School of Pharmacy and Biomedical Sciences

The impact of Vitamin D on Muscle Metabolism, Bioenergetic Responses and Exercise Performance

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) – updated March 2014. The proposed research study received human research ethics approval from the Curtin University Human Research Ethics Committee (EC00262), Approval Number HRE2019-0028.

Karina Romeu Montenegro

Date: 14/01/2021

Dedication

I dedicate this thesis to all my mother's family, especially my mother Isabel Cristina, my father Fernando Nardi, my brother Thiago Romeu, my sister Fernanda Romeu, my godfather Julio Cesar Romeu, my godmother Edimari Romeu who gave me unconditional love and support and have always been there for me. I don't have enough words for all of you, thank you so much.

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List of Publications

This thesis is organised by publications (either published or submitted) which form the individual chapters of the thesis.

The publications are listed below:

1. Montenegro KR, Cruzat V, Carlessi R, Newsholme P. Mechanisms of vitamin D action in skeletal muscle. Nutr Res Rev. 2019;32(2):192-204. DOI: 10.1017/S0954422419000064

2. Romeu Montenegro K, Carlessi R, Cruzat V, Newsholme P. Effects of vitamin D on primary human skeletal muscle cell proliferation, differentiation, protein synthesis and bioenergetics. J Steroid Biochem Mol Biol. 2019; 193:105423. DOI: 10.1016/j.jsbmb.2019.105423

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Statement of Contribution by Others

I hereby declare that the work presented in this thesis was primarily designed, experimentally executed, interpreted, and written by the first author of the individual manuscripts (Karina Romeu Montenegro). Contributions by colleagues are described and signed in the statement by co-authors as in Appendix A as well as the Copyright Permissions. I also declare that there is some similarity of my thesis with my previously published papers, specifically within the methodology and results section which as stated before, were primarily designed, experimentally, executed, interpreted, and written by myself.

Additional Publications and Conferences

Iuliano, S.; Pursey, K.; Haslam, R.; Coates, A. Abstracts of the 43rd Annual Scientific Meeting of the Nutrition Society of Australia. Proceedings 2020, 43, 2. How the Active Form of Vitamin D Might Affect Skeletal Muscle? - Oral presentation. Karina Romeu Montenegro, Vinicius Cruzat, Rodrigo Carlessi, Philip Newsholme

Mark Liveris Research Student Seminar – Faculty of Health Sciences certificate of participation (oral presentation) – 2018. Karina Romeu Montenegro, Vinicius Cruzat, Rodrigo Carlessi, Philip Newsholme

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Abstract

The active form of Vitamin D (1,25(OH)2D), has been suggested to have a regulatory role in skeletal muscle function and metabolism, however, the effects and mechanisms of the most effective form of vitamin D (VitD3) action in this tissue remain to be fully established. This PhD thesis aimed to investigate the effect of VitD3 in skeletal muscle and was divided into two main research projects: 1) an *in vitro* investigation utilising primary human skeletal muscle myoblast cells (HSMM), and 2) an *in vivo* study with physically active adults. In the first study, I used primary HSMM cells that display typical characteristics of human skeletal muscle function and protein levels, to investigate the effects of VitD3 on proliferation, differentiation, protein synthesis and bioenergetics. Myoblast cells were treated with 100 nM of VitD3 after 24 h, 48 h, 72 h and five days (myotubes) and then analyses were performed. I report that VitD inhibited myoblast proliferation and enhanced differentiation by altering the expression of myogenic regulatory factors. In addition, I found that protein synthesis signalling improved in myotubes after VitD treatment in the presence of insulin, increased oxygen consumption rate after 24 h of treatment in myoblasts and after 5 days of treatment in myotubes. The second study aimed to determine if the changes I observed in HSMM cells during study one could be observed *in vivo*. Therefore, I aimed to determine the effect of VitD3 on resting metabolic rate (RMR), body composition and strength in VitD sufficient physically active adults. Participants completed pre-supplementation testing to assess RMR, whole body composition, whole body strength, diet, exercise training load and sunlight recall before being matched for sunlight exposure and randomly allocated in a counterbalanced manner to the VitD3 or placebo group. Following 12 weeks of supplementation with 50 $IU \cdot kg^{-1}$ body-mass VitD3, participants repeated the pre-supplementation testing. Thirty-one adults completed the study (19 females and 12 males; mean ± standard deviation; age = 26.6 ± 4.9 years; BMI = 24.2 ± 4.1 $kg \cdot m^2$). The VitD3 group increased serum total 25(OH)D by 30 $nmol \cdot L^{-1}$ while the placebo group decreased total serum concentration by 21 $nmol \cdot L^{-1}$, reaching 123 (IQR 51) and 53 (IQR 42.2) $nmol \cdot L^{-1}$, respectively. There were no significant changes in muscle strength or power (back squat, bench press and jump test), RMR and body composition over the 12-week period considering serum total or free 25(OH)D as the main predictors. Overall, our results suggest that young physically active VitD sufficient adults achieved no additive benefit to RMR, body composition and strength by reaching supraphysiological serum total 25(OH)D concentrations after VitD3 supplementation. Further research using large sample size-controlled trials are required to explore the temporal relationship between serum total and free 25(OH)D concentration with muscle strength and power. I believe that this will enhance our understanding of the possible therapeutic effects of VitD3 supplementation in both VitD sufficient and deficient individuals.

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Chapter 1 – Literature Review

1.1 Introduction

Vitamin D (VitD) requirements were first described in 1645, when the effects of VitD deficiency was depicted as poorly mineralized human skeletal structure and skeletal deformity (1). This condition was more prevalent when people moved from rural areas and corresponding increase in urbanization which resulted in people living in areas with low exposure to sunlight/UVB radiation. The deficiency of VitD was so called the "English disease" as the incidence was especially high (70 - 80%) in England and Scotland (2). This may have been an outcome of a daily 12 – 16-hour work in dark factories, mills or mines with little to no natural sunlight exposure. As a consequence, further extensive research in the area lead scientists to further study VitD: how it is synthesized, its importance to calcium metabolism and its physiological actions that are similar to some other Ca^{2+} regulating hormones.

Vitamin D is described as a prohormone and can be found in two main forms: vitamin D2 (VitD2) and vitamin D3 (VitD3). Vitamin D2 is sourced from the UV irradiation of ergosterol, which can be found in some plants like fungi (3). Sources of dietary VitD2 and VitD3 include fatty fish, eggs, dairy and fortified products; however, VitD2 and VitD3 consumption and intestinal absorption is generally below the requirements (4). The major production source of VitD3 is endogenous, when solar UVB radiation of 7-dehydrocholesterol present in the skin generates cholecalciferol (5). Vitamin D3 has been reported as more efficacious at raising serum 25(OH)D concentrations than vitamin D2 (3). Vitamin D also has been reported to be vital for skeletal muscle function, as a low concentration of VitD is also associated with skeletal muscle atrophy, muscle pain, fatigue and sarcopenia (4, 6, 7). The recommendation of VitD intake has tripled in the last 10 years according to medical health guidelines, illustrating the importance of this vitamin for general health (8). It is mandatory to keep VitD levels adequate because its deficiency increases the risk of developing diseases of the bone and connective tissues (e.g., rickets, osteoporosis, osteomalacia, sarcopenia and rheumatoid artrose, and other chronic diseases (e.g., type 2 diabetes, hypertension, cardiovascular disease, cancer, Alzheimer's disease, infertility and hepatic disease) (9-15).

The foundation of this thesis is the research interest in VitD action on skeletal muscle function and metabolism. There are substantial data to support the role of VitD in skeletal muscle function and bioenergetics. However, the mechanisms by which VitD

acts and the optimal dose for skeletal muscle function is still yet to be discovered (6, 16, 17). Understanding how skeletal muscle is influenced by VitD might provide insights into its role in muscle health and athletic performance. This thesis aims to evaluate the effects of the active form of Vitamin D (VitD3) on skeletal muscle function and metabolism by first employing an *in vitro* approach and then further investigating its effect on performance outcomes in humans.

1.2 Epidemiology

Vitamin D status has emerged as a worldwide public health problem (18). Observational studies have demonstrated that more than 1 billion individuals are VitD insufficient or deficient (19-22). Low serum VitD is associated with the development of many chronic diseases and also higher mortality rate (23). In Australia it is estimated that 31% of adults have inadequate total serum VitD status 25(OH)D < 50 nmol·L^{-1}] (22). The active form of VitD (1,25OH2D) has the potential to regulate over 1,000 genes in humans, and is described in the literature as a potential exercise performance modulator (24) acting on skeletal muscle function and metabolism (25).

Farrokhyar et al., (26) conducted a meta-analysis including an international sample of 23 studies and observed that vitamin D status was inadequate in more than 50% athletes, and specifically in Australia this inadequacy was of 34%. Australian athletes reported an increased risk for vitamin D deficiency especially in winter and spring (26). Specifically, for athletes and physical active individuals, low VitD levels can result in muscle fibre atrophy, musculoskeletal pain (4), slow twitch peak, long periods of muscle relaxation and might decrease resting metabolic rate (RMR) (27).

1.3 Vitamin D physiology

The first physiological role of VitD described in the literature was the regulation of Ca^{2+} and PO_4^{2-} transport and bone mineralization (28). The exposure of the skin to UVB radiation (wavelength 290 - 315 nm) results in the conversion of 7-dehydrocholesterol to cholecalciferol (pre-vitamin D3) and represents 80 - 90% of total VitD production (4, 27). Subsequently, pre-vitamin D3 is thermally isomerized to form VitD3 and then binds to the VitD binding protein (VitDBP), ultimately being transported to the liver where it is hydroxylated at the C25 position by the enzyme VitD 25-hydroxylase. After that, 25(OH)D is metabolized to its biologically active

form, $1,25(OH)_2D$ in specific tissues that express 25-hydroxyvitamin D-1 alpha-hydroxylase(6) (e.g., kidney or muscle). The major limiting step in the synthesis of $1,25(OH)_2D$ is 1*a*-hydroxylation, which is regulated by parathyroid hormone (PTH), calcium metabolism, calcitonin, growth hormone (GH), insulin-like growth factor-1 (IGF-1), fibroblast growth factor 23, and VitD3 (negative feedback) (4). Finally, $1,25(OH)_2D$ is carried by VitDBP in plasma from the kidney to target tissues where it is able to bind to VitD receptor sites (VDR), leading to conformational changes within the receptor that permits it to interact with its heterodimeric partner, retinoid X receptor. Vitamin D receptor also forms homodimers that bind DNA and control gene expression (29).

Individual VitD status is estimated by circulating serum concentration of 25(OH)D, described as a valid and reliable marker of VitD status. However, it is the biologically active form $1,25(OH)_2D3$ responsible for maintaining Ca^{2+} and PO_4^{2-} homeostasis (30). Interestingly, it has been discussed that VitD biological activity involves unbound or free fractions of this vitamin (31, 32). This hypothesis suggests that as VitD is lipophilic, and therefore has the potential to quickly and passively diffuse across cell membranes resulting in an alternate pathway for cellular uptake as other steroid hormones (31). It is recommended to measure and differentiate total serum VitD [measured as 25(OH)D] and bioavailable VitD (i.e., free VitD), as current evidence suggests that VitDBP inhibits certain actions of VitD since the bound fraction is unavailable to act on target cells (33, 34). For example, bone mineral density (BMD) has been only linked with the bioavailable fraction of circulating 25(OH)D and not with total levels in healthy adults (35). Furthermore, other studies suggest that free VitD is a better predictor of BMD especially in an ethnically diverse athletic population as different levels of VitDBP are produced and VitD synthesis changes according to skin pigmentation (36). Additionally, it correlates better with parathyroid hormone (the marker of calcium balance associated with bone health) than total serum 25(OH)D concentration does (37, 38).

The adequate level of circulating 25(OH)D required for optimal health is highly disputed in the literature (39-42). While plasma values of $25(OH)D > 50$ $nmol \cdot L^{-1}$ have been demonstrated to improve bone health and immune system function, and assist with disease prevention, it is believed that different tissues have distinct responses to different concentrations of VitD. Therefore, the optimal 25(OH)D

concentration for muscle metabolism, protein synthesis and function may not be the same considered sufficient for other organ systems (43-50). It has been proposed by Heaney and Holick (51) that serum total 25(OH)D concentrations of ~ 120 - 225 $nmol \cdot L^{-1}$ may be required for optimal skeletal muscle function in adults. Furthermore, many factors have been documented to influence VitD status, such as season, age, geographical location, adiposity, gender and ethnicity (52-54).

1.4 The impact of Vitamin D on skeletal muscle

Skeletal muscle represents approximately 35 – 42% of body mass in adults and is critical for locomotion, production of energy and heat, and the generation and transmission of force (55, 56). Muscle tissue has the ability to react to different stimulus (e.g., exercise, hormonal and feeding response), which consequently can impact muscle mass, strength and optimise physical performance. Conversely, physical inactivity and obesogenic environments can lead to a higher risk of developing chronic diseases and are becoming an important public health problem (57). In this context, the literature suggests that exercise is a medicine polypill (58). In other words, exercise generates several adaptations in the whole body, resulting in numerous beneficial outcomes and also decreases the risk of the development of chronic diseases, including improvements to muscle function and glucose levels in type 2 diabetes and muscle mass and function in sarcopenia (59, 60).

The production of force by the skeletal muscle is dependent on several factors, such as fibre composition, size and metabolic adaptations allowing rapid ATP regeneration functional capacity (6). It is well known that VitD regulates whole-body calcium homeostasis, which has a significant impact on skeletal muscle function as the key driver of muscle contraction and controller of glucose uptake (61-63). In other words, it is mainly the influx of calcium from the sarcoplasmic reticulum (SR) and binding to the troponin-tropomyosin complex that leads to the first step of muscle contraction (64). Moreover, calcium is essential for the insulin signalling pathway, glucose transporter 4 (GLUT4) translocation and insulin receptor substrate (65, 66).

Changes in skeletal muscle mass are the result of synthesis and degradation of muscle proteins, which is mainly regulated by anabolic and catabolic reactions (67). Protein synthesis regulation in skeletal muscle occurs via the insulin signalling cascade, insulin-like growth factors (e.g., IGF-1 and IGF-2) and amino acids (68). Therefore,

understanding the effect of VitD in skeletal muscle tissue could lead to the development of strategies to increase muscle mass and optimise musculoskeletal performance.

1.5 The role of Vitamin D in skeletal muscle structure and function - *in vitro* studies

Vitamin D can impact skeletal muscle structure and function through genomic and non-genomic mechanisms. The main physiological effects of VitD reported in the literature involves the possible regulation of proliferation, differentiation, protein synthesis and mitochondrial function *in vitro*. The next section will summarise the main evidence in this field.

1.5.1 Myogenesis (proliferation and differentiation)

Myogenesis can be described as the development of muscular tissue, which starts from stem cells located in somites and then develop into the first progenitor cells named myoblasts, and subsequently differentiate into mature myotubes (69). Briefly, four myogenic regulatory factors (MRFs) control the fate and differentiation of muscle cells, including: myoblast determination protein (MYOD), myogenin (MYOG), myogenic factor 5 (Myf5) and muscle-specific regulatory factor 4 (MRF4). Myogenic factor 5 and MYOD determine skeletal muscle cell identity, and are consequently considered to be the gatekeepers for entry into the terminal specification of myogenic lineage (70), whereas MYOG is essential for the differentiation of myoblasts into myotubes (71). Myogenin acts genetically downstream of MyoD and Myf5 to control muscle differentiation genes. Even though MRF4 is categorized as a differentiation gene, it is also documented that MRF4 acts as a determinant gene when it is expressed by undifferentiated cells (72).

At the cellular level, researchers have investigated the effects of VitD in C2C12 murine cell line, and established that treatment with VitD inhibits cell proliferation (73-78) and stimulates cell differentiation (75, 79-81). Previous studies have identified an increase in myogenic factors such as MYOG, Myf5 and MYC2 in response to VitD treatment (77, 79, 82). The mechanism by which VitD improves the differentiation process was demonstrated most recently by Braga and collaborators in primary cultures of satellite cells (81). In this study, myogenic differentiation was enhanced in the presence of VitD through increased myogenic marker expression (e.g., MYOD

and MYOG), myotube formation, and the modulation of pro- and anti-myogenic factors (81).

The effects of VitD on proliferation and differentiation in primary human skeletal muscle cells, are still undefined and conflicting effects have been reported (78, 80). Owens et al. isolated human primary muscle cells by biopsy from active adults and reported increased myotube fusion and differentiation (80). However, Olsson and collaborators (78), reported the opposite effect, including reduced expression of cell cycle regulators and myogenic regulatory factors (MyoD, myogenin, MEF2C and sarcomeric proteins), with associated activation in forkhead box O3 and Notch signalling pathways (78). Another potential area of investigation is regarding the effects of VitD on lipolysis within skeletal muscle. Ryan et al. were the first group to verify a dose-response effect of VitD3 modulating the capacity of C2C12 cells to transdifferentiate into adipocytes (83). A deficient VitD status is associated with adipogenesis and up-regulation of key adipogenic marker genes (PPARg2 and FABP4), whereas higher concentrations attenuated the differentiation into adipocytes.

Discrepancies exist among studies regarding the cell line used, and VitD form, dose and duration of the treatment, consequently leading to multiple outcomes. Interestingly, an important difference between murine skeletal muscle cells lines and human primary skeletal muscle cells is that the conversion of 25(OH)D to its active form $1,25(OH)_2D3$ has not been confirmed to occur locally in human primary skeletal muscle cells, whereas it does occur in the skeletal muscle murine cell line C2C12 and *in vivo* in mice (77, 78). Overall, VitD influences myogenesis through regulation of myogenic factors and proteins involved in this process, however, it is still difficult to define how VitD affects proliferation and differentiation in primary human muscle cells. Further studies are needed to clarify the cellular development stages and/or subpopulations of cells that undergo regulation by VitD and thus impact the process of muscular development.

1.5.2 Protein synthesis and myotube size

The regulation of protein synthesis in skeletal muscle involves the insulin signalling cascade, insulin-like growth factors (e.g., IGF-1 and IGF-2) and amino acids (68). These molecules induce phosphorylation and activation of sequential targets, including the insulin receptor substrate (IRS), phosphatidylinositol-3 kinase (PI3K),

phosphoinositide-dependent kinase-1 (PDK-1), protein kinase B serine/threonine kinase family (Akt/PKB), mammalian target of rapamycin (mTOR) and 70-kDa S6 protein kinase (p70S6k) (84). Mammalian target of rapamycin is the major regulator of cell growth and proliferation by controlling the initiation phase of protein translation and synthesis (85).

Salles *et al.* have confirmed that *in vitro* treatment with 10 nM $1,25(OH)_2D3$ potentiated the effects of leucine and insulin; and improved the protein synthesis rate by 14 - 16% through Akt/PKB and mTOR pathways (86). Furthermore, these researchers reported that VitD enhanced the phosphorylation of Akt/PKB and GSK3β, and consequently improved insulin signalling by increasing the concentration of insulin receptors. Further research is essential to identify whether VitD has a biological effect on amino acid transporters as a potential alternative pathway to improve protein synthesis and myotube size.

The strongest evidence associating VitD to protein synthesis is the stimulation of fibre hypertrophy (87). In C2C12, fibre hypertrophy was observed after 10 days of treatment with 100nM of $1,25(OH)_2D3$, with a significant 2-fold growth in the mean diameter of fibres, and a 2.5-fold increase in length (73). Although the authors did not measure protein synthesis directly, they attributed their results to an increase in protein synthesis. In support of these findings, Van der Meijden et al., (77) reported that treatment with 1000 nM of 25(OH)D for 3 days resulted in a 19% increase in C2C12 fibre diameter (77). However, whilst these findings are promising, there is still a lack of evidence regarding the effect of VitD on protein synthesis in primary human skeletal muscle cells.

1.5.3 Effects of Vitamin D on mitochondria

The effects of VitD are generally mediated by its interaction with a nuclear VDR, which can be translocated into the mitochondria of certain cell types, including skeletal muscle cells, and potentially act directly on cellular bioenergetics (6). One of the possible mechanisms by which VitD acts in mitochondrial metabolism is an increase in the expression of electron transport chain (ETC) proteins and the tricarboxylic acid (TCA) cycle enzymes, through genomic and non-genomic pathways (88). Garcia et al. have investigated pathways related to the TCA cycle, oxidative phosphorylation and ATP synthesis in existing gene expression databases from multiple related

monocyte models (89). In this context genes associated with ETC activity and in the conversion of acetyl-CoA to CO_2 were upregulated by VitD in three immune cell types (THP-1 monocytic cell line - THP-1; monocyte derived dendritic cells - DCs; monocytes) (89). The same results linking serum VitD status with specific markers of bioenergetic pathways were found in human peripheral blood mononuclear cells (90).

The precise function of VitD in mitochondrial metabolism remains highly elusive in skeletal muscle tissue. Ryan et al (91) have demonstrated that mitochondrial oxygen consumption rate (OCR) increased when cells were treated with 10 and 100 nM of VitD for 48 h, and was dependent of the VDR (91). The possible mechanism behind this may be an elevation in mitochondrial volume fraction and branching, which may result in mitochondrial fusion and biogenesis (91). Vitamin D treatment increased expression levels of MYC (family of regulator genes that code for transcription factors). Mitogen-Activated Protein Kinase 13 and Endothelial PAS domain-containing protein 1 mRNAs (which encode for a protein that regulates mitochondrial biogenesis) (91). New evidence recently demonstrated that treatment with 0.1 nM $1,25(OH)_2D3$ in human primary muscle cells improved mitochondrial morphology (volume and structure) and altered mRNA expression of pyruvate dehydrogenase kinase 4 (PDK4) and CPT1, important genes that control muscle glucose and lipid metabolism (92). Finally, there is still lack of evidence regarding whether exposure to VitD up - or down - regulates the genes and proteins associated with the TCA cycle and ETC in primary skeletal muscle cells (8).

1.5.4 Glucose metabolism

There is strong evidence indicating that VitD3 sufficiency is associated with normal insulin sensitivity and glucose homeostasis (93-95). The role of VitD3 on beta cells' function was confirmed after the discovery of VDR and the response element for VitD3 promoter on the beta cells (96). It was also proven that VitD3 is able to up-regulate the insulin gene in islets directly (97). A recent review has determined that VitD improves insulin sensitivity and promotes glucose homeostasis through different molecular pathways, involving beta cell function, lowering oxidative damage and suppressing inflammatory responses (95). Additionally, skeletal muscle tissue and GLUT4 play an important role in the regulation of glucose homeostasis (98). Manna et al., (98) have discovered that treatment with 50 nM of 1,25(OH)2D3, coupled with

insulin, enhanced GLUT4 translocation and glucose uptake compared to treatment with either insulin or 50 nM of 1,25(OH)2D3 alone in high glucose-treated C2C12 myotubes (98). Overall, VitD treatment affected these cells via the upregulation of the Sirtuin 1, insulin receptor substrate 1 and GLUT4 signalling cascade (98). To the best of my knowledge, there is no evidence proving the effect of VitD on glucose uptake and/or regulation by SIRT1/IRS1/GLUT4 in primary human skeletal muscle cells.

1.6 The role of Vitamin D in skeletal muscle - animal studies

The majority of animal studies have focused on the effects of VitD on muscle mass and strength (76, 99, 100). Rodman and Baker were the first authors to investigate the effect of VitD depletion on skeletal muscle for a single isometric contraction, including the time-to-peak tension as well as the time for recovery halfway to resting tension (101). They found that rats fed a VitD deficient diet had prolongation of all parameters (101). Similarly, Schubert and DeLuca confirmed a reduction in soleus muscle force in situ (kN/ m^2) in rats fed with the same diet, however they did not observe similar results in other muscles (102). In another study, VitD deficient diet reduced maximal diaphragm force production, twitch force, and fibre cross-sectional area (CSA) by 26%, 28%, and 10%, respectively in female mice (100). Skeletal muscle predominantly composed of slow twitch fibres (type I) seem to be more susceptible to VitD deficiency, possibly because of differences in mitochondrial function in oxidative (i.e., type I) and glycolytic (i.e., fast, type II) muscle fibres. *In vitro* muscle research has found that VitD3 modulates mitochondrial metabolism, stimulating respiration coupled to the generation of ATP (91) and improving muscle phosphocreatine recovery following exercise (103). In support, Oku et al. (99) found that ingesting a high-fat diet that was deficient in VitD markedly impaired muscle metabolism in Sprague–Dawley strain male rats (99). Specifically, the total volume of the femur, bone mineral density and MyoD mRNA expression was lower in the VitD deficient group compared to control group (99).

Other animal studies have investigated the effects of VitD on proliferation and differentiation markers. Similar to *in vitro* research, the majority of animal studies have found that a diet deficient in VitD reduces markers of proliferation, such as the bone morphogenetic protein family, fibroblast growth factor 2 and/or proliferating cell nuclear antigen (76, 104). Conversely, VitD treatment increased markers of myogenic

differentiation such as myostatin, atrophy marker E3-ubiqutin ligases and Myf5 in skeletal muscle tissue of mice and Wistar rats (76, 105). Overall, studies including VDR knockout (VDR-KO) mice or a VitD deficient diet have commonly reported muscular atrophy, and reduced strength and muscle fibre size when compared to control groups. However, most have not investigated the precise pathways regulating the reported outcomes (76, 99, 100, 105).

1.7 The role of Vitamin D in skeletal muscle - human studies

The role of VitD in skeletal muscle function and ultimately in whole body health is also supported by several studies, including observational, randomised controlled trials (RCT) and systematic reviews. The effects of VitD supplementation on RMR, body composition, muscle strength and power, and the relationship between serum VitD, calcium and parathyroid hormone are summarised in the next sections.

1.7.1 Vitamin D and resting metabolic rate

Skeletal muscle is the primary producer of energy in a resting state (i.e., resting metabolic rate - RMR), accounting for ~30% of total energy production (80). The maintenance of body-mass depends on the balance between energy intake and energy utilisation (i.e., resting metabolic rate + diet-induced thermogenesis and physical activities). Resting metabolic rate represents 60 – 75% of total energy expenditure and can be defined as the energy production of the respiring tissue mass at rest (106, 107). Fat-free mass (FFM) and physical activity are the major modifiable predictors of RMR (106) but as VitD has non-calcaemic activities (e.g., inhibition of adipocyte differentiation that impacts energy metabolism) (107), it has been suggested that VitD may impact RMR.

The influence of VitD on energy metabolism involves the regulation of uncoupling proteins (UCPs) and enzymes involved in oxidation and lipolysis (108). Uncoupling proteins are associated with a wide range of physiological processes, such as adaptive thermogenesis (109), and the regulation of fatty acid oxidation (110) and body-mass (111). Clinically, higher dietary VitD intake and serum 25(OH)D levels are associated with a reduction in omental adipocyte size and lower visceral adiposity in women ($r = -0.32$) (112). In the same study, serum 25(OH)D was also inversely associated with total adipose tissue area ($r = -0.44$), subcutaneous adipose tissue area ($r = -0.36$),

BMI ($r = -0.43$) and total body fat mass ($r = -0.41$, $P \leq 0.05$ for all). Cross-sectional studies report a negative relationship between overweight and/or obesity and serum levels of 25(OH)D (113), and prospective studies have reported that low 25(OH)D plasma levels may contribute to the development of obesity (114, 115). For example, total serum 25(OH)D lower than 50 $nmol \cdot L^{-1}$ was associated with a higher odds ratio for the incidence of obesity during the two-year follow-up period (odds ratio = 1.73, 95% confidence interval: 1.24, 2.41) (114). In-vitro experiments have suggested that VitD induced increases in intracellular calcium concentrations within adipocytes, resulting in decreased lipogenesis and increased lipolysis (116). However, this area has not been well explored, with only one observational study finding an association between VitD and RMR in obese adults (27). The model produced by Calton et al. predicts that for every 10 $nmol \cdot L^{-1}$ increase in serum total 25OHD, RMR would increase by 56.5 $kJ \cdot day^{-1}$ (27). However, RCTs examining the influence of VitD on energy expenditure are rare. To the best of my knowledge, only one study with a very short supplementation protocol (1 week) with 2000 IU/day reported no influence of VitD on energy or substrate utilisation and this topic has not been further explored in a physically active population (117). Importantly, even slight changes in energy expenditure over time can potentially have a large impact on fat and muscle mass (118). Furthermore, identifying variables that directly impact RMR is essential to calculate total energy expenditure, which may have a direct impact on practice for dietitians and other health scientists.

1.7.2 Vitamin D and body composition

Vitamin D has the potential to regulate energy metabolism and possibly muscle and fat mass, which could result in significant changes in body composition. Clinical studies indicate that total serum 25(OH)D is negatively associated with BMI, percentage of body fat (% body fat), visceral fat area (VFA), and subcutaneous fat area (SFA) in adults (119, 120). However, the majority of research in this field are observational studies, which have only focused on the relationship between VitD and obesity, showing a strong epidemiological association (121, 122). A recent study that investigated the relationship between serum 25(OH)D concentrations and body composition in elderly participants found that individuals in the lowest quartile for VitD concentration (4.7 – 17.5 $ng \cdot mL^{-1}$) had a higher fat mass (9.3 $kg \cdot m^2$) compared

with participants in the third (8.40 $kg \cdot m^2$; Q3 = 26.1 – 34.8 $ng \cdot mL^{-1}$; p = 0.049) and highest (8.37 $kg \cdot m^2$; Q4 = 34.9–62.5 $ng \cdot mL^{-1}$; p = 0.04) quartile (112). In addition, a prospective cohort study found that higher total serum concentration of 25(OH)D was correlated with less body and fat mass gain in adults (114). However, the effects of VitD supplementation on body fat indicators are still ambiguous, especially considering the differences in baseline characteristics of participants (i.e., obesity, healthy adults or elderly) VitD status at the baseline and methodology to measure body composition (122-125). In regards to skeletal muscle mass, Marantes and collaborators (126) found a significant association between low 1,25(OH)2D concentration and low lean mass in both men (n = 311) and women (n = 356). On the other hand, a systematic review with meta-analysis including 6 RCTs found no significant effect of supplementation with VitD3 (doses vary from 400 to 4000 $IU \cdot day^{-1}$) on muscle mass (SMD 0.058; p = 0.52) (127). More recently, a long-term double-blind randomized controlled study reported that one year of 420 IU of VitD3 supplementation improved lean body mass (pre-supplementation = 43.8 ± 9.6, post-supplementation = 44.3 ± 9.8 kg compared to the placebo group ($p < 0.001$) in healthy Asian adults who were deficient in VitD at baseline, however no changes in fat mass were observed (128). Importantly, there is lack of literature on the impact of VitD supplementation on body composition in physically active adults that are sufficient in VitD.

1.7.3 Vitamin D and muscle strength and power

If VitD potentially impacts pathways related to muscle function, it could also potentially impact muscle structure and strength. The effect of VitD supplementation on muscle strength and power have recently been reported in soccer and rugby players, elite ballet dancers, and active male adults. Haematological samples showed that they were all either VitD deficient or insufficient at baseline (129-136). Results were equivocal, with three studies (129, 131, 132) reporting that supplementation with VitD significantly increased strength [isometric force peak, 1 repetition maximum test (1-RM) for bench press, back squat and weighted reverse-grip chin up], and four studies reporting no significant effect of VitD on any of the same physical parameters (130, 133, 134, 136). Within the first study (129), VitD supplementation enhanced the recovery in peak isometric force after the damaging event ($P < 0.05$; ≈ 8% at 24 h) and attenuated ($p < 0.05$) the immediate and delayed increase in circulating biomarkers typical of muscle damage aspartate aminotransferase (AST), alanine aminotransferase

(ALT) ($p > 0.05$). In addition, the second study (131) demonstrated a decrease in 10 m sprint times (2% improvement; $P = 0.008$) and vertical jump (5% improvement; $P = 0.008$) in the VitD group. Similar results were observed in a group of elite ballet dancers; after 4 months of supplementation with 2000 IU of VitD/day, the intervention group increased isometric strength (18.7%, $p < 0.01$) and vertical jump (7.1%, $p < 0.01$). The VitD group also sustained significantly less injuries than the controls during the study period ($p < 0.01$) (132).

The variability of participant samples, VitD status, and the type, duration and type of VitD used for supplementation make-contrasts difficult. Another limitation is that to-date only one study has included both male and female participants to allow analysis of any gender effect. Wyon and collaborators (2014) reported that VitD supplementation increased isometric strength in both genders and was positively associated with gender ($p < 0.001$) and "time" – pre- vs. post-supplementation period ($p = 0.01$) (132). Although males had higher isometric strength and vertical jump heights, and a significant main effect for time it was suggested that VitD supplementation led to improvements in these variables, there was no difference in the change between genders (132).

Vitamin D supplementation in healthy subjects with low serum levels of 25(OH)D activates the VDR in skeletal muscle, which may stimulate protein synthesis and improve muscle strength in healthy young adults (25, 137, 138). Several authors have suggested that supplementation with VitD also increases the size and number of type II muscle fibres in VitD deficient individuals (24, 139, 140). However, few studies have tested these associations in humans directly by muscle biopsy. A recent systematic review summarised the effects of supplementation with VitD on muscle strength in VitD insufficient athletes and identified that four of six studies found that VitD supplementation increased muscle strength by 1.4 to 18.8% (141). Nevertheless, more studies are needed to support this outcome.

While plasma values of 25(OH)D > 50 $nmol \cdot L^{-1}$ have been demonstrated to improve bone health and immune system function and assist with disease prevention, it is believed that different tissues have distinct responses to different concentrations of VitD. Therefore, the optimal 25(OH)D concentration for muscle metabolism, protein synthesis and function may not be the same considered sufficient for other organ systems (43-50). It has been proposed by Heaney and Holick (51) that serum total 25(OH)D concentrations of ~ 120 - 225 $nmol \cdot L^{-1}$ may be required for optimal skeletal

muscle function in adults. The majority of studies into the effects of VitD on muscle strength have examined VitD deficient athletes and not those with adequate or supraphysiological concentrations of VitD. Further studies are necessary to elucidate if supraphysiological dosages of VitD have an ergogenic effect in VitD replete athletes in different sports disciplines.

1.7.4 Vitamin D, calcium and parathyroid hormone interaction

Studies with VitD supplementation generally include the assessment of calcium and parathyroid hormone to guarantee concentration within the recommended range and prevents any adverse effects most typically hypercalcemia or hyperparathyroidism. Parathyroid hormone (PTH) is a hormone secreted by the parathyroid glands in response to low serum calcium levels and regulates bone homeostasis in combination with calcium and VitD. Parathyroid hormone activates the hydroxylation of 25-hydroxyvitamin D (25(OH)D) to the active form, 1,25-dihydroxyvitamin D (1,25(OH)2D), leading to a better intestinal absorption of calcium (142, 143). In regards to calcium, studies have found that efflux from bone is increased when VitD concentration reaches toxicity (144, 145). Whilst excess VitD is a rare clinical condition that involves hypercalcemia that might lead to serious health consequences, such as nephrocalcinosis and renal failure (146), the most common clinical symptoms related to excess of VitD includes: abdominal pain, polyuria, polydipsia, and dehydration (147). A recent study established the cut-off point 26 $ng \cdot mL^{-1}$ of serum total VitD for PTH response to VitD insufficiency and, consequently, for increased risk of bone loss in an equatorial population (148). While the inverse relationship between serum total VitD and PTH levels has been well described, many confounders might influence this relationship such as age, gender, BMI, calcium intake and absorption, sex steroid concentration and kidney function (149-151). Overall, serum levels of calcium and PTH have been used as biomarkers of VitD to prevent intoxication in clinical studies (147).

In summary, the effects of vitamin D on proliferation and differentiation of skeletal muscle tissue in human and animal studies continue to be inconsistent and need to be clarified. At the moment, several studies have suggested that VitD might control protein synthesis, mitochondrial metabolism, and energy production, which may impact on muscle strength, function and performance. More studies are essential to

understand the underlying mechanisms by which VitD acts on human skeletal muscle tissue and clarify how these changes are translated into clinical effects.

The content of this chapter is partially described and discussed in Paper 1 (152): Montenegro KR, Cruzat V, Carlessi R, Newsholme P. Mechanisms of vitamin D action in skeletal muscle. Nutr Res Rev. 2019;32(2):192-204. DOI: 10.1017/S0954422419000064

1.8 Research Justification

1. To date, a comprehensive review of the literature that includes cellular, animal and human studies focused on the mechanisms of VitD action on skeletal muscle function and metabolism has not been conducted.

2. The impact of VitD on skeletal muscle tissue in human and animal studies remain, to a limited extent, contradictory, specifically in regards to proliferation and differentiation, and need to be clarified. Important research data suggests that VitD may regulate protein synthesis, mitochondrial metabolism as well as energy production. Confirming these outcomes in primary human skeletal muscle cells will help to elucidate the role of VitD on muscle metabolism and bioenergetic responses.

3. Importantly, when VitD deficient individuals are supplemented with the most efficient form of VitD (VitD3), improvements are seen in energy metabolism, muscle mass and strength. However, whether similar outcomes occur in VitD sufficient individuals' remains to be elucidated. Therefore, there was a need to investigate the impact of VitD on these outcomes in order to comprehensively assess the evidence to supplement VitD in physically active adults. In addition, long term oral intake of VitD may cause VitD intoxication ($> 200\,nmol \cdot L^{-1}$) (153), justifying the importance of establishing optimal and safe serum concentration of VitD for skeletal muscle function.

1.9 Thesis objectives

1. To carry out a comprehensive review of the literature that summarises the effects of VitD metabolites [cholecalciferol, 25(OH)D, 1,25(OH)2D] on skeletal muscle function and metabolism including animal, *in vitro* and human studies.
2. To investigate the effects of vitamin D3 on muscle metabolism and bioenergetic responses *in vitro*.
3. To investigate the effects of Vitamin D3 on resting metabolic rate, body composition and performance in VitD sufficient physically active adults.

1.10 Research hypothesis

This PhD thesis examined two main hypotheses. The first hypothesis was that VitD treatment modulates muscle metabolism and bioenergetics in primary human skeletal muscle cells. The second hypothesis was that VitD3 supplementation improves resting metabolic rate, body composition and performance in VitD sufficient physically active adults.

Chapter 2 - The effects of Vitamin D3 on human primary skeletal muscle cells

2.1 Introduction

The effects of VitD in skeletal muscle function and metabolism have been reported in various *in vitro* skeletal muscle models including C2C12, L6 cell lines and more recent, primary human skeletal muscle cells (73, 75, 77-81, 86, 91). Previous studies demonstrated that VitD has a crucial role in muscle cell proliferation and differentiation via vitamin D receptor (VDR) in murine C2C12 cells (73-75, 154). Considering animal studies, whole body and also skeletal muscle-specific VDR knockout mice (VDRKO) were identified with muscle weakness and muscle fibre atrophy (76). Taken together, these studies suggest a specific role for the VDR in skeletal muscle regulation (155, 156). Vitamin D acts through various cellular signalling cascades, including the mitogen-activated protein kinase (MAPK) and Akt/mTOR pathway (7, 13). Another pathway of VitD action is through genomic effects, which results in gene expression regulation of proteins that affect contractility and cell regeneration after muscle injury in rats (14).

Vitamin D deficiency can lead to compromised muscle function and related reduced regenerative capacity, impaired immune metabolism, poor bone health and also decreased cardiovascular function in health adults (157). *In vitro* study with human primary skeletal muscle cells could result in clarification regarding the effects of VitD3 on aerobic metabolism and protein synthesis, and could assist in identifying the best dose-effect for muscle function and metabolism. Furthermore, there is no consensus regarding the effects of VitD3 on muscle fibre size and protein synthesis. Hence, the aim of this study is to evaluate the effects of VitD3 on myogenesis, mTOR downstream pathways, protein synthesis, and bioenergetics in primary human skeletal muscle cells. We hypothesize that treating the cells with VitD3 will result in significant and positive changes in the cells metabolism and function.

2.2 Materials and Methods

2.2.1 Optimisation of growth and differentiation of HSMM cells

The recommended seeding density for HSMM cells is 3500 cells/cm^2 (Lonza, 2012, USA; SkMC – Instructions for use). The appropriate amount of medium was added to the vessels (1 ml/5 cm^2, e.g., 15 ml in 75 cm^2) and maintained in 37ºC, 5% CO_2 humidified incubator for at least 30 minutes. Firstly, the cryovial (HSMM 29715; CAT: CC-2580; LOT N° 0000542368; cryopreserved at 08 of April, 2016 and stored

at -180 ºC) was thawed in a 37 ºC bath for less than two minutes and resuspended using a micropipette in a 75 cm^2 flask with warm growth media (CC-3160, Lonza) supplemented with 0.05 ml/ml of foetal calf serum, 50 µg/ml of fetuin (bovine), 10 ng/ml of epidermal growth factor (recombinant human), 1 ng/ml of basic fibroblast growth factor (recombinant human), 0.4 µg/ml of dexamethasone and the media did not contain Vitamin D (Single Quots TM supplements, CC-3244, Lonza).

Cells were sub cultured when they were 60-70% confluent and presented many mitotic cells throughout the flask. For each 75 cm^2 flask sub culturing process, 20 ml of HEPES buffered saline solution (CC-5022) was used to rinse the cells and then 6 ml of trypsin/EDTA (CC-5012 at room temperature) was added and the flask was maintained in the incubator 37 ºC for five minutes. Cells were examined microscopically and when approximately 90 % of the cells were rounded up the flask was tapped to release the majority of cells from the surface. Then, 12 ml of trypsin neutralizing solution (CC-5002) was added at room temperature and quickly transferred to a 15 ml centrifuge tube. Subsequently the cells were centrifuged at 220x g for five minutes to pellet the cells, after that supernatant was discarded and the cells were diluted into the best volume for counting and finally resulting in seeding for experiments. Cells were counted using a haemocytometer and Trypan Blue. Human skeletal myoblasts cells used in all experiments were identified between passages 4 to 8.

The growth medium was changed every second day in order to optimise the use of cells and reagents to all experiments needed. Myoblasts started to form multinucleated myotubes when the culture reached 80-90% confluence. Myotubes are formed by the fusion of several myoblasts after the addition of the differentiation media DMEM: F12 (D8437 Sigma-Aldrich, MO, USA) plus horse serum (2%). Media was changed every second day and after 5 days of differentiation the cells were classified as myotubes (by the presence of at least 3 nuclei per myotube) and all experiments with adult cells were performed at day 6. Vitamin D3 (C1026 50µg calcitriol, Astral Scientific, NSW) was dissolved in 100% absolute anhydrous ethanol (50µg of VitD3 dissolved in 1200µl of ethanol) and aliquot in 100, 50 and 5 µl tubes. It is important to notice that the addiction of vehicle (0.1% ethanol) in the media did not have any effect on cell viability as it has been widely used in scientific research (82). The 100 nM concentration of VitD3 applied in our study was the same optimal dose established in

the majority of publications investigating the effects of the vitamin on skeletal muscle cell function (73, 76-78).

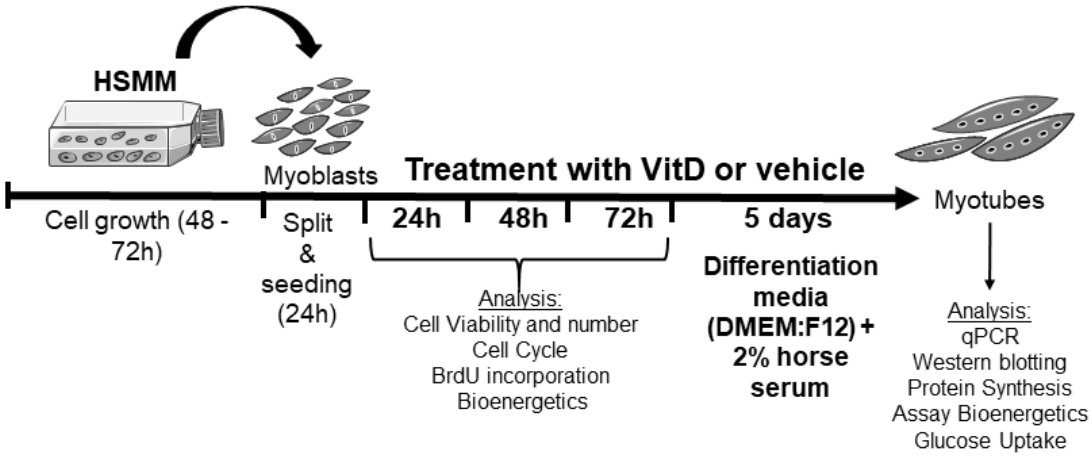


Fig. 1. A schematic presentation of the experimental design using HSMM cells. Myoblasts were grown and culture for 24 h, 48 h, 72 h or 5 days (differentiation process to form myotubes) in the presence or absence of 100 nM of VitD3 (i.e. 1α,25(OH)2D3) or vehicle (0.1% ethanol). Analyses were performed in each time point.

2.2.2 Cell Viability

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This assay involves the reaction with NADH or similar reducing molecules that transfer electrons to MTT (158). Cells were seeded at a density of 20,000 cells/well in a 96 well culture plate and treated with VitD3 (1 nM, 10 nM, 100 nM or 200 nM) or vehicle control (0.01% ethanol) during 24 h or 48 h of VitD3 treatment. For my study, 5 mg/ml of MTT was added to the culture medium at 1:10 dilution and metabolically active cells convert the water-soluble MTT to an insoluble purple formazan. Cells lose the capacity to convert MTT into formazan when they die, thus color formation is a suitable marker of only viable cells. After 4 h of incubation, supernatant was removed and formazan crystals were solubilied with 100 µl of DMSO and, finally the viable cell number was measured via plate reader at a wavelength of 540 nm using a spectrophotometer (EnSpire Multimode Plate Reader, Perkin Elmer, MA, USA). The results are expressed as a percentage of vehicle (ethanol) control.

