EFFECTS OF AGEING AND VITAMIN D DEFICIENCY ON VITAMIN D RECEPTOR (VDR) EXPRESSION IN HUMAN SKELETAL MUSCLE

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CONTENTS

CHAPTER 2: METHODS

APPENDIX

ABSTRACT

Background and aim. Vitamin D exerts its biochemical function on skeletal muscle through vitamin D receptors (VDR). Vitamin D deficiency is highly prevalent in the general population especially in the elderly. It is postulated that lower vitamin D levels lead to reduced expression of VDR in skeletal muscles. This then may lead to reduced muscle strength, function and ultimately falls in the elderly. The aim of this study was to examine the relationship between human ageing, circulating vitamin D levels and VDR expression in human skeletal muscle.

Methods. Twenty six participants were recruited to the study; 8 young participants, 8 older participants who were vitamin D sufficient (25-OH-D_3 ≥50 nmol/L) and 10 older participants who were vitamin D insufficient (<50nmol/L). Blood samples were obtained for the determination of serum 25-OH-D_3, calcium and parathyroid hormone (the latter only for older participants); and a muscle biopsy of their thigh (vastus lateralis muscle) was performed using a Magnum biopsy system. Real time quantitative PCR was used to measure the expression of VDR and some of

its target genes (myostatin, Sir1, PPARα and PPARδ) in human skeletal muscle. VDR protein content was measured using Western Blotting.

Main findings. Hypovitaminosis D was highly prevalent in the young participants recruited into the study, but it was not statistically different from the older participants who were vitamin D insufficient. Higher expression of VDR and PPARδ mRNA was observed in both older sufficient and insufficient groups when compared with the younger group ($p=0.01$). There was also higher sirt1 mRNA expression ($p=0.00$) and a tendency towards higher PPARα expression ($p=0.07$) in the older sufficient group when compared to the younger group. There was no difference in skeletal muscle content of the myostatin gene between groups. When the young group was compared with the older insufficient group, levels of VDR mRNA and PPARδ were still higher in the older group. Gene expression did not appear to be strongly influenced by circulating 25-OH-D_3 levels in any of the respective participant groups. Western blotting was unsuccessful in detecting VDR protein content despite the use of 2 different VDR antibodies. Tissue availability and time constraints precluded further work to be done.

Conclusion. Similar VDR mRNA and target gene expression levels were expressed in both older sufficient and insufficient groups; and higher VDR mRNA was seen in the older insufficient group compared with the younger participants. This suggests that the ageing process may be influencing the expression of these genes. Circulating 25-OH-D_3 levels did not appear to affect gene expression.

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DECLARATION

This study has been funded by a grant from the pump priming award of Nottingham University Hospitals NHS Charitable funds. The study design, regulatory and ethical approval were done by my supervisors, Dr. Kostas Tsintzas and Prof. Opinder Sahota. The recruitment of participants was done by myself and Dr. Kostas Tsintzas. Muscle biopsies were performed by myself and Dr. Laura Daunt. The molecular biology work presented in this thesis has been carried out by myself with the support and guidance of my supervisor, Dr. Kostas Tsintzas and Dr. Scott Cooper in the School of Life Sciences, University of Nottingham. Dr. Scott Cooper conducted the RNA and protein extraction; and western blotting.

I declare that this thesis has been prepared by me, and has not been submitted for a higher degree to any other university.

Terence Ong (2014)

ABBREVIATION

25-OH-D_3	25-hydroxycholecalciferol
1,25-(OH)2-D_3	1,25-dihydroxycholecalciferol
ATP	Adenosine triphosphate
CaBP	Calcium binding protein
ECL	Enhanced chemiluminescence
GST	Glutathione S-transferase
HRP	Horseradish peroxidase
mRNA	Messenger ribonucleic acid
NUH	Nottingham University Hospitals
PPAR	peroxisome proliferator-activated receptors
PTH	Parathyroid hormone
RANKL	receptor activator of nuclear factor (NF)-κB
RXR	Retinoid X receptor
Sirt1	Sirtuin1
UV	Ultraviolet
VDR	Vitamin D receptor
VDRE	Vitamin D response elements

LIST OF FIGURES AND TABLES

Figures

Tables

CHAPTER 1

INTRODUCTION

1.1 Background and brief history of vitamin D

Vitamins are a heterogenous group of organic substances obtained from dietary intake. They are only needed in small quantities for the maintenance of normal cell and organ function. Vitamin D is not in its truest sense a vitamin but a secosteroid as it is predominantly obtained intrinsically by the effect of ultraviolet (UV) radiation on previtamin D compounds in the skin and subsequent hydroxylation in the liver and kidneys [Basu 1996]. It was described as a vitamin during the early developments where deficiency of certain micronutrients in diet led to pathological conditions such as xerophthalmia (vitamin A deficiency), beri beri (vitamin B1 deficiency) and scurvy (vitamin C deficiency). Sir Edward Mellanby in the 1920s discovered that those suffering with rickets, a condition characterized by soft and weak bones, were cured when cod liver oil was given to them. Professor Elmer McCollum at John Hopkins University subsequently noted that after eliminating vitamin A from cod liver oil, its curative effects for rickets remained leading to the discovery

of a new micronutrient, which was called vitamin D. At the same time, researchers in Europe and Professor Harry Steenbock at the University of Wisconsin started to discover the relationship between ultraviolet light and adequate calcium homeostasis. However, it was not till the 1930s that the structure of vitamin D was isolated which perpetuated our understanding and our ongoing research of its biochemical properties and function [DeLuca 2014].

1.2 Structure, metabolism and regulation of vitamin D

Two forms of vitamin D, ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3) have most clinical relevance. Both forms of vitamin D can be obtained from dietary intake, such as oily fish, eggs and milk, and they are absorbed by the small intestine. However, predominantly human vitamin D is endogenously synthesized by the action of UV light to cholecalciferol or vitamin D_3 (Figure 1). UV light (wavelength 285-315nm) action on 7-dehydrocholesterol, a cholesterol based precursor in the skin leads to the formation of previtamin D_3. Previtamin D_3 remains in a thermal equilibrium with vitamin D_3 and its conversion is affected by the skin's properties (ethnicity, age) and the environment (UV exposure, geographical location). Both dietary and endogenous vitamin D undergoes

25-hydroxylation in the liver where a hydroxyl group is added at the carbon-25 atom to yield 25-hydroxycholecalciferol (or 25-hydroxyvitamin D, or 25-OH-D_3). This is the main circulating form of vitamin D and at normal plasma concentration of this metabolite, only small amounts of it are released into the systemic circulation to affect its target tissues. Hence, 25-OH-D_3 is the most routinely measured form of vitamin D in clinical practice. Next, there is further hydroxylation in the kidney which is a rate limiting step in the synthesis of vitamin D's active metabolite. The enzyme 1α-hydroxylase mediates the production of 1,25-dihydroxycholecalciferol (or 1,25-dihydroxyvitamin D, or 1,25-$(OH)_2$-D_3), the active hormone calcitriol. Multiple factors influence the regulation of the hydroxylation that happens in the kidney. This includes calcium, parathyroid hormone (PTH), calcitonin, growth hormone, insulin like growth factor-1 and fibroblast growth factor 23. 1,25-$(OH)_2$- D_3 itself suppresses its hydroxylation by 1α-hydroxylase ensuring optimal homeostasis within the body [Girgis 2013].

Another important enzyme in the metabolism of vitamin D is 24-hydroxylase which is highly present in the kidneys. It limits the amount of

1,25-$(OH)_2$-D_3 in target tissues by metabolising it into inactive forms (1,24,25-$(OH)_2$-D3 or 24,25-OH-D3).

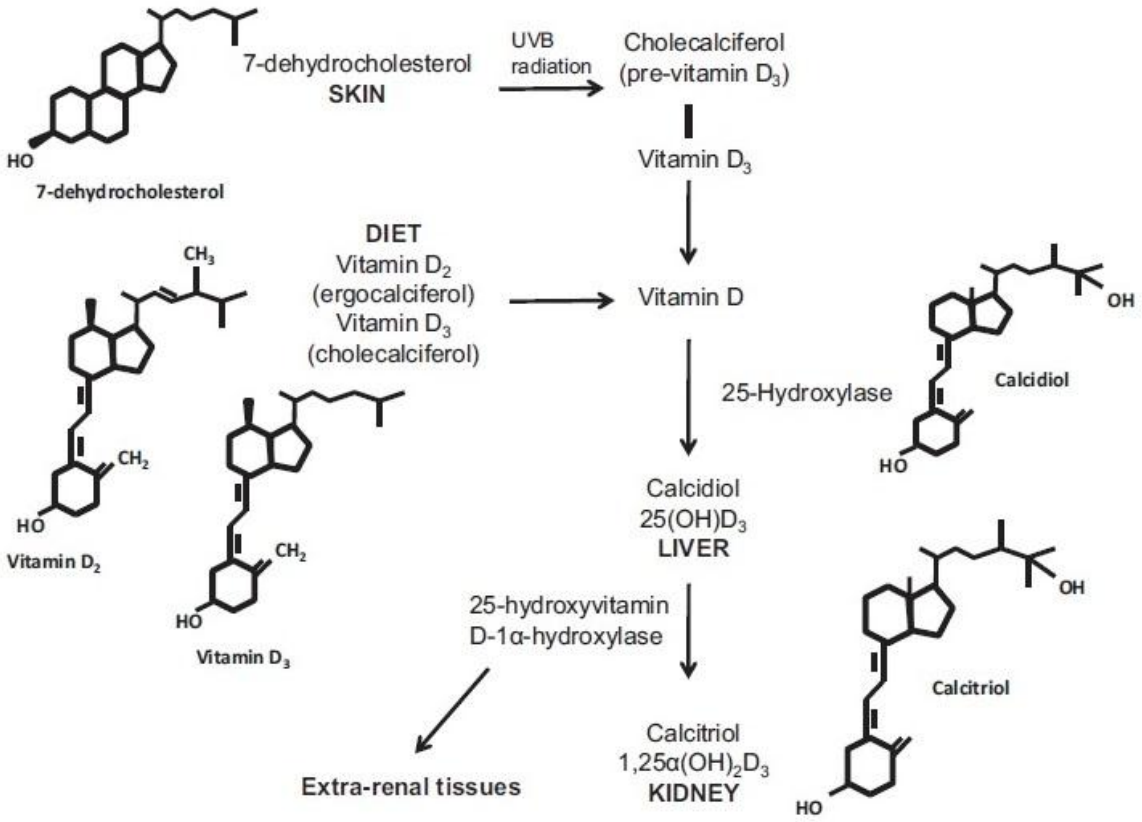


Figure 1 Vitamin D metabolism (adopted from Dirks-Naylor 2011). Metabolism of vitamin D from dietary intake and the skin precursor, 7-dehydrocholesterol by UV radiation to pre-vitamin D, and its subsequent hydroxylation in the liver and kidney to its active form.

Vitamin D regulates and is also largely regulated by calcium homeostasis. Low plasma calcium concentration activates calcium sensing receptors in the PTH gland increasing PTH release. This increases production of the active form of vitamin D, 1,25-$(OH)_2$- D_3 via the metabolic pathway described above. This active form of vitamin D_3 acts on osteoblasts by inducing the release of the receptor activator of nuclear factor (NF)-κB (RANKL) which in turn promotes osteoclastogenesis which will increases bone resorption. It also increases intestinal absorption of calcium by promoting its dietary absorption; and the activity of 1-hydroxylase in the kidneys to produce more active vitamin D. The 1,25-$(OH)_2$- D_3 synthesised and the rising calcium level will have a negative feedback on the expression of PTH which will ultimately reduce efforts to increase levels of calcium [Geissler 2005].

1.3 Function of vitamin D

Vitamin D's main role is in maintaining calcium (and phosphate) homeostasis by its action on the bone, intestine and kidneys. Vitamin D has been shown to strengthen bones. In animal models, incubation of bones with serum of animals given vitamin D has shown deposition of calcium and phosphate and strengthening of rachitic bones [DeLuca

2014]. Besides that, vitamin D has also been shown to mobilise calcium out of bones to maintain calcium homeostasis. It has also shown to be responsible for the absorption of calcium and phosphate from the intestine [Nicolayson 1953].

Vitamin D exerts its effects via genomic and non-genomic pathways. It exerts its genomic effects by regulating gene expression and modulation; and its non-genomic effects through muscle function via calcium, phosphate, insulin and glucose regulation.

Our initial understanding of the function of vitamin D started around its skeletal effects. From its initial discovery as an antirachitic element, it has been noted that a deficiency in vitamin D leads to weak, fragile and impaired growth of bones. In adults, a deficiency in vitamin D leads to osteomalacia where it is characterised by skeletal pain, muscle weakness and osteoporosis. A lack of vitamin D can also affect muscles and bones without the features of osteomalacia [Gelrup 2000]. It impairs function of osteoclasts and osteoblasts leading to a decline in bone density making bones susceptible to low trauma fractures.

In recent years, our understanding of vitamin D and its function have extended beyond its role in bone integrity and calcium homeostasis. Current literature has drawn links between vitamin D and falls [Bischoff Ferrari 2009], reduced muscle strength [Glerup 2000], cancer [Garland 2006, Deeb 2007], diabetes mellitus [Hypponen 2001, Pittas 2007], cardiovascular disease [Wang 2008], and the immune system [Cantorna 2004, Kamen 2010]. This is because vitamin D receptors (VDR) have been located in bone, gastrointestinal tract, liver, pancreas, kidney, heart, skeletal muscles, immune system, nervous system, and other endocrine organs [Coombs 1998]. This discovery adds weight to the extraskeletal effects of vitamin D and has fuelled research into better understanding of the function of vitamin D and whether either vitamin D levels or supplementation of vitamin D has beneficial clinical implications beyond bone health.

