THE ROLE OF VITAMIN D AND THE VITAMIN D RECEPTOR IN SKELETAL MUSCLE FUNCTION AND EXERCISE ADAPTATION

By

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ABSTRACT

Vitamin D deficiency remains prevalent throughout the world, with severe deficiencies resulting in skeletal muscle myopathies. Within skeletal muscle, a dynamic network of mitochondria exists primarily functioning to produce ATP via oxidative phosphorylation. Recent investigations have proposed that vitamin D related metabolites are able to modulate mitochondrial function within skeletal muscle cell lines and human populations with severe vitamin D deficiencies. Therefore, the aims of this thesis were to further explore the role of vitamin D related signalling via the VDR and diet-induced vitamin D deficiency in modulating skeletal muscle mitochondrial function. It was demonstrated that a reduction in the VDR significantly reduced mitochondrial respiration in the C2C12 skeletal muscle cell line without altering mitochondrial protein content. Furthermore, *in vivo* investigations revealed a reduction in skeletal muscle mitochondrial respiration following 3-months of diet-induced vitamin D deficiency in mice. Finally, we also demonstrated an impairment in voluntary wheel running performance and the subsequent adaptive response following diet-induced vitamin D deficiency in mice. In summary, this thesis contributes novel data towards the understanding of the role of the vitamin D and the VDR in modulating skeletal muscle mitochondrial function.

DEDICATION

In dedication to Robert ‘Bob’ Ashcroft & Paul Robert Ashcroft.

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DECLARATIONS

I declare that all the work contained within this thesis is my own with the following exceptions:

i. Vitamin D Receptor Knock-Down C2C12 cells were kindly provided by Professor Philip Atherton from the University of Nottingham and initially developed by Dr Abid Kazi from Penn State College of Medicine.

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LIST OF ABSTRACTS, CONFERENCE COMMUNICATIONS AND ARTICLES

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LIST OF ABBREVIATIONS

$1\alpha,25(OH)_2D_3$	1α,25-dihydroxyvitamin D
31-P MRS	31-phosphate nuclear magnetic resonance spectroscopy
ADP	adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
ANT1	adenine nucleotide translocase 1
ANT2	adenine nucleotide translocase 2
APS	ammonium persulfate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
$BaCL_2$	barium chloride
BCL-2	beta-cell lymphoma 2
BNIP3	BCL-2 interacting protein 3
BSA	bovine serum albumin
C15orf48	chromosome 15 open reading frame 48
$Ca2^+$	calcium
$CaCL_2$	calcium chloride
CCCP	carbonyl cyanide m-chlorophenyl hydrazone
ChIP	chromatin immunoprecipitation
$CI+II_P$	complex I and II phosphorylating
CI_L	complex I related leak
CI_p	complex I phosphorylating

CO_2	carbon dioxide
CS	citrate synthase
CYP	cytochrome P450 mixed-function oxidases
Cyt c	cytochrome c
DBP	vitamin D binding protein
DHA	docosahexaenoic acid
DMEM	dulbecco's modified eagles medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DRP1	dynamin-related protein 1
E2A	transcription factor E2-aplha
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N',- tetraacetic acid
EPA	eicosapentaenoic acid
ERp57	endoplasmic reticulum protein 57
ETC	electron transport chain
FADH	flavin adenine dinucleotide
FBS	fetal bovine serum
FCCP	carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FCR	flux control ratio
FIS1	fission 1
FOXO1	forkhead box O1
GFP	green fluorescent protein
GRP58	glucose responsive protein 58

h	hours
H^+	hydrogen ions
HCL	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF1α	hypoxia-inducible factor 1-alpha
HLB	hyperosmolar lysis buffer
HS	horse serum
HVDDR	hereditary vitamin D-resistant rickets
IP3	inositol triphosphate
IU	international units
KCL	potassium chloride
KD	knock-down
kg	kilogram
K_m	michaelis-menten constant
km	kilometers
KO	knock out
KRPH	krebs-ringer-phosphate-hepes
LC-MS/MS	liquid chromatography coupled with mass spectrometry
LC3II	lipidated microtubule-associated protein-light chain 3
LLC1	Lewis lung cancer carcinoma cell line 1
MARRS	membrane-associated rapid response steroid
MEM	minimal essential medium
MFF	mitochondrial fission factor
MFN1	mitofusin 1

MFN2	mitofusin 2
mg	milligram
ml	milliliter
mmol	millimolar
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial DNA
MURF1	muscle RING-finger protein-1
MYF5	myogenic factor 5
MyHC	myosin heavy chain
MYOD	myogenic differentiation 1
N_2	liquid nitrogen
NaCL	sodium chloride
NADH	nicotinamide adenine dinucleotide
NaH_2PO_4	sodium phosphate monobasic monohydrate
ng	nanogram
nm	nanometers
nmol	nanomolar
NNT	nicotinamide nucleotide transhydrogenase
NO	nitric oxide
O_2	oxygen
OCR	oxygen consumption rate
OE	over-expression
OPA1	optic atrophy type 1
P/O	phosphate/oxygen

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCr	phosphocreatine
PGC-1α	peroxisome proliferator-activated receptor gamma co-activator 1-alpha
PI3K	phosphoinositide-3-kinase
PINK1	PTEN-induced putative kinase 1
PKC	protein kinase c
PKCα	protein kinase c alpha
pmol	picomolar
PPAR-δ	peroxisome proliferator-activated receptor delta
PPAR-γ	peroxisome proliferator-activated receptor gamma
PS	penicillin-streptomycin
PTH	parathyroid hormone
puromycin	puromycin dihydrochloride
RCTs	randomised control trials
RDA	recommended daily allowance
RIA	radioimmunoassay
RIME	rapid immunoprecipitation mass spectrometry of endogenous proteins
ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction
RXR	retinoid x receptor
RXRα	retinoid x receptor alpha
SD	standard deviation
SDH	succinate dehydrogenase

SDS	sodium dodecyl sulfate
shRNA	short hairpin ribonucleic acid
siRNA	silencing ribonucleic acid
SOCE	calcium store-operated entry
SPF	sun protection factor
TBS-T	tris-buffered saline with tween
TCA	tricarboxylic acid
TEM	transmission electron microscopy
TFAM	transcription factor A, mitochondrial
TMRE	tetramethylrhodamine ethyl ester
TUG	timed up and go
UCP1	uncoupling protein 1
UVB	ultra violet B
VDCC	L-type voltage dependent calcium channel
VDR	vitamin D receptor
VDRE	vitamin D response element
VEGF	vascular endothelial growth factor
$\dot{V}O_2$	oxygen uptake
μg	microgram
μM	micromolar

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CHAPTER 1

REVIEW OF LITERATURE

1.1 General Introduction

Skeletal muscle is composed of cells from multiple tissues. Therefore, in this context skeletal muscle may be considered an organ. Skeletal muscle comprises ~40% of total body mass making it the largest organ within the human body [1]. The primary functions of skeletal muscle are to produce movement, maintain posture, store and move substances within the body, and generate heat. Not only does skeletal muscle account for ~30% of the resting metabolic rate [1], it is also the predominant site of glycogen storage and insulin stimulated glucose disposal [2, 3], highlighting its critical role in the maintenance of metabolic homeostasis. One of the defining features of skeletal muscle is a remarkable adaptive response (i.e. plasticity) in response to both positive and negative stimuli. For example, significant increases in skeletal muscle mass are observed during postnatal development via the process of hypertrophy [4, 5]. This process can also be induced in adult skeletal muscle via contractile activity and mechanical overload in the form of strength training [6]. Whilst skeletal muscle is able to undergo distinct periods of growth, it is also susceptible to periods of atrophy such as during the age related loss of muscle mass termed sarcopenia [7]. Given that physical inactivity is a major risk factor in the development of sarcopenia, exercise training is currently the most effective preventative treatment [8, 9]. Whilst regular physical activity plays an important role in maintaining skeletal muscle health, nutritional status is also known to play a key role. The manipulation of nutrient intake particularly surrounding acute bouts of physical activity has been studied extensively [10-13]. These interventions are designed to augment the post exercise signalling response and therefore increase the subsequent training stimulus. Whilst these interventions are designed to enhance skeletal muscle exercise adaptation, in

contrast, many individuals who suffer from poor nutritional status and dietary deficiencies may display impaired physical function and adaptation to exercise.

A common nutritional deficiency is that of vitamin D [14]. Vitamin D belongs to a group of fat-soluble seco-steroids and is primarily obtained via the exposure of the skin to sunlight [15]. Given the increase in time spent indoors, excessive use of sun screen and the reports of an increased risk in skin cancer following excessive sun exposure, it is unsurprising that the prevalence of vitamin D deficiency is still widespread [16]. Deficiencies are not only reserved to specific high risks groups, but are also evident within athletic populations [17]. Given this, the characterisation of these deficiencies and the effect that they have upon skeletal muscle health is an important area of research. A greater understanding on the impact of these deficiencies is needed in order to best optimise skeletal muscle form and function throughout lifespan to promote a greater quality of life and prevent disease.

1.2 Introduction to Skeletal Muscle

1.2.1 Skeletal Muscle Biology and Metabolism

Skeletal muscle accounts for 40% of total body mass in mammals and accounts for 30% of the resting metabolic rate in humans [1]. Playing a critical role in metabolic homeostasis and glycaemic control, skeletal muscle is the predominant site of glucose disposal under insulin-stimulated conditions [3]. In addition, skeletal muscle is the primary site of glycogen storage within the body containing approximately 80% of the total stores [18].

Mammalian skeletal muscle fibres are comprised of multiple different fibre types which are delineated by their individual biochemical properties [5]. Based upon the contractile property of “time-to-peak tension”, skeletal muscle fibres may be defined as either slow- or fast-twitch fibres [5]. There are four different subgroups of fibres, which can be classified based upon the expression of myosin heavy chain (MyHC) I, IIa, IIb, and IIx although, MyHC IIb is not detectable in human skeletal muscle [19, 20]. Type I fibres classically display slow-twitch characteristics and are red in appearance indicating a higher content of both myoglobin and mitochondria, whereas type IIx and IIb are white in colour and type IIa fibres have a more intermediate colour. Interestingly, a spectrum of fibre types with both pure or hybrid MyHC expression have been identified making the classification of skeletal muscle fibres a complex and debated issue [21-23]. The ability to classify fibres has progressed significantly via the correlation of histochemical and physiological studies of individual motor units, electron microscopy of fast and slow muscle fibres, novel procedures for myosin adenosine triphosphatase (ATPase) histochemistry, and biochemical studies on oxidative and glycolytic enzymes in different muscles [5]. Further approaches involving microarray analyses of single muscle fibres will likely shed more light on the complex issue of skeletal muscle fibre typing [24].

The primary role of skeletal muscle fibres is to generate tension via contraction. Contraction is facilitated by the binding of myosin heads to actin filaments which slide over one another according to the sliding filament theory [25]. This process begins with the myosin head binding to adenosine triphosphate (ATP). The enzyme myosin ATPase then hydrolyses ATP into adenosine diphosphate (ADP) and a phosphate

group. This results in the myosin head becoming energized which subsequently attaches to the binding site of the actin filament to form crossbridges. As a result of this binding, the phosphate group is then released. The myosin head is then able to tilt and rotate in a movement known as the power stroke. This rotation allows the myosin head to generate force and pull the actin filament towards the M-line. Following the power stroke, myosin then releases ADP and myosin remains bound to the actin filament until the next supply of ATP binds to myosin. Once ATP is bound, myosin is then released and the contraction cycle can be repeated when ATPase hydrolyses ATP. It is important to note that in order for these events to occur there needs to be a continual supply of ATP and calcium ($Ca2^{+}$) [26].

In response to exercise induced contraction, skeletal muscle is able to rapidly modulate energy production, blood flow and substrate utilisation. Indeed, during maximal exercise, whole-body metabolic rate can increase up to 20-fold and ATP turnover within skeletal muscle can increase 100-fold than that of at rest [27]. Given that intramuscular concentrations of ATP are low (20-25 nmol/kg/dry weight), alternative metabolic pathways need to be activated in order to maintain this rapid turnover of ATP [28]. The three mains sources of ATP regeneration are phosphocreatine (PCr), anaerobic glycolysis and oxidative metabolism [27]. In order to meet this increase in ATP demand, skeletal muscle contains a large pool of mitochondria and is reliant upon oxidative phosphorylation for energy production during prolonged exercise. During both submaximal and maximal exercise, it has been estimated that tricarboxylic acid (TCA) cycle flux increases up to 70- and 100-fold respectively [29]. Therefore, skeletal muscle is the primary site for the

metabolism of both carbohydrates and lipids for energy production. Substrates for oxidation can be provided from extra-muscular sources such as plasma free fatty acids or plasma glucose or intra-muscular sources such as triglycerides or glycogen [30]. The relative contribution of these is dependent on exercise intensity and duration, as well as training status and preceding dietary intake [31, 32].

1.2.2 Skeletal Muscle Plasticity in Response to Endurance Exercise

Regular physical activity in the form of endurance training results in a number of profound physiological and metabolic adaptations in a multitude of populations [33-35]. The adaptations serve to reduce the degree of perturbations to homeostasis for a given exercise intensity and therefore reduce the onset of fatigue. Functionally, these adaptations result in an increase in maximal oxygen uptake ($\dot{V}O_{2max}$) as well as rightward shift in lactate threshold [36, 37]. Whilst improvements in $\dot{V}O_{2max}$ are largely governed by adaptations to the cardiovascular system [38, 39], exercise capacity at submaximal workloads is more closely related to adaptations within skeletal muscle [40]. Alterations in substrate metabolism including a reduction in glycogen utilisation [41], a shift towards greater lipid oxidation [42] as well a reduction in glycolysis [43] are all observed with endurance exercise training. These adaptations allow for a greater absolute exercise intensity to be supported predominantly by aerobic energy production and therefore a reduction in the accumulation of lactate in both the blood and muscle [28, 44, 45].

Regular endurance training also results in a shift towards a more oxidative and fatigue-resistant phenotype in skeletal muscle [46, 47]. In humans, an increase in

both type I and IIa skeletal muscle fibres are observed in endurance-trained individuals, whereas type IIa and IIx are increased in sedentary individuals [46, 48, 49]. Hypertrophy of both type I and IIa fibres is also apparent, as well as an altered expression of MyHC isoforms to a slower phenotype [47, 50]. Given the close relationship between muscle fibre composition and the oxygen cost of locomotion, this alteration in the phenotype of skeletal muscle is of physiological importance with improved metabolic health observed across multiple populations [51-53].

Another hallmark of skeletal muscle remodelling following endurance exercise is the increase in capillary density [47, 54]. Otherwise known as angiogenesis, the formation of new blood vessels within skeletal muscle is stimulated by mechanical factors such as increased shear stress and the elevated tissue strain following repeated contractions. Increased capillarization within skeletal muscle been observed to range from 10 to 25% following 4-24 weeks of endurance training [55-58]. Both exercise duration and intensity seem to have minimal influence over exercise induced angiogenesis given that studies have observed similar increases in capillarization following both moderate and high-intensity endurance exercise [58-60]. Whilst blood flow to skeletal muscle is low at rest, during exercise, these rates can increase up to 100-fold in order to meet the increase in oxygen demand [61]. It is this large increase in blood flow that induces a significant amount of shear stress within the capillary bed and therefore promotes angiogenesis [62]. Several vasodilators are also known to induce angiogenesis [63], with the most characterised being nitric oxide (NO), which simultaneously induces vasodilation and enhances the expression of vascular endothelial growth factor (VEGF) [64]. VEGF is known to be a key

mediator or exercise induced angiogenesis with skeletal muscle and is essential for the development and maintenance of a normal muscle capillary bed [65].

Finally, aerobic exercise also promotes a large increase in mitochondrial mass, enzyme activity and oxidation efficiency [66-68]. The mitochondria undergo a large remodelling process involving coordinated events of mitochondrial biogenesis, fission, fusion and mitophagy [66, 69-73]. All of which contribute to the development of trained skeletal muscle phenotype. These processes will be discussed in more detail below (Section 1.3.2).

1.3 Introduction to Mitochondria

1.3.1 Mitochondrial Biology and Metabolism

Approximately two billion years ago, mitochondria arose from the engulfment of an α-proteobacterium by a precursor of the modern eukaryotic cell [74]. Across this time frame, the mitochondria retained its double membrane structure along with its core function of ATP production [75, 76]. This double membrane structure results in four distinct compartments within the organelle. These compartments are known as the inner membrane, the outer membrane, the intermembrane space, and the matrix [77]. The classical 'textbook' view which displays the mitochondria as a bean like structure, with the outer membrane encasing the highly folded inner membrane, has recently been challenged. These images were largely based upon two-dimensional electron micrographs however, since the introduction of three-dimensional reconstitution technologies, a more detailed picture of mitochondrial morphology has

been developed [78]. It is now accepted that the mitochondria exist within a tubular reticulum that is able to respond to the dynamic energy demands of the cell [79].

Despite the majority of the genetic material being lost to the nuclear genome following endosymbiosis, the mitochondria still possesses its own genetic material [80]. The human mitochondrial genome contains the genetic coding information for 13 proteins [81]. These proteins are core constituents of the mitochondrial electron transport chain, which are embedded within the inner membrane. These proteins, also known as complexes I to IV function together with the Krebs cycle to create an electrochemical gradient through the coupled transfer of electrons across the respiratory chain to oxygen (O_2) and the pumping of protons from the mitochondrial matrix across the inner membrane and into the intermembrane space [82]. This electrochemical gradient powers complex V, which is also known as ATP synthase. ATP synthase is an ancient rotary turbine that catalyses the synthesis of cellular ATP [82]. Commonly referred to as “the powerhouse of the cell”, the mitochondria are the engines that primarily function to produce both ATP and carbon dioxide (CO_2) at the expense of substrates and molecular O_2.

Within skeletal muscle ATP is not stored in great quantities (20-25 nmol/kg/dry weight) and during skeletal muscle contraction or a heightened metabolic demand, ATP demand can increase up to 100-fold [27]. Therefore, this results in a rapid and sustained demand for the production of ATP. In order to meet this requirement, skeletal muscle is densely populated with mitochondria, which produce ATP via a process known as oxidative phosphorylation. This process takes place in the

mitochondrial inner membrane where the individual complexes that make up the electron transport chain reside [83]. The energy conserving complexes known as complexes I, III and IV are protein-lipid enzyme complexes that function to pump protons across the inner mitochondrial membrane [84, 85]. Whilst complex II is also an important member of the electron transport chain, it is mechanistically incapable of pumping protons [86]. Under steady state conditions the total extrusion and re-entry of protons across the inner mitochondrial membrane is balanced. However, during active synthesis of ATP, the primary pathway for proton re-entry is that of ATP synthase. In brief, the TCA cycle intermediates nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH) donate electrons to the individual complexes [82, 87]. Hydrogen ions (H^+) are then extruded into the intermembrane space by proton pumping complexes. This results in the intermembrane space becoming positively charged in comparison to the mitochondrial matrix. As a result of this difference, H^+ ions diffuse through the ATP synthase enzyme, which drives the conversion of ADP to ATP [87].

Whilst the primary function of the mitochondria is the bioenergetic production of ATP, mitochondria are also involved in a number of other processes within the cell including apoptosis, the generation of reactive oxygen species (ROS) and $Ca2^+$ handling [88-90].

1.3.2 Mitochondrial Plasticity in Response to Endurance Exercise

Mitochondrial biogenesis is a well-characterised response to increased contractile activity in skeletal muscle [66, 69]. During this process, mitochondria not only

increase in number and volume, but also undergo coordinated events of fission and fusion, increases in respiratory capacity and alterations in substrate utilisation [66, 69, 70]. The magnitude of these changes are dependent on both training volume and intensity [91], whilst they are also reversible following periods of de-training [92, 93]. These processes are highly dynamic in their nature with half-lives of approximately one week having been observed for mitochondrial proteins [94, 95].

One of the key characteristics of mitochondrial biogenesis is an increase in mitochondrial mass, with regular contractile activity increasing skeletal muscle mitochondrial density by up to as much as 100% [96]. Transmission electron microscopy (TEM) is currently regarded as the gold standard technique to assess mitochondrial volume density [97], although cardiolipin content and citrate synthase (CS) activity are also viewed as valid biomarkers [98]. Studies utilising TEM have provided evidence of an increase in both the size and number of the mitochondria in response to exercise in both rats [99] and humans [100-105]. Similarly, studies employing CS activity as a surrogate marker of mitochondrial mass have reported similar findings [68, 106, 107]. Increases in the size and number of the mitochondria translate to increases in protein content of the mitochondrial fraction within skeletal muscle including key components of the electron transport chain such as cytochrome c (cyt c) [66, 70, 108]. In concordance with these changes, increases in the activity of TCA cycle enzymes CS, NAD-specific isocitrate dehydrogenase, succinate dehydrogenase (SDH) and malate dehydrogenase all increase in rat skeletal muscle following daily running exercise [70]. Enzymes within the electron transport chain

show similar responses with SDH, NADH dehydrogenase, NADH-cyt c-reductase and cyt c-oxidase all increasing up to two-fold [47].

Within skeletal muscle, the mitochondria exist within a morphologically tubular reticulum that is dynamic in its nature [79]. Due to the large size of skeletal muscle fibres, the existence of a dynamic mitochondrial reticulum may be beneficial in instances where the demand for both energy and substrates is not homogeneously distributed across the fibre [79]. Alterations within the shape and size of this reticulum are likely regulated by metabolite transport, increases in energy demand and oxygen diffusion [109, 110]. Indeed, in response to endurance exercise training, it was noted that the commonly observed increases in mitochondrial protein content are due to the proliferation of the mitochondrial reticulum [71]. Not only does this reticulum fuse and proliferate, but it also undergoes events of fission where potentially damaged or dysfunctional regions of the mitochondria are segregated and subsequently broken down [110]. These events of both fusion and fission are simultaneously regulated in order to maintain mitochondrial homeostasis and emerging evidence suggests that they are key to the mitochondrial remodelling response to endurance exercise [72, 73, 111, 112]. Several proteins are known to facilitate the processes of fusion and fission including; mitofusins 1 and 2 (MFN1 and MFN2), optic atrophy type 1 (OPA1), dynamin-related protein 1 (DRP1), fission 1 (FIS1) and mitochondrial fission factor (MFF). Whilst the precise roles of the aforementioned fusion/fission proteins across the time course of exercise adaptation are still to be elucidated, a number of studies have sought to characterise the mitochondrial remodelling process in response to chronic endurance exercise [112-116]. Current evidence points towards a shift

towards an increase in mitochondrial fusion in response to exercise or chronic contractile activity. For example, 12-weeks of aerobic exercise results in an increase in the protein expression of both MFN1 and 2, both of which are known to be involved in the fusion of outer mitochondrial membrane [113]. Additionally, increases in the expression of OPA1 have been observed when utilising electrical stimulation as a means to induce contractile activity in both rats and humans [112, 114]. OPA1 is known to modulate fusion of the inner mitochondrial membrane and regulate cristae morphology [117]. In concert with its increased expression in both rat and human skeletal muscle following chronic contractile activity, the mitochondria were observed to be larger and more reticular in their morphology [112, 114]. Whilst others have reported similar increases in pro-fusion proteins, they have also observed increases in mitochondrial fission related proteins in response to exercise training [115, 116]. Following 8-weeks of swimming exercise, an increase in Drp1 was observed as well as Mfn2 and Opa1 in mouse skeletal muscle [115]. Similar responses were observed following seven high-intensity interval training sessions with increases in the protein content of both FIS1 and DRP1, as well as MFN1 [116]. Although Drp1 is a known mediator of mitochondrial fission, an increase in protein content alone is unlikely to result in an increase in mitochondrial fission. Either mitochondrial translocation or post translational modifications such as phosphorylation are a more accurate readout of Drp1 activity [118]. For example, recent evidence suggests that the phosphorylation of Drp1 at serine 637 is increased in mouse skeletal muscle following chronic endurance exercise training [111]. A modification that has been proposed to reduce mitochondrial Drp1 translocation and mitochondrial fission [119]. Interestingly, the action of Drp1 has also been linked to mitochondrial fusion events.

Mitochondria have been reported to undergo events of both complete and transient fusion, with transient fusion events dependent on Drp1 action [111, 120]. Whilst these data indicate a pro-fusion phenotype in response to exercise, it was recently observed that the previously reported increases in pro-fusion proteins such as MFN1, MFN2 and OPA1 in response to exercise are reduced when normalised for mitochondrial protein content [72]. However, a reduction in FIS1 was independent of changes in mitochondrial protein content [72]. Although the data points towards a pro-fusion phenotype, it is difficult to interpret such studies given the dynamic nature of these mitochondrial remodelling process. Static measurements in response to exercise training only provide a snapshot of the whole story and clearly more evidence and better models to study these processes are needed for future investigations.

As mentioned previously, sections of the mitochondrial reticulum may be segregated via fission related processes and subsequently broken down by the catabolic process of autophagy [110, 115]. The selective removal of dysfunctional mitochondria is commonly referred to as mitophagy, highlighting the specific and non-random nature of this process [121]. Events of mitophagy may be upregulated in response to increased mitochondrial fragmentation, loss of mitochondrial membrane potential and increased mitochondrial ROS emission [122-124]. Currently, the most established pathway for the process of mitophagy involves PTEN-induced putative kinase 1 (PINK1) and the ubiquitin E3 ligase Parkin [125]. Under normal conditions, PINK1 is continuously degraded following translocation to the inner mitochondrial membrane [126]. However, following the depolarization of the mitochondrial membrane, PINK1

translocation is impaired, which results in its stabilization on the outer mitochondrial membrane [127]. Under these conditions, PINK1 undergoes autophosphorylation on serine residues 228 and 402, which serve to recruit and activate Parkin [128, 129]. Both Parkin and Ubiquitin are phosphorylated by PINK-1 allowing for the accretion of polyubiquitin chains on the outer mitochondrial membrane, tagging the organelle for degradation [130-132]. Polyubiquitination results in the binding of the autophagy related protein p62 which can associate with lipidated microtubule-associated protein-light chain 3 (LC3II) [133, 134]. LC3II is located on the phagophore membrane and its binding with p62 results in the autophagosomal engulfment of the mitochondria [133]. Finally, this autophagosome is completely degraded at the lysosome [133, 135]. It should be noted that whilst these pathways are well established in lower organisms, the precise mechanisms of mitophagy within skeletal muscle are still to be elucidated. The role of mitophagy in the exercise-induced mitochondrial remodelling process is an ongoing area of research with the roles of both PINK1 and Parkin becoming the focus of attention. For example, it has previously been reported that in response to a period of chronic swimming exercise, the protein content of both Pink1 and Parkin remained unchanged [115]. However, protein content alone is unlikely to be an accurate readout for mitophagy given the activity of said proteins is reliant on localisation to outer mitochondrial membrane and phosphorylation status. Interestingly, both phospho-PINK1^{Thr257} and Parkin^{Ser65} were elevated in trained human skeletal muscle when compared with sedentary individuals [136]. Additionally, following 6-weeks of voluntary wheel running, an increase in the localisation of Parkin to the outer mitochondrial membrane was observed in mouse skeletal muscle [137]. Whilst these data potentially highlight an increase in basal

mitophagy in trained skeletal muscle, it should be noted that further downstream markers of increased autophagy such as p62 or LC3II mitochondrial localisation were not apparent in trained mouse skeletal muscle [137]. In addition to the established mechanisms of PINK1 and Parkin, the beta-cell lymphoma 2 (BCL-2) related protein BCL-2 interacting protein 3 (BNIP3) has been reported to increase in both mouse and rat skeletal muscle following chronic endurance exercise [138, 139]. Due to its localisation within the mitochondria and its ability to interact with LC3, BNIP3 can be defined as a mitophagy receptor [140]. Primarily involved in hypoxia induced mitophagy, BNIP3 is transcriptionally regulated by hypoxia-inducible factor 1-alpha (HIF1α) [141] and phosphorylation promotes binding with LC3 therefore facilitating mitophagy [142]. Again, the precise mechanisms of BNIP3 mediated mitophagy within skeletal muscle are unknown and it remains to be seen whether the reported increases in BNIP3 result in an increase mitophagic activity. Clearly more research is needed to fully understand how mitophagy contributes to the exercise induced mitochondrial remodelling process. More sophisticated models to assess mitophagy such as the *mito*-Quality Control and MitoTimer mouse will aid in the development of our understanding of said processes [143, 144]. These models both utilise fluorescent probes that are sensitive to either changes in pH or oxidation allowing for the assessment of mitophagy and mitochondrial morphology in a tissue-specific manner [143, 144].

As discussed previously, the core function of the mitochondria is to generate ATP at the expense of substrates and molecular O_2. The respiratory function of the mitochondria can be assessed by measuring the oxygen consumption rate within

isolated mitochondria [145]. In response to exercise training, it is well established that the capacity to generate ATP is increased within human skeletal muscle mitochondria [93, 146]. Whilst O_2 consumption is commonly assessed in isolated mitochondria, its measurement in permeabilised muscle fibres is currently considered the gold standard [147]. Cross-sectional studies have shown that training status is associated with an increase in mass-specific mitochondrial respiration [148-151] and it was Holloszy that first demonstrated an increase in O_2 consumption in response to exercise training in isolated mitochondria from rat skeletal muscle [66]. Mitochondria from the trained cohort exhibited a high level of respiratory control and tightly coupled oxidative phosphorylation, indicating that the increase in the capacity of the electron transport chain was associated with an increase in the capacity to generate ATP [66]. Findings which were later confirmed in mitochondria isolated from guinea pig [152] and human skeletal muscle [153]. More recently, utilising protocols to assess mitochondrial respiration in permeabilised muscle fibres, it has been reported that exercise training increases human skeletal muscle mitochondrial respiratory capacity in an exercise intensity dependent manner [154-156]. Training volume on the other hand, is not a primary determinant of training-induced changes in mitochondrial respiration within human skeletal muscle [91, 157]. Differences in the ensuing adaptations following exercise training may result from differences in fibre type recruitment. Exercise undertaken at higher relative exercise intensities may result in an increase in the recruitment of type II fibres [158, 159]. Additionally, key signalling cascades that are known to be key drivers of training-induced adaptations are also known to be differentially regulated by exercise intensity with higher exercise intensities resulting in an increased signalling response [160].

Alongside gross changes in mitochondrial respiration, the sensitivity of oxidative metabolism to ADP is also altered in response to endurance exercise training. Initially postulated by Holloszy [69], this model suggests that less ADP would be required to stimulate a given rate of aerobic ATP production [69]. Typically, during exercise, free ADP concentrations rise, however, following exercise training this increase is attenuated highlighting tighter metabolic control [161, 162]. More recently, the development of a protocol utilising high-resolution respirometry to determine biologically relevant ADP Michaelis-Menten constant (K_m) values [163] and thereby assess ADP sensitivity across multiple ADP concentrations within permeabilised muscle fibres has yielded ambiguous results [150, 164, 165]. Indeed, cross sectional analyses of healthy humans across differing physical activity levels indicates a decrease in ADP sensitivity in athletic individuals [150]. Furthermore, the sensitivity of the mitochondria to ADP as measured by the apparent K_m for ADP was three-fold higher when comparing athletic to sedentary subjects [150]. Whilst similar findings have been reported in rat skeletal muscle following exercise training [165] and indirectly suggested by others [166, 167], a decrease in the sensitivity of skeletal muscle mitochondria to ADP following exercise training seems surprising. A possible means to explain this paradox involves the control of cellular respiration within different skeletal muscle fibre types. For example, the apparent K_m for ADP has been reported to be up to 30-fold higher in rat and mouse skinned fibres from the soleus when compared to the gastrocnemius [168, 169]. Additionally, whilst the sensitivity of ADP is decreased in athletic individuals and following exercise training, absolute rates of respiration are higher following training likely due to an increased

mitochondrial volume which may be more relevant to whole-body physiology [170]. Finally, given that the rates of absolute respiration are increased, an increased sensitivity to ADP and therefore lower apparent K_m in trained individuals would result in resting mitochondrial respiration of between 25-65% that of maximal [171]. Therefore, the observed increase in apparent K_m in this instance may contribute to the fine tuning of mitochondrial respiration in order to better meet the local energy demands of skeletal muscle [150]. The attenuation of mitochondrial ADP sensitivity following training is depicted below (Figure 1.1) [172].

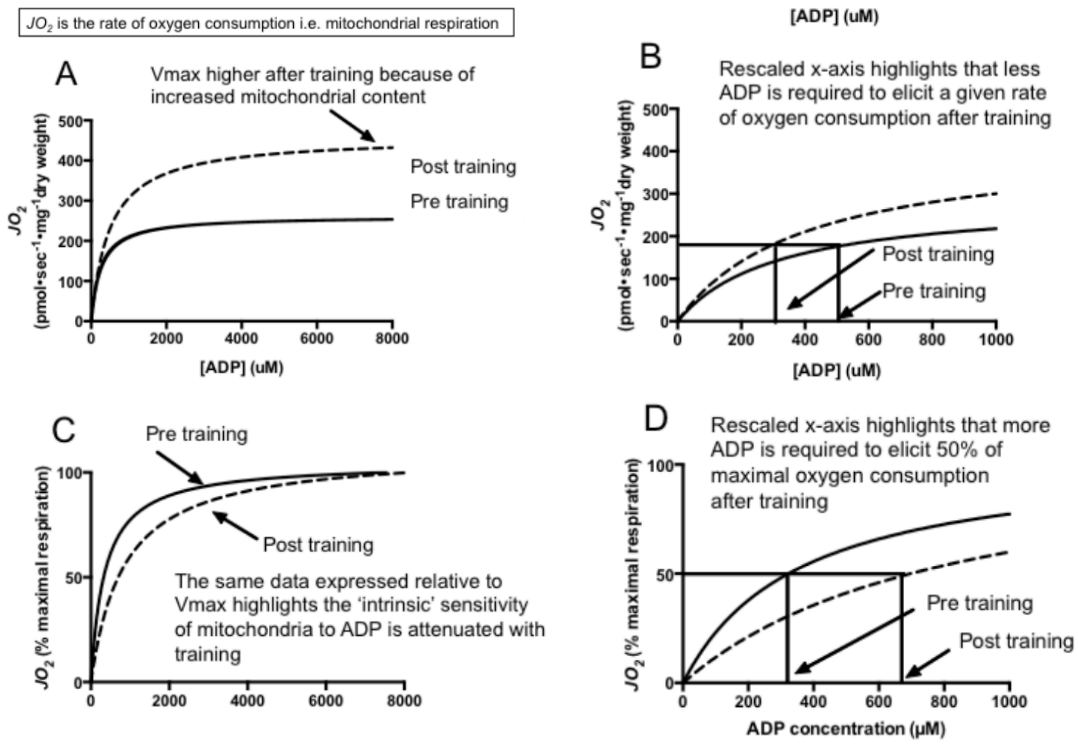

Figure 1.1. ADP respiratory kinetics in permeabilised skeletal muscle fibres in the presence of saturating concentrations of pyruvate plus malate pre and post exercise training. A) Mitochondrial respiration normalised to dry weight both pre and post training intervention. B) Mitochondrial respiration normalised to dry weight with rescaled axis highlighting that less ADP is required to elicit a given rate of oxygen consumption post training. C) The same data now expressed as percentage of maximum. D) The same data expressed as a percentage of maximum highlighting that a higher concentration of ADP is required to achieve 50% of maximal respiration. These data suggest that the intrinsic sensitivity of the mitochondria is actually decreased with training. Holloway (2017).

1.3.3 Mitochondrial Plasticity in Response to Nutritional Interventions

Primarily utilised in an additive fashion to exercise training, a number or nutritional strategies and nutrient compounds have been proposed to enhance mitochondrial function and remodelling within skeletal muscle [173]. Whilst many compounds have shown promise *in vitro* [173], this section will focus on nutritional compounds that elicit mitochondrial alterations within skeletal muscle *in vivo*.

Compounds that could potentially mimic the benefits of exercise in the absence of a training stimulus (i.e. exercise mimetics) were proposed and gained widespread attention in the media [174]. The most heralded of these approaches was the administration of the peroxisome proliferator-activated receptor delta (PPAR-δ) agonist GW501516 in combination with 4-weeks of endurance exercise resulted in a robust increase in running time (68%) and distance (70%) in mice [174]. Following this observation, the authors then substituted exercise for the administration of the 5' adenosine monophosphate-activated protein kinase (AMPK) agonist 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) [174]. The combined compound administration resulted in increase in running time (23%) and distance (44%) and the proposal that the compound combination was able to act as an exercise mimetic and enhance skeletal muscle metabolism [174]. Unfortunately, the authors did not compare the combined treatment of GW501516 and AICAR to exercise alone or in combination with exercise. Additionally, the treatment of GW501516 alone was unable to increase exercise capacity, suggesting it is not an exercise mimetic and more likely an exercise enhancer [174]. Garnering widespread attention, the feasibility of exercise mimetics has since been brought into question

[175-177]. Since the early promise, the work has failed to translate into human studies primarily due to the poor bioavailability of AICAR [178, 179]. Furthermore, the chronic activation of PPAR-δ has been linked to cancer progression, raising questions in regards to its efficacy as a proposed long-term treatment [180]. Given the widespread, multi-organ health benefits of exercise [181], an exercise mimetic targeting a single protein is unlikely to be feasible [175, 176]. More recently however, the use of bioactive compounds (vitamins or polyphenols) seems to hold promise [172, 173, 182].

One of the most well-known polyphenols is resveratrol which is found in grapes and was first isolated from red wine [183]. Resveratrol has been shown to aid in mitochondrial remodelling in mice fed a high-fat diet [184]. Indeed, mice fed a high-fat diet for 15-weeks with the addition of resveratrol show signs of increased mitochondrial volume, evidenced by increased mitochondrial size, mitochondrial DNA (mtDNA) content, SDH and CS activity [184]. Further to this, resveratrol was also shown to increase endurance capacity in mice and promote fat oxidation [185]. In contrast however, these promising reports in rodents have failed to translate to human studies and resveratrol supplementation has led to maladaptive responses to exercise training [186, 187].

Another polyphenol with a similar structure to that of resveratrol is quercetin which has been reported to promote numerous health benefits [188]. The supplementation of quercetin in mice across a 7-day period resulted in a doubling of mtDNA content in skeletal muscle as well increases in cyt c [189]. The adaptations were likely mediated

by an upregulation of the transcriptional coactivator peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α) [189]. In concert with the observed increases in mitochondrial content, mice fed quercetin exhibited increases in exercise capacity [188]. Whilst some of these findings have been replicated in human populations, including increased exercise performance and mtDNA content [190], others have shown no effect [191-193].

