The Role of Exercise and Age on Vitamin D Metabolism

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Abstract

A low vitamin D status (determined by 25(OH)D concentration) has been identified as an association risk factor in the aetiology of numerous chronic diseases, with older adults identified as generally more deficient than younger populations. Accumulating data is suggestive that physical activity and exercise may influence 25(OH)D and vitamin D metabolites downstream in the complex metabolic pathway. Specifically, exercise has been shown to act as a direct and indirect stimulus on the intracellular vitamin D receptor (VDR), which mediates the effects of vitamin D and initiates genomic and non-genomic signalling responses. The role of physical activity and exercise on 25(OH)D concentration and VDR expression is not yet recognised in a healthy human population.

This thesis aimed to establish whether there is a link between physical activity status and 25(OH)D concentration, and if there is a role of age and exercise on 25(OH)D concentration and VDR expression (as quantified in circulating systemic T lymphocytes).

The main results demonstrate that 25(OH)D concentration is not influenced by age or cardiorespiratory fitness (CRF), however VDR expression declines with older age and a higher CRF predicts a greater expression of the VDR. It was found that a single bout of exercise acutely increases VDR expression in circulating T lymphocytes, with exercise modality appearing to influence the response. There was a more pronounced response observed following an endurance compared to a resistance exercise bout. The impact of the exercise-induced lymphocyte response on the observed changes in T cell VDR expression was explored, however they appear to be independent.

In conclusion, the findings of this thesis support the notion that exercise could be used as a short-term strategy to increase cellular VDR expression in a human population.

Keywords: vitamin D, 25(OH)D, vitamin D receptor (VDR), physical activity, exercise, age.

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List of abbreviations

%	Percent
°	Degree
°C	Degree Celsius
µ	Micro
$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
AMPK	Adenosine monophosphate activated protein kinase
ANOVA	Analysis of variance
BMD	Bone mineral density
BMI	Body mass index
BP	Blood pressure
CON	Control
CRF	Cardiorespiratory fitness
CRP	C-reactive protein
CV	Coefficient of variance
DBP	Vitamin D binding protein
DEXA	Dual-energy X-ray absorptiometry
dL	Decilitre
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EE	Endurance exercise
EIA	Enzyme immunoassay
ELISA	Enzyme linked immune sorbent assay
ERK	Extracellular signal–regulated kinase

g	Gram
Geomean	Geometric mean
GLUT4	Glucose transporter type 4
GP	General Practitioner
h	Hour
HCT	Haematocrit
HGB	Haemoglobin
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
HR	Heart rate
IL	Interleukin
IU	International Units
kg	Kilogram
KO	Knock out
L	Litre
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
LPA	Light Physical Activity
m	Metre
MAPK	Mitogen-activated protein kinase
max	Maximal
MFI	Mean fluorescence intensity
mg	Milligram
min	Minute
ml	Millilitre
mmol	Millimole
mRNA	Messenger ribonucleic acid
MPA	Moderate physical activity
MVPA	Moderate-to-vigorous physical activity

MW8	MotionWatch 8
n	Number of participants
NaCl	Sodium chloride
ng	Nanogram
NK	Natural killer
nmol	Nanomole
O_2	Oxygen
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
pg	Picogram
PTH	Parathyroid hormone
PVC	Plasma volume change
RBC	Red blood cell
RE	Resistance exercise
RER	Respiratory exchange ratio
RNA	Ribonucleic acid
rpm	Revolutions/repetitions per minute
RXR	Retinoid X receptor
s	Second
SD	Standard deviation
SEM	Standard error mean
SES	Socioeconomic status
SPSS	Statistical Package for the Social Sciences
T2DM	Type 2 diabetes mellitus
TNF-α	Tumour necrosis factor – alpha
URTI	Upper respiratory tract infection
UV	Ultraviolet

VDR	Vitamin D receptor
VDRE	Vitamin D response element
$\dot{V}O_{2max}$	Maximal oxygen consumption
$\dot{V}O_{2peak}$	Peak oxygen consumption
W	Watt
w/v	Weight to volume
WBC	White blood cell
y	Years

Chapter 1: Introduction

Vitamin D is the major regulator of calcium homeostasis in the body (Sheikh et al., 1988) and is critical in the maintenance of bone mineral density (BMD) (Cauley et al., 2008). As well as this classical role of vitamin D in bone health, it has become evident that vitamin D exerts many additional effects contributing to good health functioning via both genomic and non-genomic pathways. The two main sources of vitamin D: sunlight exposure and dietary sources, contribute to whole-body vitamin D status (Holick, 2002, 2007). A low vitamin D level has been identified as a risk factor in the aetiology of numerous chronic diseases, such as diabetes (Calvo-Romero & Ramiro-Lozano, 2015; Mauss, Jarczok, Hoffmann, Thomas, & Fischer, 2015; Pittas, Lau, Hu, & Dawson-Hughes, 2007) and cardiovascular disease (Dobnig et al., 2008; T. J. Wang et al., 2008), with older adults identified as generally more vitamin D deficient than younger populations (Jacques et al., 1997; Laird et al., 2014; Touvier et al., 2015). Although a sufficient vitamin D status is important and linked to good health, it is through the cellular ligand-dependent receptor, vitamin D receptor (VDR), which receives the converted active form of vitamin D (as a ligand), that modulates the main function of vitamin D metabolism (Haussler, Jurutka, Mizwicki, & Norman, 2011; Haussler et al., 2013). The VDR is a ligand-inducible transcription factor that effectively alters gene transcription and thus protein synthesis (Bischoff et al., 2001; R. U. Simpson, Thomas, & Arnold, 1985). The upregulation or suppression of proteins at a particular time and in a specific quantity plays a role in the pathophysiology of diseases and conditions. Therefore, the bioavailability and functional capacity of the VDR is important when investigating vitamin D metabolism and health.

Scotland can be defined as a vitamin D deficient nation according to outputs from both large and small scale studies evaluating the vitamin D status of residents of Scotland (Gallacher et al., 2005; Holick, 2008; Rhein, 2008; Weiss et al., 2016; Zgaga et al., 2011). Since a low vitamin D status is linked to ill-health (Autier, Gandini, & Mullie, 2012), strategies are required to address the issue. However, vitamin D deficiency may not just be a problem at the circulatory level, but also on a cellular level. Therefore, strategies employed need to address the main mediators of the vitamin D metabolic pathway in order to improve efficiency and effectiveness of vitamin D metabolism,

presenting a solution to acquire the health-protective role vitamin D appears to provide. In addition, vitamin D supplementation, although an easy route to increase vitamin D status, is costly (around £50 per year per person) if all residents in Scotland were prescribed them. In addition, recommending increased sunlight exposure carries health risks itself: skin cancer induced by ultraviolet (UV) A radiation exposure from the sun (Armstrong & Kricker, 2001; Brash et al., 1991), and not feasible due to weather and seasonal influence (Rhodes et al., 2010). Therefore, alternative strategies are required that are low risk, plausible and low expense.

In recent studies it has been reported that mechanical stress, physical activity, and exercise may influence vitamin D metabolism at the surface and cellular level (Aly, Abdou, Rashad, & Nassef, 2016; Maimoun et al., 2005; Makanae et al., 2015; Sun, Cao, Taniguchi, Tanisawa, & Higuchi, 2017). However, there is limited research in humans, with the majority of studies conducted in murine models and not translated into the human population. According to The Scottish Healthy Survey 2016, the physical activity status of Scotland is poor, with reports that 36 % of the adult population, around 1.6 million people, are physically inactive (*The Scottish Health Survey*, 2017). The survey reports also that men are more likely to meet the recommended physical activity guidelines compared to women. If exercise is found to beneficially influence vitamin D metabolism, it could pose as a cost-effective and low risk strategy to combat the deficiency epidemic in Scotland.

Another concern surrounding vitamin D status and it's link with health, is the influence of age, with older populations exhibiting low 25(OH)D concentrations (Chapuy, Durr, & Chapuy, 1983; Jacques et al., 1997) and a reduced expression of VDR (Bischoff-Ferrari, Borchers, et al., 2004). However, it is elusive whether 25(OH)D concentration declines with age independent of other characteristics and environmental factors. Furthermore, adding to this concern, ageing is associated with a decline in physical activity levels (Sallis, 2000) and aerobic fitness (Buskirk & Hodgson, 1987; Fitzgerald, Tanaka, Tran, & Seals, 1997). Interestingly, it has been reported that higher self-reported physical activity is associated with higher circulating concentrations of vitamin D (Jacques et al., 1997; Scragg & Camargo, 2008). However, the effect of

cardiorespiratory fitness (CRF) and exercise itself on key vitamin D metabolites has not yet been investigated in a human population.

The overall aim of this thesis is to investigate the role of physical activity/exercise and age on vitamin D metabolism: primarily systemic 25(OH)D concentration, identified as vitamin D status, and circulating cellular VDR expression. The intention is to identify whether exercise could be proposed and utilised as a strategy to optimise vitamin D metabolism and potentially improve health.

To realise this aim, the following specific objectives will be addressed:

I. To evaluate the vitamin D status of older adults in Scotland and determine whether there is a link with physical activity levels.
II. Investigate whether vitamin D status is influenced by age and associated with CRF.
III. Determine whether there is a relationship between vitamin D status and baseline VDR expression, and investigate the role of age and CRF on VDR expression.
IV. Explore the effects of an acute bout of exercise on VDR expression, and identify if this response is influenced by age.

Chapter 2: Literature Review

2.1 Introduction

This literature review will cover the current main research in the areas of physical activity and exercise on vitamin D status and metabolism, specifically the vitamin D receptor (VDR), of which the function and activity will be reviewed. Both murine and human research will be reviewed, with focus on translation of the investigations into a human population. The role that the inevitable ageing process has on these areas will also be addressed, with the influence of age being a main outcome of the thesis.

2.2 Background of vitamin D

Vitamins are organic compounds, primarily obtained through the diet from dietary food sources or supplements. Vitamin D may not be a vitamin in the truest sense since it has been classified as a fat-soluble secosteroid hormone (Vieth, 2004), classically responsible for increasing intestinal absorption of calcium (Christakos, Dhawan, Porta, Mady, & Seth, 2011). More recently, non-classic roles of vitamin D have emerged in the prevention of chronic disease, assisting the immune system, and regulating the endocrine system (Holick & Chen, 2008). Vitamin D metabolism involves a substantially long and complicated pathway, exerting its effects via genomic and non-genomic mechanisms, ultimately affecting health, or more specifically preventing ill health (Autier, Boniol, Pizot, & Mullie, 2014). Studies have generally documented that vitamin D plays a crucial role in the development and maintenance of skeletal and extraskeletal health (Cauley et al., 2008; Holick, 1996; Hossein-nezhad & Holick, 2013; Wacker & Holick, 2013). Vitamin D deficiency is also linked to the prevalence of cardiovascular disease, diabetes, and autoimmune diseases (Holick, 2004a, 2004b; Hypponen, Laara, Reunanen, Jarvelin, & Virtanen, 2001; Hypponen & Power, 2007; Pittas, Lau, et al., 2007), such as multiple sclerosis (Munger et al., 2004) and Crohn's disease (Simmons, Mullighan, Welsh, & Jewell, 2000).

The two primary sources of vitamin D are dietary intake, ideally contributing to approximately 20 % of total intake (although in very minimal doses), and direct ultraviolet (UV) B radiation exposure from sunlight, optimally contributing the

other 80 % (Holick, 2002). There are two main forms of vitamin D: ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3), which should not be used interchangeably as they do not have equal nutritional values. Both forms contribute to a person's vitamin D status, identified as plasma or serum 25(OH)D concentration, determining whether a person is vitamin D deficient or sufficient (Dawson-Hughes et al., 2005). Vitamin D_2 is derived from plants and thus is consumed through the diet in relatively small quantities, whereas vitamin D_3 can be obtained from a variety of exogenous sources, such as oily fish, eggs, and green leafy vegetables, and produced endogenously by the exposure of skin to UV rays (G. Jones, Strugnell, & DeLuca, 1998). There is evidence that supplementation with vitamin D_3 is more effective at increasing overall vitamin D status than D_2 (Nimitphong, Saetung, Chanprasertyotin, Chailurkit, & Ongphiphadhanakul, 2013; Trang et al., 1998). Through the metabolic pathway, vitamin D_2 needs to be converted to vitamin D_3 in order to be further metabolised into calcidiol (25-hydroxy-vitamin D or 25(OH)D) and thus it is usually 25(OH)D (both forms combined) or 25(OH)D_3 concentration that is reported in the majority of research studies. However, vitamin D status is not only determined by the input but also the efficiency of storing and releasing 25(OH)D. Many cell types, such as skeletal muscle cells and fat cells (Abboud et al., 2014; Abboud et al., 2013), have been reported to uptake 25(OH)D for storage. However, the release of the protein hormone is dependent on the cell type: adipose tissue cells sequester and store 25(OH)D (Wortsman, Matsuoka, Chen, Lu, & Holick, 2000), whereas skeletal muscle cells store but also readily release 25(OH)D (Abboud et al., 2013) to contribute to circulating 25(OH)D concentration when required. It is suggested that this is due to the strong lipophilic properties of 25(OH)D. Compared to other constituents of blood, 25(OH)D has a relatively long half-life that varies between 15-45 days (Abboud et al., 2013; Zehnder et al., 2001; Zerwekh, 2008), although its residence is remarkably longer than its binding protein, vitamin D binding protein (DBP), which is 1-3 days. Therefore, the maintenance of vitamin D status may be attributed to more than just UV exposure, dietary intake, and lifestyle influences.

The concentration of total systemic 25(OH)D_3 has been identified as the most reliant biomarker when determining the overall vitamin D status of a person in both a clinical and research capacity (Holick, 2009). However, an observed elevation in 25(OH)D_3 does not necessarily reflect the efficiency or success of the metabolic pathway as a whole. Despite reported links between vitamin D status and health conditions, there is minimal proof of concept that vitamin D induced-health benefits are gained if the only metabolite that is measured and reported is 25(OH)D_3. Plasma or serum 25(OH)D may not be representative of vitamin D status, but a critical intermediary in the utilisation of vitamin D by the body, since the action is mediated by intracellular metabolites and receptors towards the end of the pathway. In addition, the current marker to determine vitamin D status does not take into account the distribution of 25(OH)D_3 in other stores as opposed to plasma or serum: fat cells, muscle cells and various other tissues (Heaney, Horst, Cullen, & Armas, 2009). Circulating 25(OH)D concentration may not be a reliable indicator of whole body vitamin D status, and therefore it is important to consider all key metabolites involved in the entire metabolic pathway when determining a person's vitamin D status and their capacity to effectively utilise 25(OH)D at a cellular level.

2.3 Vitamin D metabolism

2.3.1 The vitamin D pathway

Classified as an active hormone in the body, 25(OH)D is metabolised from the active pre-cursor 7-dehydrocholesterol located within the epidermis of the skin (Kumar, 1984). The basic vitamin D metabolic pathway is shown in **Figure 1**. Upon photo-activation by UVB rays from direct sunlight exposure, 7-dehydrocholesterol undergoes photo-conversion into vitamin D. This is dissimilar to oral ingestion, whereby the ingested supplement or dietary source of vitamin D is absorbed through the intestinal wall. The vitamin D from either source then circulates in the blood bound to the DBP, its carrier protein, then undergoes two consecutive hydroxylation reactions, the first in the liver and the second in the kidney (Girgis, Clifton-Bligh, Hamrick, Holick, & Gunton,

2013; Kitson & Roberts, 2012). The DBP is the primary vitamin D carrier protein, binding 80-95 % of total 25(OH)D, with the remaining 25(OH)D bound to albumin, and less than 1 % of total circulating 25(OH)D in the free form (Bikle et al., 1986). The first step in the bioactivation of vitamin D is by the hydroxylation of carbon 25, which occurs primarily in the liver by 25-hydroxylases, specific cytochrome P450 enzymes such as CYP27A (sterol 27-hydroxylase), converting vitamin D to 25-hydroxyvitamin D_3 ($25(OH)D_3$). This is then further metabolised by CYP27B1 (1α-hydroxylase) in the kidney to the bioactive form of vitamin D: 1,25-hydroxyvitamin D_3 ($1,25(OH)_2D_3$). This active form is then secreted into the bloodstream for systemic delivery to the target tissue/cells. Although, the kidney is not unique in its ability to convert $25(OH)D_3$ to $1,25(OH)_2D_3$, as numerous cells and tissues have demonstrated the capacity for local metabolism of vitamin D.

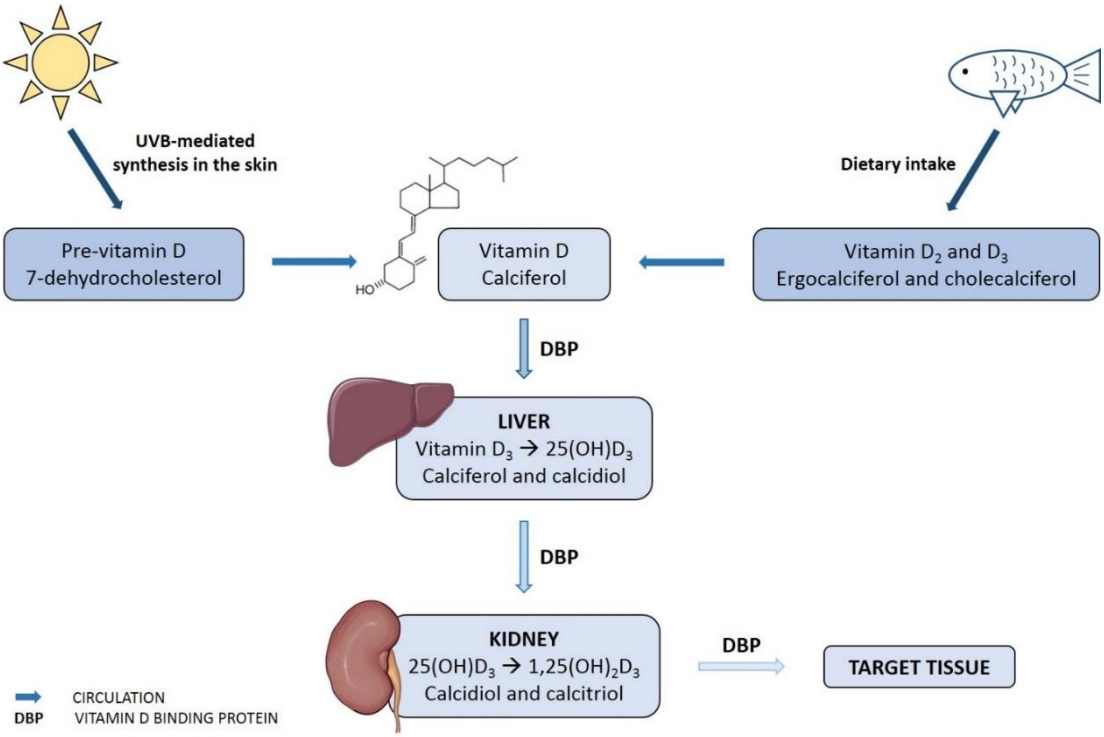

Figure 1 Vitamin D metabolic pathway adapted from Girgis et al. (2013) and Kitson and Roberts (2012).

2.3.2 The vitamin D receptor (VDR)

Located in the cytoplasm and nucleus of many cells, the VDR is a ligand-inducible transcription factor (Klopot, Hance, Peleg, Barsony, & Fleet, 2007), that was first discovered in 1974 by Brumbaugh and Haussler in animal intestines (Brumbaugh & Haussler, 1974). The target genes of VDR play a key role in cellular metabolism, bone health, mediating inflammation and muscle protein synthesis (Carlberg & Seuter, 2009). It has been reported that target genes of the VDR are associated with an individual's vitamin D status, whereby changes of their messenger ribonucleic acid (mRNA) expression significantly correlates with alterations of $25(OH)D_3$ levels (Vukic et al., 2015). Although, the metabolic pathway is closely linked and dependent upon the bioavailability of specific metabolites, vitamin D functions through the cellular action of the VDR. It is suggested that $1,25(OH)_2D_3$ acts via the VDR to have a direct effect on enzymatic activity, establishing the main control mechanism for vitamin D metabolism (Tiosano, Weisman, & Hochberg, 2001). The VDR has been found to regulate the expression of more than 900 genes involved in numerous physiological functions and pathological conditions, with more than 36 types of human tissue identified to express the VDR, including skeletal muscle (Norman, 2008). The primary role of VDR is to induce mRNA transcription and thus regulate protein synthesis (Bischoff et al., 2001; R. U. Simpson et al., 1985).

The intracellular pathway of vitamin D metabolites is displayed in **Figure 2**. The circulating unbound $1,25(OH)_2D_3$ crosses the cellular membrane of the target cell and subsequently binds to the cytoplasmic or nuclear VDR with high affinity (Pike, Meyer, & Bishop, 2012). The ligand $1,25(OH)_2D_3$ promotes heterodimerisation of VDR with retinoid X receptor (RXR), a nuclear receptor that is activated by 9-cis retinoic acid, a metabolically active form of vitamin A. Ligand binding to VDR may induce translocation of the complex to the nucleus (MacDonald et al., 1993). The VDR-RXR complex subsequently binds with high affinity to vitamin D response elements (VDRE) in the promoter region of target genes on the deoxyribonucleic acid (DNA) strand (Haussler et al., 1998). It is this binding mechanism that leads to the genomic activity of vitamin D. Once bound, VDR is then positioned such that it may recruit co-regulatory RNA

polymerase II transcriptional machinery to the target site to induce or suppress transcription of target genes (Girgis et al., 2013). These data imply a mechanism by which the VDR-RXR complex expresses promotor specificity permitting differential effects on numerous vitamin D related genes (J. Zhang et al., 2011). This ultimately induces translation of mRNA leading to de novo protein synthesis. Thus, vitamin D action depends on the delivery or metabolic production of sufficient concentrations of the $1,25(OH)_2D_3$ ligand to the target cell, and adequate intracellular expression of VDR and RXR proteins (Haussler et al., 2013).

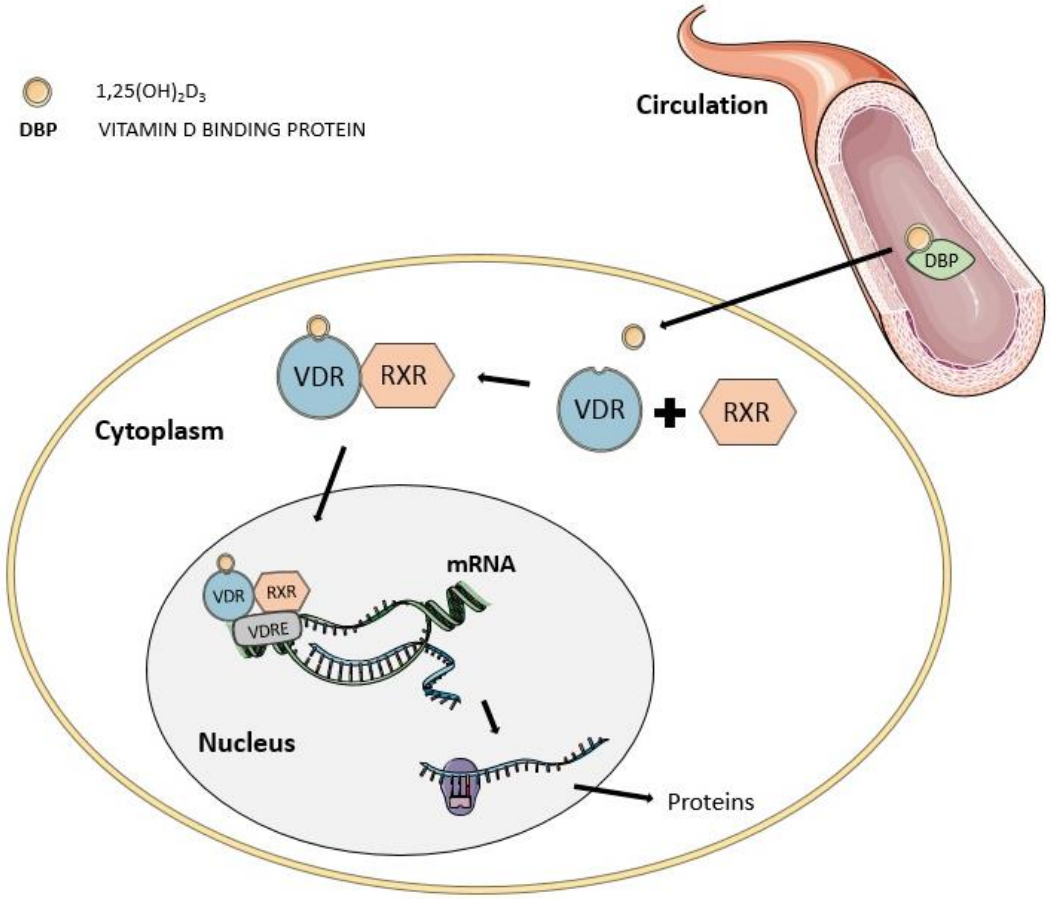

Figure 2 Intracellular vitamin D metabolic pathway adapted from Pike et al. (2012).

As with all members of the super family of steroid/thyroid hormone/retinoid nuclear receptors, the VDR is comprised of two well-conserved core domains, the ligand-binding domain, and the DNA-binding domain (Orlov, Rochel,

Moras, & Klaholz, 2012). The structure of the VDR is shown in **Figure 3**. The hinge region connecting the two active domains allows for flexibility within the structure of the receptor, allowing the receptor to bind to various DNA response elements in the DNA strand. The intricacies of the VDR structure have been well discussed and outlined by Haussler et al. (1998).

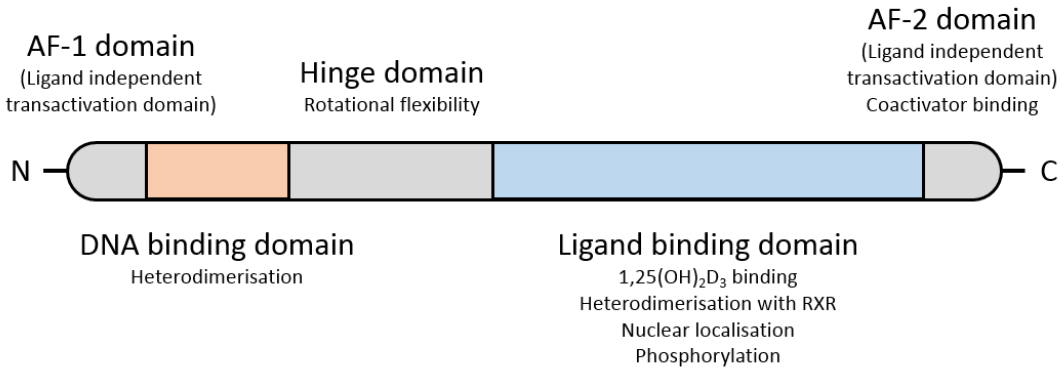

Figure 3 Structure of the VDR adapted from Orlov et al. (2012).

The positive interaction between VDR and RXR on the promotor region on a DNA strand is cyclic in nature with regards to the kinetics of receptor association (C. Zhang et al., 2003). The receptor action correlates with timely recruitment of many co-regulators, such as VDREs and the basic elements of the transcriptional machinery required for gene transcription. Following treatment with $1,25(OH)_2D_3$, binding of the VDR-RXR dimer complex has been assessed over 15 minute (min) intervals for a period of 3 hours (h) and also over longer term exposures up to 24 h (Kim, Shevde, & Pike, 2005). It appeared that VDR binding activity increased following $1,25(OH)_2D_3$ stimulation, with a peak at 45-60 min and a second wave at 135 min. Treatment with $1,25(OH)_2D_3$ also induced strong VDR and modest RXR protein upregulation, with the increase maintained to the 24 h time point. These findings support the idea that localisation of the VDR-RXR complex to the promotor region is ligand-dependent requiring the bioavailability of $1,25(OH)_2D_3$ (Pike & Meyer, 2010).

In numerous health conditions it is imperative that the functional products of a gene, primarily proteins, are consistently or intermittently expressed at a

specific concentration, for example: sufficient release of insulin to downregulate elevated glucose levels in order to achieve euglycaemia and prevent the onset of chronic hyperglycaemia: the initial pathophysiological event in the development of diabetes (Kahn, 2003). Vitamin D metabolism, specifically the VDR has been reported to regulate or induce gene expression, therefore altering protein synthesis (Haussler et al., 1998; Pike & Meyer, 2010).

2.3.3 Regulation of active vitamin D

In plasma and serum, the majority of 25(OH)D_3 is bound to the DBP (85-90 %), with a small minority (10-15 %) bound to albumin, and less than 1 % of 25(OH)D_3 circulating in the free form (Bikle, Siiteri, Ryzen, & Haddad, 1985; Chun et al., 2014). However, since the affinity of albumin to 25(OH)D is weaker than that of DBP to 25(OH)D, when bound to albumin the 25(OH)D becomes a bioavailable form, which also includes free circulating 25(OH)D (Bikle et al., 1986). Although DBP may not be directly associated with the risk of vitamin D-induced disease or health complications, the protein indirectly modulates circulating free and bioavailable 25(OH)D concentration, ultimately delivering the ligand 1,25$(OH)_2D_3$ to the target cell and VDR. Thus the concentration of bioavailable 25(OH)D depends on both the total 25(OH)D and levels of DBP and albumin. Although total 25(OH)D is recognised as the biomarker of vitamin D status (Chun et al., 2014), free or bioavailable 25(OH)D may be more relevant to determine vitamin D deficiency and the association with health complications (C. Li et al., 2017).

There is an optimal level of 1,25$(OH)_2D_3$, which requires a mechanism to promote or attenuate its activity. This physiologically active metabolite has a relatively shorter half-life (4-15 h) (Zehnder et al., 2001) than 25(OH)D_3 (15 days) (K. S. Jones et al., 2014). Although sufficient delivery of the ligand to target cells is required to have vitamin D-induced cellular transcriptional actions, a high potency of 1,25$(OH)_2D_3$ may have toxic effects. The efficacy of tissues expressing VDR positive cells to metabolise vitamin D and exert its biological effects will be dependent upon the ability of the cell to internalise the

$1,25(OH)_2D_3$-DBP complex. Cells express membrane-bound proteins that facilitate the uptake of the complex to the cytoplasm. For example, skeletal muscle tissue cells express megalin, which has been identified to play a role in mediating endocytosis of the $1,25(OH)_2D_3$ ligand (Abboud et al., 2013).

A feedback regulation by $1,25(OH)_2D_3$ has been identified, which mediates the circulating levels of 24-hydroxylase and CYP27B1, limiting an unnecessarily high abundance of the enzymes and thus strives to maintain optimal levels of the ligand. The protein 24-hydroxylase (encoded by the CYP24A1 gene) initiates the degradation of $25(OH)D_3$ and $1,25(OH)_2D_3$ at the C-24 position to produce $24,25(OH)_2D_3$ and $1,24,25(OH)_2D_3$, respective. Thus 24-hydroxylase works alongside CYP27B1 to control the systemic concentration of the non-active and active forms (Dusso, Brown, & Slatopolsky, 2005; Ohyama, Noshiro, & Okuda, 1991). It has been shown that $1,25(OH)_2D_3$ does not regulate CYP27B1 mRNA or protein expression, suggesting that $1,25(OH)_2D_3$ levels are controlled via 24-hydroylase-induced degradation of $1,25(OH)_2D_3$ (Xie et al., 2002). Thus the $1,25(OH)_2D_3$ feedback mechanism relies on $1,25(OH)_2D_3$ catabolism rather than inhibition of $1,25(OH)_2D_3$ production. For vitamin D metabolism to operate effectively, all the components and co-regulators must be orchestrated optimally. Therefore when investigating key components of the vitamin D metabolic pathway, consideration must be paid to the impact of metabolites and their potential mediating effect on protein expression and availability.

2.3.4 Physiological roles of the ligand-VDR complex

Vitamin D is not only known for its classic role in bone health and calcium regulation, but also in other non-genomic and genomic actions, such as cell proliferation and differentiation, as well as inducing and supressing gene transcription and mRNA translation leading to the synthesis and secretion of a variety of proteins. Since the VDR is identified as the controlling mechanisms of such actions, it is important to consider what ultimate outcome these VDR-induced actions have with regards to therapeutic use in human health.

2.3.4.1 Calcium regulation and bone health

A primary function of the vitamin D endocrine system is to maintain calcium homeostasis, with intestinal calcium absorption dependent on the availability of vitamin D metabolites: $1,25(OH)_2D_3$ and VDR (Sheikh et al., 1988). Intestinal calcium absorption efficiency has also been found to be attributed to a fall in circulating $1,25(OH)_2D_3$ concentration, as well as intestinal resistance to the action of $1,25(OH)_2D_3$ (Pattanaungkul et al., 2000). $1,25(OH)_2D_3$ is reported as the major hormone actively involved in the absorption of calcium from the small intestine and reabsorption in the kidney and bone. On a cellular level, $1,25(OH)_2D_3$ and VDR induce the expression of membrane calcium channels thus facilitating calcium entry into the cell, with VDR expression necessary for normal calcium homeostasis (Christakos et al., 2011; Christakos, Dhawan, Verstuyf, Verlinden, & Carmeliet, 2016). The ligand and receptor also exert control in the intestine on calcium binding once calcium enters the cell, and preventing cellular calcium concentrations from reaching toxicity (Christakos, 2012). If calcium homeostasis cannot be achieved through absorption from the intestine alone, then $1,25(OH)_2D_3$ cooperates with parathyroid hormone (PTH) to induce calcium reabsorption from the kidney and the bone, contributing to circulating calcium levels.

During the ageing process there is a decline in the intestinal absorption of calcium, which may be predetermined by $1,25(OH)_2D_3$ bioavailability (Veldurthy et al., 2016). This disturbed calcium balance is often accompanied with secondary hyperparathyroidism as a result of an overcompensated production of PTH in the presence of less $1,25(OH)_2D_3$. Hyperparathyroidism is also triggered by low concentrations of calcium in the blood, prompting the parathyroid gland to secrete more hormone (G. Jones et al., 1998), thus lowered combined action of $1,25(OH)_2D_3$ and PTH to release calcium from renal tubule or bone. Ageing has been associated with disturbed vitamin D metabolism (Chapuy et al., 1983; Jacques et al., 1997), therefore exposing older populations to calcium deficient issues, such as decreased BMD, osteoporosis and age-related bone loss (Cauley et al., 2008).

2.3.4.2 Muscle mass and strength

Vitamin D appears to have a role on several tissues in the body including skeletal muscle (Ceglia & Harris, 2013), with vitamin D deficiency associated with muscle weakness, muscle mass loss, and increased risk of falls in older populations. Fewer falls have been reported in persons who supplement with vitamin D combined with calcium verses calcium alone (Bischoff et al., 2003; Flicker et al., 2005). The risk of falls increases with changes in skeletal muscle mass, fibre type, and strength. Sarcopenia is a skeletal muscle-related syndrome characterised by low muscle mass, strength and function, which becomes increasingly prevalent as age advances (Cruz-Jentoft et al., 2010) and is linked to lower vitamin D status (Verlaan et al., 2015). Muscle mass and strength generally peak in early adult life and gradually decline with the ageing process (Frontera, Hughes, Lutz, & Evans, 1991; Hughes et al., 2001; Keller & Engelhardt, 2013), therefore maintenance of muscle mass and strength are key to promote conservation of independence and exercise capacity. Inadequate intake of vitamin D has been identified as a potentially modifiable risk factor in the development of sarcopenia (Mithal et al., 2013). It has been reported that higher baseline serum 25(OH)D concentrations have been associated with a greater muscle mass gain in sarcopenic adults (Verlaan et al., 2017). The authors suggested that sufficient baseline vitamin D status may be required for muscle mass gain or maintenance. Potential mechanisms for the action of vitamin D may be via the bioactive $1,25(OH)_2D_3$, which stimulates calcium influx into cells, phosphate transport, and muscle fibre differentiation (Garcia, King, Ferrini, Norris, & Artaza, 2011). Investigations *in vitro* have reported $1,25(OH)_2D_3$ to stimulate key cellular pathways of muscle growth and differentiation, acting primarily through the action of the VDR, to induce myogenesis (Garcia et al., 2011). VDR expression and genetic variation appears to impact human skeletal muscle strength and mass (Ceglia et al., 2013; Walsh, Ludlow, Metter, Ferrucci, & Roth, 2016). Since there is a decline in VDR expression in skeletal muscle cells with ageing (Bischoff-Ferrari, Borchers, et al., 2004), the key vitamin D-mediated molecular pathways introduce possible mechanisms for vitamin D to influence muscle mass and strength regulation.

Supplementation with 25(OH)D_3 can increase intracellular VDR concentration and muscle fibre size after a 4 month period (Ceglia et al., 2013), although research is limited within a human population. Mice with global body knockout of VDR exhibit impaired gut absorption and abnormal serum calcium levels, and differences in the phenotype of their muscles, with reduced muscle mass and smaller muscle fibres (Endo et al., 2003; Girgis et al., 2015). In addition, in skeletal muscle-specific VDR knockout mice there is a reduction in type II fibre type size (Chen, Villalta, & Agrawal, 2016). These data may suggest a vitamin D-specific effect on muscle fibre size, although the murine model studies should be explored in humans to establish whether it is a transferable finding between species.

2.3.4.3 Diabetes

Epidemiological studies have shown that vitamin D deficiency is associated with a higher prevalence of diabetes (Pittas et al., 2006; Pittas et al., 2012) and the development of metabolic syndrome (Barchetta et al., 2013). Insulin resistance and beta cell dysfunction are the main pathophysiological events in the development of diabetes (Kahn, 2003). There are several mechanisms underpinning the insulin-sensitising actions of vitamin D. Ligand bound-VDR has been identified in beta cells, where it activated intracellular signalling pathways involved in the transcription of the human insulin gene, subsequently upregulating insulin production and secretion destined to control systemic glycaemia (Guo et al., 2013; Maestro, Molero, Bajo, Davila, & Calle, 2002). 1,25$(OH)_2D_3$ has also been reported to mediate glucose transporter type 4 (GLUT4) translocation and thus cellular glucose uptake, therefore reducing the risk of developing chronic hyperglycaemia, a pre-disposed physiological state of diabetes (Manna & Jain, 2012).

2.3.4.4 Inflammation and autoimmune diseases

Vitamin D deficiency has been linked to altered inflammatory responses and chronic low-grade inflammation, leading to inflammatory and autoimmune diseases, such as diabetes, Crohns' disease, asthma, and multiple sclerosis

(Yin & Agrawal, 2014). Although investigations into the potential mechanisms involved in this association are still in their infancy, it has been proposed that the ligand binding of $1,25(OH)_2D_3$-VDR complex to VDREs alters the rate of transcription of a variety of cytokines, such as tumour necrosis factor (TNF)-α, interferon-γ, and interleukin (IL)-1 (Di Rosa, Malaguarnera, Nicoletti, & Malaguarnera, 2011). Although, *in vivo* studies have reported associations between vitamin D supplementation and a reduction in proinflammatory cytokines in patients with osteoporosis (Inanir, Ozoran, Tutkak, & Mermerci, 2004) and heart failure (Schleithoff et al., 2006), but not in healthy individuals (Gannage-Yared et al., 2003). Similarly, data from the Framingham Offspring Study, which included a healthy older population, reports no association between vitamin D and overall inflammation (Shea et al., 2008). In an *in vitro* investigation, it was demonstrated that $1,25(OH)_2D_3$ supressed the production of pro-inflammatory cytokines, which modulate the immune response and decreased overall inflammation (Colin et al., 2010). However, supplementation with vitamin D for a 3 year period reported no change on systemic inflammatory markers: plasma C-reactive protein (CRP) and IL-6 (Pittas, Harris, Stark, & Dawson-Hughes, 2007). It appears that currently the data surrounding the link between vitamin D and acute and chronic inflammation is elusive with further research required.