1.4 Vitamin D and vitamin D receptors

Vitamin D receptors (VDR) was first discovered in 1974 by Brumbaugh and Haussler in animal intestines [Brumbaugh 1974]. Since then, VDR has been detected in other tissue such as the kidneys, bone and skeletal

muscle. The integral importance of VDR in musculoskeletal function has been well demonstrated in VDR knock-out mice where these mice exhibit traits consistent with rickets. Some of these similar features were also noted in these mice and patients with vitamin D dependent rickets type 2 where the defect is within the VDR itself [Kato 2000]. VDR knock-out mice were also noted to have a lack of muscle development [Montero 2005]. Using immunohistochemical methods, Bischoff et al were able to demonstrate the presence of VDR in human skeletal muscle. They were able to demonstrate strong intranuclear immunostaining of VDR in gluteal and tranversospinalis muscles using monoclonal antibody 9A7 [Bischoff 2001]. Circulating 1,25-$(OH)_2$-D_3, bound to vitamin D binding protein is transferred to its target tissue where it exerts its biological effects through its binding with vitamin D receptors (VDR). This vitamin D-VDR ligand then activates target gene expression at the transcriptional level [Kato 2000]. Hence, it is the internal synthesis of vitamin D by the action of UV radiation on its precursor in the skin; the synthesis of its active metabolite away from its origin; its action depending on receptor proteins; and its regulation by a feedback mechanism, which makes vitamin D more akin to the action of a hormone [Basu 1996].

As described earlier, the genomic pathway from which 1,25-$(OH)_2$-D_3 works involve binding with liganded nuclear VDR. Nuclear VDR forms homodimers, or heterodimers with retinoid X-receptors (RXR). RXRs are nuclear receptors that are activated by 9-cis retinoic acid, a metabolically active form of vitamin A. The VDR homodimer or heterodimer then binds to specific enhance elements, called vitamin D response elements (VDRE) in response to activation by the circulating 1,25-(OH)2-D_3. It is this binding mechanism that leads to its ability to affect the cellular genomic pathway. Co-activators that interact with VDR have also been recently shown to be essential for the transcription process but their precise mechanism is yet to be fully understood [Kato 2000, Haussler 2008, Girgis 2013]. The vitamin D dependent modulation of mRNA subsequently leads to de novo protein synthesis which affects target genes that are associated with cell differentiation and proliferation, energy metabolism, hormonal signalling, mineral homeostasis, oncogenes, and vitamin D metabolism (Figure 2). The first gene product or protein identified was calcium binding protein (CaBP). Different forms of CaBP have later on been described and these are called calbindins [Combs 1998, Annweiler 2010].

One other protein that has recently being identified as a target of VDR expression is Sirtuin1 (sirt1). Sirt1 is a NAD dependent protein deacetylase that has a complex metabolic function which includes gene splicing, DNA repair and metabolic regulation [Price 2012]. It is found in a number of tissues, which include liver, brain, adipose tissue and skeletal muscles [Satoh 2013]. Price et al demonstrated in mice models that sirt1 overexpression had a similar effect with treatment with resveratrol (2,3,40-trihydroxystilbene), a naturally occurring substance found in certain plants that is associated with delaying the ageing process [Price 2012]. They were also able to demonstrate that resveratrol treated mice had more type 2 muscle fibres and better mitochondrial function; and that resveratrol function was dependent on sirt1 expression. Satoh et al has also shown that overexpressed sirt1 mice were shown to have significant life span and delay in the ageing process compared to control mice [Satoh 2013]. They demonstrated that brain specific sirt1 overexpressing mice had a slower rate of ageing, physiological decline and more youthful skeletal muscle structure, with better organisation of sarcomeres and less abnormal mitochondria, compared to control mice. Mitochondrial dysfunction is recognised to be pathognomonic of the ageing process and development of disease, and the findings described here highlights the crucial role sirt1 plays in the ageing process [Erol 2007]. Sirt1 is also

recognised in its role of promoting oncogenesis and also as a tumour suppressor gene, thus, raising its role in cancer on top of healthy ageing. An et al were able to show a link between vitamin D signalling via VDR and Sirt1 through its regulation of FoxO protein, a group of transcription factors that regulate cell proliferation, growth and longevity. One of the ways FoxO is regulated is by acetylation which can be reversed by sirt1. This reduces DNA binding and enhances phosphorylation and inactivation. VDR is associated directly with sirt1 in a partially hormone-dependent manner, as assessed by GST pulldown assay and coimmunoprecipitation. Vitamin D also enhances the recruitment of sirt1 to FoxO3a, as shown during coimmunoprecipitation of Sirt1 with an anti-FoxO3a antibody [An 2010].

Another target gene for vitamin D function are the peroxisome proliferator-activated receptors (PPARs). PPARs are proteins that function as transcription factors to regulate the expression of genes. It has a fairly extensive role that includes cell proliferation, differentiation, glucose homeostasis, insulin sensitivity, lipid metabolism, bone formation and tissue remodelling [Erol 2007]. PPARs are noted to be involved in the inflammatory process seen in ageing, where it suppresses the expression

of inflammatory genes, cytokines and acute phase proteins [Chung 2008]. 3 isoforms exist which are alpha (α), delta (δ) and gamma (γ). PPARα is expressed in tissues with high fatty acid catabolism such as kidney, heart and skeletal muscles; PPARδ in skin, brain, adipose tissue, kidney, heart and digestive tract; and PPARγ in adipose tissues [Sertznig 2009]. PPARs are able to form heterodimers with RXR, similar to VDR to specific DNA regions of target genes. This heterodimer of PPAR/VDR-RXR bind to their specific response elements, have interconnected pathways that regulate the target genes and its respective transcription factor mRNA levels [Matsuda 2013]. This crosstalk link between VDR and PPAR means that both is able to influence each other's pathway and that PPAR is a coactivator in the way vitamin D-VDR exerts its genomic effects. Certainly this link has been demonstrated in studies looking at breast and skin cancer [Sertznig 2009, Alimirah 2012].

1,25-$(OH)_2$-D_3 leads to uptake of calcium into muscles and this is inhibited when VDR pathways are blocked. This points towards a direct non-genomic role for VDR in muscle function [Girgis 2013]. The non-genomic effects of vitamin D arise from a series of complex intracellular signal transduction pathways after binding to a nonnuclear receptor, membrane

bound VDR [Montero 2005; Girgis 2013]. These non-genomic effects have been the subject of much research interest, especially its effect on skeletal muscle due to the high prevalence of muscle weakness, falls and subsequent fracture in a progressively ageing population.

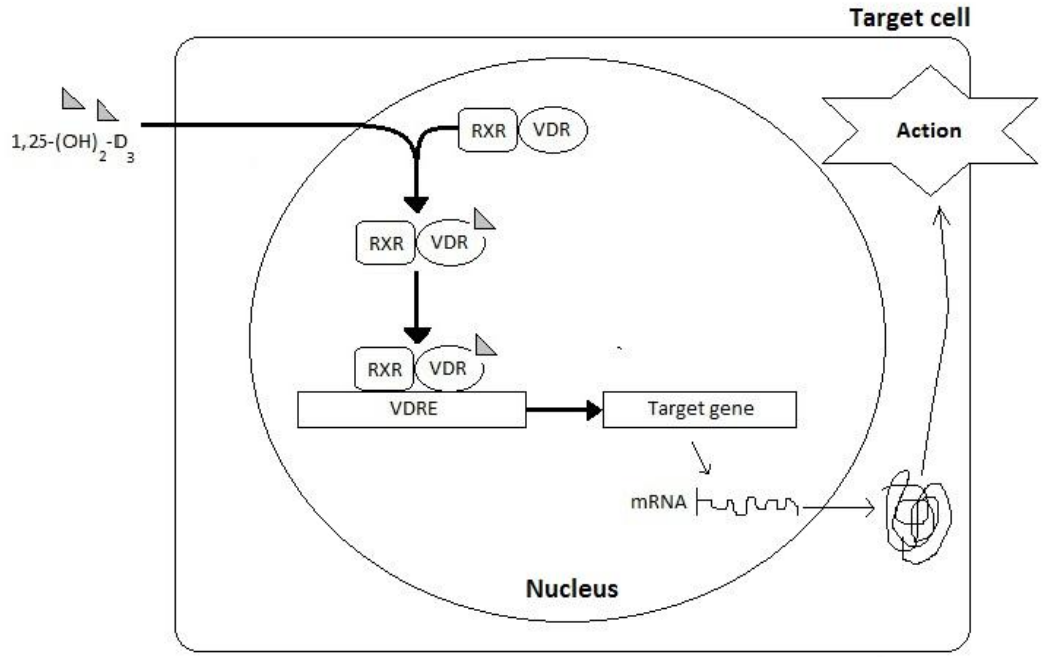


Figure 2 Molecular mechanism of vitamin D action through VDR mediated gene expression (adapted from Kato 2000). It shows 1,25-$(OH)_2$-D_3 bound to VDR-RXR heterodimers to affect the target gene, its promotion of mRNA and subsequently its action in its target tissues.

1.5 Skeletal muscle and vitamin D

Skeletal muscles are made of grouped fascicles which itself is made up of individual muscle fibres. When muscle contracts, the sarcomeres and myofibrils which make up the individual muscle fibres decreases and shortens. Despite this, the muscle length itself does not change. This is explained by Huxley's sliding filament theory of contraction. It states that during contraction, the thick and thin filaments of the muscle slide past each other and overlaps. This contraction is modulated by an electrical event, an action potential which causes a transient rise in intracellular calcium levels [Marieb 1998]. The action potential and its muscle contraction is regulated by oxidative and glycolytic metabolic pathways [Girgis 2013]. Macroscopically, muscle contraction is dependent on size, fibre composition and individual fibre functional capacity. Skeletal muscles are divided by histochemical staining that determine the pH lability of myofibril ATPase activity to myosin heavy chain (MHC) type 1 (slow activity) and type 2 (fast activity). Besides generating force and contraction, skeletal muscles are a highly metabolic organ that responds to hormones and factors, such as exercise. Calcium is noted to be an integral component in muscle contraction.

The relationship between vitamin D and skeletal muscle has been well observed in those with osteomalacia, where vitamin D deficiency leads to muscle weakness. However, those deficient may also exhibit muscle weakness without any of the features of osteomalacia [Glerup 2000]. Other patterns observed include proximal weakness and a waddling gait [Ceglia 2008]. Musculoskeletal pain has also been reported in those lacking in vitamin D [Montero 2005]. Vitamin D supplementation in those with deficiency also appears to improve muscle strength, postural stability and gait speed [Dhesi 2002, Bischoff-Ferrari 2004, Pfeifer 2009]. Research has also shown the benefits of vitamin D in preventing falls in those deficient in community dwellers and those in long term institution which support the role vitamin D plays in maintaining muscle strength and balance [Cameron 2012].

Those with vitamin D deficiency have been predominantly shown to have atrophy of type 2 fibres on muscle biopsy. It is the fast acting type 2 fibres that are called to action in the event of a fall to attempt to maintain posture and balance [Ceglia 2008]. Supplementation with vitamin D has been shown to increase the number and size of these fibres resulting in an increase in muscle strength [Sato 2005] which may explain the

improvement seen in clinical practice. Central to this is the very presence of VDR in skeletal muscle in regulating calcium homeostasis and muscle contraction. This VDR genomic pathway has been described to affect calcium uptake and transportation of phosphate in muscle cell; and to mediate cell proliferation and differentiation into mature muscle fibres [Janssen 2002].

Myostatin, a growth differentiation factor of the transforming growth factor beta (TGF β) family, is found in skeletal muscle and is recognised to play a role in inhibiting muscle growth and differentiation. Girgis et al showed in C2C12 skeletal muscle cells treated with 25-OH-D_3 that there was down regulation of myostatin with associated larger myotubules. [Garcia 2011]. This suggest the presence of CYP27B1 in converting the 25-OH-D_3 to 1,25-$(OH)_2$-D_3 and that may myostatin may be a target gene of VDR. The effects of vitamin D and VDR on other TGF β family members has been studied and shown in other organs such as how the skin reacts to injury [Luderer 2013] and inhibiting liver fibrosis [Ding 2013] supporting this pathway in skeletal muscles too.

Supplementation of vitamin D induces rapid changes in calcium metabolism of the skeletal muscle cell that cannot be explained by a slow genetic pathway suggesting a non-genomic pathway that vitamin D and VDR influences skeletal muscles [Janssen 2002].