The flavanol (-)-epicatechin a derivative of cocao has shown early promise, primarily via the consumption of small amounts of dark chocolate which has been linked to multiple health benefits including reductions in the development of cardiovascular disease [194]. In terms of its effects on the mitochondria, Nogueira and colleagues were the first to demonstrate that 15-days of (-)-epicatechin supplementation in mice increased mitochondrial volume in concert with increases in treadmill running performance, fatigue resistance and skeletal muscle capillarity [195]. Interestingly, the supplementation of (-)-epicatechin alone was able to increase skeletal muscle mitochondrial protein content as well skeletal muscle capillarity to a similar extent as the exercise alone group suggesting beneficial effects can be achieved without a training stimulus. Although the combined treatment of exercise and (-)-epicatechin resulted in further additive increases suggesting the combination treatment may be optimal [195]. Further studies examined the role of (-)-epicatechin following 5-weeks of endurance training followed by 2-weeks de-training [196]. The supplementation of (-)-epicatechin across the de-training period was able to maintain the prior training induced increases in cyt c oxidase activity and capillary to fibre ratio [196]. Whilst these data have yet to be repeated in human trials, the supplementation of flavanols

including (-)-epicatechin appears to hold promise for enhancing mitochondrial adaptations to endurance exercise training.

Dietary nitrate is primarily obtained via the consumption of green leafy vegetables or from the consumption of beetroot juice [197]. The supplementation of dietary sodium nitrate over a three-day period resulted in a reduction in the oxygen demand during submaximal workloads in healthy trained individuals [198]. Furthering this work, multiple parameters of mitochondrial respiration were altered following the same supplementation protocol [199]. Nitrate supplementation increased the mitochondrial respiratory control ratio, suggesting an improved coupling between oxidative phosphorylation and ATP production [199]. Furthermore, supplementation also resulted in an increased phosphate/oxygen (P/O) ratio at submaximal concentrations of ADP indicative of altered stoichiometry [199]. Interestingly, beetroot juice, a source of dietary nitrate, also reduces the oxygen cost of exercise at submaximal workloads [200, 201], although this does not seem to be mediated by adaptations to the mitochondria [202].

Omega-3 fatty acids, particularly those enriched with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are known to alter mitochondrial membrane phospholipid composition following supplementation [203, 204]. An increased incorporation of polyunsaturated fatty acids into the mitochondrial membrane resulted in an increase in mitochondrial ADP sensitivity in human skeletal muscle [203]. Maximal ADP stimulated respiration remained unchanged, a finding also observed in rodents [205], suggesting the function of the electron transport chain was not altered

[203]. No changes were also observed in the protein content of adenine nucleotide translocase 1 (ANT1) and 2 (ANT2) or ATP synthase although post-translational modifications of these proteins may mediate the observed increase in ADP sensitivity [203, 206]. More recently, the effects of EPA and DHA supplementation both preceding and across a period of immobilisation have been studied. Whilst 14-days of immobilisation reduced ADP stimulated respiration (-20%), the supplementation of omega-3 fatty acids during the 4-weeks preceding and during the immobilisation period attenuated these reductions [207]. Whilst similar observations were noted for respiration stimulated by submaximal ADP concentrations, the pre control group had increased respiration when compared to the pre supplementation group [207]. Therefore, the fact that omega-3 supplementation attenuated to reductions in respiration may be a product of reduced respiration in the pre-supplementation controls. Whilst interesting, the effects of omega-3 supplementation on mitochondrial adaptation to training are yet to be studied.

Given the wide variety of vitamins and bioactive compounds isolated from various fruits and vegetables there are still many questions to be answered in terms of how these compounds may enhance mitochondrial function either alone or in combination with training. Studies utilising bioactive compounds are often limited by the bioavailability of said compounds and future research should examine the extent to which the compound in question is absorbed and bioavailable to skeletal muscle [182].

1.4 Introduction to Vitamin D

1.4.1 History of Vitamin D

The idea that the adequacy of the diet could be described by the content of carbohydrate, fat, protein and minerals remained largely unchallenged until the beginning of the twentieth century when reports began to emerge suggesting that additional micro-organic nutrients may be required [208, 209].

Towards the end of the nineteenth century the industrial revolution had taken place which resulted in the urbanisation of the population. The resulting smoke from industrial plants polluted the atmosphere, and in low-sunlight countries such as England, rickets appeared in epidemic proportions [210]. Rickets is a condition by which the skeleton becomes poorly mineralised and deformed often resulting in pain and growth abnormalities [211]. The previous discovery of vitamins led to the reasoning that rickets may also be caused by some form of dietary deficiency [212]. Early investigations utilised cod liver oil to cure rickets with the vitamin A contained within cod liver oil viewed as the therapeutic. However, despite the removal of vitamin A from cod liver oil via aeration and heating, cod liver oil still retained the ability to cure rickets [213] suggesting that cod liver oil contained another active vitamin. Once characterised, this vitamin later became known as vitamin D [212, 214, 215], now viewed as an essential nutrient [215].

During the same period, it was discovered that rickets could be both prevented and cured in children by exposing them to either sunlight or artificially induced ultraviolet B (UVB) light [216]. Studies in goats also revealed that sunlight exposure was

important for the maintenance of calcium balance due to the fact that those animals that were maintained indoors lost much their calcium to lactation [217]. This work resulted in the discovery that irradiation of the animals' diets could induce vitamin D activity and thus cure rickets [218]. The discovery that UVB light could be utilised to induce vitamin D activity and fortify foods resulted in the elimination of rickets as major medical problem [218, 219]. This work also revealed that vitamin D was not a vitamin and was in fact a steroid hormone [219].

1.4.2 Forms of Vitamin D

Multiple biological forms of vitamin D are known to exist. Vitamin D_2, also known as ergocalciferol was first isolated from the irradiation of plant sterols [214]. Vitamin D_3, which is also known as cholecalciferol was first identified following the isolation of 7-dehydrocholesterol which is the precursor for vitamin D_3 within the skin. The fact that vitamin D_3 is the form that is produced in the skin was later confirmed via the chemical identification of both pre-vitamin D_3 and vitamin D_3 in the skin [220, 221]. Both vitamin D_2 and D_3 are the major sources of vitamin D that contribute to vitamin D status. Both forms are found within dietary sources however, only D_3 is produced endogenously within the skin following the exposure to UVB irradiation [221]. The chemical structure of both vitamin D forms is depicted below with the side chain of D_2 containing a double bond and the addition of a methyl group at carbon 24 (Figure 1.2).

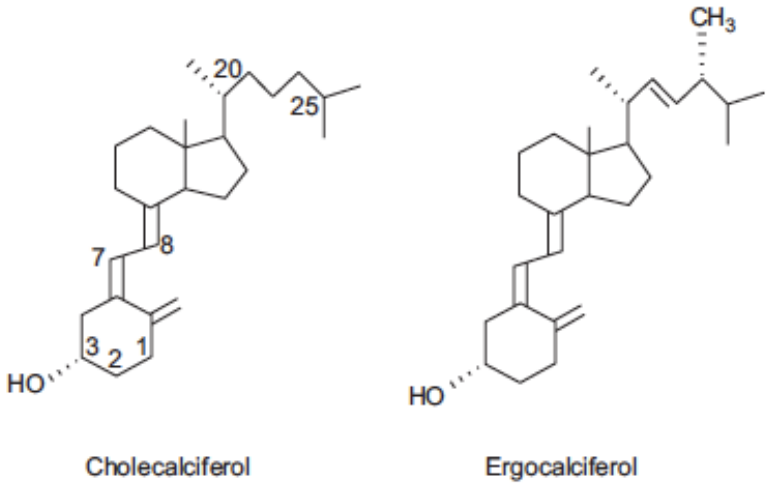

Figure 1.2. Nutritional forms of vitamin D. Vitamin D_3 also known as cholecalciferol and vitamin D_2 also known as ergocalciferol [222].

1.4.3 Synthesis of Vitamin D

Upon exposure to sunlight and more specifically UVB irradiation at a wavelength of 290-315 nm [223], human skin is able to synthesise vitamin D [224]. The photochemical process that takes place in the plasma membrane of skin cells, results in the conversion of 7-dehydrocholesterol (precursor to cholesterol and vitamin D_3) to the thermodynamically unstable pre-vitamin D_3 (pre-cholecalciferol) [224]. It is the thermal isomerisation of pre-vitamin D_3 that results in the formation of vitamin D_3, which is a seco-steroid hormone [225]. Within 8 h of exposure to UVB irradiation, ~80% of pre-vitamin D_3 is converted to vitamin D_3 [226]. When a sufficient quantity of D_3 is produced, pre-vitamin D_3 is converted to the biologically inactive photoproducts of tachysterol and lumisterol [227].

Multiple factors can influence the synthesis of vitamin D. For example, the pigmentation of the skin plays an important role in regulating the dermal synthesis of vitamin D_3 [228]. An increased melanin pigmentation will reduce the efficiency of the

sunlight mediated photosynthesis of pre-vitamin D_3 [228]. The amount of radiation that actually enters the atmosphere and is therefore available for the cutaneous production of vitamin D is also an important factor [229, 230]. This is dependent on the time of the day, latitude, and season which alter the zenith angle of the sun [231]. As well as melanin, any substance that absorbs UVB radiation such as sunblock or clothing will also reduce the cutaneous production of vitamin D_3 [232-234]. The use of sunscreen is widely used and effective method of reducing the damaging effects of chronic exposure to sunlight such as sunburn, skin cancer and skin damage. A sunscreen with a sun protection factor (SPF) of 30 will absorb up to 97.5% of UVB radiation and therefore reduce the production of vitamin D_3 [232].

1.4.4 Dietary Sources of Vitamin D

Nutrients that are not naturally synthesized will need to be obtained via an alternative source and an obvious solution to this is through dietary intake. Although there are few, some dietary sources of vitamin D do exist [235]. Dietary sources of vitamin D include oily fish, powdered milk, eggs and shitake mushrooms, as well as fortified foods such as bread, milks, cheese and breakfast cereals [235]. The current Recommended Daily Allowance (RDA) for vitamin D is 15 μg/day (600 IU) up to the age of 70 years and 20 μg/day (800 IU) thereafter [236]. Investigations into the current dietary intake of vitamin D reveal that the median intake for adults within the USA was 5.8 μg/day when both fortified foods and supplements were included [237]. Adding further difficulty to obtaining a sufficient amount of vitamin D from the diet, it has been reported that some foods stating fortification with vitamin D may contain less than 80% of the vitamin D claimed [231, 238]. Therefore, intake of vitamin D

containing foods is limited and it is unlikely that humans obtain enough vitamin D from dietary sources to compensate for a lack of sunlight exposure [237].

1.4.5 Metabolism of Vitamin D

Whether vitamin D is obtained via the exposure to sunlight or from dietary sources, both forms will enter the circulation bound to the vitamin D binding protein (DBP) [239]. The DBP is present within serum in the micromolar concentrations and is responsible for the transport of all vitamin D metabolites with the highest affinity for 25(OH)D [240, 241].

Once in circulation, vitamin D undergoes a series of hydroxylation steps including; 25-hydroxylation, 1α-hydroxylation, and 24-hydroxylation, which are all performed by cytochrome P450 mixed-function oxidases (CYPs) [242-244]. The first hydroxylation step (25-hydroxylation) occurs within the liver and converts vitamin D to 25(OH)D [245]. A number of CYPs have been established to have 25-hydroxylase activity including both CYP27A1 and CYP2R1 [242]. CYP2R1 is considered the main 25-hydroxylase as this enzyme is able to 25-hydroxylate both vitamin D_2 and D_3 with comparable kinetics where as CYP27A1 does not 25-hydroxylate vitamin D_2 [242, 246].

The second hydroxylation step, 1α-hydroxylation is performed primarily in the kidney and unlike 25-hydroxylation, only one enzyme is recognised to have 1α-hydroxylation activity [243, 244]. This enzyme, known as CYP27B1, is able to convert the 25(OH)D produced within the liver to its more biologically active metabolite 1α,25-

dihydroxyvitamin D (1α,25$(OH)_2D_3$) [243, 244, 247, 248]. Despite the kidney being known as the main site of 1α,25$(OH)_2D_3$ production, a number of other tissues or cell types have been shown to express CYP27B1 including keratinocytes, epithelial cells, pancreatic islets, cerebellum, and skeletal muscle [249-251]. The ability of the cell to bypass the kidney and perform the 1α-hydroxylation step internally will be dependent on the ability of the cell to internalize the 25(OH)D bound to the DBP [252, 253]. Within skeletal muscle, this is likely achieved via the recently identified membrane bound protein Megalin [252] (Figure 1.4).

Lastly, 24-hydroxylation is the final hydroxylation step with CYP24A1 the only established enzyme with 24-hydroxylase activity. This pathway is known to result in the conversion of the biologically active form of vitamin D (1α,25$(OH)_2D_3$) to the inactive calcitroic acid. Similar to CYP27B1, CYP24A1 is predominantly expressed within the kidney however a number of studies have also revealed its expression in other cell types and tissues including skeletal muscle [250, 251].

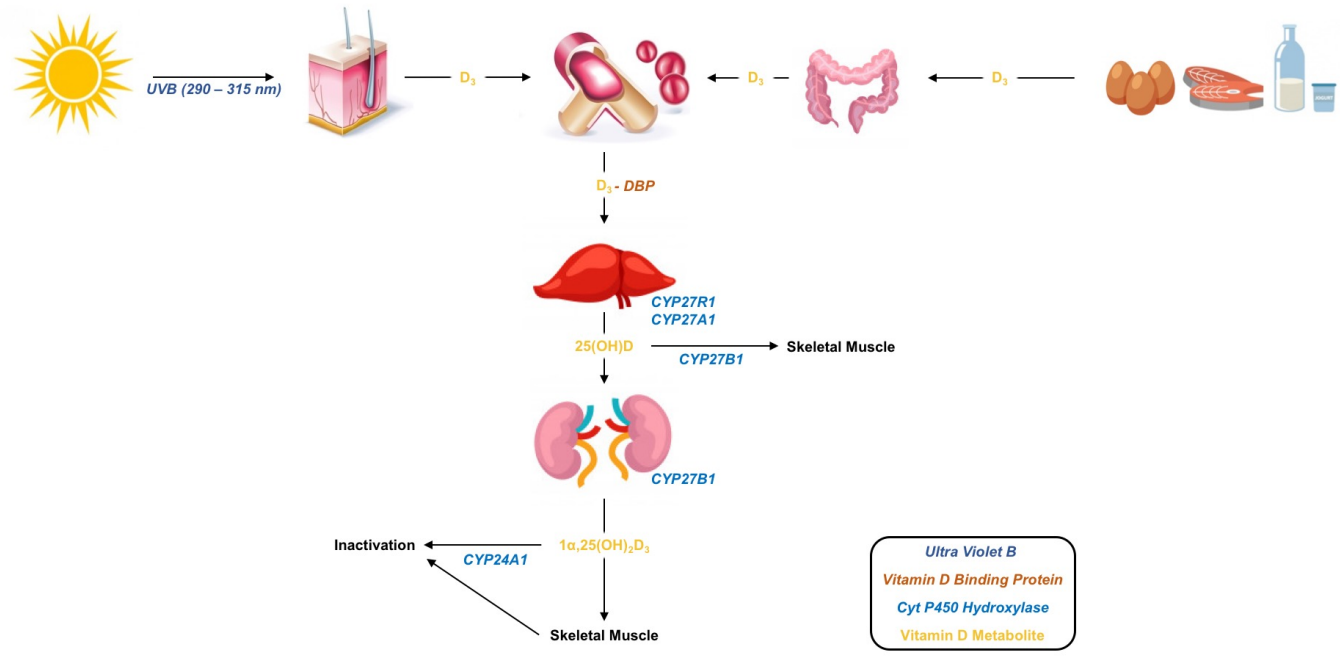

Figure 1.3. Vitamin D Metabolism. Schematic illustrating the metabolism of vitamin D obtained via the exposure of the skin to sunlight or dietary ingestion of vitamin D containing foods. Once in circulation, vitamin D is bound to the vitamin D binding protein. Vitamin D then undergoes two hydroxylation steps, the first in the liver (25-hydroxylation) and the second in the kidney (1α-hydroxylation). Note that some target tissues including skeletal muscle also express CYP27B1 allowing for the local production of 1α,25$(OH)_2D_3$. The final hydroxylation step, 24-hydroxylation, results in the conversion of 1α,25$(OH)_2D_3$ to inactive by products. Vitamin D has known actions in a number of tissues including bone, intestine, kidney, immune cells, tumour micro-environment, adipose tissue and skeletal muscle. Adapted from [254].

1.4.6 Measurement of Vitamin D Status

Over 40 metabolites of vitamin D have been identified [255] making the analysis of vitamin D metabolism challenging. These metabolites are all in circulation in a variety of concentrations and some have an extremely short half-life making them of minimal physiological relevance [256]. Currently, it is the measurement of 25(OH)D which regarded as the best representation of vitamin D exposure [257]. In comparison to its parent sterol which has a half-life of 24 h, 25(OH)D has a half-life of 21-30 days [249, 258]. Whilst $1\alpha,25(OH)_2D_3$ is the most biologically active form of vitamin D, it circulates in the picomolar ($pmol.L^{-1}$) concentrations where as 25(OH)D circulates in nanomolar ($nmol.L^{-1}$) concentrations [259, 260].

Whilst the majority of vitamin D is obtained by UVB exposure, the potential for supplementation and the fortification of foods with both vitamin D_2 and D_3 can increase the amount derived from endogenous sources [261, 262]. Therefore, it is an important consideration to make sure that the assay in question is able to determine the amount of both the D_2 and D_3 metabolites. Various methods have been utilised for the measurement of vitamin D metabolites including radioimmunoassay (RIA), vitamin D-binding protein assays, high performance liquid chromatography coupled with ultra-violet spectrophotometry, and liquid chromatography coupled with mass spectrometry (LC-MS/MS) methods [263]. Given that all RIAs evaluated have displayed a problem with the detection of $25(OH)D_2$ and under-recover this metabolite [264], isotope dilution LC-MS/MS is considered the gold standard for measuring serum total 25(OH)D [265]. Isotope dilution LC-MS/MS is able to quantify both $25(OH)D_2$ and $25(OH)D_3$ simultaneously with the sum of the two values

representing a precise and accurate measure of total 25(OH)D and therefore vitamin D status [265, 266].

1.4.7 Categorization of Vitamin D Status

The current US Institute of Medicine guidelines for vitamin D status are generally accepted worldwide (Table 1.1) with the Endocrine Society providing similar categorization guidelines [236, 267]. Despite these guidelines, there still remains conflicting views on defining vitamin D deficiencies and the concentration of vitamin D that could be deemed optimal [268-270]. It has been suggested that in the optimal serum concentrations of 25(OH)D for optimal bone mineral density, bone turnover and muscle strength should be >75 nmol.L^{-1} [268, 271]. Others have even suggested that concentrations of >100 nmol.L^{-1} should be considered optimal based upon the fact that those in such rich environments close to the equator present with 25(OH)D concentrations within this range [272]. The debate on what constitutes vitamin D deficiency will likely continue, particularly with the development of more sensitive methods to measure an increasing amount of vitamin D metabolites.

Serum 25(OH)D	Classification
<12 nmol.L^{-1}	Severely Deficient
12 – <30 nmol.L-1	Deficient
30 – 50 nmol.L^{-1}	Inadequate
>50 nmol.L^{-1}	Adequate

Table 1.1. US Institute of Medicine (2011) vitamin D concentrations for classifications of vitamin D status [236].

1.4.8 Prevalence of Vitamin D Deficiency

Even in areas with ample sunlight exposure, it has been estimated that around 1 billion people worldwide currently have insufficient serum 25(OH)D levels according to the US Institute of Medicine guidelines [273]. In support of this there are a multitude of large population based trials from across Europe that report a high frequency of individuals with serum concentrations of <50 $nmol.L^{-1}$ [274-276]. Further to this, it has been reported that 13% of individuals in Europe have vitamin D concentrations of <30 $nmol.L^{-1}$, with that figure rising to 40% including those with concentrations <50 $nmol.L^{-1}$ [14]. With similar prevalence reported within the US [277], Australia [278] and even the Middle East [279] it is clear that vitamin D deficiency is a common occurrence globally.

Whilst vitamin D deficiency is common across the general population, certain subgroups are at an increased risk [14, 16, 280]. For example, children with low birth weight, pregnant women and the institutionalised elderly often present with inadequate 25(OH)D concentrations [16, 280]. Given that dermal synthesis of vitamin D decreases with age and the fact that those who are institutionalised will spend less time outdoors due to poor health status, it unsurprising that poor vitamin D status is commonly observed within this subgroup [281]. Whilst vitamin D insufficiency is common place within the general public, it has also been reported that insufficiency is also common within professional athletes [17]. Out of the 61 athletes tested from across four differing sports, 61% had serum 25(OH)D concentrations <50 $nmol.L^{-1}$ with only one from the 61 tested having concentrations >100 $nmol.L^{-1}$ [17].

1.5 Physiological Role of Vitamin D

1.5.1 Physiological Effects of Vitamin D Deficiency

Clearly the reports of a high prevalence of vitamin D insufficiency across multiple populations and subgroups are striking however, they do not report on the physiological implications of such deficiencies. The classical role of vitamin D involves the maintenance of bone health, with severe deficiencies known to cause rickets [211], osteomalacia [282] and osteoporosis [283], as well as an increased risk of fractures [284].

Rickets is a clinical syndrome in children which results from a delay or failure in mineralisation of the growth plate within growing bones [285, 286]. Rickets caused due to nutritional deficiencies is currently the most common form of the syndrome across the globe [285, 287]. Vitamin D deficiency is a prerequisite for the development of nutritional rickets in children, associated with a lack of sunlight exposure and an inadequate intake of vitamin D from the diet [288]. Therefore, adequate skin exposure to sunlight is essential for the prevention of rickets [223]. The prevalence of vitamin D deficiency rickets is most common in children between three months and two years of age [289]. Due to the fact that 25(OH)D readily crosses the placenta, new-born infants may be provided with some protection from vitamin D deficiency providing the mother had adequate serum concentration of 25(OH)D [290]. In addition to the more classical roles of vitamin D, vitamin D deficiency has also been associated with an increased incidence of cardiovascular disease [291], diabetes [292], Alzheimer's disease [293] and chronic kidney disease [294].

1.5.2 Musculoskeletal Effects of Vitamin D Deficiency

Alongside the classical actions of vitamin D, several lines of evidence now support a role for vitamin D in the maintenance of skeletal muscle function. A prominent feature of vitamin D deficiency is proximal muscle weakness [295], whilst deficiency has also been linked to impaired physical performance, diffuse muscle pain, gait impairments and an increased risk of falls [296-299].

Multiple animal models have been utilised to study vitamin D deficiency and its role in musculoskeletal health. Vitamin D deficiency can be achieved via dietary means [300-303], a reduction in sunlight exposure [300] or by the administration of ethane 1-hydroxy-1, 1-diphosphonate which blocks the production of $1\alpha,25(OH)_2D_3$ [303]. Early research into the musculoskeletal effects of vitamin D deficiency revealed symptoms of skeletal muscle myopathy including impaired contraction kinetics and weakness in the skeletal muscle of chicks and rats [300, 301]. Despite the observed impairments in contraction kinetics, no abnormalities were reported in any histochemical measures of skeletal muscle morphology or within ATP concentrations in the skeletal muscle of vitamin D deficient chicks [300]. Similar observations have been made in rat skeletal muscle whereby diet-induced deficiency resulted in a significant decrease in muscle force [304]. Interestingly, prolonged deficiency resulted in dysregulation of both calcium and phosphate homeostasis and when phosphate levels were corrected muscle strength returned to normal [304]. In support of this, the administration of vitamin D_3 to vitamin D deficient chicks resulted in an increase in the incorporation of phosphate into skeletal muscle, suggesting that vitamin D stimulates phosphate fluxes across muscle membranes [305]. Whilst these

data suggest the effects of vitamin D deficiency on skeletal muscle may be indirect, a reduced expression of components of the sarcomere, including actin and the myosin-troponin complex were also observed in the same animals which may explain the reductions in muscle force [303, 305]. More recently, diets containing increased calcium and phosphate have been utilised in order to try to minimise the observed hypocalcemia and hypophosphatemia that are associated with the induction of vitamin D deficiency [302]. Interestingly, when fed this diet, mice have a reduced grip strength as well an increased gene expression of *Myostatin* and *muscle RING-finger protein-1 (Murf1)* [302], two known regulators of skeletal muscle mass [306, 307]. Similar impairments in physical function were observed when mice were fed a vitamin D deficient diet for longer periods (8-12 months) [308]. Whilst mice fed a vitamin D deficient diet displayed reductions in grip endurance, sprint speed and stride length, no changes were observed in absolute grip strength, lean mass or markers of muscle morphology [308].

Observational studies suggest a positive association between serum 25(OH)D levels with muscle strength and lower extremity function in older individuals [296, 309, 310]. Within a cohort of 1234 men and women aged over 65, subjects with serum concentrations of <25 nmol.L^{-1} displayed a reduction in physical capacity (timed walk and chair stands) than those with serum concentrations >75 nmol.L^{-1} [296]. In addition, individuals with a baseline serum concentration of <25 nmol.L^{-1} are reported to have a two-fold greater risk of developing sarcopenia [311]. Studies of this design however, are unable establish a causal link between vitamin D deficiency and the associated impairments in physical performance. Vitamin D deficiency also results in

a dysregulation of calcium and phosphate homeostasis which may result in indirect effects on skeletal muscle function. Furthermore, these data are often reserved to elderly populations making generalisations difficult, particularly given that these cohorts may already suffer from pre-existing conditions that involve high levels of systemic inflammation which can substantially degrade vitamin D status [312].

1.5.3 Musculoskeletal Effects of Vitamin D Supplementation

The effects of vitamin D deficiency upon the musculoskeletal system have been well studied to date [296, 300-302, 309]. Given the potential for impairments in musculoskeletal health with deficiency, many have also aimed to determine the potential health benefits of supplementation across multiple populations. Supplementation of vitamin D is commonly administered in combination with calcium due to the well-established role of vitamin D in maintaining calcium homeostasis [254].

Firstly, multiple observational studies have reported associations between serum 25(OH)D levels and markers of muscle function including, hand grip strength, lower limb strength and gait speed in elderly populations [313-315], although not all have reported such associations [316]. Similar observations have been made in athletic populations with serum 25(OH)D levels positively associating with increased muscle strength, sprint time, hand grip strength [317-320], although again others have failed to show such associations [321, 322]. Whilst the majority of such studies report a benefit of increasing serum 25(OH)D, particularly for those in the lower echelons of serum 25(OH)D, studies of this design are unable to determine causality.

Multiple randomised control trials (RCTs) have sought to establish the effect of vitamin D supplementation on skeletal muscle performance however, the results have been indifferent. The daily combination of vitamin D (800 IU) and calcium (1200 mg) over a 12-week period resulted in improvements in timed up and go (TUG) test, knee flexor strength, knee extensor strength and grip strength in cohort of elderly women [323]. Similar observations were made when daily vitamin D (800 IU) and calcium (1000 mg) supplementation was carried out over a 12-month period with improvements in TUG test and quadriceps strength reported [324]. Improvements in skeletal muscle function in both studies resulted in a reduction in the amount of falls over the study period when compared to calcium supplementation alone [323, 324]. Some improvements in muscle function have also been observed in athletic populations. Footballers randomised to receive daily vitamin D (5000 IU) for a period of 8-weeks recorded improvements in vertical jump height and 10 metre sprint time [17]. Further to this, a single dose of vitamin D (150,000 IU) resulted in improvements in quadriceps and hamstring strength in a cohort of Judoka athletes [325]. On the other hand, multiple RCTs have reported conflicting results. For example, 6-months of vitamin D (400 IU/day) and calcium (500 mg/day) resulted in no improvements in grip or knee strength in a cohort of elderly female patients [326]. Similarly, supplementation of vitamin D every 3-months (150,000 IU) for 9-months resulted in no change in grip strength and did not reduce the number of falls in a cohort of community-dwelling women [327]. Further negative results have also been reported in athletic populations with multiple studies showing no differences in muscle strength, muscle power, sprint time and hand grip strength with daily vitamin D supplementation [328-331].

Meta-analyses combining many of these RCTs have also reported indifferent results on the proposed benefits on vitamin D supplementation [332-336]. A meta-analysis combining six RCTs reported an increase in both lower and upper limb strength following vitamin D supplementation in young healthy individuals [332]. Whilst similar increases in lower limb strength were reported in athletic individuals, vitamin D supplementation did not increase upper limb strength or muscle power when eight RCTs were combined [333]. One meta-analysis containing 30 RCTs with a combined 5615 individuals reported a favourable effect of vitamin D when compared with placebo on skeletal muscle strength [334]. Supplementation was deemed more effective in adults with 25(OH)D concentration of <30 $nmol.L^{-1}$ and in individuals older individuals (>65 years). Whilst another meta-analysis reported similar findings in relation to supplementation being more effective in those with serum concentrations of <30 $nmol.L^{-1}$, they overall reported no effective of vitamin D supplementation muscle strength [335]. Interestingly, vitamin D supplementation was reported to have a positive additive effect when combined with resistance training in increasing lower limb muscle strength in older adults [336], suggesting vitamin D status may influence exercise adaptation.

Clearly, conflicting evidence exists when it comes to the proposed benefits of supplemental vitamin D on musculoskeletal health. Disparity in outcomes may in part be explained by the multitude of supplementation protocols and periods employed throughout previous studies. Whilst some studies have opted for a daily approach (400 to 800 IU) [323, 324], others have utilised large single dose bolus' (300,000 IU) [337] which could result in different outcomes. Indeed, large single dose bolus' have

been shown to have detrimental effects and actually increase the risk of falls and fractures [338-340]. A possible explanation for this involves an increase in the activation of CYP24A1, the hydroxylase responsible for the catabolism of $1\alpha,25(OH)_2D_3$ [338]. Previously, rats given a large bolus of vitamin D (75,000 IU/week) displayed a significant increase in renal CYP24A1 which resulted in a significant reduction (60%) in serum $1\alpha,25(OH)_2D_3$ [341]. Therefore, high bolus' may be counterproductive and actually dysregulate vitamin status in the short-term [338]. On the whole, vitamin D seems to be most effective in elderly populations and even more so in those that in the lower ranges of serum 25(OH)D [334, 335].

1.6 The Vitamin D Receptor

1.6.1 Discovery of the Vitamin D Receptor

The first study to propose that vitamin D acted through a specific binding protein or vitamin D receptor (VDR) was published by Haussler and Norman in 1969 [342]. In this study, the authors reported on the successful isolation of a receptor for the biologically active form of vitamin D from the chromatin fraction of the chicken intestine [342]. Subsequent work built upon the biochemical properties of the receptor with the discovery that the protein primarily resided within the cytoplasm before translocating to the nucleus upon ligand binding [343, 344]. The binding of the VDR with $1\alpha,25(OH)_2D_3$ takes place with a high affinity and high selectivity [345] although, the VDR is able to bind albeit less effectively to other vitamin D metabolites [346]. Finally, the cloning of the VDR in 1987 provided direct evidence that the VDR was indeed a member of the steroid class of nuclear receptors [347-349].

1.6.2 Function of the Vitamin D Receptor

Upon ligand binding and nuclear translocation, the VDR serves to recruit coregulatory complexes that mediate gene regulation at the transcriptional level [350]. The discovery of a number of gene candidates that were activated by $1\alpha,25(OH)_2D_3$ prompted investigations into how vitamin D related signalling could promote their respective regulation [351, 352]. Studies investigating the regulation of genes such as *Osteocalcin* [352] and *Cyp24a1* [351] suggested that the VDR bound to vitamin D response elements (VDREs) within the promoter regions of the respective regulated genes. The binding of the VDR to these VDREs is dependent upon the VDR forming a heterodimer protein complex with the steroid receptor family known as the retinoid X receptor (RXRs) [353]. Upon binding of the VDR:RXR complex to the VDREs, the complex is able to recruit multiple coregulatory complexes of unique functions that serve to remodel chromatin and subsequently influence gene transcription. These complexes remodel chromatin via the repositioning of nucleosomes via ATPase enzyme activity [354], modify lysine or arginine residues of histones 3 and 4 at specific locations via the activity of histone acetyltransferases [355] or bind with complexes that allow for the entry of RNA polymerase II into the transcriptional apparatus and the subsequent activation of transcription [356].

Alongside these classical actions of the VDR, binding of $1\alpha,25(OH)_2D_3$ to the VDR has also been proposed to result in rapid non-genomic signalling [357]. For example, a VDR distinct of the nuclear VDR has been proposed to exists on the cell membrane due to the fact that some vitamin D analogues are able to initiate rapid signalling

events without binding to the nuclear receptor [358]. A specific protein was identified that resided in the basal-lateral membranes of chick intestinal epithelium which was able to bind to 1α,25$(OH)_2D_3$ and regulate Ca^{2+} transport across such membranes [359]. This protein was initially termed the membrane-associated rapid response steroid (MARRS) binding protein however, it was later identified as a multifunctional thioredoxin-like protein known as glucose responsive protein 58 (GRP58) or endoplasmic reticulum protein 57 (ERp57) [360, 361]. Binding of 1α,25$(OH)_2D_3$ stimulates intracellular calcium flux with osteoblasts as well as the activation of protein kinase C (PKC) [362]. Whilst VDR independent signalling events involving the MARRS protein have been identified, others have also shown that the traditional VDR may also possess the ability to mediate plasma membrane signalling. Indeed, the action of 1α,25$(OH)_2D_3$ are lost in osteoblasts from VDR knock-out (KO) mice [363] whilst a 1α,25$(OH)_2D_3$ mediated translocation of the VDR has been observed within skeletal muscle cell lines [364].

1.6.3 Development of the Vitamin D Receptor Knock-Out Mouse

Our understanding of the physiological roles of the VDR has been enhanced significantly by the development of mouse models with the targeted deletion of genes encoding for the VDR [365-368]. The first, often referred to as the *Tokyo* strain, was developed via the removal of exon 2 from the VDR gene that encodes the first zinc finger of the DNA-binding domain, essential for biological function of the VDR [367]. The second strain was developed in a similar fashion, albeit this time via the removal of the second zinc finger of the DNA-binding domain [365]. The *Leuven* strain was developed by utilised a Cre-lox system and the crossing of VDR^{lox} mice with

phosphoglycerate kinase-Cre mice [366]. Finally, the München strain was developed via the targeted disruption of the first zinc finger of the DNA-binding domain which resulted in the expression of a VDR with intact hormone binding domain but lacking the first zinc-finger for DNA binding [368]. These mouse strains have been primarily utilised as a model to study hereditary vitamin D-resistant rickets (HVDDR). This autosomal recessive disorder caused by a defect in the VDR gene results in target tissue insensitivity to $1\alpha,25(OH)_2D_3$ [369].

Interestingly, mice with the targeted ablation of the VDR are unaffected at birth and are indistinguishable from their respective littermates [365]. However, as of the third week of life abnormalities begin to emerge. Most notably, impaired mineral homeostasis in the form of hypocalcemia, as well as growth plate abnormalities are observed from 21-days [365]. Later experiments revealed a significant reduction in duodenal calcium absorption in VDR-KO mice at 10-weeks of age, highlighting the critical role for the VDR in maintaining mineral homeostasis [366, 368]. Across a similar timeframe, VDR-KO mice also develop secondary hyperparathyroidism with a progressive increase in serum parathyroid hormone (PTH) levels observed from 21-days of age [365]. In correlation with increased serum PTH levels, an increase in renal phosphate losses are observed resulting in hypophosphatemia [365]. Disturbances in mineral homeostasis accompany growth plate abnormalities with an expansion, flaring and under mineralisation of the growth plate observed, characteristics that are consistent with the development of rickets [365]. As of 4-weeks of age mice begin to develop alopecia [365, 367] which progresses to complete hair loss over a 3-month period, a trait that is also observed in humans with

VDR related mutations [369]. Later consequences include significant reductions in body weight at 6-weeks as well as a reduction in bone mineral density at 7-weeks of age [367]. The development of osteomalacia is followed by the severe under mineralisation of the bone at 10-weeks of age highlighted by a coverage of 85% of the bone with osteoid [370]. Finally, mutant mice progressively deteriorate until premature death occurs around 15-weeks of age [367].

Interestingly, much of the observed phenotypes can be rescued by placing the VDR-KO mice on a rescue diet with increased calcium and phosphorus content, thereby preventing the impaired mineral homeostasis that develops in VDR-KO mice [370]. Mice placed on this diet from 16-days of age show no signs of impaired mineral homeostasis or impaired bone development [370]. Similarly, the transgenic overexpression of the VDR within the intestine of VDR-KO mice also results in the prevention of the impaired mineral homeostasis previously observed [371]. Whilst neither osteomalacia or rickets are observed in mice fed the rescue diet, the development of alopecia still persists [372]. These data demonstrate that the actions of the VDR on skeletal development are predominantly indirect and the observed phenotype of VDR-KO mice is largely a result of impaired intestinal calcium absorption [370-372]. Interestingly, the rescuing of the impaired mineral homeostasis in VDR-KO mice provides a mouse model in which it is possible to identify which phenotypic traits are secondary effects of impaired mineral homeostasis or of a direct result of the loss of the VDR.

1.6.4 Detection of the Vitamin D Receptor in Skeletal Muscle

The detection of the VDR within skeletal muscle is technically challenging due to low expression levels, the multicellular nature of skeletal muscle, differences in VDR expression across development and finally the non-specificity of antibodies [373].

One of the first reports suggesting a role for the VDR in skeletal muscle described the presence of a high affinity $1\alpha,25(OH)_2D_3$ binding protein in human derived myoblasts and myotubes [374]. However, since these early indications of positive VDR expression within skeletal muscle, following research has yielded ambiguous results [250, 375, 376]. Recently, real-time polymerase chain reaction (RT-PCR) was employed to detect VDR transcripts in a multitude of tissue extracts [250]. In comparison to the duodenum, a classical site of VDR action, skeletal muscle transcripts were detected at a substantially lower rate (4000x lower) [250]. Despite these extremely low levels, the VDR has been successfully detected in skeletal muscle cell lines [250, 375, 377] and rodent skeletal muscle [250] using the highly specific VDR-D6 antibody [373]. Whilst the VDR has also been detected in human skeletal muscle extracts, the antibody used has previously been called into question [378, 379]. In addition, the successful detection of the VDR within human skeletal muscle has been reported following a course of oral vitamin D supplementation in elderly subjects [380, 381], raising the possibility that supplementation aids in detection. On the other hand, a number of studies have failed to detect the VDR within skeletal muscle samples utilising both immunoblot and immunohistochemical approaches [375, 376]. Given the extremely low expression levels, current protein

detection methods may not be sensitive enough to reliably detect the VDR within skeletal muscle.