2.3.4.5 Immune health

Vitamin D and its metabolites are actively involved in the regulation of the innate and adaptive immune system (Aranow, 2011; Cantorna, 2000; Cantorna, Zhu, Froicu, & Wittke, 2004). It is known that the VDR and CYP27B1 are expressed in peripheral blood mononuclear cells (PBMCs), specifically immune cells, including: B cells, T cells, and antigen presenting cells, and that $1,25(OH)_2D_3$ signalling modulates both innate and adaptive immune responses (Baeke, Korf, et al., 2010; Baeke, Takiishi, Korf, Gysemans, & Mathieu, 2010; Bhalla, Amento, Clemens, Holick, & Krane, 1983; Brennan et al., 1987; Provvedini, Tsoukas, Deftos, & Manolagas, 1983; Vukic et al., 2015). These immune cells are also capable of internally converting 25(OH)D to $1,25(OH)_2D_3$, demonstrating the ability of immune cells to internally metabolise

25(OH)D (Kongsbak, von Essen, Levring, et al., 2014). In addition, immune cells are responsive to $1,25(OH)_2D_3$, however the route by which $1,25(OH)_2D_3$ regulates adaptive immunity utilising these cells remains unclear. The immune system plays the role of protector in the body, defending against invading organisms, and building immunity to foreign bodies. In a state of vitamin D deficiency there appears to be a compromised immune defence, which increases susceptibility to infection and increased autoimmunity (White, 2008). In addition to vitamin D's more classic endocrine role, it can also act in a paracrine or autocrine manner in an immune environment.

Traditionally vitamin D was prescribed in the form of cod liver oil, a rich source, to prevent upper respiratory tract infections (Ginde, Mansbach, & Camargo, 2009; Laaksi et al., 2007), influenza, and as a treatment for tuberculosis. Studies date back to the 80's and 90's, whereby vitamin D deficiency was associated with increased risk of infection, even after adjusting for seasonal variations and age. This has also been found more recently in epidemiology research (Cannell et al., 2006).

Vitamin D also has numerous effects on the immune cells within the adaptive immune system: B cells, T cell subsets (T_{helper} and $T_{cytotoxic}$ cells) (Cantorna, Snyder, Lin, & Yang, 2015), and natural killer cells. Primarily vitamin D supresses or promotes immune cell proliferation or differentiation, mediating control over the T cell response (Jeffery et al., 2009; Jeffery et al., 2012). The effects of which induce the production of inflammatory and anti-inflammatory cytokines, which modulate the function of the immune system. As a result this enhanced control of the immune systems function can prevent the development of autoimmune diseases.

However, the mechanisms and direct influence of vitamin D within these infectious and autoimmune diseases is not clear and at present the relationship is unsubstantiated. The *in vivo* effects of vitamin D on immune function also depend on the immunological challenge presented (Cantorna et al., 2004), therefore, further investigations are required to identify the cellular and molecular targets of active vitamin D within the immune system, and the mechanisms involved and recruited.

2.3.4.6 Cancer

Epidemiological observations have reported an association between inadequate concentrations of 25(OH)D and increased risk of different types of cancers, such as colon, prostate, and breast cancer, shown through *in vivo* an *in vitro* systems (Deeb, Trump, & Johnson, 2007). Potential mechanisms of action include genomic activity through the VDR, apoptosis, cell signalling, angiogenesis, and cell-cell interactions (Deeb et al., 2007), although the mechanisms have not extensively been explored.

2.4 Vitamin D status in Scotland

In regions of low UVB radiation, such as Scotland, individuals are at risk of vitamin D deficiency as the primary source of vitamin D that should contribute 80 % of our total intake is via skin synthesis following UVB exposure (Holick, 2002). The high northern latitude of Scotland (55°-59° North) and the prevalent cloud cover and poor weather conditions, does not facilitate sufficient vitamin D skin synthesis (Rhodes et al., 2010) to increase or maintain a sufficient vitamin D status. In addition to these limiting factors, the UVB radiation must be of a specific wavelength (290-315 nm) to promote dermatologic vitamin D metabolism to then contribute to the body's overall vitamin D pool (Chapuy et al., 1997). The classifications for vitamin D status are displayed in **Table 1**. The Institute of Medicine (IOM) has defined vitamin D deficiency as <20 $ng \cdot ml^{-1}$, insufficiency as 20-30 $ng \cdot ml^{-1}$, and sufficiency as ≥30 $ng \cdot ml^{-1}$ (A. C. Ross et al., 2011).

Table 1 Current classifications of vitamin D status and equivalences of units (Bischoff-Ferrari, Giovannucci, Willett, Dietrich, & Dawson-Hughes, 2006; Holick, 2009; Holick et al., 2011; A. C. Ross et al., 2011).

Serum/plasma 25(OH)D concentration		Classification
< 12 ng·ml^{-1}	< 30 nmol·L^{-1}	Severely deficient
13-20 ng·ml^{-1}	31 - 50 nmol·L^{-1}	Deficient
21-29 ng·ml^{-1}	51- 75 nmol·L^{-1}	Insufficient
> 30 ng·ml^{-1}	> 75 nmol·L^{-1}	Sufficient
> 100 ng·ml^{-1}	> 250 nmol·L^{-1}	Toxicity

Equivalences: 1 ng·ml^{-1} = 2.5 nmol·L^{-1}.

In countries of a high latitude the provision of sunlight exposure to daily vitamin D uptake is significantly reduced or diminished. When considering the overall vitamin D status of Scotland, it has been reported that during all seasons' vitamin D insufficiency and deficiency is common (Hypponen & Power, 2007; Kelly et al., 2015; Zgaga et al., 2011). The prevalence of vitamin D deficiency has been reported to be high, particularly in the winter and spring months, in the UK (Hirani, 2013; Hypponen & Power, 2007). The investigation by Hypponen and Power (2007) included men and women from the 1958 British birth cohort, all aged 45 years old and born in England, Scotland or Wales, and resided in the UK at time of data collection. Almost all of the participants (87.1 % of n=7437) were vitamin D deficient or insufficient despite the seasonal effect, with a mean serum 25(OH)D concentration of 16.5 ng·ml^{-1} in the winter and spring months, and 24.2 ng·ml^{-1} in the summer and autumn months, the lowest reported concentrations were prevalent in February. Interestingly, in the latter investigation Scotland had a significantly lower vitamin D status compared to other areas of Britain: South England, Midlands and Wales, and North England, with a mean 25(OH)D of 14.2 ng·ml^{-1} compared to 16.3-17.1 ng·ml^{-1} in the summer and autumn months and 20.4 ng·ml^{-1} compared to 24.2-25.0 ng·ml^{-1} in the winter and spring months. Hirani et al. (2013) reported the prevalence of vitamin D insufficiency and deficiency in men and women (n=2070) aged 65 years and older in England to be 33.8 % and 51.7 %,

indicating only 14.5 % of this population exhibited a sufficient status. Similarly, an adult population including older adults (n=222; 48-94 years) living in sheltered housing in East London, England, also reported a high prevalence of vitamin D deficiency, with a mean 25(OH)D_3 concentration of 17.2 ng·ml^{-1} (Jolliffe et al., 2016). They highlighted a seasonal variation on 25(OH)D_3 concentration, where vitamin D status was reported to peak in July to September and trough in January to March, consistent with reports from other studies in the UK (Hypponen & Power, 2007; Zgaga et al., 2011) and out-with the UK (K. Brock et al., 2010).

A study evaluating the vitamin D status of men and women (n=2235, 61.3 ± 10.7 years) in Scotland reported a high prevalence of severe deficiency (34.5 %) and deficiency (28.9 %), with a mean 25(OH)D_3 concentration of 14.4 ng·ml^{-1} (Zgaga et al., 2011). The same research group also conducted an investigation comparing vitamin D status of mainland Scotland to the Northern Scottish Isle Orkney, establishing the main determinants of vitamin D status in Orkney (Weiss et al., 2016). The mean 25(OH)D_3 concentration (n=1453, 16.5-100.2 years) in Orkney was 14.5 ng·ml^{-1}, which was similar to that reported in mainland Scotland. However, when observing age-matched data, Orkney had a significantly higher status (14.1 ng·ml^{-1}) than mainland Scotland (12.7 ng·ml^{-1}), with mean 25(OH)D_3 higher in every month of the year except for August. It was reported that older age, farming occupations and going on foreign holidays (holiday outside of the UK at least once per year) were associated with higher 25(OH)D_3 concentrations. It should be noted that the status in Orkney was classified as deficient despite the influence of any of the lifestyle determinants.

Despite a small sample size (n=99) to be reported from a medical practice, a General Practitioner in Scotland has reported findings that vitamin D deficiency was high in patients aged 15-85 years, with only 2 % reporting levels of sufficiency (Rhein, 2008). Severe deficiency was reported at 47 % of the cohort. Similarly in Scotland, results of a hospital based study in 548 older adult patients (94 % were aged over 60 and 74 % were aged over 75 years) reports universal vitamin D inadequacy, with a mean concentration of 9.9 ng·ml^{-1}, with no seasonal influence (Gallacher et al., 2005). Another hospital

based group in Scotland reported low vitamin D levels of older patients (n=102, 79.6 ± 7.3 years), at 16.6 $ng \cdot ml^{-1}$ (Burleigh & Potter, 2006). These data support findings that vitamin D deficiency is grossly high in the UK, in particular in Scotland, throughout the calendar year, with low levels observed in older adults.

2.4.1 Determinants of vitamin D status

The prevalence of a low vitamin D status or the risk of developing vitamin D deficiency can be attributed to the contribution of numerous environmental, physiological and lifestyle characteristics (Ginde, Liu, & Camargo, 2009; Thuesen et al., 2012). Since an estimated 80 % of the contribution to vitamin D status is ideally meant to come from UVB radiation from sunlight exposure, it must be considered that in countries lacking prevalent sunlight, such as Scotland, strategies to reduce vitamin D deficiency must focus on other modifiable determinants of vitamin D status. The recommendation to increase sunlight exposure, is not only not possible during winter months, but may carry dangers, such as increased risk of skin cancer. Therefore, it is important to identify determinants that can potentially be modified in order to reduce deficiency, especially in countries of high latitude with drastic seasonal variations. Identifying other potential determinants of vitamin D status can also assist in suitable recommendations to increase vitamin D levels. Many determinants of vitamin D status are (or can be) modifiable factors, such as UVB exposure, diet, physical activity levels, and body composition, therefore there is scope for altering lifestyle or behaviour in order to preserve or increase vitamin D status and metabolism.

2.4.1.1 Supplementation with vitamin D

It has been demonstrated through numerous studies, systematically reviewed, that oral vitamin D supplementation successfully increases systemic $25(OH)D_3$ concentration (Autier et al., 2012). This is a result also found in our own laboratory (Lithgow, Florida-James, & Leggate, 2018). Contributing to dietary intake with vitamin D supplements, most commonly cholecalciferol, is an

effective strategy to increase vitamin D status. Depending on the dose, 25(OH)D_3 concentration can rise to the level associated with sufficiency. Vitamin D can be supplemented in two forms: vitamin D_2 or vitamin D_3, although it has been reported that vitamin D_3 supplementation increases serum 25(OH)D concentration more effectively than vitamin D_2, by as much as 70 % more (Nimitphong et al., 2013; Trang et al., 1998). However, although sufficiency is associated with the health benefits discussed at the beginning of this review, simply raising vitamin D status does not necessarily directly influence the driving force for health benefits.

Cashman *et al*. (2008) have proposed a vitamin D intake of 7.2-41.1 µg/day to ensure maintenance of vitamin D status in adults (aged 20-40 years). The current intake of vitamin D in adults in Europe is 2.3-7.1 µg/day, therefore below requirements and recommendations (Spiro & Buttriss, 2014). Data solely on the intake of vitamin D supplements of the population is not known, although it is reported that 34.6 (male) – 46.9 % (female) of the UK population consume dietary supplements (including all vitamins, minerals, and oil-based supplements) (Skeie et al., 2009). The Endocrine Society suggest that a daily dose of 1500-2000 IU/day or more may be required to elevate blood 25(OH)D concentration above 30 $ng \cdot ml^{-1}$ (Holick et al., 2011). The IOM have recommended an upper level tolerable intake of 4000 IU/day to prevent any associated harm or risk from potentially excessive or high vitamin D consumption (G. Jones, 2008). A serious risk of vitamin D toxicity is hypercalcaemia, which can lead to vascular and tissue calcification and subsequent damage to blood vessels, the heart, and the kidneys (Tebben, Singh, & Kumar, 2016). Several systematic reviews have been published concerning and evaluating the influence of vitamin D supplementation on circulating 25(OH)D levels (Autier et al., 2012; Zittermann, Ernst, Gummert, & Borgermann, 2014). Although supplementation is effective at achieving an elevated 25(OH)D concentration, the absolute between-study variation in circulating concentration induced by similar doses, demonstrates the inter-individual variation in absorption and metabolism of cholecalciferol. As discussed in this review, the orchestration of the metabolic pathway that supersedes the established circulating 25(OH)D level, is complicated and

dependent on many factors, therefore the scope for inter-individual and inter-study variation is high. As a result, a ‘one size fits all’ approach for recommending or prescribing a supplementation dose may not be a practical method to guarantee a vitamin D status in the adequate range for all individuals.

2.4.1.2 Physical activity status

It is widely recognised that regular physical activity can provide health benefits in all ages. Living an active lifestyle predominantly reduces the healthcare burden and promotes healthy ageing, primarily through preventing or delaying the onset of non-communicable diseases and maintaining musculoskeletal health. The current physical activity guidelines set by the UK Government Department of Health ("Physical activity guidelines for adults (19-64 years)," 2011), recommend a minimum of 150 mins/week of moderate intensity exercise, or 75 mins/week of vigorous exercise, in combination with resistance exercises (recruiting each major muscle group) on 2-3 days/week, and flexibility and strength exercise training on 2-3 days/week, in order to maintain or achieve optimal cardiorespiratory and musculoskeletal health. It should be noted that the more specific recommendations for exercise prescription are dependent on age, and not all intricacies of optimal frequency and intensity have been determined yet. However, data from the World Health Organisation (WHO) global health observatory data repository in 2012 estimates that worldwide, 31.1 % of adults are physically inactive, determined from self-reported and monitored physical activity data, and thus are not meeting the recommended guidelines (Hallal et al., 2012). In Scotland, 36 % of the adult population are not meeting the recommended physical activity guidelines (*The Scottish Health Survey*, 2017). Furthermore, physical activity levels have been reported to decline with age, assessed through objectively measured physical activity (hip-worn accelerometers), with only 85-90 % of older adults aged 70 years and above in Scotland achieving the recommended activity requirements (Jefferis et al., 2014). Although it is ideal to measure physical activity levels with both subjective (self-reported) and objective (monitored via accelerometers) measures, the cut off points used to define moderate to

vigorous physical activity can differ between studies. Some studies use age-appropriate cut off points, which will influence the reported percentage of the population that achieve the guidelines.

Physical activity is related to circulating plasma/serum 25(OH)D, with higher self-reported activity linked with higher concentrations of 25(OH)D, reported in the established third National Health and Nutrition Examination Survey (NHANES III), as well as in other studies (Jacques et al., 1997; Lucas et al., 2005; Scragg & Camargo, 2008; Scragg, Holdaway, Jackson, & Lim, 1992; Scragg et al., 1995). Physical inactivity has also been reported to be a modifiable predictor of low vitamin D status in healthy middle-aged men and women in the United States of America (USA) (K. Brock et al., 2010). This has been attributed to increased sunlight exposure due to the nature of physical activity often occurring outdoors (Kluczynski et al., 2011). However, an investigation in the USA measuring both indoor and outdoor activity demonstrated that the relationship with vitamin D status is not stronger for self-reported outdoor activity compared to indoor activity (Wanner, Richard, Martin, Linseisen, & Rohrmann, 2015). Moreover, it was hypothesised that active people are usually active both indoors and outdoors. In support of this, investigations in the USA, Finland, and France where both exposure to UV radiation and physical activity were measured, it has been reported that the relationship between vigorous activity and vitamin D status persists when adjusting for UV exposure (K. Brock et al., 2010; K. E. Brock et al., 2010; Touvier et al., 2015). Similarly, age- and UV exposure-adjusted 25(OH)D concentrations in a USA population are associated with recreational physical activity, with season accounting for much of the observed activity-25(OH)D associations (Kluczynski et al., 2011). Taken together, these data suggest that physical activity and exercise may play a role in vitamin D metabolism regulation independent of sunlight exposure. However, self-reported physical activity measures should be interpreted with caution, as the scope for error is usually higher than objective measures, particularly for physical activity. This may disrupt the true association between physical activity status and vitamin D concentrations.

Low serum 25(OH)D has also been associated with poorer physical performance, particularly among an older population (Wicherts et al., 2007). This may suggest that either physical performance is linked to outdoor activity behaviours and thus an increase in potential UVB radiation, or that vitamin D has a role in the musculoskeletal system, therefore potential to improve physical capacity.

However, there is conflicting evidence surrounding the link between physical activity levels and the influence on vitamin D status. Serum vitamin D levels have been reported not to differ between physically active and inactive female health care workers (Lehnert et al., 2018). Although baseline 25(OH)D concentrations were relatively low (n=67, median of 20 $ng \cdot ml^{-1}$), they were unaffected by activity level, but were affected by season (Germany - 50°N latitude, therefore similar to Scotland: 55°-59° North). Therefore, the direct relationship between physical activity levels and vitamin D status may not stand when adjusted for confounding factors such as latitude and season.

2.4.1.2.1 Influence of seasonality on physical activity levels

Natural environmental factors, such as seasonality (Leger-Guist'hau et al., 2016) and weather conditions, have been shown to influence physical activity behaviours, specifically day length, precipitation level, and temperature. The unpredictable weather in Scotland, marks it as a country for unpredictable physical activity engagement, especially if the particular activity is outdoors-specific or associated with outdoors, i.e. hiking. If the winter months restrict outdoor activity, activities may be less accessible and thus require facilities such as a gym. Therefore, physical activity status becomes dependent on available funds to access such facilities. In addition, it was found that after controlling for individual characteristics, closer proximity and more accessible exercise facilities were associated with increased frequency of physical activity and exercise (Sallis et al., 1990). Furthermore, day length plays a similar role in that it can restrict outdoor activity, influenced by seasonality.

2.4.1.3 Body composition

Total body fat percentage and body mass index (BMI) has been inversely associated with serum 25(OH)D concentrations in older persons aged 55-74 years (K. Brock et al., 2010) and 65 years and older (Oliai Araghi et al., 2015), suggesting obesity is a risk factor for vitamin D deficiency. The association between vitamin D concentration and fat mass is attributable to the fat-soluble property of Vitamin D. If adipocytes sequester systemic vitamin D at a rate that exceeds the rate of synthesis of vitamin D, this renders the body as a whole deprived of vitamin D (Bolland, Grey, Ames, Mason, et al., 2007; Wortsman et al., 2000). It has been proposed that there is an increase in catabolism of vitamin D in adipose tissue (Wortsman et al., 2000). Another mechanism by which high body mass is associated with lower 25(OH)D concentration, is a greater tissue volume within which vitamin D metabolites are distributed, successively affecting measured systemic concentrations: suggesting a dilutional model (Drincic, Armas, Van Diest, & Heaney, 2012). The latter investigation demonstrated that the type of adiposity accounts for the relationship between body mass and 25(OH)D concentration, specifically visceral adiposity. A higher body fat percentage has been inversely associated with vitamin D status in an older population aged 70.5 ± 5.9 years (Vitezova et al., 2016). This was reported from data taken from the Rotterdam study: an ongoing prospective population-based follow-up study comprising of people aged ≥ 45 years in the Netherlands. They found an association between 25(OH)D and android fat but not with gynoid fat, even though it was not independent of BMI. This coincides with findings of another investigation into the relationship between vitamin D status and fat distribution, where 25(OH)D was inversely associated with all regions of fat mass, but positively associated with fat-free mass (Moschonis et al., 2009). Although, this represents the findings of an overweight postmenopausal health study, and thus differs to the ageing population in the Rotterdam Study.

Adipocytes have been found to express the VDR and CYP27B1, which is needed for the production of $1,25(OH)_2D_3$, suggesting a contribution of fat mass to vitamin D metabolism (Wamberg et al., 2013). In addition, $1,25(OH)_2D_3$ has been linked to the inhibition of the expression of genes

involved in the genesis of adipocytes (Kong & Li, 2006). Although these were reported from *in vitro* studies, it suggests a negative feedback loop is associated with excess fat mass accumulation.

Lean mass also contributes to vitamin D status and metabolism, with growing evidence demonstrating the prevalence of the VDR in skeletal muscle tissue thus mediating the vitamin D endocrine system (Bischoff et al., 2001). Despite the contribution of fat mass to overall BMI, which is known to sequester 25(OH)D_3, the quantity of lean mass may be just as important when considering the effects of body composition on vitamin D metabolism. Myocytes have a role in the orchestration of vitamin D bioavailability since myocytes have been found to store and protect vitamin D (Abboud et al., 2014; Abboud et al., 2013). Murine skeletal muscle cells have been found to act as a functional extravascular store of 25(OH)D that can uptake and release it back into the circulation, thereby preventing it from degradation (Abboud et al., 2013). Muscle tissue/mass loss is reported as a prominent feature of the clinical syndrome of vitamin D deficiency, with age-related decline in muscle mass, known as dynapenia, also associated with reduced VDR expression (Bischoff-Ferrari, Borchers, et al., 2004). The rate of loss of muscle mass with age has been summarised from literature and reported as 4.7 % in men and 3.7 % in women per decade (Mitchell et al., 2012). Therefore, a loss of muscle mass can directly impact the myocyte stores of vitamin D, and thus the capacity of extravascular stores to contribute to vitamin D status.

Low concentrations of 25(OH)D_3 have also been associated with numerous obesity-related disorders, such as diabetes, cardiovascular disease, and metabolic syndrome (Chowdhury et al., 2014; Holick, 2007). Although, the more classic role of vitamin D is in maintaining bone health, with an elevated vitamin D status linked to improvements in BMD and muscle mass and strength (Bartoszewska, Kamboj, & Patel, 2010). BMD is associated with numerous metabolic bone disorders, such as osteoporosis, and is dependent on the presence of adequate vitamin D to avoid depletion (Mezquita-Raya et al., 2001). A positive association between 25(OH)D concentration and (hip) BMD was observed in both young (34.8 ± 8.1 years) and older (69.0 ± 10.9

years) white adults (Bischoff-Ferrari, Dietrich, Orav, & Dawson-Hughes, 2004). This finding was found regardless of ethnicity/race and sex.

It has been reported that vitamin D insufficiency is associated with reduced spinal BMD but not with lower limb BMD (Mezquita-Raya et al., 2001), hypothesising that lower limb BMD is also maintained through other factors such as higher mechanical use (physical activity and exercise) of lower limbs. If physical activity status influences BMD, with premise that it may be through the vitamin D metabolic pathway, improvements in bone health may be stimulated via mechanical stress, i.e. an exercise bout.

2.4.1.4 Race and ethnicity

Race/ethnicity has been found to influence vitamin D metabolism, with a lower vitamin D status in black people than white people (Ginde, Liu, et al., 2009; Holick et al., 2011; Powe et al., 2013). Skin colour and pigmentation may explain the variation in vitamin D status, due to differential UV exposure and cultural behaviours. Melanin in the skin acts as a natural barrier to the UV light, affecting the cutaneous production of vitamin D_3 (Holick, MacLaughlin, & Doppelt, 1981). An investigation by Powe et al. (2013) also reported a lower concentration of circulating vitamin DBP in black people compared to white people, suggesting a reduced capacity to transport vitamin D in the circulation. Additionally, combined with previous data (Powe et al., 2011), they also reported black people to exhibit a higher BMD than white people, however, vitamin D status did not differ between races. These data suggest that low total 25(OH)D concentrations do not uniformly indicate vitamin D deficiency, supporting the notion that the entire metabolic pathway from 25(OH)D to cellular action should be considered.

2.5 The role of ageing

2.5.1 Ageing and physical activity status

It is well established that physical activity and exercise are key components in the management of many chronic disease associated with ageing. Activity

levels decline with advancing age (Sallis, 2000), reported to be attributable to a variety of factors and barriers to engagement in exercise: possibly environmental, psychological, or biological factors (Schutzer & Graves, 2004). Participation in leisure time activities in Scotland declines with increasing age with many deterrents identified, such as lack of interest and physical symptoms (Crombie et al., 2004). Given the extending life expectancy in the UK, thus expanding the proportion of older adults, the suboptimal physical activity status of all adults presents a public health concern. Maintaining sufficient physical activity levels throughout life, particularly when entering older adult age and onwards (60 + y) may attenuate or delay the onset of chronic diseases and health complications. Physical inactivity may also shorten life expectancy, suggesting a substantial impact of a physically inactive lifestyle on the development of the world's major non-communicable diseases, such as cardiovascular disease, coronary heart disease, and diabetes. The Lancet Physical activity Working Group reported that physical inactivity is responsible for between 6 % and 10 % of the burden of these diseases, varying between countries, and increasing activity status could extend life expectancy (Lee et al., 2013). It is therefore crucial to maintain a healthy active lifestyle as age advances in order to prevent or delay the onset of health issues. In addition, an active lifestyle helps to preserve functional ability across the lifespan, improving quality of life and independence, particularly amongst the older adult population (McPhee et al., 2016).

An investigation in a large older adult population (n=2621; 55-74 y) across the USA has found that a low vitamin D status is correlated with a low physical activity status, a higher BMI, and poor dietary vitamin D and calcium intake (K. Brock et al., 2010). Consistent with other studies, vigorous physical activity has been identified as a modifiable predictor of 25(OH)D concentration in numerous countries (K. Brock et al., 2010; K. E. Brock et al., 2010; Scragg & Camargo, 2008; van Dam et al., 2007). The relationship between physical activity status and vitamin D status has been attributed to UV exposure, since many physical activities and sports are outdoors. However, there has also been reports that mechanical stress alters vitamin D metabolites and vitamin D cell signalling pathways (Aly et al., 2016; Makanae et al., 2015), therefore

physical activity may influence vitamin D status through multiple routes as opposed to solely UV exposure.

If regular physical activity is associated with vitamin D status, and ageing is linked to a decline in both physical activity status and vitamin D status, it can be hypothesised that maintaining a physically active lifestyle may influence 25(OH)D concentrations. Therefore, identifying predictors of reduced vitamin D status, such as physical inactivity or lack of mechanical stress, is important to develop health interventions to potentially increase 25(OH)D_3 or augment the efficiency of the metabolic pathway.

2.5.2 Ageing and vitamin D metabolism

Vitamin D concentrations are inversely associated with advancing age (Chapuy et al., 1983; Chapuy et al., 1997; Jacques et al., 1997), this is largely due to low UVB exposure (Thomas et al., 1998) and low dietary vitamin D intake (Lips, 2010). In an older population there also appears to be a decline in cutaneous synthesis of pre-vitamin D (Holick & Chen, 2008; MacLaughlin & Holick, 1985). Advancing age has been reported to inversely affect the concentration of the pre-cursor 7-dehydrocholesterol in the epidermis, with exposure to UV radiation increasing 7-dehydrocholesterol and pre-vitamin D_3 in the epidermis and dermis, respectively, largely dependent on age (MacLaughlin & Holick, 1985). This suggests that age affects the cutaneous capacity for the initial metabolic conversion in the vitamin D pathway: 7-dehydrocholesterol into pre-vitamin D. In addition, the ability of the gut to absorb cholecalciferol declines with age (Barragry et al., 1978).

It appears that the relationship between stimulation of a cell and alterations in vitamin D metabolism may be tissue specific, which would coincide with the local processing of vitamin D metabolites that has been demonstrated in several studies. Likewise, specific cells may participate in vitamin D metabolism in response to stimulations. For example, it has been demonstrated that T cells are targets of 1,25$(OH)_2D_3$ and accommodate vitamin D metabolism (Baeke, Korf, et al., 2010; Cantorna, 2011). It has also been discovered that in addition to immune cells, other cells such as human

marrow stromal cells, have the molecular machinery and capacity to enzymatically convert $25(OH)D_3$ to $1,25(OH)_2D_3$ by the action of CYP27B1 (Zhou, LeBoff, & Glowacki, 2010). Skeletal muscle cells express VDR, with alterations in the concentration of VDR after administration of $1,25(OH)_2D_3$ (Garcia et al., 2011), although the exact mechanisms and signalling of VDR-mediated vitamin D action in human skeletal muscle is not well established. The level of expression of CYP27B1 also correlated to the vitamin D status of the clinical deficient population group. This may suggest that cell-localised metabolism may influence whole body metabolism, or that an elevated vitamin D status is associated with or mediates cellular metabolism. The previous study was unpowered and thus age-related changes were not assessed. However, in a follow up study, the research group investigated the effect of age (young, n=12: ≤ 50 y; older, n=15: ≥ 55 y) on the expression and activity of CYP27B1 in this cell type and reported an inverse correlation between CYP27B1 expression and age (Geng, Zhou, & Glowacki, 2011). However, they also observed a similar expression of the VDR in the young and older cells, contrasting with other findings in different cell types, including skeletal muscle tissue (Bischoff-Ferrari, Borchers, et al., 2004). Therefore, the responsiveness of cells to vitamin D or stimulation to then alter vitamin D metabolism may be dependent on age, although there is not a consensus based on current published literature.

Expression of the VDR in skeletal muscle tissue has been reported to decline with age (Bischoff-Ferrari, Borchers, et al., 2004; R. U. Simpson et al., 1985). In the investigation by Bischoff-Ferrari et al. (2004), circulating blood and skeletal muscle tissue samples were taken from female patients undergoing hip and spinal surgery (71.6 ± 14.5 y verses 55.2 ± 19.6 y, respectively): serum samples were analysed for $25(OH)D_3$ and $1,25(OH)_2D_3$ concentration, and the myocytes were cultured and subsequently analysed for VDR-positive nuclei. They reported that younger cultured myocytes expressed more VDR than older myocytes, possibly attributable to a reduction in the functional response of cells to $1,25(OH)_2D_3$ in an older cohort, or the observed decrease in $25(OH)D_3$, reported with age (Jacques et al., 1997; Lips, 2010). This may lead to a decrease in the expression of the VDR due to reduced stimulation of the

receptor by 1,25$(OH)_2D_3$ and thus an ultimate down-regulation of the VDR present intracellularly. However, no relationship between 25(OH)D and 1,25$(OH)_2D_3$ and VDR expression was shown.

Conversely, other reports have demonstrated no age-dependent change in peripheral cell VDR gene expression, through correlation and linear regression analysis (Coleman et al., 2016). However, this study was restricted to an older age range (50-69 y and ≥70 y, n=20 in each group) and thus not comparing relatively young to relatively old. The contradicting findings between studies may be because regulation of expression of VDR is tissue or cell specific, or how intracellular VDR was quantified. In the study by Bischoff et al. (2004), VDR protein concentration was measured, whereas Coleman et al. (2016) analysed VDR mRNA expression, which may not be representative of protein expression. Within a cell, the concentrations of proteins is not proportional to the levels of corresponding mRNA, although the mRNA expression indicates the rate at which proteins may be produced. Therefore, measuring mRNA expression of VDR will provide insight into the rate of production of VDR, but not specifically the available receptors to receive the ligand 1,25$(OH)_2D_3$ and subsequently feed VDR-induced transcription and translation of other proteins associated with disease. Although, VDR mRNA may not be increased just because there are receptor-ligand complexes acting on the VDRE. It may be the case that the transcriptional machinery induces gene expression of other proteins and not those required to produce VDR proteins. It has also been hypothesised that this post-transcriptional regulation of expression can be affected by ageing (Coleman et al., 2016).

All components of the vitamin D pathway can be affected by age, including the availability of the DBP and the availability and activation status of the nuclear co-regulators. There is little evidence of the expression of the DBP with advanced age, however the limited current reports are controversial, either demonstrating the expression to be compromised by ageing or showing no significant relationship (Bolland, Grey, Ames, Horne, et al., 2007).

2.6 The role of exercise on vitamin D metabolism

2.6.1 Exercise and vitamin D

Recent research has focussed on the effect of vitamin D supplementation on physical performance and/or in combination with exercise interventions (Close et al., 2013; Lithgow et al., 2018; Owens et al., 2015), in athletes, overweight individuals, and in clinical populations at risk of falls and fractures (Latham et al., 2003). Owens et al. (2015) reported 6 weeks of supplemental vitamin D_3 (4,000 IU/day) to increase serum 25(OH)D and improve skeletal muscle regeneration post-exercise in human males (n=20). Close et al. (2013) documented that 6 and 12 weeks of weekly vitamin D_3 supplementation (20,000 or 40,000 IU/day) increased serum 25(OH)D concentrations in all club-level athletes (n=30) but did not induce any alterations in measures of muscle function and physical performance (bench press, leg press, and vertical jump height). Similarly, our previous investigations (Lithgow et al., 2018) demonstrated that supplementing with vitamin D_3 (4,000 IU/day) over a 6 week period elevates 25(OH)D concentrations (n=20). However, the supplemental course in combination with high intensity intermittent training (HIIT) did not provide any additional improvements in measures of performance ($\dot{V}O_{2max}$ test) compared to the consumption of a placebo. Alongside these mixed findings, other investigation have reported that 24 weeks of vitamin D supplementation (800 IU per day) does not affect physical performance in older persons (n=59) (Vaes et al., 2018), and vitamin D deficiency is not linked to reduced performance in young persons (n=80) (Dubnov-Raz, Livne, Raz, Cohen, & Constantini, 2015). These data may suggest that a cross-over exists between vitamin D and physical activity and exercise, but the conclusions remain inconclusive.

Studies have indicated that mechanical stress can alter the expression and action of key vitamin D metabolites: 25(OH)D_3, 1,25$(OH)_2D_3$ and the VDR (Aly et al., 2016; M. Li, Li, Meikle, Islam, & Cao, 2013; Makanae et al., 2015; Sun et al., 2017). Key vitamin D metabolites have been reported to be present in skeletal muscle cells, with the capacity to locally metabolise 25(OH)D to 1,25$(OH)_2D_3$ via CYP27B1. The ligand may then form a complex with VDR in the myocyte to elicit vitamin D-induced changes in transcription, or the

myocyte may release $1,25(OH)_2D_3$ back into the circulation to be transported by DBP to a different type of cell, focussed on a different target outcome. There is data to support the uptake of 25(OH)D into skeletal muscle cells, murine model, where it is held by the DBP (Abboud et al., 2013). They suggest that although DBP is primarily a circulatory protein binding nearly all of 25(OH)D in blood, the DBP has a high affinity for actin in skeletal muscle. Following uptake, the 25(OH)D remains bound to DBP, which crosses the membrane via low density lipoprotein-related protein 2 (LRP2), known as megalin, and is retained by binding to actin. As a result, the residence time of 25(OH)D in the blood has been reported to differ based on physical activity status, with a higher half-life of 25(OH)D in exercised mice (25 days) compared to non-exercised mice (23 days) (Abboud et al., 2013). This suggests that exercise may play a role in prolonging the maintenance of vitamin D status.

It has been demonstrated that serum 25(OH)D concentrations can increase after a single bout of continuous aerobic exercise (30 min cycling at 70% $\dot{V}O_{2max}$) in a young adult cohort (Sun et al., 2017). Although, previous reports have also found no alterations of 25(OH)D concentrations in response to a single bout of exercise (a standard walking $\dot{V}O_{2max}$ test) in young adults (Maimoun et al., 2009), and a slight, but significant, decrease in 25(OH)D concentration following a maximal walking incremental exercise test in an older adult population (Maimoun et al., 2005). The latter study reported disturbances in calcium homeostasis in active older males and females (73.3 ± 9.1 years). Since calcium and vitamin D are co-regulators, changes in calcium regulation can directly influence vitamin D metabolic intercellular signalling pathways. Concentrations of ionised serum calcium and 25(OH)D decreased after exercise compared to pre-exercise, however $1,25(OH)_2D_3$ remained unchanged. If VDR expression is increased and no change in systemic $1,25(OH)_2D_3$ concentration is observed, it may be hypothesised that the renal conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ increases simultaneous to an increased cellular uptake of $1,25(OH)_2D_3$ and thus the efficiency of binding to VDR. This may result in no change in $1,25(OH)_2D_3$, however this is speculative and must be explored in response to exercise.

Although circulating 25(OH)D concentration is used as the biomarker for vitamin D status, it is the VDR that provides the functional platform for vitamin D-induced alterations in gene transcription (Haussler et al., 1998). In a recent investigation, an acute bout of resistance exercise (isometric exercise – 5 sets of 10 x 3 second (s) contractions, with 7 s intervals between repetitions and 3 min rest intervals between sets) has been reported to increase VDR protein expression in rat skeletal muscle, although there was no accompanying increase in 25(OH)D_3 concentration (Makanae et al., 2015). This study compared two modes of exercise, resistance and endurance, although there was no reported change in VDR expression in response to the endurance exercise bout (60 min running at a speed corresponding approximately to lactate threshold). Interestingly, CYP27B1 expression also increased, again this was only following the resistance exercise bout and not the endurance exercise bout. However, a limitation of the study is that 1,25$(OH)_2D_3$ was not measured and thus restricts understanding of what phase of the metabolic pathway is less efficient in response to exercise. Furthermore, no change in VDR expression was observed following the endurance exercise bout, indicating that the response is specific to muscle fibre type recruitment and contraction and greater mechanical stress. Therefore, mechanical stress may affect skeletal muscle tissue metabolism in contracting muscles but not resting muscles. It was hypothesised that local metabolism occurred within skeletal muscle, which did not contribute to whole body 25(OH)D_3 status. It should be noted that the resistance exercise bout involved maximal involuntary contractions in rats, and thus the outcome may not be comparable to voluntary contractions, which would be measured in humans.

Recent investigative findings support the hypothesis that vitamin D has a direct positive effect on skeletal muscle metabolism. It has been found that skeletal myocytes express VDR (Bischoff et al., 2001), and moreover, vitamin D signalling has been found to alter muscle protein synthesis (Girgis et al., 2013; Girgis, Clifton-Bligh, Mokbel, Cheng, & Gunton, 2014). However, the vice versa relationship, that skeletal muscle contraction has a direct effect on vitamin D metabolism, has only been investigated in the rat model by Makanae et al. (2015).

In an investigation into the combined effect of vitamin D supplementation and regular resistance training for 12 weeks on skeletal muscle function, it was reported that vitamin D intake did not change VDR mRNA expression (Agergaard et al., 2015). This was regardless of age, with a comparison between young (20-30 y) and older (60-75 y) healthy untrained adults. Furthermore, the CYP27B1 expression was decreased in the older adult vitamin D supplementation group post-training intervention. Although an increase in this key enzyme may be beneficial, as it converts $25(OH)D_3$ to $1,25(OH)_2D_3$, a negative feedback mechanism may exist. In the presence of a high circulating concentration of the active form, there is a lessened requirement of the enzymatic activity of CYP27B1, and thus a reduction in the availability of the converting enzyme. In addition, the authors reported an improvement in muscle quality and fibre type morphology in the older adult cohort, suggesting an effect of vitamin D on skeletal muscle modelling. However, the mechanisms were not explored.

It must be considered whether the relationship between exercise and vitamin D is association or causation. It has been hypothesised that possible explanations are: those with a high vitamin D status have a greater capacity for exercise, or that exercise can raise systemic $25(OH)D_3$ concentration (Birrell & Francis, 2008). There is an argument that regular exercise may increase $25(OH)D_3$ concentration because of the outdoor nature of many exercises and sports thus increasing potential opportunity for sunlight exposure. However, the investigations into the effects of exercise on vitamin D metabolism are usually performed indoors in laboratories with no exposure to UV light and thus eliminates the possible contribution of skin synthesis of vitamin D_3. Therefore, the second explanation seems plausible based on the literature discussed, as the observed effects of exercise to alter vitamin D metabolism are stratified by the mechanical intervention or stimulation (Aly et al., 2016; Makanae et al., 2015; Sun et al., 2017). Moreover, the acute UV exposure from performing exercise outdoors will not immediately raise 25(OH)D concentration, as this is a gradual elevation over time with regular exposure necessary. Therefore, investigations into the effect of acute exercise,

whether performed indoors or outdoors should be unaffected by the short-term exposure to UVB rays.