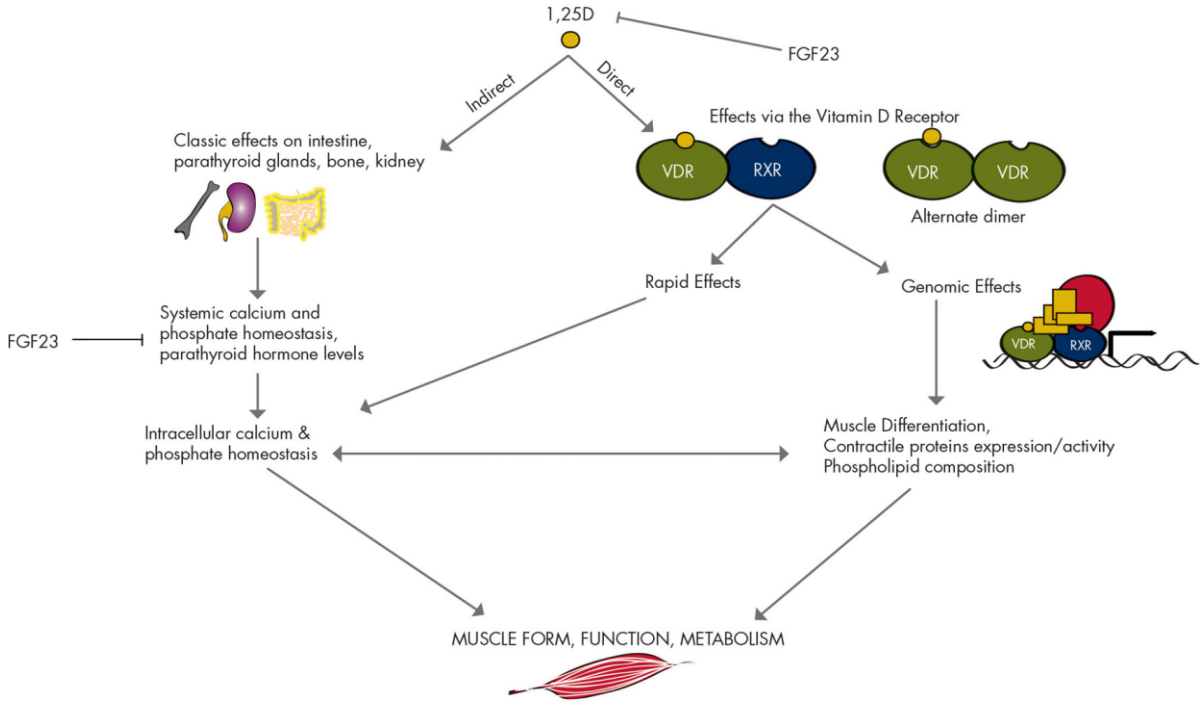


Figure 3 Genomic and non-genomic effects of vitamin D on muscle. Genomic effects are mediated by the formation of VDR-RXR heterodimers or VDR homodimers to affect muscle structure, function and metabolism. The non-genomic effects are through calcium and phosphate homeostasis (adapted from Girgis 2013)

One big challenge in all this is the accurate identification of VDR in the target tissues, in this case skeletal muscles. Early studies began with using radio-labelled vitamin D and autoradiography [Stumpf 1995]. This radio-labelled assay is injected and followed via radiation signal to get an idea of its distribution within the body. Although sensitive, errors in VDR detection occurred as a result of receptor stability, ligand/receptor dissociation, or the presence of an endogenous ligand. Also, VDR expression is determined by vitamin D status and those deficient will expect a down regulation of VDR expression and this method of identification of VDR in vitamin D deficient animals may not accurately represent normal physiological VDR expression [Wang 2010]. More recently, immunoassays using VDR antibody has been used to detect VDR in target tissues. More than 10 VDR antibodies are commercially available but none has been systematically analysed for its sensitivity and specificity. Studies so far have not been conclusive in detecting VDR expression. For example, Bischoff et al using VDR 9A7 antibody showed the presence of VDR in skeletal muscles [Bischoff 2001] but Wang et al using VDR D6 antibody was unable to confirm the presence of protein for VDR, although VDR mRNA levels were detected [Wang 2011]. Wang et al in their review used multiple immunoassays with different antibodies

(IVG8C11, XVIE6E6, C20, D6, N20, H81, 9A7) and control samples on VDR knockout mice to study this further. They demonstrated that of the antibodies tested, the D6 had the ideal combination of high specificity and high sensitivity (Figure 4). They also conclude that due to a lack of well characterised VDR antibody, proper controls and standardised protocol have led to the contradictory findings from such studies on identifying VDR in target tissues, such as in skeletal muscles [Wang 2010].

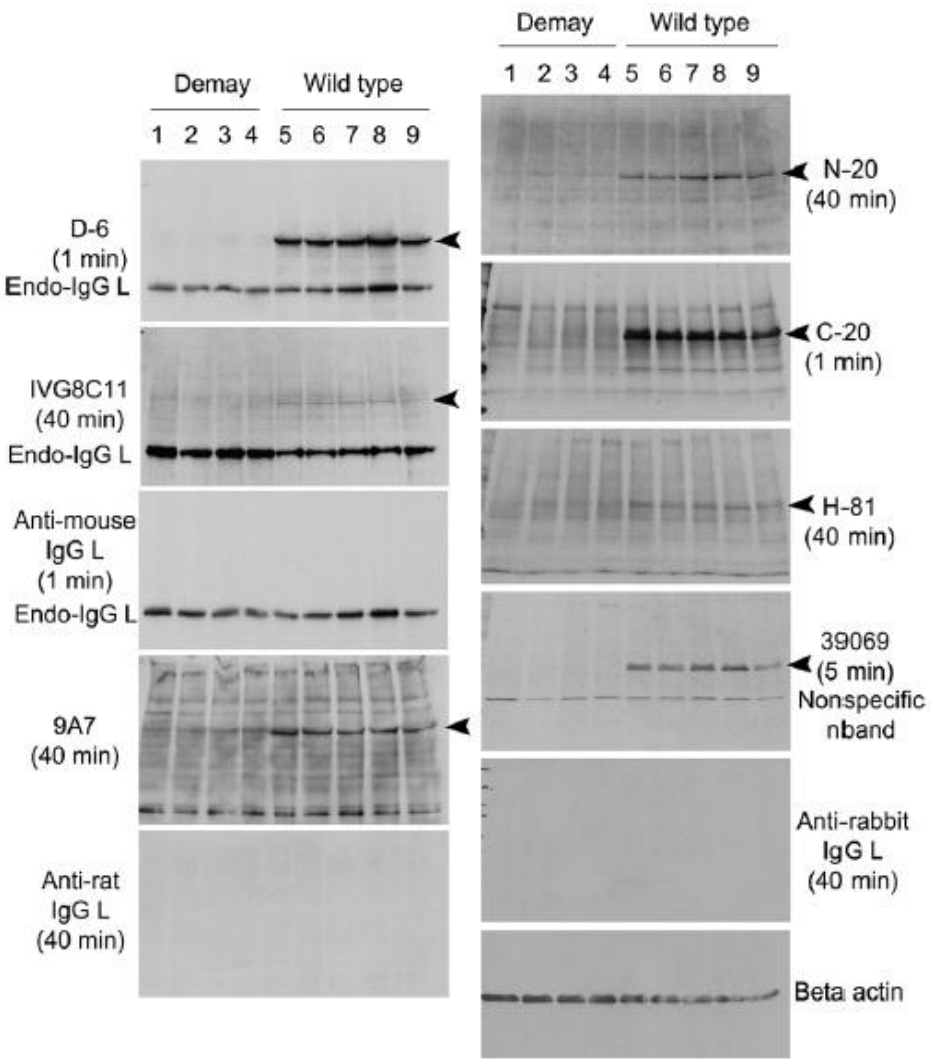


Figure 4 Immunoblotting of mouse duodenal lysates from VDR KO Demay (lanes 1-4) or wild type (lane 5-9) mice. Blots were incubated with VDR antibodies (D6, IVG8C11, 9A7, N20, C20, H81, 39069). The blots were incubated with HRP-conjugated secondary antibodies. For the blots used to evaluate nonspecific reactions of secondary antibodies with tissue, the primary antibody was omitted. The HRP signal was developed by incubation of the blot with ECL solution and captured by X-ray film for increments of a variable time indicated in this figure. The VDR band on each blot was denoted by the arrowhead. The blots were also reprobed with an antibody to beta actin as a loading control (Adopted from Wang et al 2010)

1.6 Vitamin D and ageing

Vitamin D deficiency is prevalent in older people. In the United Kingdom, the prevalence of vitamin D deficiency (25-OH-D_3 less than 25nmol/L) of older people living in the community was as high as 15% and those in institutions was around 30% [Hirani 2005]. This prevalence is known to increase further especially in the winter. An older person produces less than 30% of the amount of vitamin D of a younger person when exposed to the same amount of sunlight [Holick 1989]. Low vitamin D levels in this group is multifactorial in nature through a combination of reduced sunlight exposure due to reduced mobility; reduced dietary intake; malabsorption; low skin thickness reducing previtamin D synthesis; and impaired hydroxylation in the liver and kidneys.

We have established so far that the presence of VDR is paramount in the genomic and non-genomic function of vitamin D and its effect on skeletal muscle. However, not many studies have looked at the relationship between circulating vitamin D, VDR in skeletal muscle, human ageing and how it affects skeletal muscles. Rat models have shown that with age,

there is a decline in VDR expression in intestine, bone and kidneys [Montero 2005, Horst 1990]. Bischoff et al was able to demonstrate that VDR expression in human skeletal muscle declines with age using monoclonal VDR antibody 9A7 and that VDR expression was not related to serum 25-OH-D_3 levels [Bischoff 2004]. Clinically, human ageing is associated with a progressive reduction in muscle mass, strength and function. The reduced VDR expression will reduce the functional response of the muscle cells to vitamin D. The presence of vitamin D insufficiency which is highly prevalent in older people has clinically been shown to aggravate the problem further. This is likely due to the already impaired functional response due to limited VDR expression, but also the low circulating vitamin D will lead to further down regulation of VDR [Bischoff 2001]. Over a period of time, this ongoing muscle loss of strength and function may lead to a phenomenon called sarcopenia that is currently stimulating a lot of research interest as it appears to be a key presence in frailty syndromes of older people, as well as being an outcome of the ageing and frailty process. Studies have shown that those deficient with 25-OH-D_3 levels less than 25nmol/L have lower muscle strength, especially in the proximal lower limbs [Visser 2003; Stockton 2011]. Glerup et al showed that reduced muscle strength and function begins even before bone disease sets in those who are vitamin D deficient

[Glerup 2000]. Muscle biopsies of patients deficient in vitamin D show atrophy of the type 2 fast acting muscle fibres. These muscle fibres were noted to have enlarged interfibrillar spaces and infiltration with fat, fibrosis and glycogen granules [Montero 2005]. The type 2 muscles are needed to react in the event of a loss of upright posture. This perhaps goes some way to explaining the increase incidence of falls in older people who are deficient in vitamin D. One study has shown that replacement with vitamin D increases the number of this type 2 muscle fibres [Sorensen 1979].

Another explanation to the reduced muscle strength and function is the higher PTH levels in those who are vitamin D deficient. In rat models, PTH has been demonstrated to impair energy production, transfer and utilisation in muscle [Baczynski 1985]. PTH induces IL-6 production [Mitnick 2001] and raised IL-6 levels in older people are associated with lower muscle mass and strength [Visser 2002], and may influence sarcopenia [Payette 2003]. Secondary hyperparathyroidism has been noted in clinical studies to be a marker for reduced lower limb strength and that correcting it will restore muscle function [Vissier 2003]. However, this phenomenon may ultimately be related to the underlying

cause for the secondary hyperparathyroidisim which is vitamin D deficiency and correcting the deficiency also corrects the high PTH concentration.

Falls in the elderly is associated with worse healthcare outcomes, i.e. recurrent falls, hospitalisation, institutionalisation, fracture, and mortality. Hence, much research has been done looking at the role of vitamin D supplementation to improve muscle strength to ultimately reduce to likelihood of suffering a fall. There is certainly good evidence now supporting the role of vitamin D in doses between 700 – 1000 IU in prevention of falls in those that are deficient in vitamin D [Bischoff-Ferrari 2009; Cameron 2012]. This further supports the role that vitamin D plays in maintaining muscle function integrity.

1.7 Thesis aims

There is a paucity of studies into how human ageing and serum vitamin D levels affect VDR expression in skeletal muscle. Only one study that has been described earlier has so far examined VDR expression in human muscle tissue in relation to age using 2 different muscle groups (gluteus medius or transversospinalis muscle) [Bischoff 2004]. This present thesis aims to examine the relationship between human ageing and circulating vitamin D level to VDR expression. It is hypothesised that ageing and lower circulating levels of vitamin D is associated with lower expression of VDR and the genes dependent on vitamin D in human skeletal muscles. This study will contribute to our current understanding of whether VDR expression is affected by either the ageing process or low serum vitamin D levels. Besides that, the presence of VDR in skeletal muscle has been subject to much debate mostly due to the lack of specificity of the antibodies used to detect it either by Western blotting or immunohistochemistry. Hence, this thesis aims to contribute to this debate demonstrating VDR to be central in the way vitamin D influences skeletal muscle function. By relating vitamin D levels (serum 25-OH-D_3 concentrations) to age and expression of VDR, and some of its target genes (sirt1, PPARα, PPARδ, and myostatin) in skeletal muscle, this thesis aims to increase our understanding of the function of skeletal muscle. In

the near future, a better understanding of this can hopefully be translated into a clinical setting that will further guide how we treat either vitamin D deficiency to improve muscle function, reduce falls and fractures.

CHAPTER 2

METHODS

2.1 Study design

This is a prospective cross-sectional study involving blood and muscle sampling of young and older groups of participants.

2.2 Participants

Twenty six participants were recruited to the study. Eight young participants (18 – 25 years) were recruited from the University of Nottingham student population through the human physiology lab and notice board advertisements. Eighteen older participants (>65 years) were recruited from the bone health clinic, Queens Medical Centre, Nottingham University Hospitals NHS Trust. The older group was divided into two groups consisting of those vitamin D sufficient, defined as a

circulating level of 25-OH-D_3 >50nmol/L, and those vitamin D insufficient participants, defined as a circulating level of 25-OH-D_3 ≤50nmol/L.

Potential participants were invited to participate in the study voluntarily. After written consent was obtained, all participants underwent a medical screening which included a medical questionnaire for any potential exclusion criteria and having a blood sample taken. Blood pressure measurements were also performed during the screening process and the young participants had an electrocardiogram performed. Those excluded were taking corticosteroids, had a diagnosis of chronic kidney disease (defined by a serum creatinine level >250mmol/L), had a diagnosis of a neuromuscular disease, overt diabetes mellitus, or a condition that is known to affect vitamin D metabolism.