One such method that has been described to aid in the detection of the VDR within skeletal muscle involves the use of a hyperosmolar lysis buffer (HLB). In comparison to more commonly used sucrose based lysis buffers, a urea based lysis buffer resulted in a much more robust detection of the VDR in mouse skeletal muscle extracts [250]. Given the tight binding of the VDR to DNA, it is hypothesised that the HLB may facilitate the release of DNA-bound proteins and be more effective in protein unfolding and denaturation [382, 383]. In support of this method, similar urea concentrations have aided in the detection of other transcription factors within skeletal muscle, including heat-shock proteins [384]. Despite the reported success using this method, others have reported conflicting results. Utilising the same method, it was reported that the VDR was undetectable in human skeletal muscle extracts despite loading up to 150 μg of protein [375]. Failure to detect the VDR, a predominantly nuclear protein, was also reported even when the nuclear fraction from whole skeletal muscle homogenates was probed [381]. Clearly issues still persist in the reliable detection of the VDR within skeletal muscle. Whilst the detection of the VDR *in vitro* does not seem to be an issue, its detection in adult skeletal muscle extracts remains problematic.

1.6.5 The Vitamin D Receptor in Skeletal Muscle

Despite the difficulties surrounding the detection of the VDR within skeletal muscle, our understanding on the role of the VDR within skeletal muscle has been enhanced

primarily via the use of *in vitro* models of myogenesis and the development of VDR-KO mouse models [365-368, 370, 375, 377, 385]. Functionally, VDR-KO mice display reductions in grip strength, impaired swimming performance and increased fatigue indicating impaired skeletal muscle performance [302, 386]. Whole-body impairments in skeletal muscle function are supported by morphological changes within the skeletal muscle of VDR-KO mice. Animals at 3-weeks of age, prior to the onset of the secondary metabolic changes such as hypocalcemia, displayed a shift in muscle fibre diameters as well as a 20% decrease in size [387]. Morphological changes are still observed at both 8- and 12-weeks of age and persisted even with the administration of a rescue diet containing increased mineral content [302, 387]. Interestingly, these changes are observed across multiple muscles suggesting the actions of the VDR are not limited to skeletal muscle of specific fibre types [302, 387]. A number of myogenic regulatory factors including *myogenic factor 5 (Myf5)*, *transcription factor E2-aplha (E2A), myogenic differentiation 1 (MyoD)* and myogenin were all increased [302, 387], whilst the expression of immature forms of MHC were also observed in the small muscle fibres of VDR-KO mice [387]. *Myostatin* mRNA, a negative regulator of muscle mass, was also increased within the skeletal muscle of VDR-KO mice, possibly explaining the observed reductions in muscle fibre size [302]. Given the ubiquitous expression of the VDR, including within the central nervous and vestibular system, the assessment of the direct effects of the loss of the VDR within skeletal muscle are difficult within this mouse model [388]. Recently, mice with the tissue-specific knockout of the VDR within skeletal muscle have been generated however, limited research has been performed to date utilising this model [385, 389]. Interestingly, these mice develop a number of metabolic defects including increased

serum insulin levels, insulin resistance and glucose intolerance [389]. Whilst the morphological characterisation of these mice was not the primary aim of the study, they did report a slight decrease in muscle fibre size concomitant with increases in forkhead box O1 (FOXO1) at both the gene and protein level [389]. The increased activation of FOXO1 could potentially explain the reductions in fibre size observed in both whole-body and skeletal muscle specific VDR-KO mice given its role in skeletal muscle atrophy [390, 391].

The regeneration of skeletal muscle following an insult or injury is a complex process and recent evidence suggests that VDR activation may be increased during times of regeneration. Interestingly, a significant increase in the activation of both the VDR and CYP27B1 were observed following skeletal muscle injury induced via the injection of barium chloride ($BaCL_2$) or freeze crush [251, 392, 393]. The increase in VDR expression was localised to the myonuclei of regenerating muscle fibres however, co-localisation was also observed with satellite cells suggesting an increase in activation specifically during the regenerative processes [251, 393]. Similar observations were reported following an acute bout of resistance exercise performed via electrical stimulation in rodent skeletal muscle [394]. Both VDR and CYP27B1 expression increased both immediately and 3 h post electrical stimulation, however endurance exercise failed to stimulate corresponding increases in expression [394]. Similarly, an acute bout of high-intensity treadmill based exercise in rats failed to increase VDR expression alone, although exercise in combination with vitamin D supplementation resulted in an increase in VDR expression [395]. Whilst these data highlight an increase in VDR expression during times of skeletal muscle

regeneration, it could be argued that the means to induce said processes are supraphysiological. Given endurance exercise failed to increase VDR expression alone, significant muscle damage may have to take place to induce VDR activation with skeletal muscle.

Alongside its proposed roles in skeletal muscle regeneration, the VDR has also been studied in the context of skeletal muscle development [251, 377, 387, 396-399]. The VDR appears within 13-days of gestation in rats and resides within the mesoderm, a precursor to the musculoskeletal system [396]. Additionally, components of vitamin D related signalling including the VDR and CYP24A1 are expressed within mesenchymal stem cells [397], whilst the expression of the VDR within skeletal muscle decreases across development in mice [250]. Multiple studies have utilised the myogenic C2C12 skeletal muscle cell line to study the role of vitamin D and its related signalling on myogenesis [251, 377, 387, 398, 399]. The treatment of this cell line with 25(OH)D and $1\alpha,25(OH)_2D_3$ results in a reduction in proliferation [251, 377, 398], an inhibition of myotube formation during serum starvation and an increase in individual myotube size [377]. Alterations in key markers of cell cycle progression including *Rb*, *myc*, *ATM*, and *cyclin D1* as well the phosphorylation status of Rb contribute to the vitamin D induced anti-proliferative effects within the C2C12 cell line [377, 398]. Whilst vitamin D treatment resulted in a decrease in overall myotube number, individual myotube size was increased, in concordance with a downregulation of myostatin [377]. In contrast, others have reported that $1\alpha,25(OH)_2D_3$ treatment in C2C12's stimulates myotube formation when a high serum model of myogenesis is employed [398]. It is likely that the different models

employed to study myogenesis resulted in a different differentiation time course leading to conflicting effects of $1\alpha,25(OH)_2D_3$. Whilst the increased expression of the VDR is observed following treatments with 25(OH)D and $1\alpha,25(OH)_2D_3$ [377, 398] these models cannot confirm whether the effects on myogenesis observed are mediated by ligand dependent or independent roles of vitamin D. One study examining myogenesis following siRNA-mediated knock-down of the VDR observed similar effects on proliferation and differentiation [400] suggesting a direct role for the VDR in mediating myogenic signalling. However, given the transient nature and partial deletion often seen with siRNA approaches, improved *in vitro* models are needed to more clearly elucidate the role of the VDR in the development of skeletal muscle.

As previously discussed, the VDR also possesses non-genomic roles involving transient signalling events [357]. In support of a non-genomic role for the VDR within skeletal muscle, the VDR rapidly translocates (1-10 minutes) from the nucleus to the cytoplasm upon exposure of cultured chick myoblasts to vitamin D [364]. Similar translocation to the plasma membrane was reported in C2C12s, with translocation dependent on intact microtubular transport and caveolae structure [401]. Binding of $1\alpha,25(OH)_2D_3$ with the VDR at the plasma membrane in turn activates c-SRC, phosphoinositide-3-kinase (PI3K) and inositol triphosphate (IP3) which in turn leads to the release of $Ca2^+$ from the sarcoplasmic reticulum [402, 403]. Furthermore, the actions of vitamin D signalling have been proposed to result in the translocation of PKCα from the cytosol to the cell membrane [404]. PKCα activates the L-type voltage dependent $Ca2^+$ channel (VDCC) and $Ca2^+$ store-operated entry (SOCE)

channel resulting in an increase in $Ca2^{+}$ flux within the cell [405]. These translocation events likely govern $1\alpha,25(OH)_2D_3$ induced increases in intracellular calcium flux within skeletal muscle cell lines [406-408] and within chick skeletal muscle [409] (Figure 1.4). Following longer periods of exposure, the VDR appears to return to the nucleus, possibly to carry out its genomic actions [398].

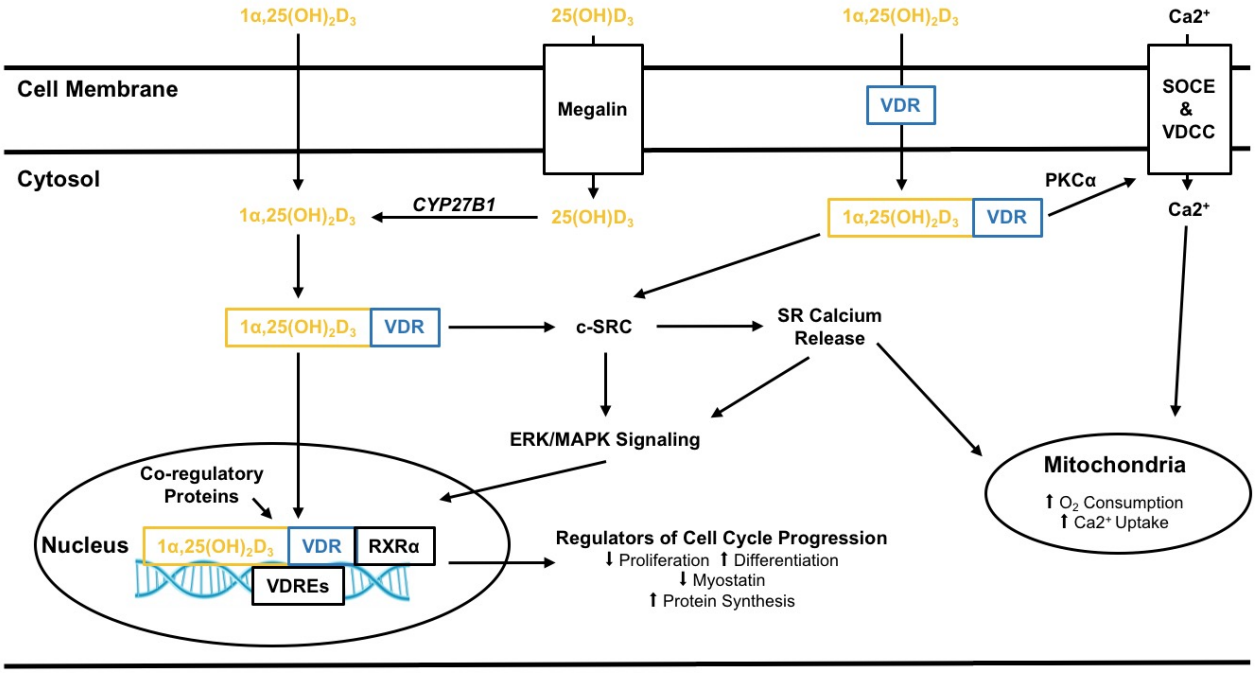

Figure 1.4. Genomic and non-genomic actions of vitamin D related signalling within skeletal muscle. $1\alpha,25(OH)_2D_3$ enters the cell prior to ligand binding to the VDR. Upon translocation to the nucleus, the VDR binds with RXRα forming a heterodimer protein complex that recruits co-regulatory binding partners and influences genomic transcription. $1\alpha,25(OH)_2D_3$ ligand binding also initiates transient signalling events mediated by the VDR that stimulate intracellular calcium uptake. Adapted from [410].

1.6.6 The Vitamin D Receptor in Mitochondria

Over the last decade, evidence has begun to emerge suggesting a novel role for the VDR within the regulation of mitochondrial energy metabolism [411]. The localisation of the VDR within the mitochondria has been confirmed within a number of cell types including platelets, megakaryocytes, and keratinocytes [412, 413]. Whilst the detection of the VDR within the mitochondria has opened up a new line of questioning in terms of its precise role, the current evidence is conflicting and primarily based upon *in vitro* observations.

Firstly, the treatment of human skeletal muscle myoblasts with $1\alpha,25(OH)_2D_3$ resulted in an increase in multiple parameters of mitochondrial function including basal, maximal and ATP dependent respiration as measured by extracellular flux analysis [414]. Similar observations were made following the treatment human skeletal muscle myoblasts with conditioned medium from the Lewis lung cancer carcinoma cell line 1 (LLC1). Whilst the conditioned medium reduced the spare capacity of the human derived myoblasts, the co-treatment with $1\alpha,25(OH)_2D_3$ was able to rescue observed impairments [415]. Increases in mitochondrial function following $1\alpha25(OH)_2D_3$ treatment were reported to be mediated by increases in the mitochondrial volume fraction and OPA1, a key mediator of mitochondrial fusion [414]. In concert, decreases were observed in mediators of mitochondrial fission including Fis1 and Drp1, as well as a decrease in the amount of phosphorylated pyruvate dehydrogenase, which could increase the amount of acetyl-CoA entering the TCA cycle [414]. Interestingly, the positive effects of $1\alpha,25(OH)_2D_3$ were

abolished following the silencing of the VDR via siRNA suggesting a direct role for the VDR in mediating these effects within the mitochondria [414].

Conversely, silencing of the VDR within the human keratinocyte cell line HaCaT and human breast cancer cell line MCF7, resulted in an increase in mitochondrial membrane potential and increased transcription of proteins involved in the mitochondrial electron transport chain [416, 417]. Whilst the authors suggested VDR silencing resulted in an increase in mitochondrial respiration, respiration was not directly measured and was indirectly assessed via measurements of membrane potential and ROS production [416, 417]. Additionally, similar analysis of human primary fibroblasts was reported, however data from the previously mentioned mitochondrial analysis was not reported, raising the question of whether similar effects of VDR silencing were observed [417]. Despite the questionable aspects of the aforementioned data, a study that did directly assess cellular bioenergetics in response to $1\alpha,25(OH)_2D_3$ reported a suppression of respiration in human brown adipocytes [418]. Both maximal and mitochondrial proton leak were suppressed in response to treatment with $1\alpha,25(OH)_2D_3$, whilst the overexpression of the VDR resulted in a decrease in mRNA expression of uncoupling protein 1 (UCP1), PGC-1α, as well as the expression and transactivation of peroxisome proliferator activated receptor gamma (PPARγ) [418].

In order to try to explain the discrepancies observed, it is interesting to note the localisation of the VDR within the cell types studied. Localisation of the VDR was observed within the mitochondria of the HaCaT cell line [412, 413] however, the VDR

was not detected within the mitochondria of human skeletal muscle myoblasts [414]. Therefore, the potential role of the VDR in modulating mitochondrial metabolism may rely on its subcellular localisation. The positive effects observed within skeletal muscle myoblasts may be as a result of the nuclear actions of the VDR or possibly due to its non-genomic actions in regulating $Ca2^{+}$ flux.

Currently, *in vivo* data regarding the precise role of the VDR within the mitochondria is lacking although, some evidence exists regarding the relationship between vitamin D and energy metabolism. Fatigue is a symptom commonly associated with vitamin D deficiency and markers of fatigue are negatively correlated with serum 25(OH)D levels [419], whilst supplementation also improves markers of fatigue in severely deficient individuals [420]. Interestingly, a beneficial effect of vitamin D supplementation in a group of severely deficient individuals was reported following the assessment of mitochondrial function via 31-phosphate nuclear magnetic resonance spectroscopy (31-P MRS) [420]. Whilst some parameters were unchanged, the time needed to recover PCr stores after exercise as well as the disappearance rate of ADP were significantly reduced following vitamin D supplementation [420]. Whilst these data are promising and potentially indicate an improvement in mitochondrial ATP production via oxidative phosphorylation following supplementation, a number issues exist within the study [420]. For example, the study design lacked proper randomisation and blinding and therefore adequate control. In addition, the control group did not have adequate serum 25(OH)D levels (18 $ng.ml^{-1}$) themselves making comparisons difficult.

1.7 Summary

Skeletal muscle is a highly plastic tissue with the ability to respond to both positive and negative stimuli. Within skeletal muscle there exists a morphologically tubular network of mitochondria that primarily functions to produce ATP at the expense of substrates and molecular O_2 [76, 79]. In response to both exercise and nutritional interventions, this network undergoes remarkable remodelling processes including events of biogenesis, fusion, fission and mitophagy [66, 69-73]. Whilst a number of nutritional interventions are known to positively affect these processes [172], nutritional deficiencies can have opposing effects. A common deficiency is that of vitamin D [14, 277], with severe deficiencies resulting in skeletal muscle myopathies [421]. Interestingly, recent evidence has linked vitamin D to increased mitochondrial function with human skeletal muscle myoblasts [414, 415] and the supplementation of vitamin D in severely deficient patients improved markers of ATP production as well as reducing symptoms of fatigue [420]. In addition, diet-induced vitamin D deficiency in mice results in impaired physical function, highlighting a prominent role for vitamin D in maintaining skeletal muscle function [308]. Whilst these data are interesting, little is known about the precise role of the VDR in the maintenance of skeletal muscle mitochondrial function. Furthermore, whilst severe vitamin D deficiencies are known to impair physical performance, it is unknown how such deficiencies affect the process of exercise adaptation within skeletal muscle. Therefore, this thesis will explore the role of vitamin D and the VDR within skeletal muscle. The aims of this thesis are briefly outlined below and will be discussed in more detail in each respective chapter.

1.8 Thesis Aims

The aims of this thesis are as follows;

1. Develop a reliable method for the detection of the VDR within skeletal muscle samples (Chapter 3).

2. Determine the role of the VDR within the maintenance of skeletal muscle mitochondrial function and protein content (Chapter 4).

3. Examine the role of diet-induced vitamin D deficiency on anthropometric measures of body composition and mitochondrial function in C57BL/6J mice (Chapter 5).

4. Determine whether diet-induced vitamin D deficiency impairs the positive impact of exercise on body composition and mitochondrial function in C57BL/6J mice (Chapter 6).

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CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Ethical Approval

2.1.1 Wistar Rats

Ethical approval for rat studies was granted by the Royal Veterinary Colleges Ethics and Welfare committee. Experiments were carried out under UK Home Office license 70/7437 and 70/25526 in order to comply with the Animals Scientific Procedures Act (1986). Male Wistar rats at 8-weeks of age were housed communally in a temperature controlled environment (22 $\pm$ 0.5°C) with a 12 h light-dark cycle.

2.1.2 C57BL/6JAusb

Ethical approval for mouse studies was granted by the Garvan Institute and St. Vincent's Hospital Animal Experimentation Ethics Committee (approval number 18/19). Ethical approval fulfils all the requirements of the NHMRC and the NSW State Government, Australia. All animal handling was carried out by trained personnel and all procedures were carried out according to the Australian code of practice for the care and use of animals for scientific purposes 8th edition [1]. Male C57BL/6JAusb mice at 3- and 10-weeks of age were housed communally in a temperature controlled environment (22 $\pm$ 0.5°C) with a 12 h light-dark cycle.

2.2 Tissue Culture

2.2.1 Tissue Culture Maintenance

Tissue culture experiments were conducted on low passage number cells (<20). Tissue culture was carried out under sterile conditions in a class II safety cabinet. Cells were incubated in a HERAcell 150i CO_2 Incubator (Thermo Scientific Inc, Chesire, UK) maintained at 37°C with 5% CO_2. A VACUSAFE aspiration system

(Integra Biosciences Ltd, Berkshire, UK) was used to discard all waste media and liquids. Necessary solutions were made using ultra-pure water (Type 1) from a Milli-Q water purification system (Merck, Darmstadt, Germany). General cell populations were maintained on 15 cm^2 plates (Corning Inc., Massachusetts, US).

2.2.2 Tissue Culture Reagents

All reagents were purchased from Gibco (Life Technologies, California, US) unless otherwise stated. Dulbecco's modified eagles medium (DMEM) with added GlutaMAX^{TM} and high glucose (4.5 $g.L^{-1}$) was used as base for both growth and differentiation medium. Growth medium contained DMEM with the addition of 10% fetal bovine serum (FBS) purchased from GE Healthcare Life Sciences (GE Healthcare Bio-sciences, Pittsburgh, US), 1% penicillin-streptomycin (PS) and 2 $\mu g.ml^{-1}$ puromycin dihydrochloride (puromycin) purchased from Sigma-Aldrich (Sigma-Aldrich, Gillingham, UK). Differentiation medium contained DMEM with the addition of 2% horse serum (HS) purchased from Sigma-Aldrich and 1% PS. Phosphate buffered saline (PBS) was used to wash cell monolayers. Tryspin-EDTA (0.05%) with the addition of phenol red was used to dissociate cell monolayers. Frozen cells were stored in freeze medium containing FBS with 10% dimethyl sulfoxide (DMSO) purchased from Sigma-Aldrich (Sigma-Aldrich, Gillingham, UK).

2.2.3 Generation of Vitamin D Receptor Knock-Down C2C12 Cell Line

The lentiviral plasmid used (pLKO.1 backbone) was designed in-house and was based on (Clone ID: RMM3981-201757375) and targeted the (3' UTR) mouse sequence 5′- TTA AAT GTG ATT GAT CTC AGG-3′ of the mouse *Vdr* gene; the

scramble short hairpin ribonucleic acid (shRNA) was used as a negative control as previously reported [2] with a hairpin sequence: CCT AAG GTT AAG TCG CCC TCG CTC TAG CGA GGG CGA CTT AAC CTT AGG (Addgene plasmid 1864, Cambridge, MA, USA). Oligos were obtained from ITDDNA USA and suspended, annealed and cloned into pLKO.1 at EcoRI and AgeI restriction sites as per the pLKO.1 protocol from Addgene. Resultant plasmids were transformed in DH5α cells for amplification and isolated. The actual DNA sequence was confirmed at the Pennsylvania State University College of Medicine DNA sequence core facility. Packaging plasmids psPAX2 and envelope protein plasmid pMD2.G were a gift from Trono Lab (Addgene plasmids 12260 and 12259 respectively). HEK293FT cells (Invitrogen, Carlsbad, CA, USA) were grown in DMEM; 80–85% confluent plates were rinsed once with Opti-MEM (Invitrogen, Carlsbad, CA, USA) and then incubated with Opti-MEM for 4 h before transfections. psPAX2 and pMD2.G along with either scramble or pLKO.1 clones targeting mouse Vdr (three clones) were added after mixing with Lipofectamine 2000 as per the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Opti-MEM was changed after overnight incubation with DMEM containing 10% FBS without antibiotics to allow cells to take up the plasmids and recover. Culture media were collected at 36 and 72 h post-transfection for viral particles. Viral particles present in the supernatant were harvested after a 15-minutes spin at 1,500 *g* to remove cellular debris. The supernatant was further filtered using a 0.45-μm syringe filter. Supernatant-containing virus was either stored at −80°C for long-term storage or at 4°C for immediate use. C2C12 cells at 60% confluence were infected twice overnight with 3 ml of viral supernatant containing 8 $\mu g.ml^{-1}$ polybrene in serum-free–antibiotic-free DMEM. Fresh DMEM media containing 10% FBS,

antibiotics and 2 μg.ml^{-1} puromycin (Sigma-Aldrich, St. Louis, MO, USA) were added the next day. Cells that survived under puromycin selection were either harvested (as stable cells), stored under liquid nitrogen (N_2) or used as either myoblasts or myotubes depending on the experiment. Successful VDR knock-down (VDR-KD) was confirmed via the determination of VDR protein content via immunoblot.

2.2.4 C2C12 Growth and Differentiation

VDR-KD and respective scramble control C2C12s were plated at 1.0 x 10^5 cells/well in 2 ml of growth medium in 6-well plates (Nunc, Roskilde, Denmark). Upon reaching 90% confluence, cells were either harvested for myoblast experiments or switched to differentiation medium. Before switching to differentiation medium, cells were washed once with PBS. Differentiation medium was changed every other day and myotube formation was monitored over a period of 7-days.

2.3 Sample Lysis and Homogenization

2.3.1 Cell and Tissue Lysis

Upon collection, both myoblast and myotube cell monolayers were washed twice with ice-cold PBS. Each well was actively scraped and collected in 100 μl of sucrose lysis buffer. Tissue samples were powdered on dry ice using a Cellcrusher^{TM} tissue pulverizer (Cellcrusher Ltd, Cork, Ireland) and homogenized in a 10-fold mass of ice-cold sucrose lysis buffer. The constituents of the sucrose lysis buffer are described below (section 2.3.2)

2.3.2 Sucrose Lysis Buffer

Cell and tissue lysis was completed using sucrose lysis buffer (50 mM Tris pH 7.5; 270 mM sucrose; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 50 mM sodium fluoride; 5 mM sodium pyrophosphate decahydrate; 25 mM beta-glytcerolphosphate). Relevant inhibitors were added fresh on the day of use and included 1 cOmplete^{TM} protease inhibitor cocktail EDTA free tablet (cat. 1183617001) and Phosphatase Inhibitor Cocktail 3 (cat. P0044) both purchased from Sigma-Aldrich (Sigma-Aldrich, Gillingham, UK).

2.3.3 Urea Lysis Buffer

With the aim of detecting the VDR in whole skeletal muscle extracts (Chapter 3), tissue samples were also lysed in a hyperosmolar lysis buffer (6.7M urea; 10% glycerol; 10mM Tris-HCl, 1% sodium dodecyl sulfate; 1mM dithiothreitol; 1mM phenylmethylsulfonyl-fluoride). One Protease Inhibitor Cocktail tablet was added fresh upon the day of use.

2.3.4 Cell and Tissue Homogenization

Cell and tissue lysates were homogenized via shaking in a FastPrep 24 5G (MP Biochemicals, Santa Ana, California, USA) at 6.0 $m \cdot s^{-1}$ for 80 s. Samples were then centrifuged for 10 min at 8,000 *g* and at 4°C to remove any insoluble material. The resulting supernatant was removed and stored at -80°C for further analysis.

2.3.5 Determination of Protein Content

Cell and tissue samples were diluted in a 1:5 and 1:20 ratio respectively and protein concentrations were determined using the DC protein assay as per manufacturer's instructions (Bio-Rad, Hercules, California, USA).

2.4 Immunoblotting

2.4.1 Sample Preparation

Total protein lysates of a known concentration were mixed 3:1 with 4x Laemmli sample loading buffer to generate polyacrylamide gel loading samples of a known concentration. Prior to gel loading samples were boiled at 95°C for 5 minutes (samples were un-boiled when using MitoProfile OXPHOS antibody). Prepared samples were stored at -80°C until analysis.

2.4.2 Gel Preparation and Electrophoresis

Gels (8-15%) were prepared by mixing relative amounts of 30% acrylamide/bis-acrylamide (Bio-Rad, Hercules, California, USA), Tris-SDS (pH 8.8) and ultra-pure water. Subsequently, both 10% ammonium persulfate (APS) and TEMED were added in order to initiate polymerization. Gels were allowed to polymerize for ~30 minutes prior to the addition of 5% stacking gel prepared as above. Gels were prepared using 10- or 15-well combs dependent upon the number of samples loaded. An equal volume of protein (10-75 μg) alongside a molecular weight marker (Precision Plus Protein™ Dual Colour Standards, Bio-Rad, Hercules, California, USA) was separated by SDS-PAGE at a constant current of 23 mA per gel for ~60 minutes.

2.4.3 Transfer and Blocking

Proteins were then transferred on to BioTrace NT nitrocellulose membranes (Pall Life Sciences, Pensacola, Florida, USA) using a wet transfer system at 100 V for 1 h. Membranes were then stained in Ponceau S (Sigma-Aldrich, Gillingham, UK) and imaged to check for even loading and transfer. Membranes were then blocked for 1 h in 3% dry-milk in tris-buffered saline with tween (TBS-T). Membranes were then incubated overnight in primary antibodies at 4°C. Following primary antibody incubation, membranes were washed three times in TBS-T and subsequently incubated in the appropriate horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Membranes were again washed three times in TBS-T prior to imaging.

2.4.4 Antibodies

All primary antibodies were used at a concentration of 1:1000 in TBS-T unless otherwise stated. Antibodies for the VDR were used at a concentration of 1:500 in TBS-T when aiming to detect the VDR in whole skeletal muscle lysates (Chapter 3). Antibodies for dynamin-1-like protein (DRP1; 8570) and vitamin D3 receptor (VDR; 12550) were from Cell Signaling Technology; MitoProfile OXPHOS antibody cocktail (110413), vitamin D receptor (VDR; 109234) and mitofilin (110329) were from Abcam; dynamin-like 120 kDa protein (OPA1; CPA3687) was from BD Biosciences; peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α; AB3242) was from Merck-Millipore; citrate synthase (CS; SAB2701077) and mitochondrial fission protein 1 (FIS1; HPA017430) were from Sigma-Aldrich; vitamin D receptor (D-6) (VDR; 13133) was from Santa Cruz Biotechnology. Secondary

antibodies were used at a concentration of 1:10,000 in TBS-T. Anti-mouse (7076) and anti-rabbit (7074) were from Cell Signaling Technology.

2.4.5 Image Capture and Analysis

Antibody detection was performed via enhanced chemiluminescence horseradish peroxidase substrate detection kit (Millipore, Watford, UK). Subsequent imaging and band quantification were performed using a G:Box Chemi-XR5 system (Syngene, Cambrige, UK).

2.5 Extracellular Flux Analysis

2.5.1 Cell Seeding and Maintenance

Both scramble control and VDR-KD cells were seeded in XFe24-well cell culture microplates (Seahorse Bioscience, North Billerica, USA) at 3.0 x 10^5 cells/well in 100 μl of growth medium and allowed to rest at room temperature for 1 h in order to promote an even cell distribution [3]. For myoblast experiments, cells were incubated at 37°C and 5% CO_2 for 3 h in order to allow sufficient time for adherence to cell culture microplate and subsequently assayed. For myotube experiments, cells were incubated for a period of 24 h resulting in a confluence of 90%. Cells were then washed once with PBS and medium changed to differentiation medium. Differentiation was changed every other day for a period of 7 days.

2.5.2 Mitochondrial Stress Test

Prior to the assay, cells were washed once and placed in 500 μl of Seahorse XF Base Medium (glucose 10 mM, sodium pyruvate 1 mM, glutamine 1 mM, pH 7.4) pre-

warmed to 37°C. The plate was then transferred to a non-CO_2 incubator for 1 h. Following calibration, cell respiratory control [4] and associated extracellular acidification were assessed following the sequential addition of oligomycin (1 µM), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (CCCP) (1 µM) and antimycin A with rotenone (1 µM). Upon completion of the assay, cells were lysed in sucrose lysis buffer and subjected to one freeze thaw cycle. Upon thawing, cell lysate was centrifuged for 10 minutes at 8,000 *g* and the supernatant was removed for protein determination. Protein concentration was determined using the DC protein assay (Bio-Rad, Hercules, California, USA) as previously described (section 2.3.5). Oxygen Consumption Rate (OCR) is reported relative to protein content (pmol/min/µg). Calculation for respiratory parameters derived from the mitochondrial stress test can be found below (Table 2.1).

Respiratory Parameter	Calculation
Basal Respiration	Last Rate Measurement Before Oligomycin Injection - Non-Mitochondrial Respiration Rate
Coupled Respiration	Last Rate Measurement Before Oligomycin Injection – Minimum Rate Measurement After Oligomycin Injection
Maximal Respiration	Maximum Rate Measurement After CCCP Injection – Non-Mitochondrial Respiration
Spare Respiratory Capacity	Maximal Respiration – Basal Respiration
H^+ (Proton) Leak	Minimum Rate Measurement After Oligomycin Injection – Non-Mitochondrial Respiration

Table 2.1. Calculations for respiratory parameters derived from mitochondrial stress test.

2.5.3 Estimation of ATP Production

Estimation of intracellular rates of glycolytic and oxidative ATP production were performed as previously described [5]. Briefly, scramble control and VDR-KD cells were seeded in XFe24-well cell culture microplates (Seahorse Bioscience, North Billerica, USA) at 3.0 x 10^5 cells/well in 100 μl of growth medium (high glucose DMEM, 10% FBS, 1% PS, puromycin 2 $\mu g.ml^{-1}$). Prior to the assay, cells were washed once and then incubated in 500 µl of Krebs-Ringer phosphate HEPES (KRPH) medium (2 mM HEPES, 136 mM NaCl, 2 mM NaH_2PO_4, 3.7 mM KCl, 1 mM $MgCl_2$, 1.5 mM$CaCl_2$, 0.1% (w/v) fatty-acid-free bovine serum albumin (BSA), pH 7.4 at 37 °C). Cell respiratory control [4] and extracellular acidification were assayed following the addition of glucose (10 mM), oligomycin (1 µM), CCCP (1 µM) and antimycin A/rotenone (1 µM). Upon completion of the assay cells were collected in sucrose lysis buffer (50 mM Tris pH 7.5; 270 mM sucrose; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 50 mM sodium fluoride; 5 mM sodium pyrophosphate decahydrate; 25 mM beta-glytcerolphosphate; 1 cOmplete™ protease inhibitor cocktail EDTA free tablet) and centrifuged for 10 minutes at 8,000 *g* and the supernatant was removed for protein determination. Protein concentration was determined using the DC protein assay (Bio-Rad, Hercules, California, USA). Following correction for the buffering power of the medium, rates of ATP production are reported relative to protein content (pmol/min/µg). Rates of ATP production are converted to the same unit accounting for the different amounts of ATP produced via glycolysis (2 ATP/Glucose) and oxidative phosphorylation (31.45 ATP/Glucose) [5].

2.6 Mitochondrial Membrane Potential

Both scramble control and VDR-KD cells were plated at 1.0 x 10^5 cells/well in 100 μl of growth medium and incubated for 24 h. A black 96-well plate with a clear bottom (Corning, Costar, NY, US) was used in order to minimise well-to-well interference. Cells were subsequently incubated for 30 minutes with 100 nM of tetramethylrhodamine, ethyl ester (TRME). A set of cells were also incubated with 20 μM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) in order to completely depolarise the mitochondrial membrane and act as a negative control. Following incubation, cells were washed with phosphate buffered saline (PBS) with 0.2% BSA and then read at 549 nm using a CLARIOstar microplate reader (BMG Labtech, Victoria, Australia) in 100 μl of PBS and 0.2% BSA.

2.7 Rodent Studies

2.7.1 Transient Electroporation in Male Wistar Rats

Eight-week-old male Wistar rats were housed a described above (section 2.1.1) and fed a standard chow diet (18% fat, 33% protein, 48% carbohydrate) for one week. VDR-KD (n=7) was achieved in right hind leg *tibialis anterior* muscle through *in vivo* electrotransfer of four unique rat VDR pGFP-C-shLenti plasmid constructs driven by a U6 promoter [6]. These constructs spanned multiple regions of rat VDR mRNA (NM_017058.1) to ensure effective coverage and knockdown. Left leg *tibialis anterior* muscles received scramble cassette sequence controls (OriGene, Rockville, USA). Plasmid DNA amplification was undertaken within JM109 *Escherichia coli* cells under puromycin selection (0.5 μg/ml) overnight. Plasmid DNA was purified using an endotoxin free plasmid Maxi Kit (Qiagen, Manchester, UK) as per the manufacturers

instructions. VDR over-expression (VDR-OE) (n=7) was achieved in right leg *tibialis anterior* muscle through in vivo electrotransfer of pCAGGS-mVDR vector constructs. The mouse cDNA sequence utilised was 92% homologous to rat VDR mRNA. To obtain pCAGGS-mVDR, myc-DDK-mVDR was subcloned in the pCAGGS vector. Left leg *tibialis anterior* muscles received empty pCAGGS vector controls. Plasmid DNA amplification was undertaken within JM109 *Escherichia coli* cells under puromycin selection (0.5 μg/ml) overnight. Plasmid DNA was purified using an endotoxin free plasmid Maxi Kit (Qiagen, Manchester, UK) as per the manufacturers instructions.

2.7.2 Mouse Diets

C57BL/6J mice were received at 10-weeks of age. Following 1-week of acclimation in which mice were fed standard chow, mice were place on either a vitamin D-control diet or a vitamin D-deplete diet [7]. The vitamin D deplete contains no vitamin D but increased calcium (2%), magnesium (0.2%), and phosphorous (1.2%) in order to maintain normal mineral homeostasis (SF085-003, Speciality Feeds, Glen Forest, NSW). The vitamin D control diet contains vitamin D (cholecalciferol 2.2 IU/g), calcium (1%), magnesium (0.2%), and phosphorous (0.7%) (SF085-034, Speciality Feeds, Glen Forest, NSW). Mice were maintained on the respective diets for a period of either 1-, 2- or 3-months. Mice subjected to voluntary wheel running maintained their respective vitamin D replete or deplete diets across the 20-day running period.

Nutrient	Standard Chow	VitD Replete	VitD Deplete
Protein	23%	19.4%	19.4%
Fat	6%	7%	7%
Fibre	5%	4.7%	4.7%
Digestible Energy	13 MJ/kg	15.8 MJ/kg	15 MJ/kg
Vitamin D	200 IU/kg	2,200 IU/kg	0 IU/kg
Calcium	1.1%	1%	2%
Magnesium	1.8%	0.2%	0.2%
Phosphorous	0.77%	0.7%	1.2%

Table 2.2. Nutrient composition of mouse diets.

2.7.3 Voluntary Wheel Running

Mice were housed individually and given access to an upright, free-spinning running wheel (diameter 10.16 cm, Columbus Instruments, Columbus, OH, US) for 20 days. Wheel revolutions were recorded every hour via a digital recorder (Columbus Instruments, Columbus, OH, US) and the distance ran per day was calculated and reported in kilometres per twenty-four hours (km/24 h).

2.7.4 EchoMRI Assessment of Body Composition

Prior to each assessment of body composition, mice were briefly weighed. Body composition was assessed upon arrival (10-weeks of age) and then following 1-, 2- and 3-months of dietary intervention and post 20-days of voluntary wheel running using quantitative EchoMRI (EchoMRI LLC, Houston, USA). Both absolute and percentage of body weight fat and lean mass' are reported.

2.8 Sample Collection and Processing

2.8.1 Tissue Collection

Following anesthetization under isoflurane (induction 4%, maintenance 1%, flow rate 1 L/min), tissues were excised from fasted (2 h) mice in order to minimise effects of

prior food intake. Mice subjected to voluntary wheel running had no access to a running wheel for 24 h prior to tissue collection. Tissues collected include; gastrocnemius, quadriceps, triceps, liver, kidney, white adipose tissue and tibia. Following tissue collection, a blood sample was taken via cardiac puncture. End stage termination was completed via cervical dislocation. All tissues were rinsed in sterile saline, blotted dry, weighed, and frozen in liquid N_2. A small portion (~20 mg) of the gastrocnemius was removed before freezing and used for high-resolution respirometry. All tissues were stored at -80°C for subsequent analysis.

2.8.2 Blood Processing

Blood samples were allowed to coagulate at room temperature for 10 minutes before being placed on ice. Samples were then centrifuged at 14,000 *g* for 10 minutes. The resulting supernatant was removed and stored at -80°C prior to further analysis.

2.9 Serum Calcium

Serum calcium was measured using a Calcium Detection Assay kit (Abcam, Cambridge, UK, cat. ab102505). Serum samples were diluted 1:10 and manufacturers instructions were followed. The microplate was read at 575 nm using a CLARIOstar microplate reader (BMG Labtech, Victoria, Australia). Calcium concentrations are reported in mM.