Furthermore, the current literature involves rat model investigations and thus are not wholly applicable to the human physiological environment. There is a requirement for investigations into the effects of mechanical stress in the form of physical activity and/or exercise on vitamin D metabolites in a human population.

2.6.2 Vitamin D metabolism in immune cells

Expression of the VDR has been identified in a variety of cells of the immune system including macrophages, T lymphocytes, dendritic cells and monocytes (Laird et al., 2014). Naive T cells have the potential to orchestrate and determine the nature of immune and inflammatory responses, and with the presence of VDRs in activated T cells (Joseph et al., 2012), this proposes additional mechanisms for $1,25(OH)_2D_3$ and VDR to directly regulate T cell responses (Cantorna, 2011). Moreover, activation of the VDR has the capacity to alter transcription, proliferation, and differentiation of immune cells (Adorini & Penna, 2008). This role in the immune system is linked to risk of infections and risk of developing autoimmune diseases, such as Crohn's disease, Multiple Sclerosis, and diabetes mellitus (Yin & Agrawal, 2014). Vitamin D has been reported to have a potential protective effect on autoimmune diseases and immune suppression, with reports that $1,25(OH)_2D_3$ triggers non-classical immunomodulatory responses (J. S. Adams & Hewison, 2008; Baeke, van Etten, Gysemans, Overbergh, & Mathieu, 2008). The literature reports contradictory results on the expression of the VDR in T cells, with some studies demonstrating that only stimulated T cells express VDR (Bhalla et al., 1983; Kongsbak, von Essen, Boding, et al., 2014), and others reporting unstimulated T cells express VDR (Veldman, Cantorna, & DeLuca, 2000) but at lower levels (Correale, Ysrraelit, & Gaitan, 2009). The latter investigation reported $1,25(OH)_2D_3$ to increase VDR expression in both inactive and activated T cells, indicating a role of vitamin D in T cell homeostasis independent of environment/stimuli. The contradictory findings may be explained by the

different subpopulations of T cells studied and the different methods for detection of the VDR: Western Blot, flow cytometry, or enzyme linked immune sorbent assay (ELISA), and the specific outcome reported: protein expression or mRNA expression.

In a recent investigation into VDR expression in human T cells, it was reported that naïve $CD4^+$ T cells do not express the VDR, however following stimulation, activated T cells start to express the VDR, independent of $1,25(OH)_2D_3$ concentration (Kongsbak, von Essen, Boding, et al., 2014). However, increased $1,25(OH)_2D_3$ induced VDR protein expression in activated T cells, independent of $25(OH)D_3$ concentration. Furthermore, $1,25(OH)_2D_3$ can also induce a substantial redistribution of intracellular VDR, with a shift in the percentage of VDR in the cytoplasm to the nucleus. This is beneficial for the formation of the VDR-RXR complex and binding to the VDRE to elicit an upregulation or suppression in gene transcription.

It is well established that the vitamin D metabolic pathway involves two hydroxylation reactions, in the liver and kidney, to convert vitamin D into the active form. It has been reported that T cells express CYP27B1, the key enzyme for the conversion to $1,25(OH)_2D_3$ (Baeke, Korf, et al., 2010; Kongsbak, von Essen, Boding, et al., 2014). However, there is debate as to whether T cells can convert $25(OH)D_3$ to $1,25(OH)_2D_3$ in physiological environments. Human T cells have been identified as direct targets for $1,25(OH)_2D_3$, with activation of T cells inducing an increase in VDR expression (Baeke, Korf, et al., 2010; Cantorna et al., 2015). Moreover, Correale et al. (2009) found that 25(OH)D could be metabolised to $1,25(OH)_2D_3$ in CD4+ T cells, representing local metabolism and production of active vitamin D. These data suggest there is a local processing of $1,25(OH)_2D_3$, creating a localised metabolic mechanism to ultimately induce immunomodulatory actions. It was also demonstrated that the timing of delivery and the presence of $1,25(OH)_2D_3$ to T cells is critical for efficient activation of intracellular VDR signalling pathways. However, the latter investigation by Baeke et al. (2010) was an *in vitro* model and thus does not replicate physiological relevant concentrations in humans.

2.6.2.1 T lymphocytes

Classically the immune system consists of two systems: innate and adaptive, which differ in their functional roles within the body (Rabb, 2002). Although born with both systems, the adaptive system comes into play later in life. The innate system is natural protection from disease and can be present at very short notice to protect from foreign invaders. Whereas the adaptive system has the capacity to mount a response to foreign bodies, but requires a change in the number or phenotype of B (bone) and T (thymic) cells in order to adapt and build an immune defence. The primary lymphocyte subpopulation express CD3+ molecules on their cell surface, and either CD4+ or CD8+ molecules, which define them as helper T cells (T_H) or cytotoxic T cells (T_C) cells, respectively. Furthermore, CD8+ T cells are affected by age, inducing the development of an inverted CD4:CD8 T cell ratio and thus contributing to immune incompetence (Effros, Cai, & Linton, 2003). Throughout life, the immune system gradually deteriorates causing impairment of its defence capability, known as immunosenescence. The innate and adaptive immune systems are differently affected by ageing, whereby innate immunity appears to be better preserved while adaptive immunity exhibits age-dependent depreciation. Age-dependent regression of the thymus, thymic atrophy, defined as the loss of thymic mass, induces a decline in the output of naïve T cells (Palmer, 2013). Therefore, as age advances fewer T cells are developed and exported into the vascular pool (Lazuardi et al., 2005), directly impacting on the peripheral naïve T cell repertoire and altering white blood cell subset diversity, and thus the cells that are circulated to the target tissues (Aw, Silva, & Palmer, 2010; Palmer, 2013). The age-related changes in lymphocyte subpopulations appear to have functional consequences. Therefore, comparing peripheral blood samples between young and older adults should consider the different T cell population and thus the altered expression of cellular components, such as metabolites and receptors. For example, a smaller peripheral blood T cell pool due to ageing, may transcend into a decline in the expression of the VDR.

2.6.3 Exercise and immunosenescence

The beneficial effect of exercise became apparent in the early work of David Neiman in the 1990's who demonstrated that individuals who exercise are at less risk of upper respiratory tract infections (URTI) (Nieman, 1994), which are a major cause of visits to and treatment from physicians. However, there is a hyperbolic relationship between intensity and volume of exercise and the risk of URTIs, suggesting that excessive or too intense exercise can be detrimental to effective immunity by supressing immune function (Malm, 2006). There are both acute and chronic effects of exercise on immune function. In response to an acute bout of exercise, one of the major changes that occurs is a change in the number of leukocytes (Gleeson & Bishop, 2005; Robson, Blannin, Walsh, Castell, & Gleeson, 1999), with a biphasic response induced (Nieman et al., 1991). The redeployment of lymphocytes from tissues or the blood vessel wall with exercise consists of an initial increase, known as is followed by a significant transient drop in lymphocyte number, known as lymphocytopenia. Immediately upon cessation of exercise the rise in lymphocyte and neutrophil number usually precedes a reduction to below baseline levels, creating a pocket period of reduced immune protection, known as exercise-induced immunosuppression. This is comprehensively reviewed by Gleeson and Bishop (2005).

Each of the individual cell types respond differently to exercise as they all perform different tasks to achieve sound immune function, however it is the Natural Killer (NK) cells and the cytotoxic T cells that display the largest response (R. J. Simpson, Florida-James, Whyte, & Guy, 2006). Exercise-induced immunosuppression can also be altered by cytokines, the signalling molecules of the immune system. Circulating concentrations of cytokines have numerous responsibilities and roles in the inflammatory profile and protection against pathogens, directly and indirectly.

Ageing is recognised to strongly affect the redeployment of lymphocytes with particular subsets not mobilised in the bloodstream: although the relative numbers of T cells are similar between young and old, it is the absolute numbers that change. The process of age-associated deterioration of immune cells and thus the operation of the system, is referred to as

immunosenescence (Castle, 2000). This causes a rise in senescent T cells that are mobilised and thus circulate around the body unable to play an efficient role in immune function and protection (R. J. Simpson et al., 2012). This age-related accumulation of senescent T cells lowers the naïve T cell stock and can increase host infection risk. This is also due to older individuals having less naïve and low differentiated cells in the circulation and peripheral tissues for redeployment (Provinciali, Moresi, Donnini, & Lisa, 2009). Exercise can potentially override the age-related impairments in T cell subset redeployment, specifically CD8+ T cells (Spielmann et al., 2014), as aerobic fitness level, achieved through regular exercise, is inversely associated with the proportion of senescent T cells, with the relationship withstanding adjustment for age (Spielmann et al., 2011). Regular exercise therefore appears to alleviate some of the deleterious effect of ageing on the immune system.

Programmed cell death, or apoptosis, is an important mechanism in the mediation of the immune response, serving as a key role in the removal of damaged, infected, exhausted or redundant cells. This orchestrated system then allows for alterations in the proportion of cells that make up the bloodstream repertoire of T cells. Acute bouts of exercise have been shown to induce increases in both senescent and naïve T cells and elevate apoptotic lymphocytes (Mooren & Kruger, 2015). Since ageing induces an accumulation of senescent T cells, it is imperative for effective immune function to induce apoptosis in specific cell types, preferentially the older less functional cells, to allow for naïve T cells to be exported into the circulation, favourably altering the bloodstream repertoire. Exercise has been associated with an increase in apoptotic cells, although the mechanisms are not yet fully understood. In addition, despite the modality of exercise, there is no evidence to suggest that lymphocyte-apoptosis contributes to exercise-induced lymphocytopenia (R. J. Simpson, Florida-James, Whyte, et al., 2007).

In summary, immunosenescence contributes to increased susceptibility to acute and chronic conditions placing the ageing population at a disadvantage. However, exercise can orchestrate a protective effect on immune function through phenotypic and functional changes in the T cell component of the immune profile.

2.6.4 VDR-induced cellular signalling

There are genomic and non-genomic roles of the secosteroid hormone vitamin D and it's metabolism, specifically through the action of the VDR (Haussler et al., 2011). The genomic role in the endocrine system is predominantly through the classic binding of the ligand, $1,25(OH)_2D_3$, to its receiving receptor, VDR, modulating the transcriptions of genes, which has been discussed earlier in this literature review. The non-genomic effects are mediated through $1,25(OH)_2D_3$, which initiates many biological responses via the VDR, through cellular rapid response signalling pathways. This has been reviewed and outlined well by Haussler et al. (2011).

In relation to the occurrence of VDR-mediated cellular signalling responses, this has been found to manifest in T cells. Recent data has shown that cytoplasmic VDR upregulation is a specific marker of T cell activation (Joseph et al., 2012), suggesting that a cooperative mechanistic relationship occurs between $1,25(OH)_2D_3$ and T cells. A molecular mechanism proposed is through the intracellular extracellular signal-regulated kinase 1/2 (ERK1/2) and mitogen-activated protein kinase (MAPK) signalling pathway, with ERK1/2 phosphorylation transiently induced following activation. Joseph et al. (2012) found an association between VDR upregulation and ERK1/2 phosphorylation. This relationship was examined given the importance of MAPK signalling in human T cell activation. It was suggested that the ERK1/2 pathway plays a role in the regulation of VDR, based on the finding that phosphorylation of ERK1/2 preceded VDR expression, but only on phospho-ERK1/2 positive cells: the T cells had induced phospho-ERK1/2, which was followed by a gradual rise in the expression of VDR.

Another form of stimulation on the ERK/MAPK pathway and the signalling proteins is muscle contraction (Kramer & Goodyear, 2007), which may then link into vitamin D metabolism. In response to exercise, the ERK/MAPK relationship has been shown to be dependent on either exercise intensity, muscle fibre type recruitment or rate of contraction. Resistance training but not endurance aerobic training has been demonstrated to increase ERK1/2

phosphorylation immediately post-exercise, although the effect was attenuated at 1 h post-exercise (Makanae et al., 2015). There also appears to be different mechanisms underlying the skeletal muscle adaptations to exercise with regards to age, with MAPK proteins differentially activated at rest and in response to exercise in the skeletal muscle of young and old men (Williamson, Gallagher, Harber, Hollon, & Trappe, 2003). Subsequently, this may affect intracellular VDR expression and thus key cellular processes, such as transcription and translation and thus gene expression.

There appears to be an exercise modality-dependent relationship between muscle contraction and upregulation of intracellular VDR expression, primarily demonstrated in skeletal muscle tissue (Makanae et al., 2015; Widegren, Wretman, Lionikas, Hedin, & Henriksson, 2000). It has not yet been investigated whether this is attributed to exercise intensity or recruitment of specific fibre type and metabolic system: aerobic or anaerobic, and thus endurance and resistance exercise. In a study investigating the effect of 25(OH)D supplementation on VDR expression in the two muscle fibre types, it was found that an increase in VDR was only observed in type II fibres and not type 1 (Ceglia et al., 2013). Therefore, the changes in vitamin metabolism response to a stimuli or intervention could be type II specific.

2.7 Summary

In summary, upon review of the current literature it appears that both the vitamin D status and physical activity status of the UK, in particular Scotland, is poor and requires attention and action. However, the relationship between environment- and behaviour-related measures has not been extensively explored, with a particular gap regarding the influence of the ageing process. The development of appropriate strategies to correct vitamin D deficiency and/or improve the efficiency of vitamin D metabolism are particularly important considering the links between low vitamin D levels and the prevalence of numerous chronic diseases. Although exercise has been linked to alterations in vitamin D status (25(OH)D concentration), active vitamin D ($1,25(OH)_2D_3$) and cellular VDR expression, the current investigations are

limited to murine models or the inclusion solely of the inactive metabolite: 25(OH)D. Therefore, it is of interest to investigate whether these findings are applicable to a human population. If this is the case, as hypothesised, the findings could support exercise as a strategic intervention to adopt in order to beneficially manipulate vitamin D metabolism.

Chapter 3: General Materials and Methods

3.1 Ethical approval

Ethical approval for each study was granted by the School of Applied Sciences Research Integrity Approvals Group at Edinburgh Napier University. All samples and data were treated and stored in line with the Declaration of Helsinki and according to the 'Edinburgh Napier University Code of Practice on Research Integrity October 2013'.

3.2 Study participants

3.2.1 Vitamin D Status (VDS) study

The study population comprised of healthy Caucasian males and females, aged 60-80 y with a BMI between 18.5-35 $kg \cdot m^{-2}$ that resided in Scotland at the time of testing and had done so for the 6 months prior to initial contact with the researcher regarding the study. Participant recruitment numbers and methods are displayed in **Figure 4**. Participants were recruited via posters, flyers, magazine advertisement (Konect Magazine), Gumtree, and the use of social media (Facebook and Twitter). Edinburgh based groups/committees were contacted regarding recruitment, including: Rotary Clubs, the University of the Third Age (U3A), Edinburgh Leisure (Ageing Well), the Eric Liddell Centre, and the NHS Retirement Fellowship.

The exclusion criteria excluded persons who regularly use tanning beds or undergo UV light therapy, had been on a 'sun holiday' in the past 8 weeks, or consume vitamin D tablets or any multivitamins containing vitamin D (concentration > 10 $\mu g \cdot day^{-1}$).

Six volunteers were excluded based on their assessment of eligibility, due to: BMI < 18.5 $kg \cdot m^{-2}$ (n=1), BMI > 35 $kg \cdot m^{-2}$ (n=1), and regular consumption of vitamin D supplements (n=4).

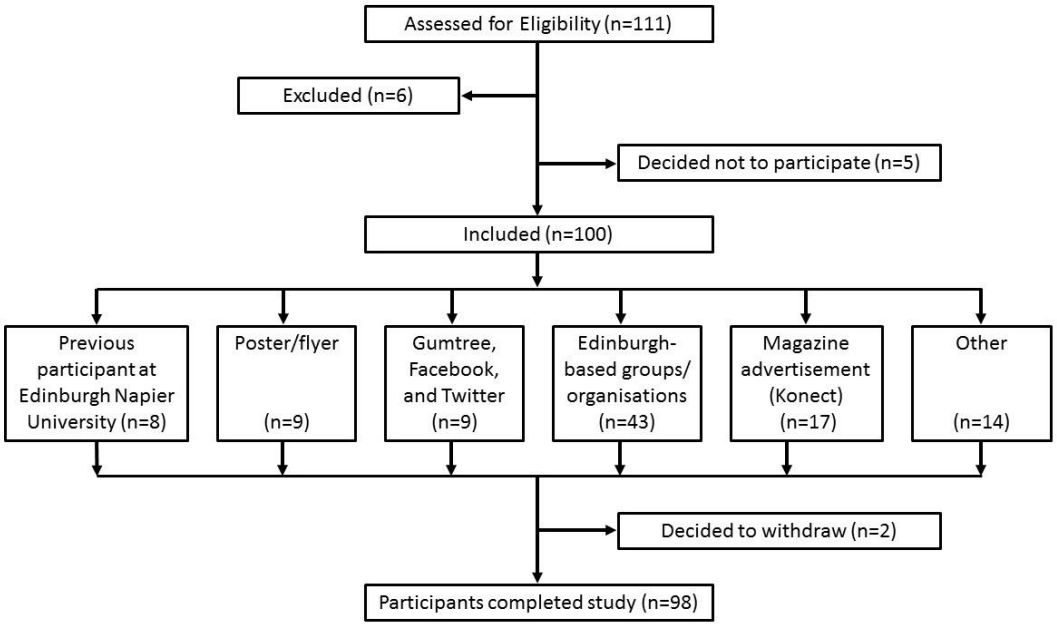

Figure 4 Participant recruitment flow diagram for the Vitamin D Status (VDS) study.

3.2.1.1 Power analysis calculation

An a priori power analysis using GPower software 3.1.9.2 was performed for sample size estimation based on a multiple regression analysis (F test) to test whether physical activity (number of predictors = 5) predicted 25(OH)D concentration. Based off the data in the study by Jacques et al. (1997), it was estimated that there would be a small effect size (Cohen's f - 0.15). The projected sample size required for the statistical test was n=89, to achieve statistical power >0.95 (α and β error probability – 0.05).

3.2.2 Exercise and Vitamin D (ExVD) study

The study population comprised of healthy Caucasian males, aged 18-45 and 60-75 y with a BMI between 18.5-30 $kg \cdot m^{-2}$ that resided in Scotland at the time of testing and had done so for the 6 months prior to initial contact with the researcher regarding the study. The recruitment process of participants is outlined in **Figure 5**. Participants were included if they were recreationally active according to the moderate-to-vigorous physical activity (MVPA)

guidelines, as defined in the UK Physical Activity Guidelines: more than 150 min of moderate activity per week, or 75 min of vigorous activity per week ("Physical activity guidelines for adults (19-64 years)," 2011).

The exclusion criteria excluded persons who have cardiovascular or respiratory diseases, autoimmune diseases, diagnosed diabetes, previous stroke or cardiac events, chronic or debilitating arthritis, hypertension, use tanning beds or undergo UV light therapy, had been on a 'sun holiday' in the past 8 weeks, or routinely take vitamin D supplements (dose >10 µg/day). If blood pressure (BP) was high, defined by the American Heart Association (AHA) as systolic BP >140 mmHg or diastolic BP >90 mmHg (Pickering et al., 2005), participants were excluded from the study and informed to contact their General Practitioner (GP).

Six volunteers were excluded based on their assessment of eligibility, due to: BMI > 35 $kg \cdot m^{-2}$ (n=1), hypertension (n=2), and regular consumption of vitamin D supplements (n=3).

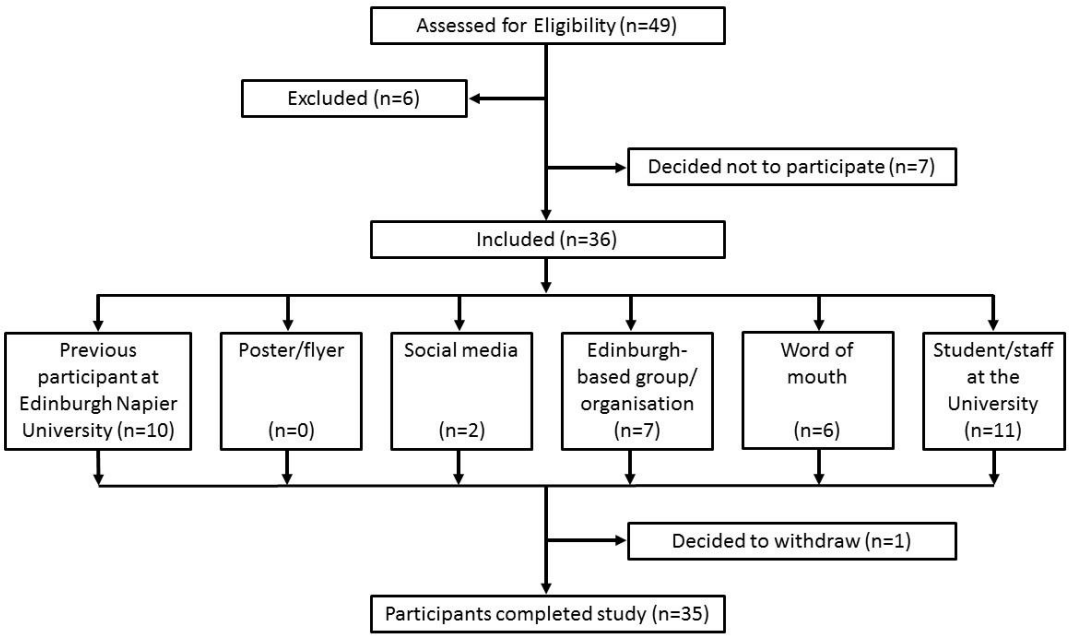

Figure 5 Participant recruitment flow diagram for the Exercise and Vitamin D (ExVD) study.

Upon inclusion, the participants were separated into three groups based on their age: young (18-30 y), young/mid (31-45 y), and older adults (60-75 y).

3.2.2.1 Power analysis calculation

An a priori power analysis using GPower software 3.1.9.2 was performed for sample size estimation based on an ANOVA: repeated measures, within-between interaction statistical test (F test). The main outcome variable was CD3+ T cell VDR expression expressed as fold change. The number of groups equals 3 to represent the three age groups within each trial: young, young/mid, and older. Number of repeated measurements equals 4 to represent the 4 time points: pre-, 0 h, 1 h, and 3 h post-exercise/control. Based off the data in the studies by Makanae et al. (2015) and Aly et al. (2016), it was estimated that there would be a large effect size (Cohen's f - 0.40). The projected sample size required for the statistical test was n=21, to achieve statistical power >0.95 (α and β error probability – 0.05).

3.3 General screening of participants and inclusion criteria

Participants who volunteered to take part in the investigations involved in this thesis provided verbal and written consent (Appendix 1) prior to participation. Participants were screened with relevant medical history questionnaires and physiology screening questionnaires prior to inclusion: physiology screening questionnaire (Appendix 2), the American College of Sports Medicine (ACSM) Physical Activity Readiness Questionnaire (PAR-Q) (Appendix 3), and the American Heart Association (AHA)/ACSM Health/Fitness Facility Pre-participation Screening Questionnaire (Appendix 4).

3.4 Anthropometric measurements

Height and body mass were measured via a Stadiometer (Harpenden Portable, Holtain Limited, UK) and scales (Seca, 808, Germany), respectively.

Waist and hip circumference were measured using a standard measuring tape (Seca, Germany) with the participant standing in the upright neutral position. Waist circumference was measured at the mid-point between the iliac crest and the lowest palpable rib. Hip circumference was measured at the widest part of the hips (WHO, 2008).

3.5 Blood pressure measurement

Arterial BP was measured on the participant's non-dominant arm using a digital automatic BP monitor (Nonin, Avant 2120, USA). Participants remained in a supine position on a laboratory bed for 5 min prior to the first measurement. Blood pressure was measured 3 times and an average of the second and third readings for systolic and diastolic BP was reported.

3.6 Peak oxygen uptake test

Peak oxygen uptake ($\dot{V}O_{2peak}$) was determined using an incremental step-protocol exercise test on an electromagnetically-braked cycle ergometer (Lode, Corival CPET, Netherlands), performed to volitional exhaustion (Poole, Wilkerson, & Jones, 2008; Yoon, Kravitz, & Robergs, 2007). An on-line breath-by-breath gas analysis system (Cortex, MetaLyzer 3B, Germany) was used to measure expired air continuously throughout the test. Calibration of the flow sensor and gas analysis device was performed 30 min prior to each use. Heart rate (HR) was continuously monitored throughout the test using a HR monitor (Polar, RS400, Finland) and the HR sensor linked to the gas analysis system. After a 5 min warm up at 80 W, the intensity was increased by 30 W every 2 min until volitional exhaustion. Participants were instructed to maintain a minimum pedalling rate of 70 revolutions per min (rpm) and were verbally encouraged to perform to volitional exhaustion, at which point the test was terminated (rpm <60). A 5 min cool down at 50 W was then immediately commenced. Peak oxygen uptake was identified as the highest $\dot{V}O_2$ over a 30 s period during the test.

3.7 Blood sampling

Participants were in a semi-supine position on a laboratory bed for all blood sampling. Blood samples were collected in 4-6 ml capacity BD (BD Biosciences, Becton, Dickinson and Company, US) vacutainers: lavender-topped vacutainers (4 ml) containing K_3 ethylenediaminetetraacetic acid (EDTA) (approximately 1.0 mg) for plasma separation, grey-topped vacutainers (4 ml) containing sodium fluoride/potassium oxalate to obtain plasma for glucose determinations, pink-topped vacutainers (6 ml) containing K_3 ethylenediaminetetraacetic acid (EDTA) (approximately 1.0 mg) to collect whole blood, and gold-topped vacutainers (5 ml) containing a clot activator and gel for serum separation. All vacutainers were inverted 8 times immediately after blood sampling.

3.7.1 Venepuncture

Peripheral venous blood samples were taken from an antecubital vein of the forearm via a 21-gauge needle and collection set with 7 inch tubing and a BD vacutainer Safety-Lok™ Luer Adapter (BD Biosciences, USA).

3.7.2 Cannulation

Venous blood samples were taken from the antecubital vein in the forearm via cannulation using a 22-gauge BD Venflon™ Pro Safety I.V. Cannula (BD, USA). Connecta tubing (Nu-Care Products Limited, Bedfordshire, UK) was used to prevent movement of the positioning of the needle. The cannula and tubing were kept patent via syringe flushing between samples with 8 ml of 0.9 (w/v) saline solution from pre-filled syringes (BD PosiFlush™ SP, BD, USA). After insertion and flushing of the cannula, at each time-point for blood collection, a 5 ml syringe (Terumo, Japan) was used to extract the first 3 ml of blood, which was discarded prior to sample collection.

3.7.3 Control measures

Participants were asked to abstain from consuming caffeine and alcohol and engaging in strenuous exercise in the 24 h prior to blood sampling. Participants were asked to maintain their normal diet and activity during involvement in either study.

3.7.4 Centrifugation of blood samples

For plasma analysis, the whole blood was centrifuged (Satorius Universal, 320R, Germany) at 1500 rpm for 15 min at 4 °C within 30 min of collection. To obtain serum, whole blood was incubated at room temperature for 30 min prior to centrifugation at 1000 x g for 10 min at 4 °C.

The resulting plasma and serum was aliquoted into 1.5 ml Eppendorf tubes and stored at minus 80 °C for later analysis.

3.8 Haematological parameters

Whole blood was analysed for full blood cell count in vacutainers containing K_3 EDTA using a haematology analyser (Sysmex Automated Haematology Analyser, XS 1000i, Sysmex, Japan). Samples were measured immediately following blood collection in triplicate and averaged. Quality control samples were used prior to each blood sample being quantified for differential white blood cell count (e-Check XS, Sysmex, Japan). Blood components measured were: white blood cell (WBC) count, red blood cell (RBC) count, haemoglobin (HGB) content, haematocrit (HCT) content, and WBC subsets: neutrophils, lymphocytes, monocytes, eosinophils, and basophils.

3.9 Quantification of 25(OH)D_3 concentration

For the analysis of total serum 25(OH)D concentration, high-pressure liquid chromatography tandem mass spectrometry (LC-MS/MS) was implemented. The analysis was carried out by Mr Gary Duncan at The Rowett Institute in Aberdeen, Scotland, UK. The LC-MS/MS method of analysis has been

validated against other commercially available assays and is regarded as the most valid and reliable technique for the assessment of vitamin D metabolites (Snellman et al., 2010). Plasma samples from the ExVD study (n=35) were also analysed using enzyme immunoassay (EIA) (IDS-PLC, Tyne & Wear, UK). EIA has been reported to underestimate blood 25(OH)D_3 concentrations in comparison to the gold standard LC-MS/MS. The coefficient of variation (CV) for the LC-MS/MS and EIA assays are displayed in **Table 2**.

Table 2 Intra-assay coefficients of variance (CV) for 25(OH)D analysis.

Study	Analysis method	Intra-assay CV (%)
VDS	LC-MS/MS	1.89
ExVD	EIA	2.65
ExVD	LC-MS/MS	3.04

VDS; Vitamin D Status study (n=97). ExVD; Exercise and Vitamin D study (n=35).

3.9.1 LC-MS/MS principle

LC-MS/MS combines the capability of liquid chromatography with the mass analysis ability of mass spectrometry. Human serum must undergo preparation prior to injection in order to clean the sample, thus purifying the vitamins from the other components in serum. Following sample preparation, the process can be generally separated into three main steps: the separation step (step 1), ionization step (step 2), and mass analysis (step 3). In step 1 the cleaned up sample is forced at high pressure by a liquid (mobile phase) into a column (solid phase) to separate the analytes. In step 2, the sample is charged via atmospheric pressure chemical ionisation (APCI), whereby a solvent reagent ionizes the analyte. In the final step, the ions are separated according to their mass-to-charge ratio by electromagnetic fields in an analyser then detected by a quantitative method. The signal is then processed into mass spectra.

3.9.2 LC-MS/MS method

Prior to injection into the mass spectrometer (Shimadzu Nexera X2 LC Binary Pump Model), 25(OH)D_3 and deuterated internal standard (QMX Laboratories Ltd, UK) were extracted from serum samples via protein precipitation sample preparation. The internal standard (25-hydroxyvtamin D_3-[d_3]) was added to serum samples at a concentration of 400 pg·µl^{-1}, then 300 µl of acetonitrile was added to 100 µl of spiked serum samples and vortexed. Solutions were incubated on ice for 30 min prior to centrifugation at 13,000 rpm for 15 min. The supernatant was removed via drying under a stream of nitrogen and dried extracts were reconstituted in the mobile phase (15% 5mM Ammonium Acetate + 0.1% Acetic Acid:85% Methanol) prior to injection (5 µl) into an LC-MS/MS in the multiple reaction mode at a flow rate of 500 µl·min^{-1}. The retention time of the injected sample in the column was 2.01 min. The accuracy of the assay is provided in **Table *3***.

Table 3 Accuracy of the LC-MS/MS assay.

VDS study (n=97)			
Standards (pg·µl^{-1})	**Concentration (pg·µl^{-1})**	**Accuracy (%)**	**Intra-CV (%)**
1000	986.7 ± 10.3	98.67	0.70
500	526.2 ± 1.2	105.23	0.68
250	252.7 ± 1.0	101.07	0.52
100	96.6 ± 0.8	96.67	0.56
50	48.0 ± 01	95.93	0.45
10	9.3 ± 0.3	92.50	3.92
5	4.7 ± 0.2	94.57	4.19
ExVD study (n=35)			
Standards (pg·µl^{-1})	**Concentration (pg·µl^{-1})**	**Accuracy (%)**	**Intra-CV (%)**
1000	984.4 ± 6.6	98.47	1.01
500	529.4 ± 3.4	105.87	1.09
250	255.0 ± 1.7	102.00	1.03
100	96.9 ± 0.3	96.90	2.13
50	49.3 ± 0.6	98.60	1.55
10	9.5 ± 0.3	94.70	4.63
5	4.8 ± 0.2	96.03	4.96

The concentration data is presented as mean ± SD.

3.9.3 EIA

Plasma 25(OH)D concentration was quantified using a 25-Hydroxy Vitamin D EIA kit (IDS-PLC, Tyne & Wear, UK). Low (10 $ng \cdot ml^{-1}$, measured at 10.1 $ng \cdot ml^{-1}$) and high (45 $ng \cdot ml^{-1}$, measured at 44.8 $ng \cdot ml^{-1}$) controls were used and a standard calibration curve (R^2=0.986) applied to the plotted concentrations of the standards. Absorbance was measured at the recommended 450 nm wavelength.

3.9.4 Comparison of LC-MS/MS and EIA

There is a diverse array of vitamin D testing methods routinely used in laboratories (Enko, Kriegshauser, Stolba, Worf, & Halwachs-Baumann, 2015). In comparative and reliability studies, LC-MS/MS is widely used as the reference method (Carter, 2012), and is a reliable diagnostic tool with the ability to distinguish between 25(OH)D_2 and 25(OH)D_3 (van den Ouweland, Beijers, Demacker, & van Daal, 2010). Although LC-MS/MS is classified as the gold standard measurement, other competitive assays have been developed that are less expensive and time-consuming compared to mass spectrometry. One such assay is EIA (or ELISA). Depending on the analysis method used, a clinical concern is the possible misdiagnosis of vitamin D concentration and thus classification. Therefore, it is important to determine whether methods that are not the gold standard have the presence of significant variance and/or proportional bias compared to the gold standard mass spectrometry.

Within this PhD thesis methodology, the two clinical measurements: EIA and LC-MS/MS have been compared via a Bland-Altman plot and statistics have been performed on the comparison. The LC-MS/MS method was defined as the reference method. The CV between the LC-MS/MS and EIA assays (n=35) was 5.16 %.

The null hypothesis was that there will be 0 difference (variance) between EIA and mass spectrometry analysis of serum sample for 25(OH)D_3 concentration. **Figure 6** shows the Bland Altman plot of the mean of the two measurements (x axis) and the difference between the two measurements (y axis). A one-sample t-test was conducted on the difference between the measurements,

with the ‘test value’ set to 0 as it was hypothesised that there will be zero systematic difference between the two measurements. Results of the one-sample t-test reported there was no significant difference (*P*=0.348) between 0 and the mean difference (-0.1914) of our measurements. Therefore, there is agreement between the methods for analysis of serum 25(OH)D_3 concentration. Upper and lower confidence levels (95 %) have been added to the plot to show how well the data fits within the normal level of agreement.

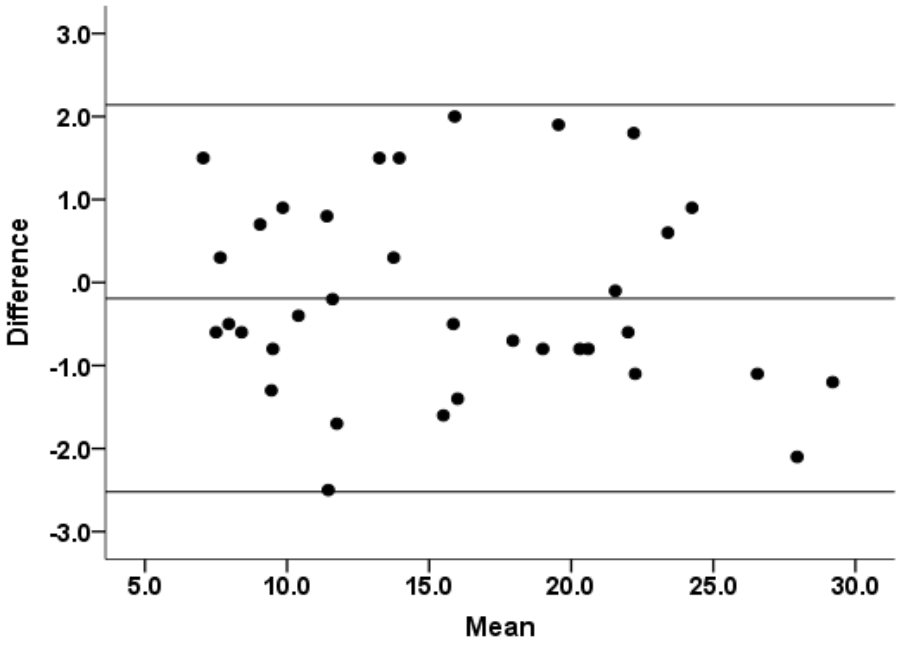

Figure 6 Bland-Altman difference plot for the proposed gold standard LC-MS/MS and EIA. Each marker represents an individual’s data (n=35). Difference: the difference between the means for LC-MS/MS and EIA. Mean: mean 25(OH)D concentration ($ng \cdot ml^{-1}$).

A linear regression analysis was then performed on the data to test the null hypothesis, presented in **Figure 7**. The results of the regression (F=0.573, t=-0.757, *P*=0.454) report that the test is non-significant and we can accept the null hypothesis. Therefore, there is no proportional bias in the data and there appears to be a level of agreement.

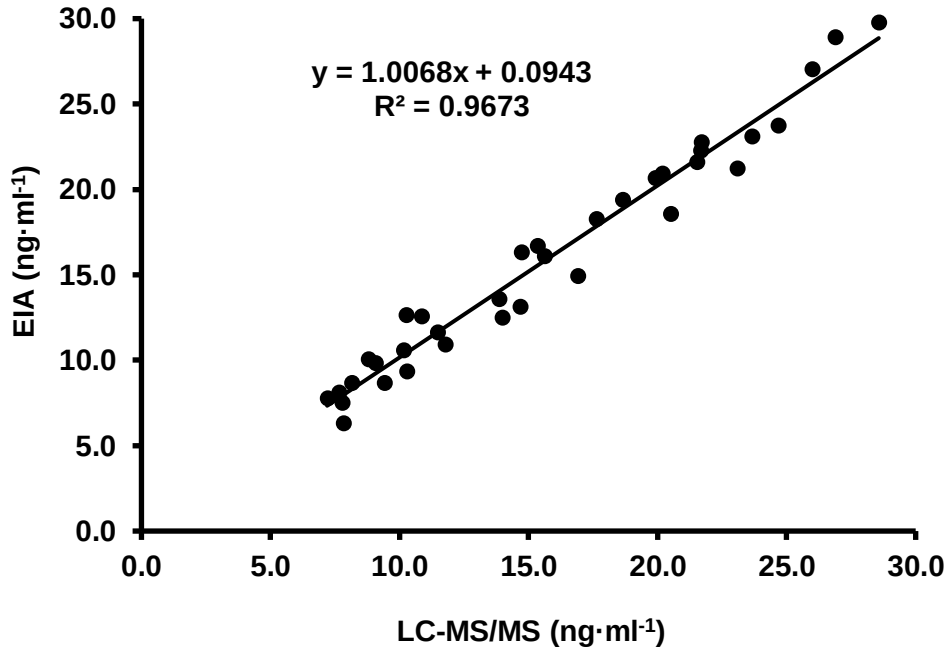

Figure 7 Comparison between mean 25(OH)D concentration measurements assessed via LC-MS/MS and EIA. Each marker represents an individual's data (n=35).

It can be concluded that there is no trend in the data or proportional bias, and no variance between the two measurements: EIA and LC-MS/MS, that were used to analyse serum for 25(OH)D_3 concentration. Therefore, EIA could be used as a reliable method to report 25(OH)D concentrations.

However, upon inspection of the data, an interesting finding was the difference in individual diagnosis of vitamin D status classification (see **Table 1**) as a result of measured plasma/serum 25(OH)D concentration. As shown in **Table 4**, the number of participants who would be diagnosed as severely deficient, deficient and insufficient is dependent on the method used to assess 25(OH)D concentration. This is clinically relevant, as this may impact upon classification and subsequent treatment.

Table 4 The number of participants in each vitamin D classification determined by LC-MS/MS and EIA (n=35).

Classification	25(OH)D concentration	LC-MS/MS	EIA	Diff
	$ng \cdot ml^{-1}$	n	n	n
Severely deficient	< 12	14	12	-2
Deficient	13-20	10	13	+3
Insufficient	21-29	11	10	-1
Sufficient	> 30	0	0	0

Diff, difference of EIA compared to gold standard LC-MS/MS.

