

In our results, we were only able to demonstrate an association between intramuscular VDR and IL-6. We did not find a relationship between serum 25OHD or intramuscular VDR with regard to intramuscular NFκB or MAPK p38, although relationships between NFκB and VDR did trend toward significance. While our sample size prohibits us from ruling out 25OHD or these signaling pathways as a potential link between intramuscular inflammation and VDR, our results may suggest that any relationship may be outside this particular p38 MAPK pathway in human skeletal muscle. As this result may be due to a small sample size, this relationship should be reevaluated in a larger sample set.

If our results are repeated, however, it is possible that vitamin D is interacting with IL-6 through VDR in human skeletal muscle via a unique mechanism. Based upon conclusions from further investigation, it is possible that a MAPK p38 mechanism could be ruled out as a downstream player in intramuscular VDR signaling. It is possible, as has been noted in other studies in human skeletal muscle[8], VDR and IL-6 may be taking on a secondary role to inflammation, specifically with regard to regeneration. As such, our findings should be considered hypothesis generating and need to be confirmed in randomized trials specifically designed to examine intramuscular inflammation. Specifically, future studies are needed to examine the relationship between protein concentrations of VDR and IL-6 in skeletal muscle after supplementation, related signaling pathways and how this interaction may affect muscle function.

Lastly, literature that identifies the response by skeletal muscle to vitamin D with regard to the location of intramuscular VDR remains limited. Specifically, it is unclear whether the potential downstream effects are due to the nuclear VDR or perhaps though non-genomic signaling events mediate by a membrane bound receptor. While the VDR has traditionally been understood as a nuclear receptor, the characterization and mechanism of action of non-nuclear VDR are becoming more established. This putative non-nuclear entity may act as a novel membrane receptor[9], membrane-associated calcium-binding protein[10] or potentially as the nuclear VDR itself, which translocates from the nucleus to the plasma membrane[11]. Recently, Buitrago and colleagues[12] utilized a VDR silencing model in $C_2C_{12}$ cells to demonstrate that participation of the VDR, a membrane entity, is required for rapid actions after treatment with $1,25OH_2D_3$ that would

intervene in mediating hormone-triggered fast effects leading to cellular responses such as myocyte survival, proliferation and differentiation. While we did not note an alteration in the location of human skeletal muscle VDR after 16 weeks of vitamin $D_3$ supplementation, the possibility that there may be a more immediate non-nuclear VDR effect or acute translocation of the VDR require further investigation.

**Future Direction**

Despite the recent advances in our understanding of the molecular actions of vitamin D in skeletal muscle, there are still many gaps in the knowledge that require further investigation. Significantly more research is required to better define the biological pathways involved in vitamin D and skeletal muscle interactions and whether the effects are mediated by the VDR in human skeletal myocytes. In addition, as molecular and cellular mechanisms become better understood, the next step should be to examine how these tissue-level events translate into changes in overall muscle mass, strength, and function.

One overlying gap in the knowledge of the interaction between vitamin D and skeletal muscle is with regard to the specific metabolite, $1,25OH_2D_3$ or 25OHD, that is responsible for alterations in VDR and downstream effects in vivo. While we were able to demonstrate a direct effect of $1,25OH_2D_3$ on primary myoblasts in vitro, we were unable to characterize the metabolite responsible for alterations in VDR after vitamin $D_3$ supplementation. A component of this lack of understanding derives from the fact that we were unable to analyze VDR protein concentration from our in vitro model. In order to

determine which metabolite is directly affecting changes in VDR gene expression and protein concentration, further study examining differing interactions between treatment with $1,25OH_2D_3$ and 25OHD could be conducted on human primary myoblasts, given that this is the closest model to in vivo supplementation as is currently available. Moreover, outside of VDR expression and concentration, the *CYP27B1* hydroxylase enzyme, which has been isolated in C2C12 and murine skeletal muscle[3] should be examine for expression and activity in this model and from muscle biopsies of deficient, insufficient, and sufficient humans. In this way, we could determine if simply providing adequate dietary vitamin D (vitamin $D_3$ or 25OHD) is sufficient for local effects on intramuscular VDR or if tissue specific conversion mechanism is also important.