2.10 High-Resolution Respirometry

2.10.1 Tissue Preparation

Small portions of gastrocnemius muscle (~20 mg) were removed and placed in ice-cold BIOPS buffer (2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2$-$6H_2O$, 20 mM Taurine, 15 mM Na_2Phosphocreatine, 20 mM Imidazole, 0.5 mM Dithiothreitol, 50 mM MES Hydrate, pH 7.1, 290 mOsm). Muscle fibres were trimmed of connective tissue and fat and separated into small bundles of approximately 1.0-2.5 mg wet weight. These bundles were subsequently teased apart using needle tipped forceps under magnification. Following separation, fibres bundles were placed in BIOPS buffer (2 ml) containing saponin (50 μg/μl) and gently rocked for 30 min at 4°C. Saponin, a cholesterol-specific detergent is used in order to permeabilize the sarcolemmal membranes while keeping the mitochondrial membranes intact. Following permeabilization, fibre bundles were placed in mitochondrial respiration medium (MiR05) (0.5 mM EGTA, 3 mM $MgCl_2$-$6H_2O$, 60 mM Lactobionic Acid, 20 mM Taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM D-Sucrose, 1 g/l BSA, pH 7.1) and gently rocked for 10 min at 4°C.

2.10.2 High-Resolution Respirometry

High-resolution respirometry was conducted in MiR05 (2 ml) with the addition of blebbistatin (25 μM) using the OROBOROS Oxygraph-2K (Oroboros Instruments, Corp., Innsbruck, AT) with stirring at 750 rpm at 37°C. Oxygen within the chamber was maintained between 150-220 μM for each experiment. Prior to the addition of the fibre bundles to the chamber, samples were blotted dry and weighed. Bundles totalling 2.5-5.0 mg were added to the chamber. Firstly, pyruvate (10 mM) and

malate (2 mM) were added as complex I substrates. Subsequently, ADP was titrated in step-wise increments (100-6000 μM) followed by the addition of glutamate (10 mM) and succinate (10 mM) as complex I and II substrates respectively. Cyt c (10 μM) was added in order to check outer mitochondrial membrane integrity [8]. The partial loss of cyt c during fibre preparation may limit respiration. Fibre preparations that exhibited an increase of >10% were removed from final analysis. CCCP was titrated in a step-wise manner (0.5 to 2.5 μM) until the maximal capacity of the electron transport chain was reached. Finally, antimycin A (2.5 μM) was injected in order to inhibit mitochondrial respiration.

2.10.3 Determination of Michaelis-Menten Enzyme Kinetics

The apparent K_m for ADP was determined through the Michaelis-Menten enzyme kinetics – fitting model ($Y = Vmax*X/(K_m + X)$), where X = (free ADP; ADP_f), using Prism version 7 (GraphPad Software, Inc., La Jolla, CA).

2.10.4 Flux Control Ratios

Flux control ratios (FCR) provide a method of internal normalization were by respiration is normalized to maximal and minimal respiration by setting CCCP stimulated respiration as one and antimycin A respiration as zero.

2.11 Statistical Analysis

Statistics were performed using the Statistical Package for the Social Sciences (SPSS) version 24.0 and Prism version 7 (GraphPad Software, Inc., La Jolla, CA). Data are presented as means with standard deviation (SD). Statistical significance

was accepted as $P < 0.05$. Additional statistical tests are discussed in each respective chapter.

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CHAPTER 3

METHODOLOGICAL CONSIDERATIONS FOR DETECTING THE VITAMIN D RECEPTOR IN C2C12 MYOBLASTS AND MOUSE SKELETAL MUSCLE

3.1 Introduction

Once in circulation, vitamin D undergoes a series of hydroxylation steps. The first, occurs within the liver and coverts vitamin D to 25(OH)D via 25-hydroxylation [1]. The second, occurs within the kidney and converts 25(OH)D to the biologically active $1\alpha,25(OH)_2D_3$ via 1α-hydroxylation [2, 3]. In order to carry out the biological actions of vitamin D, $1\alpha,25(OH)_2D_3$ binds to the VDR, which subsequently binds to the RXR forming a heterodimer protein complex [4, 5]. This protein complex then binds to VDREs within the promotor regions of vitamin D regulated genes [6, 7]. Whilst the VDR is said to be ubiquitously expressed [8], its detection within skeletal muscle has remained problematic due to extremely low expression levels, the use of non-specific antibodies and the multicellular nature of skeletal muscle [9-11].

Current *in vitro* evidence suggests that the positive detection of the VDR protein within skeletal muscle cell lines is consistent and less problematic than its respective detection in adult skeletal muscle [9, 12]. One of the first reports of positive VDR expression within skeletal muscle described the presence of a high affinity $1\alpha,25(OH)_2D_3$ binding protein in human derived myoblasts and myotubes [13]. Further to this, the positive detection of the VDR within skeletal muscle cells derived from chicks [14-16], mice [9, 17-19] and humans [12, 13, 20, 21] has been reported on multiple occasions. Within C2C12 myoblasts, the VDR was detected at the transcript and protein level, both of which increased in response to the treatment with 25(OH)D and $1\alpha,25(OH)_2D_3$ [19] and decreased during myogenesis [9]. Similarly, the detection of the VDR in C2C12 skeletal muscle cells has also been reported utilising immunohistochemistry with time-dependent localisation to the cell membrane or

nucleus in response to $1\alpha,25(OH)_2D_3$ [18, 22]. Multiple studies have also confirmed the expression of the VDR within human primary myoblasts [12, 20, 21]. Clearly, the detection of the VDR within skeletal muscle *in vitro* is reliable with consistent reports of a successful detection utilising a multitude of techniques.

The detection of the VDR in whole skeletal muscle lysates however, remains a contentious issue with multiple studies reporting conflicting results [9, 11, 12, 23, 24]. For example, VDR at the protein level was reportedly undetectable in rat and mouse skeletal muscle via both immunoblot and immunohisotchemical approaches [11]. In addition, whilst VDR transcripts were detected at an extremely low level, in comparison to the duodenum a classical site of VDR action, skeletal muscle transcripts were detected at a substantially lower rate (4000x lower) [9, 11]. Given this low expression profile, the use of a HLB (6.7M urea; 10% glycerol; 10mM Tris-HCl, 1% sodium dodecyl sulfate; 1mM dithiothreitol; 1mM phenylmethylsulfonyl-fluoride) has been reported to improve the detection of the VDR [9]. The VDR is known to tightly bind to DNA and it is hypothesised that the use of HLB may be effective in reducing VDR/DNA association [25, 26]. Whilst this method has proved successful in mouse skeletal muscle [9], other studies have reported that the VDR was undetectable in human skeletal muscle extracts when employing this method [12]. In addition, the VDR remained undetectable even when a purified nuclear fraction was probed [21]. Despite the successful detection of the VDR in mouse skeletal muscle, its expression was reduced across development and decreased significantly when tissue from 3-month old mice was compared to tissue from mice at 3-weeks of age [9]. Within human skeletal muscle samples, the positive expression

of the VDR has been reported utilising immunohistochemical [27, 28] and immunoblot approaches [21]. Despite this, the antibody utilised to detect the VDR via immunohistochemistry, Affinity BioReagents 9A7, has previously been called into question due to non-specific binding [29]. Similarly, whilst the successful detection of the VDR was reported utilising the VDR NR1I1 antibody (Perseus Proteomics), it was validated in comparison to the Santa Cruz D6 antibody and was not tested in VDR-KO tissue [28].

Despite the consistent detection of the VDR *in vitro*, its detection within whole skeletal muscle remains problematic. Within skeletal muscle cell lines, the VDR is known to play a role in proliferation, differentiation and cell cycle progression [19]. In addition, both whole body and skeletal muscle specific VDR-KO mice display impairments in skeletal muscle structure and function, suggesting a direct role for the VDR within said tissue [17, 23, 30-32]. Therefore, a protocol that results in the reliable and consistent detection of the VDR within skeletal muscle lysates would be significant addition to this thesis and the field as a whole. Expanding upon previously described methods [9], we sought to detect the VDR in skeletal muscle lysates from both young (3-weeks) and adult (10-weeks) mice. Comparisons were made across differing skeletal muscle samples, lysis buffers and antibodies with the aim of positively detecting the VDR.

3.2 Methods

3.2.1 Tissue Culture

VDR-KD and respective scramble control C2C12s were plated at 1.0 x 10^5 cells/well in 2 ml of growth medium in 6-well plates (Nunc, Roskilde, Denmark). Upon reaching 90% confluence, cells were either harvested for myoblast experiment or switched to differentiation medium. Before switching to differentiation medium, cells were washed once with PBS. Differentiation medium was changed every other day and myotube formation was monitored over a period of 7 days.

3.2.2 Mouse Characteristics and Tissue Collection

Both 3- (n=3) and 10-week old (n=3) C57BL/6J mice were housed as previously described (Section 2.1.2). Within the first week of arrival, mice were anesthetized under isoflurane and tissues were excised following a 2 h fast. Tissues collected included; gastrocnemius, quadriceps, triceps, kidney and liver. Following tissue collection, a blood sample was taken via cardiac puncture. End stage termination was completed via cervical dislocation. Samples were handled and stored as previously described (Section 2.8).

3.2.3 Cell and Tissue Lysis

Upon collection, both myoblast and myotube cell monolayers were washed twice with ice-cold PBS. Each well was actively scraped and collected in 100 μl of sucrose lysis buffer. Tissue samples were powdered on dry ice using a Cellcrusher™ tissue pulverizer (Cellcrusher Ltd, Cork, Ireland) and homogenized in a 10-fold mass of ice-cold sucrose or urea lysis buffer. Cell and tissue lysates were homogenised via

shaking in a FastPrep 24 5G (MP Biochemicals, Santa Ana, California, USA) as previously described (Section 2.3.1). The constituents of the sucrose and urea lysis buffers are described below (Section 3.2.5).

3.2.4 Lysis Buffers

Cell and tissue lysis was completed using sucrose lysis buffer (50 mM Tris pH 7.5; 270 mM sucrose; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 50 mM sodium fluoride; 5 mM sodium pyrophosphate decahydrate; 25 mM beta-glytcerolphosphate). Relevant inhibitors were added fresh on the day of use and included 1 cOmplete^{TM} protease inhibitor cocktail EDTA free tablet and Phosphatase Inhibitor Cocktail 3 both purchased from Sigma-Aldrich. With the aim of detecting the VDR in whole skeletal muscle extracts, tissue samples were also lysed in a urea lysis buffer (6.7M urea; 10% glycerol; 10mM Tris-HCl, 1% sodium dodecyl sulfate; 1mM dithiothreitol; 1mM phenylmethylsulfonyl-fluoride). One Protease Inhibitor Cocktail tablet was added fresh upon the day of use.

3.2.5 Immunoblotting

Total protein lysates of a known concentration were mixed 3:1 with 4x Laemmli sample loading buffer to generate polyacrylamide gel loading samples of a known concentration. Prior to gel loading, samples were boiled for 5 minutes unless otherwise stated. An equal volume of protein (10-75 μg) was separated by SDS-PAGE on 10% gels at a constant current of 23 mA per gel. Proteins were then transferred on to BioTrace NT nitrocellulose membranes (Pall Life Sciences, Pensacola, Florida, USA) using a wet transfer system at 100 V for 1 h. Membranes

were then stained in Ponceau S (Sigma-Aldrich, Gillingham, UK) and imaged to check for even loading and transfer. Membranes were then blocked for 1 h in 3% dry-milk in TBS-T prior to incubation overnight in primary antibodies at 4°C. Following primary antibody incubation, membranes were washed three times in TBS-T and subsequently incubated in the appropriate horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Antibody detection was performed via an enhanced chemiluminescence horseradish peroxidase substrate detection kit (Millipore, Watford, UK). Subsequent imaging and band quantification were performed using the G:Box Chemi-XR5 system (Syngene, Cambrige, UK).

3.2.6 Antibodies

Three different antibodies for the VDR were utilised and the characteristics of each are displayed below (Table 3.1). All primary antibodies were used at a concentration of 1:500 in TBS-T. Secondary antibodies were used at a concentration of 1:10,000 in TBS-T. Anti-mouse (7076) and anti-rabbit (7074) were from Cell Signaling Technology.

Antibody	Manufacturer	Host	Isotype	Epitopes
D6 SC-12133	Santa Cruz Biotechnology	Mouse	Monoclonal	Human VDR 344-424
D2K6W	Cell Signaling Technology	Rabbit	Monoclonal	Human VDR N-terminal
AB109234	Abcam	Rabbit	Monoclonal	Human VDR

Table 3.1. Antibody characteristics utilised for the detection of the vitamin D receptor. The exact sequence for the Abcam antibody is proprietary.

3.2.7 Statistical Analysis

Statistical analysis was performed using the SPSS version 24.0. Control myoblasts and myotubes were compared to VDR-KD myoblasts and myotubes respectively via independent t-tests. Data presented as mean ± SD. Statistical significance set as $P < 0.05$.

3.3 Results

3.3.1 Detection of the VDR in C2C12 Myoblasts

Antibodies from Abcam, Cell Signaling Technology and Santa Cruz all successfully detected the VDR protein in C2C12 myoblasts. Positive detection of the VDR was confirmed through immunoblotting of C2C12 myoblasts in which VDR protein was abolished via shRNA interference (See chapter 4 for detailed characterisation of this cell line). Minimal signal was detected in VDR-KD myoblasts and no non-specific binding was detected for each antibody (Fig. 3.1A-B) suggesting that all three antibodies are specific to the VDR in C2C12 cells.

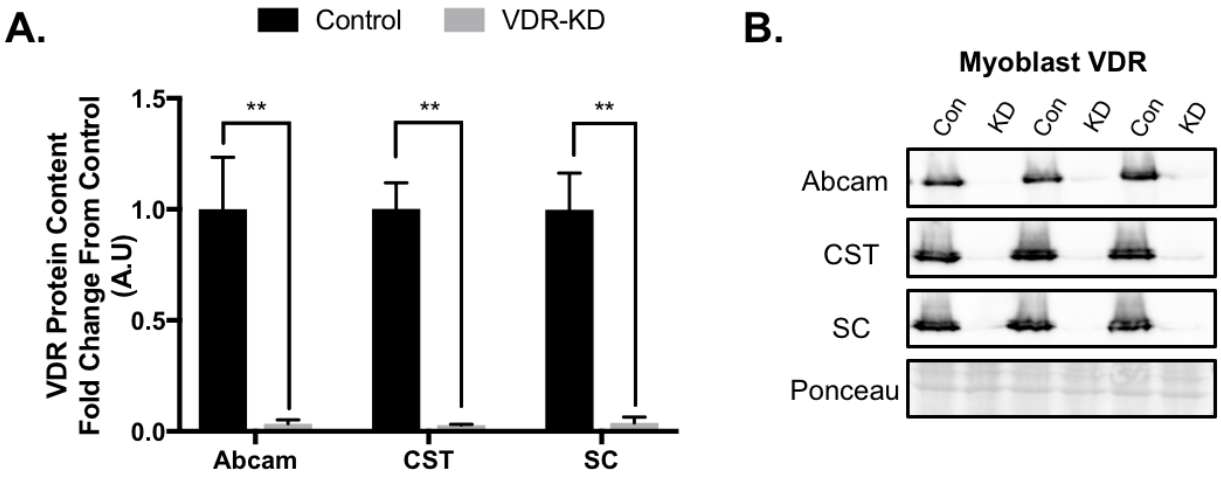

Figure 3.1. Detection of the VDR in control and VDR-KD C2C12 myoblasts. A) Detection of the VDR within control and VDR-KD C2C12 skeletal muscle myoblasts utilising antibodies from Abcam, Cell Signaling Technology (CST) and Santa Cruz (SC). B) Representative images of VDR protein expression. Data mean ± SD (n=6). $^{**}P < 0.005$.

3.3.2 Detection of the VDR in C2C12 Myotubes

VDR protein content was detected in C2C12 myotubes when antibodies from Abcam, Cell Signaling Technology and Santa Cruz were utilised. Positive detection of the VDR was confirmed via the use of the VDR-KD C2C12 cell line (See chapter 4 for detailed characterisation of this cell line). Minimal signal and no non-specific binding was detected for each antibody (Fig. 3.2A-B).

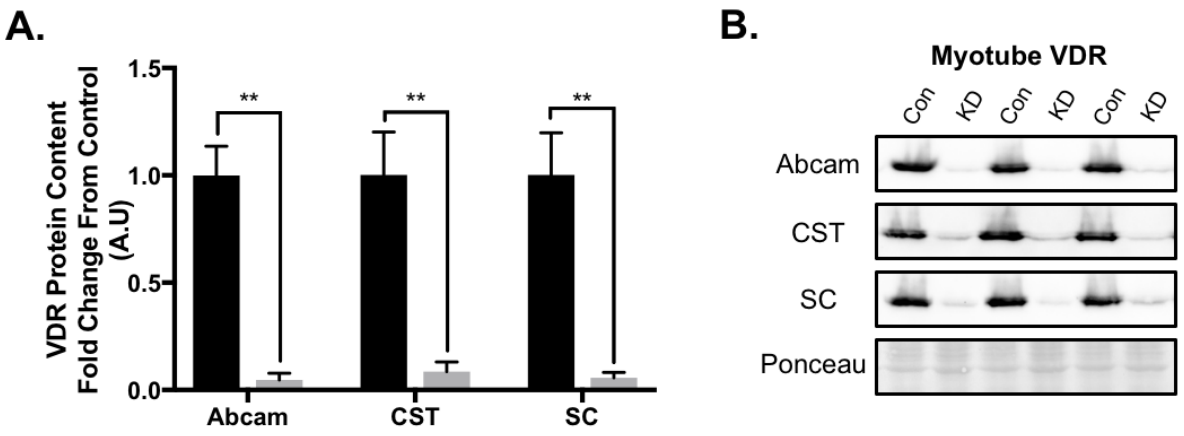

Figure 3.2. Detection of the VDR in control and VDR-KD C2C12 myotubes. A) Successful detection of the VDR protein within control and VDR-KD C2C12 skeletal muscle myotubes utilising antibodies from Abcam, Cell Signaling Technology and Santa Cruz. B) Representative images of VDR protein expression. Data mean ± SD (n=6). $^{**}P < 0.005$.

3.3.3 Detection of the VDR in Mouse Kidney

We next aimed to detect the VDR in 3- and 10-week old mouse kidney lysates using antibodies from Abcam, Cell Signaling Technology and Santa Cruz. The kidney is a known site of action for the VDR and kidney lysates are commonly recommended as a positive control for VDR detection. We successful detected the VDR using all three antibodies. All three antibodies were successful in detecting the VDR following tissue lysis with either the urea or sucrose lysis buffer (Fig. 3.3A).

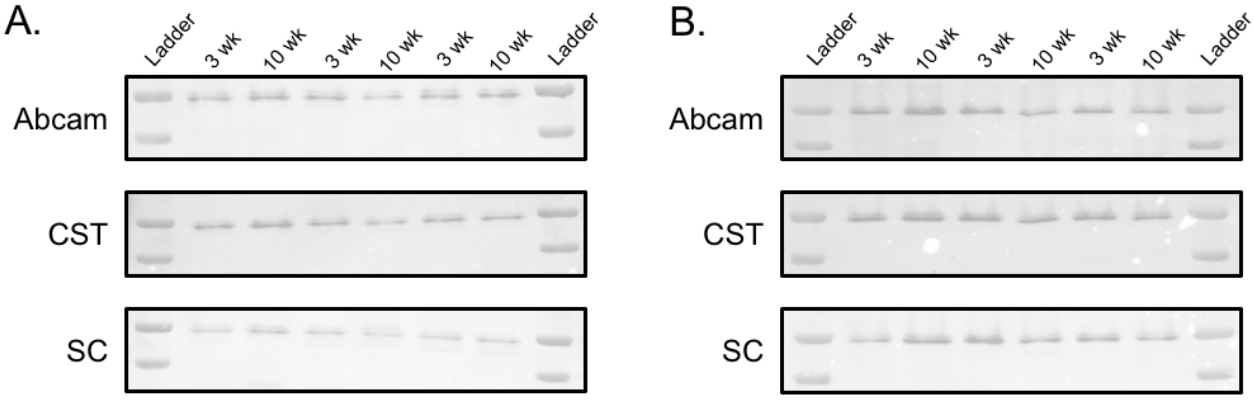

Figure 3.3. Successful detection of the VDR in mouse kidney. A) Successful detection of the VDR in 3- and 10-week old mouse kidney samples lysed in sucrose lysis buffer. B) Successful detection of the VDR in 3 and 10-week old mouse kidney samples lysed in a urea lysis buffer. Ladders represent 50 and 37 kDa.

3.3.4 Detection of the VDR in Mouse Gastrocnemius

The detection of the VDR in gastrocnemius lysates was unsuccessful whether samples were lysed in sucrose (Fig. 3.4A) or urea lysis buffer (Fig. 3.4B). Antibodies from Abcam, Cell Signaling Technology and Santa Cruz were unable to detect the VDR in 3- and 10-week old mouse gastrocnemius lysates despite loading ~75 μg of protein. A mouse kidney sample was used as a positive control (10 μg) suggesting that the immunoblotting procedure was successful.

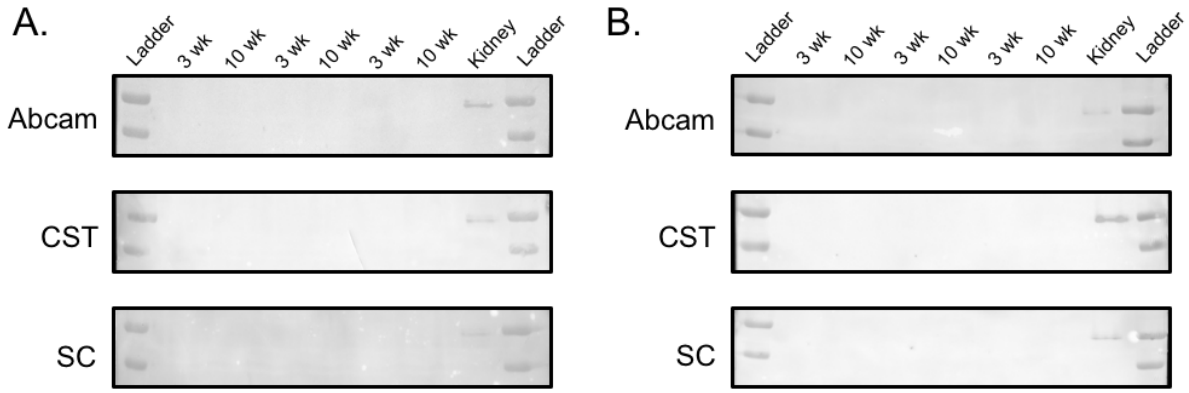

Figure 3.4. Unsuccessful detection of the VDR in mouse gastrocnemius. A) Unsuccessful detection of the VDR protein in 3- and 10-week old mouse gastrocnemius when lysed in sucrose lysis buffer. B) Unsuccessful detection of the VDR protein in 3- and 10-week old gastrocnemius when lysed in a urea lysis buffer. Ladders represent 37 and 50 kDa.

3.3.5 Detection of the VDR in Mouse Quadriceps

We also aimed to detect the VDR in mouse quadriceps samples following lysis in sucrose and urea lysis buffers. Both lysis buffers combined with the use of antibodies from Abcam, Cell Signaling Technology and Santa Cruz were unsuccessful in VDR detection within mouse quadriceps samples despite loading ~75 μg of protein (Fig. 3.5A-B). A mouse kidney sample was utilised in order to act as a positive control (10 μg).

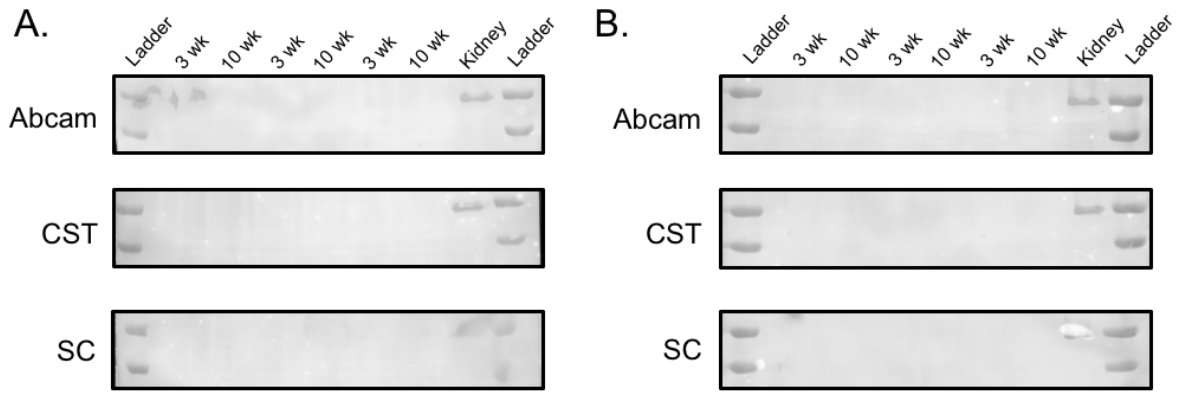

Figure 3.5. Unsuccessful detection of the VDR in mouse quadriceps. A) Unsuccessful detection of the VDR in mouse quadriceps lysed in sucrose lysis buffer. B) Unsuccessful detection of the VDR protein in mouse quadriceps lysed in urea lysis buffer. Ladder represents 37 and 50 kDa.

3.4 Discussion

The detection of the VDR within skeletal muscle samples is a contentious issue. Whilst some have reported positive VDR expression [9], others have reported a lack of VDR protein expression within skeletal muscle samples [11, 12]. *In vitro*, a prominent role for the VDR in skeletal muscle proliferation, differentiation and myogenesis has been reported [19]. In addition, both whole-body and skeletal muscle specific VDR-KO mouse models display an altered skeletal muscle phenotype [17, 23, 30-32]. Although the current evidence points towards a significant role for the VDR within skeletal muscle development and function, its detection within skeletal muscle is problematic. This primarily caused by low expression levels, the multicellular nature of skeletal muscle, differences in VDR expression across development and the use of non-specific antibodies [29].

Similar to previous studies, we report a successful detection of the VDR protein within C2C12 myoblasts [18, 19, 22, 33]. In order to confirm specificity of our antibodies, we also utilised a VDR-KD C2C12 cell line in which the protein content of the VDR is reduced following lentivirus mediated shRNA interference. The Santa Cruz D6 antibody has previously been reported to be highly specific for the VDR [29] however, we also show that antibodies from both Abcam and Cell Signaling Technologies also result in a robust detection of the VDR with C2C12 myoblasts and myotubes.

Whilst the detection of the VDR within skeletal muscle cell lines is reliable, its detection in whole skeletal muscle lysates is more contentious [11]. In order to detect

the VDR, the use of a HLB has previously been recommended [9]. This buffer contains high amounts of urea which aids in separating the VDR from its tight binding to DNA [25, 26]. Given that the kidney is a known site of positive expression for the VDR [29] we first sought to detect the VDR in mouse kidney samples. It should be noted that prior analysis and optimisation experiments were conducted comparing blocking reagents as well as multiple concentrations of primary (1:1000 & 1:500) and secondary (1:10,000 & 1:20,000) antibodies. The detection of the VDR was successful using antibodies from Abcam, Cell Signaling Technology and Santa Cruz irrespective of lysis buffer used. Finally, we sought to detect the VDR in whole skeletal muscle lysates from both young and adult mice. Within skeletal muscle, the VDR has been proposed to play a more prominent role within the early stages of muscle development [9]. Previously, the protein content of the VDR has been reported to decrease when comparing skeletal muscle samples from new-born, 3-week and 3-month old C57BL/6J mice [9]. Therefore, we also aimed to detect the VDR with skeletal muscle samples from both 3 and 10-week old C57BL/6J mice. Similar to others [12], we were unable to detect the VDR with skeletal muscle samples derived from the gastrocnemius and quadriceps irrespective of age. The detection of the VDR was unsuccessful irrespective of antibody or lysis buffer utilised. Although others have detected the VDR within skeletal muscle samples using similar methods [9], were unable to detect the VDR despite loading ~75 μg of protein and using primary antibodies at a concentration of 1:500.

Whilst some have reported a positive detection of the VDR within skeletal muscle [9], we and others [12] have been unable to replicate such findings. The results

described within this chapter only raise further questions in regards to the relevance of the VDR with skeletal muscle *in vivo*. If the VDR is not expressed within adult skeletal muscle then the positive effects of vitamin D and the skeletal muscle phenotypes arising in VDR-KO animals may result indirectly from alterations in mineral homeostasis. However, in support of a direct role for the VDR within skeletal muscle, skeletal muscle specific VDR-KO mice do display an alerted phenotype [30, 31]. Clearly, further research is needed to understand the role of the VDR within skeletal muscle and the development of more reliable methods to detect the VDR would be a significant addition to the field. A more comprehensive comparison of sample lysis and immunoblot protocols alongside the use of multiple antibodies would further the development of a protocol for VDR detection within skeletal muscle. Purification and subcellular fraction approaches may aid in the detection of the VDR in whole skeletal muscle lysates however, purification of the nuclear fraction also resulted in the unsuccessful detection of the VDR [12]. In addition, the analysis of VDR mRNA expression in the same samples utilised within this chapter may offer further insight into the relative expression levels of the VDR. Given the extremely low expression levels of the VDR within skeletal muscle, current methods may not be sensitive enough to reliably detect the VDR within whole skeletal muscle lysates on a consistent basis.

3.5 References

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CHAPTER 4

THE ROLE OF THE VITAMIN D RECEPTOR IN REGULATING MITOCHONDRIAL FUNCTION IN C2C12 CELLS AND RAT SKELETAL MUSCLE

4.1 Introduction

Vitamin D deficiency is characterised by serum 25(OH)D levels of <50 nmol.L^{-1} [1] Based on these numbers, it has been reported that approximately 40% of adults in both the USA [2] and Europe [3] can be classified as deficient, with 13% of Europeans deemed severely deficient [3]. The classical actions of vitamin D are well established, primarily functioning to maintain calcium and phosphate balance in order to prevent rickets [4], osteomalacia [5] and osteoporosis [6]. Vitamin D carries out its actions via its active metabolite 1α,25$(OH)_2D_3$ which binds to the ubiquitously expressed VDR [7]. Part of the nuclear receptor superfamily, the VDR together with its binding partner RXRα recruits both coactivators or repressors to exert its effects on genomic transcription [8, 9].

As well as its role in the maintenance of bone health, vitamin D has been shown to play a much wider role within the body, and more specifically, within skeletal muscle [10]. Multiple observational studies have reported positive associations between serum 25(OH)D levels and lower extremity muscle strength and function in older individuals [11-13], whilst those with serum concentrations of <25 nmol.L^{-1} are at a two-fold greater risk of developing sarcopenia [14]. Additionally, a prominent feature of vitamin D deficiency is proximal muscle weakness [15, 16] and chronic deficiencies are often accompanied by severe skeletal muscle myopathies which can be rescued via vitamin D supplementation [17]. Furthermore, markers of skeletal muscle function, including grip strength, grip endurance and sprint speed, are impaired in mouse models of vitamin D deficiency [18, 19].

Given that vitamin D exerts its biological actions through binding to the VDR, multiple studies have sought to elucidate the role of the VDR specifically within skeletal muscle. Investigations utilising multiple mouse models with the global deletion of the VDR have identified skeletal muscle-specific actions [20-23]. From an early age, muscle fibre atrophy, reduced grip strength and impaired motor function are all present within VDR-KO mice [18, 24]. However, the global deletion of the VDR results in a dysregulation of both calcium and phosphate homeostasis [20, 21, 23]. Given the importance of intracellular calcium and phosphate in skeletal muscle contraction [25, 26] and ATP production [27], a dysregulation in mineral homeostasis could mediate the observed impairments in skeletal muscle function indirectly within VDR-KO mice. Although, despite the administration of a rescue diet containing increased calcium and phosphate, the observed impairments in muscle function persist, suggesting a direct role for the VDR within skeletal muscle [20]. More recently, skeletal muscle specific VDR-KO mice have been developed [28, 29]. These mice exhibited a number of metabolic defects including increased serum insulin levels, insulin resistance, glucose intolerance, slight decreases in muscle fibre size [28] as well as reductions in voluntary wheel running capacity and lean mass [29].

Evidence is now emerging to suggest that the deleterious effects of vitamin D deficiency within skeletal muscle may be mediated by impairments in mitochondrial energy metabolism [30, 31]. In support of this, the repletion of vitamin D status in severely deficient humans resulted in improvements in oxidative phosphorylation (assessed via P-31 MRS) as well as reductions in symptoms of fatigue [30].

Additionally, the treatment of human primary skeletal muscle myoblasts and C2C12 myotubes with vitamin D metabolites resulted in an increase in mitochondrial respiration [32-34]. Furthermore, vitamin D treatment of human primary myoblasts increased mitochondrial volume (as assessed via MitoTracker staining and confocal microscopy), as well as increasing the expression of >80 mRNAs encoding for mitochondrial proteins [32]. Whilst the VDR was not detectable within the mitochondria of human skeletal muscle primary myoblasts [32], it is expressed within the mitochondria of other cell types [35, 36] making the precise role of the VDR within the mitochondria unclear. Whilst these data highlight a potential role for vitamin D in modulating mitochondrial function, they do not directly examine the precise role of the VDR in mediating these changes. Although a reduction in the VDR was reported to abolish the $1\alpha,25(OH)_2D_3$ mediated increases in mitochondrial respiration it was not the primary focus of the study [32]. Additionally, the knock-down of the VDR was achieved by siRNA approaches which are transient and the effects of a reduction in the VDR across skeletal muscle development cannot be established.

Taken together, these data suggest a role for vitamin D, $1\alpha,25(OH)_2D_3$ and the VDR in the maintenance of skeletal muscle mitochondrial function. However, the specific role of the VDR within mitochondrial regulation in skeletal muscle remains largely underexplored. Therefore, the aims of this chapter were as follows; 1) Examine whether stable knock-down of the VDR within C2C12 cells affects mitochondrial respiration across myogenesis 2) determine how loss of VDR affects mitochondrial protein content across myogenesis 3) determine whether manipulating VDR expression in rat skeletal muscle alters mitochondrial protein content.

4.2 Materials and Methods

4.2.1 Tissue Culture

All tissue culture experiments were carried out under sterile conditions in a class II safety cabinet. Cell cultures were maintained in a HERAcell 150i CO_2 Incubator (Thermo Scientific Inc., Chesire, UK) maintained at 37°C with 5% CO_2. Growth medium consisted of DMEM with added GlutaMAX^{TM} and glucose (4.5 g.L^{-1}) as well as the addition of 10% FBS, 1% PS and 2 μg.ml^{-1} puromycin. Differentiation medium consisted of DMEM with added GlutaMAX^{TM} and glucose (4.5 g.L^{-1}) as well as the addition 2% HS and 1% PS.

4.2.2 shRNA Mediated Knock-Down of the Vitamin D Receptor

The lentiviral plasmid used (pLKO.1 backbone) was designed in-house and was based on (Clone ID: RMM3981-201757375) and targeted the (3' UTR) mouse sequence 5′- TTA AAT GTG ATT GAT CTC AGG-3′ of the mouse *Vdr* gene; the scramble shRNA was used as a negative control as previously reported [37] with a hairpin sequence: CCT AAG GTT AAG TCG CCC TCG CTC TAG CGA GGG CGA CTT AAC CTT AGG (Addgene plasmid 1864, Cambridge, MA, USA). Oligos were obtained from ITDDNA USA (Integrated DNA Technologies, Inc. Iowa, USA) and suspended, annealed and cloned into pLKO.1 at EcoRI and AgeI restriction sites as per the pLKO.1 protocol from Addgene. The resultant plasmids were transformed in DH5α cells for amplification and isolated. The actual DNA sequence was confirmed at the Pennsylvania State University College of Medicine DNA sequence core facility. Packaging plasmids psPAX2 and envelope protein plasmid pMD2.G were a gift from Trono Lab Addgene plasmids 12260 and 12259 respectively. HEK293FT cells

(Invitrogen, Carlsbad, CA, USA) were grown in DMEM; 80–85% confluent plates were rinsed once with Opti-MEM (Invitrogen, Carlsbad, CA, USA) and then incubated with Opti-MEM for 4 h before transfections. psPAX2 and pMD2.G along with either scramble or pLKO.1 clones targeting mouse Vdr (three clones) were added after mixing with Lipofectamine 2000 as per the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Opti-MEM was changed after overnight incubation with DMEM containing 10% FBS without antibiotics to allow cells to take up the plasmids and recover. Culture media were collected at 36 and 72 h post-transfection for viral particles. Viral particles present in the supernatant were harvested after a 15-min spin at 1,500 *g* to remove cellular debris. The supernatant was further filtered using a 0.45-μm syringe filter. Supernatant-containing virus was either stored at −80°C for long-term storage or at 4°C for immediate use. C2C12 cells at 60% confluence were infected twice overnight with 3 ml of viral supernatant containing 8 $\mu g.ml^{-1}$ polybrene in serum-free–antibiotic-free DMEM. Fresh DMEM media containing 10% FBS, antibiotics and 2 $\mu g.ml^{-1}$ puromycin (Sigma, St. Louis, MO, USA) were added the next day. Cells that survived under puromycin selection were either harvested (as stable cells) and stored in liquid N_2 or used as either myoblasts or myotubes depending on the experiment.

4.2.3 Extracellular Flux Analysis

Both scramble control and VDR-KD cells were seeded in XFe24-well cell culture microplates (Seahorse Bioscience, North Billerica, USA) at 3.0 x 10^5 cells/well in 100 μl of growth medium and allowed to rest at room temperature for 1 h in order to promote an even cell distribution [38]. For myoblast experiments, cells were

incubated at 37°C and 5% CO_2 for 3 h in order to allow sufficient time for adherence to cell culture microplate and subsequently assayed. For myotube experiments, cells were incubated for a period of 24 h, resulting in a confluence of 90%. Cells were then washed once with PBS and medium changed to differentiation medium. Differentiation media was changed every other day for a period of 7 days. Prior to the assay, cells were washed once and placed in 500 µl of Seahorse XF Base Medium (glucose 10 mM, sodium pyruvate 1 mM, glutamine 1 mM, pH 7.4) pre-warmed to 37°C. The plate was then transferred to a non-CO_2 incubator for 1 h. Following calibration, cell respiratory control [39] and associated extracellular acidification were assessed following the sequential addition of oligomycin (1 µM), CCCP (1 µM) and antimycin A with rotenone (1 µM). Upon completion of the assay, cells were collected in sucrose lysis buffer and subjected to one freeze thaw cycle. Upon thawing, cell lysate was centrifuged for 10 minutes at 8,000 *g* and the supernatant was removed for protein determination. Protein concentration was determined using the DC protein assay (Bio-Rad, Hercules, CA). OCR is reported relative to protein content (pmol/min/µg).