2.2.3 Cell Cycle

Measuring cell cycle distribution is vital for studying cell growth, senescence and apoptosis. The DNA of mammalian, yeast, plant or bacterial cells can be stained by a variety of DNA binding dye. Cells that are in S phase will have higher amount of DNA than cells in G1 phase. In other words, they will be incorporated correspondingly more dye resulting in higher fluoresce. Specifically, the cells in G2 will present nearly a two-fold difference as cells in G1(159). Cells were seeded at a density of 200,000 cells/well in a 75 cm^2 flask culture and treated with VitD3 100nM or vehicle (0.01% ethanol) or control (growth media only) during 48 h with VitD3. After the treatment with VitD3 (100 nM for 48 h), cells were washed, trypsinised, collected and permeabilised with cold 70% ethanol for 1 h. These cells were subsequently washed and incubated with RNAse (2.0 mg/ml) and propidium iodide (PI, 40 mg/ml) for 40 min at room temperature in microtubes. DNA content was determined by flow cytometry running in a low flow rate, and the proliferation phases (sub-G0, G0/G1, S, G2/M) were determined by FlowJo software.

2.2.4 Bromodeoxyuridine incorporation

Bromodeoxyuridine (BrdU) incorporation assays have been used for a long time to quantify DNA synthesis *in vivo* and *in vitro*. This molecule is incorporated as a thymidine analog into nuclear DNA during S-phase of a cell cycle which can be identified by anti-BrdU antibodies (160). For this study, we have used 100 mmol/ml of the reagent 5 Bromo-2′-Deoxyuridine (Sigma-Aldrich B5002) that is designed for flow cytometry analysis. Cells were treated during 48h with 100 nM of VitD3 or with vehicle (0.1% ethanol) and a control group was maintained with only growth media. Then, after 4 h of treatment with BrdU reagent, cells were washed, trypsinised and fixed with 70 % ethanol for 5 minutes. Subsequently, 500 ul of HCl (1.5 M) was incubated for 30 minutes and rinsed by centrifugation twice (2000 rpm, 5 minutes, 4°C). Then, cells were blocked with 100µl incubation buffer for 10 minutes at room temperature. FITC-conjugated antimouse secondary antibody (150 µl) was added (Alexa fluor 488, green) for 30 minutes. Flow cytometric data was acquired using a FACS LSR Fortessa flow cytometer and data were analysed in the FlowLogic FCS analysis software. Fluorochromo/filter was BrdU FITC B530/30.

2.2.5 Myotubes number and size

Myoblasts were treated with 100 nM of VitD3 or vehicle control (0.1% ethanol) throughout differentiation process (5 days). Next, myotubes were fixed with PFA 4%, then stained during one minute with eosin and subsequently with haematoxylin, washed with Milli-Q water and then counted using 1 Olympus BX-51 upright fluorescent microscope. The diameter was assessed using Image J (diameter measurement, 20 fields with fibres were randomly selected per 6 well plate, 2 wells per sample and the experiment was repeated four times) with 20x magnification and the results were compared. Cells were classified as myotubes when they have at least 3 nuclei within the same cell membrane.

2.2.6 Quantitative real-time PCR

The real-time reverse transcription polymerase chain reaction (RT-qPCR) is currently used for quantitative data analysis in molecular medicine and has become the main technique for the quantification of mRNA (161). This method combines three steps: 1) the reverse transcriptase (RT) conversion of RNA into cDNA; 2) the amplification of the cDNA via the polymerase chain reaction and 3) the quantification of products in real time (161). Cells were seeded in 6 well plate (300.000 cells/well) and treated with 100 nM of VitD3 or vehicle control (0.01% ethanol) during 5 days of differentiation. Firstly, components PCR mix were centrifuge in a 5 ml tube (RT^2 SYBR green mastermix, cDNA synthesis reaction, RT^2 qPCR primer assay and RNase-free water). RNeasy kit (Qiagen, USA) was applied to isolate total RNA from the cells and cDNA synthesis was achieved with the Reverse Transcriptase Mix (Qiagen, USA). The thermal cycle was 42ºC for 15 min, followed by 95ºC for 5 min and mRNA expressions of differentiation genes were detected via SYBR Green based real-time PCR. Validated primers used in this study and pre coated 384-well plate were customised RT2 Profiler PCR Array from Qiagen. The gene panel included: Myogenin, Myosin Heavy Chain 2, Troponin 3, Beta-2 macroglobulin (control). The cycling procedure was as follows: 95 ºC for 10 min, and 40 cycles of 95 ºC for 15 s, followed by 60 ºC for 1 min. Data analysis was performed using the $\Delta\Delta C_\gamma$ method and relative gene expression was determined by normalizing the expression of each target

gene to beta-2 macroglobulin (control) which was stable in the different culture conditions.

2.2.7 Determination of Insulin Responsiveness by Western blotting

Western blotting is a technique by which researchers can identify the presence and amount of specific proteins from a mix of proteins extracted from cells or tissues (163). The process consists in three main parts: 1) separation by molecular weight after denaturation; 2) transfer to a solid support (gel electrophoresis), and 3) and subsequent application of a primary and secondary antibody to detect and visualize the target protein (162). Human skeletal muscle myoblasts cells (5×10^5) were differentiated as described above (section 2.2.1) and they were treated with 100 nM of VitD3 or vehicle control (0.1% ethanol). After five days, cells were washed with PBS and treated with 100 nM insulin from bovine pancreas (I6634 Sigma- Aldrich, MA, USA) or without (control group). Myotubes cells were lysed in RIPA buffer with protease and phosphatase inhibitors (Cell Signaling Technology, MA, USA) and were measured using BCA assay method (Pierce™, Thermo Scientific, MA, USA). The same amount of protein was mixed with lithium dodecyl sulphate (LDS) sample buffer and the reducing agent dithiothreitol (DTT, Novex, Life Technologies, CA, USA). Proteins (20 μg/lane) were exposed to SDS–PAGE, using 4 – 20% Tris-glycine gels, and the wet method was used to transfer onto nitrocellulose membrane. This method consists the immersion of a gel-membrane sandwich in an upright tank of transfer buffer with vertical platinum electrodes. The was used for Membrane was blocked with 3% BSA (Amresco, OH, USA) and PBS-Tween (PBST, 1% w/v) buffer using the quick immunoblot vacuum system. The antibody incubation was performed using SNAP i.d (EMD Millipore, MA, USA), in which the membranes were blocked for 45 min and then incubated overnight at 4ºC, with specific primary antibodies. All the antibodies were purchased from Cell Signalling AKT (#9272S), p-AKT (Ser473, #9271S), GAPDH (#D16H11), mTOR (#2972S), (P-mTOR Ser2448, #2971S), S6 (#2217S), P-S6 (Ser235/ 236, #4858S), GSK3β (#9315S), P-GSK3β (Ser9, #9336S), except for VDR (#13133, Santa Cruz). Finally, protein bands were then visualised and quantified based on densitometry analysis using Molecular Imager® Gel Doc™ XR System v5.2.1 (Bio-Rad Laboratories, CA, USA). here were no differences in the expression of GAPDH (protein control) between the experiments.

2.2.8 Bioenergetics (Seahorse XFe96 flux measurements and analysis)

Myoblasts and myotubes cells were seeded at an optimised density of 20,000 cells/well in a XFe96 cell culture plate and left overnight to adhere and recover following trypsinisation. Myoblasts were treated with 100 nM of VitD3 or vehicle control (0.01% ethanol) for 24 h or differentiated for 5 days with VitD3 or vehicle control. To measure oxygen consumption rate (OCR) we used the Agilent Seahorse XF Cell Mito Stress Test Kit (Seahorse Bioscience, USA) which works by measuring basal respiration initially, followed by adding a series of mitochondrial modulators and measuring the cellular responses. Firstly, the ATP synthase inhibitor oligomycin is added, followed by Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) which is an uncoupling agent. Lastly, a combination of Antimycin A and Rotenone is added (inhibitors of mitochondrial complexes I and III, respectively). The optimised injection strategy consisted of vitamin D3 (100 $nmol \cdot L^{-1}$), oligomycin (1 µM), FCCP (0.75 µM), and rotenone/antimycin A in combination (1 µM each). The acute injection strategy consisted of Vitamin D3 (100 $nmol \cdot L^{-1}$), glucose (25 mM), oligomycin (5 µM), followed by 200 mM 2-deoxyglucose (2DG).

The basal OCR represents the net sum of all mitochondrial processes in the cell capable of consuming O2 and was determined by subtracting the minimum OCR following addition of rotenone/antimycin A from the last OCR measurement recorded prior to addition of oligomycin. Then proton leak was determined as the minimum measurement after oligomycin injection through to the measurement prior to FCCP) – minimum non mitochondrial respiration. OCR related to ATP production was calculated by the difference between the proton leak and basal respiration. Coupling efficiency percentage was calculated by the rate of ATP production dependent OCR by the basal respiration and multiplied by 100. Maximal respiration is the result of maximum OCR in response to FCCP (subtracting the non-mitochondrial respiration). Spare respiratory capacity can be described as maximal respiration subtracted by basal respiration. Basal glycolysis in the presence of 0 mM glucose was determined by the last PPR measurement recorded prior to addition of 25 mM glucose.

To measure extracellular acidification rate (ECAR) we used the Agilent Seahorse XF Glycolysis Stress Test Kit (Seahorse Bioscience, USA) and cells are incubated in the medium with pyruvate and glutamine and without glucose, then the basal PPR is measured.

The first injection is a saturating concentration of glucose, where the cells catabolize using the glycolytic pathway. Glycolytic response to 25 mM glucose was calculated by subtracting the maximum PPR following addition of glucose from the last PPR measurement prior to addition of glucose. Glycolytic capacity was measured by subtracting the minimum PPR following 2DG addition from the maximum PPR after injection of oligomycin. Glycolytic reserve was calculated glycolytic capacity-glycolytic response to 25 mM glucose. Each treatment was measured in at least triplicate wells.

2.2.9 Protein synthesis assay

15×10^4 myoblasts were seeded in a 6 well plate with coverslips coated with 0.05 mg/ml of poly-d-lysine (Sigma-Aldrich) for 1 h. These cells were treated with 100 nM of VitD3 or vehicle for 5 days and differentiated into myotubes. ClickiT ® OPP (O-propargyl-puromycin) kit was performed in myotubes, adding 20 μM of OPP (Thermofisher Scientific) to the cells and incubated for 30 min. Cells were washed in PBS and then fixed and permeabilised using 3.7% formaldehyde in PBS followed by a permeabilization step using 0.5% Triton® X100. A reaction cocktail was prepared to contain Alexa-Fluor-594 (Thermofisher Scientific) and was conjugated to OPP as described in the manufacturer's instructions. Cells were washed again with a reaction rinse buffer and then stained with HCS NuclearMask™ Blue Stain. Cells were washed with PBS, water and then prepared in slides with a drop of ProLong™ Diamond Antifade Mountant (Thermo Fisher Scientific) protected from the light. Images were acquired in an A1+ confocal microscope (Nikon, Instruments, Tokyo, Japan) and to observe cells and capture images I used a Plan Apo 60X objective lens. Myotube was defined by the existence of at least two nuclei within a continuous cell membrane and at least 30 pictures were taken in randomly chosen microscope fields. Data analysis was performed using Image J software (National Institutes of Health; available at http://rsb.info.nih.gov/ij). The mean fluorescence of each myotube was individually selected and counted to calculate and compared with the average of the vehicle group.

2.2.10 Glucose Uptake unstimulated or stimulated with insulin

This assay is based on direct incubation of mammalian cells with a fluorescent deoxyglucose analogue 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-

deoxyglucose (2-NBDG) followed by flow cytometry detection of fluorescence produced by the cells. This technique allows direct and sensitive measurement of glucose uptake cell by cell (163). One hundred thousand cells were seeded in a 24 well plate with growth medium on day 1 and treated with 100 nM of VitD3 or vehicle control (0.01% ethanol) for 24 h while myotubes were differentiated for 5 days with VitD or vehicle control. Then, cells were washed with PBS and treated for 20 minutes with or without 100 nM insulin and 500 µl of 2-NBDG. Media with NBDG and media control was removed from the wells and washed with PBS. Then, cells were trypsinised and transferred to and Eppendorf with 5µl of PI (0.5 mg/ml) for a minute. Finally, tubes were centrifuged and supernatant was discarded and cells were diluted in 150 µl of PBS. The intensity of fluorescence was detected by FACS LSR Fortessa flow cytometer and data were analysed in the FlowLogic FCS analysis software. Median 2-NBDG fluorescence were obtained after gating for PI negative cells. Glucose uptake of the cells treated with VitD3 and challenged for 20 minutes with 100 nM insulin were compared with unstimulated cells.

2.2.11 Glycogen content

Glycogen content can be measured by an acid-hydrolysis method (164). Basically, the hydrolysis product of glycogen also known as glucose, is converted into glucose-6-phosphate (G-6-P) by hexokinase using ATP. With the supply of NADP, G-6-P is further converted into 6-phosphogluconic acid by G-6-P dehydrogenase (G-6-PDH), while production of NADPH can be measured spectrophotometrically. A research laboratory in the USA (Zhang, 2012) has used this method to demonstrate for example that liver glycogen levels are significantly elevated in diabetic Perk knockout mice (165). In summary, I have adapted this protocol to measure glycogen content in primary human skeletal muscle myoblasts and myotubes. Cells were seeded at an optimised density of 2×10^6 in a 75 cm^2 flask for each group (vehicle control, 10 nM VitD3, 100 nM VitD3 and glucose starvation). Myoblasts were treated 22 h with 0.1% ethanol (vehicle control) or VitD3 while myotubes were treated for 5 days. Then, for 2h cells were exposed to a media without glucose or were left with the same media in order to achieve glucose starvation. Cells were trypsinized and removed from the flask, washed and transferred to a 1 ml Eppendorf tube. Subsequently, 300 µl of HCl or NaOH were added to the cells to start the breakdown acid-hydrolysis reaction. After

1 h boiling in water, NaOH and HCl were used to neutralize the reaction. Finally, the supernatants were then collected and assayed by Amplex Red Glucose/Glucose Oxidase Assay Kit (Life Technologies, Gaithersburg, MD, USA) to measure total glucose. Final fluorescence was detected at 545 nm using a fluorescence capable spectrophotometer (Enspire Multimode Plate Reader, PerkinElmer, USA) for glucose. A glucose standard control curve was also performed to allow the deriving of glucose concentrations of the unknown samples from the fluorescent readings.

2.2.12 Statistical analysis

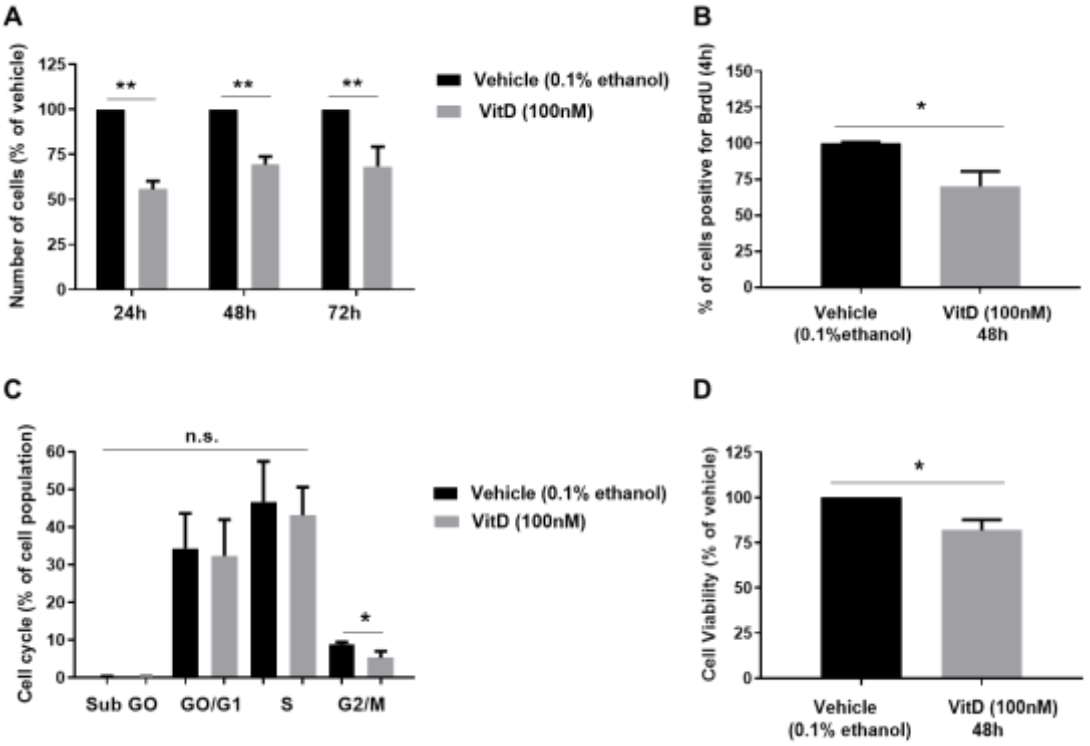
Student's t-test was applied when only two experimental groups were compared to evaluate statistical significance (i.e., VitD3 group vs. vehicle group). One-way ANOVA with a Bonferroni post hoc test was applied when more than two experimental groups were compared, and two-tailed statistical significance was set at (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$). All analyses and graphs were performed using GraphPad Prism 6 software (GP Prism, San Diego, CA, USA).

2.3 Results

2.3.1 Effects of VitD3 on proliferation in HSMM

The effects of VitD3 in primary human skeletal muscle cells proliferation were evaluated by four different methods: cell counting, cell viability (MTT assay), cell cycle and BrdU incorporation. VitD3 treatment decreased ($p < 0.01$) by 30% the number of cells when compared with vehicle group during 24 h, 48 h and 72 h (Figs. 2A-D, 3 A-B and E). To confirm these results cellular metabolic activity was performed by MTT assay. Cells treated with VitD3 reduced ($p < 0.05$) the metabolic activity when compared with vehicle group (Fig. 2D). Cell cycle analysis was also performed to identify if VitD3 has any effect on the cells proliferation. Treatment with VitD3 did not change the sub-GO, GO/G1 and S phases of the cell cycle, however, we observed a significant decrease ($p < 0.05$) in G2/Mitosis, a phase that prepares cells for mitosis when compared with vehicle (Fig. 2C). The quantification of cell proliferation was measured by BrdU assay. BrdU is incorporated into active dividing cells and characterizes cell proliferation. Cells treated with VitD3 significantly decrease ($p < 0.05$) BrdU incorporation when compared with vehicle (Fig. 2B).

Fig. 2. Anti-proliferative effects of VitD3 in HSMM cells. Myoblasts were treated during 24 h, 48 h or 72 h with 100 nM of VitD3 or vehicle (0.1% ethanol) were counted using a hematocytometer (A). Quantification of cell proliferation in HSMM using BrdU assay (percentage of BrdU positive cells were counted) in cells treated with VitD3 or vehicle (0.1% ethanol) during 48 h (B). Cell cycle analysis measured by flow cytometry with PI staining after 48 h of treatment with VitD3 or vehicle (0.1% ethanol) (C). Cellular metabolic activities of myoblasts treated with VitD3 or vehicle (0.1% ethanol) during 48 h measured by the MTT (D). All the experiments were repeated ≥3 times and compared using a t-test. The data are presented as mean ± S.E.M. *p < 0.05;



**p < 0.01 n.s. = non-significant.

2.3.2 Effects of VitD3 on differentiation in myotubes

Human skeletal muscle myoblasts were categorised for their ability to differentiate into multinucleated myotubes (Fig. 3C and D). Cells treated with VitD3 over five days of differentiation had an increase in the number of cells per field ($p < 0.05$), and also in diameter ($p < 0.001$), when compared with cell treated with vehicle (Fig. 3F and G). Myotubes were further examined for the expression of various markers of adult skeletal muscle cells such as Myogenin (MYOG), Troponin T type 1 (TNNT), and

Myosin heavy chain-2 (MYH2). Myosin heavy chain (MHC) isoforms are markers of differentiation expressed during muscle development (99). Myotubes were treated during differentiation (5 days) with 1α,25(OH)2D3 or vehicle and markers of differentiation were assessed by qPCR. VitD increased ($p < 0.01$) the gene expression of Myogenin and Troponin T type 3 (Fig. 4A and B) when compared with vehicle group. On the other hand, levels of myosin heavy chain 2 reduced ($p < 0.01$, Fig. 4C).

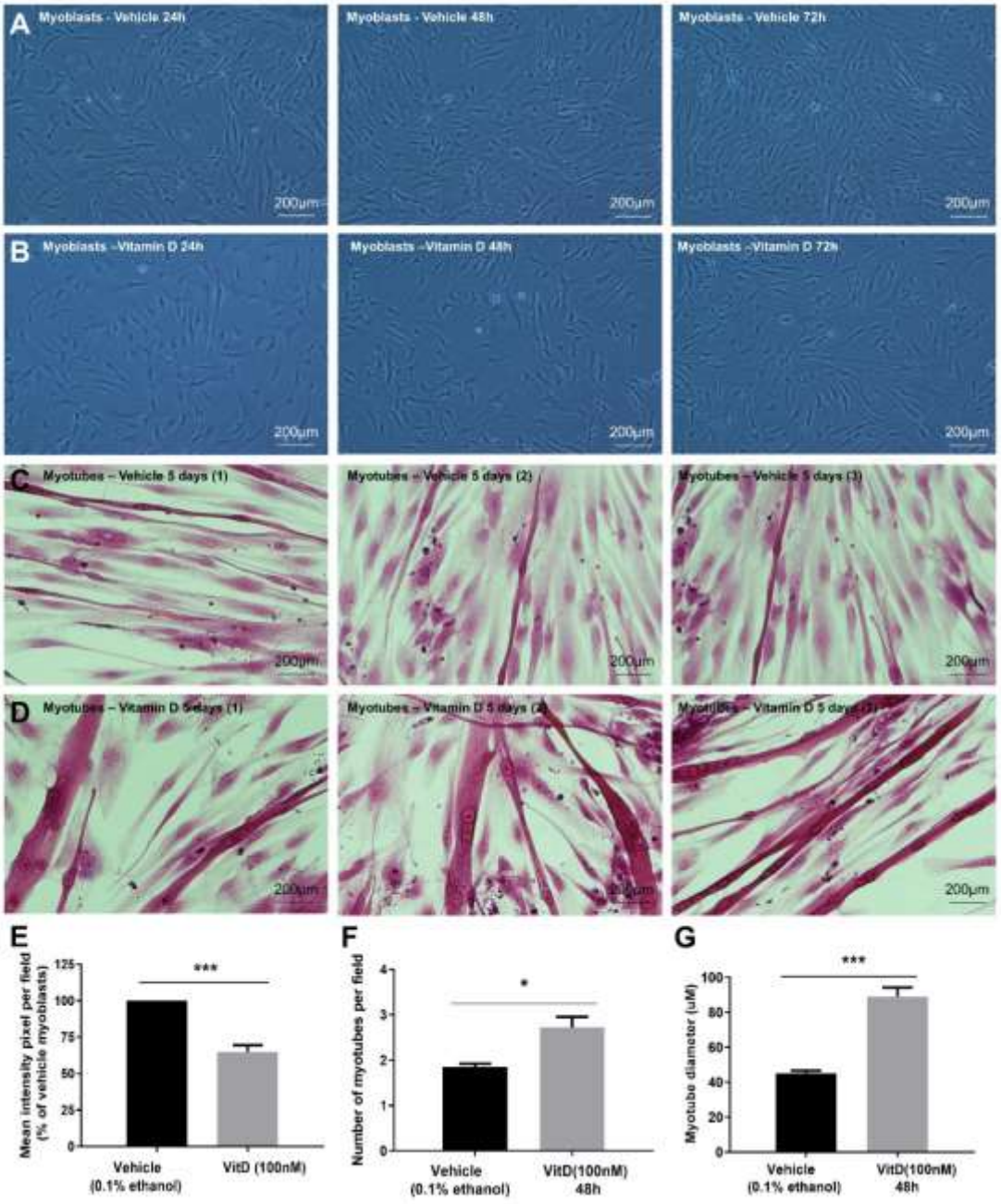


Fig. 3. Cultures of myoblasts and myotubes in the presence of VitD3. HSMM propagated in growth medium day 2 after thawing and then were treated with vehicle

(0.1% ethanol, 3A) or 100 nM VitD3 (3B) for 24 h, 48 h and 72 h - 20x magnification are illustrated in Fig. 3 (n=3). Percentage of confluence was calculated considering the mean intensity of pixels per field using image J. I have analysed 6 fields for each group (24 h, 48 h and 72 h VitD3 or Vehicle) and combined the time-points for the final analyses and compared using a t-test (E). Effects of Vitamin D on human skeletal muscle myotubes number and size are illustrated in Fig. 3C and D (Vehicle - 0.1% ethanol and myotubes treated during 5 days with 100 nM of VitD3. At day 6, there were notably higher number (D) and diameter of myotubes per field (20x magnification) (F and G) in VitD3 group compared with vehicle group using t-test *p < 0.05; ***p < 0.001.

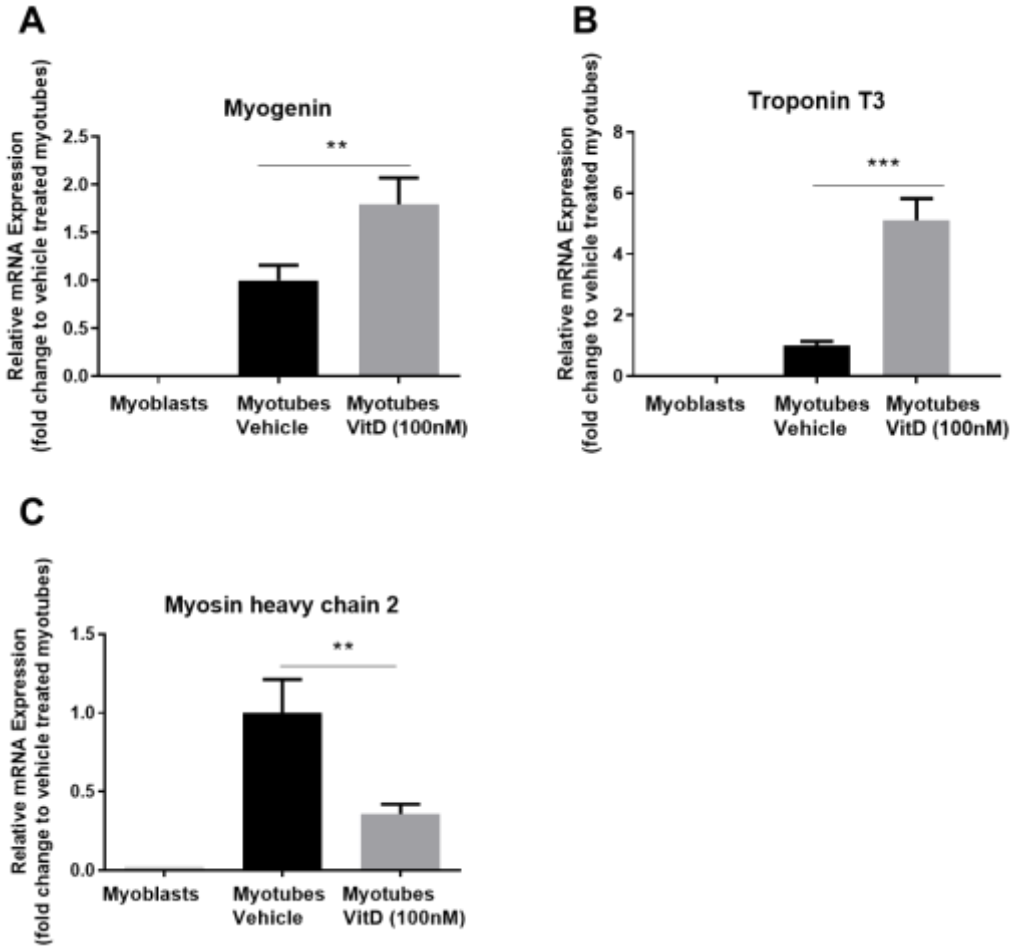
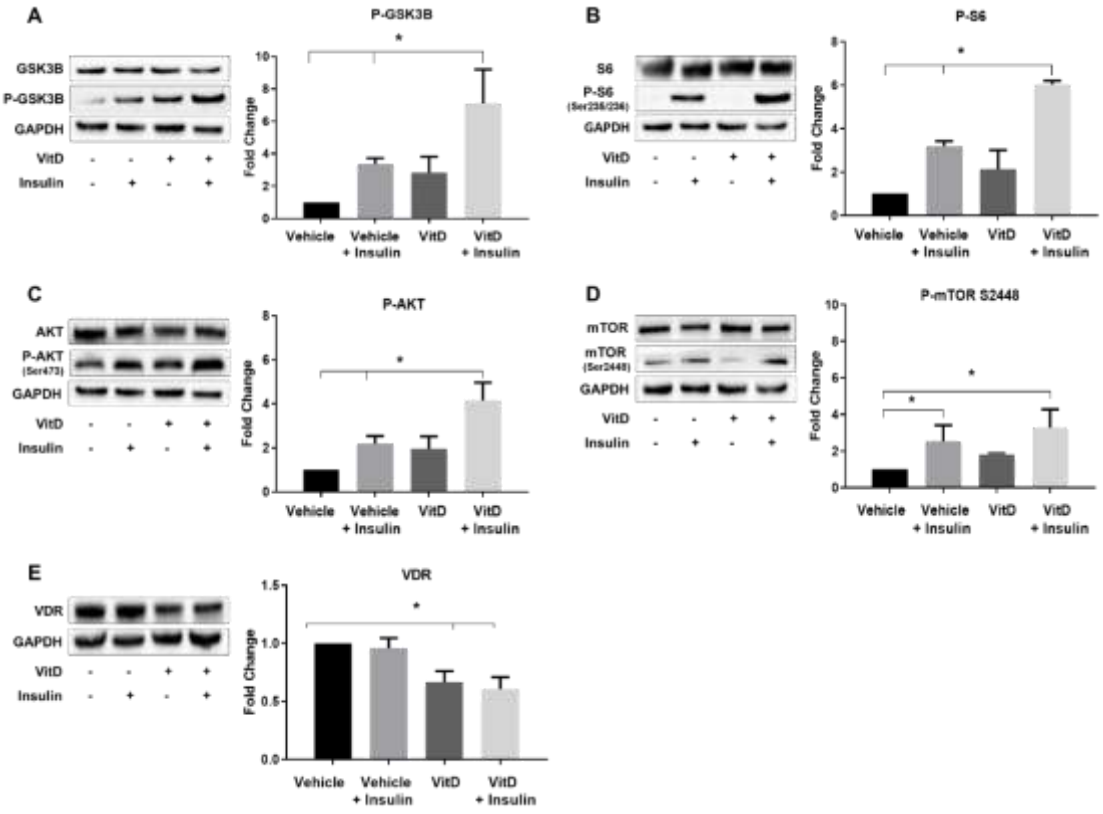


Fig. 4. VitD3 promotes myogenic differentiation in HSMM cells. Myoblasts were treated with 100 nM of VitD3 or vehicle (0.1% ethanol) for 5 days and skeletal muscle (i.e. Myotubes) gene expression of differentiation factors were investigated. Myogenin (A), Troponin T3 (B) and Myosin Heavy Chain 2 (C) were detected by qPCR. The

data is presented as mean ± S.E.M. **p < 0.01, ***p < 0.001 (n=3) and was compared using t-test.

2.3.3 Effects of VitD3 in insulin responsiveness in myotubes

Insulin regulates several intracellular pathways by stimulating AKT and mTOR downstream signalling cascades, which results in an increase in protein synthesis (166). Consequently, I investigated whether VitD3 could enhance the anabolic pathway mediated by insulin. Human skeletal muscle myoblasts cells were treated



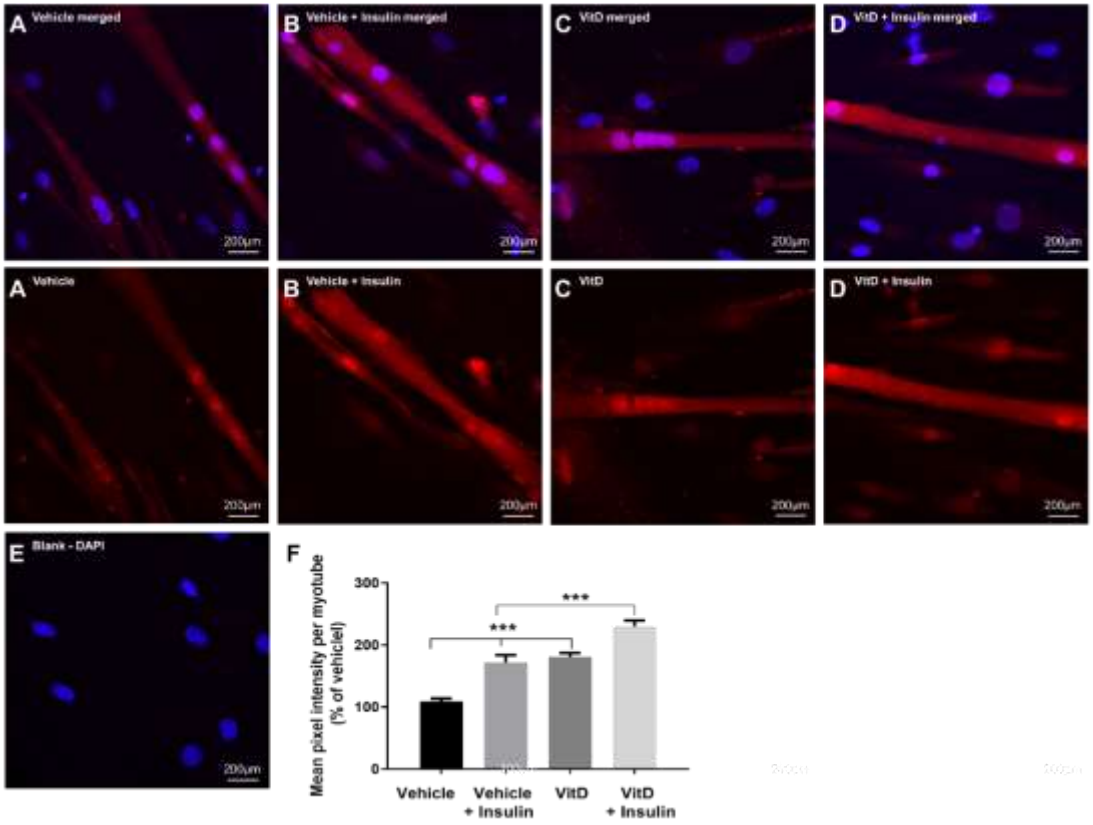
with 100 nM of VitD3 for 5 days until the myotubes formation. Then, myotubes were stimulated with 100 nM of insulin during 20 minutes. In the presence of VitD3, myotubes had higher ($p < 0.05$) phosphorylation level of GSK3β (Fig. 5A), ribosomal S6 protein (Fig. 5B), and AKT (S473, Fig. 5C), than vehicle + insulin group. The phosphorylation of mTOR (S2448) was higher ($p < 0.05$) in the vehicle + insulin and also VitD3 + insulin groups than vehicle group only (Fig. 6D). Myotubes treated with VitD3 had a reduced ($p < 0.05$) expression of VDR (Fig. 5E).

Fig. 5. mTOR downstream pathway is enhanced by VitD3 in myotubes. HSMM cells were treated with 100 nM of VitD3 or vehicle (0.1% ethanol) for 5 days and then

stimulated with insulin for 20′). Phosphorylation level of GSK3β (Fig. 5A), ribosomal S6 protein (Fig. 5B), mTOR (Fig. 5D), AKT (S473, Fig. 5C) and VDR (Fig.E) were compare between vehicle + insulin group vs. VitD3 + insulin group. Protein levels were evaluated in myotubes by immunoblot analysis and compared using one-way ANOVA. Data are presented as mean ± SEM. *$p < 0.05$; ($n \geq 3$).

2.3.4 Effects of VitD3 in protein synthesis

Protein expression was identified by the addition of Click-iT® OPP (Opropargyl-



puromycin) to actively growing cells and marked with Alexa Fluor® 594 (red). Insulin ($p < 0.001$) and VitD3 group ($p < 0.001$) rise mean pixel intensity of fluorescence per cell, suggesting an increase in protein synthesis, when compared with vehicle group only (Fig. 6A, 6B and 6C). Also, it was observed a synergistic effect of VitD3 + insulin, with respect to increased protein synthesis when compared with vehicle + insulin group ($p < 0.001$) (Fig. 6D).

Fig. 6. VitD3 increases protein synthesis in myotubes.

Representative immunofluorescence analysis of myotubes protein synthesis after 5 days with 100 nM of Vitamin D or vehicle (0.1% ethanol) (n=3). Changes in protein levels were detected by the addition of Click-iT® OPP (O-propargyl-puromycin) to

actively growing cells and stained with Alexa Fluor® 594 (red). A) Vehicle; B) Vehicle + 100 nM of Insulin; C) 100 nM of VitD3; D) VitD3 + 100 nM of insulin; E) Blank + Dapi (nuclear stain blue). The graph quantifies the mean pixel intensity of Click-iT® OPP signal per cell in comparison with percentage of vehicle (F). The data is presented as mean ± S.E.M and was compared using one-way ANOVA ***$p < 0.001$ (n=3).

2.3.5 Effects of VitD3 in mitochondrial oxygen consumption in myoblasts and myotubes human skeletal muscle cells

Myoblasts were treated with 100 nM of VitD3 or vehicle (0.1% ethanol) for 24 h or for 5 days in myotubes. Cellular oxygen consumption rate (OCR) was measured in myoblasts (Fig. 7A) and myotubes (Fig. 8A). Myoblasts treated with VitD3 during 24 h increase ($p < 0.01$) maximal respiration, spare respiratory capacity and ATP production, when compared with vehicle group (Fig. 7D, E and G). No significant changes were observed in basal OCR, proton leak and glycolytic activity after 24 h of VitD3 treatment (Fig. 7B, C, F and H). Myotubes treated with VitD3 also had a higher basal respiration ($p < 0.001$) (Fig. 8B), maximal respiration and spare respiratory capacity also increased ($p < 0.01$) when compared with vehicle (Fig. 8C and E; $p < 0.05$). Non-mitochondrial respiration, proton leak and ATP production did not change between groups (Fig. 8D, F and G).

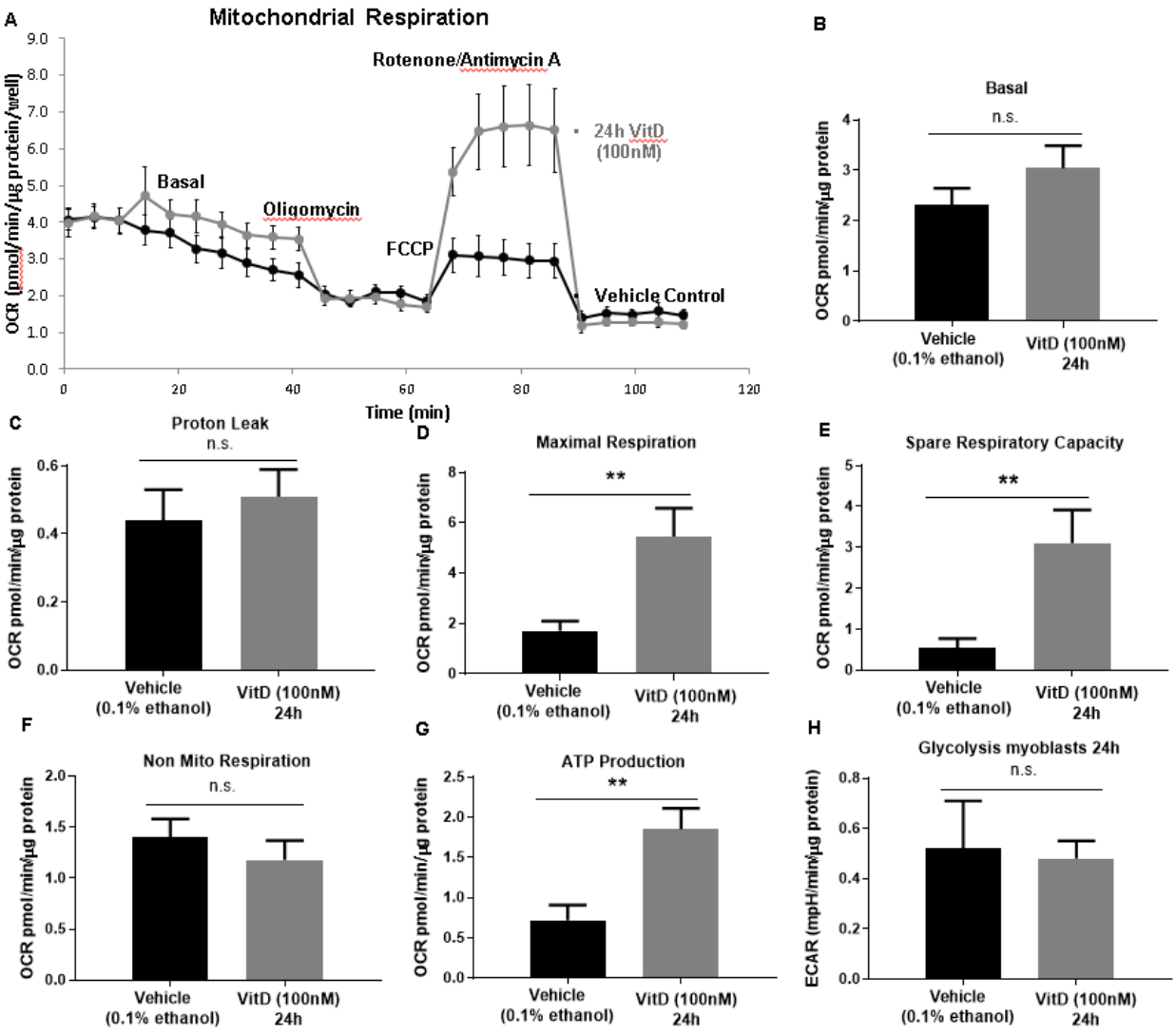


Fig. 7. Bioenergetics in myoblasts is affected by 100 nM of VitD3 during 24 h. Following this, cells were subjected to extracellular flux analysis using Seahorse Biosciences XFe96 Flux Analyzer. Oxygen consumption rate (OCR) profiles were determined after injection of 25mM glucose, followed by 2 µM oligomycin, 1 µM FCCP and 1 µM each of rotenone and antimycin A, enabling generation of a mitochondrial stress profile (A–G). Glycolysis was then determined (H). Data is presented as mean ± SEM compared using a t-test (n=3); *p < 0.05, **p < 0.01; n.s. = non-significant.

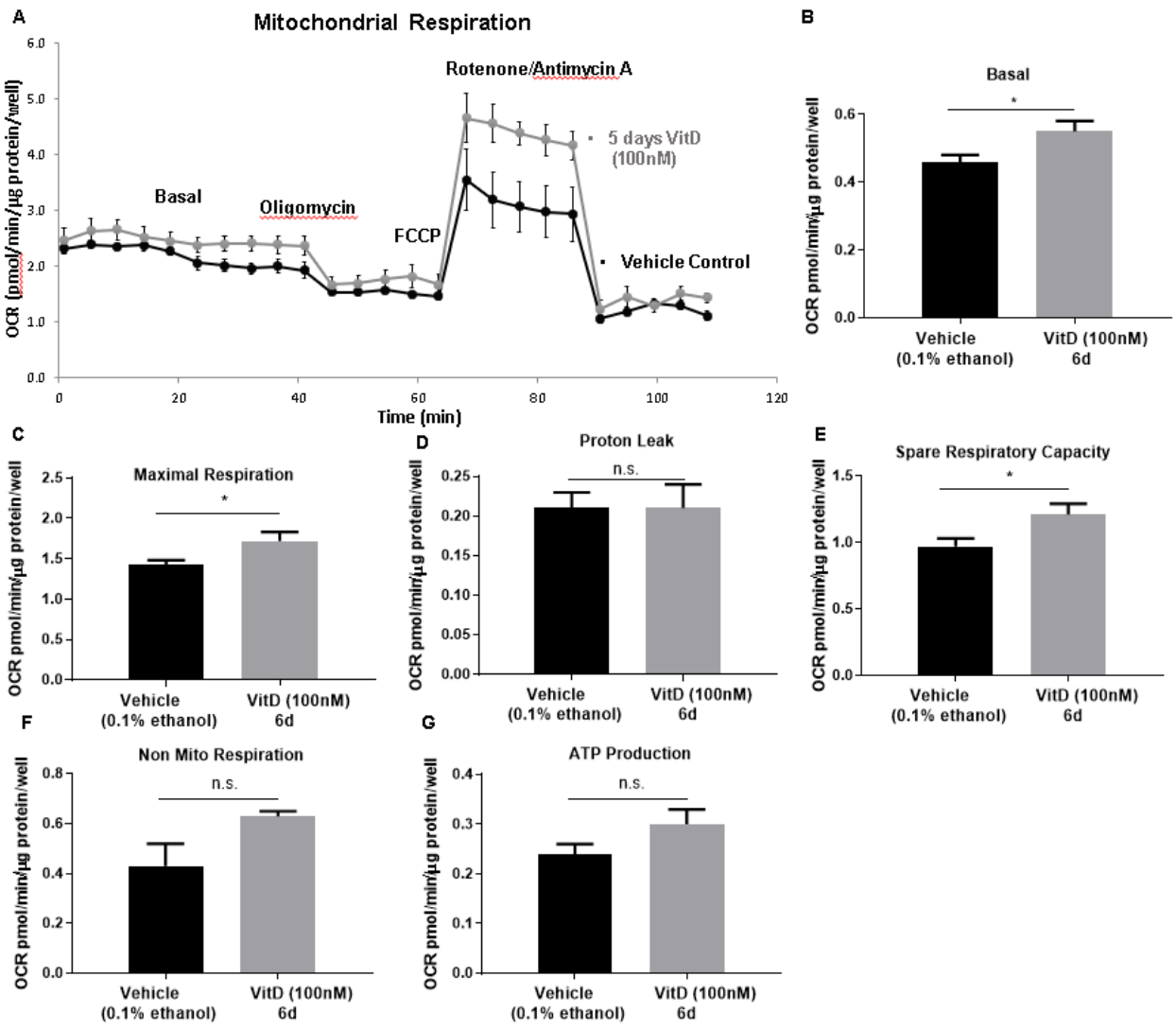


Fig. 8. Bioenergetics in myotubes is affected by 100 nM of VitD3.