In light of these findings and for the purpose of this thesis, all 25(OH)D concentrations reported in the chapters are quantified by LC-MS/MS.

3.10 Flow cytometry

The flow cytometry protocol primarily followed a previously established protocol that is validated against confocal microscopy and has been reported to stain for both cytoplasmic and nuclear VDR (Bendix et al., 2015). Following PBMC isolation from whole blood, non-permeabilised and permeabilised cells were stained for VDR to analyse both cell surface and internalised VDR expression and analysed via a flow cytometer (BD FACSCelesta™, San Jose, USA).

3.10.1 PBMC isolation

For PBMC isolation, 6 ml of peripheral blood was diluted 1:1 with sodium chloride (0.9 % NaCl) in a 50 ml centrifuge tube. The mixture was then layered on top of 12 ml of Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) in a 50 ml centrifuge tube, without disturbing the interface. The sample was then spun down at 700 x g for 30 min at 20 °C. Following centrifugation, the upper layer of plasma was carefully removed using a plastic pasture pastette and

discarded, leaving the lymphocyte layer undisturbed at the interface, which was aspirated and transferred to a new centrifuge tube. The cells were washed with 6 ml saline at 250 x g for 10 min at 20 °C, and the supernatant removed and the cell pellet washed again. The supernatant was decanted and the pellet re-suspended in 1 ml phosphate buffered saline (PBS) to create a PBMC suspension, stored at 4°C.

3.10.2 Cell count

The cooled PBMC suspension for each time point was counted for cells per µl, to calculate the volume of suspension to be added to each flow cytometry tube. Ten microliters of PBMC suspension were added to 50 µl Trypan Blue solution (Sigma-Aldrich, Missouri, USA) on Parafilm® (Bemis NA, Wisconsin, USA) and mixed thoroughly by drawing in and out of a pipette tip. Ten microliters of the stained solution was transferred onto a Neubauer counting chamber, and the 4 corner squares were counted. Only the unstained cells were counted to avoid counting dead (stained) cells.

Calculations:

For the number of PBMCs in sample:

PBMC count/ml = Mean of four squares x 10^5 x dilution factor (5)

For 500,000 PBMCs:

1000 ÷ [(PBMC count/ml) ÷ 500,000]

3.10.3 VDR staining

A control sample (PBMCs taken from the pre-trial time point and stained for CD3+, CD4+, and CD8+ T cells but not VDR) was analysed using both the permeabilised and non-permeabilised method.

3.10.3.1 Non-permeabilised method

Based on the cell count, 500,000 cell suspension was added to each tube and stained with 2 µl of anti-CD3 antibody fluorescein isothiocyanate (FITC) (BD Bioscience), anti CD4 antibody Brilliant Violet 786 (BV786) (BD Bioscience), anti-CD8 antibody phycoerythrin with the cyanide dye Cy5 (PE-Cy5) (BD Bioscience), 1 µl anti-VDR antibody (VDR Polyclonal Antibody, ThermoFisher Scientific, Massachusetts, US) and 1 µl of the secondary antibody (PE) (BD Bioscience) were added to the VDR stained tubes only and cells were incubated at 4°C for 30 min. Following incubation, 1 ml PBS was added to all tubes and the cells were washed at 250 x g for 10 min at 20 °C. The cell pellet was re-suspended in 500 µl PBS via vortex and flow cytometry was performed within 1 h.

3.10.3.2 Permeabilised method

Based on the cell count, 500,000 PBMCs suspension was added to each tube and stained with 2 µl of anti-CD3 antibody FITC (BD Biosciences), anti CD4 antibody BV786 (BD Biosciences), and anti-CD8 antibody PE-Cy5 (BD Biosciences), and incubated for 15 min at 4 °C. Following incubation, 250 µl of Fixation/Permeabilisation Solution (BD Biosciences) was added and incubated for 20 min at 4 °C. One millilitre of Perm/Wash Buffer (10x) (BD Biosciences) was added and the cells were washed at 250 x g for 10 min at 20 °C. Following centrifugation, the cell pellet was re-suspended in 500 µl Perm/Wash Buffer (10x) (BD Biosciences) via vortex, and 1 µl anti-VDR antibody and 1 µl of the secondary antibody PE was added to the VDR staining tubes only. All tubes were incubated for 30 min at 4 °C and flow cytometry was performed within 1 h.

3.10.3.3 VDR data analysis

The VDR positive gate was set on the basis of the isotype PE control in each experiment: permeabilised and non-permeabilised method for each trial. VDR expression is reported as fold change, calculated from VDR geometric mean

(geomean). Absolute T cell number was calculated and quantified using the lymphocyte concentration obtained via haematological analysis (section 3.8).

3.10.3.4 Flow cytometric gating

The colour-dot plots of flow cytometric enumeration of T lymphocytes and the subsets: CD3+, CD4+, CD8+ T cells, and permeabilised and non-permeabilised CD3+VDR+ T cells are shown in **Figure 8**.

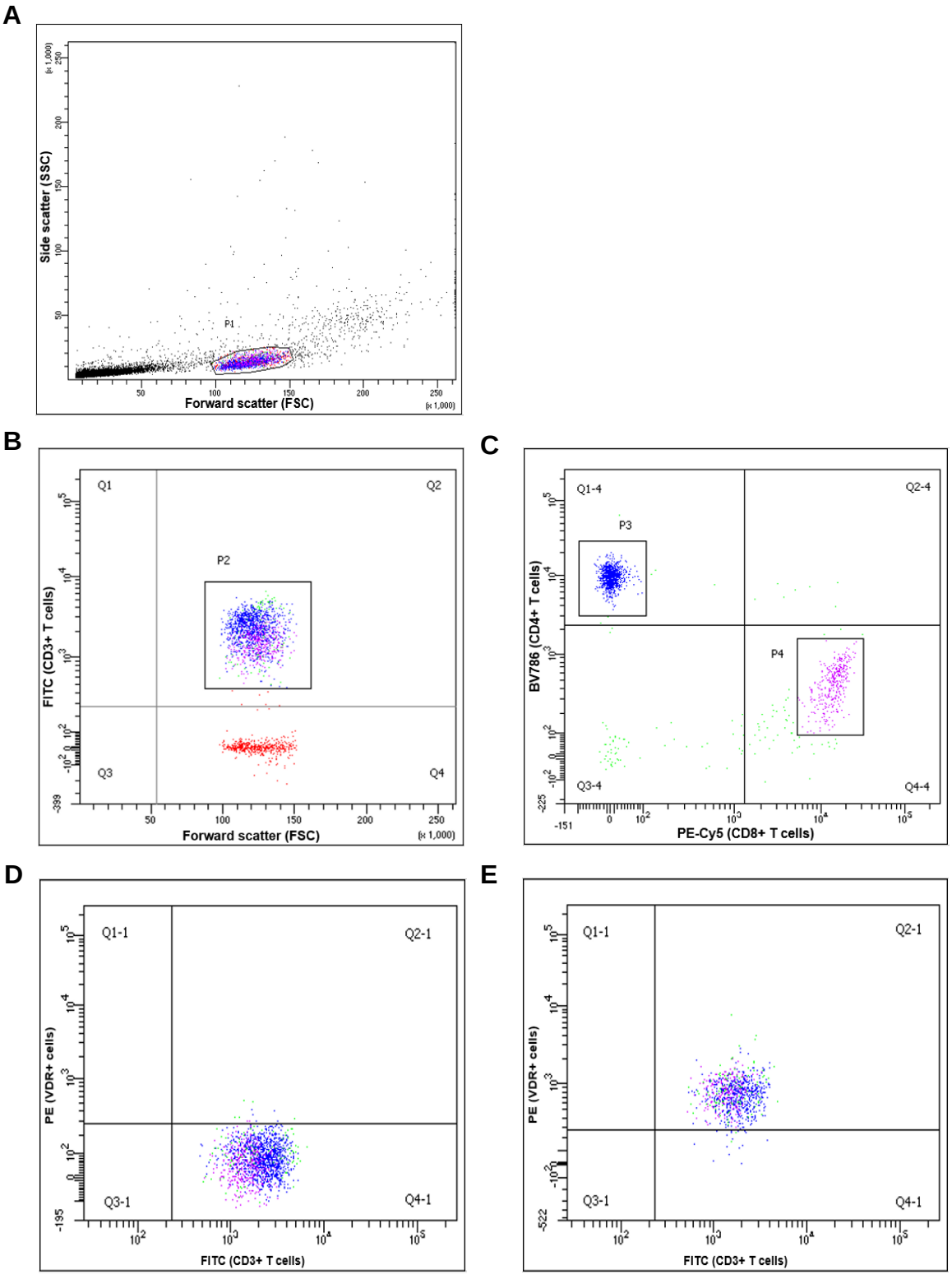

Figure 8 Flow cytometric enumeration of T lymphocytes: (**A**) side scatter vs. forward scatter for identification of lymphocyte gate, (**B**) CD3+ gating of lymphocytes, (**C**) CD3+CD4+ and CD3+CD8+ gating of lymphocytes, (**D**) non-permeabilised identification (CD3+VDR+), (**E**) permeabilised identification (CD3+VDR+).

3.10.3.5 Cell surface and intracellular expression of VDR

The T cells analysed for VDR expression were first analysed for cell surface expression and then the cells were permeabilised to analyse intracellular VDR expression. As shown in **Figure 9**, the percentage of cells (50,000 cells) expressing VDR internally was almost 100 % (CD3+: 98.0 ± 2.3 %, CD4+: 98.9 ± 1.3 %, CD8+:97.9 ± 2.0 %), whereas the cells expressing VDR on the cell surface was less than 3 % for all T cell subsets. The difference between the percentage of cells expressing VDR for non-permeabilised and permeabilised samples was significant (P<0.001) for the three T cell subsets. This data is based on the gating strategy and thus may identify false positives due to non-specific binding or doublets. This demonstrates and confirms that the receptor is predominantly an intracellularly expressed and bioavailable protein, therefore only intracellular VDR geomean is presented in the results sections to follow.

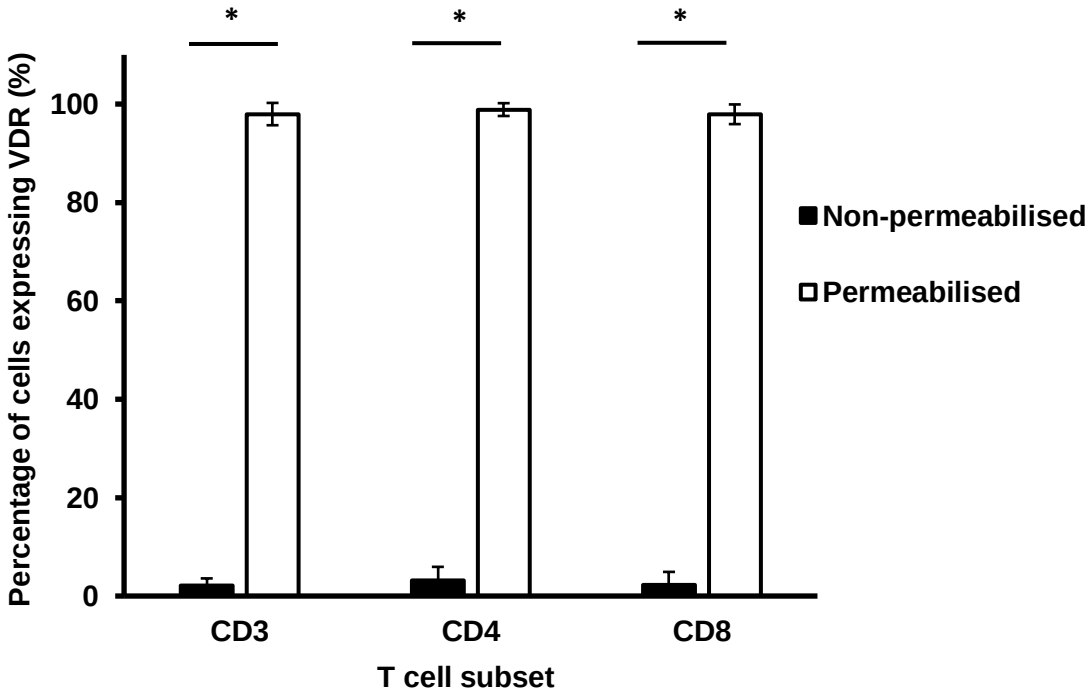

Figure 9 The percentage of T cell subsets expressing VDR, on the cell surface (non-permeabilised) and within the cell (permeabilised). * represents a significant difference between non-permeabilised and permeabilised (P<0.001). Data presented as mean ± SD.

3.11 Data analysis

All statistical analysis was performed using SPSS 23.0 software (Statistical Package for the Social Sciences Inc., IBM Corporation, Chicago, Illinois, USA). The Shapiro Wilk test was used to test for normal distribution of all data prior to main analysis. If the data was not normally distributed, the data was transformed and the check for normality was repeated. For analysis involving more than 3 comparisons, Mauchly's Test of Sphericity (P>0.05 sphericity assumed) was checked, with Greenhouse-Geisser correction applied where appropriate. Where significant main effect and interactions were present, the Bonferroni post-hoc pairwise comparisons test was used to detect where significances lay between paired comparisons, an analysis that includes correction for an ANOVA's multiple comparisons. The data is presented as mean ± standard deviation (SD) or mean ± standard error mean (SEM). Statistical significance was accepted at P≤0.05. All figures were designed in Microsoft Excel v.2011.

Independent samples t-tests were used to ascertain the difference between two independent variables, and one-way Analysis of Variance (ANOVA) were used when there were more than 2 groups in the comparison. Paired samples t-tests were used to ascertain difference between two paired variables.

Regression models were used to analyse what independent variables predict the dependent variable. The multivariate model was used to provide the results of the ANOVA, correlation significance, adjusted regression coefficients (Beta), 95 % confident intervals, and adjusted R square (adjusted to account for sample size). Where regression models were not required, Pearson correlations were conducted to examine associations between two variables.

Chapter 4: Vitamin D Status and Physical Activity of Older Adults

4.1 Abstract

Research shows that individuals residing in Scotland have a strikingly low vitamin D status, with an emerging association between physical activity levels and 25(OH)D concentrations. This association has not yet been investigated in older adults (60-80 years).

A total of 98 (39 male, 59 female) older adults (age: 67 ± 5 y, body mass: 73.4 ± 14.9 kg, BMI: 26.1 ± 3.7 $kg \cdot m^{-2}$) were included in the study. Participants provided 7-day self-reported (International Physical Activity Questionnaire, IPAQ) and objectively measured (accelerometer) physical activity levels, and fasted blood samples that were analysed for 25(OH)D concentration (measured by LC-MS/MS).

The population was defined as vitamin D deficient, with a mean 25(OH)D concentration of 14.5 ± 3.9 $ng \cdot ml^{-1}$ and reported a high prevalence of severe deficiency (26 %), deficiency (64 %), and insufficiency (10 %). A seasonal influence was observed, although there was no influence of gender (P=0.063) on vitamin D status. In line with the literature, there was a decline in vigorous physical activity with age (P=0.004). However, only time spent engaged in vigorous activity was associated with 25(OH)D concentration (P=0.034), with a surprising negative correlation observed (r=-.203). Physical activity did not predict 25(OH)D concentration (P=0.542).

In conclusion, the severely low 25(OH)D concentrations confirm that Scotland is a vitamin D deficient nation. The data suggests that 25(OH)D concentrations may be influenced by intense physical activity in older adults that reside in Scotland.

4.2 Introduction

In an older population there appears to be a decline in skin synthesis of vitamin D (Holick & Chen, 2008; MacLaughlin & Holick, 1985), reported to be attributable to a decrease in the enzyme (CYP27B1) responsible for the initial conversion reaction in the skin following exposure. Moreover, recent systematic reviews and meta-analyses report an association between high levels of vitamin D and a lower prevalence of cardio-metabolic disorders (Parker et al., 2010), particularly in older adult populations (Pittas et al., 2010; Rendina et al., 2014). Therefore, ensuring an adequate vitamin D status, assessed by 25(OH)D concentration, is identified as important for older populations to slow or delay the decline in cardio-metabolic health that accelerates with the ageing process (Ford, Giles, & Dietz, 2002). The initial step towards achieving an adequate and/or sufficient status is to identify the mean baseline 25(OH)D concentration in Scotland and the potential lifestyle determinants that may influence this: sedentary behaviour and physical activity levels in older adults.

Physical activity has been reported to decline with advancing age (Sallis, 2000), however it is not known whether this is primarily attributed to environmental or biological factors. Participation in leisure time activities in Scotland decreases as age advances with many deterrents identified, such as lack of interest and physical symptoms (Crombie et al., 2004). Physical inactivity has been identified as a modifiable predictor of vitamin D status (K. Brock et al., 2010; K. E. Brock et al., 2010), although it has not been determined whether this is due to internal vitamin D metabolism or environmental factors, for example the outdoor nature of most physical activity and thus influence of UV exposure.

Since there is a decline in physical activity with age, and a potential for decreased synthesis of vitamin D in older adults, the physical activity status and 25(OH)D concentration of older adults should be explored to identify whether these two lifestyle-induced factors are: (1) low, and (2) linked.

Since Scotland has previously been reported as a vitamin D deficient nation across all ages (21-82 years old) (Zgaga et al., 2011), it is important to evaluate

the determinants in specific age groups such as older adults that may be at risk of lower vitamin D levels. In addition, this may provide insight into whether cause and effect relationships exist. Furthermore, physical activity levels remain low in Scotland (*The Scottish Health Survey*, 2017), presenting an avenue for behavioural change and thus a potentially modifiable route for intervention.

The primary aim of this study is to determine the baseline vitamin D status (25(OH)D concentration), and evaluate the physical activity levels of older adults who live in Scotland. The association between 25(OH)D concentration and physical activity categories will then be explored.

It is hypothesised that older adults will have a low baseline 25(OH)D concentration, which will inversely correlate with physical activity status. Due to the northern latitude and characteristic weather of Scotland, it is hypothesised that a seasonal influence on 25(OH)D concentrations will be observed.

4.3 Materials and methods

4.3.1 Participants

Ninety-eight male (n=39) and female (n=59) adults (aged 60-80 y) participated in this study (see section 3.2.1: age 67 ± 5 y, body mass 73.4 ± 14.9 kg, height 1.67 ± 0.10 m, BMI 26.1 ± 3.7 $kg \cdot m^{-2}$).

Participants were recruited on a rolling basis over the projected 12 month time frame for the VDS study to ensure that seasonal influence could be investigated. Based on the calculation determining that a sample size of n=85 was required to achieve statistical power, the aim was to recruit a minimum of 8 volunteers per month. However, this was not always possible due to the nature of volunteer-based recruitment, closure of the University over seasonal periods (such as Christmas/New Year), timing of participants' holidays and thus a lag time before inclusion in the study, and screening deeming six recruited volunteers as ineligible to participate. The number of participants recruited per month for the 12 month study time frame is displayed in **Figure 10**, with a mean of n=8 (8.17) per month. All participants attended the laboratory on two occasions: visit 1 and visit 2, as outlined below.

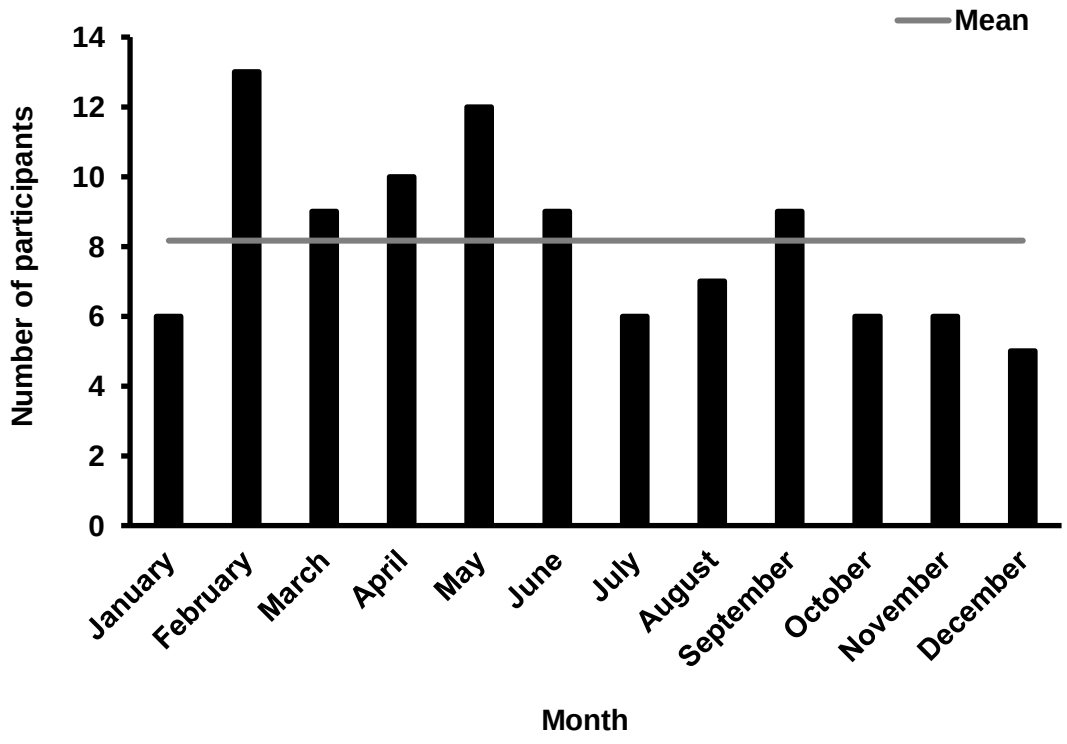

Figure 10 Monthly recruitment of participants (n=98) in the VDS study.

4.3.2 Visit 1

Written and verbal consent were obtained on arrival to the laboratory and all participants completed the screening questionnaires (see section 3.3). Anthropometric measurements and BP were then taken (see section 3.4 and 3.5). Each participant was issued with a wrist worn accelerometer (MotionWatch 8 (MW8), CamNTech Ltd and CamNTech Inc, UK) and instructed to wear it for 7 full days and nights to monitor their habitual activity. Seven days of objective monitoring has been identified as sufficient to reliably predict physical activity and sedentary behaviour of older adults (Hart, Swartz, Cashin, & Strath, 2011).

4.3.3 Visit 2

The second visit was scheduled 8-10 days after Visit 1 to allow 7 full days of data on the wrist worn accelerometer. Participants attended the laboratory between 7.30-9.30am following an overnight fast (10 h) and a fasted blood sample was collected via venepuncture (see section 3.7.1). Following blood sampling, the International Physical Activity Questionnaire (IPAQ) Long Form (Appendix 5) was completed.

4.3.4 Blood sample analysis

Whole blood was analysed for haematological parameters (see section 3.8). Serum 25(OH)D_3 concentration was determined by LC-MS/MS (see section 3.9.2).

4.3.5 Accelerometer data analysis

MotionWare Software was used for the set-up, download, and analysis of data captured with the accelerometer, and to quantify the intensity and duration of daily physical activity and night time activity (sleep duration). Recordings were for 7 days, 24 hours where possible. All activity counts were separated into 5 categories: sleep, sedentary (SED) time, light intensity physical activity (LPA), moderate intensity physical activity (MPA), and vigorous intensity physical

activity (VPA), using the cut-off points outlined specific for older adults (Falck, Landry, Brazendale, & Liu-Ambrose, 2017; Landry, Falck, Beets, & Liu-Ambrose, 2015).

4.3.6 IPAQ

The IPAQ (Appendix 5) was used to gather data on self-reported physical activity categories and sedentary time.

4.3.7 Statistical analysis

Data was analysed as outlined in section 3.11. A total of 98 participants (39 males, 59 females) were included in the analysis for all variables aside from the MW8 physical activity data analysis, which included 82 participants (34 males, 48 females) due to 16 corrupted data files.

4.4 Results

4.4.1 Participant characteristics

Full participant characteristics of all the participants and separated by sex are presented in **Table 5**. The post-hoc power calculation performed revealed that the study is statistically powered (*P*>0.95) based on a sample size of 98.

There was no difference in age but there was a difference in height and mass between sexes, with males taller and heavier compared to females. Although there was no statistically significant difference in BMI, there is a tendency for males to have a higher BMI compared to females, with a close to significant *P* value (*P*=0.051). Waist circumference and hip circumference were both significantly lower in females compared to males, as was the waist-to-hip ratio. Diastolic BP was lower in females compared to males.

Table 5 VDS study participant characteristics (n=98).

	All (n=98)	**Males (n=39)**	**Females (n=59)**	***P* value**
Age, y	67 ± 5	66 ± 4	68 ± 5	0.080
Height, m	1.67 ± 0.10	1.77 ± 0.07	1.60 ± 0.05 *	<0.001
Body mass, kg	73.4 ± 14.9	85.0 ± 13.2	65.8 ± 10.3 *	<0.001
BMI, kg·m^{-2}	26.1 ± 3.7	27.0 ± 3.4	25.5 ± 3.7	0.051
Waist circ., cm	84.4 ± 12.4	93.3 ± 9.0	78.5 ± 10.7 *	<0.001
Hip circ., cm	98.5 ± 8.9	101.2 ± 7.0	96.8 ± 9.7 *	0.011
Waist-to-hip ratio	0.86 ± 0.09	0.92 ± 0.06	0.81 ± 0.07 *	<0.001
Resting HR, bpm	67 ± 9	65 ± 8	67 ± 9	0.216
Systolic BP, mmHg	135 ± 18	134 ± 14	136 ± 20	0.522
Diastolic BP, mmHg	78 ± 9	81 ± 9	77 ± 9 *	0.040

Data presented at means ± SD. * denotes a significant difference from males.

4.4.2 Vitamin D status

We investigated baseline serum 25(OH)D_3 concentrations in 97 healthy older males and females who live in Scotland. The mean 25(OH)D_3 concentration of the population was 14.5 ± 3.9 ng·ml^{-1}, which is classified as deficient according to the current classifications (Holick, 2009). There was no difference in 25(OH)D_3 concentration between males and females, as shown in **Table 6**.

Table 6 Vitamin D status, as defined by systemic 25(OH)D_3 concentration, of participants (n=97).

	All (n=97)	**Males (n=39)**	**Females (n=58)**	***P* value**
25(OH)D_3, ng·ml^{-1}	14.5 ± 3.9	13.6 ± 3.7	15.0 ± 3.9	0.063
25(OH)D_3, nmol·L^{-1}	36.1 ± 9.7	33.9 ± 9.4	37.6 ± 9.6	

Data presented at means ± SD.

The most striking finding was the very high prevalence of severe deficiency (26 %) and deficiency (64 %), see **Table 7**. The remaining 10 % of the cohort were at risk of deficiency, while there were no individuals determined as having a sufficient vitamin D level.

Table 7 Categorical vitamin D status of participants (n=97).

Classification	**25(OH)D_3 Concentration (ng·ml^{-1})**	**N**	**%**
Severely deficient	<12	25	26
Deficient	12-20	63	64
Insufficient	20-30	10	10
Sufficient	>30	0	0

4.4.2.1 Season

The distribution of vitamin D classifications for each month is presented in **Figure 11**. The number of the individuals who were severely deficient or deficient changes in summer compared to winter months, with winter more strongly associated with lower 25(OH)D_3 concentrations.

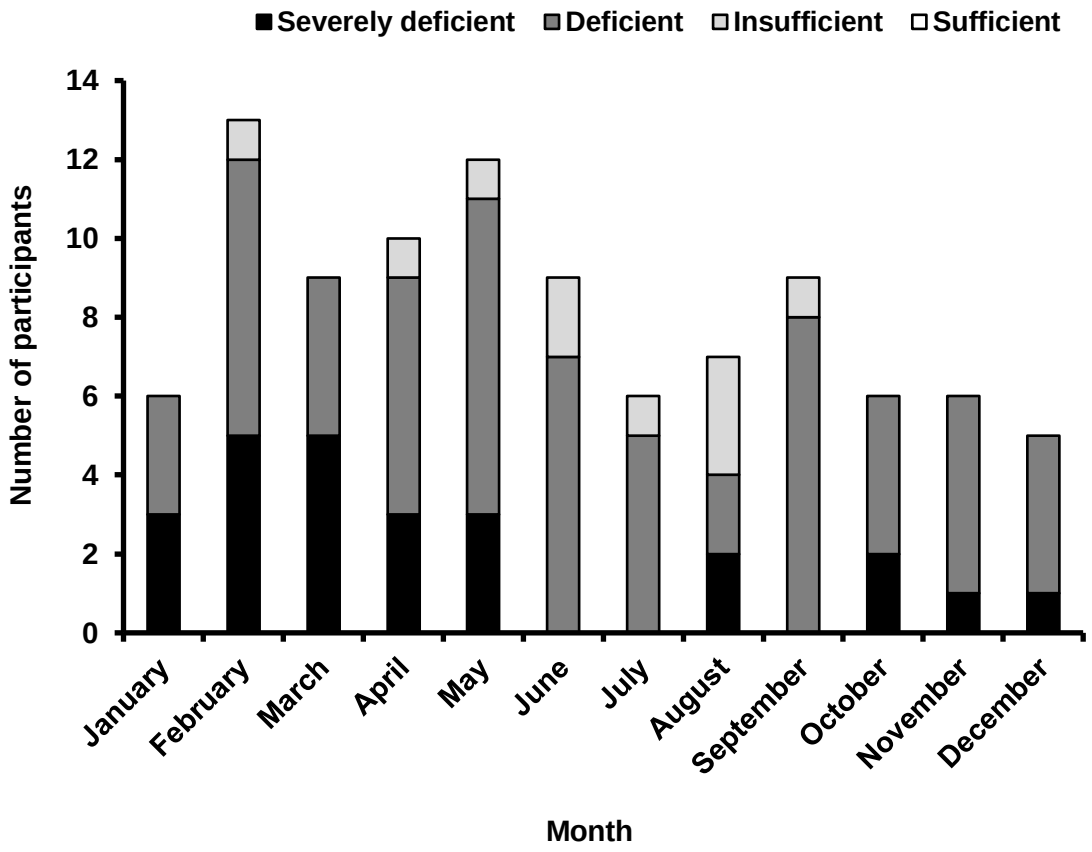

Figure 11 Monthly 25(OH)D_3 distribution in older adults in Scotland (n=97).

4.4.2.2 Characteristics

When investigating the difference in systemic 25(OH)D_3 concentration below and above the mean of participant characteristics variables (age, body mass, BMI, waist-to-hip ratio, resting HR, systolic and diastolic BP), there was no observed differences, as shown in **Table 8**.

Table 8 Descriptive analysis of 25(OH)D_3 concentrations in relation to participant characteristics (n=97).

Variable	Mean	N	25(OH)D_3 (ng·ml^{-1})		*P* value
		<,>	< mean	> mean	
Age, y	67	61,37	14.1 ± 3.8	15.0 ± 4.0	0.270
Body mass, kg	73.4	57,41	14.9 ± 3.9	13.8 ± 3.7	0.190
BMI, kg·m^{-2}	26.1	51,47	14.5 ± 3.6	14.4 ± 4.1	0.866
Waist circ., cm	84.4	47, 51	15.0 ± 4.0	14.0 ± 3.7	0.209
Hip circ., cm	98.5	47, 51	14.8 ± 3.9	14.1 ± 3.8	0.344
Waist-to-hip ratio	0.86	50,48	14.8 ± 3.9	14.1 ± 3.4	0.343
Resting HR, bpm	67	52,46	14.6 ± 3.9	14.3 ± 3.8	0.707
Systolic BP, mmHg	135	52,46	14.2 ± 4.0	14.7 ± 3.7	0.502
Diastolic BP, mmHg	78	47,51	15.1 ± 3.9	13.8 ± 3.7	0.087

Data presented as mean ± SD. BP, blood pressure. For each test variable, the sample was split into two groups: above and below the mean.

4.4.2.3 Age

Figure 12 shows there is no correlation relationship between baseline systemic serum 25(OH)D_3 concentration and age from 60 to 80 years (n=98, r=.071, *P*=0.486).

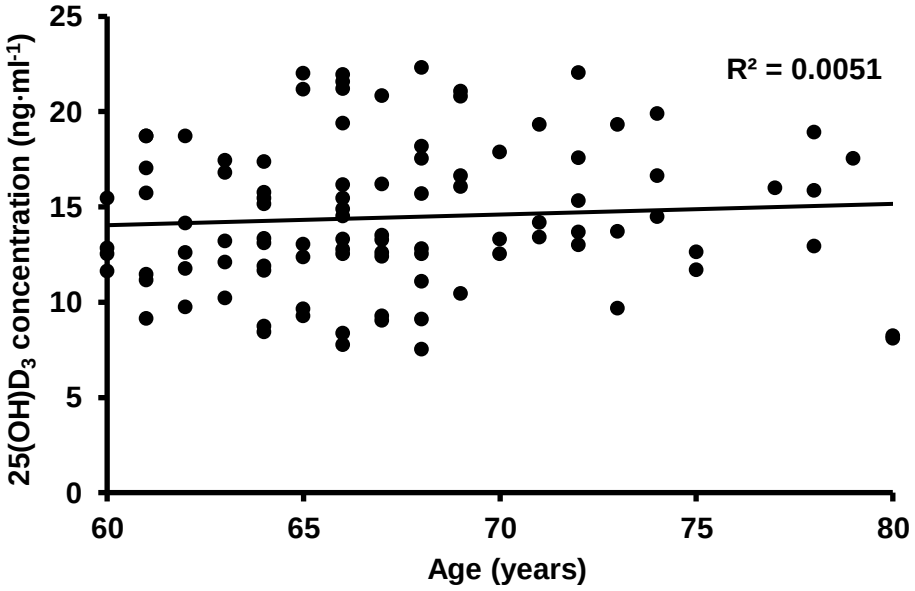

Figure 12 The relationship between baseline 25(OH)D_3 concentration and age (n=97). Each marker represents an individual participants' data.

4.4.3 Physical activity and sedentary behaviour

Of the 98 participants, 25.5 % did not meet the guidelines for moderate (150 min/week) and vigorous physical activity (75 min/week).

As shown in **Table 9**, there was a significant difference between self-reported and objectively measured sedentary time and LPA and MPA, both per day and per week. Participants were sedentary for 13 h per day compared to the 6 h self-reported sedentary time (sitting time + time spent in transportation) recorded in the IPAQ, concluding that participants underestimated their daily sedentary time by 7 h. Participants underestimated daily LPA by 1 h, and overestimated daily MPA by 37 min. VPA was not observed to be significantly different between the methods of assessment.

Table 9 The time spent sedentary or engaged in each category of physical activity per day and week.

Category	IPAQ (n=98)	MW8 (n=82)	P value
Per day			
Sedentary, h	6.30 ± 2.12	13.22 ± 1.56 *	<0.001
LPA, h	1.61 ± 1.47	2.61 ± 1.40 *	<0.001
MPA, h	1.26 ± 1.45	0.65 ± 0.43 *	<0.001
VPA, h	0.34 ± 0.65	0.21 ± 0.19	0.078
Per week			
Sedentary, h	44.09 ± 14.83	92.56 ± 10.93 *	<0.001
LPA, h	11.25 ± 10.28	18.26 ± 9.79 *	<0.001
MPA, h	8.81 ± 10.18	4.56 ± 3.04 *	<0.001
VPA, h	2.39 ± 4.56	1.44 ± 1.31	0.077

IPAQ, International Physical Activity Questionnaire (self-reported data); MW8, MotionWatch8 (objectively measured accelerometer data). Data presented as mean ± SD. * denotes a significant difference from the number of hours reported via the IPAQ.

Since the self-reported data either overestimates or underestimates time engaged in each category of activity/behaviour (excluding VPA), the physical activity data reported in the following results is the objectively measured data collected from the MW8 accelerometers.

The number of hours per week spent sleeping (6.71 ± 0.90 h per night), sedentary or engaged in LPA, MPA, and VPA, assessed via the MW8 accelerometers, is presented in **Figure 13**. There were no differences between males and females for time spent in any category of activity: sleep (*P*=0.429), SED (*P*=0.328), LPA (*P*=0.898), MPA (*P*=0.393), VPA (*P*=0.910).

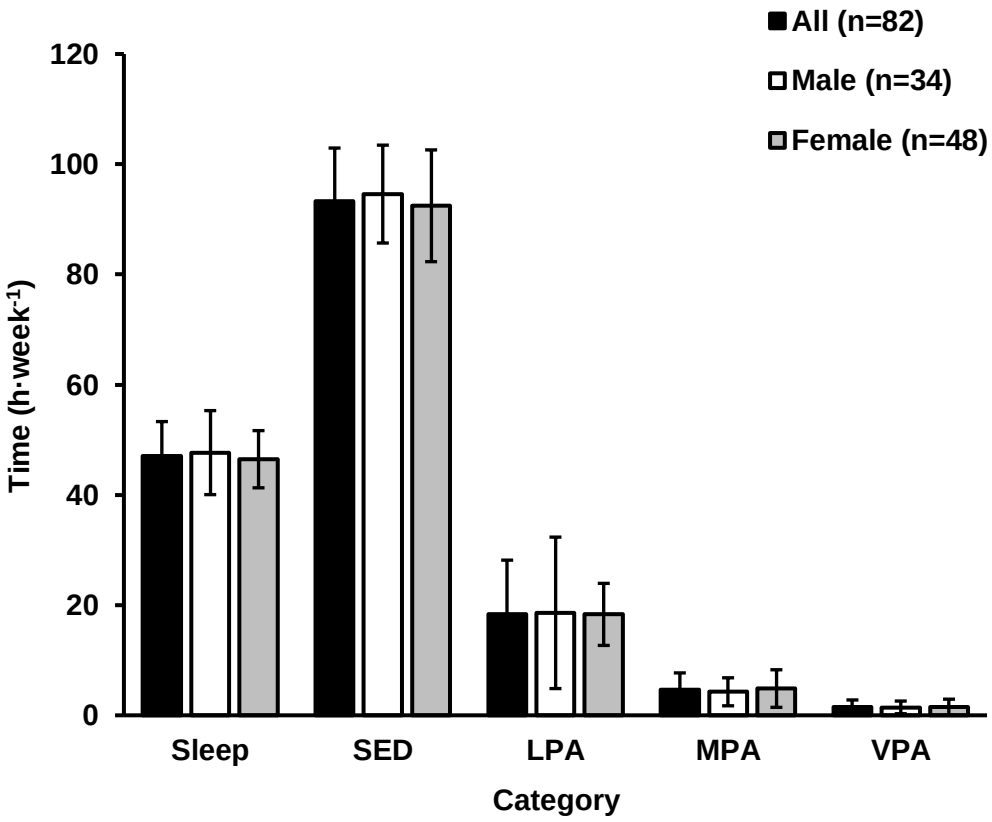

Figure 13 Hours spent in each category of daily behaviour for all participants (n=82) and males and females assessed via the MW8 accelerometer. Data presented a mean ± SD.

4.4.3.1 Age

Objective physical activity data collected from the MW8 accelerometer were used to analyse the relationship between age and physical activity levels. The results of the single linear regression models (**Figure 14**) indicated that age was a predictor of time spent engaged in VPA per week, explaining 10 % of the variance (R^2=.100, F(1,80)=8.925, β=-.317, *P*=0.004). However, sedentary (R^2=.015, F(1,80)=1.192, β=.121, *P*=0.278), LPA (R^2=.035, F(1,80)=2.936, β=-.188, *P*=0.090), and MPA (R^2=.044, F(1,80)=3.704, β=-.210, *P*=0.058) were not correlated with age.