4.2.4 Estimation of ATP Production

Estimation of intracellular rates of glycolytic and oxidative ATP production were performed as previously described [40]. Briefly, scramble control and VDR-KD cells were seeded in XFe24-well cell culture microplates (Seahorse Bioscience, North Billerica, USA) at 3.0 x 10^5 cells/well in 100 µl of growth medium (high glucose DMEM, 10% FBS, 1% penicillin/streptomycin, puromycin 2µg.ml^{-1}). Prior to the assay cells were washed once and then incubated in 500 µl of KRPH medium (2

mM HEPES, 136 mM NaCl, 2 mM NaH_2PO_4, 3.7 mM KCl, 1 mM $MgCl_2$, 1.5 mM$CaCl_2$, 0.1% (w/v) fatty-acid-free BSA, pH 7.4 at 37 °C). Cell respiratory control [39] and extracellular acidification were assayed following the addition of glucose (10 mM), oligomycin (1 μM), CCCP (1 μM) and antimycin A with rotenone (1 μM). Upon completion of the assay cells were collected in sucrose lysis buffer (50 mM Tris pH 7.5; 270 mM sucrose; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 50 mM sodium fluoride; 5 mM sodium pyrophosphate decahydrate; 25 mM beta-glytcerolphosphate; 1 cOmplete™ protease inhibitor cocktail EDTA free tablet) and centrifuged for 10 minutes at 8,000 *g* and the supernatant was removed for protein determination. Protein concentration was determined using the DC protein assay (Bio-Rad, Hercules, CA). Rates of ATP production are reported relative to protein content (pmol/min/μg).

4.2.5 Measurement of Mitochondrial Membrane Potential

Both scramble control and VDR-KD cells were plated at 1.0 x 10^5 cells/well in 100 μl of growth medium and incubated for 24 h. A black 96-well plate with a clear bottom (Corning, Costar, NY, USA) was used in order to minimise well-to-well interference. Cells were subsequently incubated for 30 minutes with 100 nM of TRME. A set of cells were also incubated with 20 μM FCCP in order to completely depolarise the mitochondrial membrane and act as a negative control. Following incubation cells were washed with PBS with 0.2% BSA and then read at 549 nm using a CLARIOstar microplate reader (BMG Labtech, Victoria, Australia) in 100 μl of PBS and 0.2% BSA.

4.2.6 Transient Electroporation in Male Wistar Rats

Eight-week-old male Wistar rats were housed as described above (section 2.1.1) and fed a standard chow diet (18% fat, 33% protein, 48% carbohydrate) for one week. VDR-KD (n=7) was achieved in right hind leg *tibialis anterior* muscle through *in vivo* electrotransfer of four unique rat VDR pGFP-C-shLenti plasmid constructs driven by a U6 promoter [41]. These constructs spanned multiple regions of rat VDR mRNA (NM_017058.1) to ensure effective coverage and knockdown. Left leg *tibialis anterior* muscles received scramble cassette sequence controls (OriGene, Rockville, USA). Plasmid DNA amplification was undertaken within JM109 *Escherichia coli* cells under puromycin selection (0.5 μg/ml) overnight. Plasmid DNA was purified using an endotoxin free plasmid Maxi Kit (Qiagen, Manchester, UK) as per the manufacturers instructions. VDR-OE (n=7) was achieved in right leg *tibialis anterior* muscle through in vivo electrotransfer of pCAGGS-mVDR vector constructs. The mouse cDNA sequence utilised was 92% homologous to rat VDR mRNA. To obtain pCAGGS-mVDR, myc-DDK-mVDR was subcloned in the pCAGGS vector. Left leg *tibialis anterior* muscles received empty pCAGGS vector controls. Plasmid DNA amplification was undertaken within JM109 *Escherichia coli* cells under puromycin selection (0.5 μg/ml) overnight. Plasmid DNA was purified using an endotoxin free plasmid Maxi Kit (Qiagen, Manchester, UK) as per the manufacturers instructions.

4.2.7 Immunoblotting

Total protein lysates of a known concentration were mixed 3:1 with 4x Laemmli sample loading buffer to generate polyacrylamide gel loading samples. Prior to gel loading, samples were boiled for 5 minutes. An equal volume of protein (10-30 μg) was separated by SDS-PAGE on 8-15% gels at a constant current of 23 mA per gel. Proteins were then transferred on to BioTrace NT nitrocellulose membranes (Pall Life Sciences, Pensacola, Florida, USA) using a wet transfer system at 100 V for 1 h. Membranes were then stained in Ponceau S (Sigma-Aldrich, Gillingham, UK) and imaged to check for even loading and transfer. Membranes were blocked for 1 h in 3% dry-milk in TBS-T prior to overnight incubation in primary antibodies at 4°C. The following day, membranes were washed three times in TBS-T and subsequently incubated in the appropriate horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Antibody detection was performed with an enhanced chemiluminescence horseradish peroxidase substrate detection kit (Millipore, Watford, UK). Subsequent imaging and band quantification were performed using the G:Box Chemi-XR5 system (Syngene, Cambrige, UK).

4.2.8 Antibodies

All primary antibodies were used at a concentration of 1:1000 in TBS-T unless otherwise stated. Antibodies for dynamin-1-like protein (DRP1;8570) and vitamin D3 receptor (VDR; 12550) were from Cell Signaling Technology; MitoProfile OXPHOS antibody cocktail (110413), vitamin D receptor (VDR; 109234) and mitofilin (110329) were from Abcam; dynamin-like 120 kDa protein (OPA1; CPA3687) was from BD Biosciences; peroxisome proliferator-activated receptor gamma coactivator 1-alpha

(PGC-1α; AB3242) was from Merck-Millipore; citrate synthase (CS; SAB2701077) and mitochondrial fission protein 1 (FIS1; HPA017430) were from Sigma Aldrich; vitamin D receptor (D-6) (VDR; 13133) was from Santa Cruz Biotechnology. Secondary antibodies were used at a concentration of 1:10,000 in TBS-T. Anti-mouse (7076) and anti-rabbit (7074) were from Cell Signaling Technology.

4.2.9 Statistical Analysis

Statistical analysis was performed using the SPSS version 24.0. Differences between scramble control and VDR-KD C2C12s were determined by independent t-tests and two-way analysis of variance (ANOVA) where appropriate, with Bonferroni correction for multiple comparisons. Differences between control and VDR-KD or VDR-OE legs were determined by independent t-tests. All data is presented as mean ± SD. Statistical significance was set at $P < 0.05$.

4.3 Results

4.3.1 Knock-Down of the Vitamin D Receptor

Following shRNA interference, VDR protein content was reduced by ~96 and ~94 % within C2C12 myoblasts (Fig. 4.1, A and B) and myotubes (Fig. 4.1, C and D) respectively. VDR protein content was significantly reduced in myoblasts when detected with antibodies from Abcam ($P < 0.001$), CST ($P < 0.001$) and Santa-Cruz ($P < 0.001$). Similar reductions were observed in myotubes with antibodies from Abcam ($P < 0.001$), CST ($P < 0.001$) and Santa-Cruz ($P < 0.001$).

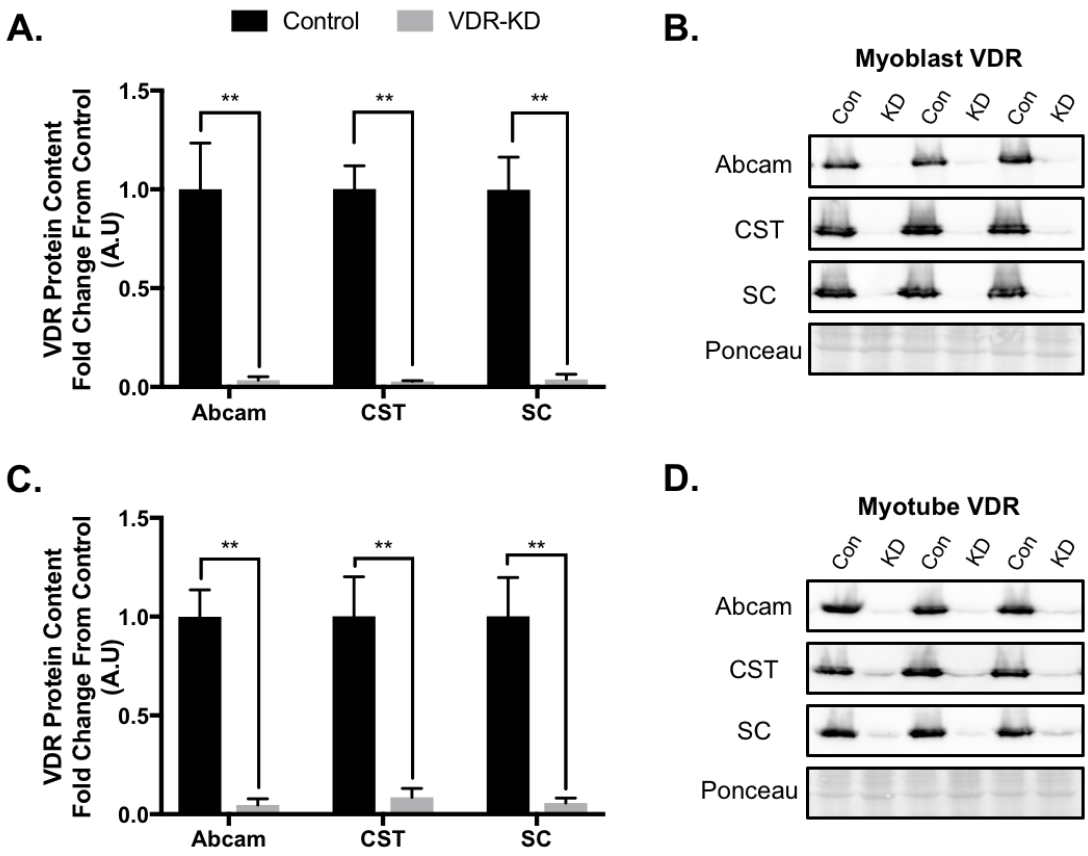

Figure 4.1. Successful knock-down of the VDR in the C2C12 skeletal muscle cell line. A) Knock-down of the VDR in C2C12 skeletal muscle myoblasts (n = 6/group). B) Representative images of VDR protein abundance in control and VDR-KD C2C12 myoblasts. C) Knock-down of the VDR in C2C12 skeletal muscle myotubes (n = 6/group). D) Representative images of VDR protein abundance in control and VDR-KD C2C12 myotubes. Data mean ± SD. ***P* < 0.005. *Repeat of Figures 3.1 and 3.2.*

4.3.2 Extracellular Flux Analysis

VDR-KD within C2C12 myoblasts reduced basal respiration by 30% (Control 9.86 ± 3.41 vs. VDR-KD 6.93 ± 1.03 pmol/min/µg, *P* = 0.034), in addition to reducing coupled respiration by 30% (Control 8.38 ± 2.69 vs. VDR-KD 5.87 ± 0.56 pmol/min/µg., *P* = 0.023). Further, VDR-KD reduced maximal respiration by 36% (Control 30.76 ± 10.42 vs. VDR-KD 19.65 ± 1.24 pmol/min/µg, *P* = 0.013) and reduced spare capacity by 39% (Control 20.90 ± 7.01 vs VDR-KD 12.72 ± 1.17 pmol/min/µg, *P* = 0.008) when compared to control (Fig. 4.2, A and C). Similarly,

VDR-KD within C2C12 myotubes reduced basal respiration by 34% (Control 17.06 ± 2.76 vs. VDR-KD 11.22 ± 1.24 pmol/min/µg, $P < 0.001$), as well as reducing coupled respiration by 33% (Control 11.40 ± 2.10 vs. VDR-KD 7.69 ± 0.96 pmol/min/µg, $P < 0.001$). Furthermore, VDR-KD in C2C12 myotubes also reduced maximal respiration by 48% (Control 58.89 ± 9.83 vs. VDR-KD 30.86 ± 6.04 pmol/min/µg, $P < 0.001$) and spare capacity by 53% (Control 41.83 ± 7.45 vs. VDR-KD 19.65 ± 5.06 pmol/min/µg, $P < 0.001$) when compared to control (Fig. 4.2, B and D). Whilst proton leak remained unchanged in myoblasts (Control 1.49 ± 0.74 vs. VDR-KD 1.06 ± 0.62 pmol/min/µg, $P > 0.05$) (Fig. 4.2C), VDR-KD resulted in a decrease in proton leak by 67% in myotubes (Control 2.78 ± 0.56 vs. VDR-KD 0.91 ± 0.35 pmol/min/µg, $P < 0.001$) (Fig. 4.2D).

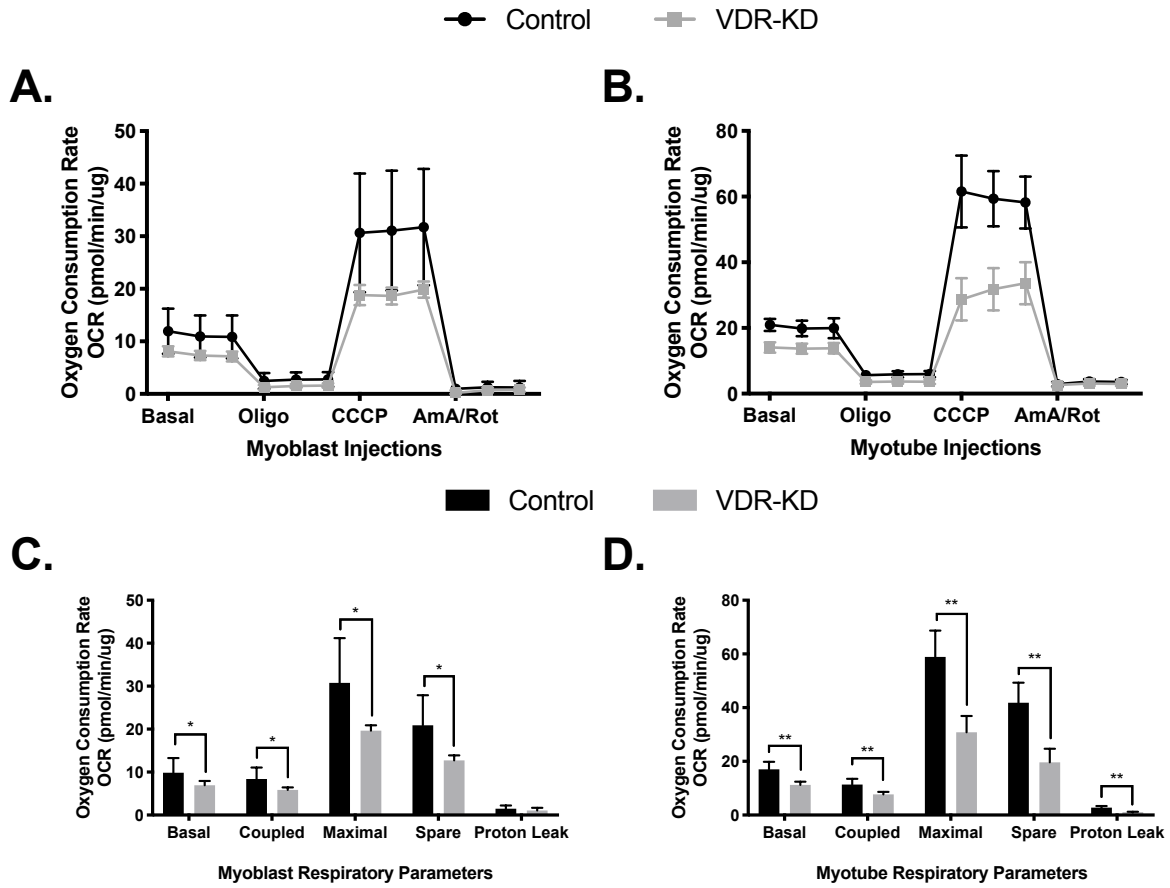

Figure 4.2. Knock-down of the VDR in C2C12 myoblasts and myotubes reduces mitochondrial respiration as measured by extracellular flux. A-B) Mitochondrial stress test traces from both control and VDR-KD myoblasts and myotubes (n = 9-10/group). C-D) Respiratory parameters derived from the mitochondrial stress test from both control and VDR-KD myoblasts and myotubes (n =9-10/group). Data mean ± SD. *P < 0.05, **P < 0.005.

4.3.3 Estimation of ATP Production

VDR-KD reduced total ATP production by 18% in C2C12 myoblasts (Control 55.15 ± 8.13 vs. 45.03 ± 3.01 pmolATP/min/µg, P = 0.002) when compared to controls (Fig. 4.3A). In addition, estimated ATP production derived from oxidative phosphorylation (ATP_{ox}) was reduced by 20% in VDR-KD myoblasts (Control 46.13 ± 8.41 vs. VDR-KD 36.87 ± 3.53 pmolATP/min/µg, P = 0.007). ATP production derived from glycolysis (ATP_{glyc}) on the other hand remained unchanged (Control 9.04 ± 4.29 vs. 8.13 ± 2.76 pmolATP/min/µg, P > 0.05) (Fig. 4.3A).

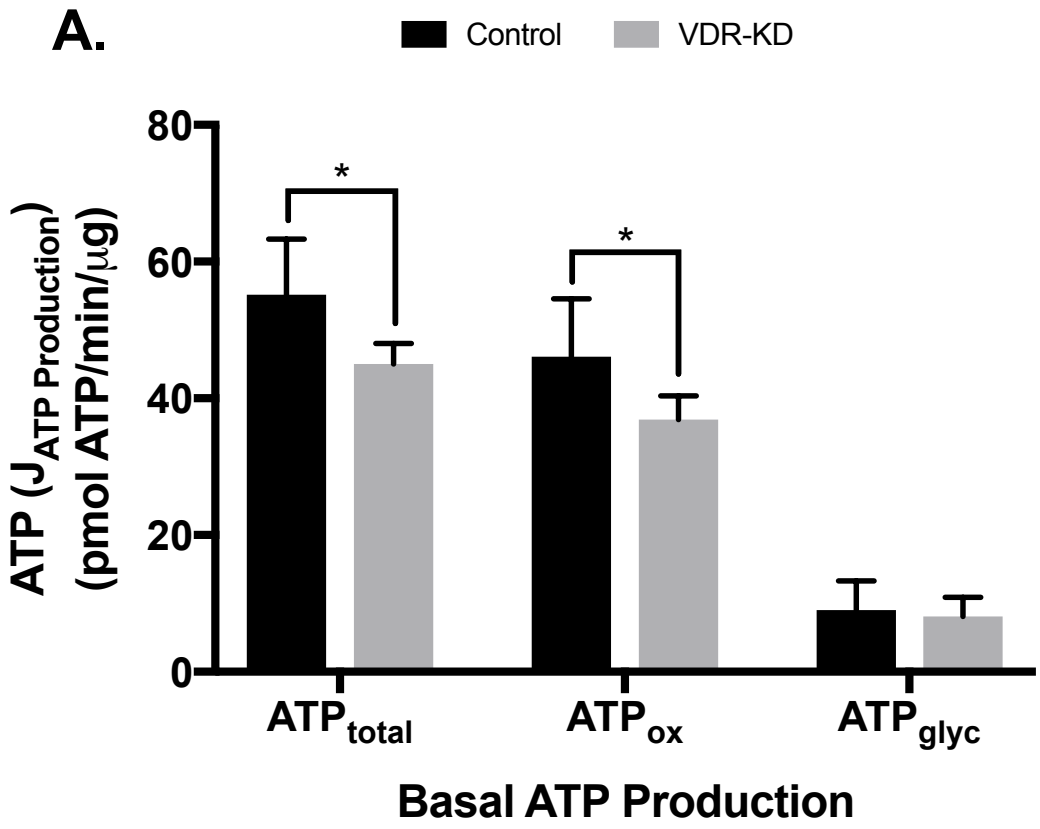

Figure 4.3. Reduction in ATP production via oxidative phosphorylation as opposed to glycolysis following VDR-KD in C2C12 myoblasts. A) Total ATP production and ATP production derived from oxidative and glycolytic means in control and VDR-KD myoblasts (n = 10/group). Data mean ± SD. *P < 0.05.

4.3.5 Membrane Potential

No main effect for group (P > 0.05) was observed for mitochondrial membrane potential although there was a main effect for treatment (P < 0.001) and group X treatment interaction (P = 0.001) indicating treatment with FCCP successfully depolarised the mitochondrial membrane. Post-hoc comparisons revealed a significant reduction in mitochondrial membrane in basal conditions (P = 0.001) when VDR-KD cells were compared with controls (Fig. 4.4A).

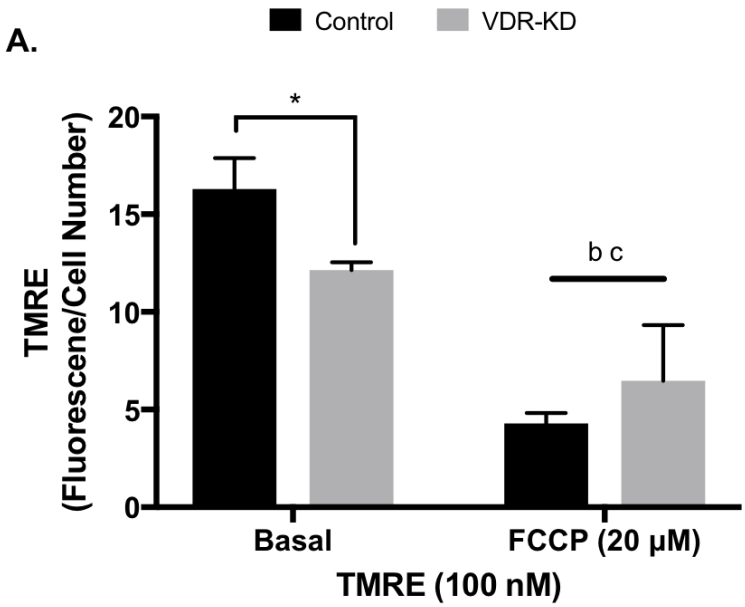

Figure 4.4. Mitochondrial membrane potential is reduced in VDR-KD C2C12 myoblasts. A) Mitochondrial membrane potential assessed via TMRE in both basal control and VDR-KD myoblasts as well as with the pre-treatment with FCCP (n = 5/group). Data mean ± SD. *P < 0.05, [b]main effect for treatment, [c]main interaction effect.

4.3.5 Mitochondrial Related Protein Content

No differences were observed in mitochondrial protein content including CI (NDUFB8), CII (SDHB), CIV (MTCO1) and CV (ATP5A), citrate synthase and cytochrome c following the knock-down of the VDR in both C2C12 myoblasts and myotubes (Fig 4.5. A-D., P > 0.05).

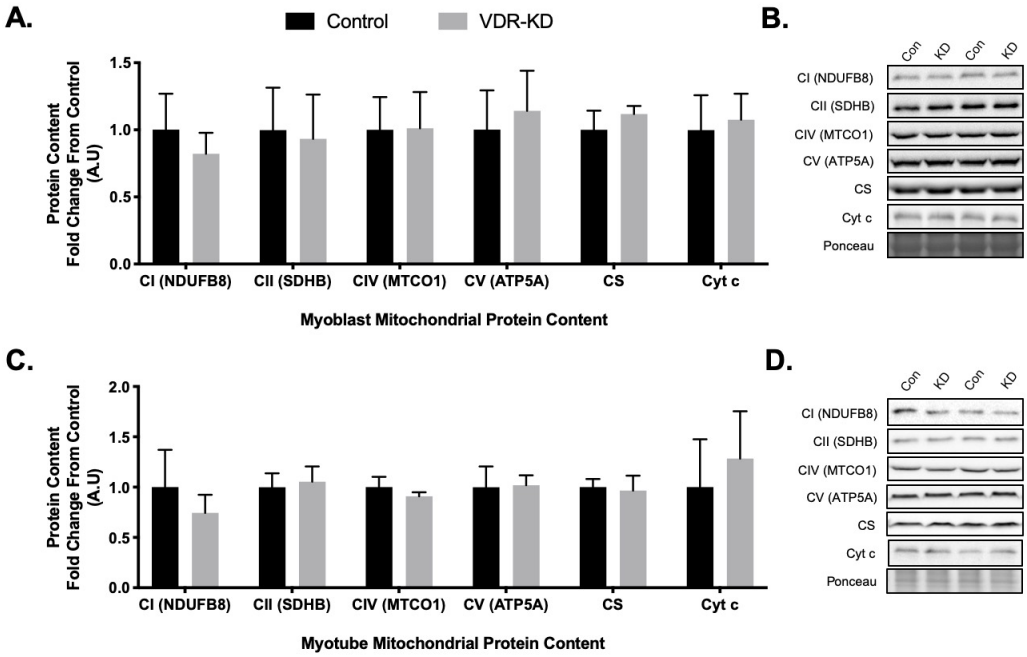

Figure 4.5. No change in markers of mitochondrial protein content following VDR-KD in both C2C12 myoblasts and myotubes. A) Protein abundance of mitochondrial electron transport chain subunits, CS and cyt c in control and VDR-KD C2C12 myoblasts (n = 6/group). B) Representative images of markers of mitochondrial protein content in control and VDR-KD C2C12 myoblasts. C) Protein abundance of mitochondrial electron transport chain subunits, CS and cyt c in control and VDR-KD C2C12 myotubes (n = 6/group). D) Representative images of markers of mitochondrial protein content in control and VDR-KD C2C12 myotubes. Data mean ± SD and represented as a fold change from control.

4.3.6 Mitochondrial Fusion and Fission Related Protein Content

Mitochondrial fusion related proteins MFN2 and mitofilin were unchanged following VDR-KD in both myoblasts and myotubes (Fig 4.6. A-D., $P > 0.05$). However, the mitochondrial fusion related protein, OPA1, was greater compared to CON by ~15% in both VDR-KD myoblasts (Fig. 4.6A-B., $P = 0.021$) and myotubes (Fig. 4.6C-D., $P = 0.046$). Mitochondrial fission related proteins Fis 1 and DRP1 remained unchanged in both VDR-KD myoblasts and myotubes (Fig. 4.6A-D., $P > 0.05$).

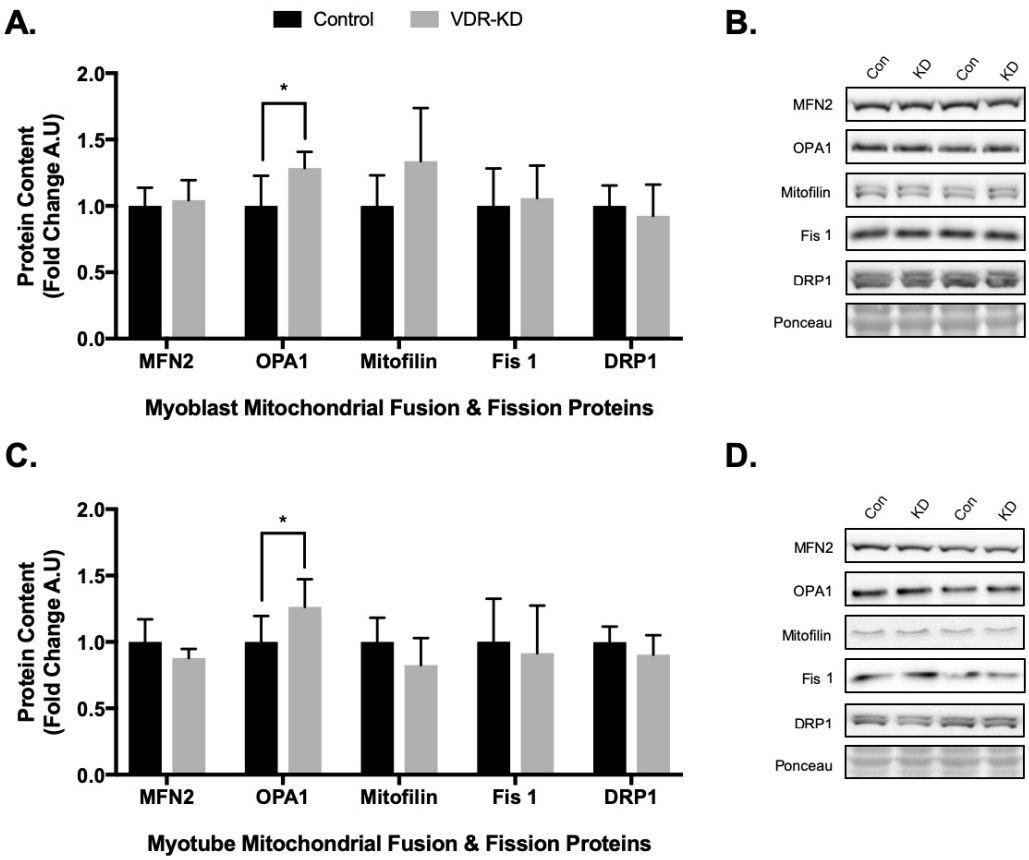

Figure 4.6. Knock-down of the VDR in C2C12 myoblasts and myotubes results in an increase in OPA1 protein abundance but no change in markers of mitochondrial fission. A) Protein abundance of markers of mitochondrial fusion and fission in control and VDR-KD C2C12 myoblasts (n = 6/group). B) Representative images of markers of mitochondrial fusion and fission in control and VDR-KD C2C12 myoblasts. C) Protein abundance of markers of mitochondrial fusion and fission in control and VDR-KD C2C12 myotubes (n = 6/group). D) Representative images of markers of mitochondrial fusion and fission in control and VDR-KD C2C12 myotubes. Data mean ± SD and reported as a fold change from control. *P < 0.05.

4.3.7 Mitochondrial Related Protein Content in VDR-KD Rat Skeletal Muscle.

VDR-KD in rat skeletal muscle via electroporation resulted in no changed in markers of mitochondrial protein content (Fig 4.7. A-B, P > 0.05). Trends were observed for decreases in the content of mitochondrial CI (NDUFB8) (P = 0.055) and CIV (MTCO1) (P = 0.056) following VDR-KD (Fig. 4.7A-B). No changes were observed in markers of mitochondrial fusion and fission when VDR-KD was compared to control leg (Fig. 4.7C-D, P > 0.05).

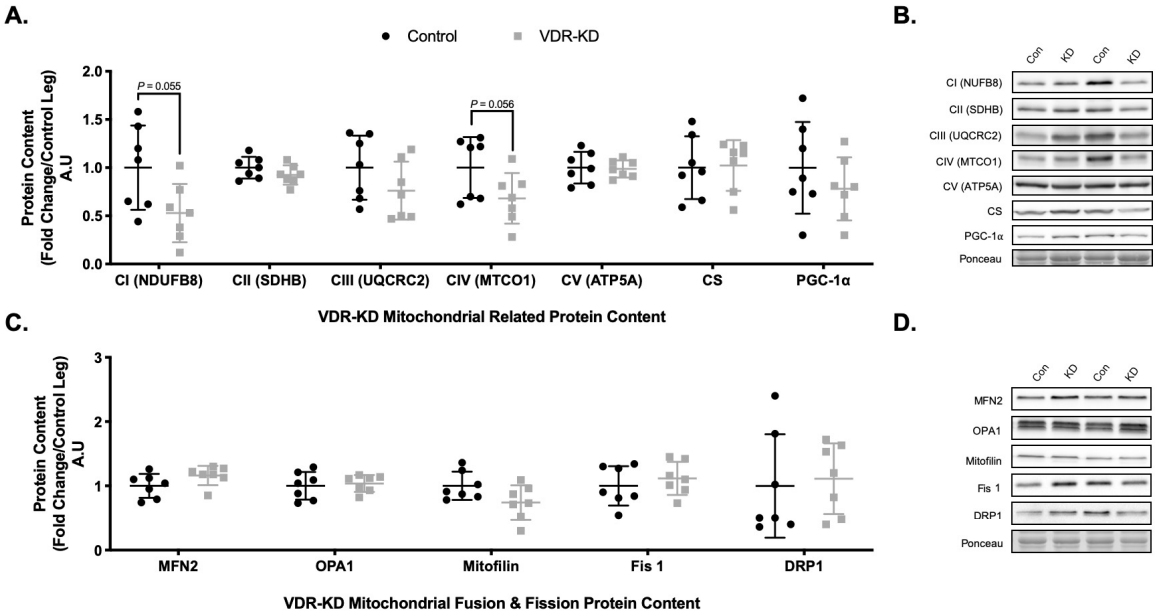

Figure 4.7. No change in markers of mitochondrial protein content, fusion or fission following the knock-down of the VDR in rat skeletal muscle. A and C) Protein abundance of markers of mitochondrial protein content, fusion and fission in the tibialis anterior following VDR-KD via electro-transfer (n = 7/group). B and D) Representative images of markers of mitochondrial protein content, fusion and fission. Data mean ± SD and reported as a fold change from control leg.

4.3.8 Mitochondrial Related Protein Content in VDR-OE Rat Skeletal Muscle

Overexpression of the VDR resulted in no change in markers of mitochondrial protein content or markers of mitochondrial fusion and fission (Fig. 4.8A-D, $P > 0.05$).

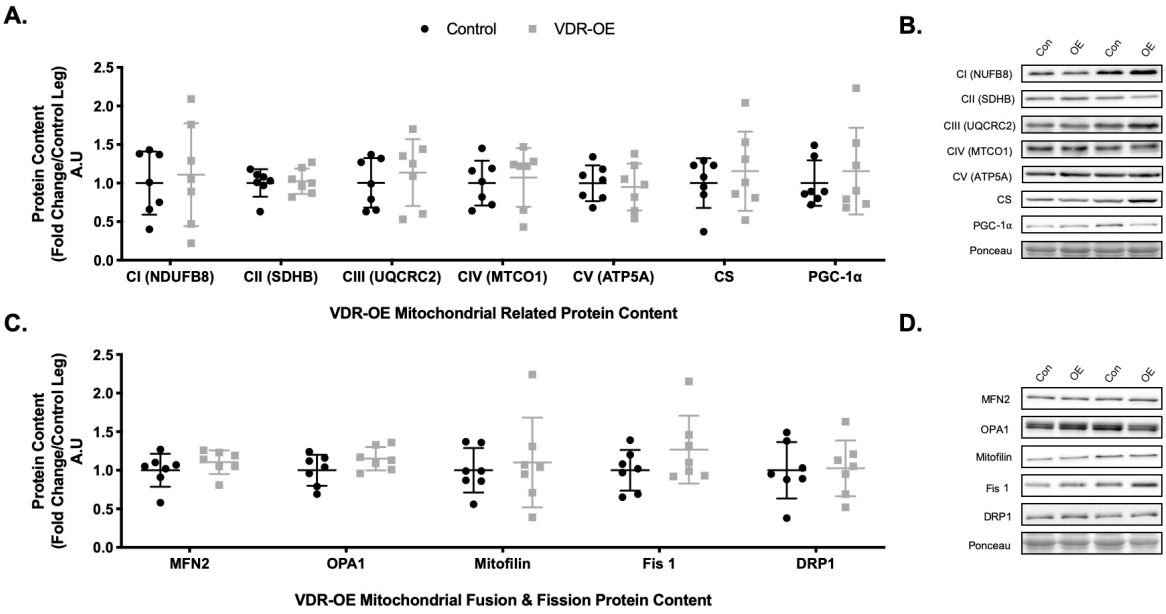

Figure 4.8. No change in markers of mitochondrial protein content, fusion and fission following over-expression of the VDR in rat skeletal muscle. A and C) Protein abundance of markers of mitochondrial protein content, fusion and fission in the tibialis anterior following VDR-OE via electro-transfer (n = 7/group). B and D) Representative images of markers of mitochondrial protein content, fusion and fission. Data mean ± SD and reported as a fold change from control leg.

4.4 Discussion

The role of vitamin D within skeletal muscle has received considerable interest in recent years and current evidence suggests that vitamin D related metabolites are able to increase mitochondrial function within skeletal muscle [30, 32-34, 42]. Building upon previous studies, we demonstrate that a loss-of-function of the VDR in C2C12 cells results in significant reductions in mitochondrial function in both myoblasts and myotubes (Fig. 4.2A-D). Impairments were specifically observed in respiration derived from oxidative phosphorylation (Fig. 4.3A) although, this was not as a result of decreases in mitochondrial protein content (Fig. 4.5A-D). Furthermore, both the knock-down and over-expression of the VDR in rat skeletal muscle resulted in no change in markers of mitochondrial protein content or fission (Fig. 4.7 & 8A-B).

Previously, difficulties in the detection of the VDR within skeletal muscle have been reported [43, 44]. Therefore, we utilised multiple antibodies including the reportedly highly specific D6 antibody in order to detect the VDR across skeletal muscle myogenesis [43, 45]. Following shRNA interference targeting the mouse VDR gene and puromycin selection, we observed a significant reduction in VDR protein content within both C2C12 skeletal muscle myoblasts and myotubes. Indicative of a successful and stable loss-of-function of the VDR. Further analysis revealed a significant reduction in mitochondrial respiration in both C2C12 myoblasts and myotubes following VDR-KD. Previously, it has been reported that the treatment of both human primary skeletal muscle myoblasts and C2C12 skeletal muscle cells with vitamin D metabolites resulted in an increase in mitochondrial respiration [32-34]. Whilst the observed increases in respiration were abolished following the siRNA mediated knock-down of the VDR in human skeletal muscle myoblasts [32], transient siRNA approaches are unable to determine the role of the VDR in mediating these changes across development. Therefore, our results build upon previous findings and indicate that mitochondrial impairments persist across development following VDR-KD in skeletal muscle. Furthermore, we utilised a recently developed assay in which mitochondrial respiration coupled to the production of ATP can be separated into ATP production derived from either oxidative phosphorylation or glycolysis [40]. We observed significant reductions in ATP production derived from oxidative phosphorylation, suggesting impairments are intrinsic to the mitochondria following VDR loss-of-function. Although the supplementation of vitamin D_3 in severely deficient individuals improves oxidative phosphorylation [30], it is yet to be

established if the loss of the VDR within skeletal muscle alters skeletal muscle mitochondrial function *in vivo*.

In order to determine whether the observed impairments in mitochondrial respiration were related to reductions in mitochondrial volume, we assessed multiple markers of mitochondrial protein content [46]. Interestingly, we observed no change in mitochondrial protein content in both myoblasts and myotubes. Therefore, the reductions in mitochondrial function following VDR loss-of-function are not mediated by a decrease in mitochondrial content *per se*. Corresponding findings have been reported previously in both human skeletal muscle myoblasts following the treatment with $1\alpha,25(OH)_2D_3$ and within the quadriceps of mice with a myocyte specific deletion of the VDR, both resulting in no change in the protein content of members of the electron transport chain [29, 32]. Similarly, we observed no changes in mitochondrial protein content in rat skeletal following both VDR-KD and VDR-OE. Although, we did observe trends for a reduction in both complex I (P = 0.055) and IV (P = 0.056) following VDR-KD. Interestingly, morphological alterations such as decreased muscle fibre size within the skeletal muscle of VDR-KO mice are not limited to muscles of specific fibre types [18, 24]. Therefore, it could be reasoned that the observed trends may have been more apparent if the VDR-KD was administered to a skeletal muscle of a more oxidative phenotype.