Further study must also be conducted within the realm of the noted inflammatory and signaling cascades, namely those related to NFκB. While we noted an association in a small sample size between intramuscular VDR and IL-6 gene expression and protein expression, the outcomes were inverse. Namely, as VDR increased, IL-6 gene expression increased and IL6 protein concentration decreased. As such, we hypothesize that there may be a trigger or signal to inhibit IL6 protein translation with increasing intramuscular VDR protein expression, however without further study, this is yet uncertain. While we did not notice a statistically significant relationship between VDR and MAPK p38 or NFκB phosphorylation, this could be due to a small sample size, as NFKB in particular tended toward significant association. Therefore, future studies examining these pathways should be conducted via immunoblot and RT-PCR techniques. In this way, phosphorylation patterns in the signaling cascades can be tied to alterations in protein

concentration and gene expression of intramuscular VDR. Lastly, although it was outside the scope of this study, the potential influence of skeletal muscle mediated inflammation on systemic inflammation should be further investigated in relationship to vitamin D treatment and alterations of intramuscular inflammation.

With regard to determining the relative actions of nuclear versus non-nuclear VDR in skeletal muscle, the actions of each local VDR should be assessed. As immunohistochemistry tends to be the predominantly utilized tool to isolate the location of VDR in human skeletal muscle, it would be important to fractionate whole cell lysates into nuclear and membrane fractions and analyze VDR with a highly sensitive antibody in an immunoblotting technique to determine quantifiable changes in local VDR concentration. Further, as both $1,25OH_2D_3$ and 25OHD have been shown to alter intramuscular VDR gene expression and protein concentration in mice[3], a supplementation model with a stable isotope labeled $1,25OH_2D_3$ and 25OHD with Mass Spectrometry (MS) detection could be utilized to determine and quantify particular ligand binding at each VDR site with subsequent fractionation.

Lastly, the relationship between skeletal muscle VDR and related genomic transcription and signaling cascades should be investigated with regard to physical function and inflammatory disease. While it has been noted that physical function is associated with vitamin D status[13-17], alterations in skeletal muscle VDR concentration and related physiologic pathways are yet unreported in humans. As such, longitudinal studies with large sample sets examining vitamin D supplementation and skeletal muscle biopsies should be conducted to determine relevant intramuscular alterations and

associations with physical function. This would be particularly important with regard to the current practice of clinically prescribed vitamin D supplementation and beneficial physiologic results.

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**APPENDIX A: Power Calculations**

Power calculations were carefully considered in an a priori manner due to the novelty of this investigation. At the time of proposal only one previous study had specifically examined VDR concentration in skeletal muscle (via immunohistochemistry), and we used those authors' results to estimate means and standard deviation[6]. It should be noted, however, that study conducted by Bishcoff et al.[6] examined patients with osteoporosis, osteoarthritis, and young subjects undergoing back surgery. As the groups in our proposed designs are not directly comparable, an average of the three studied cohorts was utilized in calculations. According to Bischoff et a.[6], the assumed baseline mean positive nuclei (per 500 counted) of 256.45 +/- 50.28 was utilized (calculated from older cohorts in the reference study). An estimate of 125 VDR positive antigens was used for a mean difference between groups (vitamin D-deficient and sufficient). As such, we expected a mean difference of 125 VDR positive antigens between the treatment and control groups and a within group SD of 50. A sample size of n=4 gives an 80% chance of rejecting the two-sided null hypothesis of no difference between the group means at the 0.05 level of significance if our expectations are met. Dr. Gerald Dallal's online sample size calculator (http://www.jerrydallal.com/LHSP/SIZECALC.HTM) was used to assess appropriate power.

| Outcome Variable | Estimated Mean Difference | Estimated Standard Deviation | Sample Size Calculated | Estimated Sample Size Available |
|---|---|---|---|---|
| VDR Expression[6] | 125 | 50 | 4 | 30 (12+ per group) |
| Intranuclear VDR Expression[9] | 29.7% | 11.7% | 4 | 30 (12+ per group) |

Further, our own preliminary data at the time[9] demonstrated a 29.7% +/- 11.7% increase in nuclear VDR in those women receiving vitamin D supplementation. According to the power calculations from Bishoff et al, 2001 and Ceglia et al, 2013, a sample size of n=4 for is confirmed for each group (placebo vs. supplemented). Given this sample size, the study was adequately powered to detect a true change in VDR concentration.