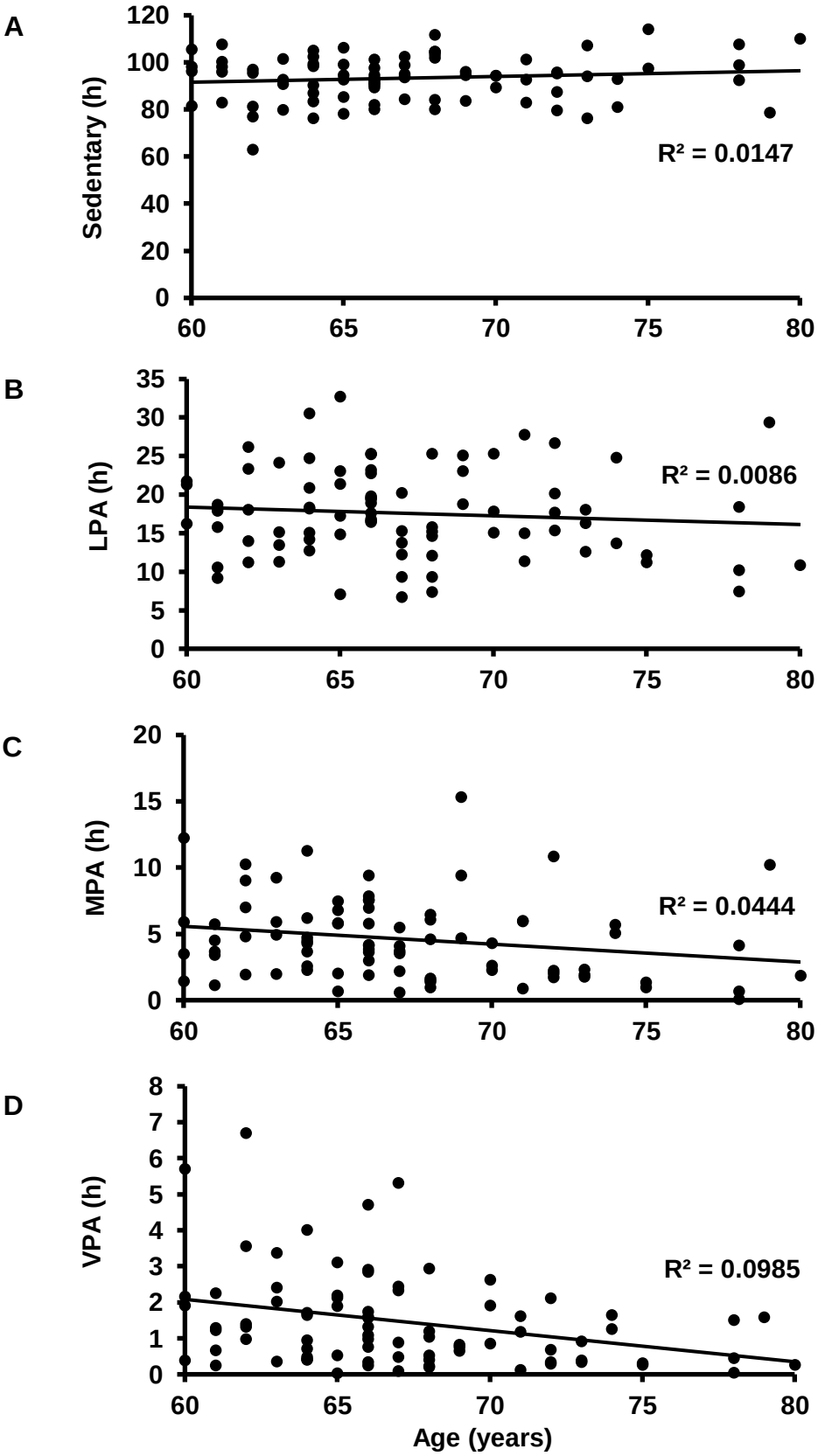

Figure 14 Time spent sedentary (**A**) or engaged in LPA (**B**), MPA (**C**), and VPA (**D**) correlated with age (n=82). Each marker represents an individual participants' data.

4.4.4 Vitamin D and physical activity

When comparing the 25(OH)D_3 concentration below the mean to above the mean of each physical activity category, it was found that there were no differences (shown in **Table 10**).

Table 10 Descriptive analysis of 25(OH)D_3 concentrations in relation to sleep and daily activity characteristics (n=82).

Variable	N	25(OH)D_3 concentration (ng·ml^{-1})		*P* value
	<,>	< mean	> mean	
Sleep, h	38,44	14.2 ± 4.2	14.5 ± 3.6	0.669
SED, h	37,45	14.2 ± 3.8	14.5 ± 3.9	0.721
LPA, h	49,33	14.3 ± 3.8	14.4 ± 3.9	0.879
MPA, h	47,35	14.6 ± 3.6	14.1 ± 4.2	0.562
VPA, h	50,32	14.3 ± 3.8	14.4 ± 3.9	0.913

Data presented as mean ± SD. For each test variable, the sample was split into two groups: above and below the mean. SED, sedentary; LPA, light physical activity; MPA, moderate physical activity; VPA, vigorous physical activity.

A regression model was implemented to analyse whether the five physical activity categories predicted 25(OH)D_3 concentration. A non-significant regression equation was found ($F(5,81)=0.817$, $R^2=0.051$, $P=0.542$). The correlation analysis for the five physical activity categories and 25(OH)D_3 concentration is shown in **Table 11**. There appears to be an inverse correlation between systemic 25(OH)D_3 concentration and the number of hours per week spent engaged in VPA ($r=-0.203$, $P=0.034$) as shown in **Figure 15**. However, there was no association with the other categories.

Table 11 Correlation analysis of 25(OH)D_3 concentration with sleeping, sedentary and physical activity behaviour (n=82).

	Correlation with 25(OH)D_3 concentration	
	r	***P* value**
Sleep, h	-.036	0.374
SED, h	.074	0.254
LPA, h	-.045	0.344
MPA, h	-.073	0.257
VPA, h	-.203	0.034

SED, sedentary; LPA, light physical activity; MPA, moderate physical activity; VPA, vigorous physical activity.

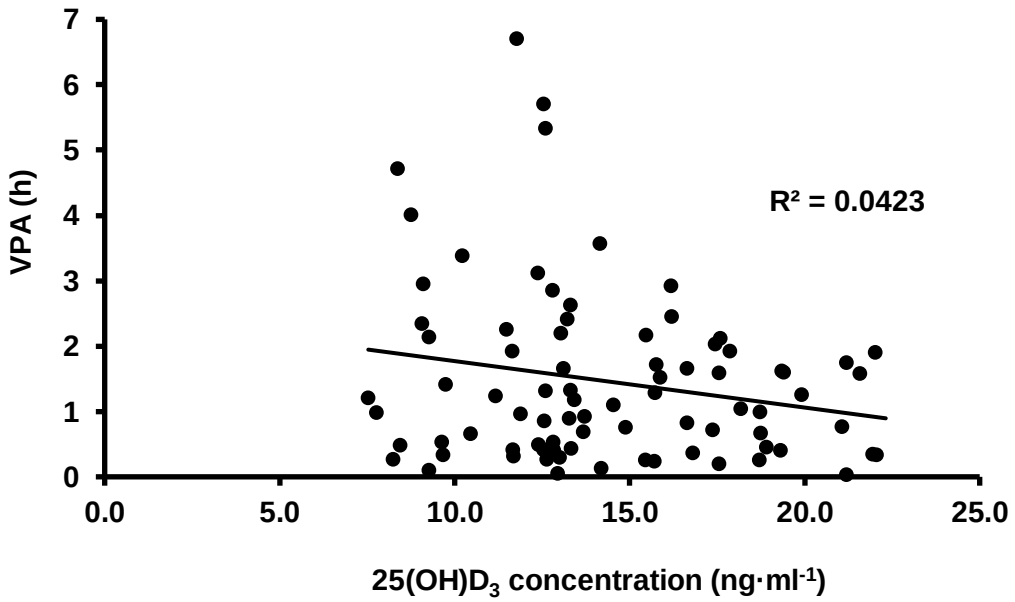

Figure 15 Association between 25(OH)D_3 concentration and VPA (n=82).

4.5 Discussion

The results of this study show that there is a high level of severe deficiency (26 %) and deficiency (64 %), with only 10 % defined as insufficient in terms of vitamin D serum concentration in the current participant population. Importantly, no individuals were classified as having a sufficient vitamin D status. The mean $25(OH)D_3$ concentration was 14.5 $ng \cdot ml^{-1}$, which is similar to published findings in Scotland by Zgaga et al. (2011) (14.4 $ng \cdot ml^{-1}$) and Weiss et al. (2016) (12.7 $ng \cdot ml^{-1}$). This consolidates the finding that Scotland is largely a vitamin D deficient nation. Our data shows a higher percentage of deficiency compared to the other studies, however the eligibility criteria only included those that did not consume vitamin D supplements, therefore negating a proportion of the population who may alter the distribution of the sample population in each category of vitamin D status. Moreover, the current study only included older adults aged 60-80 years, whereas the previous mentioned studies included populations with a broader age span. Although, in line with our findings, there are no reports that age affects vitamin D status.

In the current study, it was found that vitamin D status did not differ between males (13.6 ± 3.7 $ng \cdot ml^{-1}$) and females (15.0 ± 3.9 $ng \cdot ml^{-1}$) in an older adult cohort. However, a previous study that investigated the relationship between sex and vitamin D status reported that females have lower $25(OH)D_3$ concentrations compared to males (14.5 ± 10.9 vs. 15.9 ± 9.5 $ng \cdot ml^{-1}$) (Verdoia et al., 2015). The age of the sample population (females: 71 ± 11 y, males: 66 ± 12 y) is similar to the current study (females: 68 ± 5 y, males: 66 ± 4 y), and although Verdoia et al. (2015) reported a difference in age between male and females, when adjusting for age the difference between sexes remained. In a study in a non-Caucasian population, males were consistently found to have lower $25(OH)D_3$ concentrations compared to females (11.1 ± 9.2 vs. 12.8 ± 10.5 $ng \cdot ml^{-1}$) (Sanghera, Sapkota, Aston, & Blackett, 2017). In another study, obese males had a lower $25(OH)D_3$ concentration accompanied with a higher rate of vitamin D deficiency compared to obese females (20.0 ± 8.8 vs. 21.4 ± 9.0 $ng \cdot ml^{-1}$) (Johnson et al., 2012). These inconsistent findings may be a result of sample size, since the variance in the measured concentrations are large in comparison to the current study, or attributed to the population characteristics,

such as body mass, age, and race. Investigations and reports on sex differences in vitamin D status should be specific to the characteristics of the cohort.

When investigating the sample population as a whole, 25.5 % of the older adults did not meet the current physical activity guidelines set by the UK Government ("Physical activity guidelines for adults (19-64 years)," 2011). This finding is reported by numerous studies and has been identified as an issue in Scotland and globally (Hallal et al., 2012; *The Scottish Health Survey*, 2017). However, the percentage that is physically inactive (25.5 %) is lower than that reported by The Scottish Healthy Survey in 2016 (36 %). This may be explained by the volunteering nature of a research study: members of the public may be more inclined to volunteer for a study involving a physical activity assessment if they are currently active.

An interesting observation from the data was that compared to objective, accelerometer-derived measures, participants' self-reported activity measures via the IPAQ led to a substantial under or overestimation of time spent sedentary or physically active. This has previously been reported in males and females aged 18-73 years, however their data suggested a stronger correlation between methods for sedentary activity and a weak correlation for indices of physical activity (Celis-Morales et al., 2012). The current data set shows that individuals drastically under-estimate sedentary behaviour by as much as 7 hours per day, and over-estimate physical activity but to a lesser degree. Current epidemiological data shows that older adults are the most sedentary segment of the population, spending on average 70 % of their waking hours sedentary (Matthews et al., 2008; Stamatakis, Davis, Stathi, & Hamer, 2012). Sedentary behaviour refers to activities that do not increase energy expenditure substantially above the resting level and thus includes activities such as sitting and lying down (Pate, O'Neill, & Lobelo, 2008). According to the current cohort of older adults, 76 % of waking hours were spent sedentary (when assessed objectively), therefore similar to the larger scale epidemiological studies conducted in the US. The duration that participants believed they were sedentary per day was drastically underestimated by 7 hours, therefore perception of the time spent inactive

appears to be distorted. This may be due to social desirability whereby being physically active is a socially desirable behaviour and thus people may over-report their activity inducing bias in order to gain social approval (S. A. Adams et al., 2005; Motl, McAuley, & DiStefano, 2005).

Although studies have investigated the feasibility of interventions to reduce sedentary behaviour in older adults (Gardiner, Eakin, Healy, & Owen, 2011), if the general perception of time spent sedentary is distorted and vastly underestimated, there may not be awareness of the requirement for these physical or behavioural change interventions. Therefore, the problem may persist. This is an important finding, which may contribute to public health in light of reports that sedentary behaviour has a deleterious effect on a person's health, independent of the amount of physical activity (Tremblay, Colley, Saunders, Healy, & Owen, 2010).

Interestingly, LPA was underestimated by 1 hour and MPA was overestimated by 37 minutes daily, which then accumulates over the course of a week (7 hours and 4.25 hours, respectively). However, VPA was reported as similar via both methods of assessment. It should be noted that the objective measurement includes 24 hours' worth of activity accounted for and disseminated into a category, whereas the questionnaire asks specific questions with regards to sedentary time and time spent engaged in each physical activity category, therefore the IPAQ does not account for all hours of the day. As a result there may be gaps and sedentary or activity time unaccounted for throughout a day. This may explain the difference in sedentary time, however the under and overestimated of light and moderate activity, respectively, may be attributed to perception of intensity of an activity. The method of assessment appears to be the source of the controversial results regarding physical activity levels. There are numerous methods to assess physical activity, both subjective and objective (Ainsworth et al., 2000; Helmerhorst, Brage, Warren, Besson, & Ekelund, 2012), with the general conclusion that self-report methods (questionnaires) may provide an approximation of physical activity levels, however they may not determine whether an individual is meeting the physical activity guidelines (Loney, Standage, Thompson, Sebire, & Cumming, 2011). Although the IPAQ is the

most commonly used subjective tool for evaluating physical activity (Rangul, Holmen, Kurtze, Cuypers, & Midthjell, 2008), largely due to its cost effectiveness and relative ease of use, it may underestimate physical activity due to the greater level of variability. This may lead to false-negative and inconsistent results of absolute physical activity levels and the association with other factors/variables. The low level of agreement between the methods of assessment (Monyeki, Moss, Kemper, & Twisk, 2018) should be taken into consideration when digesting the results, with objective methods used where possible.

Upon evaluation of the physical activity data, it was observed that there was no influence of sex on physical activity or sedentary behaviour. In addition, there was no impact of age on sedentary time, or the lower intensity categories of physical activity. Interestingly, the data revealed there to be a decline in the number of hours spent engaged in VPA with older age between 60-80 years. This may be attributed to the decline in functional and physical capacity due to the ageing process (Milanovic et al., 2013). Thereby disrupting the ability to carry out strenuous physical activities such as exercise, which constitutes the VPA as assessed and reported from the objectively measured accelerometer data.

The current study also reports that age and sex did not influence baseline 25(OH)D_3 concentration of the cohort. These data suggest that it is perhaps environmental or behavioural factors, such as UVB exposure, diet and physical activity status that predict vitamin D status. Although the current study did not investigate UVB exposure and diet, the primary aim of the investigation was to evaluate the physical activity status of older adults, and to explore whether there is an association between 25(OH)D concentration and physical activity categories including sedentary behaviour. The main finding was that the time spent engaged in vigorous activity was a predictor of systemic 25(OH)D_3 concentration. However, this was an inverse correlation, with a decline in 25(OH)D_3 with increased hours per week of vigorous activity. Although this finding may appear to contradict previously published data reporting higher levels of activity are linked to higher 25(OH)D_3 concentrations (Jacques et al., 1997; Lucas et al., 2005; Scragg & Camargo, 2008; Scragg et al., 1992;

Scragg et al., 1995), the time engaged in vigorous activity does not represent a measure of overall physical activity. Therefore, the current finding is specific to vigorous activity and is not wholly comparable. Furthermore, it may be that vigorous activity, such as exercise and sports, are performed indoors and thus reduces UVB exposure. Although, it has been reported that physical activity often occurs outdoors and thus increases UVB exposure and subsequently 25(OH)D concentrations (Kluczynski et al., 2011). However, this association is not supported by all reports, with the nature of activity reported not to influence UVB exposure (Wanner et al., 2015). Therefore, it may be that factors other than sun exposure may be responsible for a higher vitamin D status, requiring further investigation.

Vitamin D status is known to be influenced by season due to the alterations in UVB radiation throughout the calendar year. Specifically in Scotland, there is impaired UVB exposure due to the high latitude of the country (Gies et al., 2004), reducing the skin synthesis source that contributes to vitamin D status. The current data observed a seasonal influence on 25(OH)D_3 with concentrations lower in winter compared to summer months, similar to other investigations conducted in Scotland and the UK (Hypponen & Power, 2007; Kelly et al., 2015; Zgaga et al., 2011). Therefore, since season is a determinant of vitamin D status and physical activity status (Tucker & Gilliland, 2007) independently, it may follow that a relationship occurs between the three factors, complicating the associations further.

In conclusion, there is an association between VPA and 25(OH)D concentration, however the link is not observed with lower intensities or sedentary time. Contrary to the hypothesis, there was no influence of age on 25(OH)D concentration within an older adult cohort (60-80 years). In order to investigate whether age influences 25(OH)D status and draw any conclusions, a broader age span must be investigated.

Chapter 5: Influence of Age and CRF on 25-hydroxyvitamin D Concentration and VDR Expression

5.1 Abstract

It is well established that vitamin D plays a key role in calcium absorption and the prevention of ill-health, with blood 25(OH)D concentration used as the identifier of a person's vitamin D status. However, the literature identifies the VDR as the main mediator of the vitamin D system. Therefore, the aim of this study is to investigate whether baseline 25(OH)D concentration is linked to cellular VDR expression, and subsequently whether these important metabolites are associated with age and CRF (a reliable surrogate for physical fitness).

Thirty-five male adults participated in this study (age 44 ± 17 y, body mass 82.5 ± 11.4 kg, height 1.79 ± 0.08 m, BMI 25.7 ± 3.1 $kg \cdot m^{-2}$). The participants were separated into three groups based on their age: young (18-30 y; n=12), young/mid (31-45 y; n=11), and older adults (60-75 y; n=12). CRF and peak power were assessed via a cycling $\dot{V}O_{2peak}$ test, and baseline blood samples were collected and analysed for serum 25(OH)D concentration (LC-MS/MS) and T cell VDR expression (flow cytometry).

As predicted and in line with the literature, CRF and peak power declined with age (P<0.001). Baseline 25(OH)D concentration was not influenced by age (P=0.774) or CRF (P=0.624). A correlation analysis was used, which reported no relationship between the two metabolites (P=0.793). Moreover, VDR expression declined with age (P=0.015), and a higher CRF was linked to a greater expression of VDR (P=0.048).

The results indicate that intracellular VDR expression appears to be susceptible to influence by age and CRF, whereas the commonly reported measure of vitamin D metabolism and status: 25(OH)D concentration, is not.

5.2 Introduction

Vitamin D_3 is an important secosteroid hormone derived from direct sunlight exposure (UVB radiation) and food sources (Holick & Chen, 2008), playing an important role in most tissues and cells on a genome and transcription-wide level (M. J. Campbell, 2014; Carlberg, 2014). A low vitamin D status, assessed as 25-hydroxyvitamin D (25(OH)D), is associated with the development and prevalence of numerous age-related chronic diseases and conditions, as discussed in the literature review (Chapter 2). Observational studies have indicated that higher levels of physical activity are associated with higher circulating 25(OH)D concentrations, even after adjusting for UVB exposure (Kluczynski et al., 2011; Scragg & Camargo, 2008; Scragg et al., 1992). This suggests that independent of sun exposure, physical activity and exercise may directly influence 25(OH)D concentrations. This relationship between CRF and vitamin D status has been previously explored in a population living in the US, reporting that 25(OH)D concentrations are positively associated with CRF in men (Farrell, Cleaver, & Willis, 2011) and women (Farrell & Willis, 2012). However, since the UVB exposure is different according to latitude and thus country, this relationship may not stand in Scotland: a country with a remarkably low annual UVB exposure level. Furthermore, the effect of age on 25(OH)D concentration was not explored.

Although 25-hydroxyvitamin D_3 (25(OH)D_3), is used as the biomarker for vitamin D status, it is the biologically active form of vitamin D, 1,25$(OH)_2D_3$, and the VDR that provide the functional platform for vitamin D metabolism function (Haussler et al., 1998). Therefore, instead of debating the optimal systemic concentration/threshold level of 25(OH)D_3 and 1,25$(OH)_2D_3$, it can be suggested that due to the human variance in vitamin D intake and predictors of vitamin D status (age, BMI, season, UVB exposure, location, training status) human individuals should be tested for vitamin D responsiveness. Since the action of vitamin D is through the transcription factor VDR (Carlberg & Molnar, 2012), expression changes of VDR and target genes can serve as a direct measure for the functional outcome of vitamin D metabolism. It has been found that following a 24 h stimulation with 1,25$(OH)_2D_3$, more than 1,000 genes alter their mRNA expression (Heikkinen et al., 2011; Hossein-nezhad, Spira, &

Holick, 2013; Nurminen et al., 2015; Ramagopalan et al., 2010). This response relies on the ligand binding to the VDR, and subsequent binding of the ligand-VDR-RXR complex to the VDRE on the DNA strand. The availability of unbound VDR to receive $1,25(OH)_2D_3$ is thus imperative for this process to be functional and efficient. Therefore, focus on measuring baseline VDR and in response to stimuli may provide greater insight into the functional capacity of vitamin D metabolism, and the potential health benefits that can then be induced through vitamin D.

It has previously been reported that VDR protein expression decreases in human skeletal muscle tissue as age advances (Bischoff-Ferrari, Borchers, et al., 2004). Whereas, in human systemic PBMCs it has been reported that there is no influence of age on baseline VDR levels, however this mixed sex cohort was limited to 50 years+ (Coleman et al., 2016). In addition, the measure of VDR levels reported differs between studies, whereby Bischoff-Ferrari et al. (2004) measured VDR protein levels, whereas Coleman et al. (2016) measured VDR mRNA expression. Pre- and post-transcriptional expression and regulation of receptors and proteins can be affected by age. In the current investigation, the age span extends from 18-45 and 60-75 years to evaluate whether VDR expression in PBMCs is lower in older adults compared to younger populations, in healthy active humans.

The aim of this investigation was to determine whether age and the CRF of a healthy male population have an influence on vitamin D status and T cell VDR expression. A secondary aim is to determine whether there is a relationship between 25(OH)D and VDR at baseline.

5.3 Materials and methods

5.3.1 Participants

Thirty-five male adults participated in this study (see section 3.2.2: age 44 ± 17 y, body mass 82.5 ± 11.4 kg, height 1.79 ± 0.08 m, BMI 25.7 ± 3.1 $kg \cdot m^{-2}$). The participants were separated into three groups based on their age (see section 3.2.2): young (18-30 y; n=12), young/mid (31-45 y; n=11), and older adults (60-75 y; n=12). Following consent and screening (see section 3.3), anthropometric measurements and BP were taken (see section 3.4 and 3.5).

5.3.2 Blood sampling and analysis

Baseline blood samples were collected via cannulation (see section 3.7.2), the number of participants sampled per month is displayed in **Figure 16**. Serum $25(OH)D_3$ concentrations were quantified using liquid chromatography mass spectrometry (LC-MS/MS) (see section 3.9.2). Baseline T cell VDR expression was analysed via flow cytometry using PBMCs isolated from EDTA treated whole blood (see section 3.10).

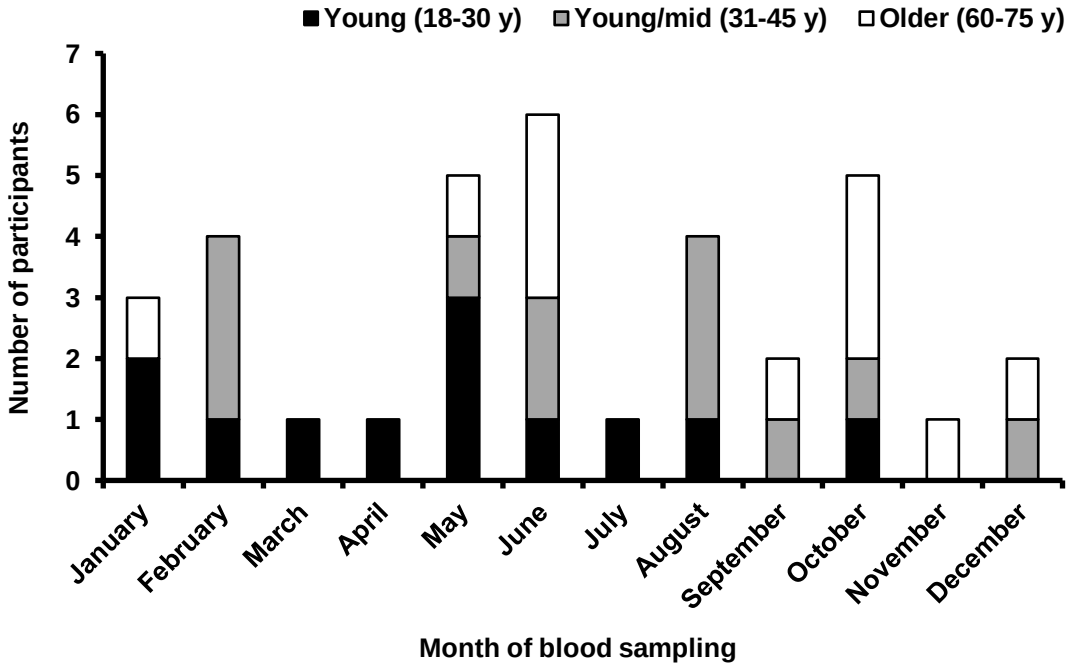

Figure 16 The number of participants sampled per month for the cohort (n=35).

5.3.3 Cardiorespiratory fitness assessment

All participants completed a peak oxygen uptake ($\dot{V}O_{2peak}$) test (see section 3.6) on a cycle ergometer as an assessment of CRF and peak power.

5.3.4 Statistical analysis

Data was analysed as outlined in section 3.11.

5.4 Results

5.4.1 Participant characteristics

Table 12 describes the characteristics of all the participants (n=35) within the study, and of each age group. One participant withdrew their data. Adherence to trials was reported as 100%. The post-hoc power calculation performed revealed that the study is statistically powered (*P*>0.95) based on a sample size of 35 with an effect size of 0.732 and Eta^2 of 0.349.

There were no significant differences between age groups for baseline characteristics except for diastolic BP (*F*(2,32)=7.447, *P*=0.002), whereby the young/mid group had a lower diastolic BP compared to the older group (*P*=0.001). Since there were no differences in body composition, as measured by body mass, BMI calculation, and waist and hip circumference, the groups can be appropriately compared in the analysis.

Table 12 ExVD study participant characteristics (n=35).

	All (n=35)	Young (n=12)	Young/mid (n=11)	Older (n=12)	df	F	*P* value	*P* value	*P* value	*P* value
								Y v Y/M	Y v O	Y/M v O
Age, y	44 ± 17	27 ± 2	38 ± 6 *	67 ± 4 †, ‡	2, 32	284.735	<0.001	<0.001	<0.001	<0.001
Height, m	1.79 ± 0.08	1.81 ± 0.06	1.77 ± 0.09	1.79 ± 0.09	2, 32	0.753	0.479	-	-	-
Body mass, kg	82.5 ± 11.4	83.8 ± 13.0	80.9 ± 11.0	82.7 ± 10.8	2, 32	0.180	0.836	-	-	-
BMI, kg·m^{-2}	25.7 ± 3.1	25.6 ± 3.9	25.8 ± 2.5	25.9 ± 2.9	2, 32	0.030	0.970	-	-	-
Waist circumference, cm	89 ± 9	87 ± 10	87 ± 7	93 ± 10	2, 32	1.923	0.163	-	-	-
Hip circumference, cm	95 ± 7	93 ± 9	94 ± 5	98 ± 6	2, 32	1.971	0.156	-	-	-
Waist-to-hip ratio	0.94 ± 0.06	0.93 ± 0.04	0.93 ± 0.05	0.95 ± 0.08	2, 32	0.320	0.728	-	-	-
Resting HR, beats·min^{-1}	59 ± 9	58 ± 11	58 ± 5	61 ± 9	2, 32	0.493	0.615	-	-	-
Systolic BP, mmHg	122 ± 11	124 ± 10	119 ± 12	122 ± 11	2, 32	0.739	0.486	-	-	-
Diastolic BP, mmHg	73 ± 8	73 ± 7	67 ± 5	78 ± 8 ‡	2, 32	7.447	0.002	0.129	0.154	0.001

All data is presented as mean ± SD. * represents a significant difference between young and young/mid (P<0.001); † represents a significant difference between young and older (P<0.001); ‡ represents a significant difference between young/mid and older (P<0.01).

5.4.2 Baseline 25-hydroxyvitamin D concentration and age

The vitamin D status, determined by serum 25(OH)D concentration ($ng \cdot ml^{-1}$, $nmol \cdot L^{-1}$), of participants is shown in **Table 13**. There was no difference in vitamin D status between age groups ($F(2,34)=0.258$, $P=0.774$). Collectively, participants as a whole and in each age group, are defined as vitamin D deficient.

Table 13 Systemic 25(OH)D concentration of participants (n=35).

	All (n=35)	**Young (n=12)**	**Young/mid (n=11)**	**Older (n=12)**
25(OH)D, $ng \cdot ml^{-1}$	15.7 ± 6.4	15.1 ± 7.0	15.2 ± 5.6	16.8 ± 6.7
25(OH)D, $nmol \cdot L^{-1}$	39.3 ± 15.9	37.7 ± 17.6	38.1 ± 14.0	42.0 ± 16.8

Data shown as mean ± SD.

Upon inspection, using the categories defined in the literature review (Chapter 2), 40% of participants were severely deficient, 29% were deficient, and 31% were defined as insufficient (shown in **Table 14**). None of the participants had a vitamin D status in the sufficient category.

Table 14 Categorical vitamin D status of participants (n=35).

Category	**Concentration ($ng \cdot ml^{-1}$)**	**N**	**%**
Severely deficient	<12	14	40
Deficient	12-20	10	29
Insufficient	20-30	11	31
Sufficient	>30	0	0

5.4.3 Baseline VDR expression and age

As shown in **Figure 17**, age was found to influence baseline VDR expression in circulating T lymphocytes (F(2,34)=4.763, *P*=0.015), with older adults expressing less intracellular VDR compared to the younger cohort (*P*=0.014). There was no difference in VDR expression between the young/mid cohort and young (*P*=0.656) or older adults (*P*=0.113).

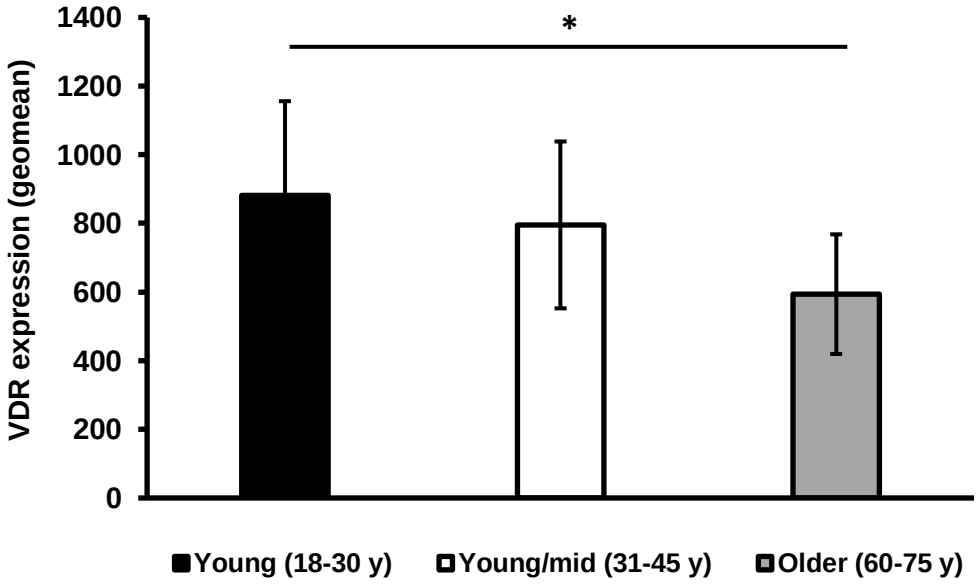

Figure 17 VDR expression in circulating CD3+ T lymphocytes for each age group: young (n=12), young/mid (n=11), and older (n=12). Data presented as mean ± SD. * denotes a significant difference between the young and older cohort (*P*≤0.05).

Figure 18 shows the correlations between baseline VDR expression of each T cell subset against age (n=35). There is a significant inverse relationship between VDR expression and age for CD3+ T cells (**Figure 18 A**; r=-0.39, *P*=0.022), CD4+ T cells (**Figure 18 B**; r=-0.39, *P*=0.020), and CD8+ T cells (**Figure 18 C**; r=-0.38, *P*=0.025). As age advances, there is a decline in density of the receptor within the cells.

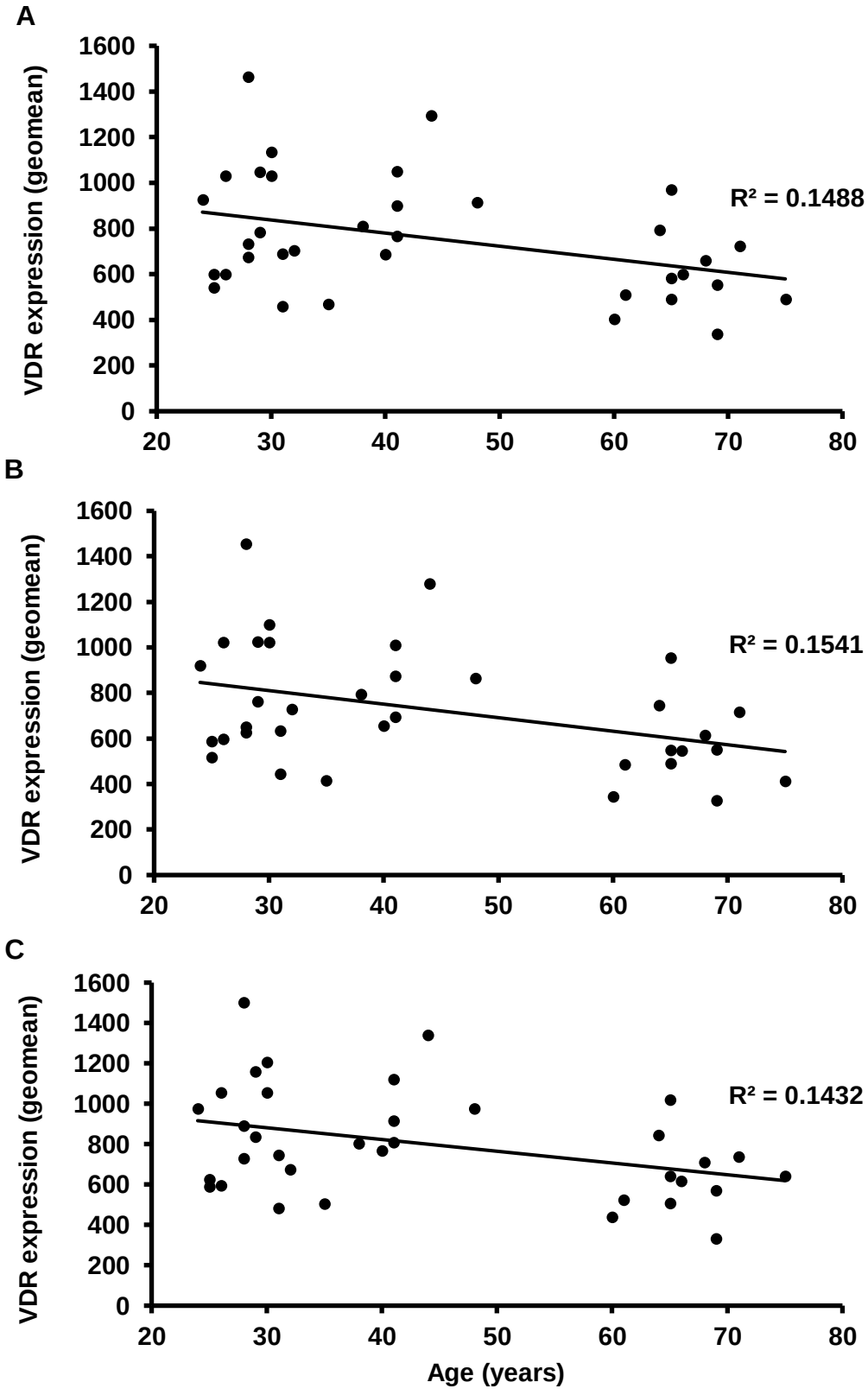

Figure 18 Correlation between intracellular VDR expression and age in (**A**) CD3+ T cells (P<0.05), (**B**) CD4+ T cells (P<0.05), (**C**) CD8+ T cells (P<0.05). Each marker represents an individual participant's data.

5.4.4 25-hydroxyvitamin D concentration and VDR expression

As shown in **Figure 19** there was no association between baseline 25(OH)D_3 concentration and T cell VDR expression (n=35, r=0.046, *P*=0.793).

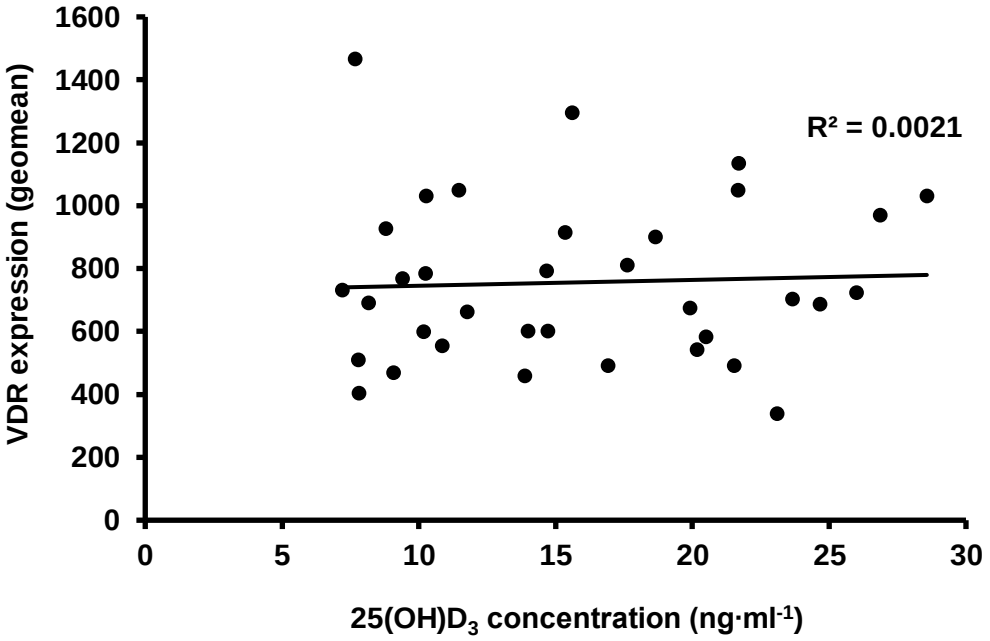

Figure 19 Correlation between baseline 25(OH)D_3 concentration (ng·ml^{-1}) and CD3+ T cell VDR expression (geometric mean) at baseline (n=35). Each marker represents an individual participants' data.

5.4.5 Influence of CRF

The main outcome variables of the $\dot{V}O_{2peak}$ test for all participants and each age group are shown in **Table 15**. Maximum HR (HR_{max}) recorded during the test differed between age groups ($F(2,31)=13.041$, $P<0.001$), with the older group reaching a lower HR_{max} compared to the young group ($P<0.001$) and the young/mid group ($P=0.009$). The respiratory exchange ratio (RER) achieved during the test was similar between groups ($F(2,32)=0.2309$, $P=0.796$), with a mean of 1.19 ± 0.05 (n=35), indicating participants performed to maximum capacity (Edvardsen, Hem, & Anderssen, 2014).