Although we observed no differences in mitochondrial protein content, an increase in the abundance of OPA1 was observed following VDR-KD in both myoblasts and myotubes. In contradiction to our results, OPA1 is also increased in response to

1α,25$(OH)_2D_3$ treatment in human skeletal muscle myoblasts [32] suggesting the increase in OPA1 following VDR-KD may be compensatory. OPA1 is known to possess a number of roles including modulating the fusion of the inner mitochondrial membrane and cristae remodelling [47, 48], in addition to protecting from apoptosis by reducing mitochondrial fragmentation [49]. OPA1 exists in both long and short isoforms, with the long isoforms undergoing proteolytic processing under conditions of cellular stress such as reduced mitochondrial membrane potential or increased ROS production [50, 51]. Given that we also report a loss of mitochondrial membrane potential following VDR loss-of-function, we may be observing an increase in OPA1 proteolytic processing. A mechanism which has previously been described to reduce mitochondrial fusion and promote to sequestering of fragmented mitochondria for degradation [50]. In support of this, the loss of the VDR in cancer cell lines has been reported to increase the production of ROS which could also contribute to the processing of OPA1 [52]. In addition, the treatment of human skeletal muscle myoblasts with 1α,25$(OH)_2D_3$ resulted in a decrease in mitochondrial fragmentation [32]. Therefore, it is possible that the large functional decrements observed following VDR-KD may be as a result of a fragmented mitochondrial network and the increase in OPA1 is a compensatory mechanism. Further analysis examining the organisation of the mitochondrial network via the use of mitochondrial labelling techniques would help to shed light upon this [53]. Despite *in vitro* observations, we report no change in OPA1 protein abundance in rat skeletal muscle following both VDR-KD and VDR-OE.

Whilst we and others have shown distinct changes in skeletal muscle mitochondrial function in response to VDR loss-of-function and treatment with vitamin D related

metabolites [32-34], the exact manner by which the VDR mediates this is unclear. Multiple studies have utilised microarray analyses to identify VDR target genes from multiple tissues [54-58]. However, the number of overlapping genes were low, indicating VDR target genes respond to $1\alpha,25(OH)_2D_3$ in a tissue-specific manner [59, 60]. Previously, a number of mRNAs encoding for mitochondrial proteins have been shown to be upregulated in human primary skeletal muscle myoblasts following the treatment with $1\alpha,25(OH)_2D_3$ [32] however, it is unclear whether these genes contain specific VDREs. Further studies combining microarray analysis, chromatin immunoprecipitation (ChIP) and rapid immunoprecipitation mass spectrometry of endogenous proteins (RIME) are much needed within skeletal muscle in order to identify VDR target genes and coregulatory binding partners [61, 62]. A potential candidate for further exploration, PPARδ has been shown to be responsive to the treatment of $1\alpha,25(OH)_2D_3$ and contain a functional VDRE, albeit within cancer cell lines [63]. Similar to the VDR, PPARδ forms a heterodimer protein complex with RXRs [64] and is a known regulator of fatty acid oxidation and mitochondrial content within skeletal muscle [65]. The potential exploration of the VDRs interaction with PPARδ within skeletal muscle may provide explanation towards the functional decrement observed following VDR-KD.

In addition to its genomic actions, the VDR is also involved in non-genomic transient signalling events [66, 67]. The treatment of skeletal muscle cell lines with $1\alpha,25(OH)_2D_3$ results in an increase in intracellular $Ca2^+$ flux likely mediated by the VDR [68-70]. Intracellular and mitochondrial $Ca2^+$ handling involving uptake, buffering and extrusion of $Ca2^+$ is tightly coupled to the energetic state of the

organelle [71, 72]. Given the observed decrements in mitochondrial function, oxidative phosphorylation and membrane potential in response to VDR loss-of-function, the exploration of $Ca2^{+}$ handling dynamics should be an avenue for further exploration.

In summary, for the first time, we build upon previous links between vitamin D and mitochondrial function [32-34, 42] by reporting a novel role for the VDR in the regulation of mitochondrial function in the C2C12 mouse skeletal muscle cell line. Reductions in mitochondrial function were as a result of reduced ATP production via oxidative phosphorylation whilst markers of mitochondrial protein content, fusion and fission were unchanged. Finally, markers of mitochondrial protein content, fusion and fission remain unchanged following the knock-down and over-expression of the VDR within rat skeletal muscle although, the role of the VDR within skeletal muscle *in vivo* requires further examination.

4.5 References

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CHAPTER 5

THE EFFECT OF DIET-INDUCED VITAMIN D DEFICIENCY ON BODY COMPOSITION AND SKELETAL MUSCLE MITOCHONDRIAL FUNCTION IN C57BL/6J MICE

5.1 Introduction

Vitamin D deficiency, characterised by serum 25(OH)D levels of <50 nmol.L^{-1}, remains a widespread issue across the world [1, 2]. Although the classical actions of vitamin D are well established [3-5], a number of non-classical actions have recently been identified including; the prevention of some cancers [6], the maintenance of immune function [7] and the maintenance of skeletal muscle function [8].

Multiple studies have also sought to assess the effects of vitamin D deficiency upon skeletal muscle function within human populations [9-12]. Of note, observational studies have reported a positive association between serum 25(OH)D levels and muscle strength and lower extremity function in older individuals [9-11], with vitamin D supplementation able to increase skeletal muscle strength in this population [13, 14]. Despite these associations, studies of this design are unable to infer causality. In addition, isolating the effects of vitamin D status within older populations is often difficult given individuals may suffer from a number of pre-existing conditions that may interfere with vitamin D status [15]. These difficulties highlight the importance of model systems that allow for the manipulation and isolation of vitamin D status in order to study the precise role of vitamin D within skeletal muscle.

In order to study the impact of vitamin D deficiency on skeletal muscle function, a number of animal models have been utilised. A dysregulation of vitamin D status can be achieved via dietary means [16-19], a reduction in sunlight exposure [16] or by the administration of ethane 1-hydroxy-1, 1-diphosphonate which blocks the production of $1\alpha,25(OH)_2D_3$ [19]. Diet-induced vitamin D deficiency has been shown to result in

symptoms of skeletal muscle myopathy including impaired contraction kinetics, skeletal muscle weakness and decreases in muscle force in both chicks and rats [16, 17, 20]. In order to isolate the effect of vitamin D and offset the observed hypocalcemia and hypophosphatemia that are associated with the induction of vitamin D deficiency [20], diets with increased calcium and phosphate have also been utilised [18]. However, despite the administration of this rescue diet, mice still display reduced grip strength and an increase in *Myostatin* gene expression [18], a known negative regulator of muscle mass [21]. Similarly, mice fed this diet chronically (8-12 months) show similar impairments in physical performance including; reduced grip endurance, sprint speed and stride length [22].

The observed impairments in physical performance with vitamin D deficiency may be linked to impairments within skeletal muscle mitochondrial function [12, 23]. *In vitro*, vitamin D related metabolites are able to increase mitochondrial function in both immortalised and primary skeletal muscle cell lines [24-27]. In addition, the supplementation of vitamin D within a cohort of severely deficient individuals resulted in an increase in oxidative phosphorylation, as measured non-invasively by P-31 MRS [12]. Whilst skeletal muscle mitochondrial content seems to remain unchanged following diet-induced vitamin D deficiency in mice [22], the functional characteristics of the mitochondria remain largely underexplored. Despite previous observations, current evidence is limited, specifically in relation to the *in vivo* effects of vitamin D deficiency upon skeletal muscle mitochondrial function. Therefore, the aims of this chapter are as follows; 1) Determine the effects of vitamin D deficiency upon body composition in C57BL/6J mice. 2) Determine the effects of vitamin D deficiency upon

skeletal muscle mitochondrial respiration in permeabilised skeletal muscle fibres from C57BL/6J mice.

5.2 Methods

5.2.1 Ethical Approval

Ethical approval for mouse studies was granted by the Garvan Institute and St. Vincent's Hospital Animal Experimentation Ethics Committee (approval number 18/19). Ethical approval fulfils all the requirements of the NHMRC and the NSW State Government, Australia. All animal handling was carried out by trained personnel and all procedures were carried out according to the Australian code of practice for the care and use of animals for scientific purposes 8th edition [28]. C57BL/6J mice were received at 10-weeks of age and housed communally in a temperature controlled environment (22 $\pm$ 0.5°C) with a 12 h light-dark cycle.

5.2.2 Composition of Diet

Following 1-week acclimation in which mice were fed a standard chow diet, mice were placed on either a vitamin D-control diet or a vitamin D-deplete diet (Table 2.2) [18]. The vitamin D deplete contains no vitamin D but increased calcium (2%), magnesium (0.2%), and phosphorous (1.2%) in order to maintain normal mineral homeostasis (SF085-003, Speciality Feeds, Glen Forest, NSW). The vitamin D control diet contains vitamin D (cholecalciferol 2,200 IU/kg), calcium (1%), magnesium (0.2%), and phosphorous (0.7%) (SF085-034, Speciality Feeds, Glen Forest, NSW). Mice were maintained on the respective diets for a period of either 1- (n=10/group), 2- (n=10/group), or 3-months (n=6/group).

5.2.3 Assessment of Body Composition

Prior to each assessment of body composition, mice were briefly weighed. Body weight was also obtained on a weekly basis throughout dietary intervention. Body composition was assessed upon arrival (10-weeks of age) and then following 1-, 2- and 3-months of dietary intervention using the EchoMRi (EchoMRI LLC, Houston, USA).

5.2.4 Tissue Collection

Tissue collections were completed following 1-, 2- and 3-months of dietary intervention. All samples were excised from fasted (2 h) mice following isoflurane (5%) anesthetization. Tissues collected include; gastrocnemius, quadriceps, triceps, liver, kidney, white adipose tissue and the tibia. Following collection, a blood sample was taken via cardiac puncture and animal terminated via cervical dislocation. All tissues were rinsed in sterile saline, blotted dry, weighed, and frozen in liquid nitrogen. A small portion (~20 mg) of the gastrocnemius was removed before freezing and used for high-resolution respirometry. All tissues were stored at -80°C for subsequent analysis.

5.2.5 Tissue Processing

Small portions of gastrocnemius muscle (~20 mg) were removed and placed in ice-cold BIOPS buffer (2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2$-$6H_2O$, 20 mM Taurine, 15 mM Na_2Phosphocreatine, 20 mM Imidazole, 0.5 mM Dithiothreitol, 50 mM MES Hydrate, pH 7.1, 290 mOsm). Blood samples were allowed to coagulate at room temperature for 10 minutes before being placed

on ice. Blood samples were then centrifuged at 14,000 *g* for 10 minutes. The resulting supernatant was removed and stored at -80°C for further analysis.

5.2.6 Analysis of Serum Calcium

Serum calcium was measured using a Calcium Detection Assay kit (Abcam, Cambridge, UK, cat. ab102505). Serum samples were diluted 1:10 and manufacturers instructions were followed. The assay plate was read at 575 nm using a CLARIOstar microplate reader (BMG Labtech, Victoria, Australia). Serum calcium concentrations are reported in mM.

5.2.7 High-Resolution Respirometry

High-resolution respirometry was conducted in MiR05 (2 ml) with the addition of blebbistatin (25 μM) using the OROBORS Oxygraph-2K (Oroboros Instruments, Corp., Innsbruck, AT) with stirring at 750 rpm at 37°C. Oxygen within the chamber was maintained between 150-220 μM for each experiment. Prior to the addition of the fibre bundles to the chamber, bundles were blotted dry and weighed. Bundles totalling 2.5-5.0 mg were added to the chamber. Firstly, pyruvate (10 mM) and malate (2 mM) were added as complex I substrates. Subsequently, ADP was titrated in step-wise increments (100-6000 μM) followed by the addition of glutamate (10 mM) and succinate (10 mM) as complex I and II substrates. Cyt c (10 μM) was added in order to check outer mitochondrial membrane integrity. The partial loss of cyt c during fibre preparation may limit respiration. No fibre preparation exhibited an increase of >10%. CCCP was titrated in a step-wise manner (0.5 to 2.5 μM) until the

maximal capacity of the electron transport chain was reached. Finally, antimycin A (2.5 μM) was injected in order to inhibit mitochondrial respiration.

The apparent K_m for ADP was determined through the Michaelis-Menten enzyme kinetics – fitting model ($Y = Vmax*X/(K_m + X)$), where X = (free ADP; ADP_f), using Prism (GraphPad Software, Inc., La Jolla, CA). Flux control ratios (FCR) was calculated by setting CCCP stimulated respiration as 1 and antimycin A respiration as 0.

5.2.8 Statistical Analysis

Statistical analysis was performed using Prism version 7 (GraphPad Software Incorporated, La Jolla, CA, USA). Differences between 1-, 2- and 3-month vitamin D replete and deplete mice were determined by two-way ANOVA with Bonferroni correction for multiple comparisons. Differences between vitamin D deplete and replete mice in mitochondrial respiration in response to ADP titration were determined by multiple t-test. For baseline comparisons of body weight and composition, vitamin D replete and deplete mice were pooled and compared via independent t-test. All values are presented as mean ± SD. Statistical significance was set at $P < 0.05$.

5.3 Results

	Dietary Group		
	VitD Replete	**VitD Deplete**	***P***
Body Weight (g)	27.2 ± 1.3	27.2 ± 1.5	*0.88*
Lean Mass (g)	24.0 ± 1.3	23.5 ± 1.3	*0.31*
Lean Mass (% of BW)	88 ± 2	89 ± 2	*0.10*
Fat Mass (g)	2.0 ± 0.6	1.8 ± 0.5	*0.19*
Fat Mass (% of BW)	7 ± 2	7 ± 2	*0.23*

Table 5.1. No differences in baseline measurements of body composition in mice prior to dietary intervention. Data mean ± SD (n=22/group).

5.3.1 Body Weight

Mice were matched for body weight ($P > 0.05$) at baseline when separated into individual groups (Table 5.1). Overall, body weight increased in both dietary groups when assessed over the 3-month dietary period, main effect for time ($P < 0.001$), with a significant increase as of week 2 when compared to baseline ($P < 0.001$) (Fig. 5.1A). Similarly, body weight increased when mice were compared at 1-, 2- and 3-months of dietary intervention, main effect for time ($P < 0.001$) (Fig. 5.1B). Body weight was increased at the 3-month time point when compared to mice at 1- and 2-months respectively ($P < 0.001$) (Fig. 5.1B). No differences in body weight were observed when comparing vitamin D replete and deplete mice ($P > 0.05$) (Fig. 5.1A-B).

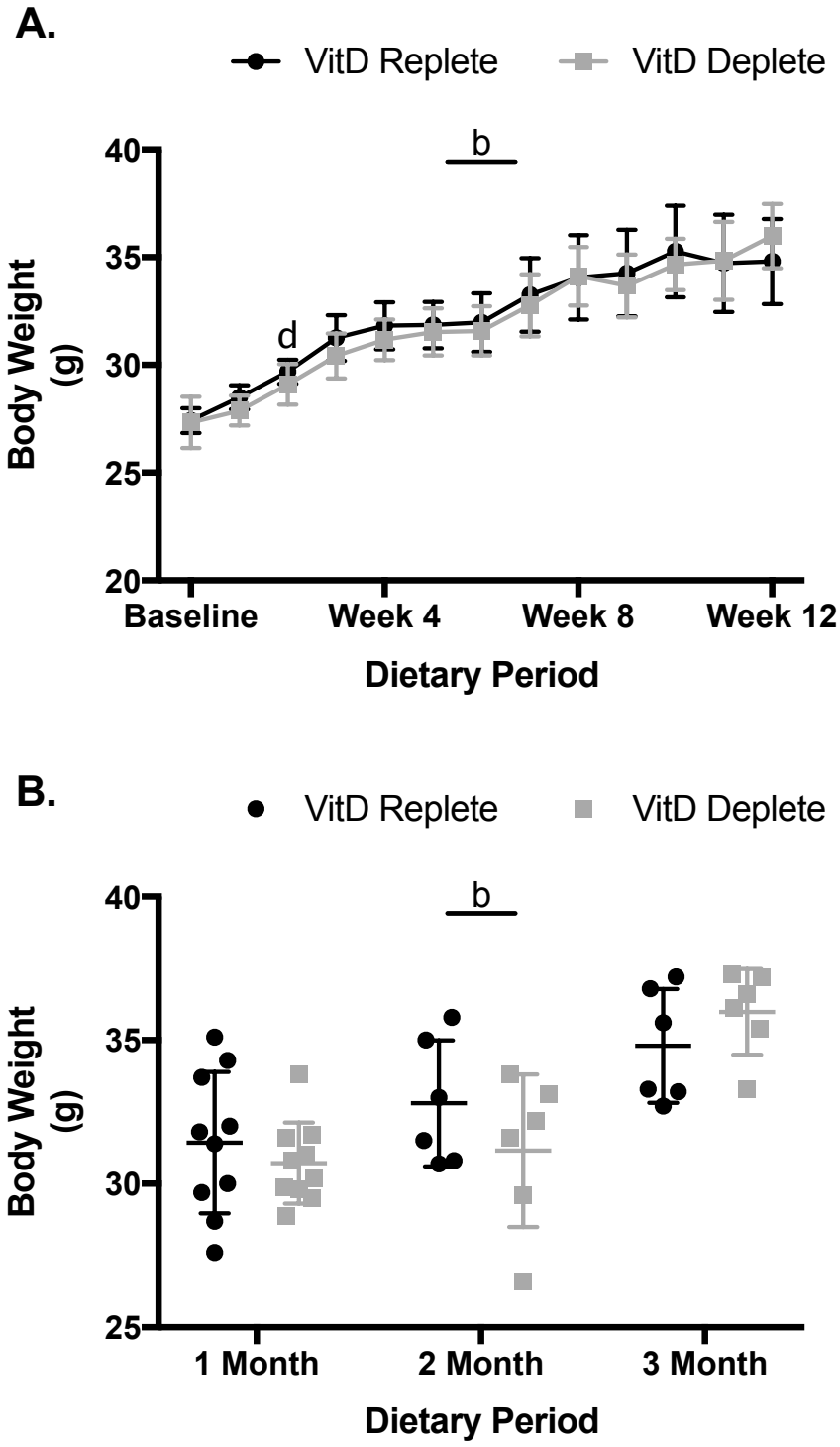

Figure 5.1. Body weight increases during 3-month dietary intervention irrespective of vitamin D status. A) Weekly assessment of body weight over 3-month dietary period. B) Body weight at 1-, 2- and 3-month dietary time point. Data mean ± SD (n=6-10/group). [b]Main effect for time; $P < 0.05$. [d]Significantly different from baseline; $P < 0.05$.

5.3.2 Lean Mass

Mice were matched for lean mass ($P > 0.05$) at baseline when separated into individual groups (Table 5.1). Absolute lean mass increased across the 3-month dietary period, main effect for time ($P < 0.001$), with a significant increase as of week 4 ($P = 0.004$) (Fig. 5.2A). Despite this, due to the increased body weight, lean mass as a percentage of body weight decreased, main effect for time ($P < 0.001$), with a significant decrease as of week 4 ($P < 0.001$) (Fig. 5.2B). No differences were observed in absolute lean mass when compared at the 1-, 2- and 3-month time points ($P > 0.05$) (Fig. 5.2C). When expressed as a percentage of body weight, lean mass decreased, main effect for time ($P < 0.001$) and group x time interaction ($P = 0.035$), when compared across the 1, 2 and 3-month time points (Fig. 5.2D). Further analysis revealed a significant decrease in lean mass as a percentage of body when the 3-month (71 ± 7%) vitamin D deplete mice was compared with the 1-month (85 ± 3%), whereas vitamin D replete remained unchanged ($P > 0.05$) (Fig. 5.2D). At the 1-month time point, lean mass as a percentage of body weight was increased in the vitamin D deplete group (85 ± 3%) when compared to vitamin D replete (79 ± 5%) ($P = 0.039$) (Fig. 5.2D).

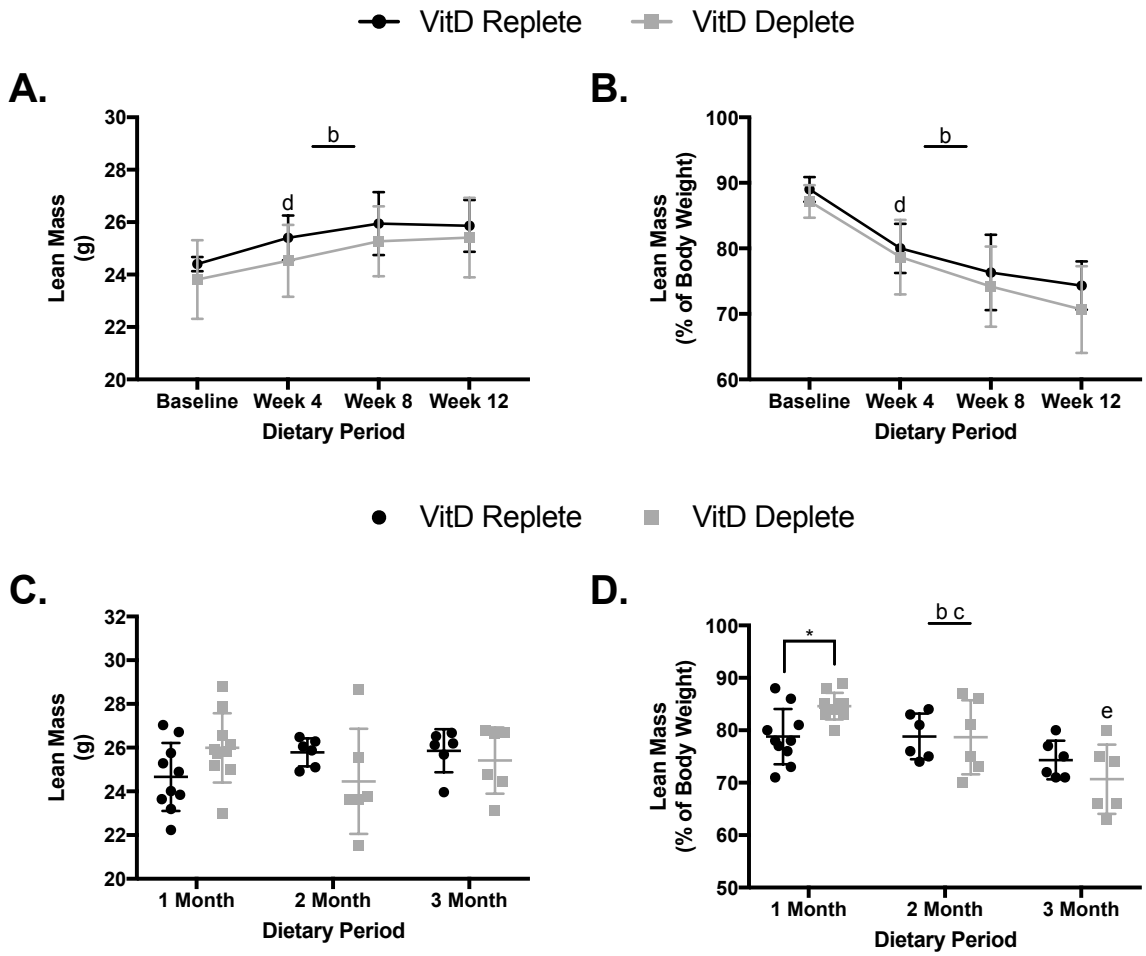

Figure 5.2. Lean mass as a percentage of body weight decreases in vitamin D deplete mice with no differences in comparison to replete mice. A) Repeated measurement of absolute lean mass (grams) across 3-month dietary intervention. B) Repeated measurement of lean mass as a percentage of body weight across 3-month dietary period. C) Absolute lean mass (grams) following 1-, 2- and 3-months of dietary intervention. D) Lean mass as a percentage of body weight following 1-, 2- and 3-months of dietary intervention. Data mean ± SD (n=6-8/group). [b]Main effect for time; $P < 0.05$, [c]group x time interaction effect; $P < 0.05$, [d]Significantly different from baseline; $P < 0.05$, [e]Significantly different from 1-month time point of same dietary group; $P < 0.05$, *$P < 0.05$.

5.3.3 Fat Mass

At baseline, mice were matched for fat mass when split into individual groups ($P > 0.05$) (Table 5.1). Overall, absolute fat mass increased across the 3-month dietary period, main effect for time ($P < 0.001$), with a significant increase as of week 4 ($P < 0.001$) (Fig. 5.3A). Similarly, when expressed as a percentage of body weight, fat

mass increased in both groups, main effect for time ($P < 0.001$), with a significant increase as of week 4 ($P < 0.001$) (Fig. 5.3B). When compared across the 1-, 2- and 3-month time points, absolute fat mass increased, main effect for time ($P < 0.001$) (Fig. 5.3C). Similarly, as a percentage of body weight, fat mass increased across the 1, 2 and 3-month time points, main effect for time ($P < 0.001$) (Fig. 5.3D). An increased in fat mass as a percentage of body was observed in the vitamin D replete (16 ± 5%) when compared with vitamin D deplete (10 ± 2%) at the 1-month time point ($P = 0.044$) (Fig. 5.3D).

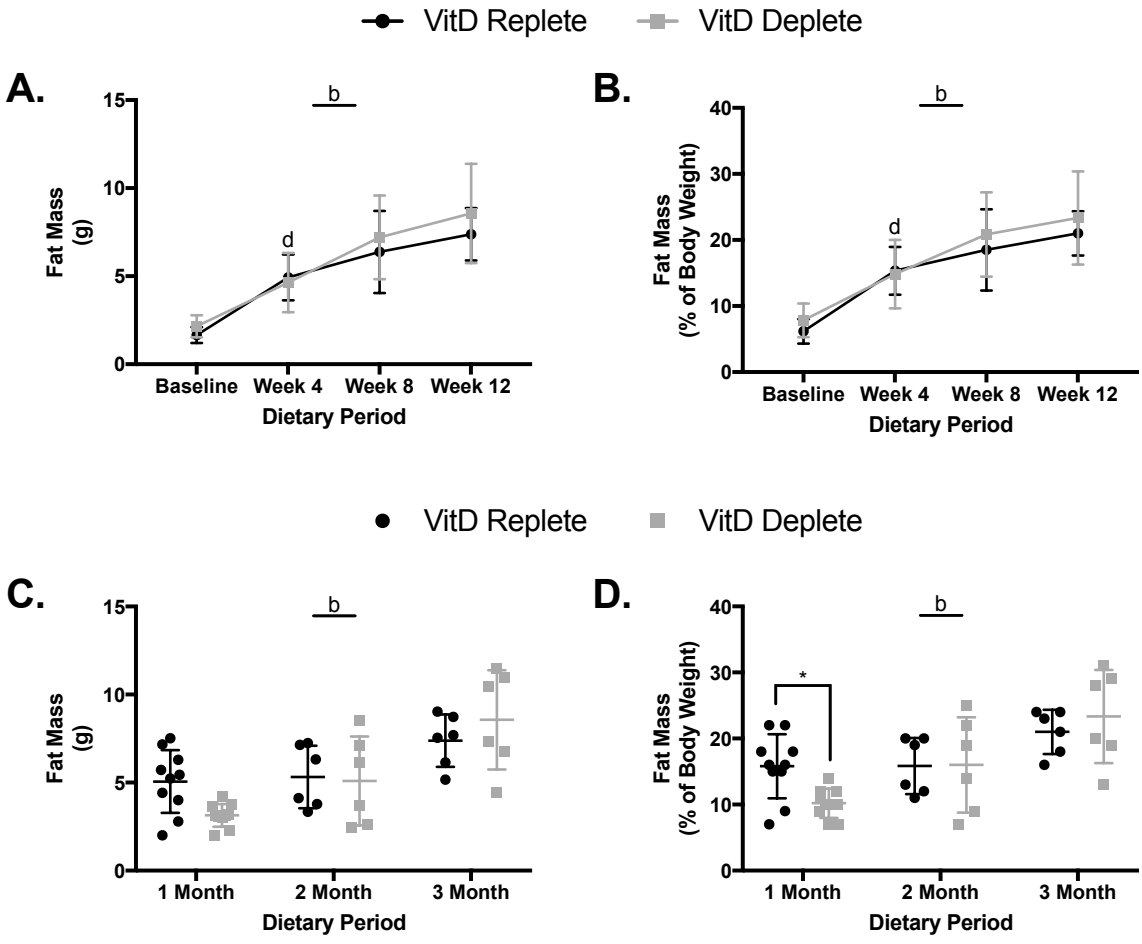

Figure 5.3. Fat mass increases across dietary period irrespective of vitamin D status. A) Repeated measurement of absolute fat mass (grams) across 3-month dietary period. B) Repeated measurement of fat mass as a percentage of body weight across 3-month dietary period. C) Assessment of absolute fat mass (grams) at 1-, 2- and 3-months of dietary intervention. D) Fat mass as a percentage of body weight at 1-, 2- and 3-months of dietary intervention. Data mean ± SD (n=6-8/group). [b]Main effect for time; $P < 0.05$, [c]group x time interaction effect; $P < 0.05$, [d]Significantly different to 1-month time point of same group; $P < 0.05$, *$P < 0.05$.

5.3.4 Skeletal Muscle Mass

No changes were observed in gastrocnemius mass in response to either dietary intervention (P = 0.408) or time point (P = 0.103) (Table 5.2). Overall, the mass of the quadriceps increased over time (P = 0.004) however, this was not changed by dietary intervention (P = 0.951) (Table 5.2). Collectively, triceps mass was increased when vitamin D replete mice were compared with deplete (P = 0.041) although, post-hoc analysis revealed no difference between groups at individual time points (P > 0.05) (Table 5.2).

	Dietary Period (Months)				
	1	**2**	**3**	***P***	
Gastrocnemius (mg)				*VitD*	*Time*
Replete	143 ± 14	166 ± 25	161 ± 14		
Deplete	152 ± 16	149 ± 12	156 ± 15	*0.408*	*0.103*
Quadriceps (mg)					
Replete	160 ± 14	189 ± 12	182 ± 20		
Deplete	169 ± 18	178 ± 22	184 ± 19	*0.951*	*0.004*
Tricep (mg)					
Replete	111 ± 20	113 ± 14	109 ± 10		
Deplete	106 ± 9	96 ± 19	101 ± 13	*0.041*	*0.695*

Table 5.2. Increased tricep and no differences in quadriceps or gastrocnemius mass when vitamin D replete mice are compared with deplete mice. Data mean ± SD (n=6-8/group). Significant set at P < 0.05.

5.3.5 Serum Calcium

Serum calcium remained unchanged irrespective of dietary group or time point (P > 0.05) (Fig. 5.4).

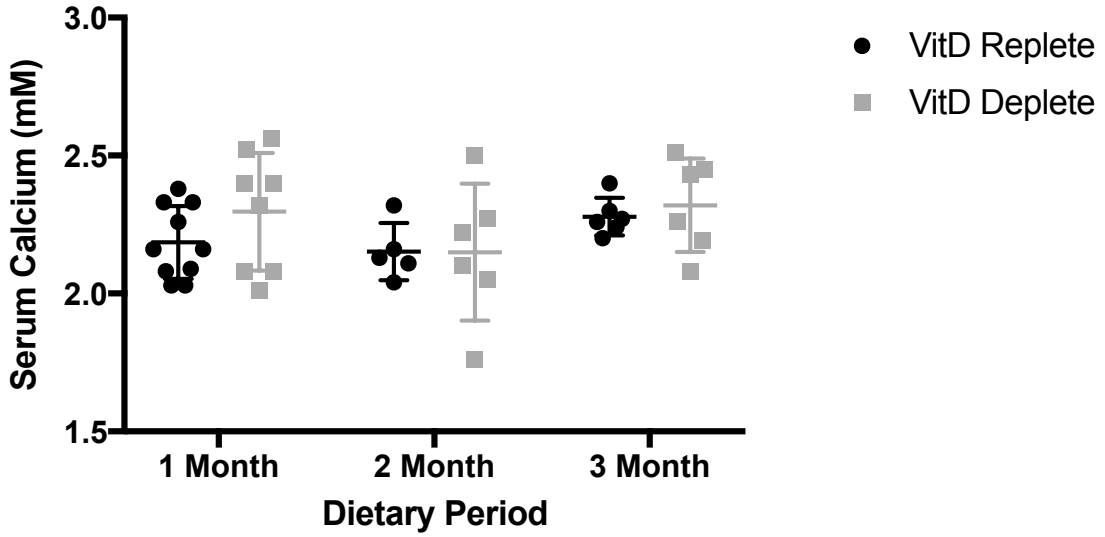

Figure 5.4. No change in serum calcium following manipulation of vitamin D status. Data mean ± SD (n=6-10/group).

5.3.6 Skeletal Muscle Mitochondrial Function

No changes were observed in complex I related leak in response to either differing vitamin D diets ($P > 0.05$) or time points ($P > 0.05$) (Fig. 5.5A). Complex I respiration increased across the 1, 2 and 3-month time points, main effect for time ($P = 0.048$) and group x time interaction ($P = 0.035$) (Fig. 5.5B). Further analysis revealed a significant increase in 2-month (319.97 ± 75.81 pmol.sec^{-1}.mg^{-1}.dry weight) and 3-month (338.08 ± 170.46 pmol.sec^{-1}.mg^{-1}.dry weight) vitamin D replete groups when compared to the 1-month (172.41 ± 51.60 pmol.sec^{-1}.mg^{-1}.dry weight), whereas the vitamin D deplete groups remained unchanged ($P > 0.05$) (Fig. 5.5B). Group x time interaction effects were observed for both complex I and II phosphorylating ($P = 0.035$) and maximal respiration ($P = 0.017$) (Fig. 5.5C-D). Complex I and II phosphorylating respiration increased in 2 (416.17 ± 94.96 pmol.sec^{-1}.mg^{-1}.dry weight) and 3-month (451.37 ± 196.40 pmol.sec^{-1}.mg^{-1}.dry weight) vitamin D replete groups when compared with the 1-month (274.86 ± 47.57 pmol.sec^{-1}.mg^{-1}.dry weight) whilst vitamin D deplete remained unchanged ($P > 0.05$) (Fig. 5.5C). Similar

increases were apparent in maximal respiration when the 2 (502.22 ± 109.35 pmol.sec^{-1}.mg^{-1}.dry weight) and 3-month (560.58 ± 205.09 pmol.sec^{-1}.mg^{-1}.dry weight) vitamin D replete groups where compared with the 1-month (323.19 ± 48.68 pmol.sec^{-1}.mg^{-1}.dry weight) and again, vitamin D deplete remained unchanged (P > 0.05) (Fig. 5.5D). At the 3-month time point, phosphorylating respiration supported via complex I and II was significantly decreased in the vitamin D deplete mice (291.76 ± 48.66 pmol.sec^{-1}.mg^{-1}.dry weight) when compared to vitamin D replete (451.37 ± 196.40 pmol.sec^{-1}.mg^{-1}.dry weight) (P = 0.035) (Fig. 5.5C). Similarly, respiration supported via the maximal capacity of the electron transport chain was reduced in the vitamin D deplete group (352.90 ± 46.56 pmol.sec^{-1}.mg^{-1}.dry weight) when compared with vitamin D replete (560.58 ± 205.09 pmol.sec^{-1}.mg^{-1}.dry weight) at the 3-month time point (P = 0.015) (Fig. 5.5D).

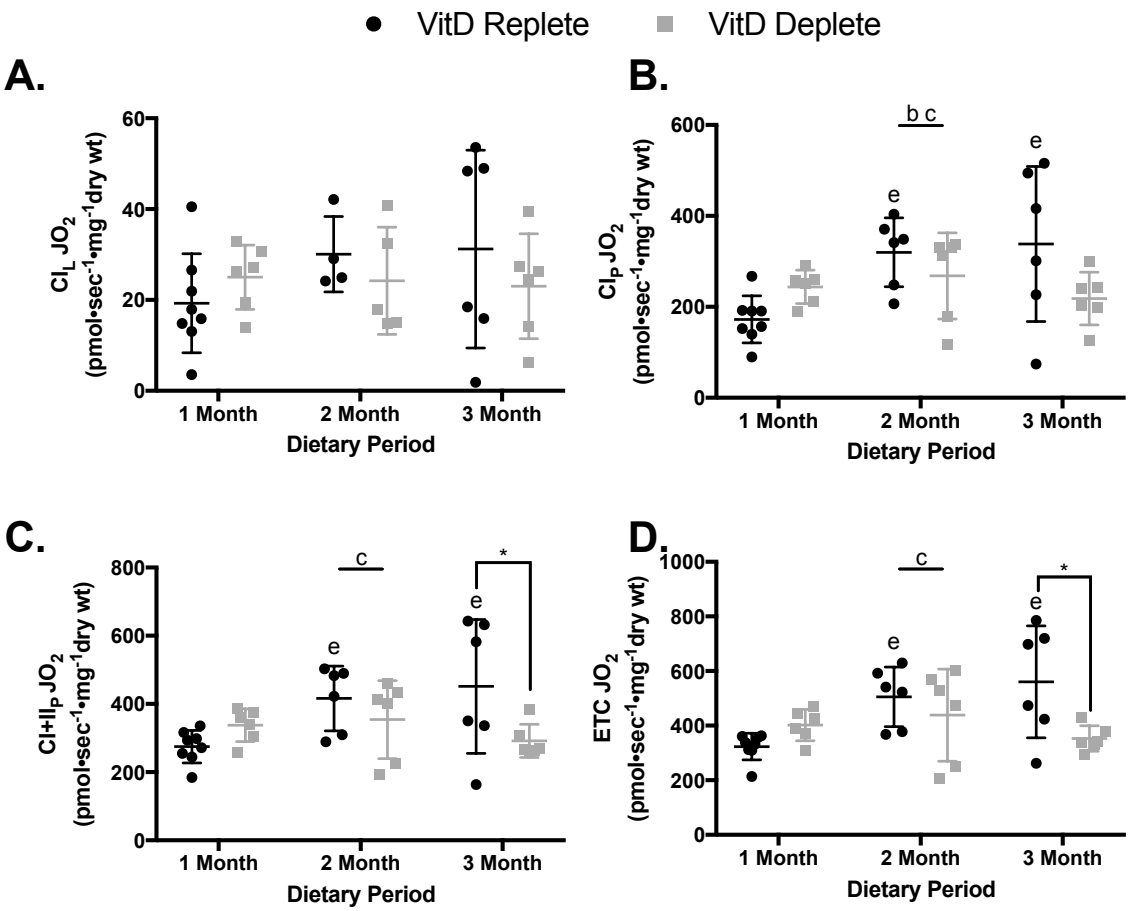

Figure 5.5. Skeletal muscle mitochondrial respiration is reduced following 3-months of diet-induced vitamin D deficiency in mice. A) Complex I related leak at following 1-, 2- and 3-months of dietary intervention. B) Complex I phosphorylation respiration following 1-, 2- and 3-months of dietary intervention. C) Complex I and II phosphorylating respiration following 1-, 2- and 3-months of dietary intervention. D) Maximal capacity of the electron transport chain following 1-, 2- and 3-months of dietary intervention. Data mean ± SD (n=6-8/group). [b]Main effect for time; $P < 0.05$, [c]group x time interaction effect; $P < 0.05$, [d]Significantly different from baseline; $P < 0.05$, [e]Significantly different from 1-month time point of same dietary group; $P < 0.05$, *$P < 0.05$.

5.3.7 ADP Sensitivity

No differences were observed in the apparent K_m for ADP in response to either dietary intervention ($P > 0.05$) or time point ($P > 0.05$) (Fig. 5.6D).