Following this, cells were subjected to extracellular flux analysis using Seahorse Biosciences XFe96 Flux Analyzer. Oxygen consumption rate (OCR) profiles were determined after injection of 25mM glucose, followed by 2 µM oligomycin, 1 µM FCCP and 1 µM each of rotenone and antimycin A, enabling generation of a mitochondrial stress profile (A–G). VitD3 treatment increased Maximal Respiration and Spare Respiratory Capacity OCR. Data is presented mean ± SEM compared using t-test (n=3). *$p < 0.05$; n.s. = non-significant.

2.3.6 VitD3 does not significantly alter glucose uptake and glycogen content in myotubes cells

Myotubes treated with VitD3 or vehicle were challenged for 20 min with 100 nM of insulin. Glucose uptake was determined and was higher in the vehicle + insulin group ($P < 0.01$), when compared with vehicle (Fig. 9B). However, no significant changes were observed in cells treated with VitD3 (Fig. 9A and B). Also, VitD3 treatment did not affect glycogen content in myoblasts and myotubes measured by and acid-hydrolysis reaction (Additional results – figure 12). Significant difference was found between vehicle vs glucose starvation group, confirming that the starvation protocol was efficient (*$p<0.05$).

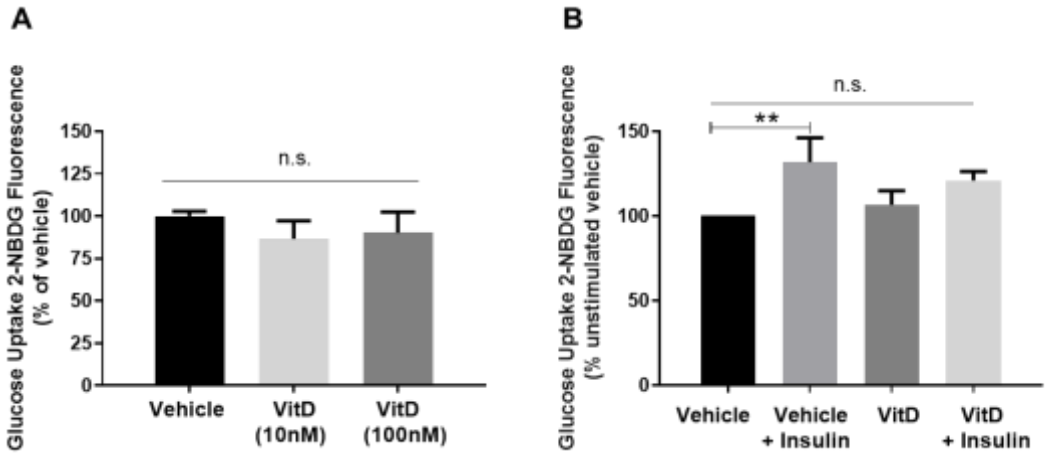


Fig. 9. VitD3 does not affect glucose uptake in myotubes.

2-NBDG uptake was determined after 5 days of treatment with VitD3 100 nM or vehicle (0.1% ethanol) Fig. 9A. Myotubes were also challenged with 100 nM of insulin for 20 min after 5 days of treatment with 100 nM VitD3 or vehicle (0.1% ethanol) (Figure B). The data is presented as mean ± S.E.M and was compared using one-way ANOVA. **$p < 0.01$; n.s. = non-significant (n=3).

2.3.7 Additional results

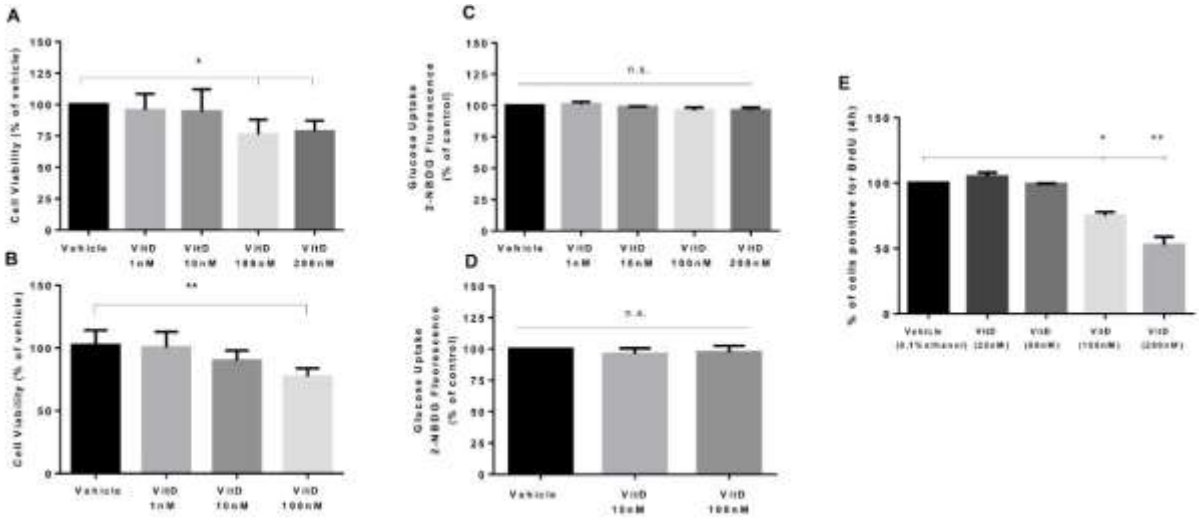


Figure 10. Dose response experiments with VitD3 in HSMM cells. Cellular metabolic activities of myoblasts (A) and myotubes (B) treated with VitD3 or vehicle (0.1% ethanol) during 48h measured by the MTT (B). Experiments were repeated three times and compared using a t test. 2-NBDG uptake was determined in myoblasts after 48h (C) and myotubes 5 days (D) of treatment with VitD3 100nM or vehicle (0.1% ethanol. The data is presented as mean ± S.E.M (n=3) and was compared by one-way ANOVA. n.s. = non-significant. Quantification of cell proliferation in HSMM using BrdU assay (percentage of BrdU- positive cells were counted) in cells treated with VitD3 or vehicle (0.1% ethanol) during 48h (E).

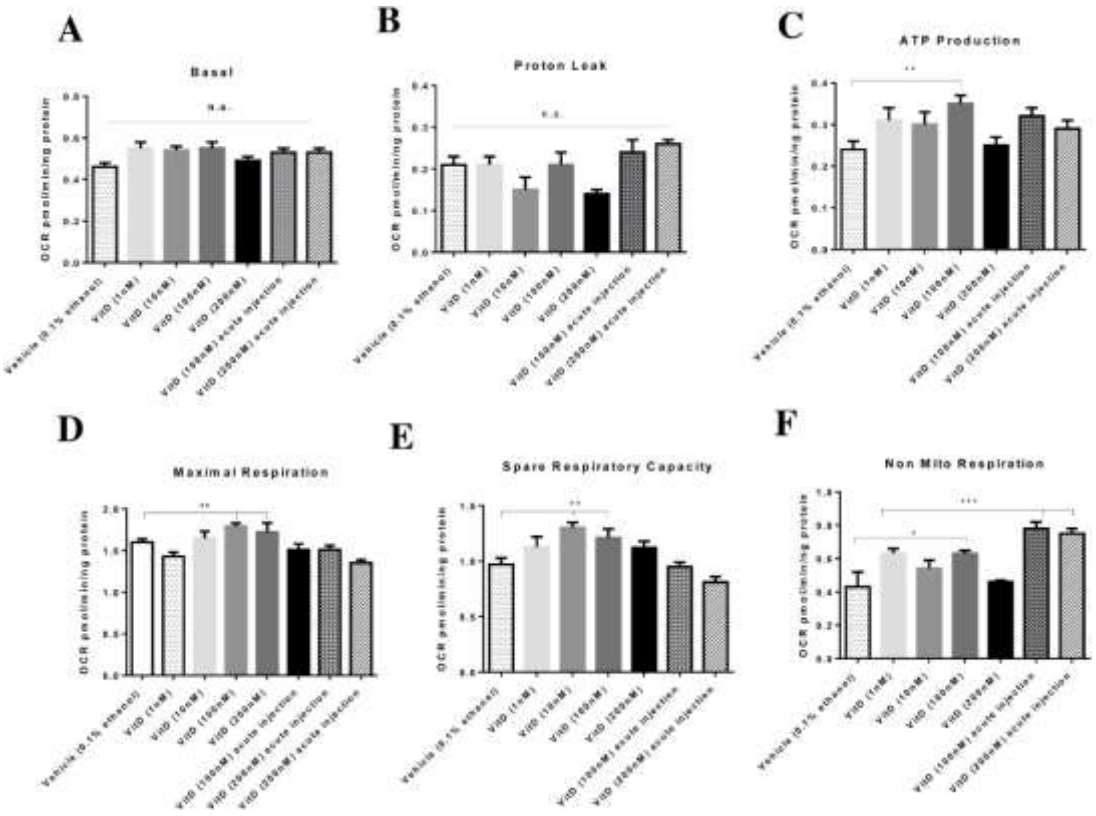


Figure 11. Bioenergetics in myotubes is affected by 100nM of VitD3.

VitD3 increases maximal OCR, spare respiratory capacity and ATP production in myotubes after 5 days of treatment. Data represent mean ± SEM (% of vehicle), ($n \geq 3$). Cells were subjected to extracellular flux analysis using Seahorse Biosciences XFe96 Flux Analyzer. Oxygen consumption rate (OCR) profiles were determined after injection of 25 mM glucose, followed by 2 μM oligomycin, 1 μM FCCP and 1 μM each of rotenone and antimycin A, enabling generation of a mitochondrial stress profile (A-F); *$p<0.05$, ** $p<0.01$; ***$p<0.001$; n.s.= non-significant.

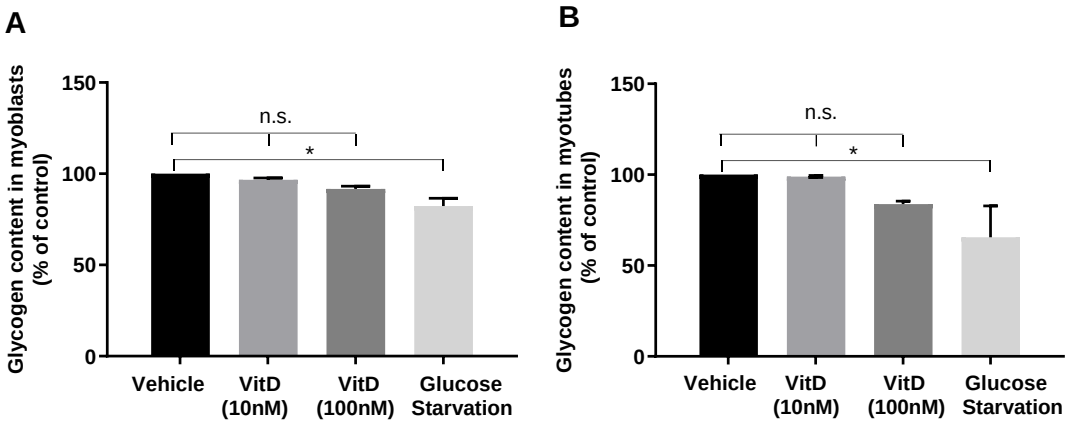


Figure 12. VitD3 does not affect glycogen content in myoblasts and myotubes. Glycogen content was measured after 24h or 5 days of treatment with VitD3 100 nM or vehicle (0.1% ethanol) in myoblasts (A) and myotubes (B). The data is presented as mean ± S.E.M and was compared during one-way ANOVA. No difference was found between vehicle and VitD3 groups; n.s. = non-significant); vehicle vs glucose starvation group *$p<0.05$ (n=3).

2.4 Discussion

The current knowledge concerning VitD3 actions in the skeletal muscle system goes much further than its conventional roles established in Ca^{+2} and phosphate homeostasis. In the study reported here, I hypothesised that primary skeletal muscle cells treated with VitD3 would have a significant change in muscle cell metabolism and in the development of muscle fibres. The effects of VitD3 on proliferation, differentiation, protein synthesis and bioenergetic parameters in primary human skeletal muscle myoblasts and myotubes cells were examined. The main results are summarised as four key outcomes: 1) proliferation (Figures 2 and 3); 2) differentiation (Figures 3 and 4); 3) expression of proteins involved in mTOR pathway (Figure 5 and 6); and 4) bioenergetics (Figure 7 and 8).

The first initial stage of myogenesis and the final stage proliferation (myoblasts) and differentiation (myotubes) respectively were influenced by the treatment with 100nM of VitD3 to the culture media. Proliferation was assessed by cell counting, cell viability, cell cycle (DNA content), and BrdU incorporation method (Figures 2A-D and 3A-B, 3E). Overall, myoblasts proliferation was significantly inhibited by the treatment with VitD3 during 24 - 72 h. Comparable antiproliferative effects were also

found by Olsson et al., (78) after treating human myoblasts with 1 or 100 nM of VitD3 during 48 h. Similar results were observed in other studies that investigated the effects of VitD3 in murine cell lines, such as the C2C12 (73-78). It is important to notice, as we have tested cell viability and counted the number of death cells, it was confirmed that the inhibition of proliferation by VitD3 was not related to enhanced toxicity, an outcome also reported by others (82).

One possible explanation for the antiproliferative effects of VitD3 might be the maintenance of cell quiescence, conserving a stem cell population and stimulating self-renewing capacity specially for damage-repair process (167-169). For instance, Owens et al., (80) have found important effects of VitD3 in muscle regeneration after damage. In this last study, human skeletal muscle derived myoblast cells from biopsies were subjected to mechanical wound injury, allowing the study of cell migration, regeneration and hypertrophy. The results demonstrated that 10 nM of VitD3 enhanced muscle cell migration velocity, distance and increased myotube hypertrophy at 7- and 10-days post-damage (80).

The results on the cell cycle illustrated that VitD3 acts by inhibiting cell proliferation precisely on G2/Mitosis phase. Similarly, 100 nM of VitD treatment inhibited proliferation of thyroid cancer stem-like cells (170). Generally, G2/Mitosis phase is the most affected in the cell cycle, specifically the phosphorylation of histones and the condensed chromosomes (170).

The development of myotubes through the fusion of myoblasts into multinuclear myotubes is confirmed by higher levels of late differentiation transcription markers, such as Myogenin (Figure 4A) and Troponin T type 3 (Figure 4C) was confirmed in this study (167-169). On the other hand, the levels of myosin heavy chain 2 significantly decreased. Jiang and collaborators (171) have reported that MyHC2 promoter activity is positively controlled by an increased activity of nuclear factor of activated T cells transcription factor (NFAT) through calcineurin. Comparing with my study, it is possible that GSK3 activity inhibited MyHC2 gene expression by reducing NFAT. Owens, Sharples (80) also found an increased in myotube fusion and differentiation after VitD3 treatment. This last study has used human primary muscle cells obtained by biopsies from active adults (80).

Contrasting results were reported in another study where VitD3 inhibited myoblast differentiation by reducing MyoD, myogenin, MEF2C and sarcomeric proteins, and at the same time activating forkhead box O3 (FOXO3) and NOTCH self-renewal

signalling pathway (e.g., target gene HES1) (78). A possible explanation for this difference between studies is the differentiation protocol. On the last study, the differentiation was performed during 8 days, so all the experiments were performed after 8 days (78). The majority of studies in this field and validated protocols indicate the period between 3 to 5 days for the completion of skeletal muscle maturation (172). For that reason, depending on the duration of the differentiation process and the day of the experimental analysis, VitD3 could enhance or inhibit cell cycle regulators, resulting in controversial results.

Skeletal muscle cell development and hypertrophy are highly dependent on protein synthesis and degradation (86). Growth factors, insulin, and amino acids stimulate protein synthesis through mTOR, PKB/Akt pathway proteins (171). It was observed increase in the phosphorylation of mTOR (Ser 2448) (Figure 5D) in my study, however it was not statistically significant when cells were supplemented with VitD3 alone. A possible explanation for that is that perhaps VitD3 phosphorylates other mTOR sites as I could not explore all of them in this study. Overall, mTOR downstream targets were stimulated by VitD3, such as P-S6 (Ser235/236) (Figure 5B), P-Akt (Ser473) (Figure 5C), and P-GSK3β (Ser9) (Figure 5A). In other words, phosphorylation increased after VitD3 treatment when compared with vehicle + insulin group. When GSK-3 is phosphorylated it prevents the inhibition of eukaryotic translation initiation factor 2B (eIF2B), potentially regulating protein synthesis (173). Furthermore, the activation of PKB/Akt and/or MAP kinase pathways are well known important regulators of cell growth (85). In summary, treatment with VitD3 associated with insulin positively impacts mTOR pathway and open a possible role for VitD3 in the protein synthesis cascade.

After finding significant results of VitD3 in the mTOR downstream pathway, I decided to test if these results could actually enhance protein synthesis. Vitamin D3 treatment alone and VitD3 in combination with insulin enhanced the rate of protein synthesis, when compared to the vehicle group only (Figure 6C, 6D and 6F). In agreement, Salles, Chanet (86) demonstrated that 10 nM of VitD3 potentiated the effects of leucine and insulin by increasing the rate of protein synthesis via Akt/PKB and mTOR pathways.

Another key result observed was an increase in the number and size of myotubes significantly after VitD3 treatment. This field was also explored in animal studies, using VDR knockout mice (VDR-KO) or a VitD deficient diet, where it was reported

significant muscular atrophy, weakened strength, lower bone mineral density and dysregulation of myogenic regulatory factors when compared to the control group (76, 100, 105). One study for example, have reported a negative impact of VitD deficiency in the expression of MyoD in skeletal muscle tissue, confirming the regulation of myogenesis and muscle maintenance by Vitamin D (99).

This current study also found a significant increase in mitochondrial OCR induced by VitD3 in skeletal muscle myoblasts and myotubes cells (Figures 7 and 8). It was observed an increased maximal respiratory capacity in the VitD3 group, which link oxygen consumption and associated mitochondrial respiration to the generation of ATP, validating a role of VitD3 in muscle cell energy production. These results might be explained by the possible rise in the expression of the electron transport chain (ETC) proteins and the tricarboxylic acid (TCA) cycle enzymes through genomic and non-genomic pathways (88). In accordance with my results, Ryan et al., (91) have found that mitochondrial OCR increased when myoblasts cells were treated with 10 nM of VitD3 for 48 h (91). Another mechanism of VitD3 action is the increase of proteins that regulate mitochondrial biogenesis such as MYC, Mitogen-Activated Protein Kinase 13 and Endothelial PAS domain-containing protein (91). The experiments that assessed the mitochondrial metabolism in my study have confirmed that myoblasts and myotubes produce energy predominately via mitochondria since glycolysis activity was particularly low in my experiments. This can be explained because skeletal muscle produces energy mainly via mitochondrial metabolism (174). Consequently, we confirmed that glycolytic activity and glycogen content was not changed by VitD3 treatment and these results are in accordance with a similar study (91).

The classic effects of VitD3 are mediated by its interaction with a nuclear VitD3 receptor (VDR) which is part of the nuclear receptor superfamily of ligand-activated transcription factors. Interestingly, the same receptor can also be translocated into the mitochondria of certain cell types, including the skeletal muscle cells, and in theory act directly on gene transcription (167). We have found in this study that treatment with VitD3 during 5 days decrease the expression of the VDR in myotubes (Figure 5E). Comparing with the literature, only two other studies that have investigated the effects of VitD3 on VDR expression in human myotubes and they found a higher expression of the receptor in response to 24h and 72 h exposure to 1 or 100 nM of VitD (78, 91). The difference between studies might be explained by the fact that

VitD3 actions are dependent on a number of factors, such as the type of skeletal muscle cells, cells maturation, duration of VitD3 treatment and culture (serum) condition employed in each experiment. For example, a long period of VitD3 treatment (5 days) might lead to a negative feedback of the VDR, which can be translocated from the nuclei to the cytoplasm. More studies are needed to clarify the chronic effects of VitD3 treatment on VDR expression and location. At the initial phase and optimization of the experiments, the authors have attempted many different doses of VitD3 (such as 1nM, 10nM, 100nM and 200nM) and statistically significant effects were only observed with the higher dose 100nM also applied in the majority of studies in skeletal muscle cell lines (73-81).

Finally, new evidence has suggested another possible role for skeletal muscle as a storage of VitD3 to maintain VitD status during winter (175). The possible mechanism is mediated by muscle cell uptake of circulating vitamin D–binding protein (DBP) through a megalin-cubilin membrane transport process. The uptake and retention of labelled 25(OH)D was measured in 1) undifferentiated C2C12 myoblasts, 2) C2C12 myotubes and these mature cells were the only cell type to accumulate 25(OH)D (176). Furthermore, evidence that muscle accumulates 25(OH)D in winter as VitD status is falling, and that this higher concentration of 25(OH)D declines to the concentrations of summer was found in sheep (177). Identification of the pathway that allows uptake and release of VitD by skeletal muscle cells awaits further confirmation *in vivo*.

2.5 Conclusion

Vitamin D is associated with enhanced metabolism and function in primary human skeletal muscle cells. In this study we confirmed the anti-proliferative effects concomitantly with an increase in the differentiation process in HSMM cells. VitD3 also increased the expression of several key proteins responsible for insulin action on protein synthesis. Additionally, mitochondrial oxygen consumption rate enhanced in myoblasts and myotubes, which lead to an increased energy production. In conclusion, these findings validated a physiologically significant effect of VitD3 in proliferation, differentiation, protein synthesis and mitochondrial metabolism in primary human skeletal muscle cells.

The content of this chapter is partially described and discussed in Paper 2 (178): Romeu Montenegro K, Carlessi R, Cruzat V, Newsholme P. Effects of vitamin D on primary human skeletal muscle cell proliferation, differentiation, protein synthesis and bioenergetics. J Steroid Biochem Mol Biol. 2019; 193:105423. DOI: 10.1016/j.jsbmb.2019.105423

Chapter 3 -The impact of vitamin D3 supplementation on resting metabolic rate, body composition and strength in physically active adults

3.1 Introduction

Vitamin D is a dietary supplement commonly used worldwide and is described as the most prevalent supplement taken by athletes who suffer from any physical impairment (157, 179). The classical role of VitD is to regulate Ca^{+2} and PO_4^{2-} homeostasis to sustain bone health and prevent bone illnesses (180). More recently, VitD receptors (VDR) have been found in nuclei and membranes of primary human skeletal muscle cells proving potential for VitD to influence tissues other than bone, regulating cell proliferation, differentiation, atrophy and anti-inflammatory processes (8, 181). Deficiency and insufficiency of VitD is prevalent worldwide (182). It is important to notice that athletes have a high prevalence of VitD insufficiency, which might be explained by the fact they commonly have a higher metabolic rate and spend substantial time indoors training (26, 183). Specifically, in Australia, despite having a temperate climate and many hours of sunshine year-round, one in four adults have a suboptimal VitD status [serum 25(OH)D concentration < 50 $nmol \cdot L^{-1}$] (22). Low concentration of VitD might be the result of insufficient sunlight exposure due to geographical location, not spending much time outdoors and also consuming a VitD deficient diet (184). Although it is possible to find two types of Vitamin D (VitD2 and VitD3) in fish oils, mushroom, fatty fish and fortified products, exposure to the UVB radiation represents 80 - 90% of the total VitD production (27). Both VitD2 and VitD3 acquired from food, sunlight exposure or supplementation are able to form 25(OH)D in the liver and then be converted into the bioactive compound calcitriol [1,25(OH)2D] in the kidneys (142, 185).

Importantly, when adults become deficient in VitD it has been reported that significant reductions in skeletal muscle mass and function can result (186). Nevertheless, when VitD sufficient levels individuals are supplemented with VitD to sufficient levels (≥ 50 $nmol \cdot L^{-1}$), improvements are observed in myofiber cross-sectional area (CSA) and function in older adults (187, 188) and athletes (131). A systematic review found that different doses of the most effective form of VitD (3) (VitD3 - ranging from 400 to 8,500 $IU \cdot day^{-1}$) resulted in 1.4 - 18.8% increase in muscle strength in athletes classified as VitD sufficient at the baseline (141). In contrast, a more recent systematic review and meta-analysis (RCT = 5) did not find a significant efficacy of VitD3 supplementation on muscle strength (SMD -0.75, 95% CI: − 1.82 to 0.32, P = 0.17) in a mixed population (insufficient and sufficient)(189). The effects of VitD3

supplementation on muscle strength and power has been investigated in a wide range of sports and population, such as elite ballet dancers (132), soccer (131, 136), rugby players (135), swimmers (133) and active adult males (129, 130), with mixed results. Considering these studies, four (129, 130, 132, 136) reported that VitD3 supplementation improved muscle strength (specifically isometric force peak, one-repetition maximum [1-RM] bench press, back squat and weighted reverse-grip chin up). However, three studies did not find any effects of VitD3 on muscle strength and power (131, 133, 134). A possible explanation for these mixed results is significant heterogeneity of VitD levels at baseline, which confounds efforts to understand the impact of VitD on muscle strength and power (141). In addition, the majority of these studies have included VitD deficient and insufficient individuals at the start of the study (129-136), leaving a gap in the literature regarding whether VitD supplementation can be beneficial in participants that are already sufficient in VitD.

Vitamin D3 is described as a potential skeletal muscle modulator, and seems to not only influence skeletal muscle mass and strength (24), but also aerobic energy production and lipid metabolism, which could have an impact on factors related to endurance exercise performance and body composition (specifically muscle and fat mass) (25). For example, in elite rowers, maximal oxygen uptake improved after supplementation with 6000 $IU \cdot day^{-1}$ of VitD3 during 8-weeks of training, possibly due to the role of VitD stimulating oxygen uptake in skeletal muscle (190). One study found significant inhibition of adipocyte differentiation and remodelling of adipose tissue after VitD treatment, which might impact energy metabolism and resting metabolic rate (RMR) (191). In addition, VitD3 seems to accelerate lipolysis [16] in the TCA cycle (192). Finding variables that affect energy metabolism is important, since changes over time in RMR might have a significant effect on fat and muscle mass (118).

Another important VitD metabolite to be investigated is the bioavailable free form of VitD [free 25(OH)D]. The latest evidence suggests that VitD binding protein (VDBP) inhibits VitD action, as the bound fraction is not available to act on target cells (33, 34). For example, Free 25 (OH) is more strongly associated with bone mineral density (BMD), parathyroid hormone (PTH) and bone turnover markers than the total VitD levels in healthy adults (193-196). However, there is still lack of understanding of how the free 25(OH)D might impact muscle function in humans (31). In summary, researchers suggest that free25(OH)D might be a better marker of VitD status (197).

Whilst the position statement by the working Group of the Australian and New Zealand Bone and Mineral Society, Endocrine Society of Australia and Osteoporosis Australia (198) recommends a serum 25-OHD concentration ≥ 50 nmol·L^{-1} at the end of winter for optimal musculoskeletal health, it has been suggested that higher concentrations (i.e., ~ 120 - 225 nmol·L^{-1}) may be required for optimal skeletal muscle function in active adults and athletes (51). However, chronic supplementation with VitD may cause intoxication when serum concentration reach values higher than 200 nmol·L^{-1}. Possible adverse effects include gastrointestinal disorders, bone pain, drowsiness and headaches (153), confirming the importance of finding an optimal and safe serum concentration of VitD for the skeletal muscle system. Hence, the aim of this study was to investigate the effects of VitD3 supplementation on total and free serum concentrations of 25(OH)VitD, resting metabolic rate, body composition and strength in VitD sufficient physically active adults.

3.2 Materials and Methods

3.2.1 Participants

The initial idea was to recruit high level athletes, however specialists in the sports area found that the majority of athletes are already taking VitD3 or other multivitamin supplementation, excluding the possibility to investigate the effects of VitD3 supplementation on this population.

Forty-two physically active adult males and females met the inclusion criteria and were recruited to participate in this study. To meet the inclusion criteria, participants had to: 1) be healthy (no clinical conditions diagnosed), 18-35 years old and to be already exercising at least three times per week including a minimum of two sessions with resistance training; in other words, participants continued their normal exercise regimen 2) not have any history of VitD3 supplementation in the last month; 3) not have any current injuries that would prevent them from completing strength testing; 4) not be using any multivitamins, medication or other supplements that are related with VitD metabolism and body composition [for example calcium, thyroxine, creatine and thermogenic supplements]). During the follow-up, four participants had sports injuries unrelated to the study protocol and seven were not able to complete the minimum training required or the post-supplementation tests, and then were excluded

from this study. The final sample analysed in my study was composed of 31 participants (19 females and 12 males; mean ± SD; age = 26.6 ± 4.9 y; height = 170.0 ± 8.4 cm; body-mass = 71.7 ± 11.8 kg). The study protocol was conducted according to the Declaration of Helsinki, was approved by the Curtin University Human Research Ethics Committee (approval number: HRE 2019-0028) and registered by the Australian New Zealand Clinical Trials Registry (ACTRN12620000896976). To be part of the study, every individual had to complete an orientation session where the participant information statement was covered, availability to complete all the tests was confirmed and written informed consent obtained.

CONSORT 2010 Flow Diagram

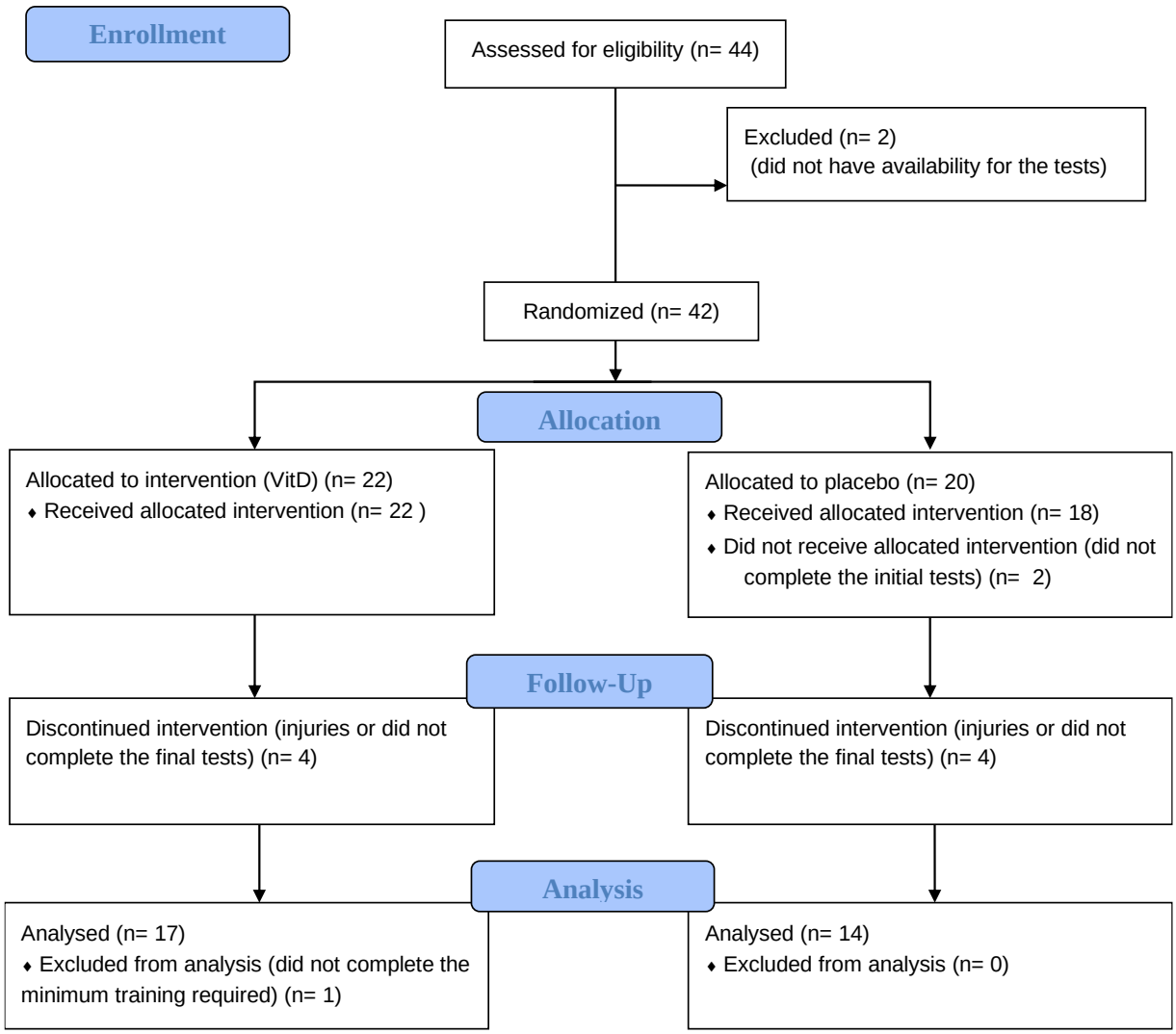


Figure 1. Flow of participants recruited

3.2.2 Study overview

Pre- and post-supplementation tests were completed over approximately 13 weeks. In the pre-supplementation period, participants had their resting metabolic rate (RMR), body composition, muscular strength and power and haematological markers [total and free plasma 25(OH)VitD, Ca^{2+} and parathyroid hormone] assessed between week 0 and 1 (see Figure 1). These first assessments were completed straight after the summer season (March/April/May, Western Australia) to ensure that participants had a higher chance of being VitD sufficient pre-supplementation and to achieve a higher concentration of VitD during the intervention period (≥ 120 $nmol \cdot L^{-1}$). After pre-testing, participants were matched for sunlight exposure and randomly allocated in a double-blind and counterbalanced manner to the VitD3 group (total n = 17, female n = 11; 50 $IU \cdot kg^{-1}$body-mass [BM]$\cdot day^{-1}$ Elite Vitamin D3, Healthspan Ltd®, United Kingdom [UK], batch tested by Informed Sport, LGC Limited, UK) or placebo group (total n = 14, female n = 8; dextrose, Glucodin, iNova Pharmaceuticals, Australia) for 12 weeks. Daily dosing has been claimed to provide a more stable amount of VitD and could act more consistently across the supplementation period than bolus or weekly dosing (199). Both supplements were concealed in opaque gelatin capsules and organized in sequentially code numbered envelopes to ensure that participants and the testing researcher were blinded to group allocations. The supplementation dose was chosen as it has been associated with a positive effect on strength performance in previous studies (132, 200).

Haematological markers were assessed again at week 7 to measure VitD concentration and to assist with identifying any possible adverse effects from the supplementation. Then, at the end of the intervention (week 12 - 13) participants repeated the pre-supplementation testing at the same time of day and referring back to a three-day food diary to ensure that they were similarly prepared to perform. Adequate VitD status was considered as 25(OH)D between 50 - 100$nmol \cdot L^{-1}$ and optimal VitD status was defined as > 100 $nmol \cdot L^{-1}$. My definition of optimal VitD status was based on previous research that suggests that a higher concentration may be related to optimal skeletal muscle outcomes (51, 201). To be able to ensure training and supplementation adherence and report any perceived side-effects from the supplementation, the main researcher was in contact with all the participants weekly. The intervention and placebo supplementation were distributed to participants fortnightly and the capsules left by the VitD group were counted to determine compliance.

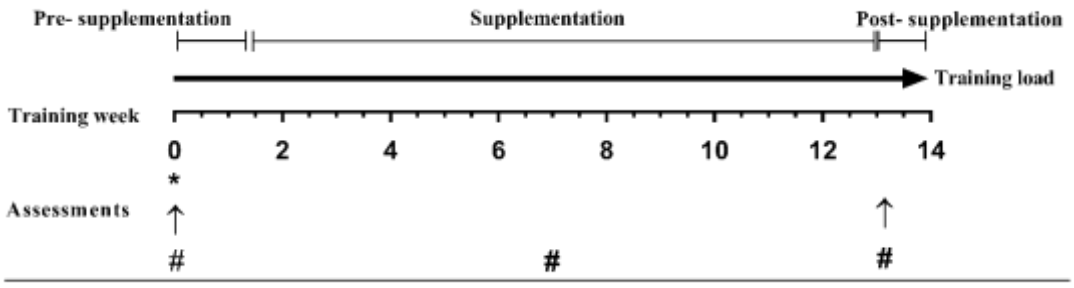


Figure 2. Study Design

***** Familiarisation with test procedures**;** ↑ Resting metabolic rate (RMR), body composition and strength and jump tests; # Food intake and venous blood sample.

3.2.3 Resting metabolic rate

Indirect calorimetry is one of the most accurate and non-invasive method of energy expenditure used in research (202). Overall, this method measures the amount of heat generated and is calculated by measuring the amount of oxygen used ($\dot{V}O_2$), and carbon dioxide released ($\dot{V}CO_2$) by each individual (203). In my study, energy expenditure was measured by indirect calorimetry, following a standard protocol that emphasised an overnight fast, abstinence for 24 h from heavy physical activity and a mandatory 10 min rest in the supine position prior to measurement. Participants arrived at the laboratory as soon as possible after waking up and were instructed to empty their bladder and void bowels before being weighed. Oxygen consumption was assessed when participants were resting in a supine position, in a quiet room without noise and strong light by metabolic cart (Parvo Medics TrueOne 2400, Parvo Medics, USA), using a mouthpiece and mixing chamber. Minute–minute measurements of O_2 consumption and CO_2 production were then conducted over 30 minutes. Energy expenditure modified equation – Weir (EE ($kcal \cdot day^{-1}$) = ([$\dot{V}O_2 \times 3.941$] + [$\dot{V}CO_2 \times 1.11$]) ×1440) was used to calculate RMR from the average of the last 10 min of data collection (204). The respiratory quotient (RQ = ratio of $\dot{V}CO_2$ to $\dot{V}O_2$) typically is between 0.7 to 1.0 depending on the amount of carbohydrate, fat and protein being combusted (205). None of the participants in this study had a measured RQ of < 0.7 or >1.0.

3.2.4 Haematological markers

Participants attended one of the Path West Laboratories in Perth, Western Australia at weeks 0 (pre-supplementation), 7 (middle) and 12 - 13 (post-supplementation) where a venous blood sample (~ 10 ml) was drawn to assess VitD status, Ca^{2+} and parathyroid hormone. Immunoassay method (Abbott Architect i2000sr analyser, USA) was used to quantify total serum VitD concentration [25(OH)D]. Calcium and parathyroid hormone were analysed using the Arsenazo III method and chemiluminescent microparticle immunoassay methodology respectively (Abbott, Spain). In addition, two serum aliquots (1 ml) were collected and frozen to – 80°C for measurement of free 25(OH)D concentration. After thawing, concentrations of free 25(OH)D were measured by a two-step ELISA immunoassay (DIASource ImmunoAssays, Netherlands) following the procedure described previously (206). In summary, during the incubation, a fixed amount of biotin-labelled VitD competes with the VitD in the sample, for a fixed number of binding sites on the anti-VitD antibody. Then, after a washing step, bound VitD-biotin is detected with Streptavidin-HRP.

Streptavidin binds to biotin and the conjugated HRP provides enzyme activity for detection using an appropriate substrate system (TMB chromogenic substrate is the staining). Finally, the stop reagent is used and the plate is read in a spectrophotometer at 450nm. The concentration of free 25OH ($pg \cdot mL^{-1}$) in the sample is inversely proportional to the absorbance in each sample well. Measurements from the unknown samples were read against the standard curve to derive the Free VitD concentration. Correlation between rate dialysis analysis result vs Free 25OH Vitamin D ELISA is 0.9916.

3.2.5 Body composition assessment

Dual energy X-ray absorptiometry (DXA) is divided into three main components of body composition, including fat mass, lean mass and bone mineral content (207). This method has many advantages, for instance it is suitable for most adults including athletes and physically active people, uses an extremely low radiation dose (~0.5 μSv) and is safe for speedy sequential measurements and lastly is non-invasive (207). After the assessment of RMR, participants had their whole-body composition measured by dual energy X-ray absorptiometry (DXA; GE Lunar Prodigy, General Electric, USA). On the day of the test, first, participants completed a pre-scan questionnaire to assess

their suitability for the scanning. Then, they were positioned on the midline of the scanning bed in a standardised position, having both arms by their side with the hands in a mid-prone position within the scan area and feet fixed at a 90° angle at the ankle. The assessment included: lean mass, fat mass and total bone mineral density. All scan analysis was completed using the scanners proprietary software and adjustments were made by the same qualified technician to ensure consistency between scans.

3.2.6 Strength and power assessment

Muscle strength is determined by 1-RM testing in the majority of intervention studies in the field of exercise science (208). Current literature confirmed that the 1-RM testing has excellent test–retest reliability, independently of many factors, such as the type of exercise, the resistance training experience, the number of familiarization sessions, part of the body tested, sex or age of participants (209).

After participants completed the first fasting tests (RMR and DXA), they were subsequently allowed to eat and drink for at least 1 h prior to the performance assessment to ensure they were optimally prepared to perform. Participants completed a self-selected warm-up first, which was recorded and replicated for the post-supplementation testing session. Second, they completed a test of 1-RM strength for the bench press and back squat exercises following the procedures set out by Baechle and Earle (210). Briefly, participants lifted progressively greater weights until a mass was identified that could only be lifted for one repetition for that specific exercise. Finally, participants completed three attempts of a counter-movement vertical jump, with 2 - 5 min rest between the attempts, to assess leg power (Vertec Yardstick Jumping Device, Swift Performance, Australia).

3.2.7 Food and training diary

A daily training diary was completed during the 12 weeks of supplementation so that training load could be calculated using the sessional rating of perceived exertion (sRPE) method proposed by Foster (211). Briefly, the participant described the type of exercise, duration of session and rating of perceived exertion for each session of exercise (sRPE; CR1 – 10 scale) (212). This value was multiplied by the duration of the session to provide a training load value in arbitrary units (AU).

As part of the training diary, participants also completed a 3-day food record, calcium quiz and sunlight exposure questionnaire on three occasions (baseline, 7 and 12 weeks). First, participants recorded the amount of food, fluid and supplements consumed during 3 consecutive days (2 weekdays and 1 day of the weekend), after having received detailed instructions about how to complete their dietary intake. This diet monitoring period is considered adequate for the estimation of habitual energy and macronutrient consumption (213). Each food record was reviewed by a nutritionist together with each participant to ensure that sufficient detail was captured. Food records were analysed using Foodworks® (V9, Xyris Australia). Each individual was encouraged to follow similar eating patterns throughout the study to minimise deviations in macronutrients, and vitamin and mineral intake.

3.2.8 Calcium quiz and sunlight exposure

Participants also completed a five-minute online questionnaire (Dairy Council of California (214) baseline, 7 and 12 weeks) to assess calcium intake. The quiz contains 34 calcium-containing foods and fluids along with a serving size, and participants select how many servings of each listed food were consumed the previous day. Additionally, participants answered a sunlight exposure diary on the same three occasions (baseline, 7 and 12 weeks), which asked them about the amount of time spent outdoors each day on average in the previous week (0 = ≤ 5 min; 1 = 5 – 30 min; and 2 = ≥ 30 min) and what areas of their body got exposed while outdoors (1 = face and hands only; 2 = face, hands and arms; 3 = face, hands and legs; and 4 = bathing suit) (190).

3.2.9 Statistical analysis

Continuous variables were checked for normality prior to analysis and they were summarised by means and standard deviations [age, free 25(OH)D, weekly sunlight exposure and daily calcium intake] or medians and interquartile ranges (IQR) [BMI, total 25(OH)D, parathyroid hormone (PTH), Ca^{+2} and total training load]. Categorical data were summarised using frequency distributions. Chi-squared tests was applied for categorical data to compare participants' main characteristics at pre- and post-supplementation between groups. For continuous data used t-tests or non-parametric Mann-Whitney U tests. The calculation of sample size was founded on previous

research, considering strength as the main outcome, a sample size of 30 has 99% power to detect a standardised mean difference of 0.60 (34, 58) in a mixed-model ANOVA in two groups across two time points with, an α value of 0.05 (215). However, as the variables were not normally distributed and due to the ability to adjust for factors, linear mixed models were used for the main analyses considering random subject intercepts. The effect of free and total serum 25(OH)D on pre-post supplementation differences in RMR, strength and body composition outcomes, within and between groups, were assessed by linear mixed models (LMM). This model was adjusted for gender, sunlight exposure, training load, protein, carbohydrate, fat and total energy intake. Total and serum 25(OH)D concentration was also assessed using LMM to detect possible differences within and between groups pre- and post-supplementation. This model uses maximum likelihood estimation methods which parametrize all longitudinal data regardless of missing data points. These results are summarised as estimated marginal means, mean differences and 95% confidence intervals. Statistical significance was accepted at $p < 0.05$. Statistical analyses were performed using STATA/IC 16.0 (StataCorp LLC, College Station, USA).

3.2.10 Ethical issues and data storage

All data was kept confidential and was organised and filed according to the date, sample, and number in the backed-up research drive (R drive). Extracted data was organised and sorted using Microsoft Excel and S STATA/IC 16.0 (StataCorp LLC, College Station, USA). This project was submitted to the Curtin University Human Research Ethics Office and approved. All participants were requested to sign a consent form. All data were kept confidential and data were de-identified.

Body composition assessment using DXA involves a small dose of ionising radiation (~ 0.74 μSv) that is equivalent to about one thousandth of the background radiation dose that a person would receive whilst living in Perth, Western Australia for one year. All participants completed a DXA pre-scan questionnaire to assess their suitability to complete the scan. Any female that was, or may be pregnant were excluded from participating. Occupational radiation exposure to the operators of this equipment is sufficiently low that screening is not required. Nonetheless, all operators were required to wear a radiation monitoring badge while using the DXA.

The risks of taking blood include the chance of infection or a bruise at the point where the blood is taken, redness and a rare risk of fainting. Participants had their blood sampled by Pathwest phlebotomists that were following best practice blood sampling procedures. Participants were reminded to keep the wound clean and dry after blood sampling to minimise the risk of infection at the sample site. To minimise the risk of injury from fainting, individuals were seated in a chair during blood sampling. As we were working with human blood, there was a small risk of infection by pathogens that might be present in the blood. The main researcher was trained at the safe handling of biohazardous items.