With regard to the cell culture experiments, while there were n=3 biopsies, myoblasts were treated in triplicate with 1,25-$OH_2$-$D_3$. As such, there were n=9 samples for each concentration of 1,25-$OH_2$-$D_3$ reported. Based on our calculations from above, a sample size of n=6 gives an 80% chance of rejecting the two-sided null hypothesis of no difference between four group means (Control, 1pmol/L, 10pmol/L, and 1nmol/L ) at the 0.05 level of significance if our expectations are met. Given this sample size, the study was adequately powered to detect a true change in VDR expression.

**Inflammation Power Calculations**

At the time of proposal there were no studies that had examined skeletal muscle-mediated inflammation with regard to vitamin D supplementation. As such, serum markers of inflammation were utilized to determine means and standard deviation in assessing the power calculations for the inflammation component of the study[70]. Estimated differences for serum concentrations of CRP, IL-6, and TNF-α are presented below and represent cross-sectional mean differences between vitamin D-deficient and sufficient human subjects. According to the power calculations, a sample size of n=6 is necessary for each group (placebo vs. supplemented). Given this sample size, the proposed study is adequately powered (n=5+ per group) to detect a true change in inflammatory markers.

| Outcome Variable | Estimated Mean Difference | Estimated Standard Deviation | Sample Size Calculated per group | Sample Size Available per group |
|---|---|---|---|---|
| CRP (ng/mL) | 0.54 | 0.34 | 6 | 8 |
| IL-6 | 6.5 (log units) | 4.1 | 6 | 8 |
| TNF-a | 12 (log units) | 7.22 | 5 | 8 |

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**Appendix B: Western Blot Images of VDR in skeletal muscle.**

Lanes are labeled as vitamin D Insufficient (InS; ≤20 ng/mL) or Sufficient (S; >20 ng/mL) with corresponding 25OHD values (ng/mL) beneath. Unlabeled lanes were not utilized in the cross sectional analysis.