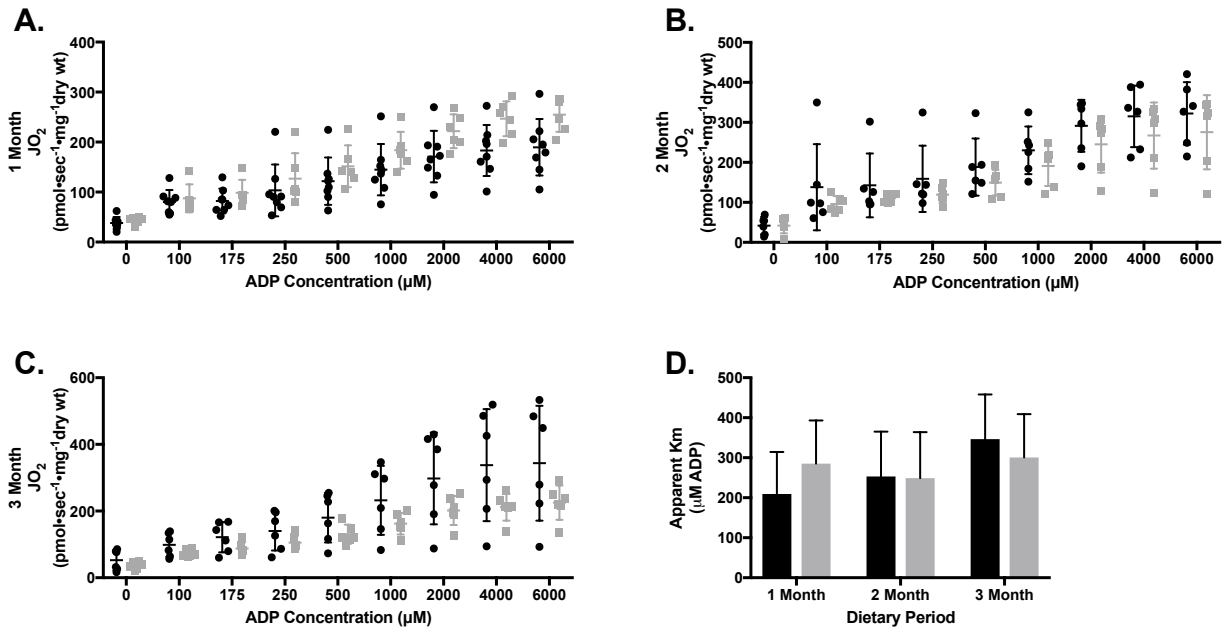

Figure 5.6. No change in mitochondrial respiration across ADP titration following manipulation of vitamin D status in mice. A) ADP stimulated respiration following 1-month of dietary intervention. B) ADP stimulated respiration following 2-month of dietary intervention. C) ADP stimulated respiration following 3-month of dietary intervention. D) Apparent K_m for ADP in vitamin D replete and deplete mice at 1-, 2- and 3-month of dietary intervention. Data mean ± SD (n=6-8/group).

5.3.8 Flux Control Ratios

When normalised to maximal respiration, the flux control ratios revealed no differences in mitochondrial respiration supported via complex I alone ($P > 0.05$) or complex I and II ($P > 0.05$) (Fig. 5.7A-B).

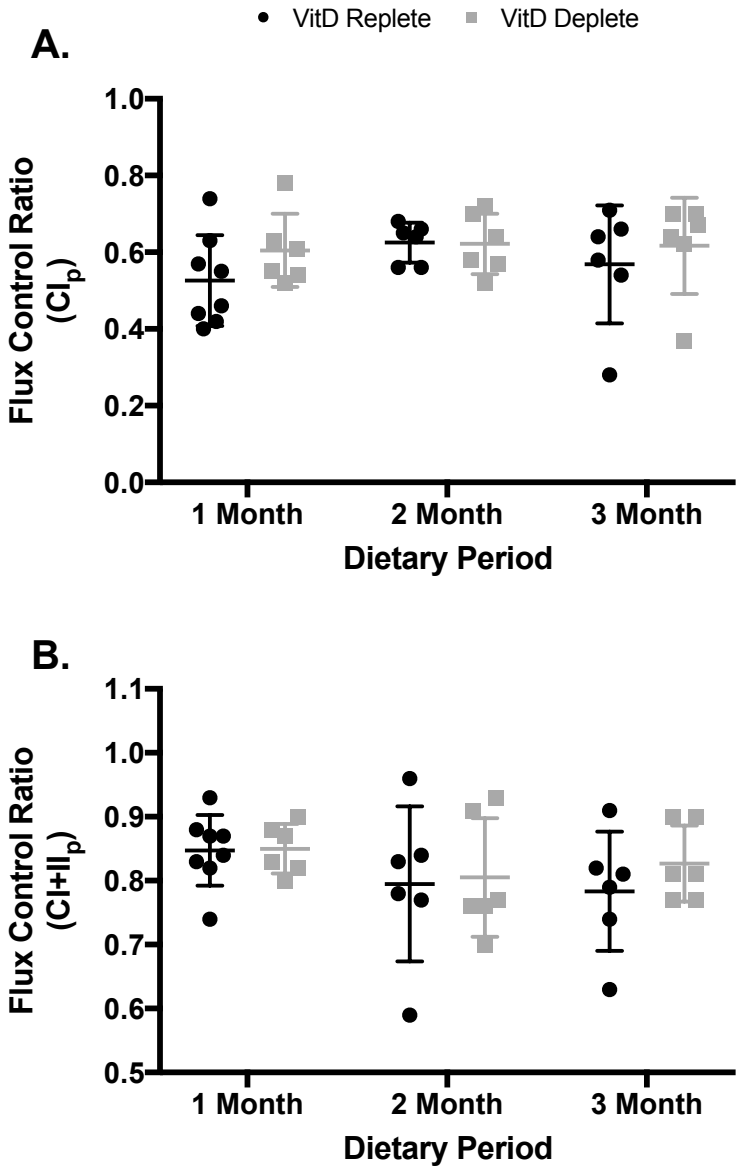

Figure 5.7. Flux control ratios remain unchanged following diet-induced vitamin D deficiency in mice. A) Complex I related flux control ration following 1-, 2- and 3-months of dietary intervention. B) Complex I and II related flux control ratio following 1-, 2- and 3-months of dietary intervention. Data mean ± SD (n=6-8/group).

5.4 Discussion

We sought to determine the effects of vitamin D deficiency upon skeletal muscle mitochondrial function in the C57BL/6J mouse line. Utilising the current gold standard method to assess mitochondrial function in permeabilised skeletal muscle fibres [29, 30], we report that 3-months of diet-induced vitamin D deficiency reduces mitochondrial respiration supported via complex I and II and the maximal capacity of the electron transport chain (Fig. 5.5C-D). However, following internal normalisation, these reductions were no longer apparent, suggesting that changes in mitochondrial respiration following dietary intervention are a result of reduced mitochondrial quantity (Fig. 5.7A-B). In addition, 1-month of diet-induced vitamin D deficiency resulted in an increase in lean mass (Fig. 5.2D) and a decrease in fat mass (Fig. 5.3D) as a percentage of body weight, although these effects were transient as they did not manifest over 2-month and 3-months of dietary intervention. Furthermore, diet-induced vitamin D deficiency resulted in a decrease in lean mass as a percentage of body weight across the 3-month time period (Fig. 5.3D). Despite this, no changes in body weight, lean mass or fat mass were apparent when comparing vitamin D replete to the deplete group following 3-months of dietary intervention.

The ability of vitamin D related metabolites to increase skeletal muscle mitochondrial function across both immortalised and primary cell lines has been well established [24-27]. Despite this, there is little evidence for the effects of vitamin D status upon skeletal muscle mitochondrial function *in vivo*. To date, just one study has sought to determine the effects of vitamin D upon skeletal muscle mitochondrial function *in vivo*. Skeletal muscle mitochondrial function was assessed non-invasively (P-31

MRS) in a cohort of severely deficient patients following the supplementation of vitamin D [12]. Whilst a decrease in PCr recovery time was reported, indicative of increased oxidative phosphorylation, the study lacked a number of experimental controls including proper randomisation and blinding [12], making the interpretation of said results difficult. Therefore, in order to assess the effects of vitamin D status upon skeletal muscle mitochondrial function, we utilised a mouse model of diet-induced vitamin D deficiency. This mouse model has been previously utilised and allows for the manipulation of vitamin D status without a dysregulation in mineral homeostasis [18]. Following 3-months of diet-induced vitamin D deficiency, we report that respiration supported via complex I and II and the maximal capacity of the electron transport chain are reduced when compared to respective controls at the same time point. In addition, vitamin D replete mice exhibited increases in multiple parameters of mitochondrial respiration (CI_L, $CI+II_P$ and ETC) across the 3-month dietary intervention period, whilst respiration in vitamin D deplete mice remained stable. Previously, both mitochondrial number and the activity of components of the electron transport chain have been reported to increase from 3 to 12 months of age within mouse skeletal muscle [31, 32]. Therefore, diet-induced vitamin D deficiency may well impair the age-related increases in skeletal muscle mitochondrial content. In support of this, we examined mitochondrial respiration as a flux control ratio, which offers a method of internal normalisation and the assessment of whether changes in respiration are as a result of mitochondrial quality or quantity [33-35]. Interestingly, the previously observed increases in mitochondrial respiration are abolished following internal normalisation, suggesting alterations in mitochondrial respiration following diet-induced vitamin D deficiency are a product of reduced mitochondrial

quantity. In contrast, no changes in mitochondrial protein content, mtDNA/nDNA ratio or citrate synthase activity have been reported following 12-months of diet-induced vitamin D deficiency in C57BL/6J mice [22]. It should be noted that diet-induced vitamin D deficiency was commenced from 6-months of age, whereas we examined a dietary period between 3- and 6-months of age. Therefore, it is possible that the effects of vitamin D deficiency upon skeletal muscle mitochondrial content and function are more potent during development. In addition, it could also be reasoned that our results may have been more prominent had vitamin D deficiency been induced in an older animal model given that in human populations vitamin D is most potent in such cohorts [14]. Unfortunately, time constraints limited the exploration of vitamin D deficiency in a setting of older age and sarcopenia. The further assessment of mitochondrial function across a longer period of diet-induced vitamin D deficiency would potentially address previous discrepancies. We also assessed the sensitivity of oxidative metabolism to ADP (apparent K_m) via the titration of ADP from biological to saturating concentrations [36]. Whilst absolute respiration was slightly increased, albeit non-significantly in the 3-month vitamin D replete mice, we observed no differences in the apparent K_m for ADP at any time point suggesting ADP sensitivity is unaffected by diet-induced vitamin D deficiency.

In addition, we sought to determine the effects of diet-induced vitamin D deficiency upon body composition within C57BL/6J mice. Following 3-months of diet-induced vitamin D deficiency we observed no differences between the vitamin D replete and deplete groups in body weight, lean mass or fat mass. We did however observe a reduction lean mass as a percentage of body weight from 1- to 3-months in vitamin D

deplete mice whereas replete mice remained the unchanged over the same time period. This may in part be driven by the fact we also observed an increase in lean mass as a percentage of body weight following 1-month of diet-induced deficiency. Previously, no differences in body weight and lean mass were observed following 12-months of diet-induced deficiency in male C57BL/6J mice [22]. On the other hand, 12-months of diet-induced vitamin D deficiency in female C57BL/6J mice results in reductions in body weight, lean mass and fat mass [37]. Given those with serum concentrations of 25(OH)D <25 $nmol.L^{-1}$ are at a greater risk of developing sarcopenia [38], vitamin D status is an important consideration for the preservation of muscle mass. Furthermore, whilst we and others observe minimal differences in lean mass following diet-induced vitamin D deficiency in male mice [22, 39], decrements have been observed in females [37]. Observations from human populations indicate that the effects of vitamin D deficiency on physical performance may be more potent in women although, this was likely mediated by increased physical activity in males [40]. Further characterisation of the sex-specific differences in skeletal muscle function and physical performance in the context of vitamin D deficiency may well be warranted.

In conclusion, we report that mitochondrial function ($CI+II_P$ and ETC) is reduced in C57BL/6J mice following 3-months of diet-induced vitamin D deficiency. These effects are no longer apparent when data is internally normalised suggesting changes are a consequence of mitochondrial quantity as opposed to quality. Similar to others, we observed minimal difference in body composition following 3-months of diet-induced vitamin D deficiency in male C57BL/6J mice [22, 39]. Our data highlight

a possible impairment in the development of skeletal muscle mitochondrial function in young C57BL/6J mice. Further analysis of mitochondrial morphology across this time period may offer more insight into how this impairment manifests.

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CHAPTER 6

THE EFFECT OF DIET-INDUCED VITAMIN D DEFICIENCY ON BODY COMPOSITION AND SKELETAL MUSCLE MITOCHODNRIAL FUNCTION FOLLOWING 20-DAYS OF VOLUNTARY WHEEL RUNNING IN C57BL/6J MICE

6.1 Introduction

Vitamin D deficiency can be characterised by serum 25(OH)D concentrations of <50 nmol.L^{-1}. Currently, it has been estimated that ~40% of individuals within Europe and the USA can be classified as deficient [1, 2]. Further to this, ~13% may be classified as severely deficient with serum 25(OH)D concentrations of <12 nmol.L^{-1} [2]. Whilst severe deficiencies are known to dysregulate mineral homeostasis and impair bone health [3-5], severe deficiencies are also associated with skeletal muscle weakness, increased muscle pain and fatigue [6-8].

In order to study the effects of vitamin D deficiency upon skeletal muscle function a number of models are available. The induction of vitamin D deficiency has previously been achieved within animal models by dietary means [9-12], a reduction in sunlight exposure [9] or by the administration of ethane 1-hydroxy-1, 1-diphosphonate [12]. Vitamin D deficiency achieved via dietary means is the most common and in such scenarios, vitamin D is completely removed from the diet [9-12]. Alongside the reduction in vitamin D, the dietary content of calcium and phosphorous is increased. The increased mineral content prevents the previously observed hypocalcemia and hypophosphatemia that are associated with the induction of vitamin D deficiency [11]. Further characterisations of vitamin D deficiency have been performed, with multiple human studies having sought to assess skeletal muscle function within individuals deemed deficient [13-15]. Within animal models, the induction of deficiency results in a reduction in skeletal muscle force, strength and endurance [9-11, 16, 17]. In addition, observational studies in humans have reported positive associations between serum 25(OH)D levels and skeletal muscle strength [13-15]. Whilst the

majority of studies focus on skeletal muscle mass and strength in association with vitamin D status, vitamin D status has also been linked to the regulation of mitochondrial function [18-21]. For example, severely deficient individuals supplemented with vitamin D for a period of 12-weeks showed improvements in markers of oxidative phosphorylation and reduced symptoms of fatigue [20].

Increases in mitochondrial function are a common adaptation to endurance exercise training (Section 1.3.2) [22, 23]. Increases in protein content and activity of enzymes involved in the mitochondrial electron transport chain are observed in rodent skeletal muscle following endurance based training [24, 25]. Alongside increases in protein content, mass specific respiration is also known to be increased in response to training, allowing for a greater production of ATP [26-28]. Despite the above evidence, limited studies have assessed skeletal muscle mitochondrial adaptations to training alongside the manipulation of vitamin D status. Given vitamin D deficiency is widespread and even common amongst athletic populations [29, 30], it may well be an important consideration for maximising the adaptive response to training. Therefore, the aim of this chapter was to determine the effects of diet-induced vitamin D deficiency upon skeletal muscle adaptation to 20-days of voluntary wheel running in C57BL/6J mice.

6.2 Methods

6.2.1 Ethical Approval

Ethical approval for mouse studies was granted by the Garvan Institute and St. Vincent's Hospital Animal Experimentation Ethics Committee (approval number 18/19). Ethical approval fulfils all the requirements of the NHMRC and the NSW State Government, Australia. All animal handling was carried out by trained personnel and all procedures were carried out according to the Australian code of practice for the care and use of animals for scientific purposes 8th edition [31]. C57BL/6J mice were received at 10-weeks of age and housed communally in a temperature controlled environment (22 $\pm$ 0.5°C) with a 12 h light-dark cycle.

6.2.2 Composition of Diet

Following 1-week of acclimation in which mice were fed standard chow (Table 2.2), mice were placed on either a vitamin D replete or a vitamin D deplete diet [11]. The vitamin D deplete diet contained no vitamin D but increased calcium (2%), magnesium (0.2%), and phosphorous (1.2%) in order to maintain normal mineral homeostasis (SF085-003, Speciality Feeds, Glen Forest, NSW). The vitamin D control diet contained vitamin D (cholecalciferol 2.2 IU/g), calcium (1%), magnesium (0.2%), and phosphorous (0.7%) (SF085-034, Speciality Feeds, Glen Forest, NSW). Mice were maintained on the respective diets for a period of either 1- or 3-months.

6.2.3 Voluntary Wheel Running

Following a 1- (n=10/group) or 3-month (n=10/group) dietary intervention period, mice were housed individually and given access to a running wheel for 20-days. Mice

continued to consume their respective vitamin D replete, deplete or standard chow diets during the 20-day voluntary wheel running period. Wheel revolutions were recorded every hour via a digital recorder (Columbus Instruments, Columbus, OH, US) and the distance ran per day was calculated and reported in kilometres per twenty-four hours. Diet only controls were maintained across the same dietary period but without access to a running wheel. Previous observations within our lab indicate that 20 days of voluntary wheel running is a suitable timeframe to induce training adaptations in mitochondrial protein content and respiration.

6.2.4 Assessment of Body Composition

Body composition was assessed upon arrival (10-weeks of age) and then following 1-, 2- and 3-months of dietary intervention as well as post 20-days voluntary wheel running using the EchoMRi (EchoMRI LLC, Houston, USA). Body weight was measured on a weekly basis throughout the dietary and voluntary wheel running period.

6.2.5 Tissue Collection

Tissue collections were completed following 1- and 3-months of dietary intervention alone and following 1- and 3-months of dietary intervention plus 20-days of voluntary wheel running. Following anesthetization under isoflurane tissues were excised from fasted (2 h) mice. Tissues collected include; gastrocnemius, quadriceps, triceps, liver, kidney, white adipose tissue and the tibia. Following collection, a blood sample was taken via cardiac puncture and mice terminated via cervical dislocation. All tissues were rinsed in sterile saline, blotted dry, weighed, and frozen in liquid

nitrogen. A small portion (~20 mg) of the gastrocnemius was removed before freezing and used for high-resolution respirometry. All tissues were stored at -80°C for subsequent analysis.

6.2.6 Tissue Processing

Small portions of gastrocnemius muscle (~20 mg) were removed and placed in ice-cold BIOPS buffer (2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2$-$6H_2O$, 20 mM Taurine, 15 mM Na_2Phosphocreatine, 20 mM Imidazole, 0.5 mM Dithiothreitol, 50 mM MES Hydrate, pH 7.1, 290 mOsm). Blood samples were allowed to coagulate at room temperature for 10 minutes before being placed on ice. Samples were then centrifuged at 14,000 *g* for 10 minutes. The resulting supernatant was removed and utilised for further analysis.

6.2.7 Analysis of Serum Calcium

Serum calcium was measured using a Calcium Detection Assay kit (Abcam). Serum samples were diluted 1:10 and manufacturers instructions were followed. The assay plate was read at 575 nM using a CLARIOstar microplate reader (BMG Labtech, Victoria, Australia). Calcium concentrations are reported in mM.

6.2.8 High-Resolution Respirometry

High-resolution respirometry was conducted in MiR05 (2 ml) with the addition of blebbistatin (25 μM) using the OROBORS Oxygraph-2K (Oroboros Instruments, Corp., Innsbruck, AT) with stirring at 750 rpm at 37°C. Oxygen within the chamber was maintained between 150-220 μM for each experiment. Prior to the addition of the

fibre bundles to the chamber, bundles were blotted dry and weighed. Bundles totalling 2.5-5.0 mg were added to the chamber. Firstly, pyruvate (10 mM) and malate (2 mM) were added as complex I substrates. Subsequently, ADP was titrated in step-wise increments (100-6000 μM) followed by the addition of glutamate (10 mM) and succinate (10 mM) as complex I and II substrates. Cyt c (10 μM) was added in order to check outer mitochondrial membrane integrity. The partial loss of cyt c during fibre preparation may limit respiration. Multiple fibre preparations from 3-month mice exhibited increases in respiration (>10%) following the addition of cyt c and were therefore removed. CCCP was titrated in a step-wise manner (0.5 to 2.5 μM) until the maximal capacity of the electron transport chain was reached. Finally, antimycin A (2.5 μM) was injected in order to inhibit mitochondrial respiration.

The K_m for ADP was determined through the Michaelis-Menten enzyme kinetics – fitting model ($Y = Vmax*X/(K_m + X)$), where X = (free ADP; ADP_f), using Prism (GraphPad Software, Inc., La Jolla, CA). FCRs were calculated by setting CCCP stimulated respiration as 1 and antimycin A respiration as 0.

6.2.9 Statistical Analysis

Statistical analysis was performed using Prism version 7 (GraphPad Software Incorporated, La Jolla, CA, USA). Differences in voluntary wheel running performance and body composition between 1 and 3-month vitamin D replete and deplete mice were determined by two-way ANOVA with Bonferroni correction for multiple comparisons. Differences between 1-month standard chow, vitamin D replete and deplete mice were determined by one-way ANOVA. Differences in

mitochondrial function between 1-month dietary intervention mice and 1-month dietary intervention plus 20-days of voluntary wheel running were determined via two-way ANOVA with Bonferroni correction for multiple comparisons. All values are presented as mean ± SD. Statistical significance was set a $P < 0.05$.

6.3 Results

6.3.1 Voluntary Wheel Running

Overall, the average running distance was greater in the vitamin D replete mice when compared to vitamin D deplete ($P = 0.041$) (Fig. 6.1A). Similarly, the total distance covered during the 20-day voluntary wheel running period was greater in the vitamin D replete mice when compared with vitamin D deplete ($P = 0.041$) (Fig. 6.1B). No differences were observed when comparing individual groups ($P > 0.05$) (Fig. 6.1A-D). We also compared the 1-month vitamin D replete and deplete mice to mice fed a standard chow diet across the same dietary and voluntary wheel running period (Fig. 6.2A-B). We observed no differences between the individual groups in either daily or total running distance ($P > 0.05$) (Fig. 6.2A-C).

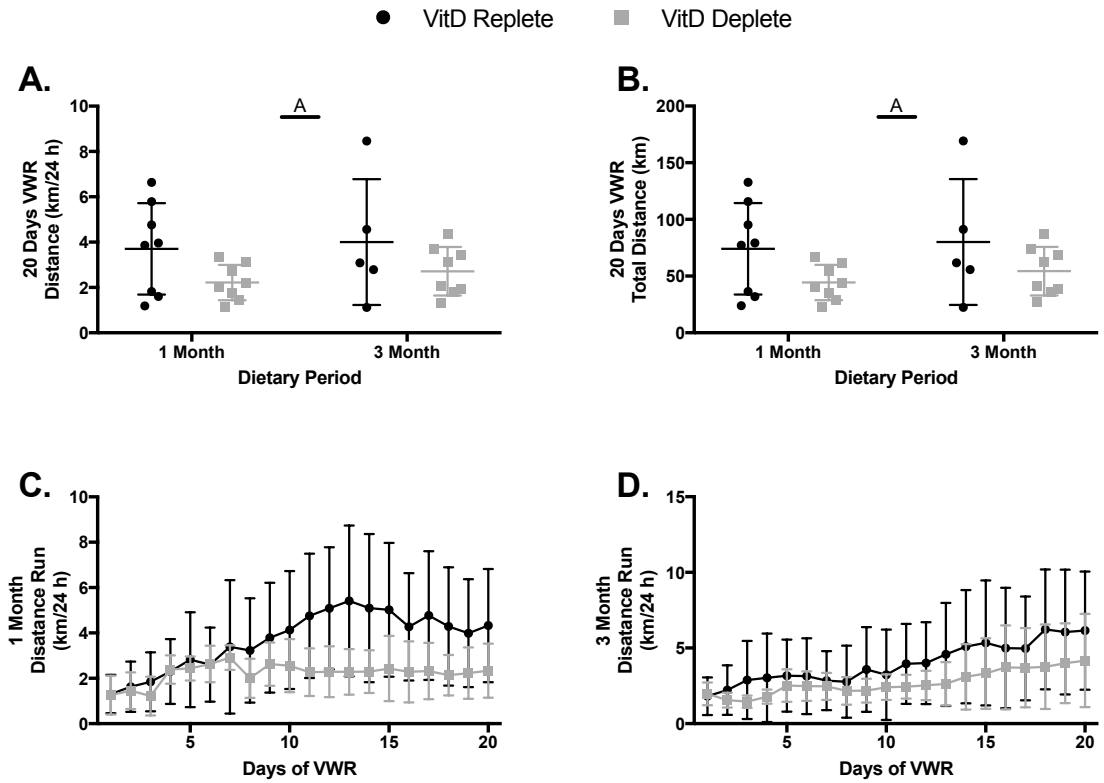

Figure 6.1. Increased running distance and volume in vitamin D replete mice when compared to vitamin D deplete. A) Daily average running distance in kilometres (km) during 20-days of voluntary wheel running (VWR) following 1- or 3-months of dietary intervention. B) Total distance run in kilometres (km) following 20-days of voluntary wheel running following 1- or 3-months of dietary intervention. C) Daily distance run (km) across 20-days of VWR following 1-month dietary intervention. D) Daily distance run (km) across 20-days of VWR following 3-months of dietary intervention. Data mean ± SD (n=5-8/group). [A]Main effect for vitamin D diet; $P > 0.05$.

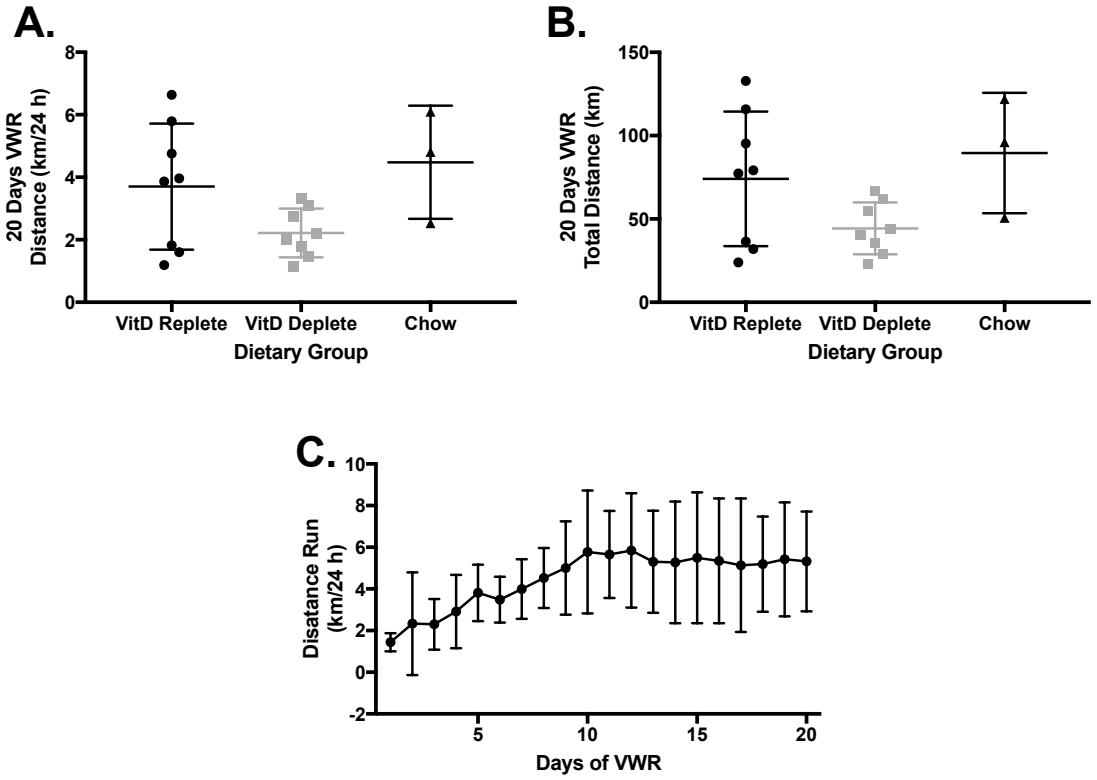

Figure 6.2. No difference in running distance or volume when vitamin D diet mice are compared to standard chow diet mice. A) Average of daily running distance in kilometres (km) following 20-days of voluntary wheel running in mice fed a standard chow, vitamin D replete and vitamin D deplete diets. B) Total running distance in kilometres (km) following 20-days of voluntary wheel running in mice fed a standard chow, vitamin D replete and vitamin D deplete diet. C) Daily running distance in kilometres in standard chow diet mice. Data mean ± SD (n = 3-8/group).

6.3.2 Body Weight

No differences were observed in body weight at baseline between each group (P > 0.05) (Table 6.1). Mice were given voluntary access to a wheel for a period of 20-days following either 1- or 3-months of dietary intervention. Following 20-days of voluntary wheel running, no differences in final body weight were observed when comparing vitamin D replete to deplete mice following 1- or 3-months of dietary intervention (P > 0.05) (Fig. 6.3A). Similarly, no changes were observed in the percentage change in body weight from pre to post voluntary wheel running (P > 0.05) (Fig. 6.3B). A slight trend however was observed in the percentage change in

body weight when vitamin D replete mice were compared with vitamin D deplete (P = 0.066) (Fig. 6.3B).

	Dietary Period (Months)			
	1	**3**	***P***	
Body Weight (g)			***VitD***	***Time***
Replete	27.7 ± 1.4	27.4 ± 0.4		
Deplete	27.8 ± 0.7	28.2 ± 0.7	*0.374*	*0.883*
Lean Mass (g)				
Replete	24.2 ± 1.4	24.4 ± 0.8		
Deplete	24.7 ± 1.7	25.3 ± 0.9	*0.149*	*0.397*
Lean Mass (% of BW)				
Replete	87.4 ± 1.8	88.8 ± 2.3		
Deplete	88.8 ± 1.3	89.4 ± 2.0	*0.168*	*0.148*
Fat Mass (g)				
Replete	1.9 ± 0.5	1.7 ± 0.7		
Deplete	1.8 ± 0.5	1.8 ± 0.4	*0.917*	*0.409*
Fat Mass (% of BW)				
Replete	7.1 ± 1.9	6.2 ± 2.8		
Deplete	6.5 ± 1.6	6.3 ± 1.7	*0.698*	*0.430*

Table 6.1. No differences in baseline body composition in mice prior to dietary and exercise intervention. Data mean ± SD (n = 5-8/group).

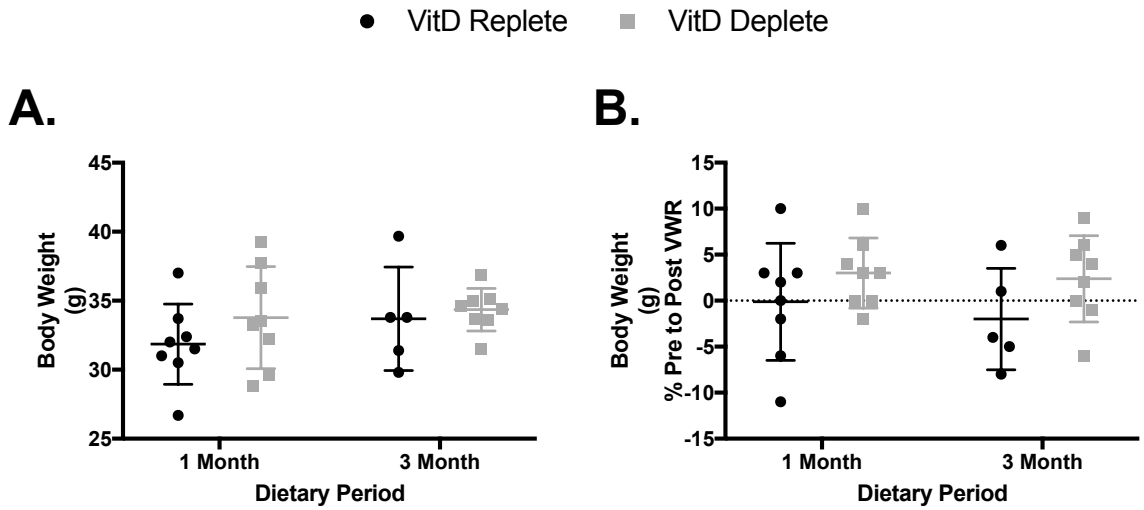

Figure 6.3. No differences in the final or percentage change in body weight following 20-days of voluntary wheel running in vitamin D replete and deplete mice. A) Final body weight following 1- or 3-months dietary intervention and 20-days of voluntary wheel running (VWR). B) Change in body weight as a percentage across 20-days voluntary wheel running period. Data mean ± SD (n=5-8/group). [A]Main effect of vitamin D diet; $P < 0.05$.

6.3.3 Lean Mass

No differences in baseline lean mass were observed at baseline in each of the groups ($P > 0.05$) (Table 6.1). Following 20-days of voluntary wheel running, absolute lean mass was similar between all groups ($P > 0.05$) (Fig. 6.4A). Similarly, absolute lean mass remained unchanged following 20-days of voluntary wheel running in all groups ($P > 0.05$) (Fig. 6.4B). In addition, no differences were observed in lean mass as a percentage of body weight ($P > 0.05$) (Fig. 6.4C). However, the change in lean mass as a percentage of body weight pre to post voluntary wheel running was greater in the vitamin D replete mice when compared to vitamin D deplete ($P = 0.013$) (Fig. 6.4D). No differences were observed when comparing the change in lean mass as a percentage of body weight when comparing individual groups ($P > 0.05$) (Fig. 6.4D).

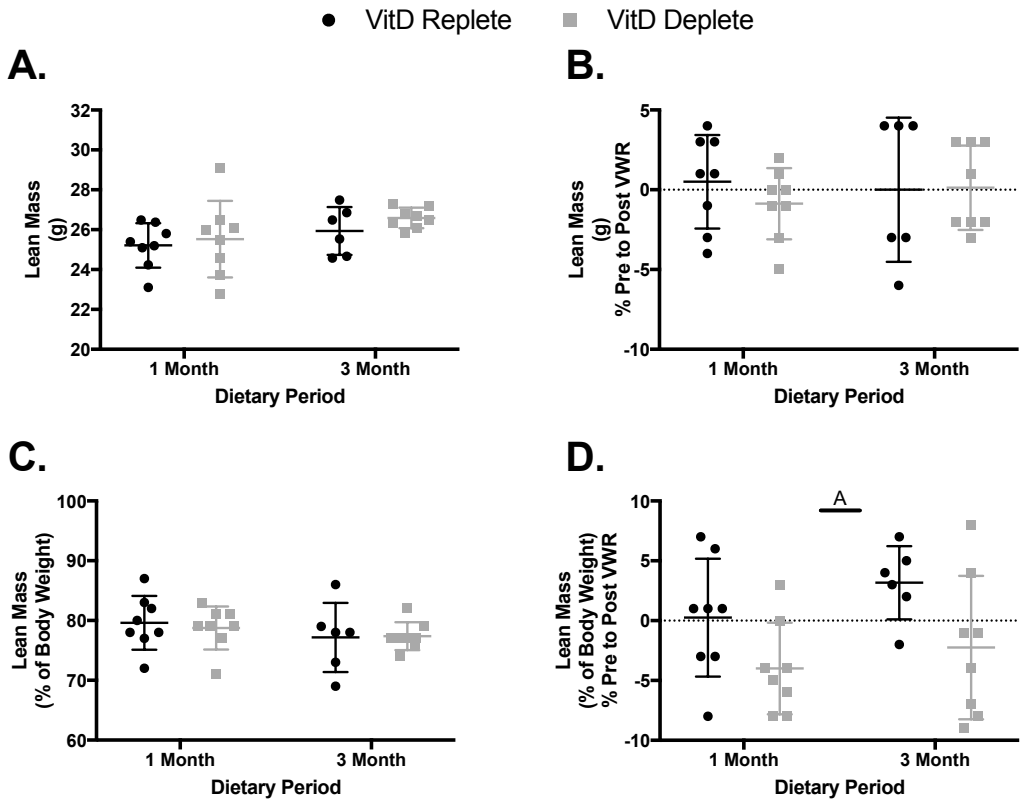

Figure 6.4. Reduction in lean mass as a percentage of body weight across 20-days of voluntary wheel running in vitamin D deplete mice. A) Final lean mass following 1- or 3-months of dietary intervention and 20-days of voluntary wheel running. B) Percentage change in lean mass across 20-days of voluntary wheel running period. C) Final lean mass as a percentage of body weight following 1 or 3-months of dietary intervention and 20-days of voluntary wheel running. D) Percentage change in lean mass as a percentage of body weight across 20-days of voluntary wheel running. Data mean ± SD (n=5-8/group). [A]Main effect of vitamin D diet; $P < 0.05$.

6.3.4 Fat Mass

No differences were observed in fat mass baseline between individual groups (P > 0.05). Following 20-days of voluntary wheel running, there were no differences in absolute fat mass ($P > 0.05$) (Fig. 6.5A). However, the change in absolute fat was greater in the vitamin D deplete mice when compared to vitamin D replete ($P = 0.003$) (Fig. 6.5B). Furthermore, the 3-month vitamin D deplete mice gained fat mass (6 ± 30%) following 20-days of voluntary wheel running whilst the vitamin D replete mice was reduced (-43 ± 50%) ($P = 0.032$) (Fig. 6.5B). No differences between groups were observed in fat mass as a percentage of body weight ($P > 0.05$) (Fig.

6.5C). However, the change in fat mass as a percentage of body weight pre to post voluntary wheel running was greater in the vitamin D deplete mice when compared to vitamin D replete (P = 0.002) (Fig. 6.5D). Furthermore, the 3-month vitamin D replete mice reduced fat mass (-38 ± 41%) as a percentage of body weight pre to post voluntary wheel running whilst the vitamin D deplete mice remained unchanged (5 ± 27%) (P = 0.030) (Fig. 6.5D).