Maximal $\dot{V}O_{2peak}$, expressed as both absolute and relative to body mass values, differed between age groups ($F(2,32)=12.877$, $P<0.001$; $F(2,32)=10.894$, $P<0.001$), whereby the older group had a lower CRF compared to the young ($P<0.001$; $P=0.001$) and young/mid groups ($P=0.001$; $P=0.002$).

Similarly, for absolute and relative peak power, there was a significant difference between age groups ($F(2,32)=9.335$, $P=0.001$; $F(2,32)=6.200$, $P=0.005$), whereby the older group has a lower peak power output compared to the young ($P=0.004$; $P=0.030$) and young/mid groups ($P=0.001$; $P=0.006$). However, there were no significant differences between the young group and young/mid group for any of the maximal data variables.

Table 15 Cardiorespiratory fitness (CRF) outcomes (n=35).

	All (n=35)	Young (n=12)	Young/mid (n=11)	Older (n=12)	*df*	*F*	*P* value	*P* value Y v Y/M	*P* value Y v O	*P* value Y/M v O
Max RER	1.19 ± 0.05	1.20 ± 0.04	1.20 ± 0.06	1.19 ± 0.05	2,33	0.230	0.796	-	-	-
HR_{max}, beats·min^{-1}	175 ± 19	189 ± 10	178 ± 8	160 ± 20 *, †	2,33	13.041	<0.001	0.182	<0.001	0.009
Absolute $V\dot{O}_{2peak}$, L·min^{-1}	3.12 ± 0.64	3.49 ± 0.45	3.33 ± 0.63	2.54 ± 0.37 *, †	2,34	12.877	<0.001	0.706	<0.001	0.001
Relative $V\dot{O}_{2peak}$, ml·kg^{-1}·min^{-1}	38.0 ± 8.2	42.1 ± 7.9	41.4 ± 7.2	30.9 ± 3.8 *, †	2,34	10.894	<0.001	0.966	0.001	0.002
Absolute peak power, W	261 ± 60	282 ± 49	293 ± 61	212 ± 34 *, †	2,34	9.335	0.001	0.852	0.004	0.001
Relative peak power, W·kg^{-1}	3.23 ± 0.88	3.45 ± 0.89	3.67 ± 0.85	2.61 ± 0.52 *, †	2,34	6.200	0.005	0.772	0.030	0.006

All data is presented as mean ± SD. RER, respiratory exchange ratio; Y, young; Y/M, young/mid; O, older. ***** represents a significant difference between young and older; **†** represents a significant difference between young/mid and older (P≤0.05).

5.4.5.1 25-hydroxyvitamin D concentration and CRF

There is no correlation between baseline 25(OH)D_3 concentration and peak oxygen uptake (r=0.086, *P*=0.624), as shown in **Figure 20**.

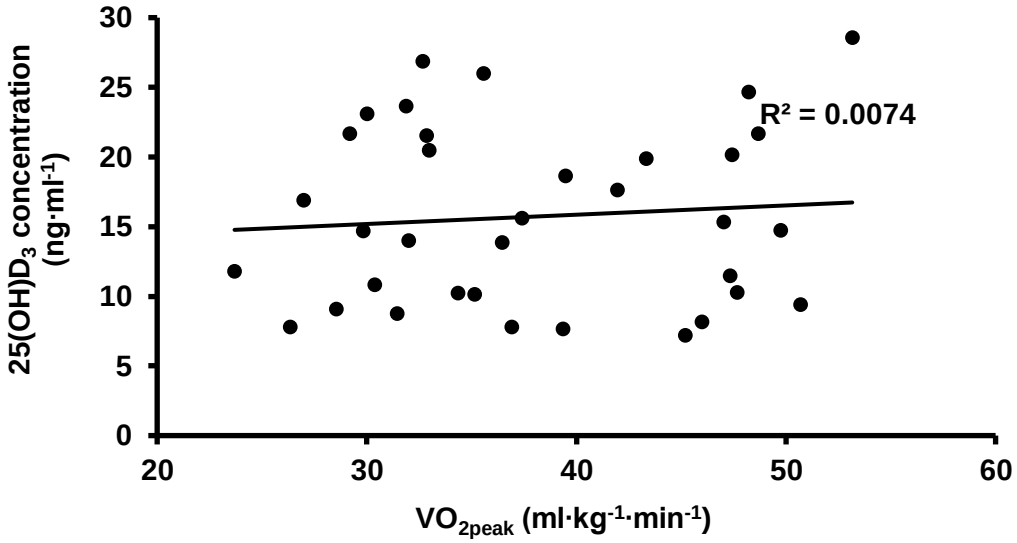

Figure 20 Correlation between 25(OH)D_3 concentration and relative peak oxygen uptake (n=35). Each marker represents an individual participants' data.

There is no significant relationship between baseline 25(OH)D_3 concentration and peak power output (r=0.145, *P*=0.407), as shown in **Figure 21**.

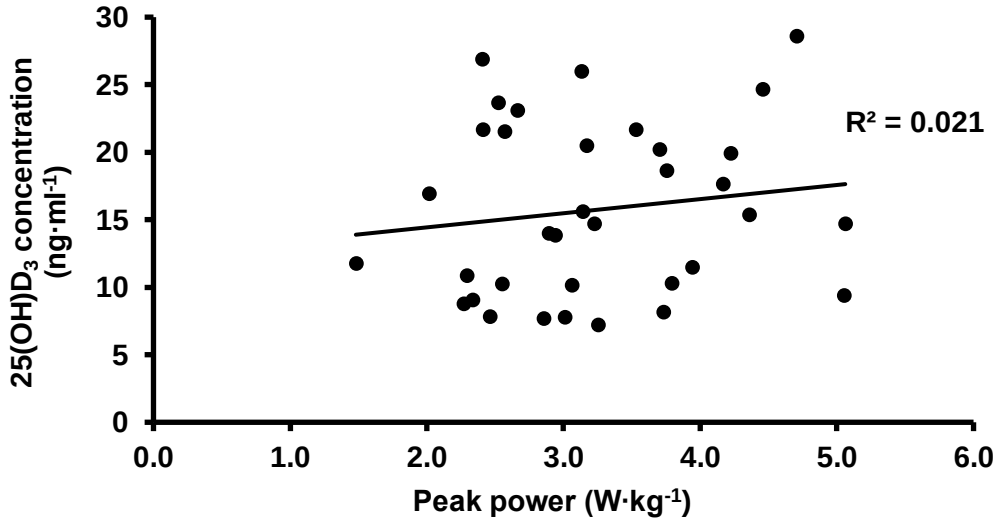

Figure 21 Correlation between 25(OH)D_3 concentration and relative peak power output (n=35). Each marker represents an individual participants' data.

5.4.5.2 VDR expression and CRF

There is a significant positive relationship between baseline VDR expression and peak oxygen uptake (r=0.336, *P*=0.048), as shown in **Figure 22**.

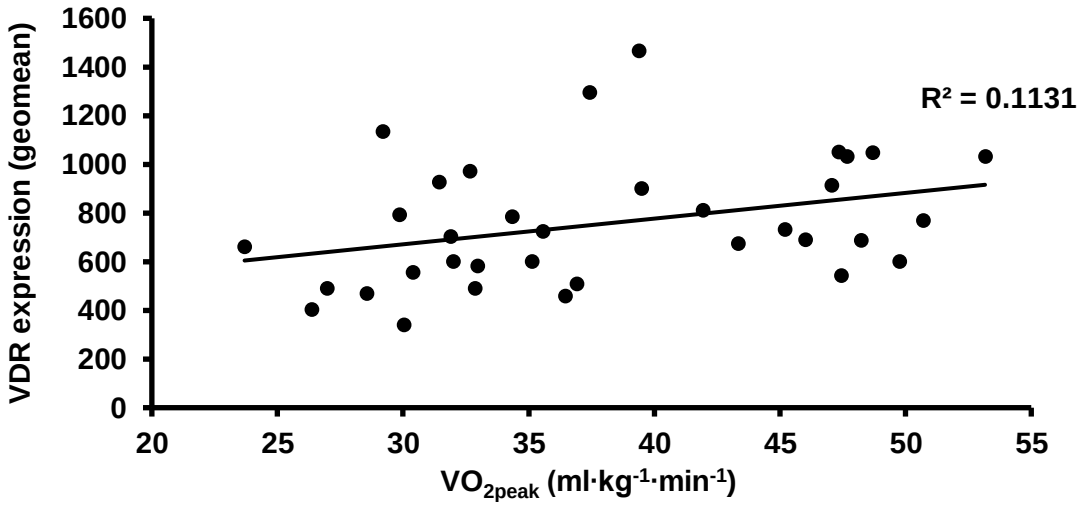

Figure 22 Correlation between baseline CD3+ T cell VDR expression and relative peak oxygen uptake (n=35). Each marker represents an individual participants' data.

There is no correlation between baseline VDR expression and peak power output (r=0.195, *P*=0.263), as shown in **Figure 23**.

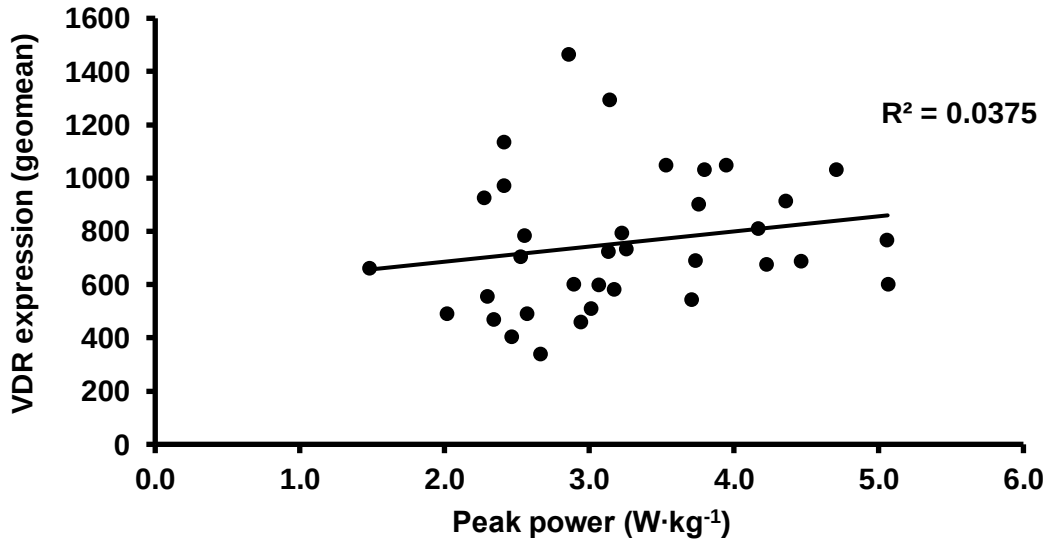

Figure 23 Correlation between baseline CD3+ T cell VDR expression and relative peak power output (n=-35). Each marker represents an individual participants' data.

5.4.5.3 CRF quartiles

Table 16 presents characteristics of the population (n=35) divided into quartiles according to peak oxygen uptake ($\dot{V}O_{2peak}$) as a marker of CRF to determine what participant characteristics are associated with a lower or higher level of fitness. A higher value for $\dot{V}O_{2peak}$ is associated with a younger population (*P*=0.005), with the top two quartiles expressing a lower age compared to the bottom two quartiles. A lower resting HR was also reported in the top two quartiles of CRF (*P*=0.026). Although, maximum HR did not statistically differ between quartiles (*P*=0.054). As expected, the higher the $\dot{V}O_{2peak}$ score the greater peak power exerted (*P*<0.001).

There was no association between $\dot{V}O_{2peak}$ and 25(OH)D_3 concentration (*P*=0.576), but there was with baseline VDR expression (*P*=0.033), with the top two quartiles linked to a higher expression.

Table 16 Characteristics of the population (n=35) divided into quartiles according to peak oxygen uptake.

Quartiles	**Q1**	**Q2**	**Q3**	**Q4**	**ANOVA**		
VO_{2peak} ($ml \cdot kg^{-1} \cdot min^{-1}$)	**23.7-31.1**	**31.1-38.4**	**38.4-45.8**	**45.8-53.2**	**df**	**F**	***P* value**
N	8	12	6	9			
Age, y	58 ± 16	49 ± 20	32 ± 8 *	34 ± 9 *	3,34	5.253	0.005
BMI, $kg \cdot m^{-2}$	27.8 ± 3.9	26.1 ± 2.2	23.9 ± 3.0	24.7 ± 2.7	3,34	2.648	0.066
Resting HR, $beats \cdot min^{-1}$	67 ± 9	58 ± 7	57 ± 6	55 ± 9 *	3,34	3.551	0.026
HR_{max}, $beats \cdot min^{-1}$	167 ± 24	169 ± 19	188 ± 8	184 ± 9	3,34	2.857	0.054
Peak power, $W \cdot kg^{-1}$	2.36 ± 0.50	2.81 ± 0.32	3.66 ± 0.53 * †	4.29 ± 0.58 * †	3,34	28.950	<0.001
25(OH)D_3 concentration, $ng \cdot ml^{-1}$	14.5 ± 5.7	17 ± 6.9	13.2 ± 6.1	17.4 ± 6.8	3,34	0.672	0.576
VDR expression, geomean	606 ± 258	721 ± 244	880 ± 299 *	853 ± 206 *	3,34	3.007	0.033

Data presented as means ± SD. * denotes a significant difference to Q1 (*P*<0.05). † denotes a significant difference to Q2 (*P*<0.05).

5.4.5.4 Regression models

A multiple linear regression was calculated to predict T cell VDR expression based on CRF variables determined by the cycle-based maximal oxygen uptake test, primarily $\dot{V}O_{2peak}$ and peak power output. The results are displayed in **Table 17**.

For CD3+ T cells, a significant regression equation was found ($F(4,32)=3.436$, $P=0.044$), with an R^2 of 0.177. Specifically, $\dot{V}O_{2peak}$ relative to body mass predicts CD3+ T cell VDR expression ($P=0.026$). Participants CD3+ VDR expression increased by 27.036 (geomean) for each measure (millilitre) of relative $\dot{V}O_{2peak}$ ($ml \cdot kg^{-1} \cdot min^{-1}$).

For CD4+ T cell, there was a non-significant regression equation reported.

For CD8+ T cell, a significant regression equation was found ($F(4,32)=4.353$, $P=0.021$), with an R^2 of 0.214. Post hoc analysis found absolute and relative $\dot{V}O_{2peak}$ predicts CD8+ T cell VDR expression ($P=0.038$; $P=0.011$). Peak power did not predict VDR expression. Participants CD8+ VDR expression increased by 31.732 (geomean) for each measure (ml) of relative $\dot{V}O_{2peak}$ ($ml \cdot kg^{-1} \cdot min^{-1}$), and by 276.141 (geomean) for each measure (L) of absolute $\dot{V}O_{2peak}$ ($L \cdot min^{-1}$).

Table 17 Multiple regression models between baseline T cell subset VDR expression and cardiorespiratory variables (relative and absolute measures).

Independent variable	Variables	df	R^2	F	*P* value
CD3+ T cell VDR expression	(regression)	2, 32	0.177	3.436	0.044
	Absolute $V\dot{O}_{2peak}$, $L \cdot min^{-1}$				0.072
	Relative $V\dot{O}_{2peak}$, $ml \cdot kg^{-1} \cdot min^{-1}$				0.026
	Absolute peak power, W				0.420
	Relative peak power, $W \cdot kg^{-1}$				0.126
CD4+ T cell VDR expression	(regression)	2, 32	0.167	3.200	0.054
CD8+ T cell VDR expression	(regression)	2, 32	0.214	4.353	0.021
	Absolute $V\dot{O}_{2peak}$, $L \cdot min^{-1}$				0.038
	Relative $V\dot{O}_{2peak}$, $ml \cdot kg^{-1} \cdot min^{-1}$				0.011
	Absolute peak power, W				0.297
	Relative peak power, $W \cdot kg^{-1}$				0.064

5.5 Discussion

This is the first study to show that there is a link between the age and CRF of participants and their baseline VDR expression levels, whereby VDR expression declines with age and a higher CRF is related to a higher density of receptors in immune cells. There was a strikingly high percentage of participants that were vitamin D severely deficient (40 %), deficient (29 %) and insufficient (31 %), contributing to literature defining Scotland as a vitamin D deficient nation (Kelly et al., 2015; Weiss et al., 2016; Zgaga et al., 2011). However, despite the low vitamin D status, it was observed that there is no association between serum 25(OH)D concentrations and circulating lymphocyte VDR expression in healthy human males.

The latter finding has been reported previously, whereby there was no relationship between serum concentrations of 25(OH)D or 1,25$(OH)_2D_3$ and intracellular VDR expression in human muscle tissue (Bischoff-Ferrari, Borchers, et al., 2004; Kinyamu et al., 1997). If the metabolites are not correlated, this may suggest that baseline expression of the VDR protein can be regulated by factors other than circulating 25(OH)D concentrations. This was observed in the current study, whereby VDR expression was associated with age and CRF whilst 25(OH)D concentrations were not.

The decline in CRF with age is an established inverse relationship reported over the past few decades (Baur, Christophi, Cook, & Kales, 2012; Buskirk & Hodgson, 1987; Fitzgerald et al., 1997; Fleg et al., 2005; Hakola et al., 2011; Pollock et al., 1997; Wilson & Tanaka, 2000; Zimmerman et al., 2014), which the current study endorses. The findings of the current investigation observed no influence of age or CRF on baseline 25$(OH)D_3$ concentration. The lack of influence of age on vitamin D status has been reported previously (Masoompour, Sadegholvaad, Larijani, & Ranjbar-Omrani, 2008). However, published investigations report a positive correlation to exist between serum 25$(OH)D_3$ concentration and CRF (Ardestani et al., 2011; Mowry, Costello, & Heelan, 2009). The contradicting results may be attributed to the nature of the physical activity or exercise, which may be performed outdoors and thus increasing exposure to UVB radiation, or indoors which may limit UVB exposure (Kluczynski et al., 2011). Therefore, the nature of physical activity

and exercise, which in turn increases CRF, may determine whether there is an association between CRF and 25(OH)D concentration, as UVB exposure may be surrogate for elevated 25(OH)D levels. As a result, the data surrounding vitamin D status and associations with lifestyle factors, such as physical activity and exercise, should be interpreted with caution. Although, it has been reported recently that vitamin D status is not influenced by indoor and outdoor activity (Wanner et al., 2015).

In line with previous data (Bischoff-Ferrari, Borchers, et al., 2004), there is an established relationship between baseline VDR expression and age, with a lesser density of the receptor found in T cells as age advances. Although, Bischoff-Ferrari et al. (2004a) reported the decline with age in skeletal muscle cells, our decline was observed in systemic T cells. Since the VDR is found in numerous tissues within the body (Y. Wang, Zhu, & DeLuca, 2012), perhaps numerous cells types exhibit this age-induced influence on VDR. For example, an age-associated decline in the intestinal concentration of VDR has been reported in both rats (Horst, Goff, & Reinhardt, 1990) and humans (female) (Ebeling et al., 1992), with no change in serum markers of vitamin D ($1,25(OH)_2D_3$). Although, this has also been contradicted showing no difference in intestinal VDR between young and older women (Kinyamu et al., 1997). A number of factors, such as sex, age, and calcium have been found to affect the expression of the VDR protein and the VDR gene in specific tissues (Y. Wang et al., 2012). Vitamin D status has been reported to influence VDR expression in the kidney (Healy, Zella, Prahl, & DeLuca, 2003) and bone (Zella, Kim, Shevde, & Pike, 2006), however not in the intestine. In addition, the cell isolation process can alter the gene transcription and expression (Ahn et al., 2007), thereby potentially altering VDR protein and gene expression. Moreover, the regulation of VDR expression has been found to differ between murine and human blood cells (Janik et al., 2017). The difference between tissue and cell sources analysed for addressing VDR expression may explain the contradictory results in the literature. Ultimately, if age induces a decline in VDR expression in cells, it follows that there are less receptors available within the cell to receive and bind to the ligand, thus reducing the capacity of the heterodimer (VDR-RXR-ligand) complex forming and translocating to the

nucleus (as outlined in the literature review). Therefore, the genomic and transcriptional alterations induced by the vitamin D complex, may be compromised. However, findings should be noted as specific to the species and tissue type analysed and the analysis methods, as all findings may not be translated into other cell types, or from murine models to humans.

In the current study, the VDR expression was measured in CD3, CD4 and CD8 T lymphocytes to identify whether there was a difference between subsets. It was found that CD3 and CD8 T cell VDR expression was predicted by CRF whereas CD4 T cell VDR expression was not. In previous studies it has been reported that training status influences the lymphocyte repertoire composition of naïve and senescent CD4 and CD8 T cells at rest (Spielmann et al., 2011) and in response to exercise (Brown et al., 2014). A higher maximal aerobic capacity exhibits a lymphocyte profile with more naïve CD8 T cells and less senescent CD4 and CD8 T cells (Spielmann et al., 2011). In support of this, it has been repeatedly demonstrated that the CD8 T lymphocyte is more responsive to exercise than its CD4 counterpart (Anane et al., 2009; J. P. Campbell, Guy, Cosgrove, Florida-James, & Simpson, 2008; R. J. Simpson et al., 2008; R. J. Simpson, Florida-James, Cosgrove, et al., 2007), with mobilisation of the senescent lymphocytes in response to exercise allowing for egress into tissues and freeing up space for naïve cells to accumulate in their place (R. J. Simpson, 2011). Although, it is not known whether there is a differing expression of VDR in naïve and senescent cells, it has been reported that 25(OH)D_3 concentrations are inversely associated with naïve T cells (Hwang et al., 2013). This suggests a link between vitamin D metabolism and the T cell pool composition. Furthermore, it has been found that in terms of the total lymphocyte population, the highest concentrations of VDR are found in CD8 lymphocytes (Veldman et al., 2000). Therefore, selective mobilisation of CD8 T cells with higher levels of VDR expression, may be beneficial for immune health. Further investigations are required in the area to aid understanding of the relationship between vitamin D metabolism and lymphocyte populations.

It should also be taken into consideration that although people in Scotland whom do not consume vitamin D supplements are deficient in vitamin D at

baseline, 25(OH)D is always present and bioavailable in the circulation, shown through the mean recorded serum 25(OH)D concentration of 15.7 ± 6.4 $ng \cdot ml^{-1}$ in the current investigation. Therefore, there is always capacity for the conversion of $25(OH)D_3$ to the ligand $1,25(OH)_2D_3$ and thus binding to the VDR within a target cell. However, the VDR must be available for binding if the vitamin D metabolic pathway is to be efficiently fulfilled. Thus strategies to increase VDR expression may be more valuable than strategies solely to increase 25(OH)D concentrations considering the metabolites may be independent of one another.

In summary, the findings show that baseline VDR expression declines with age and is positively associated with a higher CRF. There was no observed association between the two key systemic vitamin D metabolites at baseline: 25(OH)D concentration and VDR expression and circulating 25(OH)D concentrations observed no relationship with either age or CRF.

Chapter 6: Effect of Exercise on T Cell VDR Expression

6.1 Abstract

It has previously been reported that exercise can upregulate cellular VDR expression, however studies are limited to murine and in vitro models. This study aimed to determine whether a single bout of exercise upregulates VDR expression in a human population and explore whether age affects the response.

As in Chapter 5, thirty-five male adults participated in this study (age 44 ± 17 y, body mass 82.5 ± 11.4 kg, height 1.79 ± 0.08 m, BMI 25.7 ± 3.1 $kg \cdot m^{-2}$). The participants were separated into three groups based on their age: young (18-30 y; n=12), young/mid (31-45 y; n=11), and older adults (60-75 y; n=12). Participants completed three trials: control, endurance exercise and resistance exercise, with blood samples collected pre- and post-exercise bout/control rest period (pre, 0 h, 1 h, 3 h). Systemic T cells (CD3+, CD4+, CD8+) were isolated from whole blood and analysed for VDR expression (flow cytometry).

Both modalities of exercise, endurance (P<0.001) and resistance (P=0.001), induced an increase in VDR expression immediately upon cessation of the exercise, with the endurance bout promoting a greater increase in VDR expression compared to RE (P=0.048) The effect was acute, as VDR expression dropped to baseline level after 3 h post-exercise. The response was unaffected by age despite the difference in baseline VDR expression at rest (Chapter 5). Moreover, there was no correlation between change in T cells and change in VDR expression in response to exercise.

The results indicate that a single bout of exercise is an effective way to increase intracellular VDR expression in immune cells. The upregulation in VDR protein expression may be independent of T cell mobilisation in response to exercise, and independent of age.

6.2 Introduction

Vitamin D is classically regarded as a key regulator in bone health, playing a role in calcium and phosphate homeostasis. However, numerous tissues have now been identified in the literature as vitamin D active (Y. Wang et al., 2012) through the binding of the biologically active form of vitamin D, $1,25(OH)_2D_3$, to its specific receptor, the VDR. This resulting complex translocates to the nucleus where it binds to VDREs in DNA and regulates the expression of target genes and thus their products (Pike & Meyer, 2010; Pike et al., 2012), which are involved in the pathophysiology of numerous diseases. Therefore, cellular expression of the VDR is vital for this pathway to function efficiently.

Exercise has been shown to be an efficient stimulus to upregulate 25(OH)D concentrations in a human population (Sun et al., 2017). Emerging data are now suggestive that acute mechanical stress, i.e. exercise, can impact vitamin D metabolism at a cellular level, inducing an upregulation of the VDR (Aly et al., 2016; Makanae et al., 2015). To the best of our knowledge, the effects of exercise on VDR expression have not yet been investigated in a human population. Moreover, due to the differing findings surrounding exercise modality and fibre type recruitment (Ceglia et al., 2013; Makanae et al., 2015; Widegren et al., 2000), both endurance and resistance exercise will be included in the current investigation.

For the purpose of the current investigation, PBMCs, specifically T cells, which are representatives of the innate and adaptive immune system, are most suited as they are obtained through less invasive procedures to the human participant and can be easily isolated for flow cytometry staining and analysis. Moreover, exercise activates T lymphocytes (Siedlik et al., 2017), and with the VDR found in activated T cells (Baeke, Korf, et al., 2010; Baeke, Takiishi, et al., 2010; Bhalla et al., 1983; Provvedini et al., 1983), this marks them as a strong contender to determine the effect exercise may have on vitamin D metabolism.

The primary aim of this study was to investigate whether a single bout of endurance and resistance exercise can increase VDR expression in immune cells. The secondary aim was to examine if this response to exercise is

influenced by age. The tertiary aim was to explore the changes in T cell concentration in response to exercise, and whether this influences the exercise-induced increase in VDR expression. It was hypothesised that exercise will acutely upregulate VDR expression in T cells. Although, it is explorative as to whether both exercise modalities will induce alterations in VDR expression in a human population.

6.3 Materials and methods

6.3.1 Participants

Thirty-five male adults participated in this study (see section 3.2.2: age 44 ± 17 y, body mass 82.5 ± 11.4 kg, height 1.79 ± 0.08 m, BMI 25.7 ± 3.1 $kg \cdot m^{-2}$). The participants were separated into three groups based on their age (see section 3.2.2): young (18-30 y; n=12), young/mid (31-45 y; n=11), and older adults (60-75 y; n=12). Participant characteristics are presented in Chapter 5 (see section 5.4.1). Following the power analysis calculation (section 3.2.2.1), participants were recruited on a rolling basis over the projected 12 month time frame for the ExVD study. The number of participant trials per month and thus the month of blood sampling for each trial during the 12 month study time frame is displayed in **Table 18**.

Table 18 The number of participant trials per month.

Month	Total trials	CON	EE	RE
January	8	3	3	2
February	13	4	5	4
March	3	1	0	2
April	3	1	1	1
May	14	5	5	4
June	14	6	4	4
July	5	1	2	2
August	11	4	4	3
September	9	2	4	3
October	9	5	1	3
November	8	1	3	4
December	7	2	3	2
Total	104	35	35	34

6.3.2 Preliminary measurements

Participants performed a $\dot{V}O_{2peak}$ test (see section 3.6, and CRF outcomes in section 5.4.5) to set the intensity of the endurance exercise (EE), and a familiarisation of the resistance exercise (RE) protocol to allow correct positioning of the participant on the dynamometer (1 set of 4 repetitions of the RE protocol was performed, see section 6.3.3.3).

6.3.3 Trials

Participants were randomly assigned to a continuous moderate intensity EE trial or a RE trial, adapted from Makanae et al. (2015), or a control trial as shown in **Figure 24**. Randomisation was performed by an online generator that creates random permutations of treatments for situations where participants (n=36) were to receive all of the "treatments" (trials: CON, EE, RE) in random order (SEED number 22520, performed on 04/04/2017). For each visit, participants arrived to the laboratory between 7.30-8.30am in a fasted state (10 h). A baseline blood sample was collected via cannulation (see section 3.7.2), with the randomly assigned exercise performed promptly after, or a 1 h rest period during the control trial. Blood samples were collected immediately post-, 1 h post-, 3 h post-cessation of the exercise/rest period (Kim et al., 2005; Makanae et al., 2015) and were handled as previously described in sections 3.7 and 3.8. Participants remained seated and rested for the 3 hours post-exercise/rest period. HR was continuously monitored (Polar, RS400, Finland) throughout each trial. Trials were separated by at least 7 days to allow recovery.

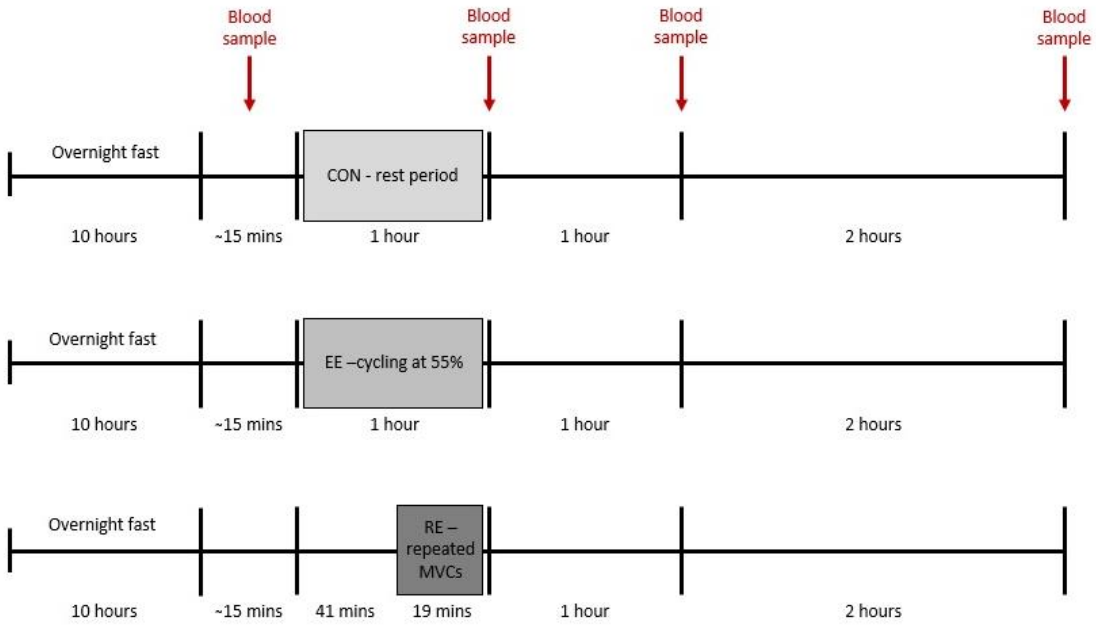

Figure 24 Schematic diagram of the protocol and time of blood sampling for each of the three trials: CON, EE, RE (top to bottom, respectively).

6.3.3.1 CON trial

During the control trial, participants remained seated for 1 h.

6.3.3.2 EE trial

The EE trial consisted of a continuous 60 min cycling bout on an electronically braked cycle ergometer (Lode, Corival CPET, Netherlands) at 55% $\dot{V}O_{2peak}$ to correspond to an intensity below estimated lactate threshold in untrained males (Joyner & Coyle, 2008). The wattage set during the protocol was based on the workload (wattage) achieved at 55 % of $\dot{V}O_{2peak}$. Participants were instructed to maintain a pedalling rate between 70-80 rpm for the duration of the trial.

6.3.3.3 RE trial

The RE trial was designed to replicate the protocol utilised in the study by Makanae et al. (2015) and consisted of 5 sets of 10 repetitions of 3 s isometric

MVCs on an isokinetic dynamometer (HUMAC Norm Cybex, CSMi Medical Solutions, USA), with 7 s rest intervals between repetitions and 3 min recovery between sets. Participants were seated with dominant leg fixed, with a knee joint angle of 90° flexion (with straight leg representing anatomical zero), and ankle attached just superior to malleoli to a strain gauge. Since the duration of the RE protocol was 19 min, the participant rested for 41 min prior to the exercise. This ensured blood sampling time points were standardised.

6.3.4 Experimental controls

Participants were asked to complete a 24 h food diary for the day prior to the trials, and asked to abstain from consuming foods that are natural or fortified sources of vitamin D_3 (Holick et al., 2011), with a list of food items provided to participants (Appendix 6). Participants were asked to maintain their habitual diet and activity in between trials, with trials separated by at least 7 days to allow for muscle recovery and vascular healing from blood collection. If participants reported they had been on a holiday outside of the UK to a sunny destination, they were not sampled for a minimum of 2 months post-holiday, to avoid any influence of increased UV exposure as a result of foreign holidays on vitamin D status and metabolism (Weiss et al., 2016). During the 12 months of data collection the weather conditions, in particular the statistical summary for 'Sunshine', was representative of the average for the UK (100% of the 1981-2010 average) as reported by the Meteorological Office (www.metoffice.gov.uk/climate/uk/summaries/2017/annual).

6.3.5 Blood sampling and analysis

Baseline blood samples were collected via cannulation (see section 3.7.2). Upon collection, EDTA treated whole blood was analysed for full haematological profile (see section 3.8). All haematological data were adjusted to account for any changes in plasma volume (PVC) from baseline using the method outlined by Dill and Costill (Dill & Costill, 1974). This procedure is required to ensure that any changes in cells in response to trials in this study is a movement of cells in/out of the circulation, rather than a

concentration/dilution effect due to alterations in plasma levels. The PVC (%) during each trial is shown in **Figure 25**, and were analysed via paired samples t-tests, with all differences reported as highly significant (*P*<0.001).

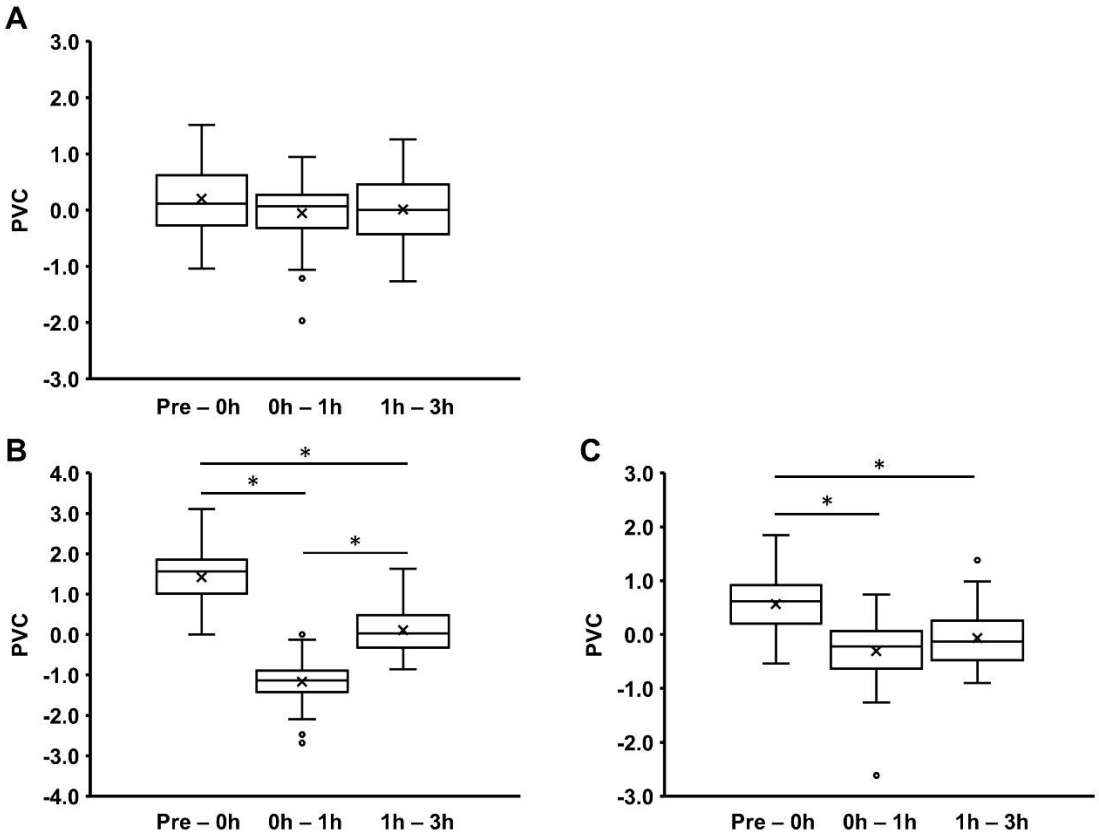

Figure 25 The change in plasma volume (%) during the three trials (n=35): CON (**A**), EE (**B**), and RE (**C**). * denotes a significant difference between changes in plasma volume (*P*≤0.05).

Whole blood was collected at each time-point, with PBMCs subsequently isolated and CD3+, CD3+CD4+, and CD3+CD8+ T cell VDR expression was analysed via flow cytometry (see section 3.10). Absolute T cell subset concentration was calculated based on the haematological data. The correction factor for PVC (percentage change) was then applied to the T cell concentration data and the T cell VDR expression data (geomean).

6.3.6 Statistical analysis

Full statistical analysis is outlined in section 3.11.

6.4 Results

6.4.1 Trial heart rate data

The mean and maximum HR response to each trial is shown in **Figure 26**. There was no difference in HR between age groups during the CON trial rest period. There was a main effect of trial (CON, EE, RE) on the mean HR recorded ($F(2,60)=463.090$, $P<0.001$), with a trial by age group interaction ($F(4,60)=4.169$, $P=0.005$), whereby mean HR was higher in the young group compared to the older group ($P=0.017$) for both EE and RE. Similar results were found for maximum HR during the sessions, with a main effect of the trial ($F(2,60)=369.925$, $P<0.001$), and a trial by age group interaction ($F(4,60)=4.086$, $P=0.005$) reported, whereby maximum HR was higher in the young compared to the older cohort ($P=0.001$) for both EE and RE.