2.3 Muscle biopsy

Participants were asked to return on a separate occasion for a muscle biopsy of the thigh (vastus lateralis) at the human physiology laboratory. Current techniques to obtain muscle specimen includes an invasive open biopsy approach, the use of a Weil-Blakesley conchotome (Figure 5, a

forcep consisting of a sharp biting tip) and the more common Bergstrom needle (Figure 6, which consists of a sharp trochar, a cutting cannula and a pushing rod to expel the muscle specimen) [Patel 2012]. These approaches involve either an incision or a semi-incision/semi-open approach that can cause pain, discomfort and leave the potential for scarring. Hence, minimally invasive approaches, similar to biopsy studies of breast, prostate or kidney tissues offer an attractive option in this research. Although this technique still involves piercing the skin and fascia to get to the muscle, the smaller incision means it is less likely to scar, cause discomfort and will heal quicker. Besides that, there is evidence that this technique of minimally invasive biopsy is comparable to the more common semi-open Bergstrom approach, which is considered the gold standard approach [Hayot 2005].

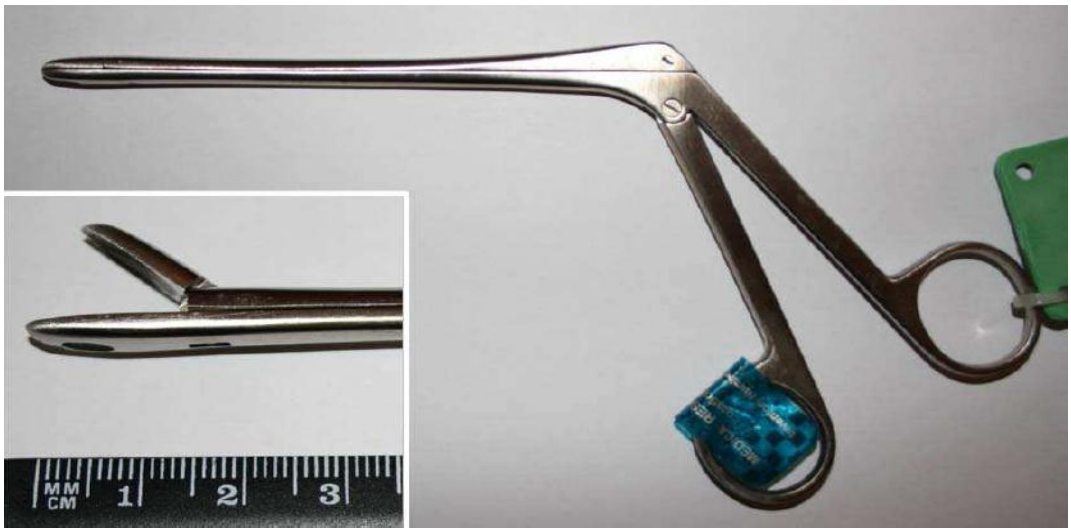


Figure 5 Weil-Blakesley conchotome for obtaining muscle biopsy (from Patel HP et al 2012)

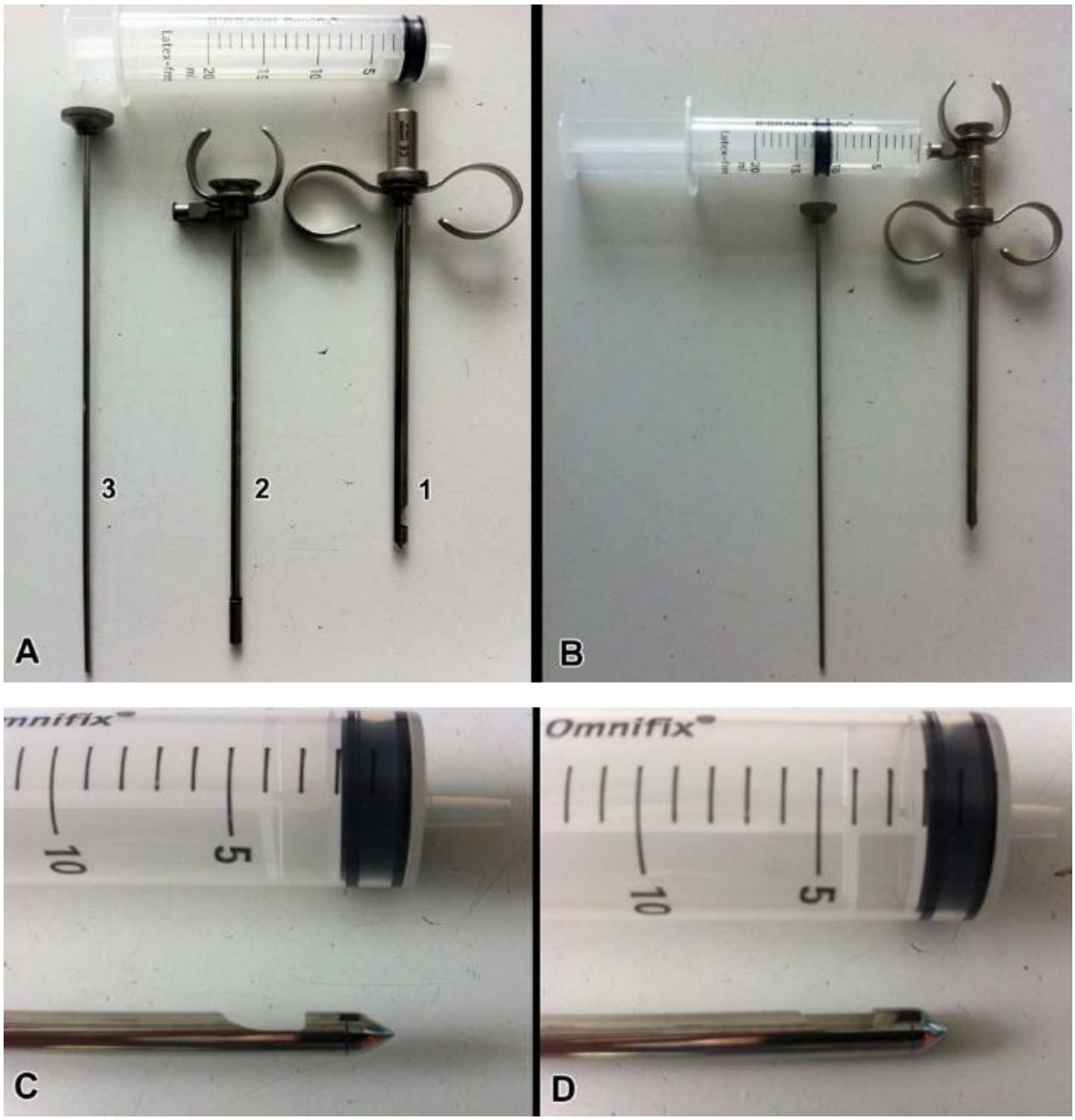
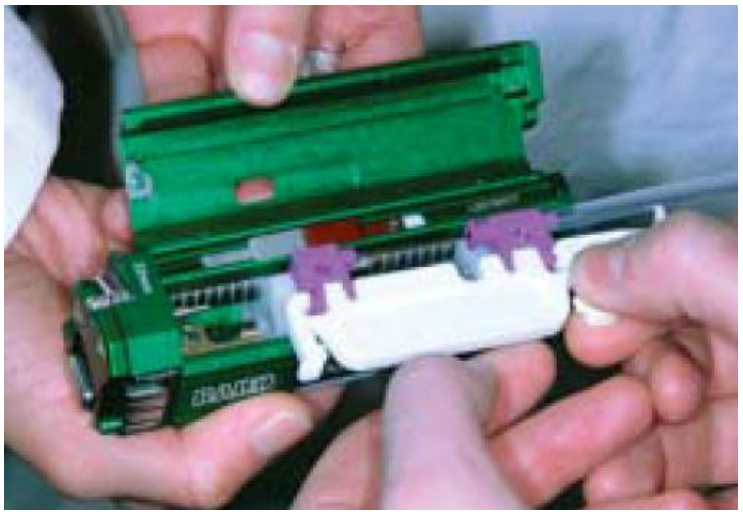


Figure 6 Bergstrom needle (from Patel HP et al 2012). **(A)** Components of the biopsy needed include (1) Trocar (2) Cutting cannula (3) Clearing probe/rod. **(B)** Assembled for biopsy, with cutting cannula inserted within the trocar. A 20ml syringe can be connected to the cutting cannula to increase the yield of tissue by suction. **(C)** Cutting window visible. **(D)** Window with cutting cannula fully depressed

This minimally invasive or microbiopsy approach uses a 16 gauge core disposable biopsy needle loaded on to a spring system reusable instrument, the Magnum® Biopsy System (Figure 7). Participants are asked to lie down with the preferred thigh for biopsied exposed. Upon cleaning the skin with an iodine based cleaning solution, local anaesthesia, i.e. 1% lignocainse, is first used to anaesthetise the skin. An incision of the skin and fascia is made. The biopsy needle inserted into the Magnum 'gun' is then inserted perpendicular to the muscle. Muscle tissue is obtained by activating the trigger button which activates the needle and collects the specimen. The muscle tissue is removed from the biopsy needle using a sterile scalpel and immediately frozen in liquid nitrogen. Pressure and a dressing were applied to the wound. There was no restriction to daily activities post procedure. Participants were asked to avoid exposing the wound to water for 48 – 72 hours after the biopsy.



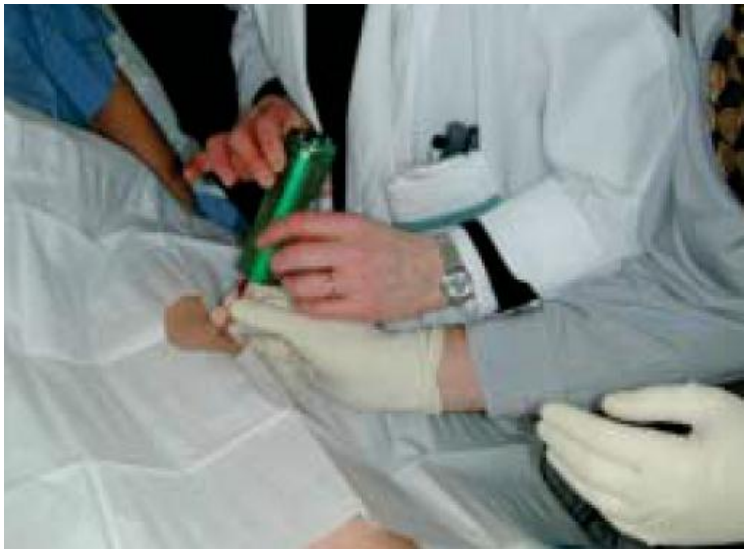


Figure 7 Magnum biopsy system (from Hayot M et al 2005). The top figure shows the disposable biopsy needle loaded on to a spring system of the Magnum gun. The bottom figure shows the Magnum 'gun' inserted perpendicular to the muscle to obtain the muscle specimen.

2.4 Blood analysis

Blood samples for the determination of serum calcium, PTH and 25-OH-D_3 concentrations were collected into EDTA tubes and allowed to clot. Plasma and serum were separated by low speed centrifugation (15 min at 3000g at 4°C) and stored at -80°until analysis. Aliquots of serum were sent for analysis to the pathology department, Nottingham University Hospitals (NUH) NHS Trust. Both 25-OH-D_3 and parathyroid hormone were quantified using two side sandwich competitive using direct chemi-lumunescent on the Siemens ADVIA Centaur system. Calcium levels were quantified using photometric colorimetric test method measured on the Beckman AU clinical chemistry analyser.

2.5 RNA extraction and quantification; and cDNA synthesis

Muscle biopsy samples were stored in liquid nitrogen. Total RNA was extracted from the frozen muscle tissue using the method of Chomczynski and Sacchi [Chomczynski 1987] using TRIzol reagent (Invitrogen, Paisley, UK). 800µL of Trizol (Invitrogen) and 20µL glycogen were added to around 15mg of tissue which was homogenised using Polytron for 30 seconds and incubated at room temperature for 5 minutes. After that,

156.8µL chloroform and 3.2µL isoamyl alcohol were added to the samples and shaken vigorously followed by vortex for a few seconds. After being left at room temperature for 2-3 minutes, the samples were then centrifuged at 12,000g for 15 minutes at 4°C. The aqueous phase (500µL) containing the RNA was transferred to a RNAse free eppendorf tube and kept overnight in 400µL isopropanol at -20°C to allow RNA precipitation. The remaining lower organic phase of the RNA extraction was kept for subsequent protein extraction (see section 2.6). The next day, the samples were centrifuged at 12,000g for 15 minutes at 4°C. Supernatant was removed and pellets were air-dried and then washed by adding 800µL of 75% EtOH and vortexing for a few seconds, and then spun again at 10,000g for 10 minutes. The supernatant was removed and any remaining EtOH was pipetted off. The pellets were air-dried for 5 minutes, re-dissolved in 30µL of RNAse-free water and quantified in duplicate using a Nanodrop ND-100 (Thermo Fisher Scientific, Delaware USA). Reverse transcription (RT) was carried out from 500 ng of total RNA using the SuperScript III cDNA kit (Invitrogen, Paisley, UK) according to manufacturer's instructions. Briefly, ten µL of RNA, 10 µl of RT Reaction mix and 2 µl of RT enzyme were mixed, centrifuged for few seconds and incubated at 25ºC for 10 minutes and then at 50ºc for 30 minutes. The reaction was terminated at 85ºc at 5 minutes; 1 µl of E.coli

RNAase H was then added and samples incubated for 20 minutes at 37ºc. 20 µl of cDNA was diluted eight times with RNAse free water.