Vitamin D supplementation is considered safe at doses < 10,000 $IU \cdot day^{-1}$ and toxicity has not been reported until doses exceed 10,000 $IU \cdot day^{-1}$, which is much higher than the dose that was prescribed in this study (e.g., for a person with a body mass = 70 kg, the dose was 50 IU x 70 kg = 3500 $IU \cdot day^{-1}$). The main pathology associated with hypervitaminosis D is hypercalcaemia and hypercalciuria, as VitD regulates the metabolism of calcium. Hypercalcaemia does not occur unless serum 25(OH)D levels reach $\geq$ 220 $nmol \cdot L^{-1}$ and is typically not observed until plasma levels reach 500 $nmol \cdot L^{-1}$ (198). Calcium and parathyroid hormone were assessed to monitor participants for toxicity. It was not expected that participants would be deficient in VitD, however, if participants were found to be deficient post-supplementation (placebo group only), they were recommended to consult their physician at the end of the study.

Strength testing does involve a small risk of musculoskeletal injury. The testing was conducted by a trained researcher who ensured that participants were warmed up and ready to participate in the tests, and were actively monitoring the activities for safety at all times. Also, these tests may cause delayed onset muscle soreness, however the participants were strength training at least two days per week and were experienced in the sort of discomfort that this type of exercise can cause. Prior soreness is also protective of future post-exercise soreness.

3.3 Results

3.3.1 Participants characteristics

Participants had similar characteristics at the start and end of the study in regards to gender, age and ethnicity, showing no significant differences pre- and post-supplementation. They also had comparable baseline characteristics such as BMI, serum total 25(OH)D concentration, serum parathyroid hormone, serum Ca^{2+}, weekly sunlight exposure, sunscreen use, training load, and daily calcium intake (Table 1). The majority of my sample was female (placebo n = 8 [57%] *vs.* VitD3 n = 11 [65%]; p = 0.67) and self-declared Caucasian and white (placebo n = 12 [86%] *vs.* VitD3 *n* = 15 [88%]) (p = 0.83). The oral VitD3 supplementation dose averaged 3205 ± 366 IU·day-1 for females and 4230 ± 548 IU·$day^{-1}$for males, respectively. During the 12 weeks of supplementation, no significant difference was observed in daily energy intake (p = 0.66), protein (p = 0.77), carbohydrate (p = 0.06) and total fat consumption (p = 0.11) between and within groups (Figure 2). Participants had a very similar training load during the study, without any statistical difference between groups (p = 0.92; Table 1).

Table 1. Pre and post supplementation characteristics of participants by study group (placebo n= 14 and VitD3 n = 17).

Parameter	**Pre supplementation (Week 0-1)**		***P*-value**	**Post supplementation (Week 13-14)**		**#*P*-value**
	Placebo n=14	**VitD3 n=17**		**Placebo n=14**	**VitD3 n=17**	
BMI (kg/m^2) med (IRQ)	**24.8 (3.6)**	**23.5 (4.6)**	**0.60**	**24.7 (4.5)**	**23.4 (4.5)**	**0.54**
Total 25(OH)D ($nmol \cdot L^{-1}$) med (IRQ)	**74.0 (44.5) [a]**	**93 (49) [a]**	**0.07**	**53 (42.2) [a]**	**123 (51) [b]**	**<0.001**
Free 25(OH)D ($pg \cdot ml^{-1}$) *mean* (*SD*)	**8.6 (5.2) [a]**	**13.1 (5.3) [b]**	**0.006**	**6.2 (4.5) [a]**	**15.8 (5.1) [b]**	**<0.001**
Parathyroid hormone ($pmol \cdot L^{-1}$) med (IRQ)	**5.1 (3.0)**	**4.9 (3.6)**	**0.68**	**6.5 (2.1)**	**3.9 (3.4)**	**0.008**
Ca^{+2} ($mmol \cdot L^{-1}$) med (IRQ)	**2.5 (0.1)**	**2.4 (0.2)**	**0.89**	**2.4 (0.1)**	**2.4 (0.1)**	**0.47**
Weekly sunlight exposure *mean (SD)* (h)	**4.4 (4.6)**	**3.8 (3.1)**	**0.95**	**0.7 (0.5)**	**0.7 (0.5)**	**1.00**
Sunscreen use (yes) n (%)	**4 (29%)**	**5 (29%)**	**0.96**	**4 (29%)**	**5 (29%)**	**0.96**
Total training load (AU) med (IRQ)	**1635 (1562)**	**1260 (1065)**	**0.54**	**18840 (16168)**	**15140 (13995)**	**0.92**
Weeks sick or injured (n) *mean* (*SD*)	**0**	**0**	**NA**	**1.00 (0.5)**	**1.18 (0.6)**	**0.42**
VitD3 supplementation dose ($IU \cdot day^{-1}$) *mean* (*SD*)	**NA**	**NA**	**NA**	**0**	**3559 ± 670**	**NA**
Daily calcium intake (mg) *mean* (*SD*)	**816 (598)**	**999 (428)**	**0.09**	**965 (447)**	**1109 (482)**	**0.54**

Different letters indicate significant differences between groups pre and post supplementation (^{a,b}). N/A: not applicable; *P-value: comparison between placebo and VitD3 pre supplementation; #P-value comparison between placebo and VitD3 post supplementation. BMI, total 25(OH)D, PTH, Ca^{+2}, total training load are represented by median (IRQ) and Free 25(OH)D, weekly sunlight exposure and daily calcium intake are represented by media ± SD.

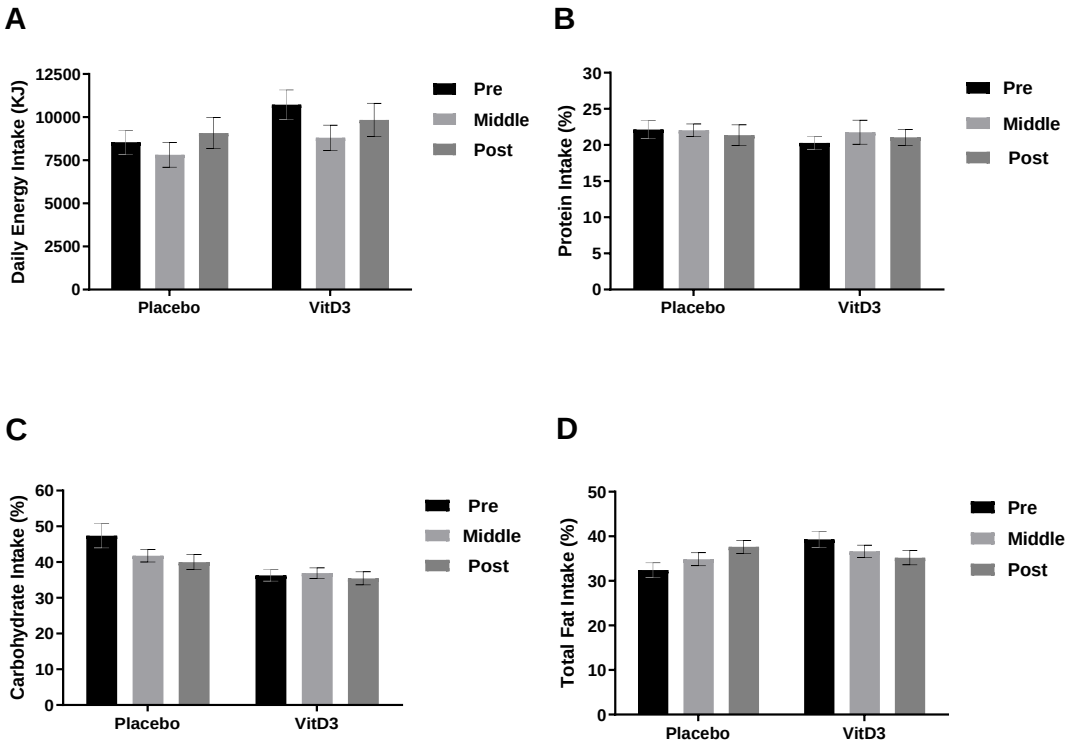


Figure 2. Mean ± (SD) daily energy intake (A), protein (B), carbohydrate (C) and total fat intake (D) pre-, mid- and post supplementation in the placebo and VitD3 groups. Daily energy intake (A; p = 0.66), protein (B; p = 0.77) carbohydrate (C; p = 0.06), and total fat consumption (D; p = 0.11) were not statistically different between and within groups pre, middle and post supplementation.

3.3.2 Serum total 25(OH)D and free 25(OH)D Concentration

Serum total 25 (OH)D was measured at week 0, 7 and 12 while free 25(OH)D was assessed at week 0 and 12. At the start of the study, all participants were classified as VitD sufficient [25 (OH)D ≥ 50 nmol·L^{-1}], with a mean serum total 25(OH)D concentration of 87.7 ± 31.4 nmol·L^{-1} (range = 50.0 – 175.0 nmol·L^{-1}) (Table 1). The only significant difference was a higher concentration of serum free 25(OH)D in the VitD3 group when compared with placebo group at the baseline of the study (Table 1). After 7 and 12 weeks of VitD3 supplementation, the VitD group increased serum total 25(OH)D by 30 nmol·L^{1} while the placebo group decreased total serum concentration by 21 nmol·L^{1}, reaching 123 (51) and 53 (42.2) nmol·L^{1}, respectively (Figure 3A). The same results were not observed when comparing the free 25(OH)D pre- *vs*. post- supplementation period (Figure 3 B). The higher concentration of Free 25(OH)D in the intervention group was sustained until the end of the study, without

significant differences within group. I excluded three participants from the final analysis (control group) as they were below the detection limit of the assay for serum free 25(OH)D concentrations (values = 0) that can be explained since they were classified as VitD deficient at the end of the study.

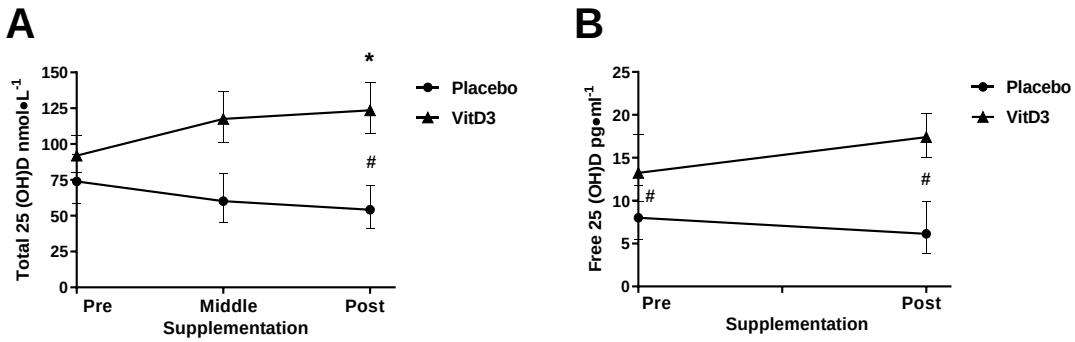


Figure 3. Serum total 25(OH)D concentration pre, middle and post supplementation (A) and free 25(OH)D concentration pre and post supplementation (B). * Difference within group (VitD3 pre supplementation *vs.* VitD3 post supplementation by ANOVA; p=0.01); #difference between groups (VitD3 pre and post supplementation *vs.* placebo pre and post supplementation; p<0.001).

3.3.3 Muscular strength and power

The results from the muscular strength and power tests are divided in two tables: Table 2 - which considers serum total concentration of 25(OH)D as the main predictor and Table 3 - which considers the main predictor as serum free 25(OH)D. Overall, muscle strength and power tests (back squat, bench press and vertical jump displacement) were not statistically different between placebo and VitD3 groups over the 12-week period considering serum total 25(OH)D as the main predictor (1-RM back squat, p = 0.54; 1-RM bench press, p = 0.38; and vertical jump p = 0.50). Within the VitD3 group increases in strength were observed for back squat (+6.9 kg; p = 0.005) and bench press (+3.2 kg; p = 0.007) (Table 2). When free 25(OH)D was considered as the main predictor, within group increases were also observed for back squat (+5.5 kg; p = 0.042 vs. +7.7kg; p = 0.003) and bench press (+2.7 kg; p = 0.020 vs. +2.9kg; p = 0.01) in both groups (placebo and VitD3, respectively).

Table 2. Estimated within group means and mean differences of outcomes (pre and post supplementation) considering serum total 25(OH)D as the main predictor

Predictor = 25(OH)D		Model adjusted for: gender, sunlight exposure, training load, protein, carbohydrate, fat and energy intake									
		Pre supplementation			Post supplementation				Pre-post change		
Outcome	**Group**	**Estimated mean**	**95%CI diff**	***P-value***	**Estimated mean**	**95%CI diff**	***ºP-value***	***#P-value***	**Estimated mean difference**	**95%CI**	****P-value***
Resting Metabolic Rate (kJ)	**Placebo**	**6372.8**	**5994.6, 6751.1**	**0.86**	**6631.5**	**6221.4, 7041.5**			**258.6**	**-48.1, 565.4**	**0.10**
	VitD3	**6327.4**	**6004.5, 6650.4**		**6632.7**	**6262.8, 7002.6**	**1.00**	**0.83**	**305.3**	**20.4, 590.2**	**0.036**
Muscle Strength and Power											
Back Squat (kg)	**Placebo**	**77.9**	**69.0, 86.9**	**0.10**	**82.6**	**73.2, 92.0**	**0.07**	**0.54**	**4.6**	**-0.6, 9.9**	**0.08**
	VitD3	**87.9**	**82.6, 94.8**		**94.8**	**86.3, 103.4**			**6.9**	**2.1, 11.8**	**0.005**
Bench Press (kg)	**Placebo**	**51.3**	**45.6, 57.1**	**0.010**	**52.9**	**47.0, 58.8**	**0.005**	**0.38**	**1.6**	**1.1, 4.2**	**0.24**
	VitD3	**61.5**	**56.4, 66.7**		**64.8**	**59.4, 70.1**			**3.2**	**0.9, 5.6**	**0.007**
Jump test (cm)	**Placebo**	**41.1**	**36.7, 45.5**	**0.17**	**41.9**	**37.4, 46.5**	**0.31**	**0.50**	**0.8**	**-0.9, 2.5**	**0.35**
	VitD3	**45.2**	**41.3, 49.2**		**45.2**	**41.1, 49.3**			**-0.01**	**-1.5, 1.5**	**0.98**
Body Composition											
Lean mass (kg)	**Placebo**	**47.9**	**47.4**	**0.09**	**48.0**	**44.9, 51.0**	**0.07**	**0.54**	**0.6**	**-0.3, 1.5**	**0.19**
	VitD3	**50.9**	**50.9**		**51.9**	**49.1, 54.6**			**1.0**	**0.2, 1.8**	**0.013**
Fat mass (kg)	**Placebo**	**22.7**	**21.4**	**0.011**	**21.4**	**18.4, 24.5**	**0.06**	**0.20**	**-1.2**	**-2.5, -0.02**	**0.050**
	VitD3	**17.5**	**17.3**		**17.3**	**14.6, 20.1**			**-0.1**	**-1.2, 1.0**	**0.83**
Bone Mineral Density (g/cm^2)	**Placebo**	**1.2**	**1.7, 1.2**		**1.2**	**1.2, 1.2**		**0.09**	**-0.0005**	**0.014, 0.013**	**0.94**
	VitD3	**1.3**	**1.2, 1.3**	**0.026**	**1.3**	**1.2, 1.3**	**0.008**		**0.02**	**0.004, 0.02**	**0.004**

P -value: difference between groups pre supplementation); ºP-value:(difference between groups post supplementation); #P-value: group-time interaction (rate of change); *P-value: within group pre-post change. Estimated means and mean differences were assessed by linear mixed models (LMM); 95% CI: confidence intervals; diff: difference.

Table 3. Estimated within group means and mean differences of outcomes (pre and post supplementation) considering Free 25(OH)D as the main predictor.

Predictor = Free 25(OH)D		**Model adjusted for: gender, sunlight exposure, training load, protein, carbohydrate, fat and energy intake**									
		Pre supplementation			**Post supplementation**				**Pre-post change**		
Outcome	**Group**	**Estimated mean**	**95%CI diff**	***P-value***	**Estimated mean**	**95%CI diff**	***ºP-value***	***#P-value***	**Estimated mean difference**	**95%CI**	****P-value***
Resting Metabolic Rate (kJ)	**Placebo**	**6361.2**	**5971.4, 6750.9**	**0.86**	**6619.6**	**6223.8, 7015.5**	**0.82**	**0.91**	**258.4**	**-39.1, 556.0**	**0.09**
	VitD3	**6409.3**	**6057, 6761.6**		**6690.1**	**6296.2, 7084**			**280.9**	**0.67, 561.1**	**0.049**
Muscle Strength and Power											
Back Squat (kg)	**Placebo**	**79.1**	**68.9, 89.4**	**0.12**	**84.6**	**74.2, 95.0**	**0.08**	**0.51**	**5.5**	**0.2, 10.7**	**0.042**
	VitD3	**90.8**	**80.9, 100.6**		**98.5**	**88.0, 11**			**7.7**	**2.7, 12.8**	**0.003**
Bench Press (kg)	**Placebo**	**53.3**	**46.8, 59.7**	**0.020**	**56.0**	**49.5, 62.6**	**0.030**	**0.93**	**2.7**	**0.4, 5.0**	**0.020**
	VitD3	**64.0**	**57.7, 70.3**		**66.9**	**60.3, 73.4**			**2.9**	**0.7, 5.0**	**0.010**
Jump test (cm)	**Placebo**	**41.3**	**36.4, 46.2**	**0.26**	**41.6**	**36.6, 46.6**	**0.234**	**0.78**	**0.3**	**-1.3, 2.0**	**0.70**
	VitD3	**45.4**	**40.5, 50.2**		**46.0**	**41.0, 51.0**			**0.6**	**-0.9, 2.2**	**0.24**
Body Composition											
Lean mass (kg)	**Placebo**	**48.0**	**44.6, 51.3**	**0.12**	**48.4**	**45.0, 51.7**	**0.10**	**0.61**	**0.4**	**-0.4, 1.2**	**0.34**
	VitD3	**51.7**	**48.4, 54.9**		**52.3**	**49.0, 55.7**			**0.7**	**-0.1, 1.4**	**0.09**
Fat mass (kg)	**Placebo**	**22.4**	**19.1, 25.7**	**0.07**	**21.6**	**18.2, 24.9**	**0.13**	**0.57**	**-0.9**	**-2.2, 0.4**	**0.19**
	VitD3	**18.1**	**14.9, 21.3**		**17.7**	**14.4, 21.1**			**-0.4**	**-1.6, 0.8**	**0.55**
Bone Mineral Density (g/cm^2)	**Placebo**	**1.2**	**1.2, 1.3**	**0.11**	**1.2**	**1.2, 1.3**	**0.12**	**0.94**	**0.007**	**-0.001, 0.023**	**0.41**
	VitD3	**1.3**	**1.2, 1.3**		**1.3**	**1.2, 1.3**			**0.061**	**-0.001, 0.021**	**0.43**

P-value: difference between groups pre supplementation); ºP-value:(difference between groups post supplementation); #P-value: group-time interaction (rate of change); *P-value: within group pre-post change. Estimated means and mean differences were assessed by linear mixed models (LMM); 95% CI: confidence intervals; diff: difference.

3.3.4 Resting metabolic rate and body composition

The results of the tests including: RMR, lean mass, fat mass and bone mineral density are described in Table 2 (serum total 25(OH)D as the main predictor) and in Table 3 where serum free 25(OH)D is main predictor. After 12 weeks of supplementation, RMR increased in both groups (estimated mean difference for placebo = + 258.6 kJ and VitD3 = + 305.3 kJ; p =1,00), however there was no significant differences between groups. When I analysed within group differences, RMR significantly increased in the VitD3 group only (RMR + 305.3kJ; p = 0.036 and + 280.9kJ p = 0.049) considering both predictors (serum total and free 25(OH)D, respectively). Similar results were observed in regards to body composition. Specifically, lean, fat mass and bone mineral density were not different between groups after the intervention (Table 2). However, significant within group increases in lean mass (+1.0 kg; p = 0.013) in the VitD3 group were detected when serum total 25(OH)D was the main predictor.

3.3.5 Calcium (Ca^{+2}) and parathyroid hormone

Serum concentration of Ca^{+2} and parathyroid hormone (PTH) was measured at weeks 0, 7 and 12 of the study. Total serum Ca^{+2} concentration was 2.5 $mmol \cdot L^{-1}$ (IRQ 0.1) vs. 2.4 $mmol \cdot L^{-1}$ (IRQ 0.2) pre-supplementation and 2.4 $mmol \cdot L^{-1}$ (IRQ 0.1) vs. 2.4 $mmol \cdot L^{-1}$ (IRQ 0.1) post-supplementation in placebo and VitD3 groups, respectively (Table 1), showing no significant differences between placebo and VitD3 groups. Serum PTH slightly increased in the placebo group and decreased in the VitD3 group at the end of the study. In the placebo group, PTH concentration was 5.1 (IRQ 3.0) pre-supplementation and 6.5 (IRQ 2.1) post-supplementation while the VitD group was 4.9 (IRQ 3.6) vs. 3.9 (IRQ 3.4) pre-post supplementation respectively. In summary, parathyroid hormone concentrations were significantly higher in the placebo group when compared to the VitD3 group (p = 0.008; Table 1) post-supplementation. Overall, participants sustained Ca^{+2} and parathyroid hormone serum concentration in the normal range during the whole study.

3.4 Discussion

In the present study, I demonstrated that supplementation over 12 weeks with VitD3, significantly improved serum total 25 (OH)D level. However, there was no change in RMR, body composition, strength and power in physically active adults already sufficient in VitD when comparing the VitD3 and placebo groups. The present data also revealed that VitD3 supplementation did not significantly change the concentration of serum free 25 (OH)D, which may explain the lack of statistical significance on the main outcomes of this study.

It has been reported that the effects of VitD on muscle strength and metabolism are mediated by molecular changes in protein synthesis, myogenesis, mitochondrial activity, muscle regeneration and glucose metabolism (78, 80, 91, 216). Several molecular pathways of VitD action have been investigated and described in cells and animal studies (152), which led to further investigation in clinical trials. The Australian Institute of Sport and other researchers in the area highlight that VitD possibly enhances the adaptive response to exercise, decreases stress fractures and improves immunity, however there is no consensus regarding the best dose-effects for athletes and physically active adults (197, 217, 218).

My data confirmed that participants started the study with a high concentration of serum total 25(OH)D (placebo = 74 nmol·L^{-1}; VitD3 = 93 nmol·L^{-1}) prior to supplementation. This fact can be explained because participants spent an average of ~ 4 h·week^{-1} exposed to UVR (mean UVR index = 11 – classified as extreme) and high temperatures (mean = 30 ºC) during summer months in Perth, Western Australia (situated 31°S) (219, 220). After 6 weeks of VitD3 supplementation, serum total 25(OH)D significantly increased in the intervention group, which was maintained through to the end of the study [123.88 (60) *vs* 123 (51)]. These results suggest a possible plateau and maximum concentration of serum total 25(OH)D after week 7 of the intervention. Likewise, another study that investigated the levels of VitD in Western Australian athletes reported similar serum total 25 (OH)D concentrations (111 ± 37 nmol·L^{-1}) (219) after summer. Following 12 weeks of supplementation, the VitD3 group increased serum total 25(OH)D by 32 nmol·L^{1} (IQR 50), while the placebo group reduced total serum concentration by 21 nmol·L^{-1} (IQR 43). Consequently, 36% of the placebo group was classified as VitD deficient [total serum levels of 25(OH) D < 50 nmol·L^{-1})]. Although the reduction in serum total 25(OH)D levels pre- to post-supplementation in the placebo group was not statistically

significant, it is clinically meaningful as it represents a decrease that may require VitD3 supplementation to maintain adequate concentration throughout the winter months. As the majority of participants completed the tests in the beginning of the winter, the serum total 25 (OH)D levels are likely to decline even more by the end of the season.

The primary marker of VitD (serum total 25(OH)D) is the metabolite with the longest half-life and is directly associated with sunlight exposure through dermal synthesis, explaining why it is considered the most stable marker of VitD status (36). An alternative pathway for cellular uptake of steroid hormones has been described in the literature as the "free hormone hypothesis"(31). As these steroid hormones and VitD are lipophilic, they have the potential to rapidly diffuse across cell membranes (31). The association between total and free 25 (OH)D and serum PTH, bone turnover markers, and bone mineral density, has been assessed in a wide range of clinical trials (193-196). These studies have shown that free 25 (OH)D might be more useful as a biomarker of VitD-mediated bioactivity than total serum 25 (OH)D. Other studies have reported that specific functions of VitD may be more closely related to the free 25 (OH)D than to the total serum concentration of VitD, for example maintenance of BMD (35, 37, 38). Accordingly, it is recommended to also measure the biological form of VitD [free 25 (OH)D]. A study based upon 109 healthy individuals have suggested the range of concentration of free 25(OH)D expected is 5.1 $pg \cdot ml^{-1}$ (between 2.4 to 17.1 $pg \cdot ml^{-1}$) (31). In agreement with my study where participants had high concentration of free 25(OH)D (8.6 ± 5.2 $pg \cdot ml^{-1}$ placebo and 13.1 ± 5.3 $pg \cdot ml^{-1}$ VitD3 group) before supplementation, Sollid et al., (196) also found similar results (13.7 ± 4.2 $pg \cdot ml^{-1}$). Unexpectedly, free 25(OH)D did not significantly increase after VitD3 intervention, possibly meaning that VitD3 group already reached optimal serum free 25(OH)D values before the start of the VitD3 supplementation.

Even though participants reached an optimal concentration (~120 $nmol \cdot L^{-1}$) of serum total 25 (OH)D after the intervention, no significant impact on strength or power was detected. All participants were already more than VitD sufficient at the beginning of the study which might partially explain why I did not see any additional benefits of the supplementation. Interestingly, this data illustrated within VitD3 group differences between VitD and RMR, back squat, bench press, lean mass and bone mineral density when serum total 25 (OH)D is considered as the main predictor. Overall, at this stage

these results should be interpreted with caution, as significant differences were not observed between groups (VitD3 *vs* placebo) in either of the outcomes discussed.

The use of different protocols to measure muscular strength and power within the literature makes comparisons with this current study difficult (130-132, 221, 222). I identified one study that used the same protocol as my study (1-RM) and they also reported no improvements in muscle strength or power in a VitD deficient, young and active cohort after 12 weeks of supplementation with 20,000 or 40,000 IU·week^{-1} of VitD3 (130). The researchers suggested that as the majority of participants reached only the VitD sufficient range (50 - 75 nmol·L^{-1}), higher doses of VitD3 might be necessary to reach optimal concentrations of serum total 25(OH)D (> 120 nmol·L^{-1}) in VitD deficient people, however my work suggests that this may not be correct.

An association between higher serum 25(OH)D concentration and reduction in omental adipocyte size and lower visceral adiposity was found in a clinical study with slightly overweight women (BMI = 27.2 ± 4.6 kg/m^2) (112). In more detail, dietary VitD intake was inversely associated with visceral adipose tissue area ($r = -0.34$, $p \leq 0.05$) and concentration of serum 25(OH)D was inversely linked with visceral adipose tissue area ($r = -0.32$), total adipose tissue area ($r = -0.44$), subcutaneous adipose tissue area ($r = -0.36$), BMI ($r = -0.43$) and total body fat mass ($r = -0.41$, $p \leq 0.05$ for all). In addition, an inverse association between obesity and serum concentration of total VitD has been reported in many studies (113-115). However, I did not observe the same effects in my study, which may be due to the fact that these studies were mostly observational in nature and VitD might have a larger effect on adipose tissue in overweight and obese individuals.

With regard to muscle mass, supplementation with VitD3 in deficient individuals seems to increase size and result in a higher number of type II muscle fibres (24, 139, 140). However, limited studies have tested these responses in humans directly by muscle biopsy. More recently, a year of VitD3 supplementation in VitD deficient participants resulted in increased lean mass from 43.8 ± 9.6 to 44.3 ± 9.8 kg in the intervention group only(128). In my study, participants maintained a healthy body composition during the 12-week supplementation period and the differences observed after supplementation in lean or fat mass between groups did not reach statistical significance.

To date, randomised clinical trials exploring the possible effects of VitD3 supplementation on RMR are scarce. A clinical study that investigated the effects of

VitD3 supplementation in physically active adults described no impact on energy or substrate utilization (117). However, this study used a very short supplementation period (one week), which maybe the reason for the lack of significant results. *In vitro* research has suggested that increased VitD concentrations increases maximal respiratory capacity, which is associated with oxygen consumption and associated mitochondrial respiration to generate adenosine triphosphate (ATP) (91, 178). These results elucidate an important function for VitD in energy production, however, the data from my study imply that changes in body composition and RMR are not observed in a population of physically active adults.

Future research should consider measuring not only total serum 25(OH)D but also the serum free 25(OH)D, as it could be more representative of the VitD biological activity and more information is needed about any potential differences. For example, individuals might have very different proportions of total and free 25(OH)D, and for that reason might not be accurately classified as VitD sufficient or deficient (Table 4). My suggestion is that VitD oral supplementation with the purpose of treating VitD deficiency should not be focused solely on total serum 25(OH)D concentration. Instead, this recommendation could be also be based on free 25(OH)D concentrations and symptoms of VitD deficiency (e.g., fatigue or tiredness, muscle weakness, and bone and muscle pain) (38). Furthermore, individuals might respond differently to VitD3 supplementation depending on the baseline concentration (59).

It is important to note that as I was not able to prescribe and control participants training it might represent a limitation of this study. Despite the fact that total training loads were the same between groups and exercise adherence was high during the whole study, any potential differences in the training design may still affect these findings. This is possible since I didn't detect statistical increases in muscle mass in VitD3 or placebo groups despite the 12 weeks of generic self-directed training in which at least 2 sessions had to be resistance. Additionally, during the supplementation period, participants only forgot to take the VitD3 supplement on average once a month and were sick and/or not training only for 1 week, confirming an appropriate adherence with supplementation and exercise routine through the full intervention. Future studies should also investigate the effects of VitD3 supplementation in endurance testing. Lastly, serum Ca^{+2} and the PTH were also measured during the 12 weeks of VitD3 supplementation to avoid possible adverse effects including hypercalcemia or hyperparathyroidism confirming that concentration was within the

recommended range. I have noticed that serum PTH concentration reduced in the VitD3 group which can be justified by the rise of serum total 25(OH)D, however no adverse effects were reported by any participant. As expected, the opposite result was observed in the placebo group as serum total 25(OH)D concentration decreased. Vitamin D concentration regulates serum PTH, assisting to control Ca^{+2} metabolism and bone function (76).

Table 4. Theoretical method to calculate the percentage of free 25(OH)D availability (example)

Case	Total Serum VitD $nmol \cdot L^{-1}$	Classification by IOM (51)	Free VitD $pg \cdot ml^{-1}$	Free VitD $nmol \cdot L^{-1}$	Free VitD in proportion to total VitD	% of free VitD	Serum Free VitD recommendation 0.03 - 0.4% (32)
1	100	sufficient	2	0.01182	100*0.01182/100	0.01%	Inadequate
2	40	deficient	100	0.0591	100*0.0591/40	0.15%	Adequate

3.5 Conclusion

This study confirms that VitD3 supplementation in physically active individuals already classified as sufficient in VitD does not result in additional benefits on muscle strength, power, RMR and body composition. However, for the autumn and winter seasons where people are at a higher risk of VitD deficiency, supplementation of VitD might still be required. Further research is required to explore the long-term relationship between serum total and free 25(OH)D concentration with energy generation. I believe that a large sample size will contribute in the understanding of the main effects of VitD3 supplementation in VitD deficiency and the possible differences in a VitD sufficient population.

The content of this chapter is partially described and discussed in Paper 3 (223): Montenegro KR, Cruzat V, Melder H, Jacques A, Newsholme P, Ducker KJ. Vitamin D Supplementation Does Not Impact Resting Metabolic Rate, Body Composition and Strength in Vitamin D Sufficient Physically Active Adults. Nutrients. 2020;12(10). DOI: 10.3390/nu12103111

Chapter 4 - Final Discussion and Conclusion

4.1 Final Discussion

Considering the two original studies of this thesis, the main findings of the first study demonstrated that VitD3 was associated with skeletal muscle cellular myogenesis and bioenergetics. This *in vitro* study (chapter 2) established that VitD3 is important for skeletal muscle function and enhances skeletal muscle growth, protein synthesis and energy metabolism using primary human skeletal muscle myoblasts and myotubes cells as an experimental model for the first time. Therefore, I subsequently performed a randomised clinical trial (*in vivo* study to investigate key outcomes such as RMR, body composition, and muscular strength and power that may be impacted by cellular processes after VitD treatment.

I have confirmed the effects of VitD in myogenesis in primary human skeletal cells, as previous studies reported effects in murine C2C12 cells or presented conflicting results. This thesis adds further evidence that VitD3 inhibits cellular proliferation in primary skeletal muscle myoblasts and stimulates differentiation in primary skeletal muscle myotubes. In addition, for the first time it was demonstrated that VitD3 significantly increased protein synthesis in primary myotubes, possibly through activation of the mTOR, PKB/Akt pathway proteins. Also, a significant increase in the size and number of myotubes was observed after VitD treatment, which to-date had only been proven in murine C2C12 cells (77). To the best of my knowledge, this study was also the first to detect an increase in mitochondrial oxygen consumption in primary myotubes and not only in primary myoblasts after VitD3 treatment (91). Overall, these results confirm an important physiological effect of VitD3 on muscle metabolism and function in primary human skeletal muscle cells. There is significant discussion whether extra-skeletal benefits of VitD exist and this first study might help to confirm its effects in primary human skeletal muscle cells.

After finding significant effects of VitD in skeletal muscle *in vitro*, I have tested whether these effects might transfer to functional markers of these same attributes *in vivo* (i.e., RMR, body composition and muscle strength and power) using VitD sufficient physically active adults. In general, researchers do not consider that physically active individuals require a greater supply of VitD3 to achieve optimal skeletal muscle health and function. In addition, VitD requirement for athletes is still unknown and VitD storage in skeletal muscle tissue seems to be different for physically active people that it is for sedentary individuals (175). This second *in vivo*

study (chapter 3) established that a VitD serum concentration ≥ 100 nmol·L^{-1} does not represent a level that may facilitate optimal skeletal muscle function or add any extra benefits in energy expenditure, muscle mass, strength and power in VitD sufficient physically active adults. This was the first study to select a sample 100% classified as sufficient in VitD3 at the baseline and to utilise a VitD dosage that is corrected for body-mass. Using a body-mass corrected dose of VitD rather than relying on varying absolute doses might help future research to assess the impact of an individualised approach to supplementation in-line with current best practice. In summary, the VitD group increased serum total 25(OH)D by 30 nmol·L^{-1} while the placebo group decreased total serum concentration by 21 nmol·L^{-1}, reaching 123 (51) and 53 (42.2) nmol·L^{-1}, respectively. However, I did not observe changes in muscle strength or power, RMR and body composition over the 12-week period. This study was the first to investigate the impact of serum free VitD concentrations with metabolic, performance and body composition parameters. Serum free VitD concentration did not significantly change after VitD3 supplementation, which might explain partially the lack of change I observed in the outcomes of this second study. More clarification on the effect of free VitD on health outcomes is necessary to characterize the true VitD status.

As discussed in Chapter 3, the majority of positive effects of VitD on sports performance are significant if athletes are deficient or insufficient in VitD at the baseline of the study. For example, recent RCT supplemented VitD3 in insufficient elite dancers with 5000 IU/day during eight weeks and observed higher vertical jump heights when compared with placebo group (224). The same authors have found a significant increase in isometric strength (18.7%) and vertical jump (7.1%) in elite ballet dancers (132) after supplementation with 2000 IU/day during 4 months (132). In a larger RCT including 61 male athletes and 30 healthy male non-athletes, 5000 IU/day of VitD3 during 8 weeks resulted in improvements in 10-metre sprint times and vertical jump. However, other RCTs were unable to observe any benefits of VitD supplementation in athletes with adequate or deficient VitD levels prior to supplementation (225). In addition, the effects of VitD on muscle strength were summarised in two main systematic reviews. The first review found that doses of VitD3 ranging from 400 to 8,500 IU·day^{-1} resulted in 1.4 - 18.8% increase in muscle strength in athletes classified as VitD sufficient at the baseline (141). The second review with meta-analysis (RCT = 5) did not find a significant efficacy of VitD3

supplementation on muscle strength (SMD -0.75, 95% CI: − 1.82 to 0.32, P = 0.17) in a mixed population of athletes (insufficient and sufficient in VitD) (189). Overall, comparisons between studies are challenging because of heterogeneity of ethnicities, sports investigated and also measures of physical performance are generally not standardised. As a result, it is problematic to establish conclusions on the VitD's clinical effects and VitD serum level recommendations for skeletal muscle function. Geographic location, skin pigmentation, indoor training and extensive sunscreen use are the most common factors known to inhibit VitD synthesis in athletes (226).

One possible theory that cannot be discarded and also justifies the variation of results in VitD supplementation studies is the individual molecular response to VitD. Carlberg and Haq suggested that VitD supplementation dose should be established according to the personal VitD response index (227). The concept is based on the fact that the active form of VitD combined with its receptor VDR can have a direct effect on the epigenome and transcriptome of many human tissues and cell types with different responsiveness (227).Two main intervention trials (VitDmet and VitDbol studies) (228, 229) have measured VitD sensitive molecular parameters, including transcription of genes of immune cells from blood, PTH and concentration of proteins and VitD metabolites in serum after VitD supplementation and then individuals were classified as high, mid or low responder to VitD. These intervention studies indicated that 25% of the participants were low responders and these results might be reproducible for other populations (228, 229). In other words, individuals with a low VitD response index should aim for a higher VitD concentration to have similar effects to the high VitD response individuals. More studies that investigate the epigenome and transcriptome in skeletal muscle cells are required to clarify if individuals have the same variation of VitD response in other tissues and cells. In summary, VitD exerts its effects in two main pathways: 1) genomic: slow process and involves the transcription and translation of target genes or 2) non-genomic: rapid process and involves the interaction between myosin and actin in the sarcomere implementing the force (230, 231).

As the majority of research with VitD supplementation has preferred to study participants during winter or autumn seasons because they are more likely to be VitD deficient at the start of the study, this thesis potentially validates the scenario that after summer, the VitD concentration is substantially elevated and physically active individuals do not need to reach supraphysiological levels to achieve optimal skeletal

muscle function, at least in temperate climates such as that found in Western Australia and in a Caucasian physically active population. On the other hand, several studies have demonstrated that dark skin pigmentation and indoor athletes also have a higher risk to develop muscle or bone disorders due to VitD deficiency (232-234). Previous research have described high rates of VitD deficiency in groups of athletes from UK (57%) (131), the Middle East (58%) (235) and Australia (33%) for example (236). In a clinical perspective, signs and symptoms of VitD deficiency are generally non-specific and include musculoskeletal pains, weakness, bone disorders and are frequently not noticed or are misdiagnosed in adults (225). As reported by Gunton and Girgis (7), VitD deficiency is detrimental for muscle function and might be independent of changes in serum calcium and phosphate levels (226).

An important limitation of this thesis that should be addressed in future research is to control the exercise stimulus in all participants. Although individuals in my study were orientated to keep a similar training during pre, middle and post-supplementation period and the total training load was monitored and not different between groups during the whole study, perhaps the training stimulus was not strong enough to impact on the outcomes measured. Future intervention studies are necessary to confirm these findings in a larger group of individuals, in a long-term manner and in other populations such as athletes or individuals who have an intense strength training routine. Also, future studies could focus solely on endurance training or resistance training. The second limitation of this thesis that should be addressed is to assess primary human skeletal muscle cells collected by muscle biopsy from the same participants of the *in vivo* study. This area of research in the field of VitD dose-effect in the whole body and at a cellular level is still developing and may encourage further studies to connect human *in vivo* and *in vitro* studies.

Another interesting area of research for future studies is the relationship between VitD serum concentration and maximal oxygen uptake (VO_2max). Positive correlations have been detected between total serum VitD concentration and VO_2max in physically inactive individuals in previous research (237-239). An observational study has demonstrated that physically active male individuals with VitD levels > 87.5 nmol·L^1 had significantly higher VO_2max levels than those with lower 25(OH)D levels (240). On the other side, no association was observed between VitD serum concentration and VO_2max in 52 professional ice hockey players (241). In summary, the possible VitD

pathway to increase VO_2max might be trough activation of cytochrome P450 (242), however, at this stage studies in this area including athletes are still inconclusive.

To better understand the difference in the results between the main two studies of this thesis (*in vitro* and *in vivo*) it is crucial to highlight that all living beings are structured according to a hierarchical level of complexity. One of the most important differences is that skeletal muscle cells are cultured in a low complexity, isolated system, without interaction with other cells, tissues, or hormones and specifically lack of factors provided by blood circulation. For example, if some change happens at the cellular level, it could consequently induce a reaction manifested or expressed in another higher level (i.e., other cells, tissue, organic or systemic levels) that tends to neutralise the change that took place at the lower level (cellular) in human body. Therefore, the in vitro approach forces the system to work in a different organisation level compared with the natural behaviour when all the systems (muscular, skeletal, nervous, endocrine and circulatory) are integrated in the human being. An absence of biokinetics (how the body metabolises and transports drugs and toxins) is one of the disadvantages of in vitro studies. Another key aspect to remember about musculoskeletal tissue is that *in vivo* cells are subject to loading forces. In my study, I did not consider a model with fluid-flow shear stress or cell stretching, representing a possible limitation (243).

The main regulation of VitD synthesis and activation will depend on factors such as PTH, fibroblast growth factors, Ca^{+2}, GH and are only able to be reproduced in animal and human studies. For this reason, *in vitro* studies may not always replicate the same results as *in vivo* studies. Importantly, I tried to use a hypothetical similar VitD dose in the in vitro and *in vivo* study, however it is challenging to compare the VitD concentration in both systems as they are not the same. For instance, the concentration of VitD treatment in the cells are equally distributed in the media, however it is not possible to guarantee that the VitD supplementation *in vivo* will reach the same concentration in the blood, tissues and specially in the skeletal muscle cellular level. To the best of my knowledge, the comparison between VitD supplementation and levels reached in different tissues and skeletal muscle cells haven't been explored. Finally, I also would like to highlight that the term vitamin D has been used to describe a wide range of VitD metabolites, such as cholecalciferol, ergocalciferol, calcitriol and the free/ biologically active form of VitD. Identifying which VitD metabolite is

biologically active and include measurements of multiple metabolites with the appropriate terminology should be prioritise in future research.

4.2 Final Conclusion

Vitamin D is essential for skeletal muscle function and impacts skeletal muscle metabolism. Myogenesis was significantly affected by VitD, as well as protein synthesis, mTOR downstream pathway(s) and energy metabolism in primary human skeletal muscle cells. In a RCT with physically active young adults sufficient in VitD, total serum VitD [25 (OH)D] concentration ≥ 100 nmol·L^1 did not affect energy metabolism, body composition and muscle strength and power. The concentration of total VitD significantly decreased in the winter season, suggesting that VitD supplementation might be beneficial. Finally, further clarification on the relationship between total and free 25(OH)D concentration is needed to better understand which metabolite is responsible for the biological effect of VitD.

4.3 References

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Appendices

Appendix A - Statement of Contribution by Others and Copyright Permissions

Paper 1

To Whom It May Concern

I, Karina Romeu Montenegro declare to have collected analysed and interpreted data from available PUBMED literature, conceptualized and wrote the manuscript entitled: **"Mechanisms of vitamin D action in skeletal muscle"**

_____

Full name: Karina Romeu Montenegro

Date: 27th May 2020

I, Dr. Vinicius Cruzat, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate and therefore give permission to use this publication as a part of Karina Romeu Montenegro' thesis.

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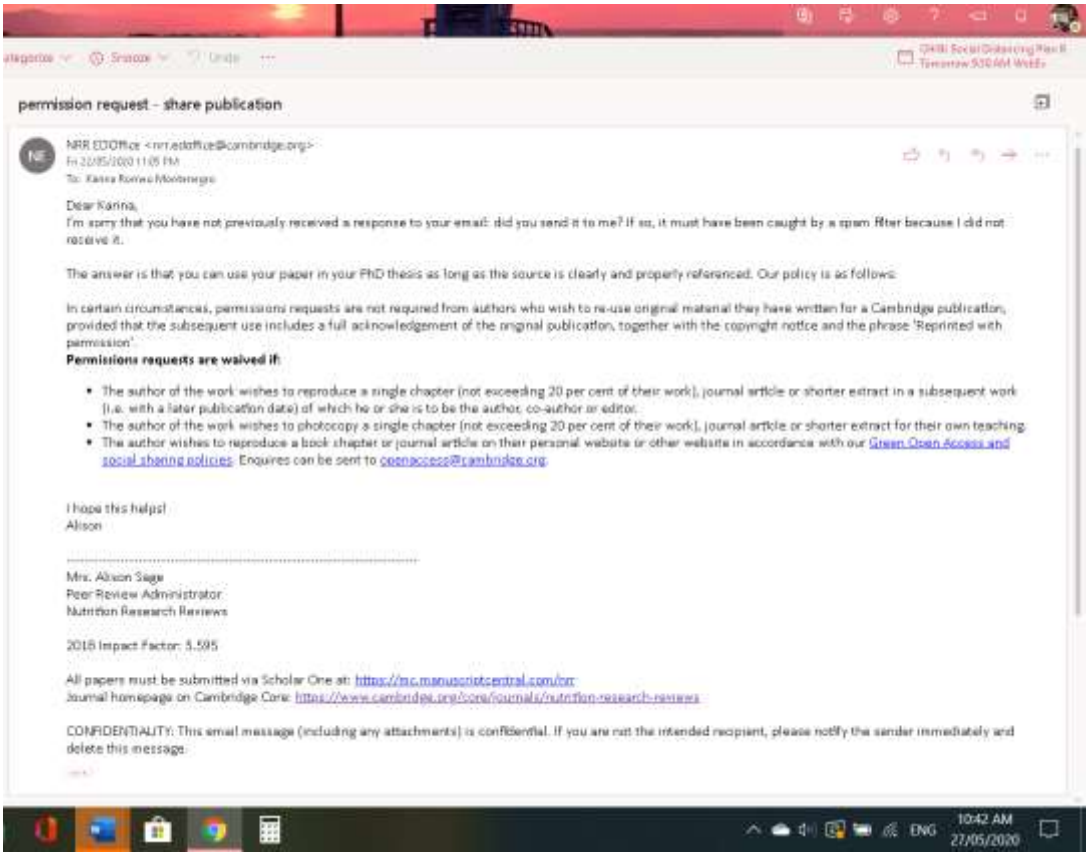

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ENG 10:42 AM 27/05/2020

Paper 2

To Whom It May Concern

I, Karina Romeu Montenegro declare to have planned, collected, analysed and interpreted data from experiments about the effects of vitamin D on primary human skeletal muscle cell, conceptualized and wrote the manuscript entitled**: "Effects of vitamin D on primary human skeletal muscle cell proliferation, differentiation, protein synthesis and bioenergetics"**

_____

Full name: Karina Romeu Montenegro

Date: 27th May 2020

I, Dr. Rodrigo Carlessi, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate and therefore give permission to use this publication as a part of Karina Romeu Montenegro' thesis.