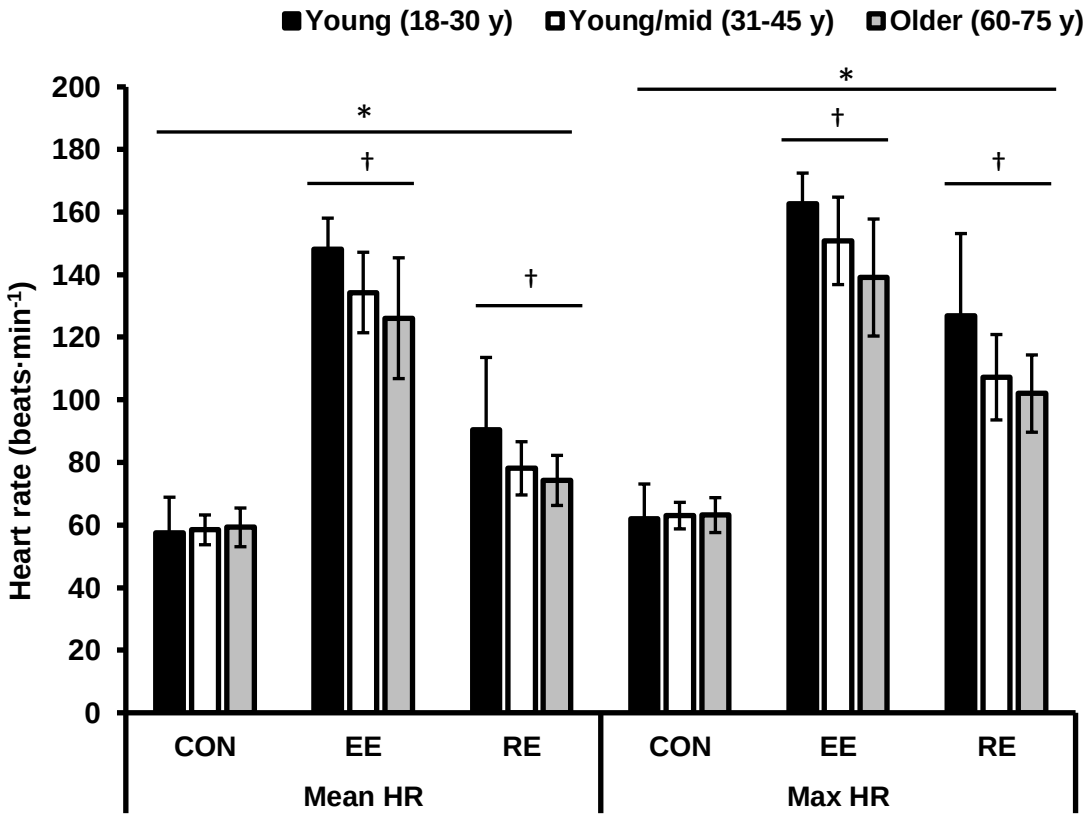

Figure 26 Mean and maximal HR data for each trial for young (n=12), young/mid (n=11), older (n=12) adults. All data are presented as mean ± SD. ***** represents a significant within subject effect of the trial ($P<0.001$). **†** represents a significant difference between young and older ($P\leq0.05$).

6.4.2 Exercise and VDR expression

The intracellular T cell VDR expression is expressed as fold change, to allow for comparison between trials and age groups relative to the change as opposed to absolute value due to baseline VDR expression differences between the age groups (as seen in section 6.4.2). The change in VDR expression (CD3 T cells) in response to EE and RE compared to the CON trial is shown in **Figure 27**. There was a main effect of time (F(3,96)=21.128, P<0.001) on the response of VDR expression to exercise, with a significant interaction effect (trial*time) observed (F(6,192)=6.241, P<0.001). There was a significant effect of the trial/modality on VDR expression (F(2,64)=7.005, P=0.002), with EE inducing a greater increase in VDR expression compared to RE (P=0.048) and the CON (P=0.002). Moreover, RE upregulated VDR expression compared to the CON (P=0.047).

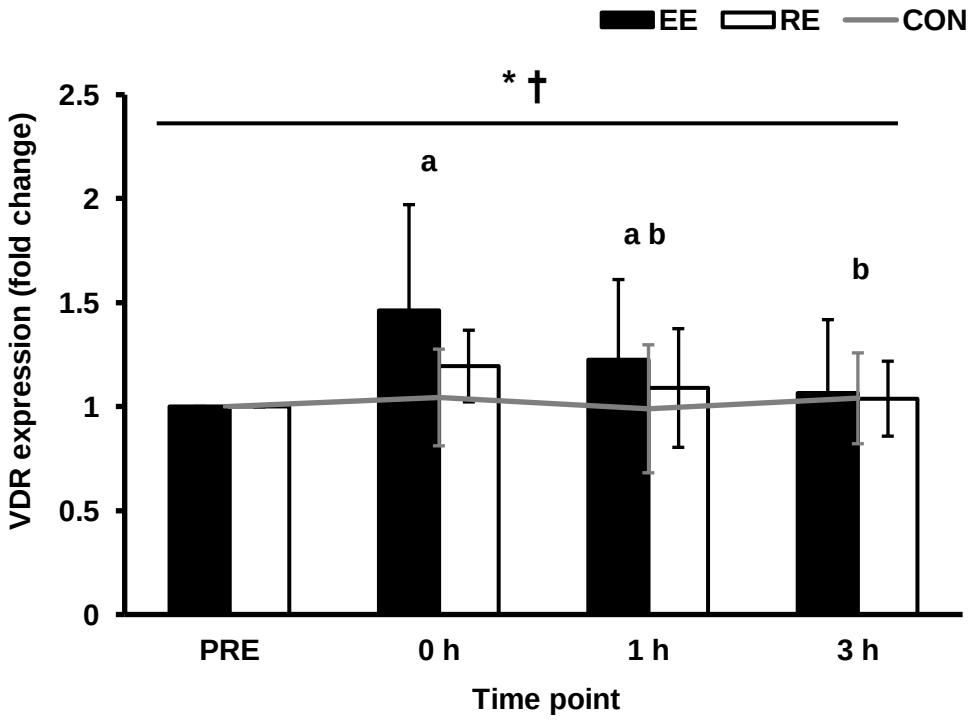

Figure 27 Effect of endurance and resistance exercise on VDR expression (n=35). Data presented as mean ± SD. * denotes a significant main effect of time (P≤0.05). † denotes a significant main effect of the trial (P≤0.05). [a] represents a significant difference from PRE; [b] represents a significant difference from 0h (P≤0.05).

6.4.2.1 CD3+ T cells

The change in CD3+ T cell VDR expression of each age group in response to the three trials is shown in **Figure 28**. There was no change in CD3+ T cell VDR expression during the 4 hour CON trial (**Figure 28 A**, $F(3,93)=0.769$, $P=0.514$).

There was a significant main effect of the EE trial on VDR expression (**Figure 28 B**, $F(3,93)=16.639$, $P<0.001$), with an increase at the 0 h time point ($P<0.001$) and the 1 h post-exercise time point, compared to baseline ($P=0.002$).

There was a main effect of the RE trial on VDR expression (**Figure 28 C**, $F(3,96)=7.401$, $P=0.001$), with an increase immediately after exercise (0 h) compared to baseline ($P<0.001$) and then a decrease at the 1 h compared to 0 h post-exercise time point ($P=0.014$).

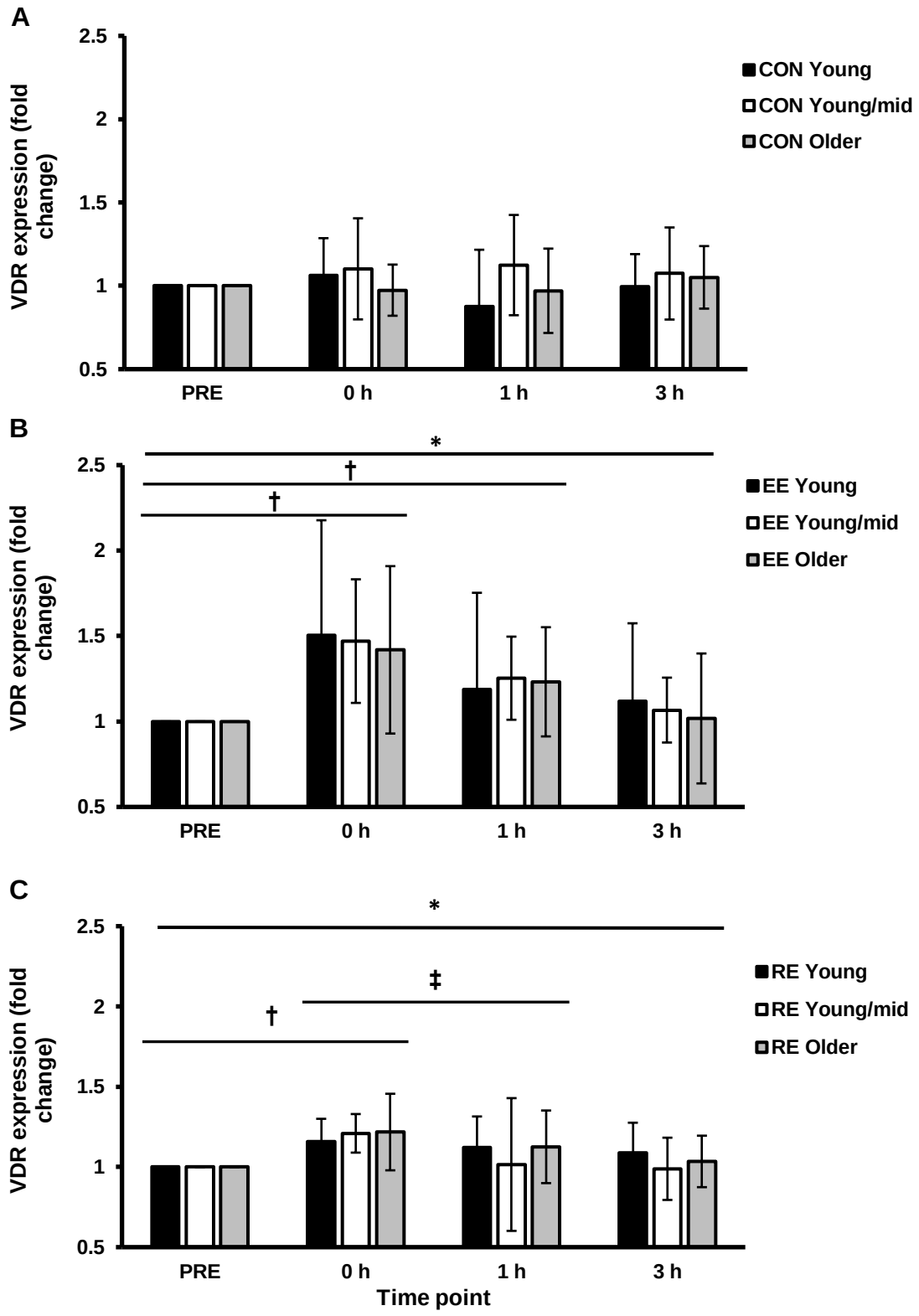

Figure 28 Changes in T cell CD3+VDR+ expression, expressed as fold change during each trial for the (**A**) CON trial, (**B**) EE trial, (**C**) RE trial. Data presented as mean ± SD. * denotes a significant main effect of the trial (P<0.05). † denotes a significant difference from PRE (P<0.05). ‡ denotes a significant difference from 0 h (P<0.05).

6.4.2.2 CD4+ T cells

The change in CD4+ T cell VDR expression in response to each of the trials is shown in **Figure 29**. There was no change in receptor expression during the CON trial (**Figure 29 A**, $F(3,93)=0.757$, $P=0.521$).

There was a main effect of the EE trial on CD4 T cell VDR expression (**Figure 29 B**, $F(3,90)=10.760$, $P<0.001$), with an increase at the 0 h time point ($P<0.001$) and the 1 h post-exercise time point, compared to baseline ($P=0.001$).

There was a main effect of the RE trial on CD4 T cell VDR expression (**Figure 29 C**, $F(3,96)=12.730$, $P<0.001$), with an increase immediately after exercise (0 h) compared to baseline ($P<0.001$) and then a decrease at the 1 h compared to 0 h post-exercise time point ($P=0.036$).

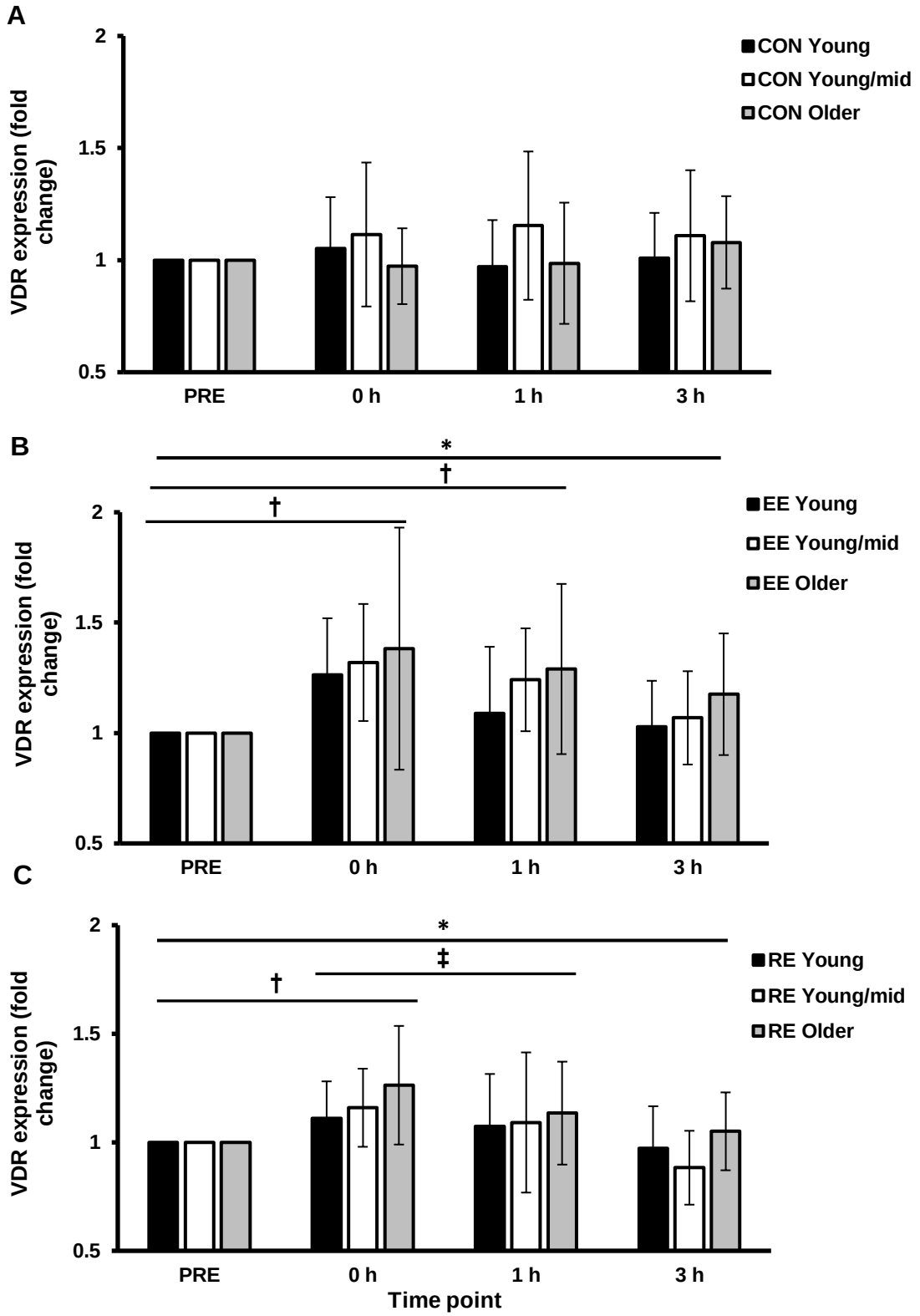

Figure 29 Changes in T cell CD3+CD4+VDR+ expression, expressed as fold change during each trial for the (**A**) CON trial, (**B**) EE trial, (**C**) RE trial. Data presented as mean ± SD. * denotes a significant main effect of the trial ($P \leq 0.05$). † represents a significant difference from PRE ($P \leq 0.05$). ‡ denotes a significant difference from 0 h ($P \leq 0.05$).

6.4.2.3 CD8+ T cells

The change in CD8+ T cell VDR expression in response to each of the trials is shown in **Figure 30**. There was no effect of time on the CD8+ T cell VDR expression fold change during the CON trial (**Figure 30 A**, $F(3,93)=0.473$, $P=0.686$). There was a main effect of the EE trial, and the RE trial on CD8+ T cell VDR expression.

There was a main effect of the EE trial on CD8 T cell VDR expression (**Figure 30 B**, $F(3,93)=8.329$, $P<0.001$), with an increase at the 0 h time point ($P=0.001$) and the 1 h post-exercise time point ($P<0.001$), compared to baseline.

Similarly, there was a main effect of the RE trial on CD8 T cell VDR expression (**Figure 30 C**, $F(3,96)=6.437$, $P=0.001$), with an increase immediately after exercise ($P<0.001$) and at the 1 h time point ($P<0.001$) compared to baseline.

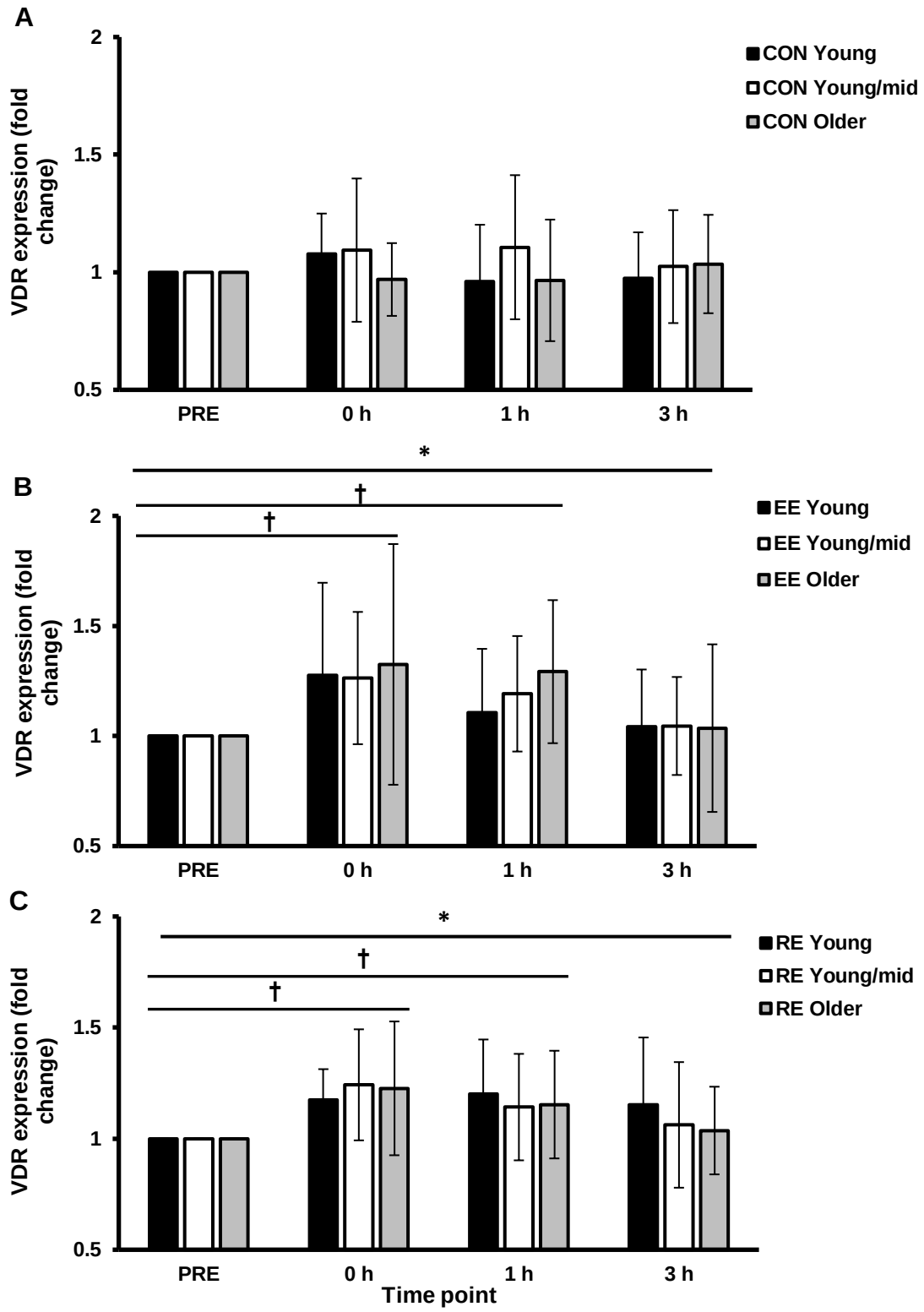

Figure 30 Changes in T cell CD3+CD8+VDR+ expression, expressed as fold change during each trial for the (**A**) CON trial, (**B**) EE trial, (**C**) RE trial. Data presented as mean ± SD. ***** denotes a significant main effect of the trial ($P \leq 0.05$). † represents a significant difference from PRE ($P \leq 0.05$).

6.4.3 Acute T cell response to exercise

There was a main effect of time on the CD3+ T cell response to the three trials ($F(3,93)=16.066$, $P<0.001$), with a significant interaction effect (trial*time) observed ($F(6,186)=13.493$, $P<0.001$), whereby there was a difference between trials (CON, EE, RE) at the 0 h time point ($F(2,100)=6.115$, $P=0.003$), but not at the other time points. At the 0 h time point, CD3 T cell number was elevated in response to the EE bout compared to CON ($P=0.002$). However, there was no difference between the other trials.

The CD3+, CD4+ and CD8+ T cell response to each trial is presented in **Table 19**. There was an increase in T cell number in response to both EE and RE, which falls in line with the classic acute response to exercise that is well established in the literature. There was also a main effect of time during the CON trial in CD3+ and CD4+ T cells. There was no interaction effect (time*age group) during any of the trials, and thus no influence of age group.

Table 19 The T cell response to each trial (n=35).

		Time points				*Main effect of time*			Interaction effect		
		PRE	**0h**	**1h**	**3h**	***df***	***F***	***P* value**	**df**	***F***	***P* value**
CON	**CD3**	1011 ± 479	1011 ± 502	1088 ± 496	1124 ± 529	3,96	3.468	0.043	6,96	0.517	0.704
	CD4	554 ± 232	543 ± 242	587 ± 218	613 ± 247	3,96	3.436	0.039	6,96	0.414	0.794
	CD8	355 ± 239	360 ± 250	384 ± 264	392 ± 275	3,96	2.327	0.106	6,96	0.597	0.732
EE	**CD3**	976 ± 506	1505 ± 913	891 ± 417	1001 ± 474	3,96	20.228	<0.001	6,96	0.977	0.409
	CD4	526 ± 238	683 ± 363	491 ± 218	544 ± 246	3,96	8.326	<0.001	6,96	0.778	0.516
	CD8	344 ± 257	611 ± 523	302 ± 185	351 ± 213	3,96	17.232	<0.001	6,96	0.493	0.656
RE	**CD3**	1012 ± 462	1204 ± 615	1027 ± 500	1106 ± 556	3,96	7.669	<0.001	6,96	1.621	0.168
	CD4	544 ± 210	589 ± 242	547 ± 233	606 ± 264	3,96	2.895	0.039	6,96	0.857	0.529
	CD8	349 ± 231	450 ± 311	356 ± 233	379 ± 254	3,96	13.912	<0.001	6,96	1.794	0.131

All data is presented as mean ± SD.

6.4.4 Change in circulating T cells

The change in circulating concentration of T cell subsets in response to a single bout of EE and RE is presented in **Figure 31**, showing the differences between age groups. This potentially represents the mobilisation of T cell subsets into the circulation and out of the circulation. There is a large increase of CD3, CD4, and CD8 T cells from pre- to immediately post-exercise (**Figure 31 A**), followed by a large decrease from post-exercise to 1 h post-exercise (**Figure 31 B**).

Although, when analysing the difference between age groups statistical significance was only observed for the egress of CD3 T cells in response to the EE ($F(2,33)=5.140$, $P=0.012$) and RE trials ($F(2,33)=5.104$, $P=0.012$), and CD4 T cells in response to the EE trial ($F(2,33)=4.022$, $P=0.028$). Following the EE bout, the younger cohort compared to the older cohort redistributed significantly more CD3 T cells ($P=0.013$) and CD4 T cells ($P=0.027$) from the circulation to surrounding tissues. In response to the RE bout, the egress of CD3 T cells was greater in the young compared to the young/mid ($P=0.023$) and older group ($P=0.030$). There was no significant difference between age groups in the CD8 T cell circulating concentration.

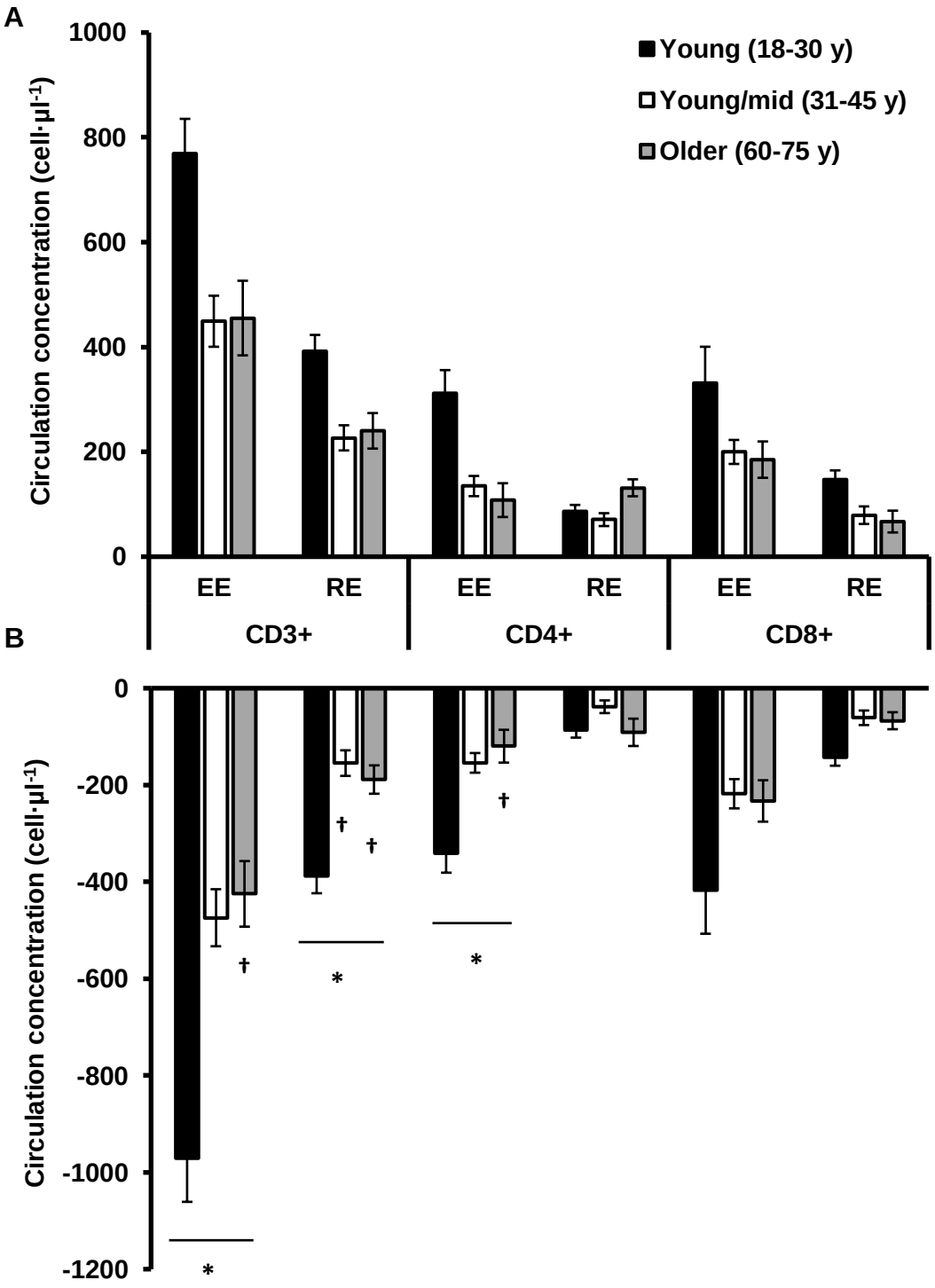

Figure 31 The change in circulating T cell subset concentration in response to exercise: (**A**) the increase immediately after exercise and (**B**) the decrease 1 h after exercise in young (n=12), young/mid (n=11), older (n=12). ***** represents a significant difference between age groups (ANOVA: $P \leq 0.05$). † denotes a significance difference compared to young (post hoc analysis: $P \leq 0.05$).

6.4.5 VDR expression and acute T cell response

Figure 32 A and **B** demonstrate the difference in cell concentration and VDR expression within the cells, respectively. There is a significant difference between ultimate cell number in circulation, and the density of the receptor within those cells, at the 0 h post-exercise (CD8+ T cells, *P*=0.036) and 1 h post-exercise (CD3+ T cells, *P*=0.003; CD4+ T cells, *P*=0.005; CD8+ T cells, *P*=0.006) time points during the EE trial, and the 3 h post-exercise time point during the RE trial (CD8+ T cells, *P*=0.031). The decline in cell number follows the biphasic response, with a substantial decrease 1 h after exercise cessation, however the VDR expression remains elevated, indicating that VDR expression is increased in T cells after exercise.

To further explore this, the correlation analyses between the delta change in cell number and the delta change in VDR expression (geomean), from each time-point to the next, are presented in **Table 20**. There is no relationship between cell number in the circulation and the expression of the receptor in CD3 and CD4 T cells during the EE trial. There is a correlation for CD8 T cells from 1 h post- to 3 h post-exercise, whereby there is an increase in cell number but a decrease in VDR expression. There were no correlations between change in cell number and VDR expression during the RE trial.

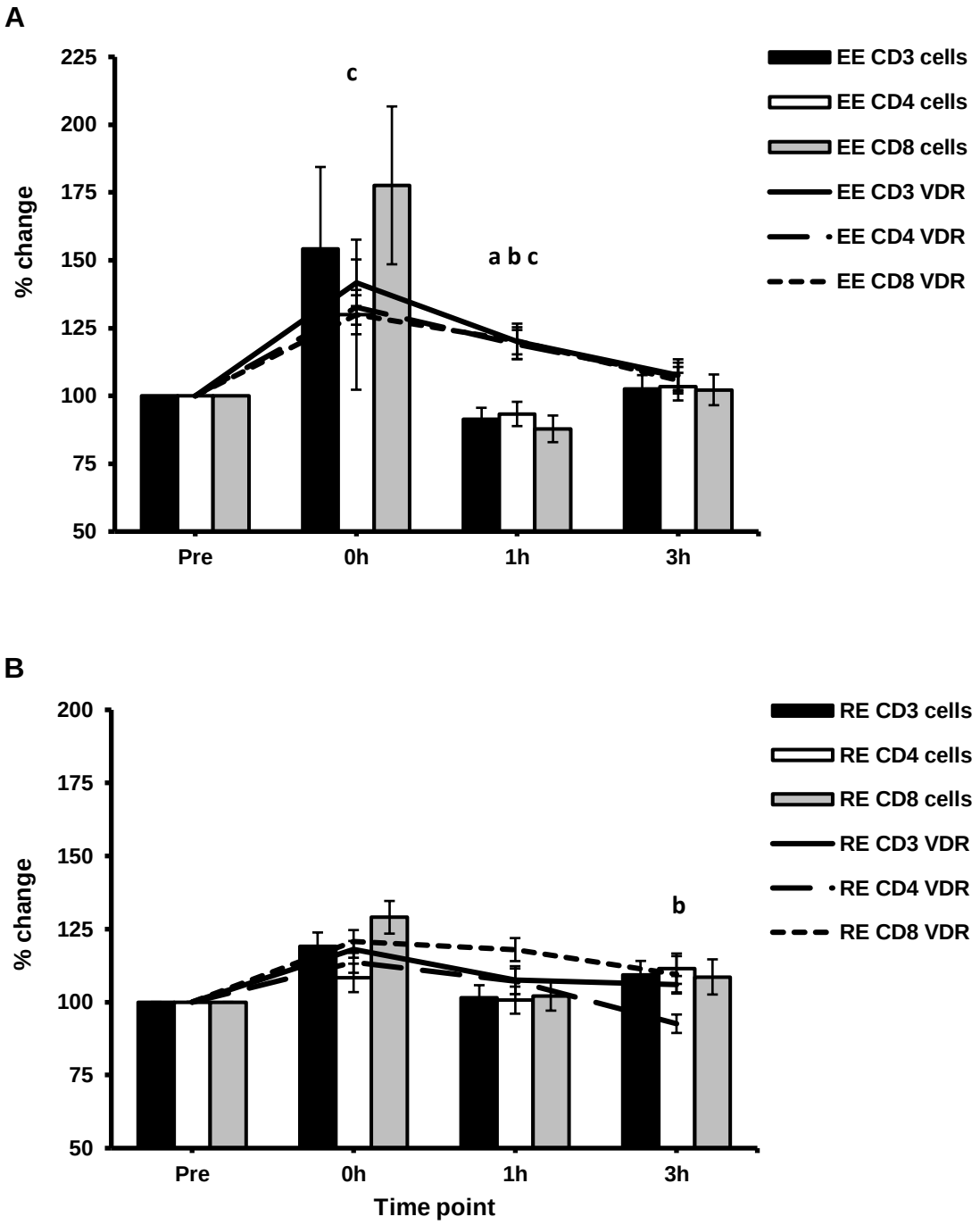

Figure 32 Percentage change of T cells (solid bars) and T cell VDR expression (lines) during (**A**) EE (n=35) and (**B**) RE (n=35) trial. Data presented as mean ± SEM. Significant differences between T cell concentration and VDR expression within the cells are denoted on the figures by: [a] CD3+ T cells; [b] CD4+ T cells; [c] CD8+ T cells (P<0.05).

Table 20 Correlations between delta change in cell number and delta change in VDR expression (n=35).

			Δ cell number	Δ VDR expression	Correlation	*P* value
EE	CD3	Pre-0h	545 ± 629	300 ± 332	-.145	0.406
		0h-1h	-631 ± 684	-155 ± 289	-.029	0.871
		1h-3h	113 ± 236	-113 ± 240	.331	0.052
	CD4	Pre-0h	158 ± 308	229 ± 284	0.558	0.558
		0h-1h	-192 ± 318	-96 ± 285	-.061	0.728
		1h-3h	53 ± 128	-104 ± 230	.114	0.515
	CD8	Pre-0h	267 ± 322	229 ± 358	-.212	0.221
		0h-1h	-309 ± 393	-74 ± 311	-.025	0.884
		1h-3h	49 ± 107	-135 ± 273	.344	0.043
RE	CD3	Pre-0h	192 ± 279	137 ± 142	-.048	0.783
		0h-1h	-177 ± 274	-80 ± 227	.055	0.752
		1h-3h	79 ± 227	-12 ± 366	-.095	0.587
	CD4	Pre-0h	45 ± 134	105 ± 166	-.136	0.438
		0h-1h	-41 ± 142	-50 ± 154	.150	0.390
		1h-3h	59 ± 146	-111 ± 185	.102	0.560
	CD8	Pre-0h	101 ± 115	157 ± 189	-.046	0.794
		0h-1h	-94 ± 119	-21 ± 157	-.030	0.863
		1h-3h	23 ± 74	-64 ± 370	-.126	0.472

All data is presented as mean ± SD.

6.5 Discussion

This is the first study to show that a single bout of endurance and resistance exercise can increase T cell VDR expression in male adults. The data shows that VDR expression increases in line with T cell elevations in circulation, which may be due to either 1) selective ingress of T cells with high levels of VDR expression, or 2) upregulation of VDR protein expression in circulating T cells. However, the changes in cell number in response to exercise are not correlated with the change in intracellular VDR expression, suggesting that the acute increase in VDR expression may be exercise-induced and not cell mobilisation-induced thus independent of the redistribution of T cells observed in response to exercise. Moreover, despite the decline in baseline VDR expression with age (found in Chapter 5), the response to exercise was not influenced by age.

Previous studies have demonstrated that exercise can increase serum 25(OH)D concentration in humans (Sun et al., 2017) and myocyte VDR expression in rats (Aly et al., 2016; Makanae et al., 2015), presenting a strategic intervention to favour metabolism of vitamin D. The current study demonstrated that an acute bout of both resistance and endurance exercise can upregulate VDR expression in systemic blood T cells from a human male population. In the study by Makanae et al. (2015), only resistance exercise augmented intramuscular VDR expression, suggesting the response may differ between species and/or cell type.

It is well established that exercise induces alterations of blood lymphocytes, with an initial rise in lymphocyte count followed by an immediate decline post-exercise in the recovery phase (Kruger & Mooren, 2007; Nieman, 1994; Nieman et al., 1991; M. Ross et al., 2016; R. J. Simpson, Florida-James, Cosgrove, et al., 2007). The current investigation observed this response with an increase in lymphocytes in response to both an acute bout of endurance and resistance exercise. The rise in lymphocytes is usually attributed to cell mobilisation and redistribution from tissues into the circulation, which can be induced by a trigger or stimuli such as exercise (McCarthy & Dale, 1988; R. J. Simpson, Florida-James, Cosgrove, et al., 2007; Witard et al., 2012). The lack of correlation between change in T cell concentration in the blood and VDR

expression in the T cells suggests that the upregulation in VDR expression may not be dependent on mobilisation of cells but an increase in the VDR protein within the cells.

Since the current investigation was conducted in T cells, it is important to determine what the physiological role of the VDR is in T cells and thus the impact of the acute upregulation. A paracrine mode of action of vitamin D in the immune system is suggested by the regulated presence of both the activating enzyme, CYP27B1, and the VDR in immune cells. The effect of ligand-activated VDR on key genes involved in the immune system, also points toward a physiological role for vitamin D in immune health. One approach to determine the role of VDR in the immune system is to study mice lacking the VDR: the VDR knock out (KO) mouse. These mice exhibit impaired bone formation (Kato et al., 1999) but no abnormalities in the numbers and types of cells present (Mathieu et al., 2001). Specifically, the latter study observed equivalent distributions of T cells (CD4 and CD8) in peripheral blood and in other lymphoid organs (bone marrow, spleen, thymus, lymph node) of wild-type mice and the VDR KO mice. There is also no effect of VDR deficiency on the number of naïve and or memory CD4 cells. Similarly, it was reported that relatively small changes in CD4 T cells do not explain the increased autoimmunity that develops in VDR KO mice. Taken together these data suggest that it is not the change in T cell number that impacts VDR expression and the genomic and non-genomic actions of the VDR. Therefore, it is reasonable to suggest that exercise-induced changes in VDR expression in T cells may not be attributable to alterations in T cell number and type *per se*, but potentially the quantity of receptors within the cells.

The VDR has a relatively short half-life of 1.7 h in untreated T cells and 2.9 h in T cells treated with 25(OH)D (Kongsbak, von Essen, Boding, et al., 2014). The VDR is degraded in the cytosol and nucleus, with $1,25(OH)_2D_3$ upregulating the VDR by increasing VDR mRNA expression and/or stabilising the VDR at the protein level by protecting it from proteasomal degradation (Kongsbak, von Essen, Boding, et al., 2014). The acute increase in VDR protein expression observed in the current study in response to exercise may be short-term due to the short half-life of the VDR. If the exercise bout

increases intracellular VDR expression with an immediate decline during the hour after the exercise, this may suggest that the VDR begins to degrade upon cessation of the physical demand. Therefore, the benefit of exercise for vitamin D and VDR metabolism may be very short-term. It is currently unknown whether regular exercise training could increase the half-life of the VDR and/or the rate of expression, thereby raising baseline VDR expression.

The response of VDR expression to an acute bout of exercise was found not to be influenced by age. Ageing is associated with an accumulation of senescent T cells. Immune cell senescence, simply the process of ageing of cells, occurs as a "consequence of chromosome telomere shortening in response to repeated antigenic stimulation and/or excessive exposure to oxidative stress" (R. J. Simpson, Florida-James, Whyte, et al., 2007). Senescent T cells do not follow the same path as younger cells, whereby they go through rounds of cell division and result in different phenotypes, they can no longer enter the cell division cycle, and thus become dysfunctional within the immune system. This may affect the T cell VDR response to exercise, as the biological ageing of cells can result in reduced expression of receptors and antigens, and thus intracellular VDR expression. Although biological ageing does not necessarily correspond with chronical ageing, older persons will likely have a greater accumulation of senescent cells (R. J. Simpson, Florida-James, Whyte, et al., 2007), which can then influence the baseline expression of key receptors, such as the VDR. It could also be hypothesised that this would then affect the response to exercise, however in the current study there was no effect of age on the response of T cell VDR expression to either modes of exercise. This may indicate that the response of cellular VDR metabolism responds to exercise regardless of chronological age of the individual. Therefore, although baseline VDR expression is lower in older adults (Chapter 5), exercise is effective at inducing a substantial increase in the expression of receptors regardless of age.