2.6 Real time quantitative PCR

Taqman primers and probes sets were designed using Primer Express version 2.0 software (Applied Biosystems, Warrington, UK) and obtained from Applied Biosystems, UK. Their sequences are presented in Table 1. Real-time PCR measurements were performed in triplicate using PCR Universal Master Mix (Applied Biosystems) in a Micro- Amp 96-well plate using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, UK). The quantification of expression of each gene relative to α-actin was determined using the method described in Appendix 2. Standard curves were produced from serial dilutions of a pooled sample of cDNA from all individual samples. Twenty five microlitre reactions were run in triplicate and each tube comprised 5 µl of cDNA template, 0.75 µl of each primer, 0.5 µl of probe, 12,5 µl of Taqman Universal PCR Mastermix and 5.5 µl RNase-free water. Cycling conditions included an initial hold of 2 min at 50°C, followed by a hold of 10 min at 95°C, and then 40 cycles of 95°C for 15 sec, 60°C for 1 min.

Table 1. Sequences for primers and Taqman probes used for real-time PCR

Gene	*Forward Primer*	*Probe*	*Reverse Primer*
VDR	GACCCCACCTACTCCGACTTCT	CCAGTTCCGGCCTCCAGTTCGTG	GGCTCCCTCCACCATCATT
Sirt1	TGCGGGAATCCAAAGGATAA	TCAGTGTCATGGTTCCT	CAGGCAAGATGCTGTTGCA
Myostatin	GATGAGAATGGTCATGATCTTGCT	TAACCTTCCCAGGACCAG	AAAAACGGATTCAGCCCATCT
PPARα	GCTTCCTGCTTCATAGATAAGAGCT T	AGCTCGGCGGCACAACCAGCA	CACCATCGCGACCAGATG
PPARδ	TGCGGCCATCATTCTGTGT	ACCGGCCAGGCCTCATGAACG	CAGGATGGTGTCCTGGATAGC
α-Actin	GAGCCGAGAGTAGCAGTTGTAGCT	CCCGCCCAGAAACTAGACACAATGTGC	GCGGTGGTCTCGTCTTCGT

2.7 Protein extraction

Protein extraction was performed from the organic phase left over after the RNA extraction method using the Trizol reagent (see Appendix 1 for the detailed method). Briefly, after removing and discarding the interphase (that contained the DNA), 1.5ml Isopropanol per ml of Trizol originally used was added to the organic phase (lower pink phase that contained the proteins) This was mixed and left at room temperature for 10 minutes to allow the protein to precipitate. Tubes were then

centrifuged at 12,000g for 10 minutes and then 2ml of wash solution (0.3M guanidine in 95% EtOH) was added before being mixed on a daisy wheel for 20 minutes at room temperature. This was followed by centrifugation at 7,500g for 5 minutes at 4^{o}C. Pellets were then washed with 100% ethanol and resuspended by adding 400µl Protein Resuspension Solution, sonicated on ice and stored for further analysis. Quantification of protein concentrations was performed using the Bovine Serum Albumin Protein Assay (Pierce, Perbio, USA) using a spectrophotometer (SpectraMAX 190 Molecular Devices).

2.8 Western Blotting

Twenty micrograms of total muscle lysate proteins were separated using 10% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). The gels were electrophoresed on a vertical dual plate unit (Fisher, Loughborough, UK) and the separated proteins were electroblotted overnight to Hybond-C nitrocellulose membranes (Amersham Biosciences, UK). After blocking with 5% (w/v) Marvel (milk) in a solution of 0,1% (w/v) Tween 20 in Tris Buffered Saline (TBS-T) for 1h at room temperature, the membranes were incubated for 1h with a

primary antibody for human VDR (dilution 1:250, GR37, Oncogene Research Products, USA or VDR-D6, Santa Cruz, sc13133) and then on a separate day for the endogenous control (α-actin, dilution 1:2,000, A-2066, Sigma-Aldrich Co). After washing for 4 x 10 minutes in 0.5% (w/v) Marvel TBS-T the membranes were incubated for 1h at room temperature with secondary antibodies linked to HRP, anti-rat (dilution 1:2,000, Amersham Biosciences, UK) and anti-rabbit (dilution 1:2,000, Amersham Biosciences, UK) detecting the primary antibodies for VDR and α-actin, respectively. After washing (as above) all immunoreactive proteins were visualised using ECL and Hyperfilm (Amersham Biosciences, UK) with the resulting bands quantified using densitometry performed using the Quantity One® 1-D Analysis Software version 4.5 (Bio-Rad Laboratories, Inc., USA).

2.9 Statistical analysis

Participant demographics were described for the young and older group. Mean and standard error or the mean (SEM) was used to describe age and calcium levels. Median and interquartile range (IQR) was used to describe the 25-OH-D_3 levels due to the skewed results obtained within

the young group. Data are presented as standard error of the mean (SEM) for VDR mRNA, myostatin mRNA, Sirt1 mRNA, PPARα and PPARγ normalised to α-actin. One way analysis of variance (ANOVA) for parametric data and Kruskal-Wallis test for non-parametric data were used to analyse differences between groups [young participants, older participants vitamin D sufficient (25-OH-D_3 >50nmol/L) and older participants vitamin D insufficient (25-OH-D_3 ≤50nmol/L)]. Statistical significance was defined as a p value of <0.05. When statistical difference was detected, post-hoc tests using t-test for parametric data and Mann-Whitney U test for non-parametric data, with Bonferroni corrections were conducted within groups (young group vs older sufficient group; young group vs older insufficient group; and older sufficient group vs older insufficient group) to locate the differences. Pearson's correlation coefficient was used to describe the strength of the relationship between circulation vitamin D and target genes.

CHAPTER 3

RESULTS

Participant characteristics are described in Table 2. The young group was made up of males only and in the older group, the participants were almost entirely made up of female volunteers. The older sufficient group had higher 25-OH-D_3 levels than the older insufficient group and the young group (P=0.00). However, there was no significant difference in its levels between the older insufficient and young groups (p=0.14). Most of the participants in the young group were deficient in vitamin D levels with a median 25-OH-D_3 of 16nmol/L (range 12 – 97). All participants had calcium levels within the normal range for the laboratory analysis with no differences observed between groups. PTH values were higher in the older 25-OH-D_3 insufficient group compared to the sufficient group (p=0.02).

Table 2 Participant characteristics (age, gender, body mass index (BMI) and circulating levels of 25-OH-D_3, calcium and parathyroid (PTH)

	Young group (n=8)	Older sufficient group (n=10)	Older insufficient group (n=8)	p-value
Age, mean (SEM)	23.5 (1.1)	76.2 (2.1)	75.9 (2.0)	0.00
Male/Female	8/0	10/0	7/1	-
Body mass index, mean (SEM)	23.09 (1.3)	25.7 (1.7)	26.8 (4.3)	0.54
25-OH-D_3, nmol/L, median (IQR)	16 (29.3)	62 (21.3)	38 (14.5)	0.001#
Calcium, mmol/L, mean (SEM)	2.36 (0.8)	2.45 (0.8)	2.40 (0.8)	0.07
PTH, pg/mL, mean (SEM)	-*	39.57 (15.0)	68.86 (26.0)	0.02

#Older sufficient group had higher 25-OH-D_3 levels than the older insufficient group and the young group. There was no statistically significant difference in its levels between the older insufficient and young groups (p=0.14). *PTH was not done in the young group as NUH Hospitals Trust laboratory only runs PTH analysis in EDTA plasma specimens (which were not collected for this group).

Table 3 describes the mean ± SEM of the 5 genes analysed within the 3 groups. Within the defined level of significance ($p<0.05$), there were differences between groups in VDR mRNA, Sirt1 mRNA and PPARδ; but not myostatin and PPARα mRNA.

Table 3 Skeletal muscle mRNA content of VDR, myostatin, Sirt1, PPARα and PPARδ relative to α-actin mRNA content in the 3 groups, young participants (18-25 years old); older participants (≥65 years old) vitamin D sufficient (25-OH-D_3 >50nmol/L) and older participants vitamin D insufficient (25-OH-D_3 ≤50nmol/L).

	Young group (n=8)	Older sufficient group (n=8)	Older insufficient group (n=10)	p-value
VDR mRNA/**α**-actin mRNA	6.84±0.93	12.66±1.53 [a]	14.01±2.08 [a]	0.01*
Myostatin mRNA/**α**-actin mRNA	10.11±0.75	9.98±1.13	11.73±1.83	0.58
Sirt1 mRNA/**α**-actin mRNA	8.58±0.52	12.62±0.81 [b]	11.26±0.95	0.00*
PPARα/**α**-actin mRNA	5.53±0.35	6.93±0.52	5.86±0.37	0.07
PPARδ/**α**-actin mRNA	4.99±0.23	8.96±0.63 [c]	7.91±0.83 [c]	0.00*

Data expressed as mean±SEM. P-value calculated based on one way ANOVA. *denotes p-value <0.05. [a] Higher expression of VDR mRNA in older sufficient and insufficient group vs younger group. [b] Higher Sirt1 mRNA expression in older sufficient group vs younger group. [c] Higher expression of PPARδ in older sufficient and insufficient group vs younger group.

There was a difference in skeletal muscle VDR mRNA content between the young participants and the older participants sufficient of vitamin D (p=0.01); and young participants and the older participants insufficient of vitamin D (p=0.01). Specifically, VDR mRNA expression was higher in the older participants compared to the younger group regardless of circulating levels of vitamin D. However, there was no difference between the two older participant groups (p=0.61) (Figure 8). When sirt1 was analysed, the only statistically significant difference was between the younger participants and the older group who were vitamin D sufficient (p=0.001) with higher expression in the older cohort (Figure 10). When the older insufficient group was compared to the younger group, there was a tendency towards a higher expression in the older group (p=0.02). There was a difference in PPARδ between the younger participants and both the older sufficient (p=0.00) and older insufficient group (p=0.00), with the young cohort showing lower levels of expression (Figure 12). There was no difference in skeletal muscle PPARα mRNA content between groups although there was a tendency (P=0.07) for higher expression in the older sufficient group when compared with the other 2 groups (Figure

11). There was no statistical difference in skeletal muscle myostatin mRNA content between groups (Figure 9).

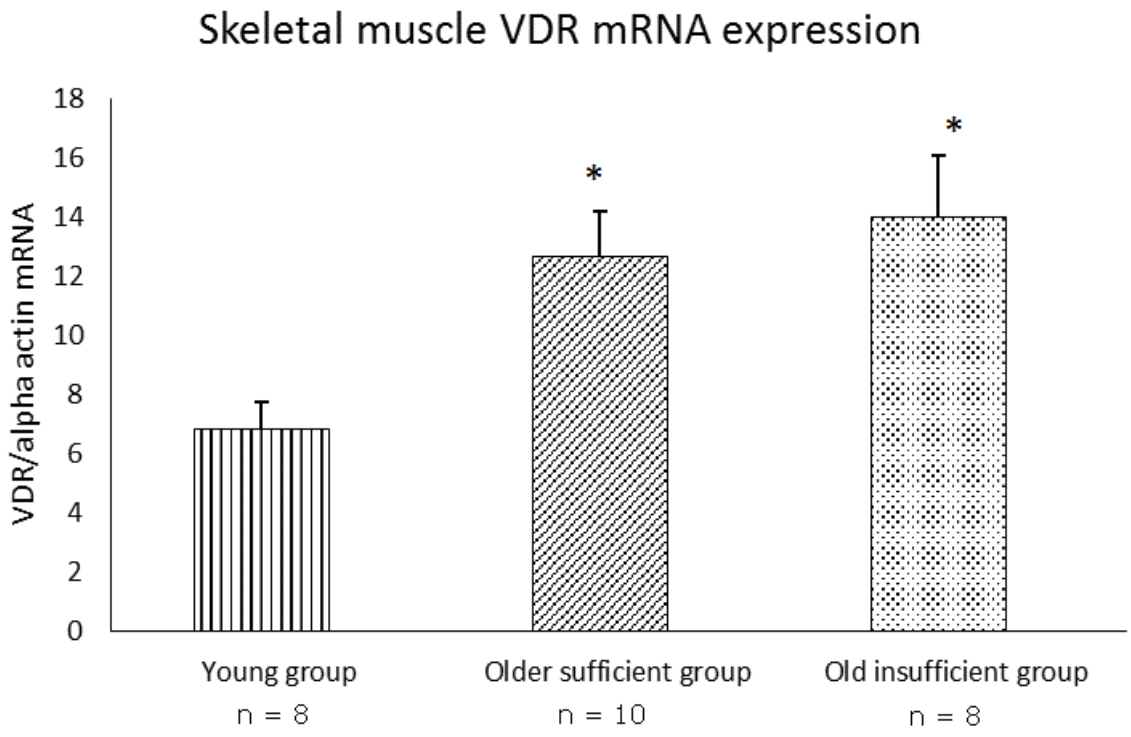


Figure 8 Bar graph representing the 3 groups with skeletal muscle VDR mRNA normalised to alpha actin mRNA content with each bar representing the respective mean. Error lines represent SEM of each group. *One-way ANOVA performed between young participants and older participants group showed statistical significance of p-value=0.01.