_____

Full name: Rodrigo Carlessi

Date: 27th May 2020

I, Dr. Vinicius Cruzat, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate and therefore give permission to use this publication as a part of Karina Romeu Montenegro' thesis.

_____

Full name: Vinicius Cruzat

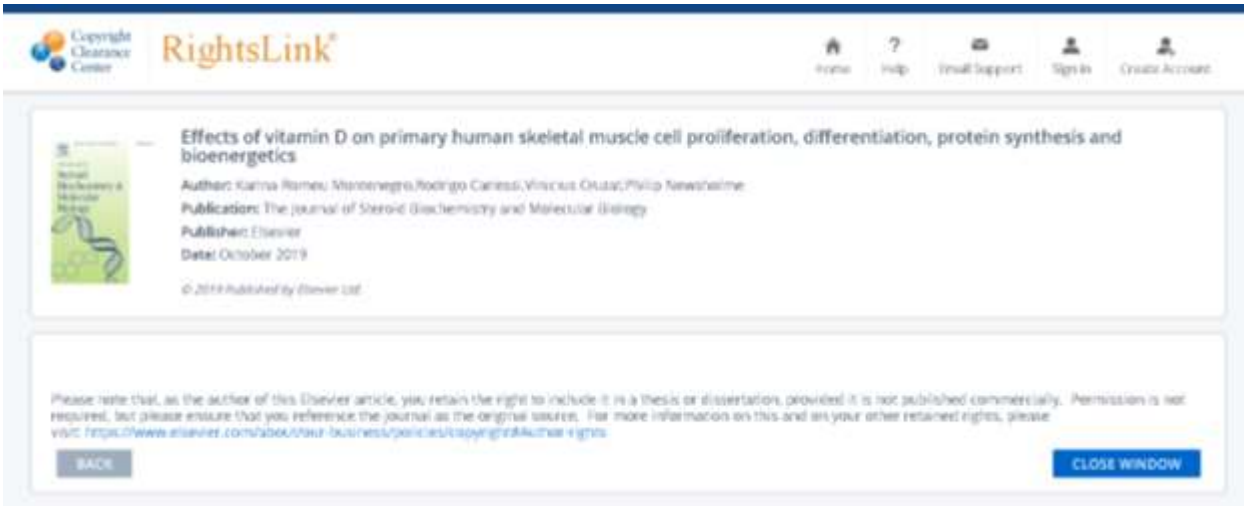
Date: 27th May 2020

I, Prof. Philip Newsholme, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate and therefore give permission to use this publication as a part of Karina Romeu Montenegro'thesis.

_____

Full name: Philip Newsholme

Date: 10th September 2020



Paper 3

To Whom It May Concern

I, Karina Romeu Montenegro declare to have planned, collected, analysed and interpreted data from my second PhD project that aimed to investigate the supplementation of Vitamin D in physically active adults. I conceptualised and wrote the manuscript entitled: **"Vitamin D Supplementation Does Not Impact Resting Metabolic Rate, Body Composition and Strength in Vitamin D Sufficient Physically Active Adults".**

_____

Full name: Karina Romeu Montenegro

Date: 14/10/2020

I, Dr. Vinicius Cruzat, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate and therefore give permission to use this publication as a part of Karina Romeu Montenegro' thesis.

_____

Full name: Vinicius Cruzat

Date: 16/10/2020

I, Hilton Melder, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate and therefore give permission to use this publication as a part of Karina Romeu Montenegro' thesis.

_____

Full name: Hilton Melder

Date: 20/10/2020

I, Dr. Angela Jacques, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate and therefore give permission to use this publication as a part of Karina Romeu Montenegro' thesis.

_____

Full name: Angela Jacques

Date: 15/10/2020

I, Prof. Philip Newsholme, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate and therefore give permission to use this publication as a part of Karina Romeu Montenegro' thesis.

_____

Full name: Philip Newsholme

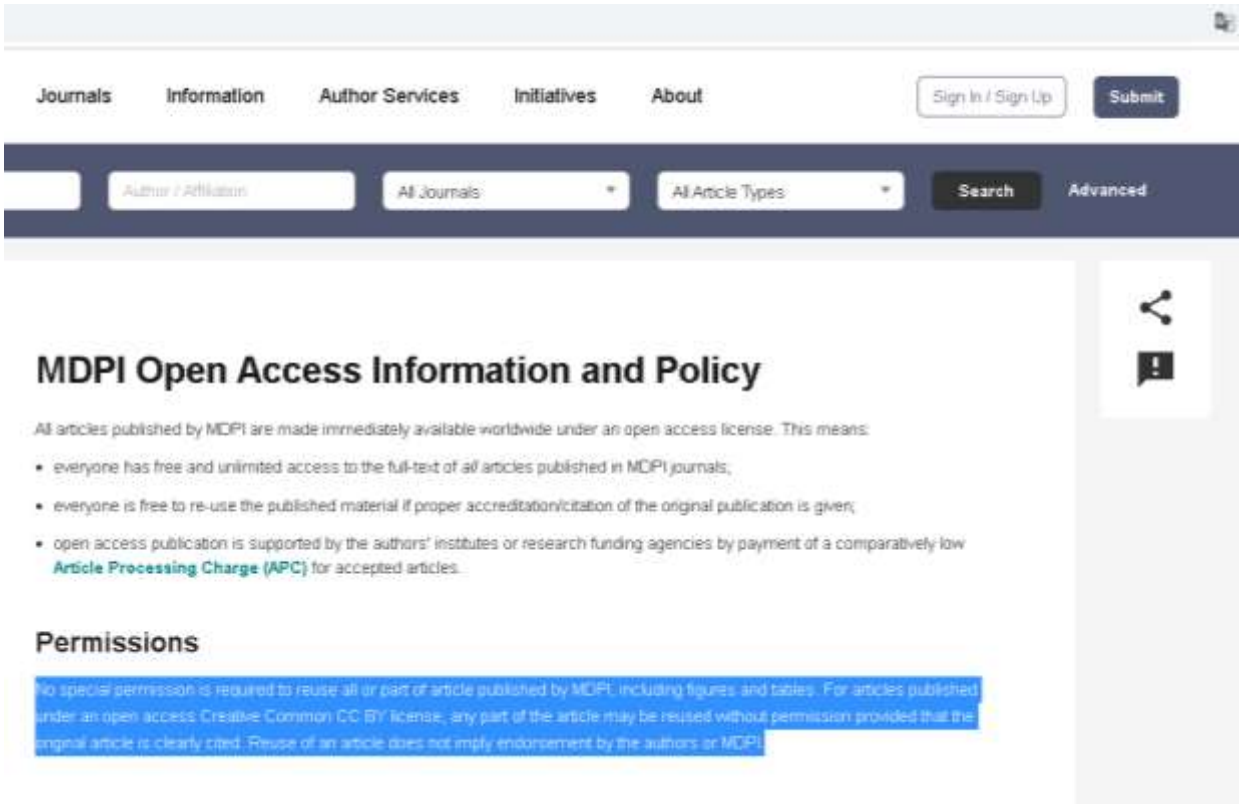
Date: 20/10/2020

I, Dr Kagan J. Ducker, as a Co-Author, endorse that the level of contribution by the candidate indicated above is appropriate and therefore give permission to use this publication as a part of Karina Romeu Montenegro' thesis.

_____

Full name: Kagan J. Ducker

Date: 20/10/2020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Appendix B – Study Forms

CONSENT FORM

HREC Project Number:	**15976**
Project Title:	***The impact of vitamin D supplementation on resting metabolic rate, body composition, and strength in physically active adults***
Principal Investigator:	***Dr Kagan Ducker, Senior Lecturer, School of Physiotherapy and Exercise Science***
Student researcher:	***Karina Romeu Montenegro***
Version Number:	***V1***
Version Date:	**08/01/2019**

- I have read, the information statement version listed above and I understand its contents.
- I believe I understand the purpose, extent and possible risks of my involvement in this project.
- I voluntarily consent to take part in this research project.
- I have had an opportunity to ask questions and I am satisfied with the answers I have received.
- I understand that this project has been approved by Curtin University Human Research Ethics Committee and will be carried out in line with the National Statement on Ethical Conduct in Human Research (2007).
- I understand I will receive a copy of this Information Statement and Consent Form.
- If you are female please tick (☑) the check box below.
  □ *I confirm that to the best of my knowledge I am not pregnant, nor attempting to become pregnant*

Participant Name	
Participant Signature	
Date	

Declaration by researcher: I have supplied an Information Letter and Consent Form to the participant who has signed above, and believe that they understand the purpose, extent and possible risks of their involvement in this project.

Researcher Name	
Researcher Signature	
Date	

Note: All parties signing the Consent Form must date their own signature.

PARTICIPANT INFORMATION STATEMENT

HREC Project Number:	15976
Project Title:	***The impact of vitamin D supplementation on resting metabolic rate, body composition, and strength in physically active adults***
Principal Investigator:	***Dr Kagan Ducker, Senior Lecturer, School of Physiotherapy and Exercise Science***
Student researcher:	***Karina Romeu Montenegro***
Version Number:	**V2**
Version Date:	**21/01/2019**

What is the Project About?

Most of our vitamin D comes from spending time in the sun, but also a little comes from our diet. Importantly, research suggests that the amount of vitamin D that you have may affect the health of your muscles and therefore your strength, body composition and the amount of energy that your body uses at rest. However, we don't know lots of things about this relationship and therefore we need to investigate it further. This is important because vitamin D may assist people to achieve a healthier body composition and perhaps help with the progression of exercise training (e.g., increasing muscle strength). The results of this study will help health professionals to clarify if higher levels of vitamin D are beneficial for achieving optimal muscle function and body composition.

Who is doing the Research?

The project is being conducted by a collaborative team of researchers from the Schools of Physiotherapy and Exercise Science and Pharmacy and Biomedical Sciences at Curtin University, and the Faculty of Health Sciences at Torrens University Australia. The team comprises Dr Kagan Ducker (principle investigator; Curtin University), Karina Romeu Montenegro (PhD student, Curtin University), Prof Philip Newsholme (main supervisor, Curtin University), and Dr Vinicius Cruzat (supervisor, Torrens University Australia). This research project is funded by Curtin University and whilst you will not be paid for participating in this project, there will be no costs to you.

Why am I being asked to take part and what will I have to do?

Given that this project is seeking to identify the effects of vitamin D supplementation in physically active adults you must be male or female aged 18 – 35 y, currently be completing exercise at least three times per week, with resistance training making up at least two of those three sessions per week, with no history of vitamin D supplementation in the last month, no current injuries that would prevent you from completing maximal strength testing, and with no current use of multivitamins, medication or other supplements that are related to vitamin D metabolism and body composition (e.g., calcium, thyroxine, whey protein, creatine and thermogenic supplements). We are looking for this population to volunteer so that we can ensure that our findings are representative of the typical physically active adult.

We need you to complete 4 testing sessions (2 pre-supplementation and 2 post-supplementation) over approximately 13 weeks (see Figure 1 below for a summary of the study design). Each session will take approximately 1 – 2 hours to complete. Additionally, there are a few smaller questionnaires and a food and training diary to complete during the study period while you are supplementing with vitamin D.

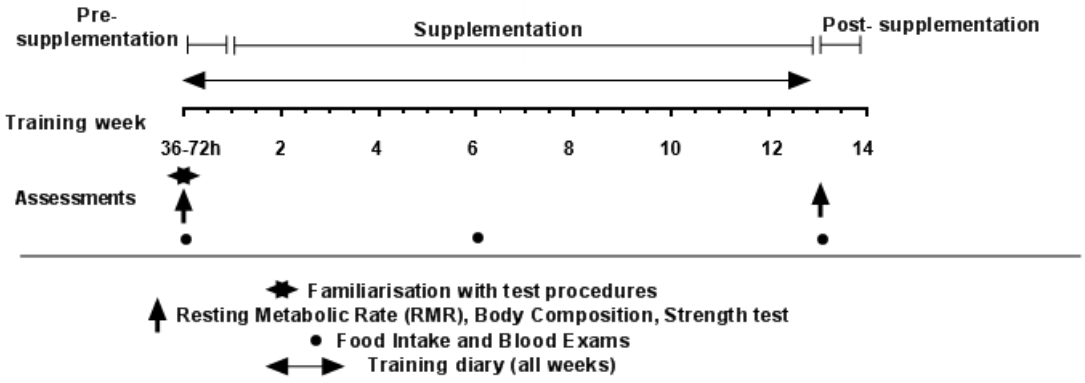
Before you start the study, we will explain all of the tests to you so that we can familiarise you with all the tests. The first session will involve the assessment of your resting energy usage (resting metabolic rate – RMR), body composition, a blood test to assess some markers related to vitamin D (completed at your local Pathwest collection point at the beginning, middle and end of the study) and familiarisation to the study diary. We will ask you to complete 3 x 3 day food diaries, at the start, in the middle (after 6 weeks) and last week of the trial to assess what you normally eat and

drink (2 days during the week and 1 day of the weekend). The training diary will have to be completed each day of the study and it includes details of the type of exercise, duration and a perceived intensity of the session. The training diary should be easy to complete because you should maintain a similar type of training/exercise during the study, if possible. We will also ask you to complete a five minute questionnaire at baseline, 7 and 12 weeks to assess your calcium intake, and another short 5 min quiz to assess your sunlight exposure at baseline, 7 and 12 weeks. You should bring the diary to testing sessions each time you come in so that we can check on your progress.

For the assessment of your resting energy usage we will ask you to come to the Exercise Physiology Research Laboratory at Curtin University as soon as possible after you have woken up in the morning, after fasting overnight. We will get you to lie down in a quiet room (30 min) and we will measure the gases that you are breathing in and out via a mouthpiece. From this we can determine how much energy you use at rest. That same morning we will take you another laboratory for a quick (approximately 10 min) scan to assess your body composition (amount of muscle and fat) using dual-energy x-ray absorptiometry (DXA – pronounced as deck-sa). This technique passes a small amount of x-rays through your body (see risk section below) so that it can determine what tissues make up your body. Finally, during the first week of the study we will ask you to attend your nearest Pathwest Collection Centre to have a small blood sample taken so that we can assess your vitamin D levels and other markers related to vitamin D. We will direct you to do this again at the middle and end of the study.

A few days later we will ask you to come back to the Strength and Conditioning Laboratory at Curtin University to complete functional strength testing. We will ask you to warm-up, then, you will lift progressively greater weights until a weight is identified that you can only lift for one repetition. We will do this for the bench press (chest) and back squat (legs) exercises. This will be a heavy weight, but we will guide you through the process. Finally, you will complete a vertical jump test, which involves you squatting down then jumping as high as you can. This is an assessment of leg power. At this session we will also review your training diary, food recall, calcium quiz and sunlight recall quickly to make sure that you have been completing it correctly – participants should organise an hour for this second session.

Figure 1. Study Design



Following the initial assessments, we will randomly put you into one of two groups for the 12 weeks supplementation period, and we will not tell you which group you are in until you finish the post-study testing. One group will ingest a dose of active vitamin D3 (50 IU·kg^{-1}body-mass·day^{-1}). This is equal to about 3500 IU·day^{-1} for someone that weighs 70 kg and is the equivalent of approximately 30 minutes in the sun. The second group is a placebo group, which means that you won't be given vitamin D supplementation. If you are allocated to this group you will ingest a small amount of dextrose (sugar) placebo for 12 weeks, which shouldn't affect you in any meaningful way. Your group will be chosen by chance, like tossing a coin so that you cannot tell which group you are allocated to. Both supplements will be placed in opaque gelatin capsules so that they look the same. The student researcher won't know which group you are in so that they will not be able to influence the research in any way, but we will keep a close eye on your health and response. The principle investigator will know which group you are in so that they can monitor you throughout the study.

At the end of the supplementation period, we will repeat the initial tests in the same manner to see if there have been any changes in your resting energy use, body composition and muscle strength. We will also ask you about which supplement group you believe that you were in and the reasons for your decision. After that, we will tell you which group you were in.

Are there any benefits' to being in the research project?

There may be no direct benefit to you from participating in this research. However, this research will provide important insights into how vitamin D levels affect your body. Furthermore, you will receive the results of all the assessments performed, like body composition, resting metabolic rate and strength tests that may be very interesting to you. Finally, if we find any issues with your vitamin D levels or other markers that seem atypical, we will tell you so that you can consult your physician to have these checked.

Are there any risks, side-effects, discomforts or inconveniences from being in the research project?

Body composition assessments using DXA involves a small dose of ionising radiation (~ 0.74 µSv µSv) that is equivalent to about one thousandth of the background radiation dose that a person would receive whilst living in Perth, Western Australia for one year. For comparison the total background radiation in Western Australia is about 2000 µSv per year, and the radiation dose from cosmic rays when flying from Perth to London is approximately 100 µSv. All participants will complete a DXA pre-scan questionnaire to assess their suitability to complete the scan. Any female that is pregnant or may be pregnant will be excluded from participating.

The risks of taking blood include the chance of infection or a bruise at the point where the blood is taken, redness and a rare risk of fainting. Participants will have blood sampled by a trained professional that will be using gloves and following hygienic procedures. Participants are reminded to keep the wound clean and dry after blood sampling to minimise the risk of infection at the sample site. To minimise the risk of injury from fainting, individuals will be seated in a chair during blood sampling.

Regarding supplementation with vitamin D, it is considered safe at doses < 10,000 $IU \cdot day^{-1}$, which is much higher than the dose that will be prescribed in this study. We will be monitoring your vitamin D status and other markers at the start, middle and end of the study to ensure that no negative effects have occurred. We will refer you to your physician if we identify any negative changes.

Strength testing does involve a rare risk of musculoskeletal injury and a small risk of a delayed onset muscle soreness. The testing will be conducted by a trained researcher who will ensure that you are warmed up and ready to participate in the tests and will

monitor the activity for safety at all times. As you regularly complete strength training, you will be familiar with the type of exercise and after effects.

Who will have access to my information?

The information collected and kept by Curtin University staff in this research will be re-identifiable (coded). This means that the stored information will be re-identifiable which means we will remove identifying information on any data or sample and replace it with a code. Only the research team have access to the code to match your name if it is necessary to do so. Any information we collect will be treated as confidential and used only in this project unless otherwise specified. The following people will have access to the information we collect in this research: the research team and, in the event of an audit or investigation, staff from the Curtin University Office of Research and Development. All information will be stored securely on backed up and password protected research hard-drives at Curtin University.

Due to the ionizing radiation of the DXA scans, the University is required to keep the data 50 years after the date of publication or completion of project whichever is later. It is a legal requirement (Radiation Safety Act, Section 36: point 2.4) that "records are maintained of the patients examined on the apparatus including their name, age, the type of examination, the date of the examination and the name of the referring medical practitioner and these records shall be available for inspection by officers of the (Radiological) Council." The Curtin University license has been endorsed to permit densitometry scans for research subjects without medical referral. Therefore, a register of participants scanned will include the study they are participating in instead of the referring Doctor's name, but a register will be maintained for the Radiological Council. If this arrangement doesn't suit you, you will be unable to participate in the study. You have the right to access, and request correction of, your information in accordance with relevant privacy laws. The results of this research may be presented at conferences or published in professional journals. You will not be identified in any results that are published or presented.

Will you tell me the results of the research?

If you give us your contact details, we will write a report that will be delivered to you at the end of the study with the results of all the assessments. We will also let you know what the study findings, however this can take 6 – 12 months after study completion to finalise.

Do I have to take part in the research project?

Taking part in a research project is voluntary. It is your choice to take part or not. You do not have to agree if you do not want to. If you decide to take part and then change your mind, that is okay, you can withdraw from the project. You do not have to give us a reason; just tell us that you want to stop. Please let us know you want to stop so we can make sure you are aware of anything that needs to be done so you can withdraw safely. If you chose not to take part or start and then stop the study, it will not affect your relationship with the University, staff or colleagues.

If you chose to leave the study, we will use any information collected unless you tell us not to.

What happens next and who can I contact about the research?

You can contact any of the following people to discuss this project.

PhD Student Karina Romeu Montenegro (Curtin University)
PH: +61 8 9266 1649
EMAIL: karina.romeumon@student.curtin.edu.au
Dr Kagan Ducker (Curtin University)
PH: +61 8 9266 4644
EMAIL: kagan.ducker@curtin.edu.au

Curtin University Human Research Ethics Committee (HREC) has approved this study (HREC number XX/XXXX). Should you wish to discuss the study with someone not directly involved, in particular, any matters concerning the conduct of the study or your rights as a participant, or you wish to make a confidential complaint, you may contact the Ethics Officer on (08) 9266 9223 or the Manager, Research Integrity on (08) 9266 7093 or email hrec@curtin.edu.au.

Ethics Project Approval

Curtin University

Research Office at Curtin

GPO Box U1987
Perth Western Australia 6845

Telephone +61 8 9266 7863
Facsimile +61 8 9266 3793
Web research.curtin.edu.au

22-Jan-2019

Name: Kagan Ducker
Department/School: School of Physiotherapy and Exercise Science
Email: Kagan.Ducker@curtin.edu.au

Dear Kagan Ducker

RE: Ethics Office approval
Approval number: HRE2019-0028

Thank you for submitting your application to the Human Research Ethics Office for the project **The impact of vitamin D supplementation on resting metabolic rate, body composition, and strength in physically active adults**.

Your application was reviewed through the Curtin University Low risk review process.

The review outcome is: **Approved**.

Your proposal meets the requirements described in the National Health and Medical Research Council's (NHMRC) *National Statement on Ethical Conduct in Human Research (2007)*.

Approval is granted for a period of one year from **22-Jan-2019** to **21-Jan-2020**. Continuation of approval will be granted on an annual basis following submission of an annual report.

Personnel authorised to work on this project:

Name	Role
Ducker, Kagan	CI
Romeu Montenegro, Karina	Student
Newsholme, Philip	Supervisor
Cruzat, Vinicius	Supervisor

Approved documents:

Document

Standard conditions of approval

1. Research must be conducted according to the approved proposal
2. Report in a timely manner anything that might warrant review of ethical approval of the project including:
    - proposed changes to the approved proposal or conduct of the study
    - unanticipated problems that might affect continued ethical acceptability of the project
    - major deviations from the approved proposal and/or regulatory guidelines
    - serious adverse events
3. Amendments to the proposal must be approved by the Human Research Ethics Office before they are implemented (except where an amendment is undertaken to eliminate an immediate risk to participants)
4. An annual progress report must be submitted to the Human Research Ethics Office on or before the anniversary of approval and a completion report submitted on completion of the project
5. Personnel working on this project must be adequately qualified by education, training and experience for their role, or supervised
6. Personnel must disclose any actual or potential conflicts of interest, including any financial or other interest or affiliation, that bears on this project
7. Changes to personnel working on this project must be reported to the Human Research Ethics Office
8. Data and primary materials must be retained and stored in accordance with the Western Australian University Sector Disposal Authority (WAUSDA) and the Curtin University Research Data and Primary Materials policy
9. Where practicable, results of the research should be made available to the research participants in a timely and clear manner
10. Unless prohibited by contractual obligations, results of the research should be disseminated in a manner that will allow public scrutiny; the Human Research Ethics Office must be informed of any constraints on publication
11. Approval is dependent upon ongoing compliance of the research with the Australian Code for the Responsible Conduct of Research, the National Statement on Ethical Conduct in Human Research, applicable legal requirements, and with Curtin University policies, procedures and governance requirements
12. The Human Research Ethics Office may conduct audits on a portion of approved projects.

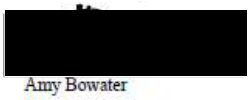
Special Conditions of Approval

None.

This letter constitutes low risk/negligible risk approval only. This project may not proceed until you have met all of the Curtin University research governance requirements.

Should you have any queries regarding consideration of your project, please contact the Ethics Support Officer for your faculty or the Ethics Office at hrec@curtin.edu.au or on 9266 2784.

Yours sincerely



Amy Bowater
Ethics, Team Lead

Radiation Project Approval

Curtin University

Research Office at Curtin

GPO Box U1987
Perth Western Australia 6845

Telephone +61 8 9266 7863
Facsimile +61 8 9266 3793
Web research.curtin.edu.au

06-Mar-2019

Name: Kagan Ducker
Department: School of Physiotherapy and Exercise Science
Email: Kagan.Ducker@curtin.edu.au

Dear **Kagan Ducker**

RE: Radiation project application approval

Thank you for submitting your application to the Radiation Safety Committee for the project **The impact of vitamin D supplementation on resting metabolic rate, body composition, and strength in physically active adults**. Your application was reviewed through the Curtin University Low Risk radiation review process against requirements under the Radiation Safety Act 1975 and the Nuclear Non-Proliferation Act 1987.

The review outcome is: **Approved**
Approval number: **RSC2018-08**

Approval is granted from **06-Mar-2019** subject to the following standard conditions of approval:
1. Work is conducted as described in the protocol.
2. Any changes to the protocol must be approved via an amendment submission before being implemented.
3. Self-audit reports are submitted in a timely manner when requested.
4. A completion report is submitted when the radiation aspects of the project come to an end.
5. If a licence holder is no longer able to take responsibility for a particular radiation substance or equipment, work on that substance or equipment must cease until a new licence holder is found. This project uses the following substances and/or equipment:

The following additional conditions of approval are also applicable:

This letter constitutes approval for the radiation aspects of the project only. This project may not proceed until you have met all of the Curtin University research governance requirements.

Should you have any queries regarding consideration of your project, please contact the Radiation Saftey Officer at radsafety@curtin.edu.au or on 9266 1708.

Yours sincerely

[redacted]

Dr Matt Carroll
Radiation Safety Officer

Training and Food Diary

Effect of vitamin D supplementation on metabolic rate, body composition and strength in physically active adults

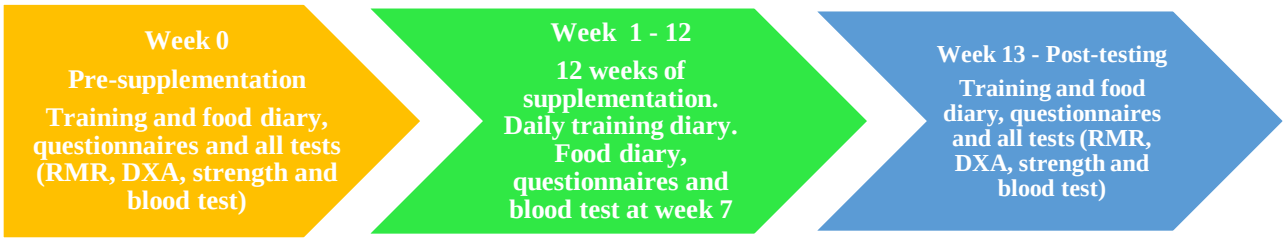
Training and Food Diary (short version)

PhD Student: Karina Romeu Montenegro
Contact details: karina.romeumon@postgrad.curtin.edu.au

Supervisor: Prof. Philip Newsholme
Co-supervisor: Dr Kagan Ducker
Co-supervisor: Vinicius Cruzat

Your research project diary

Being part of a research project can be quite a confusing experience. My hope is that this diary will help make the experience a little simpler. First of all, here is a quick reminder about the project structure;



The purpose of this diary is to assess factors that might impact your vitamin D status, strength and body composition. Hopefully this diary will guide you through what is required each day of the study.

First of all here is what you are required to fill out on each of the weeks;

Pre Supplementation period/WEEK 0-1 – Before you start the supplementation, please record your exercise/training and record your food intake. You will be required to match this as closely as possible before each test. In the three days prior to your second test record your food intake **(only 2 days of the week and 1 day of the weekend). Please also complete the online calcium quiz (link below) and sunlight questionnaire.**

Link: http://www.dairycouncilofca.org/Tools/CalciumQuiz

Week 2 – Begin supplementation Weekly record of training/exercise completed and any perceived side effects of the supplement.

Week 3 – Weekly record of training/exercise completed and any perceived side effects of the supplement.

Week 4 – Weekly record of training/exercise completed and any perceived side effects of the supplement.

Week 5 – Weekly record of training/exercise completed and any perceived side effects of the supplement.

Week 6 – Weekly record of training/exercise completed and any perceived side effects of the supplement.

Week 7 – Weekly record of training/exercise completed and any perceived side effects of the supplement. This week you also have to record your **food intake (only 2 days**

of the week and 1 day of the weekend). Please also complete the online calcium quiz and general questions (end of this document).

Link: http://www.dairycouncilofca.org/Tools/CalciumQuiz

Week 8 – Weekly record of training/exercise completed and any perceived side effects of the supplement.

Week 9 – Weekly record of training/exercise completed and any perceived side effects of the supplement.

Week 10 – Weekly record of training/exercise completed and any perceived side effects of the supplement.

Week 11– Weekly record of training/exercise completed and any perceived side effects of the supplement.

Week 12 – Weekly record of training/exercise completed and any perceived side effects of the supplement. **This week you also must record your food intake (only 2 days of the week and 1 day of the weekend). Please also complete the online calcium quiz and general questions (end of this document).**

Week 13 – Finalise the tests at Curtin University and PathWest lab.

Link: http://www.dairycouncilofca.org/Tools/CalciumQuiz

Food Diary

In this section please list exactly what you ate during each of the periods. Please estimate the breakdown of each thing eaten into its components

e.g., stir fry = 1/2 cup of chicken, 1/3 cup broccoli, 1/3 cup carrot, 1 cup of rice noodles; 1 Mars bar = 1 Mars bar etc.

I understand there will be some temptation to lie (we all do it) but there is no wrong or right things to put in this section so please ensure you tell the complete truth so that I get an accurate reflection of what you ate during these days.
On the day of strength testing ensure that you are well rested, hydrated and have eaten.

Exercise/Training Diary
In this section please list all of the exercise and training that you did that day. Please note the **modality** (e.g., row, cycle, run, etc.), **details of the session** (e.g. 5 x 1000 m), **duration of the session and then finally rate each session based upon how hard you perceived it was based upon the Borg scale below**. To use the Borg scale below simply ask yourself **"how hard was that session?"** and then choose the number that best describes how you felt after the session. Again please be as honest as possible when filling in the details of your sessions.

Rating	Descriptor
0	Rest
1	Very, very easy
2	Easy
3	Moderate
4	Somewhat Hard
5	Hard
6	
7	Very Hard
8	
9	
10	Maximal

Figure 1. Modified version of the category ratio rating of perceived exertion (RPE) scale(211).

MOST IMPORTANTLY GET IN TOUCH IF YOU HAVE ANY DOUBTS AND ENJOY BEING PART OF THE STUDY!

Thanks again for taking part!

If you have any questions at all do not hesitate to email me on karina.romeumon@postgrad.curtin.edu.au
(Karina Romeu Montenegro)

DAY 1- week 0 of the study (before supplementation) - Date:

FOOD DIARY 1 – week 0
Morning – Including snacks until lunch
Lunch – including snacks until dinner
Dinner – including snacks until bed

DAY 2 – week 0 Date:

FOOD DIARY 2 – week 0
Morning – Including snacks until lunch
Lunch – including snacks until dinner
Dinner – including snacks until bed

DAY 3- week 0 - Date:

WEEKEND 1 FOOD DIARY 3
Morning – Including snacks until lunch
Lunch – including snacks until dinner
Dinner – including snacks until bed **PS: PLEASE ALSO COMPLETE THIS QUIZ ONLINE AND SAVE THE RESULTS (picture or pint screen or copy and paste in word, whatever is easier for you)** **Link: http://www.dairycouncilofca.org/Tools/CalciumQuiz**

EXERCISE/TRAINING DIARY 1 – Week 0
DAY _____ – WEEK 0 **In this box please provide details about every session that you completed each day of this week. For each session please include:** **What type of exercise (e.g., cycling, swimming, running) did you do for the session?** **How long was the session (minutes)?** **How hard was the session (rating 1 - 10)?**
DAY _____ – WEEK 0 **What type of exercise (e.g., cycling, swimming, running) did you do for the session?** **How long was the session (minutes)?** **How hard was the session (rating 1 - 10)?**
DAY _____ – WEEK 0 **What type of exercise (e.g., cycling, swimming, running) did you do for the session?** **How long was the session (minutes)?** **How hard was the session (rating 1 - 10)?**

DAY _____ – WEEK 0

What type of exercise (e.g., cycling, swimming, running) did you do for the session?

How long was the session (minutes)?

How hard was the session (rating 1 - 10)?

DAY _____ – WEEK 0

What type of exercise (e.g., cycling, swimming, running) did you do for the session?

How long was the session (minutes)?

How hard was the session (rating 1 – 10)

DAY _____ – WEEK 0

What type of exercise (e.g., cycling, swimming, running) did you do for the session?

How long was the session (minutes)?

How hard was the session (rating 1 – 10)

DAY _____ – WEEK 0

What type of exercise (e.g., cycling, swimming, running) did you do for the session?

How long was the session (minutes)?

How hard was the session (rating 1 – 10)

General questions:

Have you been sick or injured this week?

_____

How much time you were exposed to sunlight this week (0 ≤5 min, 1 = 5–30 min, and 2 = ≥30 min)?

_____

Which area is exposed? (1 = face and hands only; 2 = face, hands and arms; 3 = face, hands and legs; and 4 = “bathing suit”)

_____

Do you use sunscreen when you are exposed to the sun? in which area?

_____

Appendix C - Original Reprints of Publications.

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Mechanisms of vitamin D action in skeletal muscle

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Abstract

Vitamin D receptor expression and associated function have been reported in various muscle models, including C_2C_{12}, L6 cell lines and primary human skeletal muscle cells. It is believed that 1,25-hydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active form of vitamin D, has a direct regulatory role in skeletal muscle function, where it participates in myogenesis, cell proliferation, differentiation, regulation of protein synthesis and mitochondrial metabolism through activation of various cellular signalling cascades, including the mitogen-activated protein kinase pathway(s). It has also been suggested that $1,25(OH)_2D_3$ and its associated receptor have genomic targets, resulting in regulation of gene expression, as well as non-genomic functions that can alter cellular behaviour through binding and modification of targets not directly associated with transcriptional regulation. The molecular mechanisms of vitamin D signalling, however, have not been fully clarified. Vitamin D inadequacy or deficiency is associated with muscle fibre atrophy, increased risk of chronic musculoskeletal pain, sarcopenia and associated falls, and may also decrease RMR. The main purpose of the present review is to describe the molecular role of vitamin D in skeletal muscle tissue function and metabolism, specifically in relation to proliferation, differentiation and protein synthesis processes. In addition, the present review also includes discussion of possible genomic and non-genomic pathways of vitamin D action.

Key words: **Calcitriol: Muscle: Mitochondria: Myogenesis: Protein synthesis**

Introduction

The 'sunshine hormone', vitamin D, is a pro-steroid hormone that is reported to be the earliest hormone to arise on earth[1]. In the last 20 years, the number of scientific studies reporting the importance of vitamin D dietary intake and supplementation for cell and tissue function has increased dramatically. However, clinical studies have indicated that more than 1 billion individuals present with vitamin D insufficiency or deficiency[2–5]. Vitamin D receptor (VDR) expression in human muscle declines with age and a reduced capacity for UV-mediated vitamin D synthesis in the elderly's skin may partly explain why the muscles of these individuals tend to be more susceptible to low vitamin D levels[6]. Hence, low vitamin D status has been considered a worldwide public health problem and is associated with the development of many diseases, such as osteoporosis, cancer, infertility, type 2 diabetes mellitus, coronary artery disease, and also has a significant impact on the immune system[7–10].

It has long been accepted that vitamin D plays a critical role in the regulation of Ca^{2+} and phosphate homeostasis and therefore is critical for impact on bone function. However, the discovery of a VDR in skeletal muscle cells provided further evidence of the important role of this hormone in skeletal muscle function and metabolism[11–14]. Low levels of vitamin D are associated with skeletal muscle fibre atrophy, muscle pain, weakness, and increased risk of sarcopenia and associated falls, in active and non-active individuals[15–18]. In athletes, low vitamin D levels are associated with poor bone health, and can impair muscle and immune functions, resulting in low muscle regenerative capacity after exercise sessions[19] and high risk of upper respiratory tract infections[20], respectively. The importance of vitamin D for skeletal muscle function and ultimately for whole body health is also supported by several randomised controlled trials (RCT) where vitamin D supplementation resulted in an increase in muscle strength in physically active and non-active individuals[21,22].

Several molecular mechanisms have been proposed to mediate the effects of vitamin D in muscle strength, function and metabolism, including changes in protein synthesis, myogenesis, mitochondrial activity, muscle regeneration and glucose metabolism[22–25]. However, the exact underlying mechanisms of vitamin D-related pathways, as well as their regulation and action in skeletal muscle, or how these pathways can be translated into clinical improvements, are unclear. Hence, the main purpose of the

Abbreviations: $1,25(OH)_2D_3$, 1,25-hydroxyvitamin D_3; 25(OH)D, 25-hydroxyvitamin D; Akt, protein kinase B; IGF, insulin-like growth factor; MAPK, mitogen-activated protein kinase; MEF, myocyte enhancer factor; MRF, myogenic regulatory factor; mTOR, mammalian target of rapamycin; Myf5, myogenic factor 5; MyoD, myoblast determination protein; MYOG, myogenin; PKB, protein kinase B; RCT, randomised controlled trial; TGF-β, transforming growth factor β; VDR, vitamin D receptor.

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present review is to discuss the evidence for vitamin D action in skeletal muscle function and metabolism, at the molecular level, citing results from studies *in vitro*, *in vivo* and clinical research.

Vitamin D physiology and synthesis

Vitamin D is a liposoluble pro-hormone mostly found in two forms (vitamin D_2 and vitamin D_3) and is obtained by a self-regulated process, as described below. Sources of dietary vitamin D include oily fish, eggs and dairy products; however, the consumption and intestinal absorption of vitamin D represent only a minor part of the total vitamin D requirement for the whole body[15]. Exposure of the skin to UV radiation (wavelength 290–315 nm) is associated with 80–90 % of total vitamin D synthesis and allows 7-dehydrocholesterol to be converted to cholecalciferol (pre-vitamin D_3)[15,26,27]. Both endogenous synthesis and exogenous dietary intake raise serum concentrations of vitamin D, which undergoes two hydroxylation steps to the final product[28]. Firstly, pre-vitamin D_3 is thermal isomerised to vitamin D_3, binds to vitamin D binding protein and is subsequently transported into the liver where it is hydroxylated by the enzyme vitamin D 25-hydroxylase to 25-hydroxyvitamin D (25(OH)D). This molecule is the predominant circulating form of vitamin D that is traditionally measured to identify vitamin D status in humans as it has a relatively long half-life in the circulation as it is established as a reliable marker of vitamin D status[29]. 25(OH)D is then metabolised primarily in the proximal tubule of the kidney, or other tissues such as the skeletal muscle, by the enzyme 25-hydroxyvitamin D-1α-hydroxylase (encoded by the *CYP27B1* mitochondrial gene) to its biologically active form 1,25-hydroxyvitamin D_3 ($1,25(OH)_2D_3$), also known as calcitriol[27,30]. Subsequently, the resultant seco-steroid is carried by vitamin D binding protein via plasma from the kidneys to target tissues where it is able to bind to the potent transcription factor VDR. Receptor binding leads to conformational changes within the receptor that allow the VDR to interact with its heterodimeric partner, the retinoid X receptor, in the nucleus. VDR also forms homodimers that bind specific regions of DNA and activate or inhibit gene transcription[31]. The biologically active form $1,25(OH)_2D_3$ is responsible for maintaining Ca^{2+} and PO_4^{2-} homeostasis[29]. Interestingly, it has been proposed that vitamin D biological activity involves unbound or free fractions of the vitamin[32,33]. The 'free-hormone hypothesis' is considered a different pathway for cellular uptake of steroid hormones, as these molecules are highly lipophilic and therefore have the potential to quickly and passively diffuse across cell membranes[32]. It seems to be important to measure and differentiate total vitamin D (measured as 25(OH)D) and bioavailable vitamin D (i.e. free vitamin D) as important evidence suggests that vitamin D binding protein inhibits certain actions of vitamin D since the bound fraction is unavailable to act on target cells[34,35]. Powe *et al.*[36] have demonstrated in healthy adults that bone mineral density was more strongly associated with the bioavailable fraction of circulating 25(OH)D than the total levels[36]. In accordance with this result, other studies suggest that free vitamin D is a better predictor of bone mineral density especially in an ethnically diverse athletic population and also correlates better with parathyroid hormone (the marker of Ca balance associated with bone health) than total serum 25(OH)D concentration[37,38].

Role of vitamin D in skeletal muscle

Proliferation and differentiation

The development of muscular tissue (myogenesis) has several phases starting from stem cells located in somites, followed by the development of the first progenitor cells named myoblasts, and finally their differentiation into mature myotubes[39]. Regulation of myogenesis depends mainly on two transcription factor systems: paired-box transcription factors, Pax3 and Pax7, and a family of basic Helix–Loop–Helix transcription factors known as myogenic regulatory factors (MRF)[40]. Myoblasts then proliferate extensively until they reach a myofibrillar protein synthesis peak and then differentiate into mature myotubes, due to activation of MRF. To maintain tissue homeostasis, there is a subpopulation of cells that resides in the quiescent state, also known as satellite cells. These cells also have the potential to differentiate into new muscle fibres (i.e. myogenesis) and maintain protein turnover[41,42]. Briefly, the fate and differentiation of muscle cells are controlled by four MRF: myogenic factor 5 (Myf5), muscle-specific regulatory factor 4 (MRF4), myoblast determination protein (MyoD) and myogenin (MYOG). Myf5 and MyoD are specifically degraded at mitosis and G1/S, respectively, and this is mediated by phosphorylation via cyclin-dependent kinases. Myf5 and MyoD determine skeletal muscle cell identity, and are consequently considered to be the gatekeepers for entry into the terminal specification of myogenic lineage[43], whereas MYOG is essential for the differentiation of myoblasts into myotubes[44]. MYOG acts genetically downstream of MyoD and Myf5 to switch on muscle differentiation genes. Although MRF4 is classified as a differentiation gene, it is also believed to act as a determinant gene when it is expressed by undifferentiated cells[45]. MRF signalling molecules induce the activation of skeletal muscle cell receptors and regulate transcription of specific target genes in order to develop the adult skeletal muscle tissue, such as SIX1–SIX6 and myocyte enhancer factor (MEF)2 proteins (MEF2A, MEF2C and MEF2D)[42].

Several animal studies have demonstrated that a vitamin D-deficient diet decreases markers of proliferation, such as bone morphogenetic protein family, fibroblast growth factor 2 and/or proliferating cell nuclear antigen, while increasing markers of myogenic differentiation such as Myf5, myostatin, atrophy marker E3-ubiqutin ligases and muscle ring-finger protein-1 (Table 1)[46]. Interestingly, in many studies the expression of VDR in skeletal muscle increases in animals receiving a vitamin D-sufficient diet and/or vitamin D-rich diet, which suggests that the effects of vitamin D are dependent on its receptor[47,48]. VDR was also detected in the nucleus of muscle fibres from middle-aged and older female patients with osteoarthritis/osteoporosis and the expression seems to decrease with age[49,50]. In accordance with these studies, Ceglia *et al.*[51] also confirmed the VDR expression in skeletal muscles from healthy

Table 1. Overview of the biomolecular role of vitamin D (VitD) in skeletal muscle (six animal studies)

Study	Species, *n*	VitD form, dose, time and diet	Findings and effects	Comments
Srikuea *et al.* (2012)[57]	C57BL/6 mice aged 12 weeks *n* 4	No VitD treatment Regeneration (injection of 1·2 % $BaCl_2$ to *tibialis anterior*) *v.* control group	↑ Expression of VDR and *CYP27B1* gene at day 7 of regeneration *v.* control ($P<0·001$)	Expression of VDR in skeletal muscle during regeneration and repair
Hutton *et al.* (2014)[47]	Male, Ross 708 broiler chicks *n* 48–52 birds per group	5000 IU/kg diet with $VitD_3$ or $VitD_3$ + 25(OH)D 49 d	↑ Myogenic factor 5, ↑ density of satellite cells ($P=0·09$), ↑ total nuclear density ($P=0·05$) and ↑ muscle fibre cross-sectional area ($P=0·09$) in *pectoralis major*	Hypertrophic response in the *pectoralis major* muscle
Domingues-Faria *et al.* (2014)[46]	15-month-old Wistar rats *n* 10 per group	AIN-93M maintenance diet (1000 IU $VitD_3$/kg) or without VitD 9 months	↓ 74 % plasma 25(OH)D with VitD-deficient diet ($P<0·01$) ↓ *Tibialis anterior* weight in VitD-depleted rats (−25 %; $P<0·05$) In VitD-depleted group, ↓ expression of three markers of proliferation (BMP4, FGF2, PCNA; $P<0·005$)	↓ Notch pathway activity and proliferation potential
Girgis *et al.* (2015)[55]	C57BL/6 mice *n* 6–12 per group	Deletion of VDR-KO *v.* wild-type littermates Diet: 2·2 IU/g or VitD-deficient 3 months	Weaker grip strength: VDR-KO and VitD-deficient mice ($P<0·005$) VitD-deficient diet: ↑ myostatin, atrophy marker E3-ubiquitin ligase and MuRF1 ($P<0·005$) VDR-KO: small muscle fibre size and dysregulation of myogenic regulatory factors	Weakness advanced with age and duration of VitD deficiency
Ray *et al.* (2016)[54]	Female A/J mice (aged 4 weeks) *n* 5 per group	AIN-93-based diets with 100, 1000 or 10 000 IU $VitD_3$/kg diet 6 weeks	Maximal diaphragm force, twitch force, and fibre cross-sectional area ↓ 26, 28 and 10 % (respectively) with VitD-deficient diet ($P<0·01$)	
Oku *et al.* (2016)[53]	Sprague–Dawley strain male rats (aged 10 weeks) *n* 6	AIN-93 diet (1000 IU $VitD_3$/kg) Control, VitD restriction, high-fat diet and high-fat diet with VitD restriction 14 d	VitD restriction: ↓ volume of the femur ($P<0·001$), ↓ bone mineral density ($P<0·05$), ↓ MyoD ($P<0·05$)	Muscle mass trending towards a ↓ in VitD restriction group ($P=0·051$)

↑, Increase; ↓, decrease; 25(OH)D, 25-hydroxyvitamin D; AIN, American Institution of Nutrition; BMP4, bone morphogenetic protein family; FGF2, fibroblast growth factor 2; IU, international units; MyoD, family of myogenic regulatory factors; MuRF1, muscle ring-finger protein-1; VDR-KO, vitamin D receptor knockout mouse; PCNA, proliferating cell nuclear antigen; VDR, vitamin D receptor.

postmenopausal women. Conversely, a recent study has reported that VDR protein is readily detected in human myoblasts and myotubes, while non-detectable in adult human skeletal muscle tissue[23]. It is important to highlight that the detection of VDR expressed in skeletal muscle cells and tissue varies according with the technique used for protein extraction and the type of primary antibody; consequently many authors have found difficulty in detecting this receptor[52].