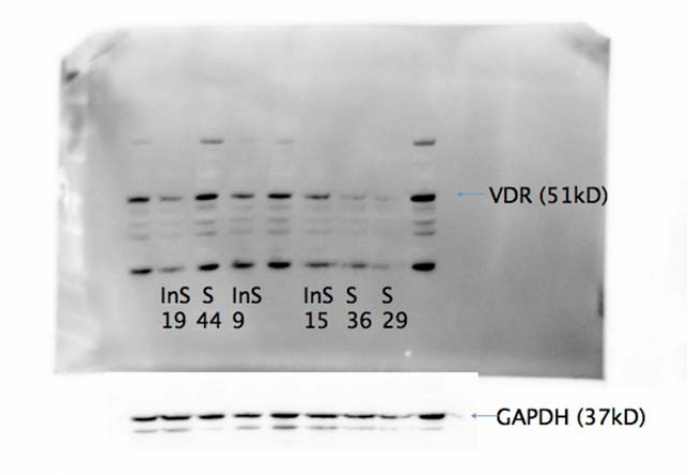



Figure A-1: VDR protein expression with GAPDH control, Gel 1

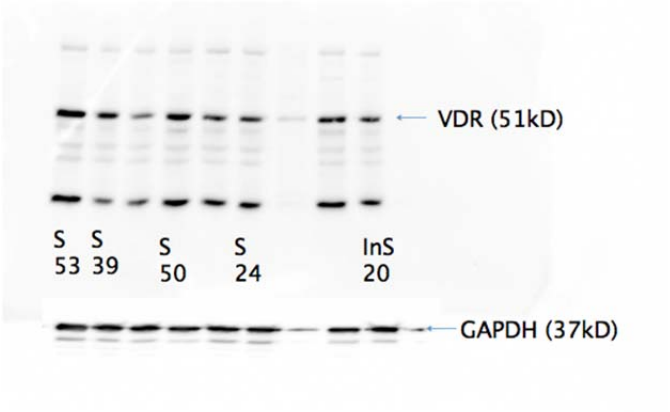



Figure A-2: VDR protein expression with GAPDH control, Gel 2

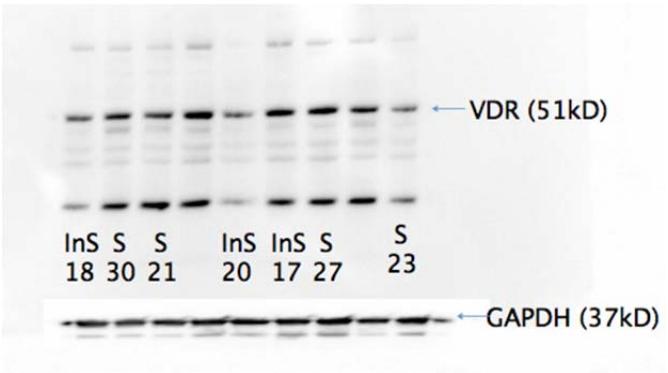
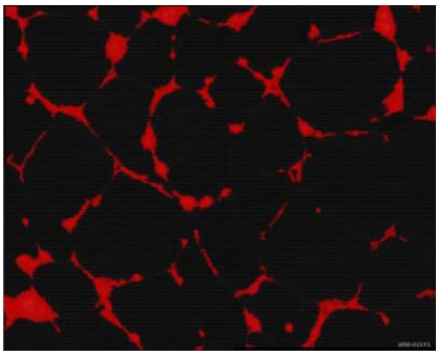
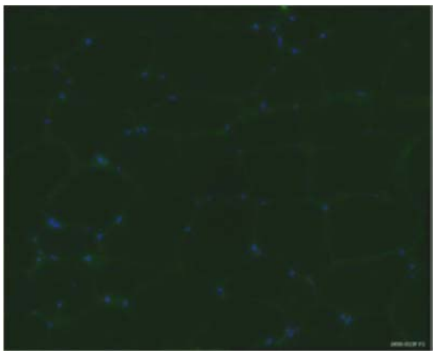
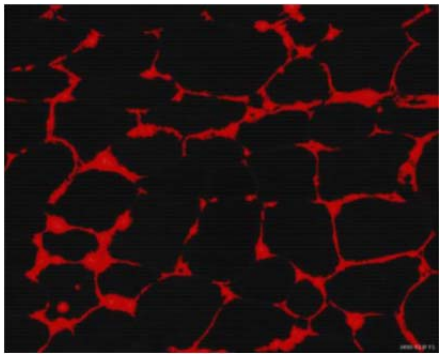
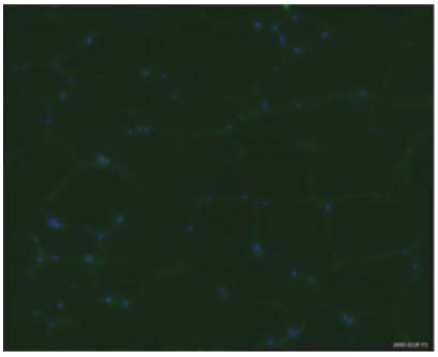



Figure A-3: VDR protein expression with GAPDH control, Gel 3

## APPENDIX C: Immunohistochemical Images For VDR Localization (Nuclear vs. Plasma Membrane)

**Figure A-4: Fluorescence of VDR at Plasma Membrane and Nucleus of Human Muscle Cell** Representative image of VDR detection in a single subject before vitamin D3 intervention at skeletal muscle (A) Cell Membrane and (B) Nuclear Colocalization and after supplementation at (C) Cell Membrane and (D) Nuclear Colocalization. A software algorithm was utilized to detect total VDR signal area and VDR-positive myonuclei across individual field images of skeletal muscle. The VDR and DAPI layer images were overlaid for detection of positive nuclei. VDR signal was aggregated across the field, then VDR signal colocalized with myonuclei (B, D) was subtracted from total VDR signal (A, C) to determine the non-nuclear fraction.