The current investigation incorporated the two exercise modalities, endurance and resistance, with the intent to recruit different skeletal muscle fibre types. The findings suggest that exercise modality appears to play a role in T cell VDR expression, with endurance exercise eliciting a greater effect compared

to a resistance exercise bout. This may be intensity or duration dependent, since the exercise bouts differed in both parameters, possibly presenting a different mechanical load. Both exercise protocols were designed to mimic those employed in the murine model by Makanae et al. (2015), however were altered to be reproducible by a human population. The HR zones differed during the exercise bouts, with the endurance bout ranging between 62-86 % HR_{peak}, and the resistance bout ranging between 50-81 % HR_{peak}. Therefore, the differing response could be attributed to the higher HR zone during the endurance bout, combined with the longer duration. In addition, only one leg was exercised in the RE trial, therefore reduced muscle recruitment compared to the EE trial. Therefore, the muscle fibre type and muscle mass recruited during exercise-induced muscle contraction could account for the difference in VDR expression observed between endurance and resistance exercise modality. Supplementation with vitamin D has previously been shown to increase myocyte VDR expression in type II fibres and not type I fibres (Ceglia et al., 2013). However, this contradicts our finding that endurance exercise, which is more focussed on type I fibre recruitment (Gollnick, Piehl, & Saltin, 1974), augments the increase in VDR compared to a more type II fibre based resistance protocol. It could also be argued that the participants are all recreationally active individuals that partake in different sports and activities and thus are not trained cyclists, therefore the muscle recruitment pattern and utilisation of aerobic and anaerobic systems may differ between participants, thereby affecting the response to the RE and EE trials. Moreover, it must be taken into consideration that these studies measure localised (to contracting skeletal muscle) metabolism, whereas the current investigation utilised a non-localised systemic model, which may account for the differing outcomes.

In summary, we have demonstrated that an acute bout of resistance and endurance exercise acutely increased T cell VDR expression in healthy males immediately upon cessation of the exercise bout, with the effect lost at 3 h post-exercise. The VDR response to exercise was found in circulating CD3 T cells, and the two subsets: CD4 and CD8 T cells. In contrast to the hypothesis, there was no observed influence of age on the response. These results suggest that exercise could be an efficient way to increase systemic cellular

VDR expression in a human population, although only on a short-term basis. Furthermore, as discussed, there may be an influence of cell mobilisation in response to exercise that may contribute to the increase in VDR expression observed. Interestingly, there was no correlation between the change in circulating cell number and VDR expression thereby concluding that exercise may have the capacity to increase intracellular VDR expression independent of T cell redistribution and mobilisation.

Chapter 7: Thesis Synthesis

7.1 Main findings

This thesis project was designed to address the main aim of exploring the role of exercise and ageing on vitamin D metabolism, investigating the relationships between physical activity and CRF, and vitamin D status and the key mediator of vitamin D metabolism: VDR.

The four aims of the thesis were:

I. To evaluate the vitamin D status of older adults in Scotland and determine whether there is a link with physical activity levels.
II. Investigate whether vitamin D status is influenced by age and associated with CRF.
III. Determine whether there is a relationship between vitamin D status and baseline VDR expression, and investigate the role of age and CRF on VDR expression.
IV. Explore the effects of acute exercise on VDR expression, and identify if this response is influenced by age.

The main findings of this thesis are:

- There is a high prevalence of severe deficiency (26 % and 40 %), deficiency (64 % and 29 %) and insufficiency (10 % and 31 %) in adults living in Scotland reporting an overall deficient vitamin D status of 14.5 ± 3.9 $ng \cdot ml^{-1}$ and 15.7 ± 6.4 $ng \cdot ml^{-1}$ (Chapters 4 and 5, respectively).
- There was an influence of season on 25(OH)D concentrations, with vitamin D status lower in winter compared to summer months (Chapter 4).
- Older adults drastically underestimate sedentary behaviour by as much as 7 h, underestimate LPA to a lesser degree, and overestimate MPA. Surprisingly, there was an inverse correlation between VPA and 25(OH)D concentration (Chapter 4).

- Baseline 25(OH)D concentration was not influenced by age or CRF, even though CRF was lower in older adults (Chapter 5). These data suggest that vitamin D status is likely dependent on environmental factors (UVB exposure) and dietary sources, as opposed to an individual's modifiable (CRF) and non-modifiable (age) characteristics.
- There is no observed relationship between the two measured key systemic vitamin D metabolites at baseline: circulating 25(OH)D concentration and T cell VDR expression (Chapter 5).
- Baseline circulating T cell VDR expression is associated with both CRF and age, with lower expression observed in older adults compared to younger populations, and a higher CRF associated with a greater density of receptors in the cells (Chapter 5).
- An acute bout of endurance and resistance exercise acutely increased circulating T cell VDR expression in healthy males (Chapter 6). Despite the difference in VDR expression at rest (Chapter 5), the short-term response to exercise was unaffected by age.
- There was a classic acute response of T cells to both endurance and resistance exercise bouts and although not entirely conclusive, the data suggests that VDR expression was upregulated in response to exercise independent of T lymphocyte mobilisation and translocation (Chapter 6).

7.2 General discussion

The main aim of this thesis was to investigate the influence of physical activity/exercise and age on vitamin D metabolism. The research questions in this thesis recognised that vitamin D plays a role in prevention of ill-health (Autier et al., 2014), primarily through the action of the VDR. However, it has been reported that there is a decline in vitamin D status (25(OH)D concentration) (Baker, Peacock, & Nordin, 1980; Maggio et al., 2005) and VDR expression with increasing age (Bischoff-Ferrari, Borchers, et al., 2004). This

presents an issue as age advances, and an avenue for intervention to improve vitamin D metabolism and promote 'healthy ageing'.

To the best of our knowledge, this is the first study to show in a human population that there is a decline in resting systemic lymphocyte VDR expression with age, and exercise alone can increase VDR expression in these cells, independent of age. Furthermore, we show that there is no association between vitamin D status and VDR expression, therefore age appears to alter vitamin D metabolism at the cellular level and not the circulating blood marker level. Moreover, a higher CRF predicts a higher expression of the VDR, suggesting aerobic fitness serves as a protective mechanism to beneficially influence vitamin D metabolism.

In Scotland, the "normal" baseline 25(OH)D concentration is far below the level classified as "sufficient" (>30 $ng \cdot ml^{-1}$) (Holick, 2009; Holick et al., 2011), with a strikingly low vitamin D status reported by several Scotland-based studies: 14.4 $ng \cdot ml^{-1}$ (Zgaga et al., 2011) and 12.7 $ng \cdot ml^{-1}$ (Weiss et al., 2016). Our data is in line with these reports, with a mean 25(OH)D concentration of 14.5 $ng \cdot ml^{-1}$ (VDS study) and 15.7 $ng \cdot ml^{-1}$ (ExVD study). Although an individual may be classified as vitamin D deficient or insufficient, there is a constant, albeit low, quantity of 25(OH)D compounds circulating in the blood (the findings are stated above). Therefore, there is a constant baseline capacity to convert 25(OH)D to the active form, $1,25(OH)_2D_3$, and thus a supply of the ligand available to enter target cells and find an available receptor. Vitamin D-induced benefits are modulated by the intracellular binding of $1,25(OH)_2D_3$ to the VDR and the translocation of this complex to the nucleus to bind to the VDRE (Pike et al., 2012). It could be suggested that the availability of VDR needs to be proportional to the number of $1,25(OH)_2D_3$ for the metabolic pathway to operate efficiently. Therefore, adequate VDRs in the cell that are available to receive the ligand, may be equally or more important as 25(OH)D concentration when determining the role of vitamin D in health and disease. Moreover, this thesis reports that there is no association between 25(OH)D concentration and VDR expression, therefore VDR expression in cells may be independent of "vitamin D status". Strategies to improve vitamin D metabolism currently identify supplementation with vitamin D as a solution to increase

25(OH)D availability (Autier et al., 2012) and thus potentially alleviate skeletal and non-skeletal disorders. However, future studies should consider the pathway as a whole when determining whether low 25(OH)D or impaired vitamin D metabolism is the cause or result of ill health.

Serum 25(OH)D concentration is measured as the main marker of vitamin D status (Holick, 2009) due to its close relationship with vitamin D_3 exposure (through dietary intake or dermal synthesis), and its relatively long half-life of around 15 days (K. S. Jones et al., 2014). However, since it is the biologically active form of vitamin D, $1,25(OH)_2D_3$, that enters the cell and binds to VDR, it may be thought to be the ideal measure to determine vitamin D status. In terms of the pathway, although an adequate supply of $1,25(OH)_2D_3$ to target cells is vital to progress towards VDR-induced gene activity, the relatively short half-life of this metabolite (4-15 hours) (Zehnder et al., 2001) renders it an unreliable and useless marker of vitamin D status and metabolism. In addition, the efficacy of tissues to exert VDR-induced biological effects will be dependent upon the ability of the cell to internalise $1,25(OH)_2D_3$. Cells express membrane-bound proteins, such as megalin, that play a facilitating role in the endocytosis of the $1,25(OH)_2D_3$-DBP complex to the cytoplasm (Abboud et al., 2013; Rowling, Kemmis, Taffany, & Welsh, 2006). In addition, circulating levels of $1,25(OH)_2D_3$ are a thousand fold less than 25(OH)D (Metzger et al., 2013). Taken together, 25(OH)D may be effectively used to help with the association of vitamin D deficiency and several disorders (Holick, 2006), but the VDR provides a better indication of the capacity to alter gene transcription. Measuring different metabolites may also be more relevant depending on the tissue type.

The antibody to the VDR used in our study does not have specific binding to unoccupied (“available”) VDR and thus binds to both occupied and unoccupied VDR, reported in other study’s (Bischoff-Ferrari, Borchers, et al., 2004). The unoccupied VDR may be a more sensitive measure to determine VDR available for binding to the ligand. It is therefore important to consider these limiting factors to the vitamin D metabolic pathway, particularly when reporting VDR expression as a marker of vitamin D metabolism-responsiveness. Nonetheless, this is the first study to employ flow cytometry analysis of

intracellular VDR protein expression in human primary cells. Therefore, in conjunction with the study by Bendix et al. (2015), it could be used to inform methods of future investigations and experiments.

This thesis reports that baseline 25(OH)D concentration is not influenced by age, however VDR expression significantly declines with age. Currently, it is not known whether low 25(OH)D concentration or another factor associated with ageing leads to decreased stimulation and expression of the VDR. In light of the results of this thesis, it could be suggested that low 25(OH)D concentrations do not influence VDR expression, in agreement with other studies (Bischoff-Ferrari, Borchers, et al., 2004; Kinyamu et al., 1997). This points towards other factors being responsible for disrupting cellular VDR expression in older persons. It should be noted that the VDR expression reported in this thesis is specific to circulating T lymphocytes, however it does represent a human cell model. This is similar to that reported in human skeletal muscle cells (Bischoff-Ferrari, Borchers, et al., 2004), whereby the authors reported decreased VDR expression with older age. These findings lead us to propose that measuring VDR expression responsiveness during investigative trials, in addition to the classically measured vitamin D status, is important for public health. Determining what exercise protocols yield the most effective increase of VDR expression may inform the design/approach of strategies and interventions aimed at improving the vitamin D endocrine system.

Although this may specifically benefit older populations, adults of all ages are defined as vitamin D deficient in Scotland. Therefore, perhaps people that reside in Scotland have a relatively low VDR expression at baseline compared to populations living at different latitudes. This is currently unknown and speculative. However, as a result there would be differing latitude-induced UVB exposure and thus skin synthesis of vitamin D contributing 25(OH)D concentrations, and since increasing 25(OH)D concentration through supplementation increases VDR expression (Ceglia et al., 2013), it follows that this may also occur in response to sunlight-induced increases in 25(OH)D concentration. Taking this into consideration, public health strategies to improve vitamin D health in adults that reside in Scotland should target all ages.

This thesis also reports that CRF is associated with, and predicts, baseline VDR expression in circulating T lymphocytes, but has no relationship with 25(OH)D concentration. This latter finding contradicts previous publications, whereby serum 25(OH)D concentrations are associated with $\dot{V}O_{2max}$ (Mowry et al., 2009) and predict $\dot{V}O_{2max}$ (Ardestani et al., 2011) in healthy adults. However, the latter studies were conducted in the US with reported baseline serum 25(OH)D concentrations of 34 $ng \cdot ml^{-1}$ and 46 $ng \cdot ml^{-1}$, which are substantially higher than that reported in our populations living in Scotland: 14.5 and 15.7 $ng \cdot ml^{-1}$. The UV Index differs between countries/latitudes, dependent on climate and weather and seasonal variations, therefore influencing vitamin D status (O'Neill et al., 2016). For that reason, the reports are specific to the country/location. Scotland is a region of low UVB radiation due to the high northern latitude (55°-59° North), with the prevalent cloud cover and poor weather conditions limiting sunlight-induced vitamin D skin synthesis (Rhodes et al., 2010). This relationship appeared in our findings and is in line with other studies in the UK (Hirani, 2013; Hypponen & Power, 2007; Kelly et al., 2015; Zgaga et al., 2011), whereby there appears to be an influence of season on 25(OH)D concentration, with vitamin D deficiency more prevalent in winter compared to summer months.

There is also an influence of season and weather on physical activity levels (Tucker & Gilliland, 2007), therefore CRF may decline in winter months. Although even in light of the influence that season likely has on these two factors, there was no association between baseline 25(OH)D concentration and CRF in the current investigations. Interestingly, it has been reported in an investigation conducted in the US, that increasing physical activity can increase circulating vitamin D independent of whether the activity is performed outdoors or indoors (Wanner et al., 2015). Thereby implying that factors other than sun exposure may be responsible for the higher 25(OH)D concentrations. It has also been reported that physically active people are active both indoors and outdoors regardless of season (Touvier et al., 2015). This suggests that physical activity may influence vitamin D metabolism, not only via sun exposure during such activities. In order to confirm, this would require both factors, physical activity and UVB exposure, to be investigated simultaneously.

This has been identified and supported as a future direction by other studies (K. Brock et al., 2010), with the feasibility of objectively measuring both physical activity and UV exposure through an app currently being investigated by researchers (Hacker et al., 2018).

If a higher CRF predicts a greater expression of VDR in cells, then methods to improve CRF may be a strategic approach in order to acquire the benefits associated with VDR-induced alterations in gene expression and protein synthesis. Particularly in older adults, since they have a lower CRF compared to younger populations. This concept was explored, whereby we investigated the responsiveness of VDR expression to a single bout of exercise, since regular exercise is associated with higher levels of CRF (Toth, Goran, Ades, Howard, & Poehlman, 1993). This study aimed to uncover the short-term response to exercise, thereby determining 1) whether exercise can increase VDR expression in humans, and 2) if there is a response, what is the duration of the effect? Previous reports in murine models have observed elevations in muscle tissue VDR expression in response to strenuous exercise (Aly et al., 2016; Makanae et al., 2015). To our knowledge, this is the first study conducted in humans showing that a single bout of exercise increases intracellular VDR expression. Following both an endurance and a resistance exercise protocol, VDR expression was immediately increased and remained elevated for 1 hour upon cessation of the exercise bout. VDR expression then declined to baseline levels after 3 hours, indicating a short-term response. The two bouts of exercise do differ with regards to work load and muscle mass recruited, therefore are perhaps not entirely comparable (as discussed in the Thesis Limitations).

Interestingly, although there is a lower baseline expression of VDR in older compared to younger individuals, age did not attenuate the responsiveness of cellular VDR expression to a bout of exercise. The biphasic pattern observed followed the same pattern regardless of the age group: 18-30 y, 31-45 y, 60-75 y. Taken together these results suggest that exercise could be an efficient way to increase systemic cellular VDR expression in all ages of a human adult male population.

7.3 Thesis limitations and future directions

There were a number of limitations in this thesis as outlined and discussed below. Limitations within research studies often present future directions. Where this is the case, suggestions for future work have been proposed.

The exercise bouts aimed to recruit the two muscle fibre types therefore inducing a different mechanical stress and metabolic demand. However, the duration and muscle recruitment of the two bouts were not matched and thus pose a limiting ability to compare the two types of exercise. Therefore, it cannot be concluded that either exercise modality induces a more pronounced response compared to the other. Future investigations should match bouts for workload and effort. Moreover, there are many different exercise modalities, which may alter the response of the vitamin D endocrine system. The protocols adopted in this thesis were designed to mimic those used by Makanae et al. (2015) in a murine model study. Ideally going forward for public health, focus should be directed towards protocols that the public are likely to engage in, in order to optimise the use of exercise as a strategy to improve vitamin D metabolic capacity. There is research on the affective responses and 'enjoyment' of specific exercise protocols, however these differ depending on the population. For example, high intensity interval training (HIIT) has received much attention over the past decade as a protocol to promote enjoyment and adherence to exercise (Heisz, Tejada, Paolucci, & Muir, 2016), yet still receives mixed feedback (Biddle & Batterham, 2015). Therefore, consideration of the population recruited (sex, health status, age, BMI, physical ability) and the potential compliance out-with a supervised/controlled circumstance (i.e. laboratory-based studies) should be at the forefront of future investigations in humans (Courneya, 2010).

In this thesis, T cells were used in the experiments, which represents a non-localised response to mechanical stress. T cells were used in the study as they are easily obtainable through a less invasive procedure, systemic blood collection, compared to muscle biopsy procedures, which would provide myocytes. In order to accompany and compliment the results of the findings of this thesis, it would be ideal to investigate VDR expression in myocytes as this

represents localised metabolism induced by exercise, particularly in the working muscles.

In the ExVD study it was found that an acute bout of exercise can increase VDR expression, however the effect was lost after 3 hours. Going forward it is of interest to determine whether a higher exercise load can elevate VDR to a higher degree, and/or for a longer duration. Moreover, if it is important to raise resting VDR expression in cells, then going forward it is of interest to investigate whether regular exercise can induce an adaptive response in cells to express VDR at a higher concentration. It may be that exercise only induces an acute response and thus exercise bouts must be repeated at regular intervals in order to benefit. Furthermore, since CRF is positively associated with VDR expression, using CRF as a measure of adaptation to regular exercise, and/or intentionally aiming to increase CRF through exercise training, may prove to be a source for elevated VDR expression and vitamin D endocrine metabolic capacity.

7.4 Conclusions and implications

As described in the Introduction and outlined in the Literature Review of this thesis, vitamin D deficiency is a worldwide epidemic, with Scotland being a prime example of a nation with low vitamin D concentrations, regardless of characteristics and lifestyle. The data in this thesis contributes to this consensus, as all individuals, whom did not consume vitamin D supplements or UVB treatment, were classified as vitamin D insufficient, deficient and severely deficient. Most tissues and cells of the body express key vitamin D metabolites and components, specifically the VDR, that mediate the vitamin D endocrine system and its functions: both genomic, mediated through the VDR transcriptional effects inside the cell nucleus, and non-genomic actions, rapid VDR-induced signalling. Another epidemic sweeping the UK and Scotland is a rise in sedentary behaviour and decline in physical activity levels.

This thesis has shown that there is a link between vitamin D metabolism and physical fitness and capacity, with the association residing with VDR expression as opposed to 25(OH)D_3 concentration (vitamin D status). The

findings are suggestive that a higher CRF is linked to a greater expression of VDR, but unfortunately VDR expression declines with age.

One of the main findings of this thesis is that exercise can acutely increase VDR expression in circulating T cells, suggesting exercise may be an effective strategy to alter the vitamin D endocrine system providing a potential platform to influence the pathophysiological development of chronic diseases. However, much research is required into the mechanisms involved and if this is a relationship and/or response seen in other cell types.

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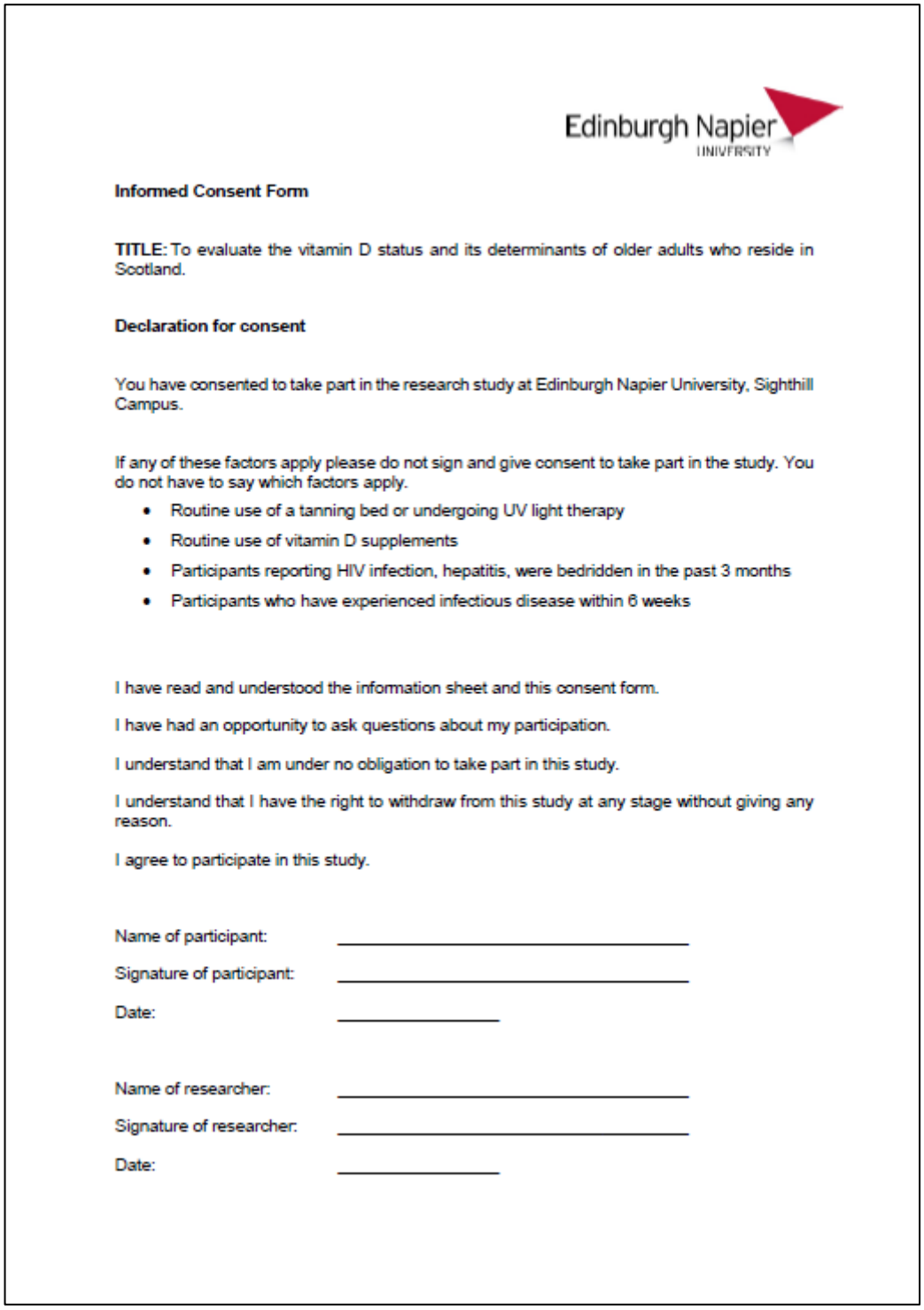
Appendices

Appendix 1 Consent Forms

VDS study



Edinburgh Napier UNIVERSITY

Informed Consent Form

TITLE: To evaluate the vitamin D status and its determinants of older adults who reside in Scotland.

Declaration for consent

You have consented to take part in the research study at Edinburgh Napier University, Sighthill Campus.

If any of these factors apply please do not sign and give consent to take part in the study. You do not have to say which factors apply.

- Routine use of a tanning bed or undergoing UV light therapy
- Routine use of vitamin D supplements
- Participants reporting HIV infection, hepatitis, were bedridden in the past 3 months
- Participants who have experienced infectious disease within 6 weeks

I have read and understood the information sheet and this consent form.

I have had an opportunity to ask questions about my participation.

I understand that I am under no obligation to take part in this study.

I understand that I have the right to withdraw from this study at any stage without giving any reason.

I agree to participate in this study.

Name of participant: _____

Signature of participant: _____

Date: _____

Name of researcher: _____

Signature of researcher: _____

Date: _____

School of Applied Sciences

Edinburgh Napier UNIVERSITY

Informed Consent Form

TITLE: The effects of a single bout of resistance or endurance exercise on 1,25$(OH)_2D_3$ bioavailability and vitamin D receptor expression in healthy young and older male adults.

Declaration for consent

You have consented to take part in the research study at Edinburgh Napier University, Sighthill Campus.

If any of these factors apply please do not sign and give consent to take part in the study. You do not have to say which factors apply.

- Routine use of a tanning bed or undergoing UV light therapy
- Routine use of vitamin D supplements
- Participants reporting HIV infection, hepatitis, were bedridden in the past 3 months
- Participants who have experienced infectious disease in the past 6 weeks

I have read and understood the information sheet and this consent form.

I have had an opportunity to ask questions about my participation.

I understand that I am under no obligation to take part in this study.

I understand that I have the right to withdraw from this study at any stage without giving any reason.

I agree to participate in this study.

Name of participant: _____

Signature of participant: _____

Date: _____

Name of researcher: _____

Signature of researcher: _____

Date: _____

Appendix 2 Physiology Screening Questionnaire

School of Applied Sciences

Edinburgh Napier UNIVERSITY

Physiology Screening Questionnaire

Please read the following carefully and answer all the questions truthfully. Information will be treated with the strictest confidence.

If you have any questions regarding the questionnaire, please ask the researcher.

To be completed by participant:

Have you ever had a cardiac-related issue such as a heart attack, hypertrophic cardiomyopathy, congenital abnormality, heart valve defect, heart failure or heart rhythm disturbance?	Yes / No
Have you ever received treatment for a heart problem such as heart surgery, the fitting of a pacemaker/defibrillator, coronary angioplasty or heart transplantation?	Yes / No
Are you currently taking medication for your heart?	Yes / No
How many units of alcohol do you consume a week? N.B One alcohol unit is measured as 10ml or 8g of pure alcohol. This equals one 25ml single measure of whisky (ABV 40%), or a third of a pint of beer (ABV 5-6%) or half a standard (175ml) glass of red wine (ABV 12%).	

Do you currently have or have you ever suffered from any of the following:	
Arthritis, osteoporosis or any other bone or joint problem?	Yes / No
Asthma, bronchitis or any other respiratory problem?	Yes / No
Coagulation disorders?	Yes / No
Diabetes (Type I or Type II)?	Yes / No
Epilepsy?	Yes / No
Hypertension (High Blood Pressure)?	Yes / No
Liver or gastrointestinal problems?	Yes / No
Kidney problems?	Yes / No
Infectious disease such as HIV, hepatitis or glandular fever?	Yes / No
Autoimmune disease?	Yes / No
Any peripheral or central nervous system disease? (e.g. Alzheimer's, Meningitis, Huntington's, Parkinson's, Tourette's)	Yes / No

Do you experience any of the following:	
Chest discomfort with exertion?	Yes / No
Unreasonable breathlessness?	Yes / No
Dizziness, fainting, blackouts?	Yes / No
Palpitations or skipped heart beats?	Yes / No
Unusual levels of fatigue?	Yes / No

Please indicate if any of the following apply to you:	
You have a close blood male relative (father or brother) who has had a heart attack before the age of 55 or a close female relative (mother or sister) who has had a heart attack before the age of 65?	Yes / No
You have elevated levels of cholesterol or are on lipid lowering medication?	Yes / No
You have or have ever suffered from depression?	Yes / No
You have known elevated levels of blood glucose?	Yes / No
You are completely inactive (do not take part in 20 minutes of moderate physical activity such as walking, 3 times per week)?	Yes / No
You have suffered a stroke or major cardiac event?	Yes / No
You have suffered from an infectious disease in last 6 weeks?	Yes / No
You use a vaporiser or an e-cigarette?	Yes / No
You are a cigarette smoker?	Yes / No
If you do smoke, how many cigarettes do you smoke per day?	

Are you currently taking any medications? (Please state)
Have you any other conditions that have not been mentioned above? (Please state)

Thank you for taking the time to complete this questionnaire. Please return the questionnaire to the researcher.

Appendix 3 American College of Sports Medicine (ACSM) Physical Activity Readiness Questionnaire (PAR-Q)

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Edinburgh Napier UNIVERSITY

Physical Activity Readiness Questionnaire (PAR-Q)

Regular physical activity is fun and healthy, and increasingly more people are starting to become more active every day. Being more active is very safe for most people. However, some people should check with their doctor before they start becoming much more physically active. If you are planning to become much more physically active than you are now, start by answering the seven questions in the box below. Common sense is your best guide when you answer these questions. Please read the questions carefully and answer each one honestly:

YES	NO		
☐	☐	1.	Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?
☐	☐	2.	Do you feel pain in your chest when you do physical activity?
☐	☐	3.	In the past month, have you had chest pain when you were not doing physical activity?
☐	☐	4.	Do you lose your balance because of dizziness or do you ever lose consciousness?
☐	☐	5.	Do you have a bone or joint problem that could be made worse by a change in your physical activity?
☐	☐	6.	Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?
☐	☐	7.	Do you know of any other reason why you should not do physical activity?

If you answered:

YES to one or more questions

Talk to your doctor by phone or in person BEFORE you start becoming much more physically active or BEFORE you have a fitness appraisal. Tell your doctor about the PAR-Q and which questions you answered YES.

- You may be able to do any activity you want – as long as you start slowly and build up gradually. Or, you may need to restrict your activities to those which are safe for you. Talk with your doctor about the kinds of activities you wish to participate in and follow his/her advice.
- Find out which community programs are safe and helpful for you.

NO to all questions

If you answered NO honestly to all PAR-Q questions, you can be reasonably sure that you can:

- Start becoming much more physically active – begin slowly and build up gradually. This is the safest and easiest way to go.
- Take part in a fitness appraisal – this is an excellent way to determine your basic fitness so that you can plan the best way for you to live actively.

Delay becoming much more active:

- If you are not feeling well because of a temporary illness such as a cold or a fever – wait until you feel better; or
- If you are or may be pregnant – talk to your doctor before you start becoming more active.

Please note: If your health changes so that you then answer YES to any of the above questions, tell your fitness or health professional. Ask whether you should change your physical activity plan.

Thank you for taking the time to complete this questionnaire. Please return the questionnaire to the researcher.

Reprinted from ACSM's Health/Fitness Facility Standards and Guidelines, 1997 by American College of sports Medicine

Appendix 4 American Heart Association (AHA)/American College of Sports Medicine (ACSM) Health/Fitness Facility Pre-Participation Screening Questionnaire

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AHA/ACSM Health/Fitness Facility Pre-participation Screening Questionnaire

Assess your health status by marking all true statements

History

You have had:

_____ A heart attack
_____ Heart surgery
_____ Cardiac catheterization coronary
_____ Angioplasty (PTCA)
_____ Pacemaker/implantable cardiac defibrillator
_____ Rhythm disturbance
_____ Heart valve disease
_____ Heart failure
_____ Heart transplantation
_____ Congenital heart disease

Symptoms:

_____ You experience chest discomfort with exertion
_____ You experience unreasonable breathlessness
_____ You experience dizziness, fainting, or blackouts
_____ You take heart medications

Other health issues:

_____ You have diabetes
_____ You have asthma or other lung disease
_____ You have burning or cramping sensation in your lower legs when walking short distances
_____ You have musculoskeletal problems that limit your physical activity
_____ You have concerns about the safety of exercise
_____ You take prescription medication(s)
_____ You are pregnant

If you marked any of these statements in this section, consult your physician or other appropriate health care provider before engaging in exercise. You may need to use a facility with a medically qualified staff.

Modified from American College of Sports Medicine and American Heart Association. ACSM/AHA Joint Position Statement: Recommendations for cardiovascular screening, staffing, and emergency policies at health/fitness facili-ties. Medicine and Science in Sports and Exercise 1998:1018.

Cardiovascular risk factors:

_____ You are a man older than 45 years

_____ You are a woman older than 55 years, have had a hysterectomy, or are postmenopausal

_____ You smoke, or quit smoking within the previous 6 months

_____ Your systolic blood pressure is >140 mmHg or diastolic blood pressure is >90 mmHg

_____ You do not know your blood pressure

_____ You take blood pressure medication

_____ Your blood cholesterol level is >200 mg/dl

_____ You do not know your cholesterol level

_____ You have a close blood relative who had a heart attack or heart surgery before age 55 (father or brother) or age 65 (mother or sister)

_____ You are physically inactive (i.e., you get <30 minutes of physical activity on at least 3 days per week)

_____ You are >20 pounds overweight

If you marked two or more of the statements in this section you should consult your physician or other appropriate health care provider before engaging in exercise.

_____ None of the above

You should be able to exercise safely without consulting your physician or other appropriate health care provider.

Thank you for taking the time to complete this questionnaire. Please return the questionnaire to the researcher.

Modified from American College of Sports Medicine and American Heart Association. ACSM/AHA Joint Position Statement: Recommendations for cardiovascular screening, staffing, and emergency policies at health/fitness facili-ties. Medicine and Science in Sports and Exercise 1998:1018.

Appendix 5 International Physical Activity Questionnaire (IPAQ) – Long Form

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** and **moderate** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?

   ☐ Yes

   ☐ No → *Skip to PART 2: TRANSPORTATION*

The next questions are about all the physical activity you did in the **last 7 days** as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, heavy construction, or climbing up stairs **as part of your work**? Think about only those physical activities that you did for at least 10 minutes at a time.

   _____ **days per week**

   ☐ No vigorous job-related physical activity → *Skip to question 4*

3. How much time did you usually spend on one of those days doing **vigorous** physical activities as part of your work?

   _____ **hours per day**
   _____ **minutes per day**

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads **as part of your work**? Please do not include walking.

   _____ **days per week**

   ☐ No moderate job-related physical activity → *Skip to question 6*

LONG LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised October 2002.

5. How much time did you usually spend on one of those days doing **moderate** physical activities as part of your work?

_____ **hours per day**
_____ **minutes per day**

6. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **as part of your work**? Please do not count any walking you did to travel to or from work.

_____ **days per week**

☐ No job-related walking → *Skip to PART 2: TRANSPORTATION*

7. How much time did you usually spend on one of those days **walking** as part of your work?

_____ **hours per day**
_____ **minutes per day**

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the **last 7 days**, on how many days did you **travel in a motor vehicle** like a train, bus, car, or tram?

_____ **days per week**

☐ No traveling in a motor vehicle → *Skip to question 10*

9. How much time did you usually spend on one of those days **traveling** in a train, bus, car, tram, or other kind of motor vehicle?

_____ **hours per day**
_____ **minutes per day**

Now think only about the **bicycling** and **walking** you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the **last 7 days**, on how many days did you **bicycle** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐ No bicycling from place to place → *Skip to question 12*

LONG LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised October 2002.

11. How much time did you usually spend on one of those days to **bicycle** from place to place?

_____ **hours per day**
_____ **minutes per day**

12. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐ No walking from place to place → *Skip to PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY*

13. How much time did you usually spend on one of those days **walking** from place to place?

_____ **hours per day**
_____ **minutes per day**

PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the **last 7 days** in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, chopping wood, shoveling snow, or digging **in the garden or yard**?

_____ **days per week**

☐ No vigorous activity in garden or yard → *Skip to question 16*

15. How much time did you usually spend on one of those days doing **vigorous** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, sweeping, washing windows, and raking **in the garden or yard**?

_____ **days per week**

☐ No moderate activity in garden or yard → *Skip to question 18*

LONG LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised October 2002.

17. How much time did you usually spend on one of those days doing **moderate** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, washing windows, scrubbing floors and sweeping **inside your home**?

_____ **days per week**

☐ No moderate activity inside home → *Skip to PART 4: RECREATION, SPORT AND LEISURE-TIME PHYSICAL ACTIVITY*

19. How much time did you usually spend on one of those days doing **moderate** physical activities inside your home?

_____ **hours per day**
_____ **minutes per day**

PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the **last 7 days** solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **in your leisure time**?

_____ **days per week**

☐ No walking in leisure time → *Skip to question 22*

21. How much time did you usually spend on one of those days **walking** in your leisure time?

_____ **hours per day**
_____ **minutes per day**

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like aerobics, running, fast bicycling, or fast swimming **in your leisure time**?

_____ **days per week**

☐ No vigorous activity in leisure time → *Skip to question 24*

LONG LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised October 2002.

23. How much time did you usually spend on one of those days doing **vigorous** physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis **in your leisure time**?

_____ **days per week**

☐ No moderate activity in leisure time → *Skip to PART 5: TIME SPENT SITTING*

25. How much time did you usually spend on one of those days doing **moderate** physical activities in your leisure time?
_____ **hours per day**
_____ **minutes per day**

PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekday**?

_____ **hours per day**
_____ **minutes per day**

27. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekend day**?

_____ **hours per day**
_____ **minutes per day**

This is the end of the questionnaire, thank you for participating.

LONG LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised October 2002.

Appendix 6 Participant Information: Sources of vitamin D_2 and D_3

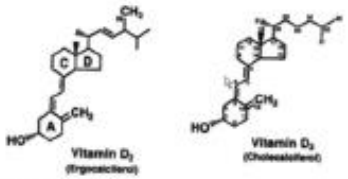
School of Applied Sciences

Edinburgh Napier UNIVERSITY

Participant Information

At stages in the study we will ask you to abstain from consuming foods that are natural or fortified sources of vitamin D_2 and D_3. We ask you to do this in the **24 hours prior to each trial and during the laboratory visits.**

Here is a list of sources to abstain from consuming:

Source	Vitamin D content
Natural sources	
	Vitamin D_2 (Ergocalciferol) Vitamin D_3 (Cholecalciferol)
Cod liver oil	~400–1,000 IU/teaspoon vitamin D_3
Salmon, fresh wild caught	~600–1,000 IU/3.5 oz vitamin D_3
Salmon, fresh farmed	~100–250 IU/3.5 oz vitamin D_3, vitamin D_2
Salmon, canned	~300–600 IU/3.5 oz vitamin D_3
Sardines, canned	~300 IU/3.5 oz vitamin D_3
Mackerel, canned	~250 IU/3.5 oz vitamin D_3
Tuna, canned	236 IU/3.5 oz vitamin D_3
Shiitake mushrooms, fresh	~100 IU/3.5 oz vitamin D_2
Shiitake mushrooms, sun-dried	~1,600 IU/3.5 oz vitamin D_2
Egg yolk	~20 IU/yolk vitamin D_3 or D_2
Sunlight/UVB radiation	~20,000 IU equivalent to exposure to 1 minimal erythemal dose (MED) in a bathing suit. Thus, exposure of arms and legs to 0.5 MED is equivalent to ingesting ~3,000 IU vitamin D_3.
Fortified foods	
Fortified milk	100 IU/8 oz, usually vitamin D_3
Fortified orange juice	100 IU/8 oz vitamin D_3
Infant formulas	100 IU/8 oz vitamin D_3
Fortified yogurts	100 IU/8 oz, usually vitamin D_2
Fortified butter	56 IU/3.5 oz, usually vitamin D_3
Fortified margarine	429 IU/3.5 oz, usually vitamin D_3
Fortified cheeses	100 IU/3 oz, usually vitamin D_3
Fortified breakfast cereals	~100 IU/serving, usually vitamin D_3
Pharmaceutical sources in the United States	
Vitamin D_2, ergocalciferol	50,000 IU/capsule
Drisdol (vitamin D_2) liquid	8,000 IU/cc
Supplemental sources	
Multivitamin	400, 500, 1,000 IU vitamin D_3 or vitamin D_2
Vitamin D_3	400, 800, 1,000, 2,000, 5,000, 10,000, and 50,000 IU

IU = 25 ng. [Reproduced with permission from M. F. Holick: *N Engl J Med* 357:266–281, 2007 (3). © Massachusetts Medical Society.]

This table is extracted from a research article titled: Evaluation, Treatment, and Prevention of Vitamin D Deficiency: an Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab.* by Holick *et al.* (2011).

If you are unsure, please contact the Primary Researcher, Hannah Lithgow on [redacted] or [redacted]