The majority of animal studies have focused on the physiological effects of vitamin D on muscle mass and strength[53–55] and most have not investigated the precise pathways regulating the reported outcomes. At the cellular level, some studies have investigated the effects of vitamin D in murine cell lines, such as C_2C_{12} (Table 2), and demonstrated that treatment with vitamin D inhibits cell proliferation[23,55–59] and stimulates cell differentiation[25,58,60,61]. More recently, Braga *et al.*[61] have shown that treatment with $1,25(OH)_2D_3$ in primary cultures of satellite cells enhances myogenic differentiation through an increase in the expression of myogenic markers, such as MyoD and MYOG, myotube formation, and the modulation of pro- and anti-myogenic factors[61]. These results are in agreement with previous studies that identified an increase in myogenic factors such as MYOG, Myf5 and MYC2 (transcription factor) in response to vitamin D treatment[48,59,60]. In addition, Ryan *et al.*[62] were the first group to prove a dose–response effect of the active form of vitamin D to modulate the capacity of C_2C_{12} cells to transdifferentiate into adipocytes. Low concentrations of vitamin D (simulating a deficient status) induced adipogenesis and up-regulation of key adipogenic marker genes (PPARγ2 and fatty acid binding protein 4 (FABP4)), whereas higher concentrations attenuated the differentiation into adipocytes. Consequently, an increase in triacylglycerol synthesis and levels within skeletal muscle is associated with a decrease in functional strength and impairment of glucose tolerance, leading to a higher risk of developing metabolic diseases, insulin resistance, obesity and type 2 diabetes[63–65]. However, in primary human skeletal muscle cells, the effects of vitamin D on proliferation and differentiation are conflicting[23,25]. Owens *et al.*[25] have investigated the effects of vitamin D during differentiation in human primary muscle cells collected by biopsies from active adults and found an increase in myotube fusion and differentiation[25]. On the other hand, Olsson *et al.*[23] reported opposite effects regarding differentiation, such as reduction of expression of cell cycle regulators and myogenic regulatory factors (MyoD, MYOG, MEF2C and sarcomeric proteins), with associated activation in forkhead box O3 and Notch signalling pathways[23].

Table 2. Overview of the biomolecular role of vitamin D (VitD) in skeletal muscle cells (eleven *in vitro* studies)

Study	Cell line/cell type	VitD form, dose and time	Significant findings and effects	Comments
Garcia *et al.* (2011)[56]	C_2C_{12} Myoblasts	100 nM-$1,25(OH)_2D_3$ 1–10 d	↓ Proliferation, ↓ IGF-1, ↑ myogenic differentiation, ↑ IGF-2, ↑ follistatin expression and ↓ myostatin	2-fold ↑ in the mean diameter of the fibres (P<0·001); 2·5-fold ↑ in size (width) (P<0·001)
Srikuea *et al.* (2012)[57]	C_2C_{12} Myoblasts, myotubes	20 nM-$1,25(OH)_2D_3$ or 2 μM-$25(OH)_2D_3$ 5 d	Inhibits cell proliferation (P<0·001)	↓ Myoblast number after treatment
Garcia *et al.* (2013)[58]	C_2C_{12} Myoblasts	100 nM-$1,25(OH)_2D_3$ 1, 3, 4, 7 or 10 d	↓ Proliferation (FGF2 and TIMP-3) (P<0·05) ↑ Differentiation (↑ VEGFα and FGF1, two pro-angiogenic growth factors) (P<0·05)	↓ Myostatin expression (negative regulator of skeletal muscle mass)
Salles *et al.* (2013)[72]	C_2C_{12} Myotubes	0, 1 or 10 nM-$1,25(OH)_2D_3$ 72 h	$1,25(OH)_2D_3$ (10 nM) + leucine and insulin, ↑ protein fractional synthesis rate (14–16 %) (P<0·01) ↑ mRNA expression of insulin receptor and VDR (P<0·001)	Akt/mTOR-dependent pathway was enhanced by $1,25(OH)_2D_3$
Girgis *et al.* (2014)[48]	C_2C_{12} Myoblasts, myotubes	100 nM-$1,25(OH)D_2$, 100 nM-25(OH)D 48 h	↓ Proliferation and differentiation (↓ myostatin, myc, myogenin, myogenic factor 5 and cyclin-D; ↑ retinoblastoma protein and ATM) (P<0·05)	1·8-fold ↑ in cross-sectional size of individual myotubes
Irazoqui *et al.* (2014)[60]	C_2C_{12} Myoblasts	C_2C_{12} wild-type or VDR (knockdown) 1 nM-$1,25(OH)_2D_3$ 6, 12 and 24 h	Induces differentiation (↑ myogenin) (P<0·05)	p38-dependent co-localisation of VDR and cyclin D3
Owens *et al.* (2015)[25]	Human myoblasts and myotubes (biopsy)	10 or 100 nM-$1,25(OH)_2D_3$ 7–10 d	With 10 nM-$1,25(OH)_2D_3$, ↑ muscle cell migration, dynamics and ↑ myotube fusion/differentiation	Humans: improves recovery of peak torque at 48 h and 7 d post-exercise
Ryan *et al.* (2016)[24]	Human myoblasts (culture)	10 nM-$1,25(OH)_2D_3$ 48 h	↑ Mitochondrial oxygen consumption rate (P=0·003) No changes in glycolysis (P=0·213)	↑ Mitochondrial volume and branching
Van der Meijden *et al.* (2016)[59]	C_2C_{12} Myoblasts,myotubes	0, 400 or 1000 nM-25(OH)D or $1,25(OH)_2D_3$ 24 h	↑ VDR mRNA expression and ↓ proliferation (P<0·01) Stimulates myosin heavy chain mRNA expression (during differentiation; P<0·001) ↑ Diameter of myotubes (P<0·05)	C_2C_{12} is able to metabolise $25(OH)D_3$ and $1,25(OH)_2D_3$
Olsson *et al.* (2016)[23]	Human myoblasts and myotubes (biopsy)	1 or 100 nM-$1,25(OH)_2D_3$ 48 h	Lack of ability to convert 25(OH)D into $1,25(OH)_2D_3$ Inhibits myoblast proliferation and differentiation (P=0·014 and P=0·012)	Changes in forkhead box O3 and Notch signalling pathways
Braga *et al.* (2017)[61]	Mouse skeletal muscle Satellite cells	100 nM-$1,25(OH)_2D_3$ 1–12 d	↑ Expression: MyoD, MYC2, myogenin, skeletal muscle fast troponin I and T, MYH1, IGF-1 and -2, FGF1 and 2, BMP4, MMP9 and follistatin (P<0·05)	↑ Myotube formation and expression of myostatin (P<0·001)

↑, Increase; ↓, decrease; $1,25(OH)_2D_3$, 1,25-hydroxyvitamin D_3; 25(OH)D, 25-hydroxyvitamin D; Akt, protein kinase B; ATM, ataxia telangiectasia mutated; BMP4, bone morphogenetic protein family; FGF, fibroblast growth factor; IGF, insulin-like growth factor; MMP9, matrix metallopeptidase 9; mTOR, mammalian target of rapamycin; MYC2, transcription factor; MYH1, heavy polypeptide 1; MyoD, family of myogenic regulatory factors; TIMP-3, metalloproteinase inhibitor 3; VDR, vitamin D receptor; VEGFα, vascular endothelial growth factor.

An important factor to consider regarding skeletal muscle cell lines is that the conversion of 25(OH)D to its active form ($1,25(OH)_2D_3$) has not been confirmed to occur locally in primary skeletal muscle cells, whereas it does occur in the skeletal muscle murine cell line C_2C_{12} and *in vivo* in mice[23,59]. In addition, discrepancies exist among studies regarding the cell line used, and vitamin D form, dose and duration of the treatment, consequently leading to different outcomes. Overall, vitamin D influences myogenesis through regulation of myogenic factors and proteins involved in this process. The main findings suggest that vitamin D promotes differentiation while reducing proliferation in murine cells; however, is still difficult to define how vitamin D affects proliferation and differentiation in primary human muscle cells. Further studies are needed to elucidate the cellular development stages and/or subpopulations of cells that undergo regulation by vitamin D and thus affect the process of muscular development.

Protein synthesis and myotube size

Cellular protein content is controlled by anabolic and catabolic mechanisms that regulate synthesis and degradation of muscle proteins, resulting in changes in muscle mass[41]. The main mechanisms that regulate protein synthesis in skeletal muscle

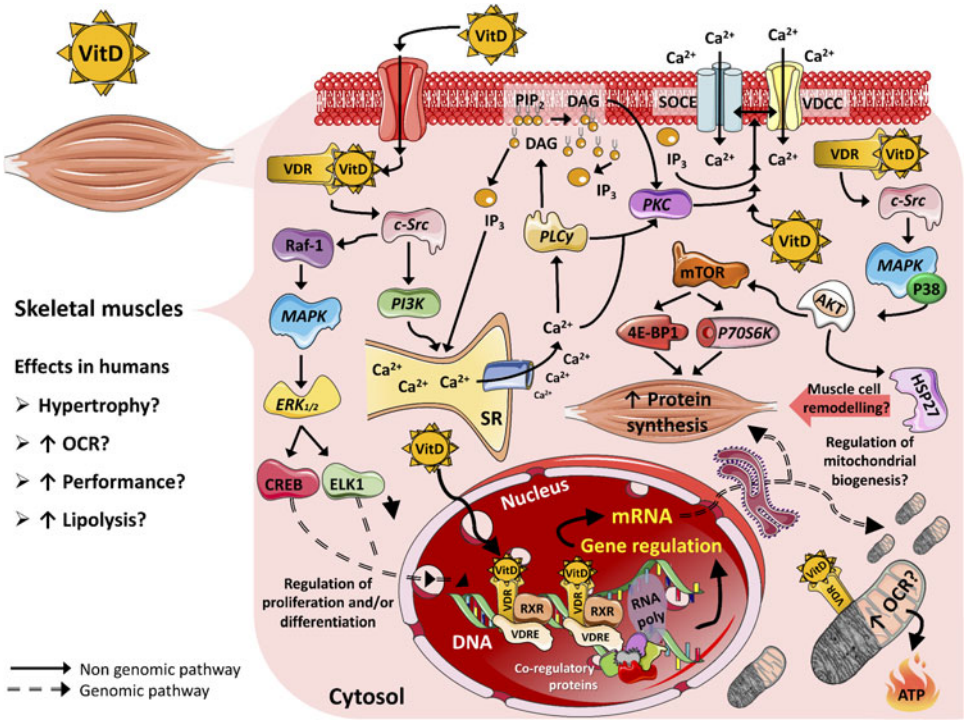

Fig. 1. Proposed mechanisms of action of vitamin D (VitD) in mammalian skeletal muscle cells. 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; AKT, serine–threonine kinase; Ca^{2+}, calcium ions; CREB, cellular transcription factor; c-Src, proto-oncogene c-Src; DAG, diacylglycerol; ELK1, ETS domain-containing protein; ERK1/2, extracellular signal-regulated kinases; HSP27, heat shock protein 27; IP_3, inositol triphosphate; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; OCR, oxygen consumption rate; P38, P38 mitogen-activated protein kinases; P70S6K, ribosomal protein S6 kinase β-1; PI3K, phosphoinositide-3 kinase; PIP2, phosphatidylinositol biphosphate; PKC, protein kinase C; PLCγ, phospholipase Cγ; Raf-1, proto-oncogene serine/threonine-protein kinase (also known as c-RAF); RNA poly, RNA polymerase; RXR, retinoid X receptor; SOCE, store-operated calcium entry; SR, sarcoplasmic reticulum; VDCC, L-type voltage-dependent calcium channel; VDR, vitamin D receptor; VDRE, vitamin D response elements.

are via the insulin signalling cascade, insulin-like growth factors (IGF, such as IGF-1 and IGF-2) and amino acids[66]. These molecules, upon receptor binding, induce phosphorylation and activation of sequential targets, including the insulin receptor substrate, phosphatidylinositol-3 kinase (PI3K), phosphoinositide-dependent kinase-1, protein kinase B serine/threonine kinase family (Akt/PKB), mammalian target of rapamycin (mTOR) and 70-kDa S6 protein kinase (p70S6k)[67]. mTOR is the major regulator of cell growth and proliferation by controlling the initiation phase of protein translation and synthesis (Fig. 1)[68]. Furthermore, it has been suggested that regulatory proteins, for example transforming growth factor β (TGF-β) family and myostatin, also play an essential role in protein synthesis and myotube size by regulating Akt/PKB, mitogen-activated protein kinase (MAPK) and mTOR pathways[69,70]. The proposed model of signalling is via activation of the TGF-β signalling pathway that initially is inhibited by follistatin (activin-binding protein essential for muscle fibre formation and growth)[69,70]. Follistatin subsequently binds to multiple extracellular ligands including myostatin which modifies the transcription of Smad target genes, consequently stimulating Akt/mTOR signalling that potentiates protein synthesis[69].

The first *in vivo* study to suggest an association between vitamin D and improvements in net muscle protein synthesis was published in 1975[71]. This first observation indicated that 25(OH)D supplementation increased [^{3}H]leucine incorporation into proteins in rat skeletal muscle and specified that the vitamin D effect was, at least in part, independent of the action of the intestinal transport of Ca and phosphate[71]. However, Ca^{2+} and phosphate (PO_4^{2-}) could be directly involved in the mechanism of action of vitamin D in muscle cells. Subsequent studies with VDR knockout (VDR-KO) mice, and with also mice subjected to a vitamin D-deficient diet (Table 1), have reported significant muscular atrophy, weakened strength, decreased muscle fibre size, lower bone mineral density and dysregulation of myogenic regulatory factors when compared with control groups, i.e. normal mice and mice receiving a vitamin D-sufficient diet[46,53–55]. Moreover, Oku *et al.*[53] have demonstrated the negative impact of vitamin D deficiency on the levels of mRNA expression of MyoD in skeletal muscle tissue, which corroborates the importance of vitamin D for the process of myogenesis, muscle maintenance and hypertrophy. Salles *et al.*[72] later confirmed that an *in vitro* treatment with 10 nM-$1,25(OH)_2D_3$ potentiated the effects of leucine and insulin; and increased the protein synthesis rate by 14–16 % through Akt/PKB and mTOR pathways. In the later study vitamin D has also been shown to enhance the phosphorylation of Akt/PKB and glycogen synthase kinase 3β (GSK3β), and consequently improve insulin signalling by up-regulating the insulin receptor. It is proposed that Akt inhibits GSK3 and, as a consequence, activates the eukaryotic translation initiation factor 2B (eIF2B), resulting in the formation of the 43S preinitiation

complex. Furthermore, mTOR phosphorylates eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and p70S6K allowing assembly of the 43S pre-initiation complex[73,74]. Further research is important to identify if vitamin D has a biological effect on amino acid transporters, as a potential alternative pathway to improve protein synthesis and myotube size. Studies also have reported the action of vitamin D on muscle proteolytic activity and regulation via proteases, specifically the ubiquitin–proteasome pathway[75,76]. In a rat muscle model, vitamin D deficiency led to an increase in activities of the glutathione-dependent enzymes and decrease in superoxide dismutase and catalase enzymes, resulting in oxidative stress and proteolysis. Rehabilitation with vitamin D could reverse the alterations in oxidative stress parameters, increase total protein degradation and muscle atrophy gene markers post-vitamin D treatment in C_2C_{12} muscle cells[77]. The same authors have also demonstrated an increase in protein degradation and decreased protein synthesis after a vitamin D-deficient diet in male rats[75].

Some of the strongest evidence linking vitamin D to protein synthesis is the stimulation of fibre hypertrophy[42]. In one study, fibre hypertrophy was observed after 10 d of treatment with $1,25(OH)_2D_3$, with a significant 2-fold increase in the mean diameter of C_2C_{12} fibres, and an increase of 2·5-fold in length[56]. These results were attributed to an increase in protein synthesis; however, the authors did not measure this parameter directly. Similar results were obtained by van der Meijden *et al.*[59] where the treatment with 25(OH)D for 3 d resulted in a 19 % increase in C_2C_{12} fibre diameter. Despite previous evidence of the impact of vitamin D on metabolic signalling pathways and phenotype in skeletal muscle cell lines, there is currently no evidence regarding the effect of vitamin D on protein synthesis in primary human skeletal muscle cells. Finally, in clinical studies, vitamin D supplementation in healthy human subjects with low serum levels activates the VDR in skeletal muscle tissue, which appears to stimulate protein synthesis and improve muscle strength following an increase in the size and number of type II muscle fibres[5,78].

Mitochondrial metabolism

Adequate mitochondrial function is essential for cellular homeostasis, especially in high energy-demanding skeletal muscle cells, as mitochondria are the main organelles responsible for generating energy in the form of ATP[79]. Typically, the effects of vitamin D are intermediated by its interaction with a nuclear VDR and is part of the nuclear receptor superfamily of ligand-activated transcription factors. VDR can also be translocated into the mitochondria of certain cell types, including the skeletal muscle cells, and potentially act directly on cellular bioenergetics[30]. This evidence led researchers to consider that mitochondrial activity could potentially be modified by a diet containing sufficient levels of vitamin D and/or supplementation, which is thought to increase cellular metabolism. Interestingly, a few studies have indicated that vitamin D supplementation modulates mitochondrial activity and enhances ATP production at rest and during exercise[80,81]. More recently, a positive association between vitamin D and RMR was observed in obese adults[26]. The latter study demonstrated that for every 10 nmol/l increase in serum 25(OH)D, the RMR increased by 56·5 kJ/d[16]. However, another study with a very short (1 week) period of vitamin D supplementation reported no influence on energy or substrate utilisation[82]. Additional randomised clinical trials examining the influence of vitamin D on energy expenditure are therefore urgently needed.

The mechanism of vitamin D action in mitochondrial metabolism currently proposed is an increase in the expression of electron transport chain proteins and the tricarboxylic acid cycle enzymes via both genomic and non-genomic pathways[83]. This theory was tested by Muñoz Garcia *et al.*[84] who investigated pathways related to the tricarboxylic acid cycle, oxidative phosphorylation and ATP synthesis in existing gene expression databases from multiple related monocyte models[84]. In this study, genes associated with the electron transport chain activity and in the conversion of acetyl-CoA to CO_2 were up-regulated by vitamin D in three immune cell types, the THP-1 monocytic cell line, monocyte derived dendritic cells and monocytes[84]. The same pattern was observed in human peripheral blood mononuclear cells linking serum vitamin D status with specific markers of bioenergetic pathways[85].

With respect to skeletal muscle tissue, the precise function of vitamin D in relation to mitochondrial metabolism remains highly elusive. Ryan *et al.*[24] has investigated the effects of vitamin D on cellular bioenergetics in primary human muscle cells (undifferentiated myoblasts). The authors have demonstrated that mitochondrial oxygen consumption rate increased when cells were treated with vitamin D for 48 h and this response was dependent of the VDR[24]. A possible mechanism for this, is an elevation in mitochondrial volume fraction and branching, which may result in mitochondrial fusion and biogenesis. Vitamin D treatment increased expression levels of MYC, MAPK13 and endothelial PAS domain-containing protein 1 mRNA (which encodes for a protein that regulates mitochondrial biogenesis)[24]. In addition, mediators of mitochondrial fusion were altered. Specifically, OPA1 expression increased following vitamin D treatment, while mediators of mitochondrial fission (Fis1 and Drp1) were decreased[24]. Furthermore, current evidence has illustrated that treatment with 0·1 nM-$1,25(OH)_2D_3$ in human primary muscle improved mitochondrial morphology (volume and structure) and altered mRNA expression of pyruvate dehydrogenase kinase 4 and carnitine palmitoyltransferase 1 (CPT1), important genes that control muscle glucose and lipid metabolism[86]. Vitamin D deficiency is known to impair muscle function and metabolism, where, in this case, skeletal muscle fibres are most likely to VDR ablation and to uptake cytoplasmic Ca^{2+} released from the sarcoplasmic reticulum during twitch responses[87]. Experiments with chick muscles deficient in vitamin D observed changes in oxidative phosphorylation and a failure of muscle mitochondria to maintain Ca^{2+}, leading to impairment of cellular metabolic homeostasis, increased reactive oxygen species and cytotoxicity due to mitochondrial dysfunction[88]. Despite previous reports, there are still no studies that have reported whether the effects of vitamin D up-regulate or down-regulate genes and proteins associated with the tricarboxylic acid cycle and electron transport chain in primary skeletal muscle cells.

Actions and targets of vitamin D

The outcomes of vitamin D action appear to be dependent on interaction with a nuclear VDR and with the retinoid X receptor. This complex is able to up-regulate and/or down-regulate target genes by binding to regulatory sequences named vitamin D_3 response elements (Fig. 1)[89]. It is estimated that approximately 3 % of the human genome is regulated, directly and/or indirectly, by the vitamin D–endocrine system[90]. Genomic mechanisms may explain how supplementation of vitamin D influences, for instance, muscle hypertrophy in adults. Gene expression was evaluated using muscle biopsies in a retrospective cohort of healthy volunteers[11]. In this study, a positive correlation between serum active vitamin D and genes encoding for transcriptional activators or co-repressors of TGF-β and myostatin were observed, resulting in anti-proliferative effects[11]. Associations between vitamin D levels and up-regulation of gene expression were also found with molecules involved in protein synthesis pathways, such as IGF-1 receptor and eukaryotic translation initiation factor 4E-binding protein 1 (EIF4BP1) and eukaryotic translation initiation factor 2B subunit α (EIF2B1), which are members of a family of translation repressor proteins[11]. Additionally, it has been established that $1,25(OH)_2D_3$ regulates proteins that effect contractility and cell regeneration in a rat model of muscle crush injury[91]. In the present study, vitamin D treatment increased cell proliferation of non-satellite cells, including fibroblasts, endothelial cells, Pax7-negative local stem cells and prolyl-4-hydroxylase-β expression (P4HB), which have a direct impact on collagen synthesis. Treatment with vitamin D also increased the production of extracellular matrix components that can similarly affect muscle contractility and force[91].

Another possible genomic mechanism of vitamin D involves the stimulation of IGF-1[92]. A study assessing rickets patients detected that circulating concentrations of IGF-1 increased significantly after vitamin D treatment and this may regulate the pituitary gland and growth hormone production[93]. More recently, a *post hoc* analysis of the Styrian Vitamin D Hypertension Trial did not find any significant effects of vitamin D supplementation on IGF-1 concentrations in hypertensive patients with low 25(OH) D levels at baseline; however, a significant effect was detected in a cross-sectional correlation between the active form of $1,25(OH)_2D_3$ and IGF-1[94]. Therefore, further studies are necessary to clarify the relationship between vitamin D and IGF-1/growth hormone. It is also known that vitamin D influences pathways of apoptosis in a variety of cells, including osteoblasts, osteocytes and tumour cells, resulting in anti-apoptotic effects[91]. These findings suggest that vitamin D acts via VDR/inositol triphosphate (IP_3) and Akt, consequently reducing caspase activity and increasing cell survival.

The typical steroid hormone (genomic pathway) is characterised by direct regulation of gene expression, which mediates the responses to the hormone some hours after hormone-receptor binding. In contrast, the non-genomic pathway is characterised by activation of a rapid second messenger which results in acute (mostly seconds) cellular responses and it is not dependent upon the immediate regulation of gene transcription[31]. $1,25(OH)_2D_3$ stimulates fast transcriptional-independent effects (seconds to minutes range) that are not feasibly explained by alterations in gene expression[17]. Although there is no consensus about the non-genomic pathways of vitamin D, studies have suggested that the actions of vitamin D start at the plasma membrane[95]. Initially, vitamin D binds to a surface or caveolae VDR, which activates c-Src and phosphoinositide-3 kinase and leads to the fast recruitment of Ca^{2+} from the sarcoplasmic reticulum into the cytosol. This results in the activation of phospholipase Cγ and release of IP_3 and diacylglycerol from the membrane. Subsequently, IP_3 facilitates the release of Ca^{2+} from the sarcoplasmic reticulum into the cytosol[96,97].

Another non-genomic mechanism of vitamin D action is promotion of the translocation of PKC-α from the cytosol to the cell membrane, which has been previously studied using chick and rat myoblasts with *in vitro* $1,25(OH)_2D$ treatment (Fig. 1)[98,99]. PKC also activates the L-type voltage-dependent Ca^{2+} channel and store-operated Ca^{2+} entry (SOCE) channel and may have a role in the activation of extracellular signal-regulated kinases (ERK1/2). Briefly, studies have found a dose-dependent increase in intracellular muscle Ca^{2+} uptake after treatment with the active form of vitamin D, which may have an impact on muscle contraction[98,100,101]. In addition, the activation of c-Src results in Raf-1 stimulation[102,103], which leads to the activation of the MAPK pathway. Subsequently, ERK1/2 activation and phosphorylation of ETS domain-containing protein (Elk-1) and cAMP response element-binding protein increases the expression of c-myc and c-fos, which are key regulators of proliferation and differentiation[104]. Finally, vitamin D also activates p38 MAPK and consequently phosphorylates heat-shock protein 27 which has a significant role in remodelling muscle cells through the actin microfilament system (Fig. 1)[105,106]. Even though this seems like a non-genomic effect of vitamin D through direct and acute stimulation of the AKT pathway, it is also known that both genomic and non-genomic effects are interdependent. For instance, extra nuclear-initiated actions may regulate gene expression indirectly through their effects in pathways that themselves control transcription[107]. New studies focusing on genomic and especially non-genomic mechanisms of vitamin D are required to better distinguish the precise contribution of each mechanism of action and optimal dose–effect for skeletal muscle tissue.

Clinical studies and perspectives

The clinical outcomes of vitamin D supplementation for skeletal muscle physiology have recently received increased attention (the last decade) and could result in a better understanding of the mechanisms of vitamin D effects when analysed from the perspective of the genomic pathways induced by the hormone. A vitamin D inadequacy or deficiency induces muscle fibre atrophy, slow twitch peak, may promote long periods of muscle relaxation and increased risk of chronic musculoskeletal pain[15]. Muscle biopsies from vitamin D-deficient adults demonstrate impairment in skeletal muscle tissue, such as fibrosis, loss of type II fibre complement and enlarged interfibrillar spaces[17,108]. Type II fibres are responsible for fast muscle contraction and are used predominantly to prevent falls and in power exercises and anaerobic activities[109].

Many studies investigated healthy patients with baseline low serum vitamin D levels followed by supplementation with vitamin D. In these studies, vitamin D supplementation led to the activation of the VDR in skeletal muscle and consequently improved protein synthesis and muscle strength, as well as increased size and number of type II muscle fibres[75,110–112]. A systematic review reported that vitamin D supplementation increased muscle strength (1·4 to 18·8 %) in vitamin D-insufficient athletes[21]. In this review, the quality of controlled trials was assessed using the PEDro scale, and five RCT were identified as excellent quality and one controlled trial as good quality. Furthermore, a reduced injury incidence was reported in a group of elite ballet dancers following 4 months of 2000 IU/d of vitamin D supplementation[113]. In a study with elite ballet dancers, significantly fewer injuries were observed after 4 months of vitamin D supplementation[113]. Interestingly, the majority of studies investigating the effects of vitamin D in exercise performance have reported positive results in vitamin D-deficient athletes and not with adequate or supraphysiological concentrations of vitamin D. Hence, further studies are required to elucidate if supraphysiological doses of vitamin D have an ergogenic effect in vitamin D-replete athletes in different sport disciplines. Chronic long-term modifications observed in clinical studies suggest that such consequences are primarily a result of genomic actions. In disparity, genomic secondary changes in gene expression can also arise from alterations in sustained non-genomic/acute signalling.

Recent findings validated the involvement of vitamin D in the regulation of numerous skeletal and extra-skeletal cellular processes that have a direct impact on muscle function and metabolism; however, the pathways involved are incompletely understood. Vitamin D has been reported to positively influence protein synthesis, as well as increase the size of adult muscle cells and muscle mass (in animal and human studies), which could consequently result in increased muscle strength, and/or performance. Moreover, increments in skeletal muscle mass can raise the RMR and consequently the daily energy expenditure, which may benefit in the reduction of body weight in obese individuals. Three meta-analyses of RCT[114–116] reported a positive effect of vitamin D supplementation on muscle function and strength in healthy adults, while five cohort studies identified a link between serum 25(OH)D concentration and muscle strength and function[117–121]. In older adults, a recent meta-analysis found that vitamin D supplementation significantly improved muscle strength of the lower limb and femoral neck bone mineral density[122]. Moreover, Chanet *et al.*[123] have proved in healthy older men that supplementing breakfast with vitamin D and leucine enhances the postprandial muscle protein synthesis and muscle mass[123]. Higher muscle mass is associated with higher muscle strength, which is an important variable to prevent sarcopenia, falls and muscle disorders, as well as being associated with enhancement of performance in athletes. Valuable nutritional interventions combining vitamin D and amino acid supplementation could positively support muscle fibre protein synthesis in specific conditions, for example sarcopenia, where vitamin D and amino acid responses are deficient[124]. Vitamin D status is also a regulator of intestinal Ca absorption and, in turn, bone mineralisation[125]. In this context, several reviews have concluded that there is a positive outcome for supplementation of Ca plus vitamin D for fracture risk reduction, osteoporosis and/or falls[6,126–128]. In contrast, recent meta-analyses have not been consistent with these outcomes, though many variables need to be considered when comparing studies, for example, the parameters analysed, age of the population, if participants live in residential institutions, dose and cut points of vitamin D, ethnicity, period of the intervention, instruments to assess Ca and vitamin D intake and sunlight exposure, and many others[129]. Another important variable to consider in supplementation studies is if the participant is classified as sufficient or deficient in vitamin D since the baseline period, as many of the biological effects of vitamin D are evident when status of vitamin D is corrected from deficient to normal levels[113,122,130–137].

Nutrition strategies to complement the adaptive response to exercise have been well investigated. The effects of supplementation of vitamin D on exercise and muscle health have recently been reported in soccer and rugby players, elite ballet dancers, and in active adult males that were vitamin D insufficient at the baseline[113,130–136]. Four studies[113,130,131,136] have found that supplementation of vitamin D significantly increased strength, power and exercise performance even with a small sample size, while three studies reported no significant effect of vitamin D on any physical parameter[132–134]. Further, a reduced injury incidence was reported in a group of elite ballet dancers following 4 months of 2000 IU/d of vitamin D supplementation[113]. Additionally, in a RCT, Owens *et al.*[25] showed that elevating serum 25(OH)D concentrations to >75 nmol/l of 25(OH)D after supplementation with vitamin D_3 at 4000 IU/d had a positive effect on the recovery of force following a protocol of damaging eccentric exercise[25]. In summary, evidence supports a role for vitamin D in muscle remodelling and function; however, further work is needed to clarify under what specific conditions vitamin D can be beneficial.

An important limitation related to the effect of vitamin D in muscle strength is the lack of agreement on the optimal range in blood. While plasma values of >50 nmol/l of 25(OH)D have been demonstrated to improve physiological function and assist with disease prevention, it is believed that different tissues have distinct responses to the levels of vitamin D. Hence, it is possible that the optimal 25(OH)D concentration for skeletal muscle metabolism is different from the optimal concentration for other organs/tissues[138,139–145]. For example, Heaney & Holick[146] proposed that a higher (120–225 nmol/l) serum total 25(OH)D concentration may be required for optimal skeletal muscle physiology in adults, whilst Bischoff-Ferrari *et al.*[147] recommended a 25(OH)D concentration of 90–100 nmol/l to enhance bone mineral density and prevent fractures in young adults. Conversely, the metabolic effects of vitamin D supplementation in non-deficient athletes remain to be clarified. In accordance with previous research, it is possible that higher serum levels of vitamin D benefit sports performance[113,130,131,136]. For example, *in vitro* treatment with high vitamin D levels (400, 1000 nM) stimulates differentiation of skeletal muscle cells and results in the maturation of myoblasts, leading to the developing of an increase in myotube fibre diameter[59].

Vitamin D can also affect mitochondrial metabolism, increasing O_2 consumption, ATP production and modulating RMR and

energy production. Vitamin D supplementation could result in significantly better efficiency regarding energy metabolism, perhaps leading to better health outcomes, for example, preventing/reducing obesity and/or weight gain. However, insufficient data exist to confirm the effects of vitamin D on mitochondrial muscle metabolism. Observational research seems promising; however, more RCT are important to assess the effects of vitamin D supplementation in the context of muscle metabolism and function.

Conclusion

Vitamin D is associated with enhanced muscle structure and function, although the mechanisms are not yet fully understood. There is a wide range of muscle disorders related to vitamin D deficiency, and supplementation with vitamin D has mostly shown beneficial effects by counteracting the progression of diseases such as myopathy, sarcopenia, rickets and muscular dystrophy. The effects of vitamin D on proliferation and differentiation of skeletal muscle tissue in human and animal studies remain partially conflicting and need to be clarified. Finally, substantial evidence suggests that vitamin D may have the potential to modulate protein synthesis, mitochondrial metabolism as well as energy production, which is likely to make an impact on muscle strength, function and performance. Further research is required to describe the underlying mechanisms of vitamin D action on human muscle tissue, to clarify how these changes are translated into clinical effects and to define the optimal dose–effect conditions for vitamin D to obtain improvements in skeletal muscle function.

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Effects of vitamin D on primary human skeletal muscle cell proliferation, differentiation, protein synthesis and bioenergetics

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ABSTRACT

The active form of Vitamin D (1,25(OH)2D), has been suggested to have a regulatory role in skeletal muscle function and metabolism, however, the effects and mechanisms of vitamin D (VitD) action in this tissue remain to be fully established. In this study, we have used primary human skeletal muscle myoblast (HSMM) cells that display typical characteristics of human skeletal muscle function and protein levels, to investigate the effects of the active form of VitD on proliferation, differentiation, protein synthesis and bioenergetics. Myoblast cells were treated with 100 nM of VitD for 24 h, 48 h, 72 h and five days (cells were differentiated into myotubes) and then analyses were performed. We report that VitD inhibits myoblast proliferation and enhances differentiation by altering the expression of myogenic regulatory factors. In addition, we found that protein synthesis signaling improved in myotubes after VitD treatment in the presence of insulin. We also report an increase in oxygen consumption rate after 24 h of treatment in myoblasts and after 5 days of treatment in myotubes after VitD exposure. VitD significantly impacted HSMM myogenesis, as well as protein synthesis in the presence of insulin.

1. Introduction

Vitamin D (VitD) is a fat-soluble secosteroid prohormone derived primarily from dermal synthesis dependent on ultraviolet B radiation exposure [1]. To a lesser extent, VitD is also found in foods, such as mushrooms, eggs, fish, and other fortified foods, as well as in nutritional supplements. Studies have described the importance of VitD in several metabolic pathways, including calcium and phosphate homeostasis in tissues such as the bones, intestine, kidneys, parathyroid glands and immune system [2]. However, after the discovery and validation of the VitD receptor in human skeletal muscle cells [3,4] the number of scientific studies reporting the effects of vitamin D on muscle function and metabolism increased significantly [5–8].

Research in the field has demonstrated that more than 1 billion adults have inadequate or deficient levels of VitD [9–12]. Observational and epidemiological studies indicate that VitD inadequacy or deficiency induces muscle fiber atrophy, slow twitch peak, long periods of muscle relaxation, and may decrease resting metabolic rate [13–15]. The risk of chronic musculoskeletal pain, sarcopenia and associated falls are also increased by VitD inadequacy or deficiency [13,15–17]. Conversely, adequate VitD levels appear to have a wide range of functional outcomes in skeletal muscle metabolism and function, such as improvements in protein synthesis, muscle mass and strength, and oxygen consumption [5,18]. However, the relationship between metabolism and the link between the latter outcomes are not completely understood. The first reported observation that indicated improvements in net muscle protein synthesis after VitD treatment reported an increased [3H] leucine incorporation into protein in rat skeletal muscle [19]. Moreover, VitD deficiency was demonstrated to reduce levels of mRNA expression of MyoD in skeletal muscle tissue, which validates the effects of VitD on myogenesis, hypertrophy and muscle turnover [20]. Salles et al. also confirmed that an in vitro treatment with VitD increased the protein synthesis rate by 14–16% through Akt/PKB and mTOR pathways [21] and enhanced the effects of leucine and insulin. Also, stimulation of protein synthesis and an increase in size and number of type II muscle fibres in healthy humans with low serum levels supplemented with VitD was reported [12,22]. Furthermore, a range of hormones have been described that impact skeletal muscle tissue, including insulin, insulin like growth factors, glucocorticoids and thyroid hormones [8]. Insulin can stimulate a specific signalling network by

Abbreviations: BrdU, bromodeoxyuridine; 2-DG, 2-deoxy-glucose; ECAR, extracellular acidification rate; HSMM, human skeletal muscle myoblast cells; MTT, 3-(45 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OCR, oxygen consumption rate; OPP, O-propargyl-puromycin; VitD, vitamin D

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activation of the well-known phosphoinositide 3-kinase (PI3K)/ protein kinase B (AKT) and in the presence of an adequate amino acid supply, insulin promotes muscle protein synthesis, synergistically with nutrient- sensing mammalian target of rapamycin (mTOR)/ribosomal protein S6K (S6K) pathway, while inhibiting protein breakdown [23,24]. Regardless of previous evidence of the effect of VitD on bioenergetic pathways in skeletal muscle cell lines, there is no evidence of the impact of VitD on protein synthesis in primary human skeletal muscle cells *in vitro*.

Studies using muscle murine cells, such as C2C12 have reported that VitD can inhibit myogenesis [6,7,25–28] and stimulate cell differentiation [21,27,29–31]. Braga, Simmons, Norris, Ferrini and Artaza [31] demonstrated that VitD (i.e. 1,25-D_3) improved differentiation through an increase in the expression of myogenic markers, such as myoblast determination protein and myogenin, myotube formation, and the modulation of pro- and anti-myogenic factors in primary cultured satellite cells. Other studies in murine C2C12 cells also described an increase in myogenic factors such as myogenin, myogenic factor 5 and myosin heavy chain II in response to VitD treatment [6,29,32].

On the other hand, in primary human skeletal muscle cells, the effects of VitD on proliferation, differentiation and bioenergetics are conflicting [28,30]. In one study [28], VitD inhibited proliferation and differentiation processes in human skeletal muscle cells. However, in another study it was found that VitD improved myotube fusion/differentiation and hypertrophy [30]. These contradictory results might be due to differences in experimental protocols, such as the form, dose and duration of VitD treatment, and the cell type used in the experiments [32]. For instance, while skeletal muscle cells in mice mainly consist of type 2B and 2X fibers (highest oxidative potential) [33], human muscles cells are mostly composed by type 1 (fibers which are abundant in mitochondria and oxidative enzymes) [8] and 2A fibres, leading to variable results in oxygen consumption and mitochondrial content [33]. Furthermore, it is also possible that *in vitro* cultures of immortalized/genetically modified skeletal muscle cells, such as C2C12 and rat L6 do not accurately reflect the physiological mechanisms observed in human skeletal muscle cells [34–36].

In vitro cultures of primary human skeletal muscle myoblasts (HSMM) have been validated and considered a gold standard alternative to study cellular and molecular pathways that regulate muscle growth and differentiation [28,37,38]. These cells differentiate rapidly, developing contractile myotubes and generate typical characteristics of human skeletal muscle proteins, such as the ability to develop hypertrophy when stimulated with insulin or insulin-like growth factor [5]. Moreover, these cells express several markers of in vivo human skeletal muscle cells, such as Myogenic Differentiation-1 (MYOD), Myocyte enhancer factor 2C (Mef2C), Myogenin (MYOG), Troponin T type 1 (TNNT), and Myosin heavy chain-2 (MYH2) [39].

Hence, the objective of this study was to investigate the effects of VitD in HSMM myogenesis, mTOR downstream pathway, protein synthesis, and bioenergetics. We hypothesized that VitD supplementation could play a key role in HSMM proliferation and differentiation, and also increase protein synthesis in the presence of insulin through the AKT/mTOR pathway.

2. Materials and methods

2.1. Cell culture

HSMM cells were cultured in different plate formats according to the requirements for each assessment. Cells were treated in the presence or absence of active form of VitD (i.e 1α,25(OH)2D3) for 24 h, 48 h and 72 h. Myotubes received the same treatment for 5 days and the media and VitD supplement were changed every 48 h. Several metabolic parameters were assessed in myoblasts and myotubes, as per described in Fig. 1. The human primary skeletal muscle myoblasts (SkGMTM-2, CC-3246, Lonza, Basel, CH) were propagated in growth medium (CC-3160, Lonza) supplemented with 0.05 ml/ml of foetal calf serum, 50 µg/ml of fetuin (bovine), 10 ng/ml of epidermal growth factor (recombinant human), 1 ng/ml of basic fibroblast growth factor (recombinant human), and 0.4 µg/ml of dexamethasone (SingleQuotsTM supplements, CC-3244, Lonza). All cells were maintained at 37°C and 5% CO2 at 60–70% confluence in T75 flasks. Then, cells were distributed into appropriate plates for each assay in growth media (myoblasts) and treated with 100 nM of the active VitD - calcitriol (C-1026-100ug, Astral Cientific, NSW) or 0.1% of ethanol (vehicle control group) for 24 h, 48 h or 72 h in accordance with each protocol. To study myotubes, after 1–2 days (when the cells reach 80–90% of confluence) of seeding with growth media, the cells were cultured in a differentiation media consisting of DMEM:F12 (D8437 Sigma-Aldrich, MO, USA) supplemented with 2% horse serum for 5 days (media was changed every 2 days). During the experimental protocol, cells were incubated with or without 100 nM of VitD (i.e. 1α,25(OH)2D3) dissolved in 0.1% ethanol for 5 days until they differentiate in myotubes. The media with VitD or vehicle was changed every 48 h. The optimal VitD dose (100 nM) was decided according to dose-response experiments perfomed (supplemental material). HSMM cells used in all experiments were between passages 4–8. The 100 nM concentration of VitD used in our study was the same optimal dose established in the majority of publications investigating the effects of the vitamin on skeletal muscle cell function [6,7,25–28].

2.2. Cell counting

For cell counting, cells cultured in 24 well plate were treated with vehicle (0.1% ethanol or 100 nM of 1α,25(OH)2D3) for 24 h, 48 h or 72 h hours (media was changed every day). Then, the cells were removed by using trypsin/EDTA solution 0.25% (CC-5012, Lonza) and stained with trypan blue solution 0.4% (Thermo Fisher Scientific, MA, USA) to identify live vs dead cells and counted using a hemocytometer. The percentage of confluence was calculated considering the mean intensity of pixels per field using image J. We have analysed 6 fields for each group (24 h, 48 h and 72 h VitD or Vehicle) and combined the time-points for the final analyses.

2.3. Cell viability determination

Cell viability following VitD treatment or vehicle control during 48 h was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, where metabolically active cells convert the water-soluble MTT to an insoluble purple formazan. MTT (5 mg/ml) was added to the culture medium at 1:10 dilution. After 4 h incubation, formazan crystals were solubilized with DMSO and absorbance was measured at 540 nm using a spectrophotometer (EnSpire Multimode Plate Reader, Perkin Elmer, MA, USA). Relative values of viable cells were determined in comparison to the untreated controls and expressed as percentage.

2.4. Cell cycle determination

Myoblasts were treated with VitD or vehicle control for 48 h, and then cells were collected and permeabilized overnight with cold 70% ethanol. The cells were subsequently incubated with RNAse (2.0 mg/ml) and PI (40 mg/ml) for 40 min at room temperature. DNA content was determined by flow cytometry, and the proliferation phases (sub-G0, G0/G1, S, G2/Mitosis) were determined by FlowJo software (FlowJo®, OR, USA).