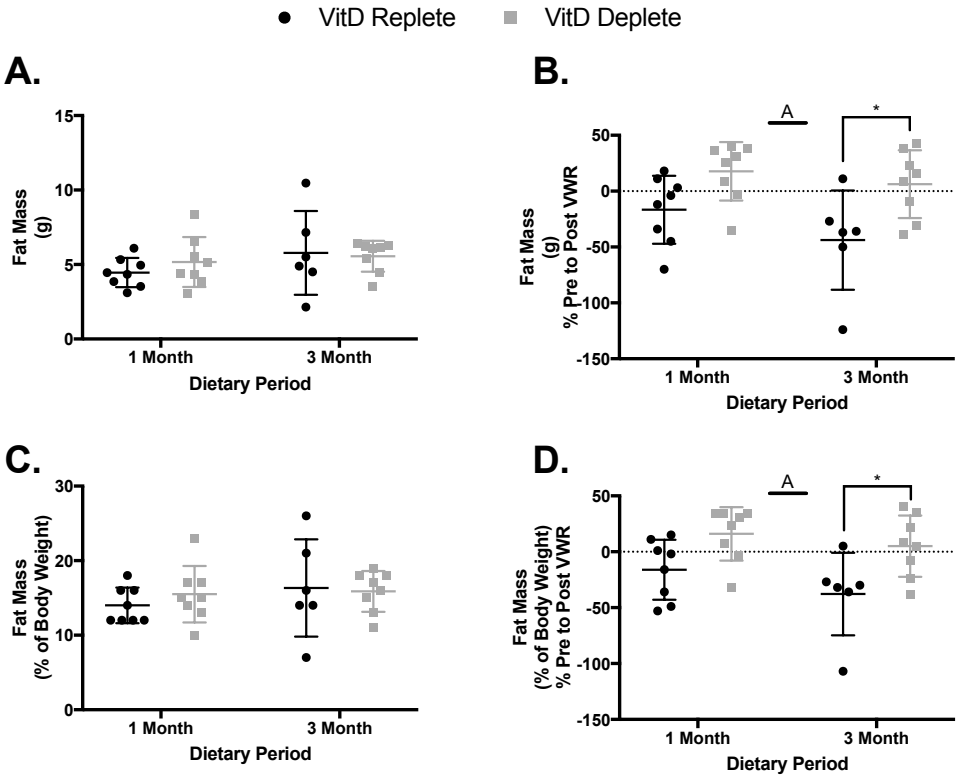

Figure 6.5. Increase in absolute fat mass and as a percentage of body weight in vitamin D deplete mice when compared to vitamin D replete following 3-months of dietary intervention and 20-days of voluntary wheel running. A) Final fat mass following 1- or 3-months of dietary intervention and 20-days of voluntary wheel running. B) Percentage change in fat mass across 20-day voluntary wheel running period. C) Final fat mass as a percentage of body weight following 1 or 3 months of dietary intervention and 20-days of voluntary wheel running. D) Percentage change in fat mass as a percentage of body weight across 20-days of voluntary wheel running. Data mean ± SD (n=5-8/group). [A]Main effect of vitamin D diet; P < 0.05.

6.3.5 Skeletal Muscle Mass

An increase in gastrocnemius (P = 0.006) and triceps (P = 0.003) mass was observed with the 3-month mice displaying greater tissue weights than those of the 1-month. In contrast, no differences were observed in quadriceps mass (P = 0.069). No differences were observed between vitamin D replete and deplete mice in gastrocnemius, quadriceps and triceps weights (P > 0.05).

	Dietary Period (Months)			
	1	**3**	***P***	
Gastrocnemius (mg)			*VitD*	*Time*
Replete	150 ± 9	159 ± 13		
Deplete	149 ± 12	166 ± 17	*0.597*	*0.014*
Quadriceps (mg)				
Replete	184 ± 20	162 ± 18		
Deplete	165 + 34	159 ± 13	*0.217*	*0.128*
Triceps (mg)				
Replete	103 ± 11	116 ± 13		
Deplete	99 ± 17	118 ± 11	*0.782*	*0.004*

Table 6.2. Increase in gastrocnemius and triceps mass in 3-month mice when compared to 1-month with no effect of diet-induced vitamin D deficiency. Data mean ± SD (n=5-8/group).

6.3.6 Serum Calcium

Serum calcium measurements were similar between vitamin D replete and deplete mice when measured following 1-month of dietary intervention and 20-days of voluntary wheel running (P > 0.05) (Fig. 6.6A).

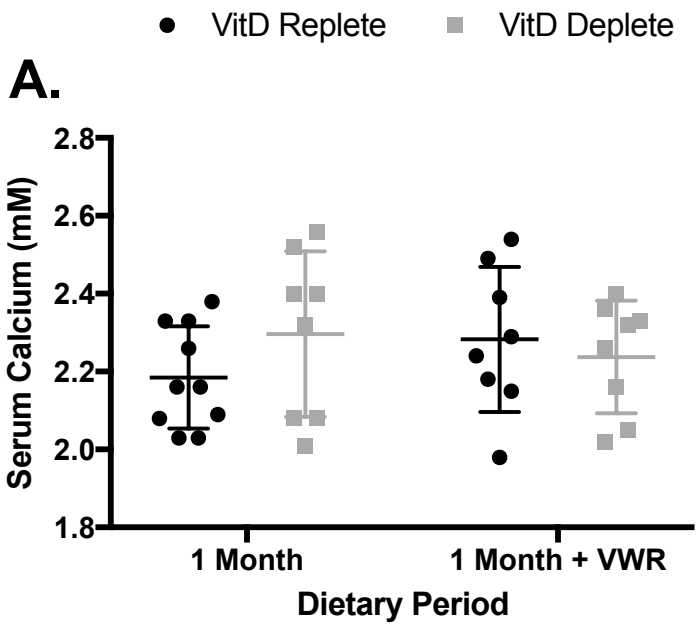

Figure 6.6. No change in serum calcium following diet-induced vitamin D deficiency and 20-days of voluntary wheel running. Data mean ± SD (n = 8-10/group).

6.3.7 Skeletal Muscle Mitochondrial Function

In response to both dietary intervention and 20-days of voluntary wheel running, complex I related leak remained unchanged ($P > 0.05$) (Fig. 6.7A). Overall, complex I phosphorylating respiration increased in response to voluntary wheel running ($P = 0.005$) and a main interaction effect was observed ($P = 0.027$) (Fig. 6.7B). A significant training response in complex I phosphorylating respiration was observed in vitamin D replete mice when comparing the diet only group (172.42 ± 51.60 pmol.sec^{-1}.mg^{-1}.dry weight) to diet and voluntary wheel running (333.32 ± 86.15 pmol.sec^{-1}.mg^{-1}.dry weight) (P = 0.004) (Fig. 6.7B). Similarly, complex I and II phosphorylating respiration increased in response to voluntary wheel running ($P = 0.007$) and an interaction effect was observed ($P = 0.039$) (Fig. 6.7C). In response to voluntary wheel running, vitamin D replete mice displayed a significant training response in complex I and II phosphorylating respiration when comparing the diet

only group (274.86 ± 47.57 pmol.sec^{-1}.mg^{-1}.dry weight) to diet and voluntary wheel running (427.33 ± 89.48 pmol.sec^{-1}.mg^{-1}.dry weight) (P = 0.008) (Fig. 6.7C). Overall, the maximal capacity of the electron transport chain increased in response to voluntary wheel running (P = 0.003) and a main interaction effect was observed (P = 0.039) (Fig. 6.7D). In response to voluntary wheel running, vitamin D replete mice increased the maximal capacity of the electron transport chain when comparing the diet only group (323.19 ± 48.68 pmol.sec^{-1}.mg^{-1}.dry weight) to diet and voluntary wheel running (517.01 ± 91.28 pmol.sec^{-1}.mg^{-1}.dry weight) (P = 0.005) (Fig. 6.7D).

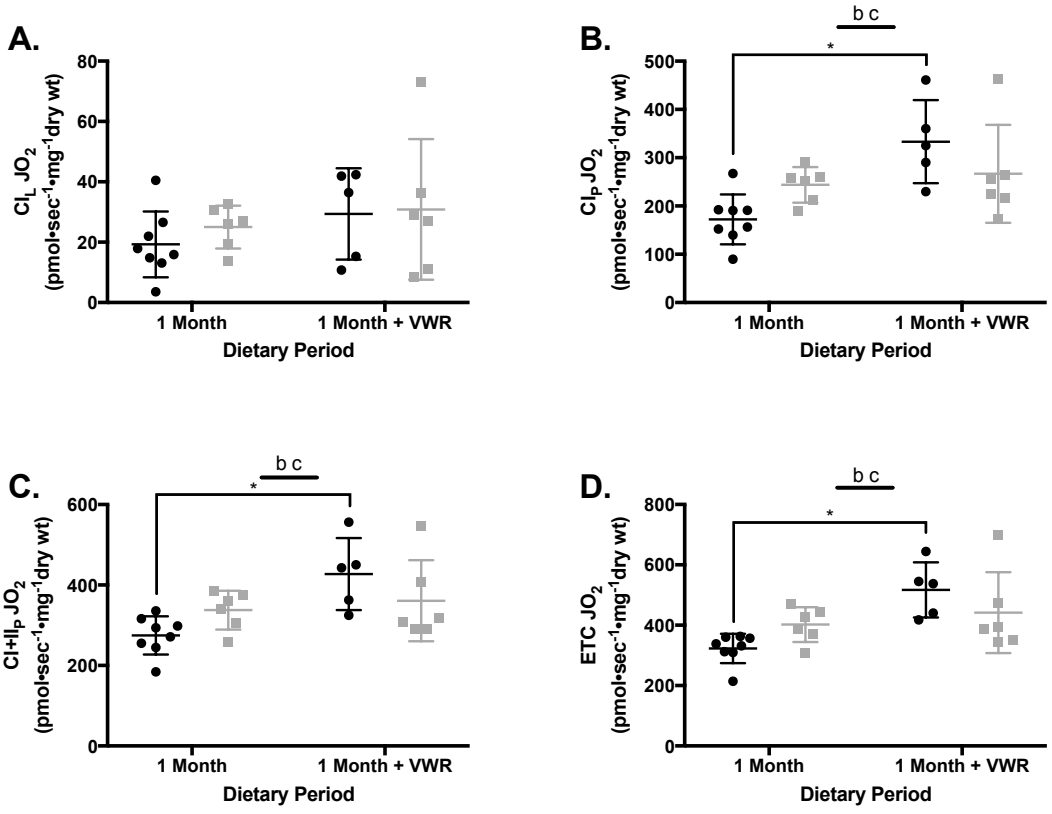

Figure 6.7. Increases in mitochondrial function following 20-days of voluntary wheel running in vitamin D replete mice. A) Complex I related leak (CI_L) in response to diet-induced vitamin D deficiency and 20-days of voluntary wheel running. B) Complex I phosphorylating respiration (CI_P) in response to diet-induced vitamin D deficiency and 20-days of voluntary wheel running. C) Complex I and II phosphorylating respiration ($CI+II_P$) in response to diet-induced vitamin D deficiency and 20-days of voluntary wheel running. D) Maximal capacity of the electron transport chain (ETC) in response to diet-induced vitamin D deficiency and 20-days of voluntary wheel running. Data mean ± SD (n=5-8/group). [b]Main effect for VWR; P < 0.05. [c]Main interaction effect; P < 0.05. *P < 0.05.

6.3.8 ADP Sensitivity

The titration of ADP across multiple concentrations (100-6000 μM) revealed no differences between vitamin D replete and deplete mice in ADP stimulated respiration following 20-days of voluntary wheel running ($P > 0.05$) (Fig. 6.8A). Overall, ADP sensitivity as measured by the apparent K_m for ADP, increased in response to voluntary wheel running ($P = 0.004$) (Fig. 6.8B). In response to 20-days of voluntary wheel running, ADP sensitivity decreased in vitamin D replete mice when comparing the diet only group (209.4 ± 37.2 μM) to diet and voluntary wheel running group (460.1 ± 71.9 μM) ($P = 0.011$) (Fig. 6.8B).

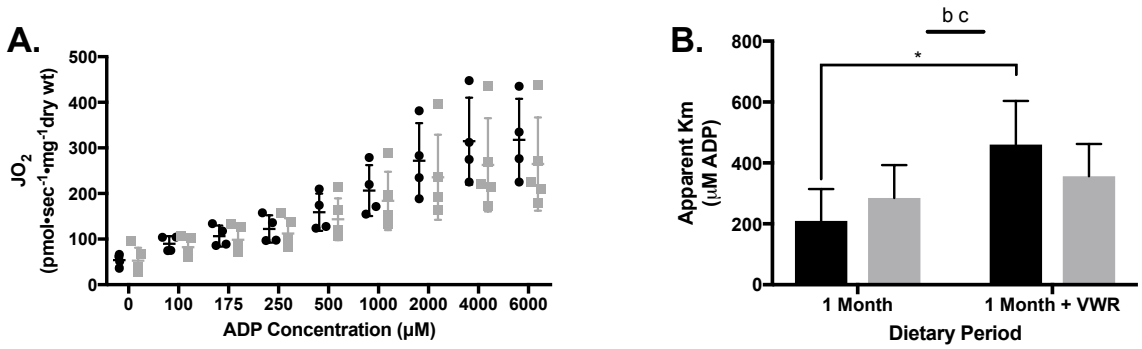

Figure 6.8. Increased ADP sensitivity in response to 20-days of voluntary wheel running in vitamin D replete mice. A) ADP stimulated respiration following 20-days of voluntary wheel running in vitamin D replete and deplete mice. B) The apparent K_m for ADP following 1-month of dietary intervention and 20-days of voluntary wheel running in vitamin D replete and deplete mice. Data mean ± SD (n=5-8/group).

6.3.9 Flux Control Ratios

When normalised to maximal respiration, there were no differences in respiration support via complex I ($P < 0.05$) or complex I and II ($P > 0.05$) combined in response to 20-days of voluntary wheel running (Fig. 6.9A-B).

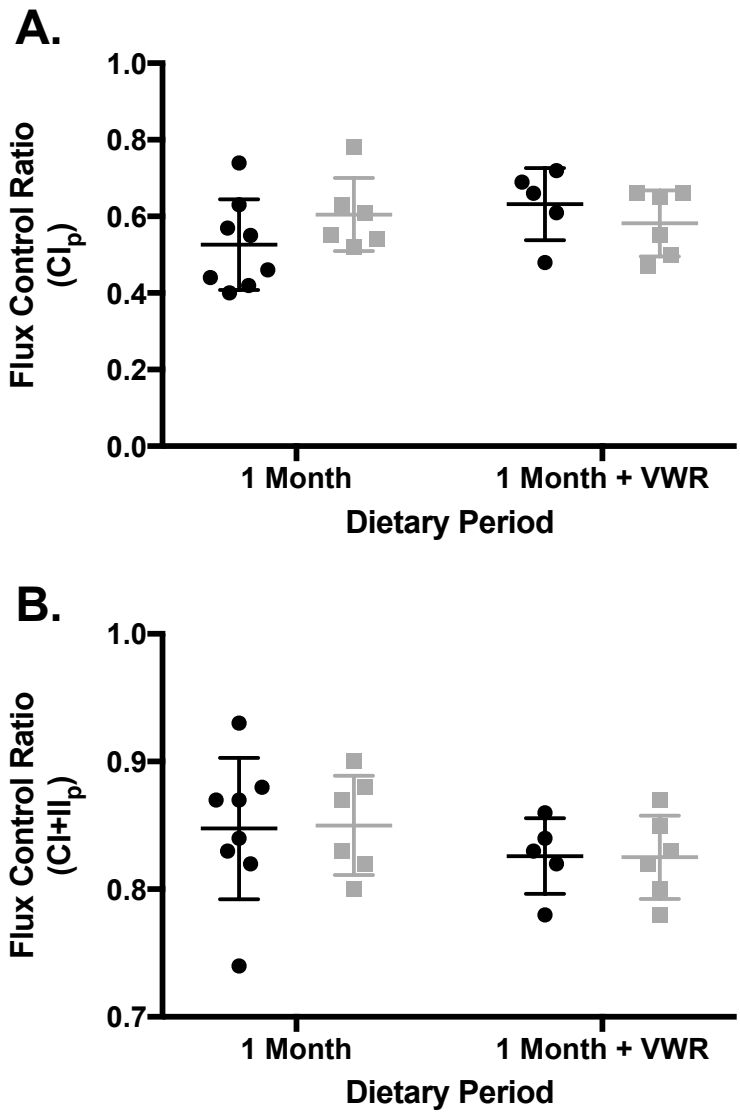

Figure 6.9. No differences in flux control ratios following 20-days of voluntary wheel running. A) Complex I related flux control ratio following 1-month dietary intervention and 20-days of voluntary wheel running. B) Complex I and II related flux control ratio following 1-month dietary intervention and 20-days of voluntary wheel running. Data mean ± SD (n=5-8/group).

6.4 Discussion

We sought to determine the effects of diet-induced vitamin D deficiency upon the adaptive responses to 20-days of voluntary wheel running in C57BL/6J mice. Overall, diet-induced vitamin D deficiency resulted in a reduced daily and total volume in running performance in comparison to vitamin D replete mice (Fig. 6.1A-B). Whilst no changes in body weight were observed (Fig. 6.3A-B), diet-induced vitamin D deficiency resulted in a diminished accretion of lean mass (Fig. 6.4D) and loss of fat mass (Fig. 6.5C) as a percentage of body weight across the 20-days of voluntary wheel running. Alongside alterations in body composition, vitamin D replete mice displayed an increase in multiple parameters of mitochondrial function (CI_P, $CI+II_P$, ETC and ADP sensitivity) following 20-days of voluntary wheel running (Fig. 6.7A-D; Fig. 6.8B).

Previously, diet-induced vitamin D deficiency has been shown to reduce physical performance within the C57BL/6J mouse line [11, 17]. Reductions in grip strength, grip endurance, sprint speed and stride length have been observed across a range of dietary intervention periods (3-12 months) [11, 17]. Furthermore, mice with a myocyte-specific deletion of the VDR display a reduction in voluntary wheel running capacity when assessed over a 24 h period [32]. Despite this, no previous study has examined the effects of diet-induced vitamin D deficiency upon voluntary wheel running capacity in C57BL/6J mice. Voluntary wheel running offers an interesting model to study physical capacity and exercise adaptation within mice. In comparison to forced treadmill exercise, voluntary wheel running offers a more natural pattern of running behaviour in mice, the exercise intervention is performed under non-stressed

conditions and it can easily be applied in longer term intervention studies [33]. Although a large degree of variation exists, which is to be expected with voluntary wheel running, vitamin D replete (1-month; 3.7 ± 2.0 km, 3-month; 4.0 ± 2.8 km) mice did run on average more than that of their vitamin D deplete (1-month; 2.2 ± 0.8 km, 3-month; 2.7 ± 1.1 km) counterparts. Similar to previous observations [11, 17], this supports the notion that vitamin D deficiency reduces physical performance in mice. Whilst strain to strain variations exist, mice have been to known to run between ~4 to 20 km per day [33-35]. Given this, our mice were on the lower end of this spectrum. In addition to these low running distances a large number of mice (11/40) were removed from the above analysis due to running distances of <1 km per day. The removal of mice was not limited to a certain cohort of vitamin D replete or deplete mice suggesting low running distances were not as a result of vitamin D deficiency. In order to further explore this issue, we also gave mice fed a standard chow diet over the same period as the 1-month dietary intervention mice access to a running wheel. We observed similar running distances (4.5 ± 1.8 km) in standard chow mice as well as a large proportion running <1 km per day (3/6). Therefore, the lower running distances we observed previously were not as a result of the vitamin D replete and deplete diets. A number of factors may be contributing to these lower running distances including; genetic influences, sex differences and running wheel design [35-38]. All of which will be discussed in more detail below (Chapter 7).

Despite the low running distances, vitamin D replete mice displayed some favourable adaptations in body composition, particularly within the 3-month mice. Whilst we and others have shown no differences in body weight or skeletal muscle tissue weight

following voluntary wheel running in C57BL/6J mice [39], we also report no difference in absolute fat and lean mass following diet-induced vitamin D deficiency and 20-days of voluntary wheel running. However, as a percentage of body weight, vitamin D replete mice displayed positive responses in the accretion of lean mass and the loss of fat mass following 20-days of voluntary wheel running. Whilst others have also reported favourable adaptations in body composition to voluntary wheel running including reductions in fat mass [40], we now highlight that these changes are dependent upon vitamin D status. It is important to note that given the reduced running distances in the vitamin D deplete mice, the favourable adaptations in vitamin D replete mice may be a consequence of an increased training volume. It would be interesting to see if these effects are more pronounced in a voluntary wheel running model that encourages increased running distances than that observed within this study.

Adaptations to the mitochondria are a prominent feature in the response of skeletal muscle to endurance training [22, 24, 25]. In addition, voluntary wheel running in mice is known to result in an increase in citrate synthase activity, mitochondrial protein content and markers of mitochondrial biogenesis (PGC-1α and TFAM) [40, 41]. Whilst diet-induced vitamin D deficiency has been shown to impair physical performance in mice [11, 17], its effects upon the adaptive response to a period of endurance exercise have not been studied. Given this, we sought to assess mitochondrial function with permeabilised skeletal muscle fibres following 20-days of voluntary wheel running in vitamin D replete and deplete mice. In response to endurance exercise, both mitochondrial mass and oxygen consumption are known to

increase in rodent skeletal muscle [22, 40-42]. Similarly, we report an increase in multiple parameters of mitochondrial function (CI_P, $CI+II_P$ and ETC) following 20-days of voluntary wheel running in C57BL/6J mice. However, these increases were only apparent in vitamin D replete mice indicating an impaired training adaptation following 1-month of diet-induced vitamin D deficiency. Similarly, the apparent K_m for ADP increased in vitamin D replete mice only following 20-days of voluntary wheel running, indicating a reduced sensitivity to ADP. Whilst this seems counter intuitive, a decrease in the sensitivity to ADP within skeletal muscle is a prominent feature of the adaptive response to endurance exercise [43-46]. Whilst absolute rates of respiration are increased, no differences were observed when respiration was reported as a ratio of maximal respiration (FCR). This likely indicates that alterations in respiration are mediated by changes in mitochondrial quantity as opposed to quality although, this would need confirming via the assessment of mitochondrial mass via TEM, CS activity or mitochondrial protein content [47]. In addition, we completed the same analysis following 3-months of diet-induced vitamin D deficiency however, technical issues with the analysis resulted in an unusable dataset. Alongside considerable increases (>10%) in respiration in response to the addition of cyt c, overall respiration values were much lower than normally observed throughout this thesis and in previous reports [48].

In conclusion, we report that diet-induced vitamin D deficiency results in a reduced capacity for voluntary wheel running in C57BL/6J mice. Vitamin D replete mice displayed favourable responses in body composition pre to post voluntary wheel running, highlighted by an accretion of lean mass and decrease in fat mass as a

percentage of body weight. Although mitochondrial function increased in the vitamin D replete mice, there was no difference in mitochondrial function when comparing vitamin D replete mice to vitamin D deplete at the same time point. Mitochondrial respiration data from the 3-month cohort of mice may have revealed more prominent differences following diet-induced vitamin D deficiency. Given that vitamin D deficiency remains widespread [1, 2] in the general population and even within athletic populations [29], vitamin D status may be an important consideration for those looking to maximise the adaptive benefits of exercise. Further characterisation of the adaptive process in models of vitamin D deficiency over longer timeframes would provide further evidence towards to determining the role of vitamin D in exercise adaptation.

6.5 References

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CHAPTER 7

GENERAL DISCUSSION

7.1 Introduction

Skeletal muscle is a highly adaptive (i.e. plasticity) tissue, capable of undergoing distinct periods of hypertrophy and atrophy in response to both positive and negative stimuli [1, 2]. Contributing to this plasticity, a dynamic network of mitochondria exists within skeletal muscle [3]. In response to physiological stressors such as contractile activity, this network undergoes events of biogenesis, fusion, fission and mitophagy [4-9]. Primarily functioning to produce ATP via oxidative phosphorylation [10], the maintenance of mitochondrial health is therefore crucial to meet the energy demands of skeletal muscle and other tissues throughout the body [11]. Indeed, mitochondrial dysfunction has been linked to numerous disease states including, the age related loss of muscle mass termed sarcopenia [12]. Whilst physical inactivity is a major risk factor for age related diseases, poor nutritional status and dietary deficiencies have also been linked to impaired physical function [13-16]. A common nutritional deficiency is that of vitamin D [17, 18]. Recent investigations have shown that vitamin D related metabolites are able to increase mitochondrial function in skeletal muscle *in vitro* [19-21] and within human populations of severe vitamin D deficiency [22]. Despite this, little is known with regard to the role of the VDR in mediating this adaptive response. Furthermore, the effects of vitamin D deficiency upon skeletal muscle mitochondrial function *in vivo* remain largely underexplored. Therefore, the aims of this thesis were as follows;

1. Develop a reliable method for the detection of the VDR within skeletal muscle samples (Chapter 3).

2. Determine the role of the VDR within the maintenance of skeletal muscle mitochondrial function and protein content (Chapter 4).

3. Examine the role of diet-induced vitamin D deficiency on anthropometric measures of body composition and mitochondrial function in C57BL/6J mice (Chapter 5).

4. Determine whether diet-induced vitamin D deficiency impairs the positive impact of exercise on body composition and mitochondrial function in C57BL/6J mice (Chapter 6).

In order to address these aims, this thesis utilised both *in vitro* and *in vivo* approaches. Firstly, methodological considerations were evaluated for the detection of the VDR within skeletal muscle. This was followed by a combination of *in vitro* and *in vivo* approaches to assess the skeletal muscle-specific role of the VDR in regulating mitochondrial function and protein content. Finally, two *in vivo* studies investigated the influence of diet-induced vitamin D deficiency upon skeletal muscle mitochondrial function. This chapter will further summarise and amalgamate the data obtained throughout this thesis.

7.2 The Vitamin D Receptor in Skeletal Muscle

Recent investigations have shown that vitamin D related metabolites are able to increase mitochondrial function in both immortalised and primary skeletal muscle cell lines [19-21]. To directly assess the role of the VDR on mitochondrial function, we utilised RNA interference approaches to generate a stable C2C12 clone in which VDR protein content was reduced by ~95%. Utilising this novel approach, we report that loss of VDR in C2C12s resulted in a significant decrement in mitochondrial function (Chapter 4). Despite this, no changes were observed in mitochondrial

protein content in both VDR-KD myoblasts or myotubes (Chapter 4). In continuation, we also studied the skeletal muscle-specific role of the VDR within rat skeletal muscle. Whilst the knock-down of the VDR resulted in a trend towards a decrease in both mitochondrial complex I and IV, overall, we observed no differences in mitochondrial protein content following both the knock-down and over-expression of the VDR (Chapter 4). Results that have recently been corroborated in a mouse model with the skeletal muscle-specific deletion of the VDR [23]. Despite this, this does not preclude a role for the VDR in mediating mitochondrial function *in vivo*. Given that we report a reduction in mitochondrial function following 3-months of diet-induced vitamin D deficiency in C57BL/6J mice (Chapter 5), the examination of mitochondrial function in mouse models with the skeletal muscle-specific deletion of the VDR may also reveal functional decrements despite no change in mitochondrial protein content. Unfortunately, we were unable to perform such measurements due to limited access to tissue of VDR-KD and –OE rats.

The precise role by which the VDR may mediate changes in skeletal muscle mitochondrial function remains unclear. Previously, microarray analysis has identified potential VDR target genes however, the overlap between tissues is minimal, highlighting that VDR target genes respond in a highly tissue specific manner [24, 25]. Within skeletal muscle, >80 mRNAs encoding for mitochondrial proteins have been shown to be upregulated in response to treatment with $1\alpha,25(OH)_2D_3$ [19] however, it is unclear whether these genes contain specific VDREs. A potential target for further exploration, the chromosome 15 open reading frame 48 (*C15orf48*) is responsive to $1\alpha,25(OH)_2D_3$ within skeletal muscle [19]. Whilst this protein remains largely uncharacterised, particularly within skeletal muscle, one study reported an interaction with both complex I and IV, suggesting that it regulates either the activity

of said complexes or potential supercomplex formation [26]. Therefore, the potential characterisation of said protein within skeletal muscle may provide an opportunity to establish a mechanism by which the VDR mediates mitochondrial function.

Due to its extremely low expression levels within skeletal muscle [27, 28], we were unable to detect the VDR in adult mouse skeletal lysates (Chapter 3). Whilst the detection of the VDR was not central to aim of each chapter (Chapter 5-6), the ability to reliably detect the VDR would have enabled further analysis throughout this thesis. Previously, the successful detection of the VDR within human skeletal muscle has been reported following a period of vitamin D supplementation [29, 30], suggesting the tissue-specific expression levels of the VDR are mediated by whole-body vitamin D status. In addition, whilst the expression of the VDR has been reported to increase in response to an acute bout of muscle damage [31], its expression in response to a period of chronic exercise has not been explored. Therefore, the ability to reliably detect the VDR within adult skeletal muscle would have enabled the further exploration of these questions and added further novelty to this thesis (Chapter 5-6). Given that we were unable to detect the VDR within skeletal muscle, this raises questions in regards to its potential relevance *in vivo*. Given the systemic changes that occur in models of vitamin D deficiency and VDR-KO mice, the phenotypes observed may be as a result of indirect effects as opposed to VDR signalling. Further characterisation of models with skeletal muscle VDR-KO will shed light upon the role of the VDR within skeletal muscle *in vivo* [23].

7.3 Vitamin D Deficiency, Body Composition and Physical Function

Previously, no changes in body composition were reported in male C57BL/6J mice following diet-induced vitamin D deficiency across a 12-month period [32]. On the

other hand, reductions in body weight, lean mass and fat mass were reported in female mice across the same time period [33]. In response to 3-months of diet-induced vitamin D deficiency, we observed no differences in body weight, lean mass or fat mass between vitamin D replete and deplete C57BL/6J male mice. Vitamin D deplete mice did display a reduction in lean mass as a percentage of body weight from 1- to 3-months of diet-induced deficiency. This however, is likely due to the observed increase in lean mass as a percentage of body weight at the 1-month time point between vitamin D replete and deplete mice. In continuation of this, we also examined body composition in response to a period of voluntary wheel running (Chapter 6). Whilst physical function has been reported to be impaired in mouse models of diet-induced vitamin D deficiency [32, 34], these effects have only been explored in the basal state and not following a period of adaptation. In order to explore the effects of diet-induced vitamin D deficiency upon exercise adaptation, we utilised a model of voluntary wheel running in which mice were given access to a wheel for a period of 20-days following either 1 or 3-months of diet-induced vitamin D deficiency. In comparison to vitamin D replete mice, diet-induced vitamin D deficiency resulted in a reduced overall running performance (Chapter 6). Reductions in both sprint speed and stride length have previously been reported and likely contribute to reduced running performance observed in our study [32, 34]. In addition, mice with the skeletal muscle-specific deletion of the VDR display similar reductions in running performance over a 24 h period, suggesting direct effects for vitamin D related signalling in mediating physical function [23]. Whilst the running distances we observed were lower than that previously observed [35-37], similar running distances in mice fed a standard chow indicate that this is a strain issue as opposed to an effect of the vitamin D diets. The further analysis of running performance and subsequent adaptation in a mouse model of increased running volume may reveal

more prominent effects of diet-induced vitamin D deficiency. Whilst, no changes in body weight were observed between vitamin D replete and deplete mice, we did observe some favourable adaptations in body composition in vitamin D replete mice. Most notably, vitamin D replete mice displayed a positive accretion of lean mass and a decrease in fat mass as percentages of body weight in response to 20-days of voluntary wheel running. These beneficial effects are likely due to an increase in running volume observed within vitamin D replete mice.

7.4 Vitamin D Deficiency and Skeletal Muscle Mitochondrial Function

Whilst vitamin D related metabolites are reported to increase skeletal muscle mitochondrial function *in vitro* [19-21], *in vivo* investigations are currently lacking. To date, one study explored skeletal muscle mitochondrial function following vitamin D supplementation in a cohort of severely deficiency patients exhibiting symptoms of skeletal muscle myopathy [22]. Skeletal muscle mitochondrial function was assessed indirectly via 31-P MRS and vitamin D supplementation was reported to reduce PCr recovery time, a marker of improved oxidative phosphorylation [22]. Despite this observation, a number of limitations were evident in this study, such as improper randomisation and blinding [22]. Therefore, in order to further examine the role of vitamin D status in modulating mitochondrial function *in vivo*, we utilised a well-established mouse model of diet-induced vitamin D deficiency [34] in combination with high-resolution respirometry, the current gold-standard method to assess mitochondrial function within skeletal muscle fibres [38]. Initially, we examined the role of diet-induced vitamin D deficiency alone upon skeletal muscle mitochondrial function. We reported that 3-months of diet-induced deficiency reduced the capacity of multiple parameters of skeletal muscle mitochondrial function to increase ($CI+II_P$ and ETC) (Chapter 5). In continuation of this, we also assessed the effects of diet-

induced vitamin D deficiency upon skeletal muscle mitochondrial function following a period of voluntary wheel running (Chapter 6). We reported that following 1-month of diet-induced vitamin D deficiency and 20-days of voluntary wheel running, only vitamin D replete mice displayed an increase in multiple parameters of mitochondrial function (CI_P, $CI+II_P$ and ETC) (Chapter 6). Therefore, we build upon previous research reporting impairments in physical function following diet-induced vitamin D deficiency [32, 34] by highlighting a role for vitamin D status in modulating skeletal muscle mitochondrial function not only in the basal state, but also following a period of adaptation.

Furthermore, we also examined the effects of diet-induced vitamin D deficiency upon mitochondrial ADP sensitivity within skeletal muscle. Whilst diet-induced vitamin D deficiency alone did not alter mitochondrial ADP sensitivity (Chapter 5), only the vitamin D replete mice displayed an increase in the apparent K_m for ADP following 1-month of diet-induced deficiency and 20-days of voluntary wheel running (Chapter 6). Whilst an increase in the apparent K_m highlights a decrease in sensitivity, this is a common characteristic of a trained skeletal muscle phenotype [39-42]. A decrease in sensitivity possibly indicates an increase in mitochondrial mass, however, time constraints limited the scope of analysis performed within these studies (Chapter 5-6). In support of this notion, the reported effects upon skeletal muscle mitochondrial function following diet-induced vitamin D deficiency and voluntary wheel running where abolished when subjected to internal normalisation (Chapter 5-6). This suggests that the alterations observed within parameters of skeletal muscle mitochondrial function are as a consequence of mitochondrial quantity as opposed to quality. The further analysis of tissues collected throughout these studies will provide further insight into the above notion.

7.5 Limitations

As with all research, a number of limitations exist within this thesis that should be considered when making interpretations in regards to the results obtained. Firstly, we were unsuccessful in our attempt to detect the VDR in adult skeletal muscle samples (Chapter 3). Whilst the detection of the VDR was not central to aims of each chapter, it did preclude the further analysis of the role of the VDR in response to diet-induced vitamin D deficiency and voluntary wheel running. Furthermore, our examination of the role of the VDR in modulating skeletal muscle mitochondrial function and protein content was only conducted in the basal state (Chapter 4). Further characterisation of such rodent models in response to physiological stressors such as exercise training and ageing may reveal further skeletal muscle-specific roles for the VDR. Unfortunately, the scope of our analysis of VDR-KD and –OE rat skeletal muscle was limited due to restrictions upon tissue availability as this tissue was utilised in previous research. The analysis of skeletal muscle mitochondrial function within these samples would have also provided further translational of *in vitro* findings (Chapter 4). Furthermore, the analysis of protein content via immunoblotting is semi-quantitative in nature which may limit the sensitivity of such analyses. Given the VDR is proposed to be highly tissue specific [24, 25], untargeted based approaches utilising 'omics' platforms may have provided broader insight into the skeletal muscle-specific role of the VDR.

As discussed previously, a large proportion of mice were removed from analyses due to running distances of <1 km/day (Chapter 6). Even with these mice removed, running distances overall were on the lower end of those previously reported in mice [35-37]. These issues resulted in a reduced sample size and training volume. This

reduced running performance may be as a result of the mouse strain chosen for such studies. The C57BL/6J mouse is known to contain a mutation in the nicotinamide nucleotide transhydrogenase (*Nnt*) gene which encodes for an inner membrane mitochondrial protein [43]. Whilst running distances have not been directly compared to mice without the *Nnt* mutation, metabolic defects in glucose homeostasis and mitochondrial redox abnormalities have previously been reported [43, 44]. In addition, the design of the running wheel is an important consideration when evaluating running performance in mice. For example, an upright wheel, as used within our study (Chapter 6), results in reduced running distances when compared to an angled wheel [35]. Furthermore, given our analyses was conducted using only adult male mice, sex or age-related differences cannot be excluded. The further comparison of mouse strains for running performance may have alleviated such issues. We also assessed skeletal muscle mitochondrial function in response to 3-months of diet-induced vitamin D deficiency and subsequently 20-days of voluntary wheel running. Unfortunately, this analysis was not included due to a number of issues. Firstly, multiple data points exhibited signs of poor fibre preparation including a cyt c response of >10%. In addition, the overall respiration rates were much lower than that previously observed for C57BL/6J skeletal muscle both within this thesis (Chapter 5-6) and by others [45]. Finally, time constraints meant that it was not feasible to repeat analyses with the above issues nor was it feasible to conduct further analysis of the multiple tissue samples that were collected during mouse *in vivo* studies (Chapter 5-6).

7.6 Future Research

Further studies in continuation of recent publications [23, 46] should examine the skeletal muscle-specific role of the VDR in response to physiological stressors such

as exercise training and ageing. In addition, given the role of the VDR is tissue specific [24, 25], the use of unbiased 'omics' approaches may aid in the characterisation of specific VDR target genes within skeletal muscle. The combination of *in vitro* models of VDR-KD with microarray analysis, ChIP and RIME would aid in the identification of skeletal muscle specific VDR targets genes and coregulatory binding partners [47, 48]. Furthermore, the translation of rodent studies of diet-induced vitamin D deficiency into human populations is particularly interesting. The utilisation of techniques such as high-resolution respirometry would shed light upon the effects of vitamin D deficiency upon skeletal muscle mitochondrial function. Such analysis could be combined with the assessment of ROS production, membrane potential or calcium concentrations via the use of O2k-fluorometry [49-51] providing broader insight into the regulation of mitochondrial function in response to vitamin D deficiency and supplementation. Furthermore, recent technical developments that allow for the measurement of multiple vitamin D metabolites provide the opportunity for the further characterisation of the vitamin D metabolome [52, 53]. Recent investigations have reported differential effects of vitamin D_2 and D_3 supplementation upon this metabolome [54]. Similar studies in the context of skeletal muscle may provide broader insight into the positive effects of vitamin D supplementation upon skeletal muscle metabolism.

7.7 Conclusions

Throughout this thesis we have provided direct evidence that loss of VDR leads to significant decrements in mitochondrial function during myogenesis, whilst diet-induced vitamin D deficiency reduces skeletal muscle mitochondrial respiration ($CI+II_P$ and ETC), voluntary wheel running performance and an impaired adaptive response in mitochondrial function (CI_P, $CI+II_P$ and ETC) and body composition in

male C57BL/6J mice. Therefore, we highlight vitamin D status as an important consideration within the maintenance of skeletal muscle mitochondrial function both in the basal state and following a period of adaptation. Given that vitamin D deficiency remains prevalent across multiple populations including both the elderly and athletes [17, 55], maintaining sufficient vitamin D levels may aid in the maintenance of skeletal muscle function. Finally, this thesis contributes novel data towards the understanding of the role of the vitamin D and the VDR in modulating skeletal muscle mitochondrial function.

7.8 References

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