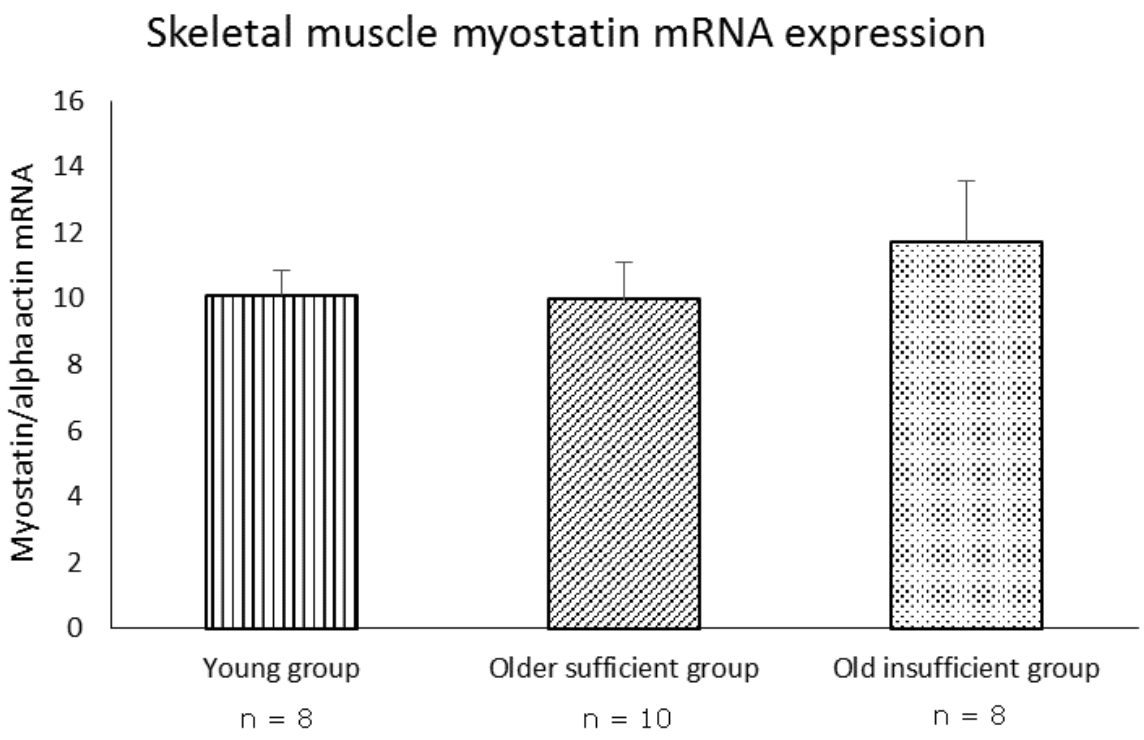


Figure 9 Bar graph showing skeletal muscle myostatin mRNA normalised to alpha actin mRNA content with each bar representing the respective mean. Error lines represent SEM of each group.

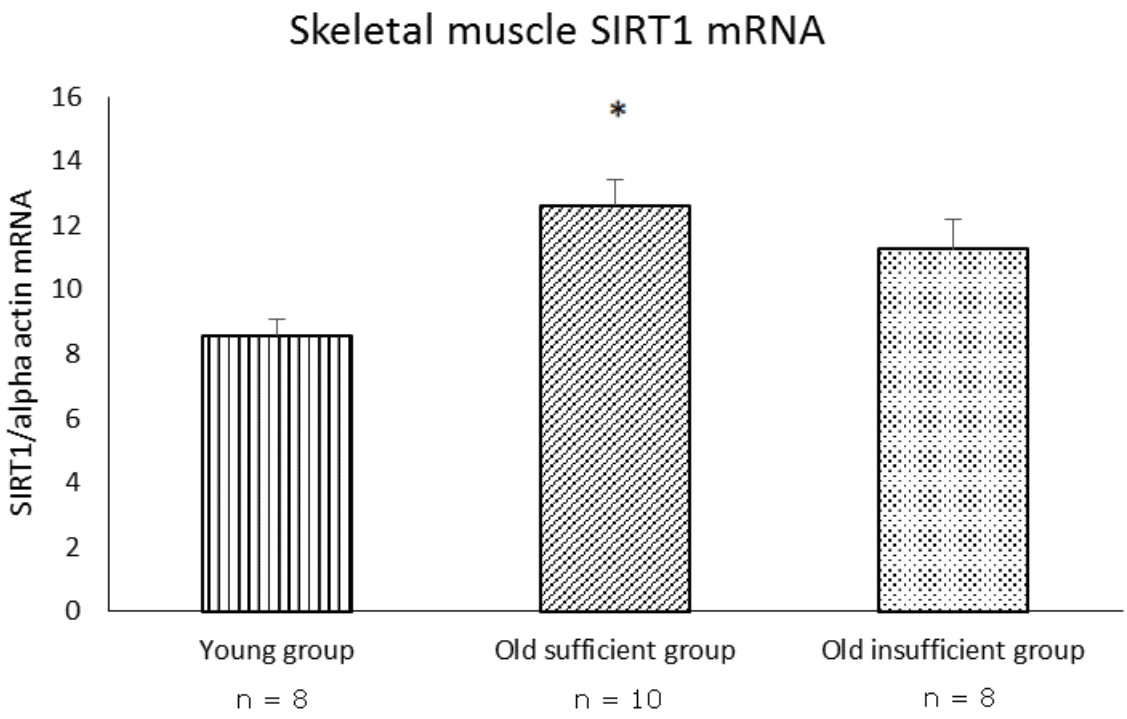


Figure 10 Bar graph representing the 3 groups with skeletal muscle sirt1 mRNA normalised to alpha actin mRNA content with each bar representing the respective mean. Error lines represent SEM of each group. *One-way ANOVA performed between young participants and older participants in the vitamin D sufficient group showed statistical significance of p-value<0.01.

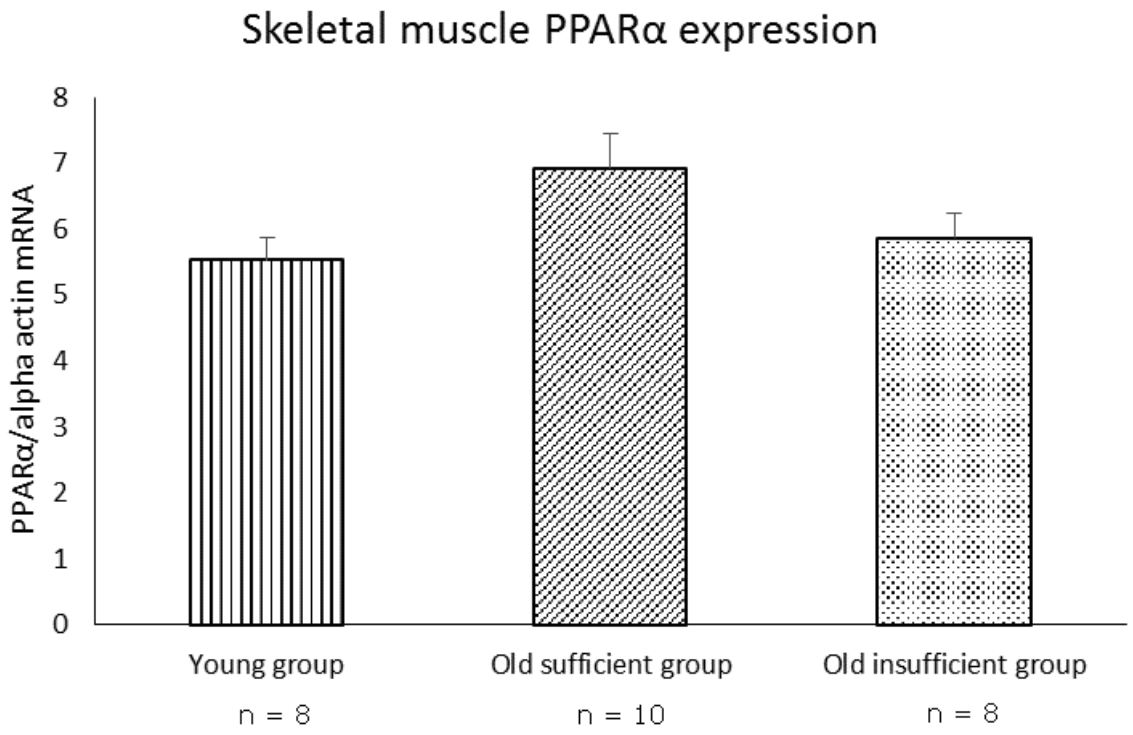


Figure 11 Bar graph showing skeletal muscle PPARα normalised to alpha actin mRNA content with each bar representing the respective mean. Error lines represent SEM of each group.

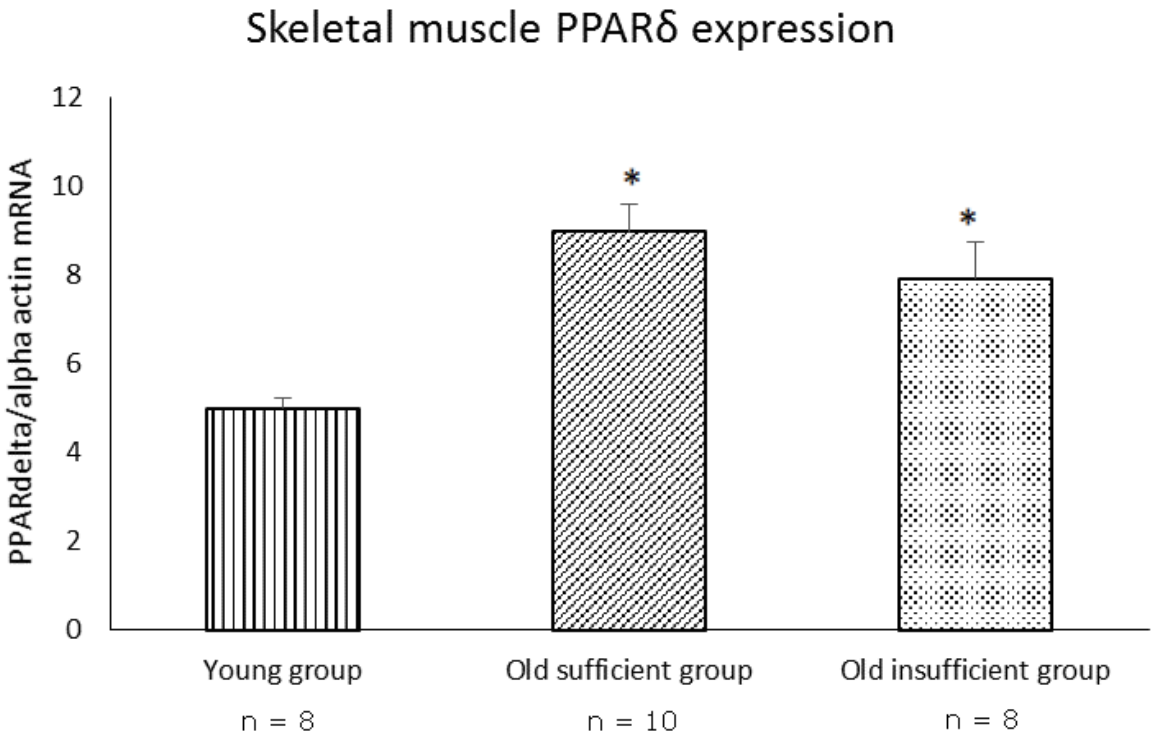


Figure 12 Bar graph representing the 3 groups with skeletal muscle PPARδ normalised to alpha actin mRNA content with each bar representing the respective mean. Error lines represent SEM of each group. *One-way ANOVA performed between young participants and both groups of older participants in showed statistical significance of p-value<0.01.

The scatter plots shown in figures 13 – 17 describe the relationship between circulating levels of 25-OH-D_3 nmol/L and skeletal muscle gene expression. There was not any overall strong relationship between circulating vitamin D levels and any of the genes expressed. When the relationship between circulating 25-OH-D_3 and VDR mRNA was examined in the older participant group as a whole (sufficient and insufficient) (Figure 13) and young participants (Figure 14) separately, it demonstrated a moderate non-significant association (r=0.56) in the young group but not in the older group. There were also moderate relationships between increasing circulating 25-OH-D_3 levels and lower myostatin mRNA content (r=-0.63) and higher sirt1 mRNA (r=0.55) in the younger participants only. With respect to PPARs, circulating 25-OH-D_3 showed weak associations with PPARα in young and older participants (r=0.45 and r=0.36, respectively), and PPARδ (r=0.43 and r=0.09, respectively).