2.5. Cell proliferation as determined by BrdU incorporation

For bromodeoxyuridine (BrdU) incorporation, using the reagent 5 Bromo-2′-Deoxyuridine (Sigma-Aldrich B5002). 100 mmol/mL of BrdU was added after 44 h of treatment with 100 nM of VitD or vehicle (0.1%

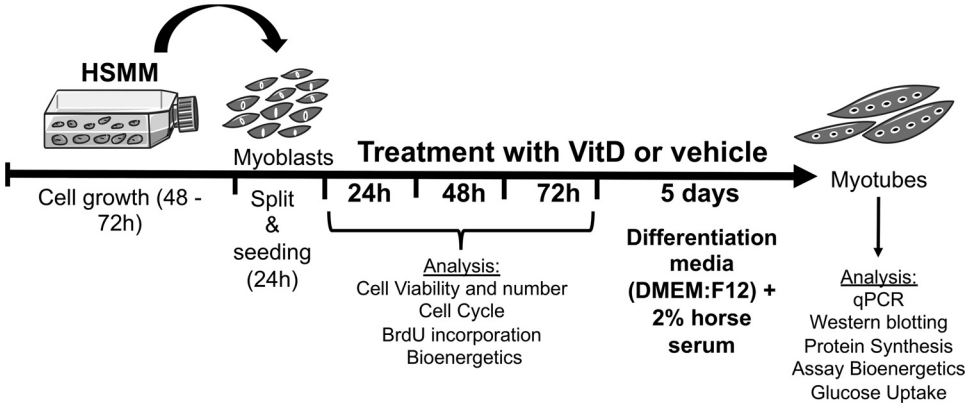

Fig. 1. A schematic presentation of the experimental design using HSMM cells.
Myoblasts were grown and culture for 24 h, 48 h, 72 h or 5 days (differentiation process to form myotubes) in the presence or absence of 100 nM of VitD (i.e. 1α,25(OH)2D3) or vehicle (0.1% ethanol). Analyses were performed in each time point.

ethanol). Then, after 4 h of treatment with BrdU, FITC-conjugated anti-mouse secondary antibodies was applied (Alexa fluor 488, green) for 30′. Flow cytometric data was acquired using a FACS LSR Fortessa flow cytometer and data were analyzed in the FlowLogic FCS analysis software. Fluorochromo/filter was BrdU FITC B530/30.

2.6. Gene expression changes as determined by quantitative real-time PCR

Cells were seeded in a 6 well plate (300.000 cells/well) and treated with VitD or vehicle control during 5 days of differentiation (media with or without VitD/vehicle was changed every 48 h). Total RNA from the cells was isolated using a RNeasy kit (Qiagen, MD, USA). cDNA synthesis was performed using the Reverse Transcriptase Mix (Qiagen, USA) by a thermal cycle of 42 °C for 15 min, followed by 95 °C for 5 min mRNA expressions of differentiation genes were detected via SYBR Green based real-time PCR. Customized RT2 Profiler PCR Array from Qiagen, with all the validated primers used in this study pre-coated on a 384-well plate was used in this study. The gene panel included: Myogenin, Myosin Heavy Chain 2, Troponin 3, Beta-2 macroglobulin (control). The cycling procedure was as follows: 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, followed by 60 °C for 1 min. Data analysis was performed using the $\Delta\Delta C_{\gamma}$ method.

2.7. Myotubes number and size

Myoblasts were treated with VitD or vehicle control throughout differentiation (i.e. 5 days), and then myotubes were fixed with PFA 4%, then stained during one minute with eosin and subsequently with haematoxylin, washed with Milli-Q water and then counted. The diameter was assessed using ImageJ (diameter measurement, 20 fields with fibers were randomly selected per 6 well plate, 2 wells per sample and the experiment was repeated four times) with 20x magnification and the results were compared.

2.8. Protein concentration as determined by Western blot analysis

5×10^5 cells were seeded in a 75 cm^2 flask with growth medium (CC-3160, Lonza) on day 1, and subsequently, when they reach confluence, the medium was changed to a differentiation medium consisting of DMEM: F12 (Sigma-Aldrich, D8437) supplemented with 2% horse serum. During the complete differentiation process (5 days), cells were exposed to VitD or vehicle control (0.1% ethanol) and incubated in the same differentiation media. The differentiation media was changed every 48 h hours and 24 h before the start of the experiment. After that, cells were washed with PBS and challenged for 20 min with or without 100 nM insulin from bovine pancreas (I6634 1Sigma-Aldrich, MA, USA). HSMM cells were lysed in RIPA buffer containing protease and phosphatase inhibitors (Cell Signaling Technology, MA, USA) and were quantified using BCA assay method (Pierce™, Thermo Scientific, MA, USA). Equal amounts of protein were combined with lithium dodecyl sulphate (LDS) sample buffer and the reducing agent dithiothreitol (DTT, Novex, Life Technologies, CA, USA). Proteins (20 μg/lane) were subjected to SDS–PAGE, using 4–20% Tris-glycine gels, and transferred onto nitrocellulose membrane was performed by wet method that involves immersing a gel-membrane sandwich in an upright tank of transfer buffer with vertical platinum electrodes. The quick immunoblot vacuum system was used for membrane blocking (3% BSA, Amresco, OH, USA) in PBS-Tween (PBST, 1% w/v) buffer and antibody incubation SNAP i.d (EMD Millipore, MA, USA). Membranes were blocked for 45 min and then incubated overnight at 4∘C, with specific primary antibodies purchased from Cell Signaling AKT (#9272S), p-AKT (Ser473, #9271S), GAPDH (#D16H11), mTOR (#2972S), (P-mTOR Ser2448, #2971S), S6 (#2217S), P-S6 (Ser235/236, #4858S), GSK3β (#9315S), P-GSK3β (Ser9, #9336S), except for VDR (#13133, Santa Cruz)- following the manufacturer's instructions. Bands were then visualized and quantified based on densitometry analysis using Molecular Imager® Gel Doc™ XR System v5.2.1 (Bio-Rad Laboratories, CA, USA). GAPDH was used as a protein control and there were no differences in the expression of protein between the experiments.

2.9. Protein synthesis assay

15×10^4 myoblasts were seeded in a 6 well plate with coverslips coated with 0.05 mg/ml of poly-d-lysine (Sigma-Aldrich) for 1 h. Click-iT® OPP (O-propargyl-puromycin) kit was performed in myotubes, adding 20 μM of OPP (Thermofisher Scientific) to the cells and incubated for 30 min. Cells were washed in PBS and then fixed and permeabilized using 3.7% formaldehyde in PBS followed by a permeabilization step using 0.5% Triton® X100. A reaction cocktail was prepared to contain Alexa-Fluor-594 (Thermofisher Scientific) and was conjugated to OPP as described in the manufacturer's instructions. Cells were washed again with a reaction rinse buffer and then stained with HCS NuclearMask™ Blue Stain. Cells were washed with PBS, water and then prepared in slides with a drop of ProLong™ Diamond Antifade Mountant (Thermo Fisher Scientific) protected from the light. Nikon Confocal A1 (Nikon, Instruments, Tokyo, Japan) was used to observe cells and capture images using a Plan Apo 60X objective lens. Myotube was defined by the existence of at least two nuclei within a continuous cell membrane and at least 30 pictures were taken in randomly chosen microscope fields. Data analysis was performed using Image J software (National Institutes of Health; available at http://rsb.info.nih.gov/ij). The mean fluorescence of each myotube was individually selected and counted to calculate and compared with the average of the vehicle

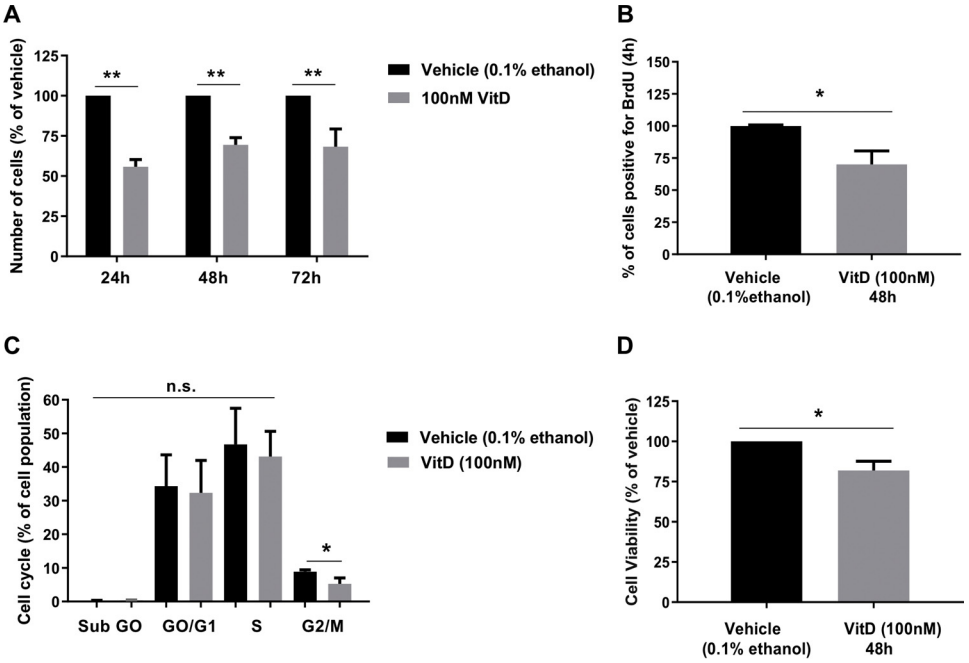

Fig. 2. Anti-proliferative effects of VitD in HSMM cells. Myoblasts were treated during 24 h, 48 h or 72 h with 100 nM of VitD or vehicle (0.1% ethanol) were counted using a hematocytometer (A). Cellular metabolic activities of myoblasts treated with VitD or vehicle (0.1% ethanol) during 48 h measured by the MTT (B). Cell cycle analysis measured by flow cytometry with PI staining after 48 h of treatment with VitD or vehicle (0.1% ethanol) (C). Quantification of cell proliferation in HSMM using BrdU assay (percentage of BrdU-positive cells were counted) in cells treated with VitD or vehicle (0.1% ethanol) during 48 h (D). All the experiments were repeated ≥3 times and compared using a *t*-test The data are presented as mean ± S.E.M. *p < 0.05; **p < 0.01 n.s. = non-significant.

group.

2.9.1. Bioenergetics measured by seahorse XFe96 flux analysis

The Seahorse Bioscience XFe96 Flux analyser and the Mito Stress Test kit (Seahorse Bioscience, USA) were used for assessment of cell bioenergetics. In summary, cells were seeded in 96-well plates (20,000 cells/well) overnight and treated with 100 nM of VitD or vehicle control (0.01% ethanol) for a further 24 h (myoblasts) or differentiated during 5 days with VitD or vehicle (media changed every 48 h and 24 h before the experiment). Next, the culture medium was changed to serum-free DMEM containing 1 mM sodium pyruvate and 2.5 mM D-glucose without bicarbonate, followed by 1 h incubation in a non−CO2 incubator at 37°C. We have recorded the basal measurements and then the injections via a pre-hydrated XFe96 sensor cartridge were carried out consecutively as follows: only media DMEM (2.5 mM D-glucose final), oligomycin (2 mM final, an inhibitor of ATP synthesis used to determine respiration linked to ATP synthesis), carbonyl cyanide p-trifluoromethoxy phenylhydrazone (FCCP, the mitochondrial uncoupler used to measure maximal respiration capacity; 1 mM final), and a combined solution of rotenone and antimycin A (1 mM final, inhibitors of mitochondrial complexes III and I, respectively). The oxygen consumption rate (OCR), which indicates the rate of mitochondrial respiration was measured at the baseline levels and after every injection. Extracellular acidification rate (ECAR), which is an index of glycolysis was measured following sequential injections of 2.5 mM glucose, oligomycin and 2-deoxy-glucose (2-DG). Normalization of the cells was performed by determining the protein content in each well using BCA assay (Thermo Scientific).

2.9.2. Glucose uptake in the absence or presence of insulin

Glucose uptake was monitored with the fluorescent deoxyglucose analogue 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) in human skeletal muscle myotubes cells (HSMM). One hundred thousand cells were seeded in a 24 well plate with growth medium (CC-3160, Lonza) on day 1, and after 1 day when they reach confluence the medium was switched to a differentiation medium consisting of DMEM:F12 (D8437, Sigma-Aldrich,) supplemented with 2% horse serum. After 5 days, the adult cells were exposed to 100 nM of 1α,25(OH)2D3 or vehicle control (0.1% ethanol) and incubated in the same differentiation media for 24 h. Subsequently, cells were washed with PBS and challenged for 20 min with or without 100 nM insulin and 2-NBDG. The intensity of fluorescence was evaluated by FACS LSR Fortessa flow cytometer and data were analyzed in the FlowLogic FCS analysis software. Median 2-NBDG fluorescence were obtained after gating for PI negative cells. Measurements of size (FSC-A) and complexity (SSC-A) were obtained in the same analysis [40]. The experiments were repeated three times and the results were compared using one-way ANOVA.

2.9.3. Statistical analysis

All analyses were performed using GraphPad Prism 6 software (GP Prism, San Diego, CA, USA). Student's *t*-test was applied when only two experimental groups were compared to evaluate statistical significance (i.e. VitD group *vs*. vehicle group). One-way ANOVA with a Bonferroni post hoc test was applied when more than two experimental groups were compared, and two-tailed statistical significance was set at *P < 0.05, **P < 0.01, and ***P < 0.001. All graphs were generated using GraphPad Prism 6 software.

3. Results

3.1. VitD inhibited proliferation in HSMM

To investigate the effects of VitD in primary human skeletal muscle cells, proliferation was assessed using four different methods: cell counting, cell viability (MTT assay), cell cycle and BrdU incorporation. VitD treatment decreased ($p < 0.01$) by 30% the number of cells when compared with vehicle group during 24 h, 48 h and 72 h (Figs. 2A-D, 3 A-B and E). To confirm these results cellular metabolic activity was performed by using MTT assay. Cells treated with VitD reduced ($p < 0.05$) the metabolic activity when compared with vehicle group (Fig. 2B). Cell cycle analysis was also performed to identify if VitD has any effect on the cells. Treatment with VitD did not change the sub-GO, GO/G1 and S phases of the cell cycle, however, we observed a significant decrease ($p < 0.05$) in G2/Mitosis, a phase that prepares cells for mitosis when compared with vehicle (Fig. 2C). The quantification of cell proliferation was measured by BrdU assay. BrdU is incorporated into active dividing cells and characterizes cell proliferation. Cells treated with VitD significantly decrease ($p < 0.05$) BrdU incorporation when compared with vehicle (Fig. 2D).

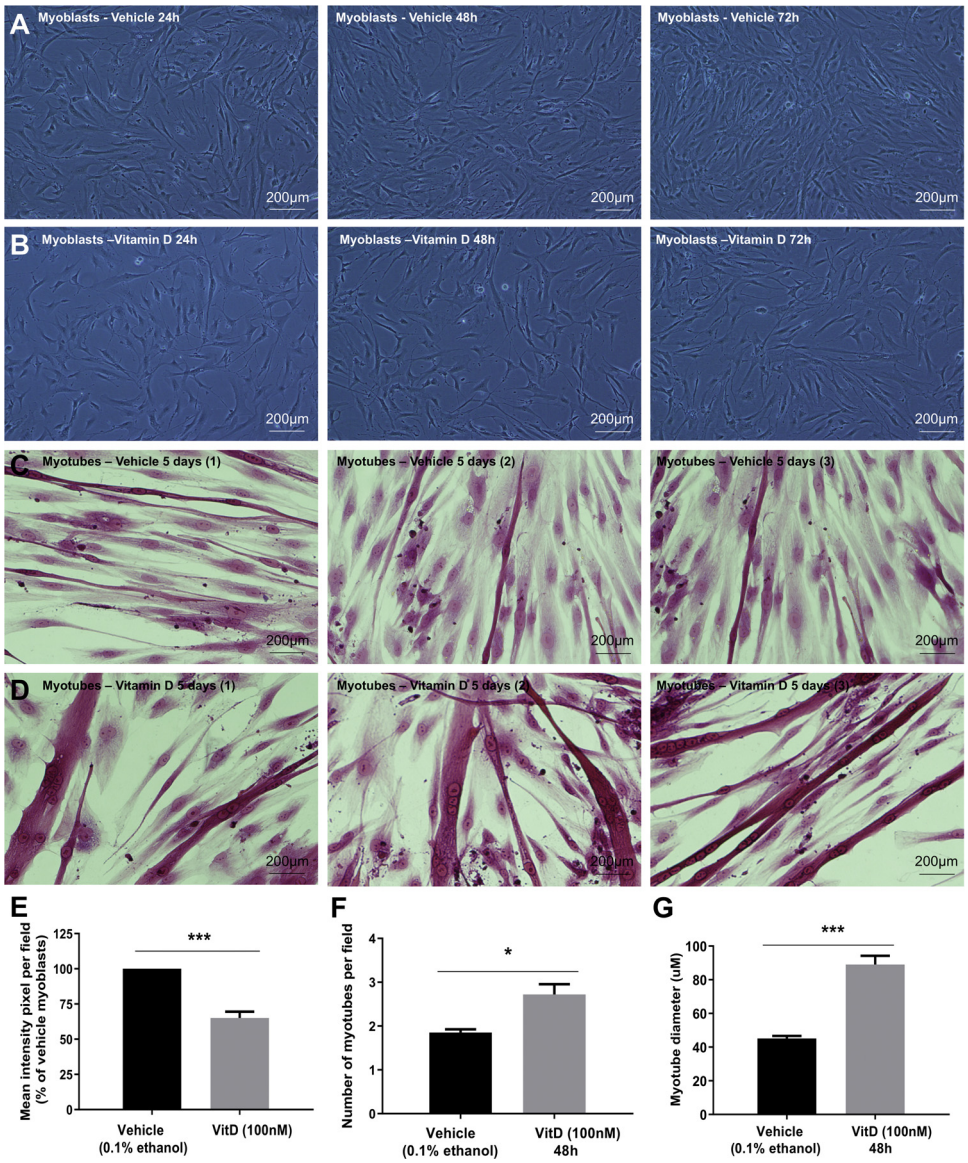

Fig. 3. Cultures of myoblasts and myotubes in the presence of VitD.
HSMM propagated in growth medium day 2 after thawing and then were treated with vehicle (0.1% ethanol, 3A) or 100 nM VitD (3B) for 24 h, 48 h and 72 h - 20x magnification are illustrated in Fig. 3 (n = 3). Percentage of confluence was calculated considering the mean intensity of pixels per field using image J. We have analysed 6 fields for each group (24 h, 48 h and 72 h VitD or Vehicle) and combined the time-points for the final analyses and compared using a *t*-test (E).
Effects of Vitamin D on human skeletal muscle myotubes number and size are illustrate in Fig. 3C and D (Vehicle - 0.1% ethanol and myotubes treated during 5 days with 100 nM of VitD. At day 6, there were notably higher number (D) and diameter of myotubes per field (20x magnification) (F and G) in VitD group compared with vehicle group using *t*-test *p < 0.05; ***p < 0.001.

3.2. VitD enhanced markers of differentiation in myotubes

HSMM were characterized for their capacity to differentiate into multinucleated myotubes (Fig. 3C and D). Cells treated with VitD over five days of differentiation exhibited an increase in the number of cells per field ($p < 0.05$), and also in diameter ($p < 0.001$), when compared with cell treated with vehicle solution (Fig. 3F and G). Myotubes were further examined for the expression of various markers of adult skeletal muscle cells such as Myogenin (MYOG), Troponin T type 1 (TNNT), and Myosin heavy chain-2 (MYH2). Myosin heavy chain (MHC) isoforms are markers of differentiation expressed during muscle development [20]. Myotubes were treated during differentiation (5 days) with 1α,25(OH)2D3 or vehicle and markers of differentiation were assessed by qPCR. VitD increased ($p < 0.01$) the gene expression of Myogenin and Troponin T type 3 (Fig. 4A and B). On the other hand, levels of myosin heavy chain 2 was reduced ($p < 0.01$, Fig. 4C).

3.3. VitD and additive effect on mTOR downstream pathway

VitD regulate a series of intracellular pathways by stimulating AKT and mTOR downstream signaling cascades, which eventually results in an increase in protein synthesis [41]. Therefore, we investigated whether VitD could enhance the anabolic pathway mediate by insulin. HSMM cells were treated with VitD for 5 days and then stimulated with 100 nM of insulin for 20′. In the presence of VitD, differentiated myotubes demonstrated higher ($p < 0.05$) phosphorylation level of GSK3β (Fig. 5A), ribosomal S6 protein (Fig. 5B), and AKT (S473, Fig. 5C), when compare to the vehicle + insulin group. The phosphorylation of mTOR (S2448) was higher ($p < 0.05$) in the Vehicle + insulin and VitD + insulin groups, when compared to the vehicle group (Fig. 6D). Myotubes treated with VitD were associated with a reduced ($p < 0.05$) expression of VDR (Fig. 5E).

3.4. VitD increased protein synthesis in myotubes

As VitD demonstrated a positive effect on mTOR downstream pathway in this study, we investigated if this signal could enhance protein synthesis in mature human skeletal muscle cells. Changes in protein expression were detected by the addition of Click-iT® OPP (O-propargyl-puromycin) to actively growing cells and stained with Alexa Fluor® 594 (red). Insulin group ($p < 0.001$) and VitD group ($p < 0.001$) increase mean pixel intensity of fluorescence per cell, suggesting an increase in protein synthesis, when compared with vehicle group (Fig. 6A and 6C). Interestingly, we also observed a

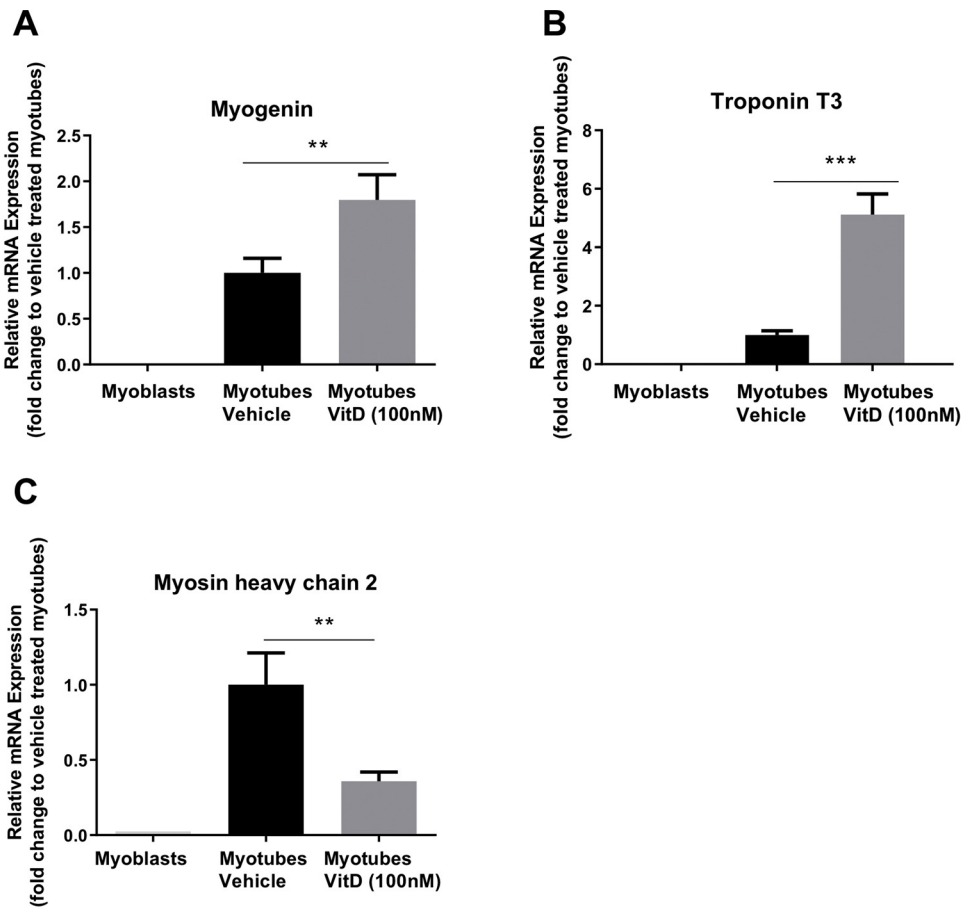

Fig. 4. VitD promotes myogenic differentiation in HSMM cells. Myoblasts were treated with 100 nM of VitD or vehicle (0.1% ethanol) for 5 days and skeletal muscle (i.e. Myotubes) gene expression of differentiation factors were investigated. Myogenin (A), Troponin T3 (B) and Myosin Heavy Chain 2 (C) were detected by qPCR. The data is presented as mean ± S.E.M. $^{**}p < 0.01$, $^{***}p < 0.001$ (n = 3) and was compared using *t*-test.

synergistic effect of VitD + insulin, with respect to increased protein synthesis when compared with vehicle + insulin group ($p < 0.001$) (Fig. 6D).

3.5. VitD increased mitochondrial oxygen consumption in myoblasts and myotubes

Cellular OCR was measured following the treatment with VitD or vehicle (0.1% ethanol) for 24 h in myoblasts (Fig. 7A) or for 5 days in myotubes (Fig. 8A). Myoblasts treated with VitD during 24 h exhibited an increase ($p < 0.01$) in maximal respiration, spare respiratory capacity and ATP production, when compared to the vehicle group (Fig. 7D, E and G). No significant changes were observed in basal OCR, proton leak and glycolytic activity after 24 h of VitD treatment (Fig. 7B, C, F and H). The effects of VitD in myotubes OCR were also investigated after 5 days of treatment. Basal respiration was higher ($p < 0.001$) from vehicle when 100 nM of VitD was supplemented for 5 days (Fig. 8B). Maximal respiration and spare respiratory capacity also increased ($p < 0.01$) after VitD treatment (Fig. 8C and E; $p < 0.05$). Non-mitochondrial respiration, proton leak and ATP production did not change between groups (Fig. 8D, F and G).

3.6. VitD did not significantly alter glucose uptake in myoblasts and myotubes

As myotubes appear to respond to VitD by increasing mTOR downstream outcomes, we investigated if an increase in mTOR stimulated processes could lead to a higher glucose uptake. Therefore, myoblasts and myotubes treated with VitD or vehicle were challenged for 20 min with 100 nM of insulin. Glucose uptake was determined and was higher in the vehicle + insulin group ($P < 0.01$), when compared with vehicle (Fig. 9B). However, no significant changes were observed in cells treated with VitD (Fig. 9A and B).

4. Discussion

In the present study, an investigation of the effects of VitD on proliferation, differentiation, protein synthesis and bioenergetic parameters in primary human skeletal muscle cells was performed. We report herein that VitD (i.e. 1α,25(OH)2D3) supplementation in HSMM cells 1) inhibited proliferation (Figs. 2 and 3); 2) increased differentiation (Figs. 3 and 4); 3) enhanced expression of proteins involved in mTOR downstream outcomes (possibly due to an increase in protein synthesis – Figs. 5 and 6); and 4) increased OCR in myoblasts after 24 h, and myotubes after 5 days (Figs. 7 and 8).

Myogenesis of skeletal muscle cells is a complex and ordered process. Myoblasts are derived from mesodermal stem cells and in this study, the first initial stage (proliferation) and the last stage (differentiation) was significantly affected by the addition of VitD to the culture media. Proliferation was inhibited by VitD, and this was tested by cell counting, cell viability, cell cycle techniques, and BrdU incorporation method (Figs. 2A-D and 3 A-B, E). Similar antiproliferative effects were also observed when Olsson et al. [28] treated human myoblasts with 1 or 100 nM of VitD during 48 h. This is also in agreement with other studies in murine cell lines, such as the C2C12, confirming the antiproliferative effects of VitD [6,7,25–28]. Interestingly, the inhibition of proliferation induced by VitD was not due to an increase in cell death, making toxicity unlikely, as reported by previous studies [32]. Nevertheless, the antiproliferative effects of VitD may indicate the promotion of cell quiescence, maintaining a stem cell population and potentiating self-renewing capacity [42–44]. Our results also demonstrated that in 48 h VitD inhibits cell proliferation acting

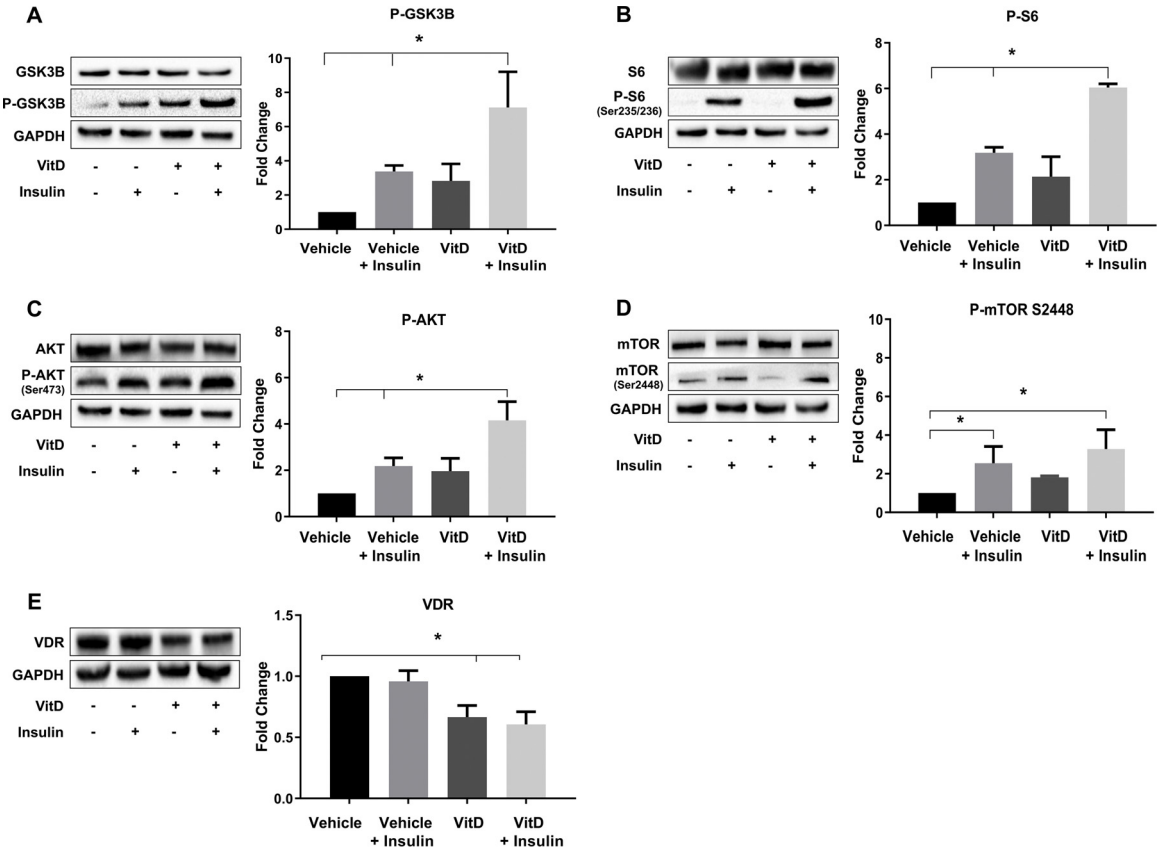

Fig. 5. mTOR downstream pathway is enhanced by vitamin D in myotubes. HSMM cells were treated with 100 nM of VitD or vehicle (0.1% ethanol) for 5 days and then stimulated with insulin for 20′). Phosphorylation level of GSK3β (Fig. 5A), ribosomal S6 protein (Fig. 5B), mTOR (Fig. 5D) and AKT (S473, Fig. 5C) were compare between vehicle + insulin group *vs.* VitD + insulin group. Protein levels were evaluated in myotubes by immunoblot analysis and compared using one-way ANOVA. Data are presented as mean ± SEM. *$p < 0.05$; ($n \geq 3$).

specifically on G2/Mitosis phase. One of the most common factors impending mitosis phase is the phosphorylation of histones and the presence of condensed chromosomes and changes specifically in G2/Mitosis phase were also evidenced in thyroid cancer stem-like cells treated with 100 nM of VitD [45]. A withdraw from the cell cycle through an increase in the expression of muscle-specific transcription factors, such as Myogenin and Troponin T type 1 indicates the fusion of myoblasts into multinuclear myotubes [42–44]. In our study, VitD increased the gene expression of myogenin (Fig. 4A) and troponin T type 3 (Fig. 4B), and at the same time decreased the levels of myosin heavy chain 2 (Fig. 4C). One possible explanation is that since MyHC2 promoter activity is positively regulated by an increased activity of nuclear factor of activated T cells transcription factor (NFAT), it is possible that GSK3 activity inhibited MyHC2 gene expression by reducing NFAT, and this is due calcineurin, as evidenced by Jiang and collaborators in 2006 [46]. Overall, our results are in agreement with Owens, Sharples, Polydorou, Alwan, Donovan, Tang, Fraser, Cooper, Morton, Stewart and Close [30] that investigated the effects of VitD during differentiation process in human primary muscle cells obtained by biopsies from active adults. The authors observed that VitD increased myotube fusion and differentiation [30].

Conversely, in another study it was observed that VitD inhibited myoblast differentiation by reducing the expression of cell cycle and myogenic regulatory factors (e.g. MyoD, myogenin, MEF2C and sarcomeric proteins), and also through the activation of the forkhead box O3 (FOXO3) and NOTCH self-renewal signaling pathway (e.g. target gene HES1) [28]. However, in this study the differentiation protocol was applied for a longer period (i.e. 8 days), and consequently, all the tests were performed on or after 8 days [28]. The majority of protocols in the literature have recommended 3–5 days for the skeletal muscle differentiation process [39]. Therefore, it is possible that depending on the day/time of differentiation and the day of the analysis, VitD could stimulate or reduce cell cycle regulators, resulting in controversial results.

Myotube growth and maintenance is mainly dependent on protein synthesis stimulus and protein turnover, which is determined by the rates of protein synthesis and degradation [21]. One of the main mechanisms of protein synthesis regulation is through the mTOR pathway dependent on growth promoting factors, such as insulin, growth factors and amino acids [46]. In our study, the increase in the phosphorylation of mTOR (Ser 2448) (Fig. 5D) was not statistically significant when cells were supplemented with VitD alone, however, this is possibly due to the wide range of mTOR target sites for phosphorylation. Interestingly the phosphorylation of mTOR downstream targets were significantly stimulated by VitD, such as P-S6 (Ser235/236) (Fig. 5B), P-Akt (Ser473)) (Fig. 5C), and P-GSK3β (Ser9)) (Fig. 5A) increased when cells were treated with VitD, as compared with the vehicle + insulin group. The phosphorylation of GSK-3 by PKB results in its inactivation, and consequently prevents the inhibition of guanine nucleotide exchange factors, such as the eukaryotic translation initiation factor 2B (eIF2B), representing one potential mechanism for the regulation of protein synthesis [47]. Additionally, the activation of PKB/Akt and/or MAP kinase pathways are considered major regulators of cell growth [48]. Overall, these results suggest an additional effect of VitD + insulin on the mTOR pathway and a possible role for VitD in insulin signaling and/or protein synthesis cascade.

As skeletal muscle protein synthesis rate is positively controlled by a wide range of postprandial factors, such as the insulinemic response, we investigated the possible additive effects mediated by VitD and insulin. The treatment with VitD and also VitD + insulin increased the rate of

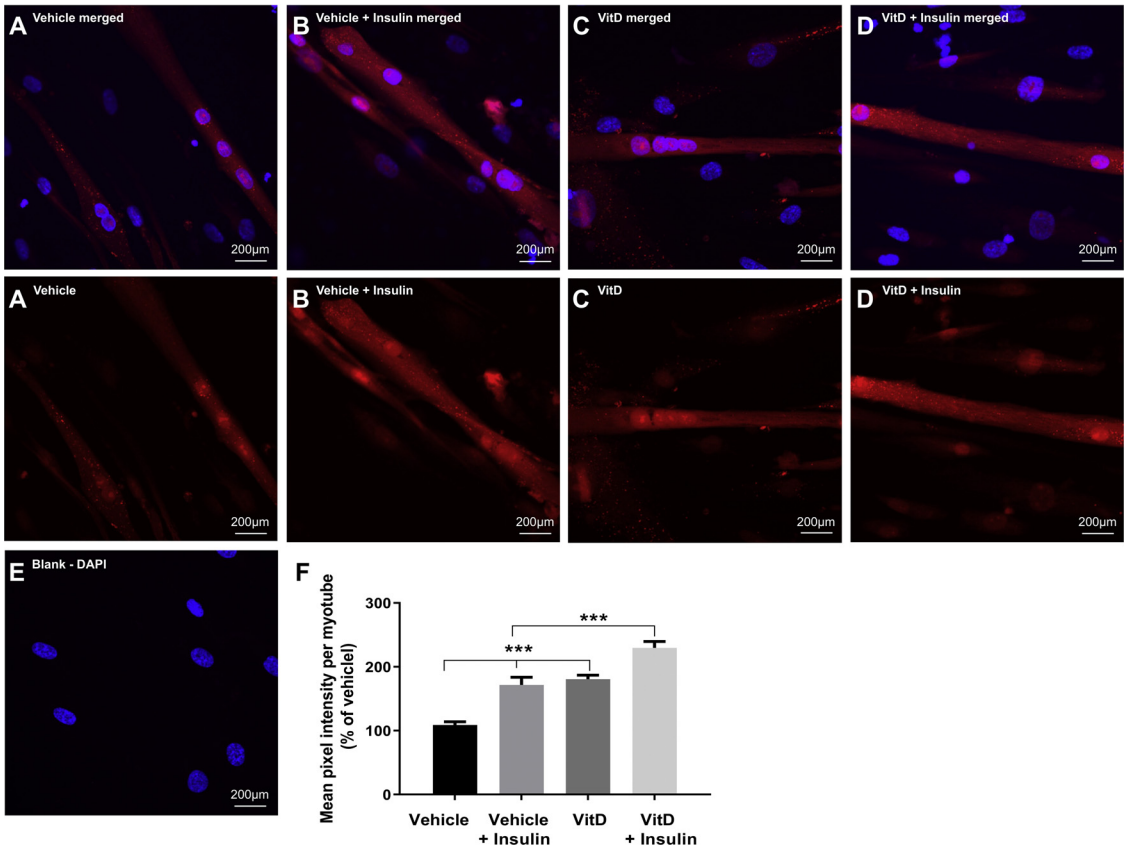

Fig. 6. VitD increases protein synthesis in myotubes.
Representative immunofluorescence analysis of myotubes protein synthesis after 5 days with 100 nM of Vitamin D or vehicle (0.1% ethanol) (n = 3). Changes in protein levels were detected by the addition of Click-iT® OPP (O-propargyl-puromycin) to actively growing cells and stained with Alexa Fluor® 594 (red). A) Vehicle; B) Vehicle + 100 nM of Insulin; C) 100 nM of VitD; D) VitD + 100 nM of insulin; E) Blank + Dapi (nuclear stain blue). The graph quantifies the mean pixel intensity of Click-iT® OPP signal per cell in comparison with percentage of vehicle (F). The data is presented as mean ± S.E.M and was compared using one-way ANOVA ***$p < 0.001$ (n = 3).

protein synthesis, when compared to the vehicle group (Fig. 6C, D and F). However, Salles, Chanet, Giraudet, Patrac, Pierre, Jourdan, Luiking, Verlaan, Migne, Boirie and Walrand [21] observed that 10 nM of VitD potentiated the effects of leucine and insulin by increasing the rate of protein synthesis through Akt/PKB and mTOR pathways

In our study, an increase in number and size of myotubes after VitD treatment during the differentiation process was observed. Several studies with VDR knockout mice (VDR-KO), and also mice subjected to a VitD deficient diet have reported significant muscular atrophy, weakened strength, decreased muscle fiber size, lower bone mineral density and dysregulation of myogenic regulatory factors when compared to wild type animals [7,33,34]. For instance, one study have observed a negative impact of VitD deficiency in the expression of MyoD in skeletal muscle tissue, suggesting a role of VitD for the process of myogenesis, muscle maintenance and hypertrophy [20]. Our *in vitro* experiments with HSMM cells also highlight the importance of VitD in the modulation for muscle function, which could lead to potential increments in muscle mass and strength. Interestingly, in clinical studies, VitD supplementation in healthy humans with low VitD serum levels activates the VDR in skeletal muscle tissue, which appears to stimulate protein synthesis and improve muscle strength following an increase in the size and number of type II muscle fibers [11,41].

Another important finding in our study is related to the increased mitochondrial OCR induced by VitD in skeletal muscle cells (Figs. 7 and 8). In particular, the control and maximal respiratory capacity which link oxygen consumption and associated mitochondrial respiration to the generation of ATP was increased, demonstrating a role of VitD in muscle cell energy production. This effect is possibly due to an increase in the expression of the electron transport chain (ETC) proteins and the tricarboxylic acid (TCA) cycle enzymes via both genomic and non-genomic pathways [49]. To the best of our knowledge, only one study investigated the effects of VitD on cellular bioenergetics in primary human muscle cells (i.e myoblasts) [5]. The authors demonstrated that mitochondrial OCR increased when cells were treated with VitD for 48 h [5]. A possible mechanism is an elevation in mitochondrial volume fraction and branching, which may result in mitochondrial fusion and biogenesis. VitD can increase the expression of MYC, Mitogen-Activated Protein Kinase 13 and Endothelial PAS domain-containing protein 1 (which encodes for proteins that regulate mitochondrial biogenesis) [5]. Our experiments confirmed that myoblasts and myotubes produce energy predominately via mitochondrial metabolism as the level of glycolysis was particularly low in our experiments. This is because skeletal muscles predominantly produce energy via mitochondrial metabolism [50]. Therefore, VitD did not changed the glycolytic activity and these results are in accordance with other studies [5].

Typically, the effects of VitD are mediated by its interaction with a nuclear VitD receptor (VDR) which is part of the nuclear receptor superfamily of ligand-activated transcription factors. VDR can also be translocated into the mitochondria of certain cell types, including the skeletal muscles, and potentially act directly on cellular bioenergetics [42]. In the current study, VitD treatment reduced the expression of the VDR in myotubes after 5 days of treatment (Fig. 5E). Only two other studies that have investigated the effects of VitD on VDR in human myotubes and they reported an increased level of the receptor in response to 24 h and 72 h exposure to 1 or 100 nM of VitD [5,28]. The latter results might be explained by the fact that VitD effects are

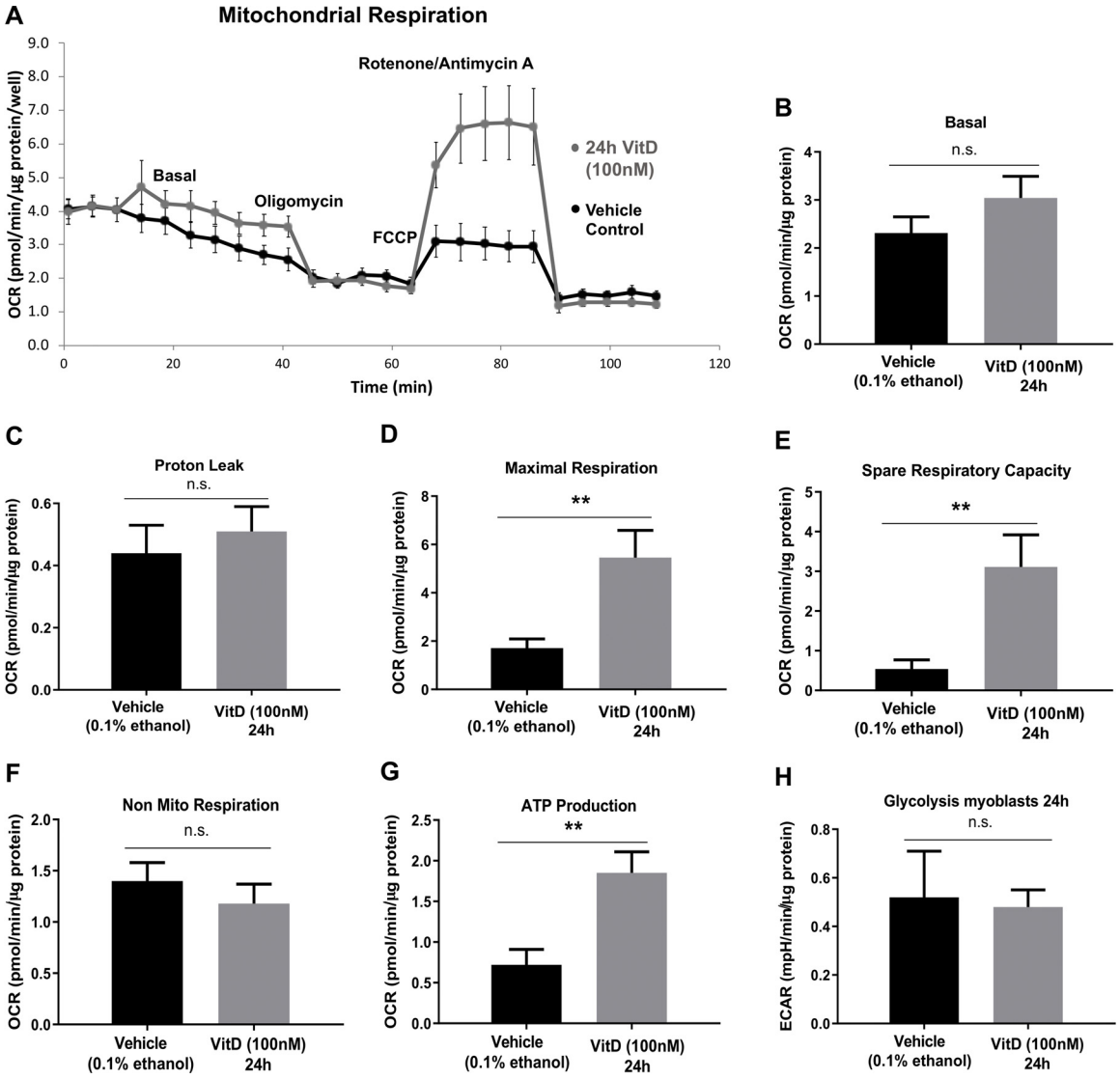

Fig. 7. Bioenergetics in myoblasts is affected by 100 nM of VitD during 24 h.
Following this, cells were subjected to extracellular flux analysis using Seahorse Biosciences XFe96 Flux Analyzer. Oxygen consumption rate (OCR) profiles were determined after injection of 25 mM glucose, followed by 2 µM oligomycin, 1 µM FCCP and 1 µM each of rotenone and antimycin A, enabling generation of a mitochondrial stress profile (A–G). Glycolysis were then determined (H). Data is presented as mean ± SEM compared using a *t*-test (n = 3); *p < 0.05, ** p < 0.01; n.s. = non-significant.

dependent on several factors, such as the type of skeletal muscle cells, stage of proliferation or differentiation, and culture (serum) condition employed in the experiment. Furthermore, VitD responses could be related with a long period of VitD treatment, leading to a negative feedback of the VDR, which can be translocated from the nuclei to the cytoplasm. More studies are required to investigate the chronic effects of VitD on VDR.