A.  B. 

C.  D. 

**APPENDIX D: Detailed Methods**

Western Blot Protocol

Preparing Samples

*Homogenization:*

1. Prepare homogenization buffer = 10mL BufferA (TrisHCL/EDTA/EGTA/Glycerol10%/Triton-ex/NaFl/NaPyrophosphate/DTT1mM)+ 1tab phostop (Roche) +1 tab cOmplete Ultra protease inhibitor (Roche) + 10uL DTT (154.25gDTT/mol$H_2O$)
2. Mass Muscle Tissue (n.b. keep samples frozen in $LN_2$)
3. Add 10x buffer to tube (i.e. for 17mg tissue, add 170uL Buffer)
4. Add half scoop of provided spoon of Zirconium Oxide Beads (Next Advance) (.55mm ZrOB05)
5. Insert into Bead Beater (5min @ speed 9; 2min@speed 10; 1min increments at speed 10 as needed; Next Advance Bullet Blender) until all tissue is homogenzed
6. Place tubes into 4C centrifuge (Eppendorf Centrifuge 5417R) @7,000rpm for 10min
7. Extract lysate into new tube, being careful not to extract pellet
8. Put on ice if preparing aliquots or flash freeze in $LN_2$ to be stored in -80C freezer

*Protein Concentration Assay:*

1. Dilute 1:10 (lysate:$H_2O$) in new tube (i.e. to make 100uL, use 10uL of sample and 90uL DD $H_2O$)

2. Get clear, flat bottomed 96-well plate, add 10uL of Pre-Diluted Protein Assay Standards: Bovine Serum Albumin (BSA) set (ThermoScientific) or dilution in triplicate (n.b. be consistent with pipetting and make sure to vortex all samples)
    a. Standard: Wells #1-7 with #8 as $H_2O$
    b. Sample: Wells #9-x
3. Add 150uL of Pierce Protein Assay Reagent 660nm with multi-channel pipette
4. Incubate 5m
5. Place loaded plate onto tray of BioKinetics Reader EL 312e (Bio-Tek Instruments, Winooski, VT) and open KCJunior Spectophotometer program on computer
    a. Choose Pierce Plate 2 protocol
    b. Choose "modify protocol" > "read plate" (make sure to name file)
    c. Let plate read and save to desktop folder and memory stick
    d. Transfer to excel template to calculate standard curve and determine volume of samples required for specified protein concentration (n.b. All protein concentrations were set to 2.5ug/uL).

*Preparing Aliquots:*

1. Use 2.5ug/uL, make sure all numbers in sheet are positive, print and put into notebook
2. Add equal amounts of lysate + Laemmli Buffer (54mg DTT+1.0mL of Laemmli, BioRad), and supplement $H_2O$ as noted on printout
3. Boil for 5 min
4. Spin in small centrifuge
5. Freeze at -80C

Running Western Blot Gel

*Loading the Gel*

1. Remove chosen gradient PreCast Gels (BioRad) from refrigerator (typically 4-20%, sometimes 10% for extra separation). Remove from wrapper and take off tape from bottom.
2. Place precast gels (BioRad) into gel holder.
3. Pour running buffer (1:10 dilution; 100mL Tris/Glycine/SDS (BioRad): 900mL dd$H_2O$) into inner chamber. Remove green comb from gel.
4. Load samples (thawed on ice) into gel from #15 --> #1 (backwards). Make sure all samples are vortexed before loading.
    a. #15 Precision Plus Protein Kaleidoscope Ladder (BioRad)
    b. #14 Skip
    c. #13-1 load as evenly as possible with samples (move pipette very slowly as to evenly fill wells with no bubbles)
5. Pour running buffer into tank and fill to flaps on holder
6. Place cover on top of gel box (red to red)
7. Plug into BioRad PowerPac HC (BioRad) machine
8. Run at 200V for 40-50 min (or until dye runs off bottom)