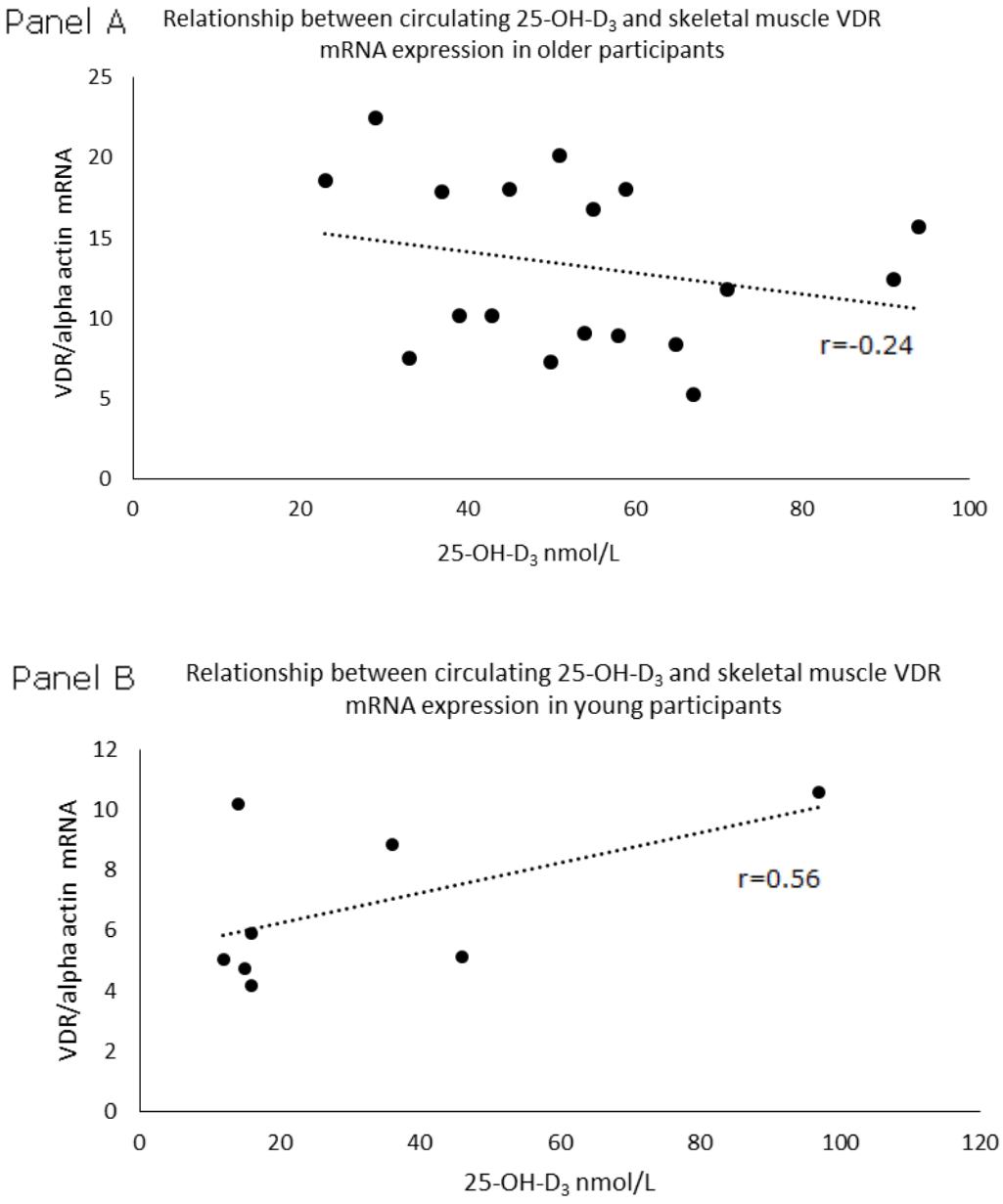


Figure 13 Relationship between skeletal muscle VDR mRNA content normalised to alpha actin mRNA content, and serum 25-0H-D_3 levels (nmmol/L) in older participants, n=18 **(A)** and young participants, n=8 **(B)**.

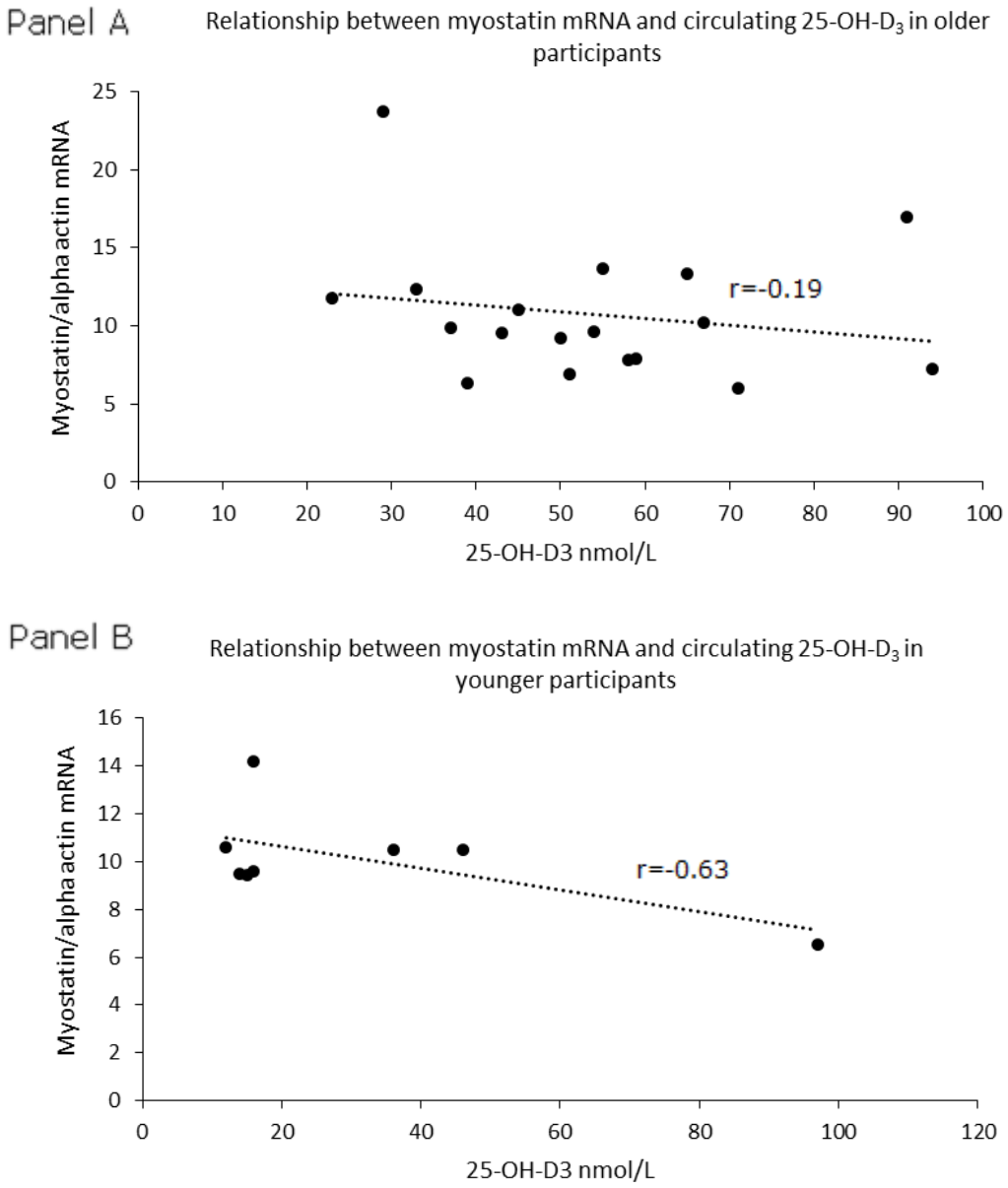


Figure 14 Relationship between skeletal muscle myostatin content normalised to alpha actin mRNA content, and serum 25-0H-D_3 levels (nmmol/L) in older participants, n=18 **(A)** and young participants, n=8 **(B)**.

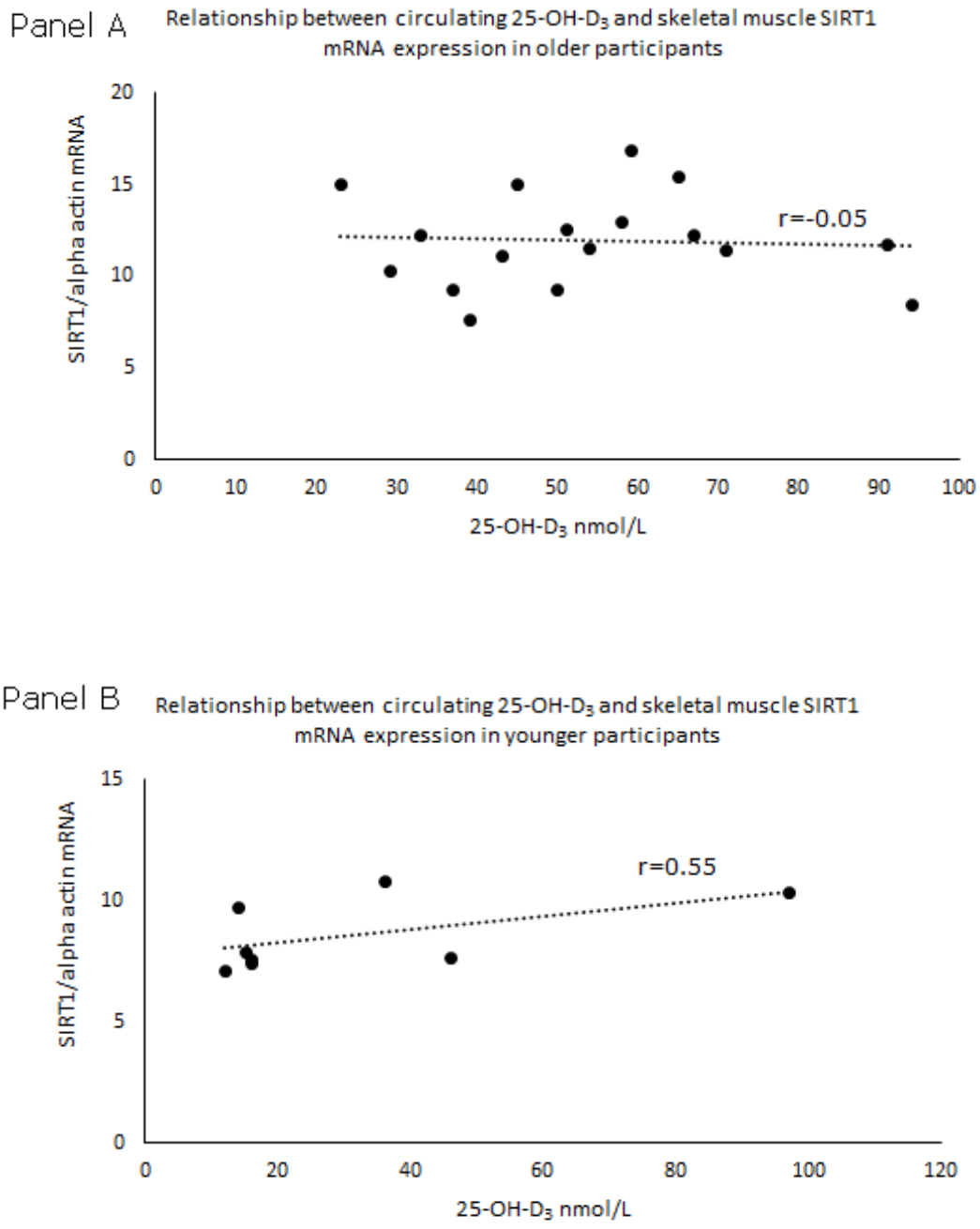


Figure 15 Relationship between skeletal muscle sirt1 content normalised to alpha actin mRNA content, and serum 25-0H-D_3 levels (nmmol/L) in older participants, n=17 **(A)** and young participants, n=8 **(B)**.

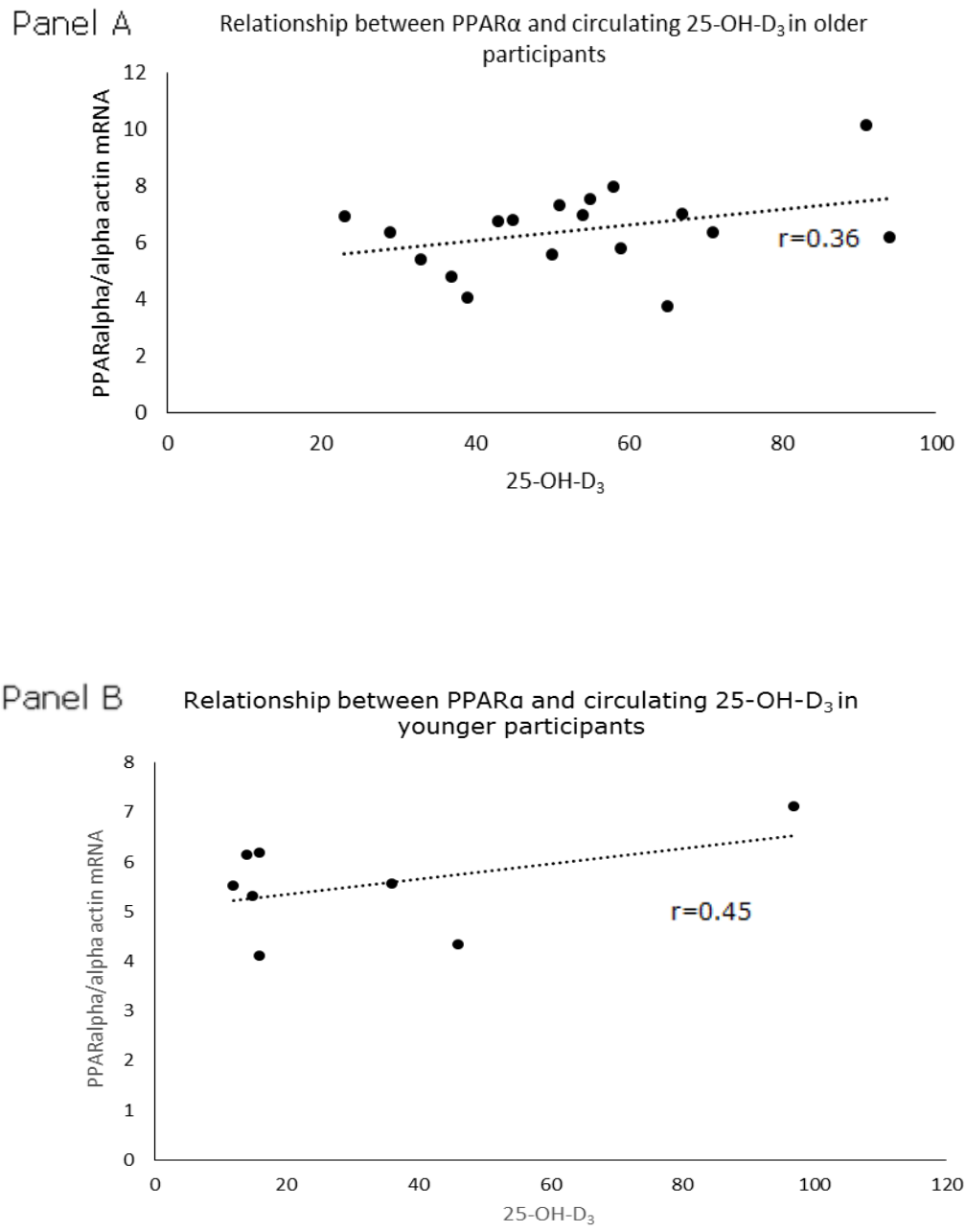


Figure 16 Relationship between skeletal muscle PPARα content normalised to alpha actin mRNA content, and serum 25-0H-D_3 levels (nmmol/L) in older participants, n=18 **(A)** and young participants, n=8 **(B)**.