In conclusion, the present study demonstrated the positive *in vitro* effects of VitD on myogenesis (i.e. anti-proliferative effects concomitantly with an increase in the differentiation process) in HSMM cells. VitD also had an additive effect with insulin, and increased the expression of several key proteins responsible for insulin action on protein synthesis. Furthermore, VitD supplementation altered mitochondrial OCR in myoblasts and myotubes, which resulted in increased energy production. Taken together, these findings demonstrated a physiologically relevant effect of VitD on muscle metabolism and function in human skeletal muscle cells.

Author contributions

The present work was designed by V.C., R.C, P.N, and K.R.M.; K.R.M and R.C performed all experiments; K.R.M, R.C, V.C. and P.N analyzed data; K.R.M and V.C. wrote the paper. Revision of the manuscript drafts was made by R.C, V.C. and P.N. All authors approved the final version of the paper.

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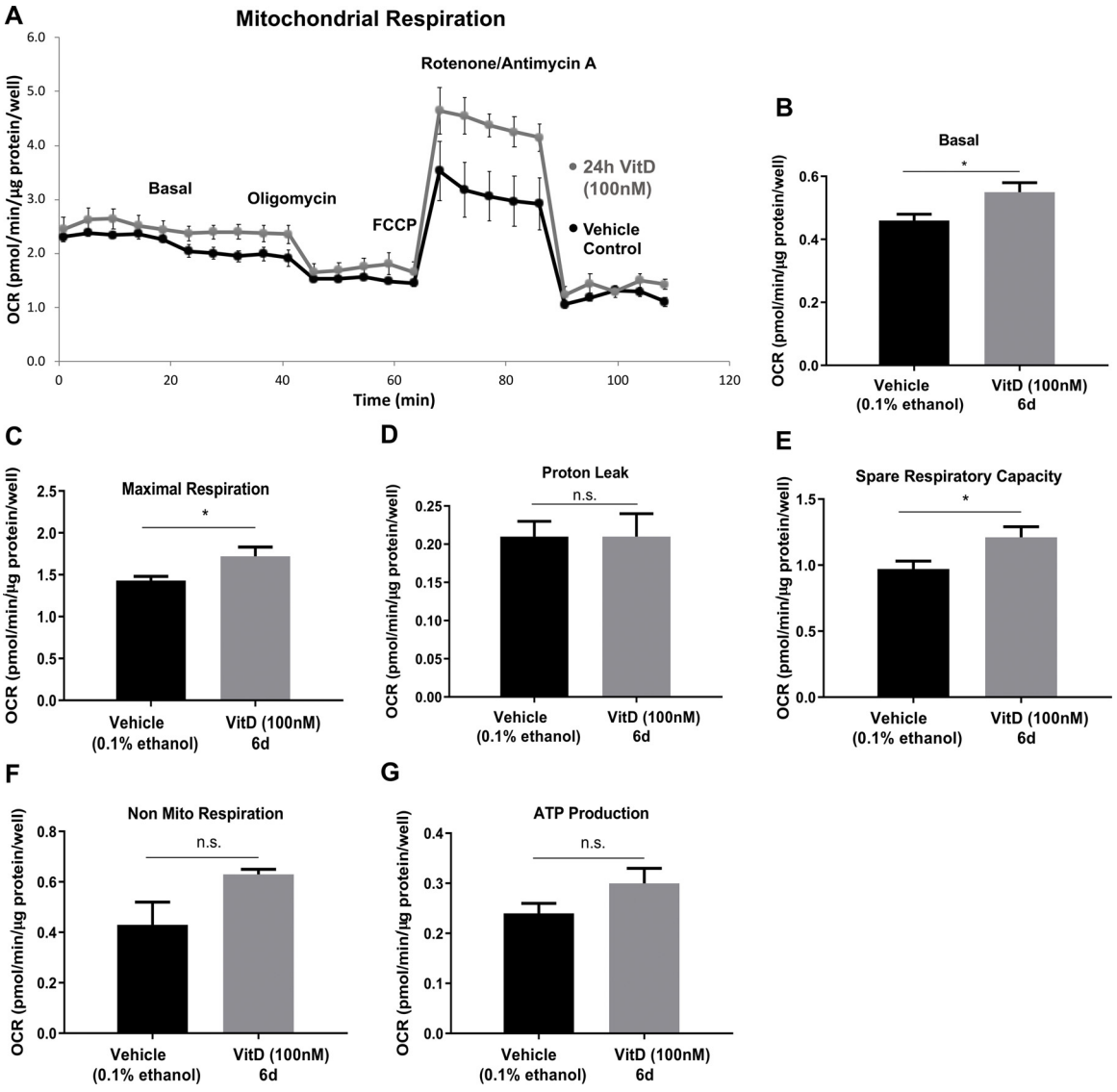

Fig. 8. Bioenergetics in myotubes is affected by 100 nM of VitD.
Following this, cells were subjected to extracellular flux analysis using Seahorse Biosciences XFe96 Flux Analyzer. Oxygen consumption rate (OCR) profiles were determined after injection of 25 mM glucose, followed by 2 µM oligomycin, 1 µM FCCP and 1 µM each of rotenone and antimycin A, enabling generation of a mitochondrial stress profile (A–G). VitD treatment increased Maximal Respiration and Spare Respiratory Capacity OCR. Data is presented mean ± SEM compared using *t*-test (n = 3). *p < 0.05; n.s. = non-significant.

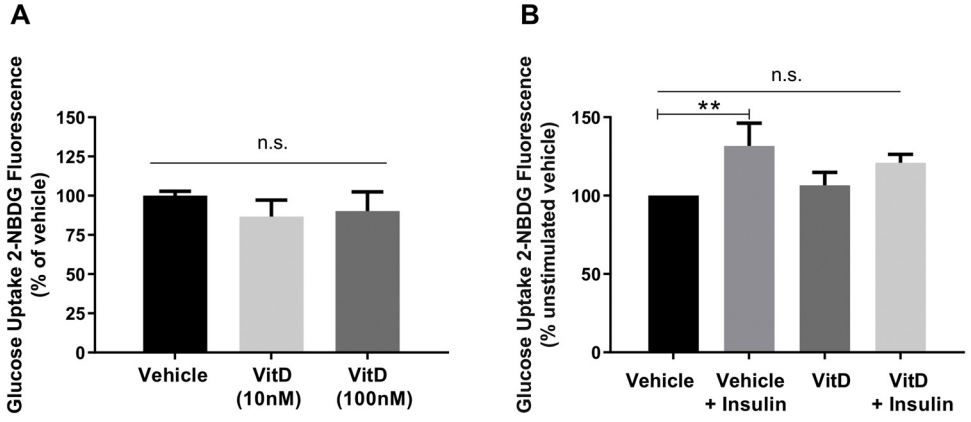

Fig. 9. VitD does not affect glucose uptake in myotubes.
2-NBDG uptake was determined after 5 days of treatment with VitD 100 nM or vehicle (0.1% ethanol) Fig. 9A. Myotubes were also challenged with 100 nM of insulin for 20 min after 5 days of treatment with 100 nM VitD or vehicle (0.1% ethanol) (Figure B). The data is presented as mean ± S.E.M and was compared using one-way ANOVA. **p < 0.01; n.s. = non-significant (n = 3).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsbmb.2019.105423.

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Article

Vitamin D Supplementation Does Not Impact Resting Metabolic Rate, Body Composition and Strength in Vitamin D Sufficient Physically Active Adults

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Abstract: Supplementation with the most efficient form of Vitamin D (VitD3) results in improvements in energy metabolism, muscle mass and strength in VitD deficient individuals. Whether similar outcomes occur in VitD sufficient individuals' remains to be elucidated. The aim of this study is to determine the effect of VitD3 supplementation on resting metabolic rate (RMR), body composition and strength in VitD sufficient physically active young adults. Participants completed pre-supplementation testing before being matched for sunlight exposure and randomly allocated in a counterbalanced manner to the VitD3 or placebo group. Following 12 weeks of 50 IU/kg body-mass VitD3 supplementation, participants repeated the pre-supplementation testing. Thirty-one adults completed the study (19 females and 12 males; mean ± standard deviation (SD); age = 26.6 ± 4.9 years; BMI = 24.2 ± 4.1 kg·m^2). The VitD group increased serum total 25(OH)D by 30 nmol/L while the placebo group decreased total serum concentration by 21 nmol/L, reaching 123 (51) and 53 (42.2) nmol/L, respectively. There were no significant changes in muscle strength or power, resting metabolic rate and body composition over the 12-week period. Physically active young adults that are VitD sufficient have demonstrated that no additional physiological effects of achieving supraphysiological serum total 25(OH)D concentrations after VitD3 supplementation.

Keywords: calcitriol; energy metabolism; muscle strength; lean mass; adults

1. Introduction

Vitamin D (VitD) is an essential pro-steroid hormone [1] responsible for the regulation of calcium and phosphate metabolism [2]. Recently, VitD has received significant scientific interest as the importance of adequate concentration of VitD for optimal skeletal muscle metabolism and function has been identified [3,4]. Interestingly, despite having a temperate climate and many hours of sunshine year-round, one in four Australian adults have a suboptimal VitD status (serum 25(OH)D concentration < 50 nmol/L)[2], which highlights the need for further research into the impact of VitD on health and exercise performance. The key source of VitD for humans comes from exposure of the skin to ultraviolet radiation (UVR), allowing the conversion of 7-dehydrocholesterol to cholecalciferol

(pre-vitamin D3) and subsequently, to 1,25(OH)2D3 (calcitriol), which is the biologically active form of VitD, representing 80–90% of total VitD production [5].

Vitamin D3 is a potential skeletal muscle modulator and has been reported to influence several muscle functions [6], including skeletal muscle mass and strength, aerobic energy production and lipid metabolism [7]. VitD3 supplementation in healthy adults with low serum concentration of 25(OH)D activates the VitD receptor (VDR) in skeletal muscle, which can stimulate protein synthesis, develop muscle tissue and improve muscle strength in healthy young adults [7–9]. Several authors have suggested that supplementation with VitD3 also results in an increase in size and number of type II muscle fibres in VitD deficient individuals [6,10,11]. However, whether these effects can be observed in participants that have adequate or high VitD concentration remains to be elucidated.

A systematic review by Chiang et al. [12] recently reported that VitD3 supplementation (ranging from 400 to 8500 IU/day) in VitD sufficient athletes resulted in an increase of 1.4–18.8% in muscle strength. The effects of VitD3 supplementation on muscle strength and power has been investigated in soccer [13,14] and rugby players [15], elite ballet dancers [16], swimmers [17] and active adult males [18,19], with mixed findings. However, the majority of these randomized clinical trials (RCT) focused on investigating the effect of VitD in individuals classified as deficient and insufficient at the baseline of the study [13–20]. For example, four RCT [13,16,18,19] reported that VitD3 supplementation increased strength (e.g., isometric force peak, one-repetition maximum (1-RM) bench press, back squat and weighted reverse-grip chin up). Conversely, three RCT reported no effects of VitD3 on any parameter of muscle strength and power [14,17,20]. It is possible that including a population with different concentrations of VitD at the baseline confounds efforts to understand the impact of VitD on muscle strength and power [12].

Currently, the concentration of VitD required for optimal skeletal muscle function is not fully agreed, with guidelines based on the United States Institute of Medicine bone health recommendation of VitD ≥ 50 nmol/L [21,22]. However, it has been suggested by Heaney and Holick [23] that serum total 25(OH)D concentrations of ~ 120–225 nmol/L may be required for optimal skeletal muscle function, particularly for optimal function. Importantly, long-term oral intake of VitD may cause VitD intoxication (> 200 nmol/L) and result in negative health consequences such as gastrointestinal disorders, bone pain, drowsiness and headaches [24], justifying the importance of establishing optimal and safe serum concentration of VitD for skeletal muscle function.

To date, the majority of intervention studies on the effect of VitD on muscle strength have examined VitD deficient athletes and not those with adequate or supraphysiological concentrations of total VitD. It also seems important to differentiate serum 25(OH)D and the bioavailable free form of VitD (free 25(OH)D), as the latest evidence suggests that VitD binding protein (VDBP) inhibits certain actions of this vitamin, since the bound fraction is unavailable to act on target cells [25,26]. However, it is not clear how the free 25(OH)D versus bound component might impact muscle strength and power in humans [27]. Furthermore, as VitD3 has non-calcemic activities (e.g., inhibition of adipocyte differentiation and remodeling adipose tissue that might impact energy metabolism) [28], it has been suggested that VitD3 may also impact resting metabolic rate (RMR). Identifying variables that affect energy metabolism is essential as changes over time in RMR can have a large impact on fat and muscle mass and vice-versa [29]. Therefore, the aim of this study is to determine the effects of VitD3 supplementation on total and free serum concentrations of 25(OH)VitD, RMR, body composition and strength in VitD sufficient, physically active adults.

2. Materials and Methods

2.1. Subjects

A total of 42 physically active adult males and females met the inclusion criteria (exercising at least three times per week with at least two of those sessions involving resistance training; no history of VitD3 supplementation in the last month; no current injuries that would prevent them from completing strength testing; no current use of multivitamins, medication or other supplements that are related with VitD metabolism and body composition (including calcium, thyroxine, creatine and

thermogenic supplements)) and were recruited to participate in this study. During the study, four participants had sports injuries unrelated to the study protocol and seven were not able to complete the minimum training required or any of the post-supplementation tests, and then were excluded from this study, leaving a final sample of 31 participants (19 female and 12 male; mean ± SD; age = 26.6 ± 4.9 years; height = 170.0 ± 8.4 cm; body-mass = 71.7 ± 11.8 kg). The study protocol was conducted according to the Declaration of Helsinki and approved by the Curtin University Human Research Ethics Committee (approval number: HRE2019-0028) and registered by the Australian New Zealand Clinical Trials Registry (ACTRN12620000896976). Informed consent was obtained from all subjects.

2.2. Study Overview

Participants completed two testing sessions (one pre-supplementation and another post-supplementation) over approximately 13 weeks (Figure 1). First, participants completed initial assessments, including an assessment of RMR, body composition, muscular strength and power and hematological markers [total and free plasma 25(OH)VitD, Ca^{2+} and parathyroid hormone] between week 0 and 1. Data collection started after the summer season (March/April/May, Western Australia) to increase the chance of individuals being VitD sufficient at the baseline and to optimize the chance of participants reaching higher serum total concentration (≥ 120 nmol/L) at the end of the supplementation period. Following pre-testing, participants were matched for sunlight exposure and randomly allocated in a double-blind and counterbalanced manner to the VitD3 group (total n = 17, female n = 11; 50 IU/kg body-mass [BM]/day Elite Vitamin D3, Healthspan Ltd.®, United Kingdom [UK], batch tested by Informed Sport, LGC Limited, UK) or placebo (total n = 14, female n = 8; dextrose, Glucodin, iNova Pharmaceuticals, Australia) supplement group for 12 weeks. All doses were concealed in opaque gelatin capsules and organized in sequentially numbered containers to ensure that participants and the testing researcher were blinded to group allocations.

This dosing strategy was selected because it has been associated with a positive effect on strength performance in previous research [16,18]. In the middle of the study (week 7), hematological markers were again assessed to measure VitD concentration and check for any possible adverse effects of supplementation. Then, following 12 weeks of supplementation, participants repeated the pre-supplementation testing at the same time of day and referred back to their three-day food diary to ensure that they were similarly prepared to perform. Adequate and optimal VitD status were defined as 25(OH)D between 50–100 nmol/L and > 100 nmol/L, respectively, based on previous research that suggests that these concentrations may be related to optimal skeletal muscle outcomes [23,30]. Participants were in contact with the main researcher weekly, to ensure training and supplementation adherence and report any perceived side-effects from the supplementation. Vitamin D3 supplementation was distributed to participants fortnightly and the capsules left were counted to ensure compliance.

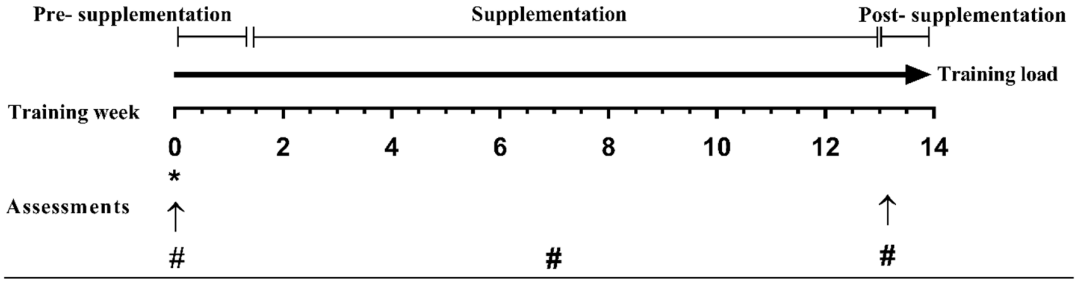

Figure 1. Study design. * Familiarisation with test procedures; ↑ Resting metabolic rate (RMR), body composition and strength and jump tests; # Food intake and venous blood sample.

2.3. Procedures

2.3.1. Resting Metabolic Rate

Participants abstained from any strenuous exercise for 24 h prior to the measurement of RMR. Participants arrived at the laboratory as soon as possible after waking following a 12 h overnight fast and were instructed to empty their bladder and void bowels before being weighed. Oxygen consumption was assessed when participants were resting in a supine position, in a quiet room without noise and strong light by metabolic cart (Parvo Medics TrueOne 2400, Parvo Medics , Salt Lake City, USA), using a mouthpiece and mixing chamber. Minute–minute measurements of O_2 consumption and CO_2 production were then conducted over a minimum of 30 min. The energy expenditure equation (Weir) was used to calculate RMR from the average of the last 10 min of data collection [31].

2.3.2. Body Composition Assessment

Immediately following the assessment of RMR, participants had their whole-body composition assessed using dual-energy X-ray absorptiometry (DXA; GE Lunar Prodigy, General Electric, Madison, USA), including lean mass, fat mass and total bone mineral density (BMD). Participants completed a pre-scan questionnaire to assess their suitability for scanning, before being positioned on the midline of the scanning bed in a standardized position with both arms by their side with the hands in a mid-prone position within the scan area and feet fixed at a 90° angle at the ankle. All scan analyses were completed using the scanners proprietary software, with adjustments made by the same experienced technician to ensure consistency between scans.

2.3.3. Assessment of Muscular Strength and Power

Having completed the fasting elements of the testing protocols, the participants were allowed to eat and drink prior to the performance assessment components of the protocol to ensure that they were optimally prepared to perform. First, participants completed a self-selected warm-up, which was recorded and replicated for the post-supplementation testing session. Second, they completed a test of (1-RM) strength for the bench press and back squat exercises following the procedures set out by Baechle and Earle [32]. Briefly, participants lifted progressively greater weights until a mass was identified that could only be lifted for one repetition for that exercise. Finally, participants completed a counter-movement vertical jump test to assess leg power (Vertec Yardstick Jumping Device, Swift, Brisbane, Australia). Participants completed three attempts with a 2–5 min rest between attempts.

2.3.4. Hematological Markers

Participants attended an external pathology laboratory (PathWest Laboratories, Perth, Western Australia) pre-, mid-and post-supplementation, where a venous blood sample (~ 10 mL) was drawn to assess VitD status, Ca^{2+} and parathyroid hormone. Vitamin D status (25(OH)D) was determined using the Immunoassay method (Abbott Architect i2000sr analyser, Abbott, Illinois, USA). This method has a correlation coefficient (r) of 0.99 with the certified reference method, Isotope Dilution-Liquid Chromatography-Tandem Mass Spectrometry (ID-LC-MS/MS) for 25 (OH)D[33]. Calcium and parathyroid hormone were analysed using the Arsenazo III method and chemiluminescent microparticle immunoassay methodology, respectively (Abbott, Spain). Additionally, two small serum aliquots (1 mL) were separated and frozen to −80 °C for subsequent measurement of the free 25(OH)D concentration. After thawing, concentrations of free 25(OH)D were measured by ELISA immunoassay (DIASource ImmunoAssays, Wijchen, Netherlands) following the procedure described previously [34]. Correlation between rate dialysis analysis results vs. Free 25OH Vitamin D ELISA is described as 0.992.

2.3.5. Training Diary and Questionnaires

As part of a training diary, participants completed a calcium quiz (Dairy Council of California) [35] and sunlight exposure questionnaire [36] on three occasions: baseline, 7 and 12 weeks. Additionally, on these three occasions, participants completed a three-day food record (2 weekdays and 1 day of the weekend), after having received detailed instructions about how to complete their dietary intake. Each food diary was reviewed in detail by a nutritionist together with each participant to ensure that sufficient detail was captured. Food records were analysed using Foodworks® V9 (Xyris Software Pty Ltd., Brisbane, Australia). Each individual was encouraged to follow similar eating patterns throughout the study to minimize deviations in macronutrient, vitamin and mineral intake. Finally, a daily training diary was completed during the 12 weeks of supplementation so that training load could be calculated using the session rating of perceived exertion (sRPE) method proposed by Foster and the adherence to exercise could be confirmed. [37]. Briefly, the participant had to describe the type of exercise, duration of session and chose the rating of perceived exertion for the session (CR1–10 scale) [38]. This value was multiplied by the duration of the session and it provided us with a training load value in arbitrary units (AU).

2.4. Statistical Analysis

Categorical data were summarized using frequency distributions. Depending on normality, continuous variables were summarized by means and standard deviations (age, free 25(OH)D, weekly sunlight exposure and daily calcium intake) or medians and interquartile ranges (IQR) [BMI, total 25(OH)D, parathyroid hormone (PTH), Ca^{+2} and total training load). Participants' main characteristics at pre- and post-supplementation were compared between groups using Chi-squared tests for categorical data and t-tests or non-parametric Mann–Whitney U tests for continuous data. Based on previous research considering strength as the main outcome, a sample size of 30 has 99% power to detect a standardized mean difference of 0.60 (33, 58) in a mixed-model ANOVA in two groups across two time points with an α value of 0.05 [39]. However, due to the ability to adjust for factors, linear mixed modelling was used for the main analyses considering random subject intercepts. Effects of free and total serum 25(OH)D on pre-post supplementation differences in strength, RMR and body composition outcomes, within and between intervention groups, were assessed by linear mixed models (LMM), adjusting for gender, sunlight exposure, training load, protein, carbohydrate, fat and total energy intake. LMM were also used to assess total and serum 25(OH)D concentration within and between groups differences pre- and post-supplementation. This model uses maximum-likelihood estimation methods that parametrize all longitudinal data regardless of missing data points. Results are summarized as estimated marginal means, mean differences and 95% confidence intervals. Statistical significance was as accepted at $p < 0.05$. Analyses were conducted using STATA/IC 16.0 (StataCorp LLC, College Station, USA).

3. Results

3.1. Participants Characteristics

Participants were equally distributed in both groups in regards to gender, age and ethnicity, showing no significant differences pre-supplementation. The majority of participants were female (n = 8 (57%) vs. n = 11 (65%); p = 0.67) and self-declared Caucasian and white (n = 12 (86%) vs. n = 15 (88%)) in placebo and VitD3, respectively (p = 0.83) (self-reported). On average, participants were 24.9 years ± 4.3 vs. 27.9 years ± 5.3 years old in placebo and VitD3, respectively (p = 0.09). Vitamin D3 and placebo group also had similar characteristics including BMI, serum total 25(OH)D concentration, serum parathyroid hormone, serum Ca^{2+}, weekly sunlight exposure, sunscreen use, training load, weeks sick or injured and daily calcium intake (Table 1). The VitD3 supplementation dose was calculated by body-mass, which averaged 3205 ± 366 IU/day for females and 4230 ± 548 IU/day for males, respectively. No difference was observed in energy intake (p = 0.66), carbohydrate (p = 0.06), protein (p = 0.77) and total fat consumption (p = 0.11) between and within groups during the 12 weeks

of supplementation (Appendix A, Supplementary Figure 1). Additionally, during the supplementation period, participants only forgot to take the VitD3 supplement on average once a month and were sick and/or not training only for 1–2 weeks, confirming an appropriate adherence with supplementation and exercise routine through the full intervention as well as having a very similar training load without any difference between groups ($p = 0.92$).

Table 1. Pre- and post-supplementation characteristics of participants by study group (placebo *n* = 14 and VitD3 *n* = 17).

Parameter	Pre-Supplementation (Week 0–1)		*p-value	Post-Supplementation (Week 13–14)		#p-value
	Placebo *n* = 14	**VitD3 *n* = 17**		**Placebo *n* = 14**	**VitD3 *n* = 17**	
BMI (kg/m^2) med (IRQ)	24.8 (3.6)	23.5 (4.6)	0.60	24.7 (4.5)	23.4 (4.5)	0.54
Total 25(OH)D (nmol/L) med (IRQ)	74.0 (44.5) [a]	93 (49) [a]	0.07	53 (42.2) [a]	123 (51) [b]	< 0.001
Free 25(OH)D (pg/mL) mean (SD)	8.6 (5.2) [a]	13.1 (5.3) [b]	0.006	6.2 (4.5) [a]	15.8 (5.1) [b]	< 0.001
Parathyroid hormone (pmol/L) med (IRQ)	5.1 (3.0)	4.9 (3.6)	0.68	6.5 (2.1)	3.9 (3.4)	0.008
Ca^{+2} (mmol/L) med (IRQ)	2.5 (0.1)	2.4 (0.2)	0.89	2.4 (0.1)	2.4 (0.1)	0.47
Weekly sunlight exposure mean (SD) (h)	4.4 (4.6)	3.8 (3.1)	0.95	0.7 (0.5)	0.7 (0.5)	1.00
Sunscreen use (yes) *n* (%)	4 (29%)	5 (29%)	0.96	4 (29%)	5 (29%)	0.96
Total training load (AU) med (IRQ)	1635 (1562)	1260 (1065)	0.54	18840 (16168)	15140 (13995)	0.92
Weeks sick or injured (*n*) mean (SD)	0	0	NA	1.00 (0.5)	1.18 (0.6)	0.42
VitD3 supplementation dose (IU/day) mean (SD)	NA	NA	NA	0	3559 ± 670	NA
Daily calcium intake (mg) mean (SD)	816 (598)	999 (428)	0.09	965 (447)	1109 (482)	0.54

Different letters indicate significant differences between groups pre- and post-supplementation ([a,b]). N/A: not applicable; *p-value: comparison between placebo and VitD3 pre-supplementation; #p-value comparison between placebo and VitD3 post-supplementation. BMI, total 25(OH)D, PTH, Ca^{+2}, total training load are represented by median (IRQ) and Free 25(OH)D, weekly sunlight exposure and daily calcium intake are represented by media ± SD (standard deviation).

3.2. Serum Total 25(OH)D and Free 25(OH)D Concentration

Mean baseline serum total 25(OH)D concentration of participants was 87.7 ± 31.4 nmol/L (range = 50.0–175.0 nmol/L), meaning that all participants were classified as VitD sufficient (serum total concentration of ≥ 50 nmol/L) prior to supplementation (Table 1). Serum free 25(OH)D concentration were higher in VitD3 group when compared with placebo at the baseline of the study (Table 1). Vitamin D3 supplementation significantly increased serum total concentration of 25(OH)D in the VitD3 group (in average by 32%) when compared with baseline (Figure 2A), while the placebo group reduced in average by 28%. A similar effect was not observed when comparing the free 25(OH)D pre- vs. post-supplementation period (Figure 2B). Free 25(OH)D concentration was higher (34%) only at the baseline in the VitD3 group when compared with placebo and this difference was sustained during the study (40%). Three participants from the placebo group had non-detectable serum free 25(OH)D concentrations and were excluded from the analysis.

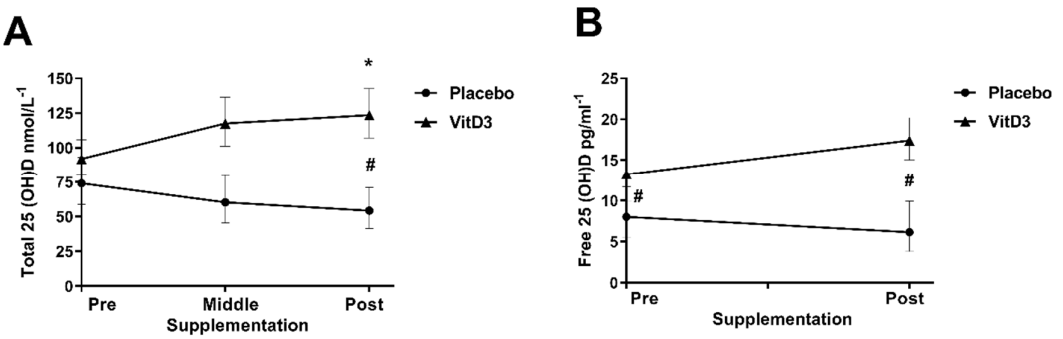

Figure 2. Serum total 25(OH)D concentration pre-, middle- and post-supplementation (**A**) and free 25(OH)D concentration pre- and post-supplementation (**B**). *Difference within group (VitD3 pre-supplementation vs. VitD3 post-supplementation by ANOVA; $p = 0.01$); #difference between groups (VitD3 pre- and post-supplementation vs. placebo pre- and post-supplementation; $p < 0.001$).

3.3. Muscular Strength and Power

All data from the muscular strength and power tests are reported in Table 2 considering the main predictor as serum total concentration of 25(OH)D and in Table 3 considering the main predictor as serum free 25(OH)D. There were no significant changes between placebo and VitD3 groups in any of the muscle strength and power tests (back squat, bench press and vertical jump displacement) over the 12-week period considering serum total 25(OH)D as the main predictor (1-RM back squat, $p = 0.54$; 1-RM bench press, $p = 0.38$; and vertical jump $p = 0.50$). Within-group increases in strength in the VitD3 group were observed for back squat (+ 6.9 kg; $p = 0.005$) and bench press (+ 3.2 kg, $p = 0.007$) (Table 2). Similar within-group increases were observed when free 25(OH)D was considered as the main predictor for back squat (+ 5.5 kg, $p = 0.042$ vs. + 7.7kg, $p = 0.003$) and bench press (+ 2.7 kg, $p = 0.020$ vs. + 2.9kg; $p = 0.01$) in both groups (placebo and VitD3, respectively).

Table 2. Estimated within-group means and mean differences of outcomes (pre- and post-supplementation) considering serum total 25(OH)D as the main predictor.

Predictor = 25(OH)D	**Model Adjusted For: Gender, Sunlight Exposure, Training Load, Protein, Carbohydrate, Fat and Energy Intake**										
		Pre-Supplementation			**Post-Supplementation**				**Pre-Post Change**		
Outcome	**Group**	**Estimated mean**	**95%CI diff**	***p*-value**	**Estimated mean**	**95%CI diff**	**°P-value**	**#*p*-value**	**Estimated Mean Difference**	**95%CI**	***p-value**
Resting Metabolic Rate (kJ)	Placebo	6372.8	5994.6, 6751.1	0.86	6631.5	6221.4, 7041.5	1.00	0.83	258.6	-48.1, 565.4	0.10
	VitD3	6327.4	6004.5, 6650.4		6632.7	6262.8, 7002.6			305.3	20.4, 590.2	0.036
Muscle Strength and Power											
Back Squat (kg)	Placebo	77.9	69.0, 86.9	0.10	82.6	73.2, 92.0	0.07	0.54	4.6	-0.6, 9.9	0.08
	VitD3	87.9	82.6, 94.8		94.8	86.3, 103.4			6.9	2.1, 11.8	0.005
Bench Press (kg)	Placebo	51.3	45.6, 57.1	0.010	52.9	47.0, 58.8	0.005	0.38	1.6	1.1, 4.2	0.24
	VitD3	61.5	56.4, 66.7		64.8	59.4, 70.1			3.2	0.9, 5.6	0.007
Jump test (cm)	Placebo	41.1	36.7, 45.5	0.17	41.9	37.4, 46.5	0.31	0.50	0.8	-0.9, 2.5	0.35
	VitD3	45.2	41.3, 49.2		45.2	41.1, 49.3			-0.01	-1.5, 1.5	0.98
Body Composition											
Lean mass (kg)	Placebo	47.9	47.4	0.09	48.0	44.9, 51.0	0.07	0.54	0.6	-0.3, 1.5	0.19
	VitD3	50.9	50.9		51.9	49.1, 54.6			1.0	0.2, 1.8	0.013
Fat mass (kg)	Placebo	22.7	21.4	0.011	21.4	18.4, 24.5	0.06	0.20	-1.2	-2.5, -0.02	0.050
	VitD3	17.5	17.3		17.3	14.6, 20.1			-0.1	-1.2, 1.0	0.83
Bone Mineral Density (g/cm^2)	Placebo	1.2	1.7, 1.2	0.026	1.2	1.2, 1.2	0.008	0.09	-0.0005	0.014, 0.013	0.94
	VitD3	1.3	1.2, 1.3		1.3	1.2, 1.3			0.02	0.004, 0.02	0.004

p -value: difference between groups pre-supplementation); °P-value:(difference between groups post-supplementation); #*p*-value: group-time interaction (rate of change); **p*-value: within group pre-post change. Estimated means and mean differences were assessed by linear mixed models (LMM); 95% CI: confidence intervals; diff: difference.

Table 3. Estimated within group means and mean differences of outcomes (pre- and post-supplementation) considering Free total 25(OH)D as the main predictor.

		Model Adjusted For: Gender, Sunlight Exposure, Training Load, Protein, Carbohydrate, Fat and Energy Intake									
		Pre-Supplementation			Post-Supplementation				Pre-Post Change		
Outcome	**Group**	**Estimated Mean**	**95%CI diff**	***p*-value**	**Estimated Mean**	**95%CI diff**	**ºP-value**	**#*p*-value**	**Estimated Mean Difference**	**95%CI**	***p-value**
Resting Metabolic Rate (kJ)	Placebo	6361.2	5971.4, 6750.9	0.86	6619.6	6223.8, 7015.5	0.82	0.91	258.4	−39.1, 556.0	0.09
	VitD3	6409.3	6057, 6761.6		6690.1	6296.2, 7084			280.9	0.67, 561.1	0.049
Muscle Strength and Power											
Back Squat (kg)	Placebo	79.1	68.9, 89.4	0.12	84.6	74.2, 95.0	0.08	0.51	5.5	0.2, 10.7	0.042
	VitD3	90.8	80.9, 100.6		98.5	88.0, 11			7.7	2.7, 12.8	0.003
Bench Press (kg)	Placebo	53.3	46.8, 59.7	0.020	56.0	49.5, 62.6	0.030	0.93	2.7	0.4, 5.0	0.020
	VitD3	64.0	57.7, 70.3		66.9	60.3, 73.4			2.9	0.7, 5.0	0.010
Jump test (cm)	Placebo	41.3	36.4, 46.2	0.26	41.6	36.6, 46.6	0.234	0.78	0.3	−1.3, 2.0	0.70
	VitD3	45.4	40.5, 50.2		46.0	41.0, 51.0			0.6	−0.9, 2.2	0.24
Body Composition											
Lean mass (kg)	Placebo	48.0	44.6, 51.3	0.12	48.4	45.0, 51.7	0.10	0.61	0.4	−0.4, 1.2	0.34
	VitD3	51.7	48.4, 54.9		52.3	49.0, 55.7			0.7	−0.1, 1.4	0.09
Fat mass (kg)	Placebo	22.4	19.1, 25.7	0.07	21.6	18.2, 24.9	0.13	0.57	-0.9	−2.2, 0.4	0.19
	VitD3	18.1	14.9, 21.3		17.7	14.4, 21.1			-0.4	−1.6, 0.8	0.55
Bone Mineral Density (g/cm²)	Placebo	1.2	1.2, 1.3	0.11	1.2	1.2, 1.3	0.12	0.94	0.007	−0.001, 0.023	0.41
	VitD3	1.3	1.2, 1.3		1.3	1.2, 1.3			0.061	−0.001, 0.021	0.43

p-value: difference between groups pre-supplementation); ºP-value:(difference between groups post-supplementation); #*p*-value: group-time interaction (rate of change); **p*-value: within group pre-post change. Estimated means and mean differences were assessed by linear mixed models (LMM); 95% CI: confidence intervals; diff: difference.

3.4. Resting Metabolic Rate and Body Composition

Resting metabolic rate, lean mass, fat mass and bone mineral density are described in Table 2 considering serum total concentration of 25(OH)D as the main predictor, and in Table 3 with serum free 25(OH)D as the main predictor. Resting metabolic rate increased in both groups following the 12-week supplementation period (estimated mean difference for placebo = +258.6 kJ and VitD3 = + 305.3 kJ); however, there were no significant differences between groups. Within-group increases in RMR in the VitD3 group were observed (RMR + 305.3kJ, $p = 0.036$ and + 280.9kJ, $p = 0.049$) considering both predictors (i.e., serum total and free 25(OH)D, respectively). Lean, fat mass and bone mineral density did not differ significantly between the groups after 12 weeks of supplementation with VitD3 or placebo (Table 2). Within-group increases in lean mass (+ 1.0 kg; $p = 0.013$) in the VitD3 group were also observed when serum total 25(OH)D was the main predictor.

3.5. Calcium and Parathyroid Hormone

Serum calcium concentration was constant during the whole study between and within groups (Table 1). Total serum calcium concentration was 2.5 mmol/L (IRQ 0.1) vs. 2.4 mmol/L (IRQ 0.2) pre-supplementation and 2.4 mmol/L (IRQ 0.1) vs. 2.4 mmol/L (IRQ 0.1) post-supplementation in placebo and VitD3 groups, respectively (Table 1), showing no significant differences between placebo and VitD3 groups. In contrast, the concentration of serum parathyroid hormone slightly decreased in the VitD group only, starting with 5.1 (IRQ 3.0) vs. 4.9 (IRQ 3.6) pre-supplementation and with concentration 6.5 (IRQ 2.1) vs. 3.9 (IRQ 3.4) post-supplementation in placebo and VitD3, respectively. Parathyroid hormone concentrations were significantly higher in the placebo group when compared to the VitD3 group ($p = 0.008$; Table 1) post-supplementation. Overall, participants maintained Ca^{2+} and parathyroid hormone serum concentration in the normal range during the study

4. Discussion

We describe herein an investigation of the effects of VitD3 on RMR, body composition, strength and power in physically active adults. Following 12 weeks of VitD3 supplementation, total serum concentration of 25(OH)D significantly increased in the VitD3 group; however, no significant differences in RMR, body composition, strength and power were identified when comparing the VitD3 and placebo groups. We also found that supplementation with VitD3 did not significantly change serum free 25(OH)D concentration, which may explain the lack of meaningful effect on the main outcomes.

In our study, the participants demonstrated high concentration of serum total 25(OH)D (placebo = 74 nmol/L; VitD3 = 93 nmol/L) prior to supplementation. These results can be partially explained by approximately 4 h/week of exposure to the intense UVR (mean UVR index = 11–classified as extreme) and high temperatures (mean = 30 °C) during summer months in Perth, Western Australia (situated 31 °S) [40,41]. Vitamin D3 supplementation resulted in a 30 nmol/L (IQR 50) increase in serum total 25(OH)D, while the placebo group reduced total serum concentration by 21 nmol/L (IQR 43) throughout the supplementation period. Therefore, 36% of the placebo group was classified as VitD deficient (< 50 nmol/L), with a serum total 25(OH)D concentration of 53 (42.2) nmol/L at the end of this study. Even though the change in serum total 25(OH)D concentration pre- to post-supplementation in the placebo group was not statistically significant, the measured decrease is clinically meaningful as it represents a decrease that may require VitD3 supplementation to maintain adequate concentration throughout the winter months.

Serum total 25(OH)D has a longer half-life than other VitD metabolites and is directly associated with sunlight exposure through dermal synthesis and also dietary intake, justifying why we used it as the primary marker of VitD status [42]. The 'free-hormone hypothesis' is considered an alternative pathway for cellular uptake of steroid hormones, as these molecules are highly lipophilic and, therefore, have the potential to quickly and passively diffuse across cell membranes [27]. The initial average range established for the free 25(OH)D concentration is 5.1 pg/mL (2.4–17.1 pg/mL) based on 109 healthy individuals [27]. In the present study, participants had a high concentration of free

25(OH)D (8.6 ± 5.2 pg/mL placebo and 13.1 ± 5.3 pg/mL VitD3 group) before supplementation, which is similar to concentration reported by Sollid et al., 2016 (13.7 ± 4.2 pg/mL) [43]. Surprisingly, free 25(OH)D concentration did not significantly increase after VitD3 supplementation, suggesting participants from the VitD3 group might have already reached optimal serum free 25(OH)D values before starting the intervention protocol.

In our study, participants had an optimal concentration of serum total VitD after supplementation (~120 nmol/L); however, no significant impact on strength or power was detected. This is possibly due to the fact that the majority of participants were already VitD sufficient at the beginning of the study, showing no additional effect of the VitD3 supplementation. Interestingly, within-group differences between VitD and RMR, back squat, bench press, lean mass and bone mineral density, were observed within the VitD3 group [considering serum total 25(OH)D as the main predictor for these outcomes]. In previous RCT studies, the protocols used to measure muscular strength and power were inconsistent, making comparisons with our study challenging [14,16,19,44,45]. Close et al. used a similar protocol to measure strength as we used in our study (1-RM) and they reported that 12 weeks of supplementation with 20,000 or 40,000 IU/week of VitD3 offered no improvements on skeletal muscle strength or power in a VitD deficient, young and active cohort [19]. The authors proposed that higher doses of VitD3 might be needed to reach optimal concentrations of serum total 25(OH)D (> 120 nmol/L), as the majority of participants reached only the VitD sufficient range (50–75 nmol/L) previously endorsed for bone health [19].

Clinically, higher dietary VitD intake and serum 25(OH)D concentration is associated with a reduction in omental adipocyte size and lower visceral adiposity in women [46]. Cross-sectional studies report a negative relationship between overweight and/or obesity and serum concentration of 25(OH)D [47], and prospective studies have reported that low 25(OH)D plasma concentration may contribute to the development of obesity [48,49]. In relation to muscle mass, several authors have suggested that supplementation with VitD also results in an increase in size and number of type II muscle fibers in VitD deficient individuals [6,10,11]; however, few RCT have tested these associations in humans directly by muscle biopsy. A recent study that investigated one year of VitD3 supplementation in VitD deficient participants found that lean mass significantly increased from 43.8 ± 9.6 to 44.3 ± 9.8 kg in the VitD group, while no change was observed in the placebo group. In our study, participants maintained a healthy body composition during the 12-week supplementation period and no significant differences were observed after supplementation in lean or fat mass between groups. Randomised clinical trials examining the influence of VitD on energy expenditure are rare. To the best of our knowledge, only one clinical study with a very short supplementation protocol (1 week) reported no influence of VitD3 on energy or substrate utilization and this topic has not been further explored in a physically active population [50]. Cellular studies have reported an increase in control and maximal respiratory capacity, which link oxygen consumption and associated mitochondrial respiration to the generation of ATP, demonstrating a role of VitD in primary human skeletal muscle cell energy production [4,51]. Our results and those of other researchers suggest that the effects of VitD3 supplementation on body composition and RMR might be observed in VitD deficient and/or obese and or/elderly populations, and not in physically active young adults.

It is important for further RCT to perform a dose-escalation study for physically active adults and also to consider the serum free 25(OH)D as part of the total serum 25(OH)D. For example, participants may show different concentration of total versus free 25(OH)D, and therefore, might not be accurately classified as VitD inadequate or adequate (Appendix A, Supplementary Table 1). Perhaps the recommendation for VitD supplementation in order to correct deficient VitD status should not be based only on total serum 25(OH)D concentration, but also on free 25(OH)D concentrations, and symptoms of VitD deficiency (e.g., fatigue or tiredness, muscle weakness, and bone and muscle pain) [52]. Furthermore, other factors influence the response to VitD supplementation, for example Sollid et al. found that individuals respond better to VitD3 supplementation when they are VitD deficient at the baseline [53].

One limitation of our study is that we were not able to prescribe and control participants' training. Whilst training loads were similar between group and exercise adherence was high, this

may have impacted our findings as we did not observe significant increases in muscle mass in both groups after 12 weeks of resistance training, which would have been expected. Finally, in order to assure that participants would not suffer any adverse effects to supplementation with VitD3 and to guarantee concentration within the recommended range, serum calcium and the PTH were also assessed during the study. Only the concentration of PTH decreased in the VitD3 group in response to the increase of serum total 25(OH)D concentration and the opposite was observed on the placebo group. It is well known that VitD concentration directly impacts serum PTH, helping to regulate calcium metabolism and bone density and function [54]. No adverse effects, including hypercalcemia or hyperparathyroidism were reported for any participant.

5. Conclusions

In conclusion, the present study demonstrates no additional benefits on muscle strength, power, RMR and body composition of VitD supplementation in physically active individuals already classified as sufficient in VitD. It is possible that the supplementation of VitD might still be necessary during autumn and winter seasons, where people are at a higher risk to be VitD deficient. Our results suggest that further research using large sample size-controlled trials are required to explore the temporal relationship between serum total and free 25(OH)D concentration with muscle strength and power. We believe that this will assist enhanced understanding of the possible therapeutic effects of VitD supplementation in VitD sufficient and deficient individuals.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6643/12/10/3111/s1 Figure S1: Mean ± (SD) Daily total energy (A) and macronutrient intake (B, C and D) pre-, mid- and post-supplementation in the placebo and VitD3 groups., Table S1: Theoretical method to calculate the percentage of free 25(OH)D availability (example).

Author Contributions: The present work was designed by K.R.M, K.J.D., V.C., and P.N; K.R.M performed all data collection; K.R.M, K.J.D, V.C., H.M. and A.J. and P.N analysed data; K.R.M, K.J.D and V.C. wrote the paper. Revision of the manuscript drafts was made by K.J.D, V.C. and P.N. All authors approved the final version of the paper.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

AU	Arbitrary Units
BMD	Bone mineral Density
IU	International Unit
RCT	Randomized Clinical Trial
RMR	Resting Metabolic Rate
VitD	Vitamin D
VitD3	Vitamin D3
VDBP	Vitamin D Binding Protein
VDR	Vitamin D Receptor
UVR	Ultraviolet Radiation
1-RM	One Repetition Maximum

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