*Transfer*

1. Get Transfer-Blot Turbo Transfer Pack (BioRad) from refrigerator (a.k.a membrane sandwich)
    a. Use Mini (0.2um PVDF) for 1 gel
    b. Use Midi (0.2um PVDF ) for 2 gels
2. Put "bottom" (filter + membrane) on cassette of Trans-Blot Turbo (BioRad) machine.
    a. Use roller (wet with running buffer) to smooth
3. Remove gel from case with green prying tool (keep gel on larger of two pieces of plastic)
4. Cut edges of gel with flat green plastic cutting tool
5. Transfer gel to membrane (ladder to the left) and use roller to smooth out any bubbles
6. Place top of "membrane sandwich" over gel and smooth again with roller
7. Place cassette in TransBlot Turbo (BioRad) and select "Mixed Molecular Weight"
8. Run for 5-7 minutes

*Antibody Application*

1. Remove top of membrane sandwich, and gel. Discard.
2. Using a new razor blade, cut membrane to size that will fit in western box. Also, make angled cuts on corners to signify blots #1-4 (one corner cut signifies blot #1, etc).
3. Add 5mL blocking buffer (5% NonFat Milk powder in TBS-T (1:10 dilution of 100mL Tris-Buffered Saline: 900mL dd$H_2O$ + 500uL Tween)) and place on rocker for 1 hour
4. Rinse 3 times with TBS-T for 10 minutes
5. Prepare Primary Antibody

a. 1:1000 dilution in 5% Bovine Serum Albumin (BSA) in TBS-T.

6. Pour 5mL Primary antibody on membrane and let incubate on rocker overnight in cold room (~16 hours)
7. Rinse 3 times for 10 minutes in TBS-T
8. Prepare Secondary antibody
    a. 1:2000 in 2.5% NonFat Milk in TBS-T
9. Incubate with secondary antibody on rocker for 1 hour
10. Rinse 3 times in TBS-T

*Imaging Blot*

1. Leave blot in Box with TBS-T from final wash
2. Take blot from western box and put into clean square plastic dish
3. Add 750 uL-1mL of SuperSignal West Femto Maximum Sensitivity Substrate + equal amount of activator solution (ThermoScientific, Rockford, IL) to plastic dish (n.b. use different pipette tip for each so as not to contaminate activator solution)
4. Wash blot 10 times with pipette (only 1 time for GAPDH)
5. Position blot on imaging tray (BioRad ChemiDoc XRS+) tray. Make sure it is in the middle, with no bubbles.
6. Press “position gel” to align.
7. Press “run protocol” to image (use 5s for 60s; “PH_5-60s” Protocol).
8. When imaging is complete, save all images to desktop and memory stick.

*Stripping Blot*

1. After imaging, rinse blot for 5 minutes in TBS-T
2. Add 5mL of Restore Western Blot Stripping Buffer (ThermoScientific) for 5-10minutes
3. Wash one time in TBS-T for 5 minutes
4. Repeat blocking procedure and then add desired primary and secondary antibodies as previously described

*Saving Blots*

1. After final imaging of blot, leave box open in hood area to dry
2. Once dry, remove blot, place between two pieces of Kimwipes (Kimberly Clark Professional) and place in manilla envelope

**PCR Protocol**

All protocols of BioRad Aurum Total RNA Fatty and Fibrous Tissue Kit Spin Protocol were followed as below:

*Preparing Samples*

1. Ground frozen muscle to fine powder with mortar and pestle under liquid nitrogen. Massed sample and transferred to microcentrifuge tube. Added 1mL of PureZOL (BioRad) and homogenized cells with bead beater (Next Advanced) as previously described.
2. After tissue was homogenized, incubate at room temperature for 5 minutes.

3. Add 0.2mL of chloroform to the lysate, cover, and shake vigorously for 15 seconds.
4. Incubate for 5 minutes at room temperature while periodically mixing the sample.
5. Centrifuge at 12,000 x g for 15 min at  4C.
    a. Reconstitute DNAse I by adding 250uL of 10mM Tris to the vial and pipetting up and down.
    b. For each column to be processed, mix 5mL of reconstituted DNAse I with 75uL of DNAse solution in a 1.5mL microcentrifuge tube.
6. Without disturbing the interphase, immediately transfer aqueous solution from centrifuged tubes to a new 2.0mL microcentrifuge tube. (n.b. It is crucial that none of the interphase or organic material is transferred).
7. Add equal amounts (~600mL) of 70% ethanol to the tube and mix thoroughly by pipetting up and down.

*Extracting RNA*

Note: All steps should be performed at room temperature.

1. Insert an RNA binding column (2.0mL capless) wash tube.
2. Pipette up to 700uL of the RNA sample into the RNA binding mini-column. Centrifuge for 60 seconds at >12,000 x g. Remove the RNA binding column from the wash tube and discard filtrate. Replace column into same wash tube.

3. Repeat Step #2 for remainder of sample.
4. Add 700uL of low stringency wash solution to the RNA binding column. Centrifuge for 30 seconds at > 12,000 x g. Discard low stringency wash solution and replace column into same wash tube.
5. Remove any contaminating genomic DNA from the RNA sample.
    a. Add 80uL of the diluted DNAse I to each column, making sure to add only to the very center of the membrane stack at the bottom of the column.
    b. Allow DNAse to digest at room temperature for 15 minutes.
6. Add 700uL of high stringency wash solution to the RNA binding column. Centrifuge for 30 seconds at >12,000 x g. Discard high stringency wash solution and place the column back in the same wash tube.
7. Add 700uL of low stringency wash solution to RNA binding column. Centrifuge for 1 minute at >12,000 x g. Discard filtrate from the wash tube and place column back into the same wash tube.
8. Centrifgule for an additional 2 minutes at > 12,000 x g to remove residual wash solution.
9. Transfer the RNA binding column to a 1.5mL capped microcentrifuge tube.
10. Pipette 40uL of the elution solution onto the center of the membrane at the bottom of the column.
11. Incubate for 1 minute for complete soaking and saturation of the membrane.
12. Centrifuge for 2 minutes at > 12,000 x g to elute the total RNA.

*Quantitate RNA*

1. Place 1uL on NanoDrop ND-1000 (ThermoFisher) Spectrophotometer
2. Determine RNA concentration in ng/uL
3. Determine amount of ng to use in reactions (typically, 500ng per 20uL reactions).
4. Enter into prepared excel spreadsheet to determine amount of RNA template to add to cDNA synthesis reaction.

*cDNA Synthesis*

All protocols of iScript Revers Transcription Supermix for RT-qPCR were followed as below:

1. Prepare the reverse transcription master mix on ice.
2. Add 15uL of master mix to 5uL RNA for each reverse transcriptase reaction. Enough should be prepared to accommodate all reactions plus excess for loss during pipetting.
3. Adjust volume of water if RNA input volume differs from the example in the table. If more reactions are required, scale up appropriately.

| Component | Volume Per Reaction |
|---|---|
| 5x iScript revers transcription supermix | 4uL |
| RNA template (1ug to 1pg total RNA) | variable |

| Component | Volume Per Reaction |
|---|---|
| Nuclease Free Water | variable |
| Total Volume | 20uL |

4. Pipette 20uL aliquots into miniature tubes and place into thermal cycler.
5. Incubate complete reaction in T100 Thermal Cycler (BioRad) using the following protocol:

| | |
|---|---|
| Priming | 5 min @ 25C |
| Reverse Transcription | 30min @ 42C |
| RT Inactivation | 5 min @ 85C |

*Real Time PCR*

1. Create control cDNA with 1uL of each sample
2. Prepare serial dilution (1:10) from control
    a. D1 = 10ng/uL (10uL Control + 90uL of nuclease free $H_2O$)
    b. D2 = 1ng/uL (10uL D1 + 90uL of nuclease free $H_2O$)
    c. D3 = 100pg/uL (10uL D2 + 90uL of nuclease free $H_2O$)
3. Prepare SsoAdvanced SYBR Green Supermix (BioRad) with GAPDH Primer (Perkin-Elmer Applied Biosystems) and water and pipette into new tube (“MasterMix”)

4. Prepare cDNA mixture by pipetting cDNA sample, MasterMix, and dd$H_2O$ into new tubes
5. Pipette 20uL D1, D2, D3, NTC in triplicate into 96 well PCR plate
6. Cover with plastic film covering, ensuring there are no bubbles or potential leaks
7. Run on BioRad CFX96 Real Time PCR system (used Expressload>RPVDR protocol).
8. After run, determine dilution to use based on best Ct output from standard curve
9. Repeat steps 3-7 for all samples with appropriate primers.