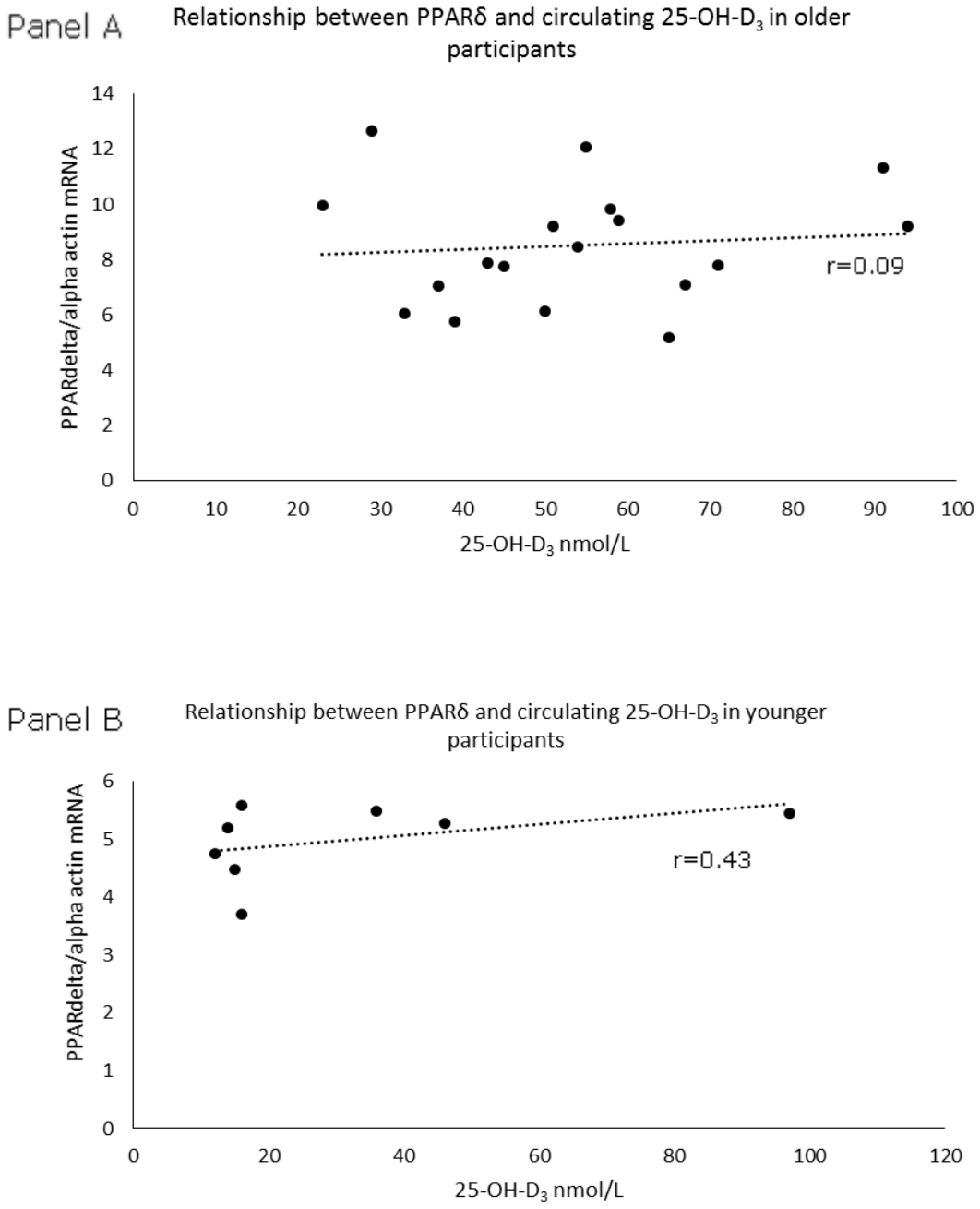


Figure 17 Relationship between skeletal muscle PPARδ content normalised to alpha actin mRNA content, and serum 25-0H-D_3 levels (nmmol/L) in older participants, n=18 **(A)** and young participants, n=8 **(B)**.

Figure 18 Scatterplot showing the relationship between sirt1 and PPARδ expression relative to VDR expression in all older participants, n=18.

Figure 18 shows the relationship between sirt1 and PPARδ with VDR. There is a moderate positive association between PPARδ and VDR mRNA (r=0.62) but not with sirt1 (r=0.15).

Western blotting to quantify VDR protein in human skeletal muscle was unsuccessful. The first antibody used VDR 9A7 did not detect any bands. Based on existing literature [Wang 2010] which identified the D6 antibody being highly specific and more sensitive, it was subsequently purchased and used in new blots generated from protein samples derived from the Trizol extraction. However, that attempt was unsuccessful in detecting the VDR protein. In further studies, we were able to detect the VDR protein as a 53kDa band in protein homogenates extracted directly from human skeletal muscle. The reason protein samples from TRIzol extraction were used in this study was because the amount of muscle sample obtained using the microbiopsy approach was limited and not sufficient to perform separate RNA and protein extractions.

CHAPTER 4

DISCUSSION

This thesis set out to investigate the relationship between circulating vitamin D, ageing and skeletal muscle VDR expression by comparing 3 different cohorts; young participants, older participants who were vitamin D sufficient and older participants who were vitamin D insufficient. It should be noted that circulating 25-OH-D_3 levels were low in the young group (which was meant to act as the control group in this study). Samples were obtained over the course of the year with 4 taken during the summer period (June – July) and the rest between October and December. There is existing literature that has shown that hypovitaminosis D is highly prevalent even in young adults, especially in the winter period [Mithal 2009, Tangpricha 2002]. One study identified that the prevalence of vitamin D deficiency is between 11 – 30% depending on the time of the year the sample was obtained [Tangpricha 2002]. In the present study, the circulating 25-OH-D_3 levels in the young subjects were not statistically different compared with the older insufficient group.

The findings from the present investigation demonstrated that skeletal muscle VDR mRNA expression was higher in the older sufficient group and the older insufficient group compared to the younger group. VDR is central to the effect that vitamin D plays within skeletal muscle and existing literature suggests that higher levels of 25-OH-D_3 upregulate VDR expression. Solvsten et al demonstrated that when keratinocytes where incubated with 1,25-$(OH)_2$-D_3, it resulted in an increase in VDR protein and mRNA levels [Solvsten 1997]. The maximal increase in protein levels peaked after 8 hours of incubation where the levels rose to 177% in undifferentiated keratinocyte cultures and 268% in differentiated keratinocyte cultures compared to the levels in unstimulated cells at the start of the incubation. This rise above basal levels were sustained even up to 24 hours post incubation. There was also a dose dependent upregulation of VDR mRNA expression with higher levels of 1,25-OH_2-D_3. The vitamin D-VDR ligand subsequently binds to VDRE response elements (VDRE) to affect gene transcription. Hence, the upregulation seen may be related to the way this ligand affects and induces upregulation of VDR mRNA; or it may just be due to the direct effect vitamin D has on the promotor of the VDR gene [Solvsten 1997]. Our study has revealed no statistically significant difference in VDR mRNA in the two older groups (sufficient and insufficient); and despite similar circulating 25-OH-D_3

levels in the young participants and older insufficient group, there was still higher content of VDR mRNA in the older group. Figure 13 demonstrated a weak negative relationship between circulating 25-OH-D_3 and VDR mRNA expression in older participants. However, in the younger participants, there was a moderate positive relationship between circulating 25-OH-D_3 and VDR mRNA expression. Hence, an alternative explanation is that VDR expression may be upregulated by the ageing process itself and that higher levels of vitamin D may be associated with higher VDR mRNA in young subjects but not the older participants. Ageing may be an intrinsic natural upregulator to improve vitamin D function in tissues, such as skeletal muscle, as lower vitamin D synthesis happens with advancing age. Due to that, an upregulation of VDR may be needed to facilitate the function of vitamin D. However, this goes against animal studies that have shown a decline in VDR expression with age. Montero et al and Horst et al have shown in rat models reduced VDR expression in intestine, bone and kidneys reduced with advancing age [Montero 2005, Horst 1990]. Bischoff in their paper also demonstrated a decline in VDR expression in human skeletal muscle with advancing age [Bischoff 2004]. However, in their study, muscle was obtained from either the gluteus medius or transversospinalis muscle; and the specimens incubated with VDR 9A7 antibody and later analysed using immunohistochemical

methods. They also had relatively younger patients within their cohort compared to our study. Another study presented recently as an abstract at the Federation of American Societies for Experimental Biology showed VDR in human skeletal muscle to be strongly associated with circulating 25-OH-D_3 levels in older people [Pojednic 2013]. Our study was unable to show a relationship between circulating levels of vitamin D and VDR in skeletal muscle biopsies of older people. Bischoff's study also failed to show and association between VDR expression and circulating 25-OH-D_3 levels [Bischoff 2004].

Mouse skeletal muscle cells C2C12 is widely used to study genes that regulate muscle growth and differentiation [Garcia 2011]. Garcia et al were able to demonstrate that C2C12 muscles cells when incubated with 1,25-$(OH)_2$-D_3, had increased expression of VDR mRNA by 15.1 and 6.4 fold at day 1 and day 4 respectively compared with controls (no 1,25-$(OH)_2$-D_3 incubation); enhanced myogenesis, as shown with an increase in myogenic markers, e.g. MyoD and desmin; reduced mRNA expression of myostatin by 2.5 and 10 fold on day 4 and 7 respectively; and an increase in follistatin expression, an inhibitor of myostatin [Garcia 2011]. These findings highlight the crucial role vitamin D play in muscle

myogenesis via its interaction with VDR. However, the present study did not show any difference in myostatin mRNA expression between the older sufficient and insufficient groups which points towards circulating 25-OH-D_3 levels not affecting its expression. Given that similar VDR levels were observed in both the older sufficient and insufficient groups, it appears that myostatin expression is associated with the level of VDR expression rather than the amount of circulating vitamin D. However, myostatin expression in skeletal muscle is also regulated by other factors, such as ageing, exercise, and other myogenic regulatory factors, although in the present study ageing per se did not appear to affect myostatin expression as the younger group had similar myostatin levels to the older participants.

When other genes that are known targets of vitamin D were studied, significant differences were detected between the older sufficient group vs the younger group for sirt1 and PPARδ. In our study, sirt1 was significantly more expressed in older sufficient group compared to the younger group and there was a tendency for it to be more expressed in the older insufficient group compared to the younger group. When both older groups with different vitamin D levels were compared, there was no

statistical difference in sirt1 expression. An et al in their study were able to link the role of vitamin D/VDR with sirt1 by showing how VDR interacts with FoXO proteins and its regulator sirt1 [An 2010]. They were able to demonstrate how vitamin D/VDR induces sirt1 and the phosphatase-dependent dephsphorylation and activation of FoxO function; and that vitamin D and FoXO share similar target genes. Existing studies have also shown that sirt1 overexpression lead to longer life span and a delay in the ageing process in mouse models [Price 2012, Satoh 2013]. One likely explanation to this is that sirt1 expression leads to better mitochondrial structure and function, as it is the mitochondrial dysfunction that has been recognized to be associated with the ageing process and the development of diseases, such as cancer [Erol 2007]. Price et al described in C2C12 muscle cells how sirt1 is associated with an increase in mitochondrial membrane potential and cellular ATP content and treatment with sirt1 inhibitor or knockdown sirt1 reduces it. Treatment with resveratrol which is dependent on sirt1 for its function increased mRNA expression of genes responsible for stimulating mitochondrial biogenesis, e.g NRF-1 and NRF-2. Hence, one can conclude that the higher sirt1 levels in older participants might be related to the ageing process itself as opposed to circulating vitamin D levels.

When PPARs were examined, there was higher expression of PPARδ mRNA in both older sufficient and insufficient groups when compared with the younger group, and there appeared to be a tendency for higher PPARα expression in older vitamin D sufficient group compared to the younger group. PPARδ is noted to be more abundant in skeletal muscle [Ehrenborg 2009]. Again, very much like sirt1, PPARs have a role in the ageing process being involved in the suppression of the inflammatory process seen with advancing age [Chung 2008]. Besides that, it is also involved in cellular growth, glucose homeostasis, insulin sensitivity and lipid metabolism [Erol 2007]. PPARs exert its effects by forming heterodimers with RXR, similar to VDR acting on specific DNA regions of target genes. This complex of PPAR/VDR-RXR bind to their specific response elements and have interconnected pathways that regulate the target genes and their transcription [Matsuda 2013]. This crosstalk link between VDR and PPAR means that both are able to influence each other's signalling pathway and that PPAR may act as a coactivator in the way vitamin D-VDR exerts its genomic effects. In one study using kidney tissue from rat models, it showed downregulation of PPAR (PPARα and γ) due to the ageing process. Compared with young rats, older rats showed decreased PPARα and PPARγ mRNA and nuclear protein levels; by 30% and 46%, and 53% and 84% respectively [Sung 2004]. The author further

highlighted the limited number of studies that have looked at the influence of ageing on PPARs. Iemitsu et al studied the influence of ageing on cardiac muscle in rats and described a reduction in PPARα with age [Iemitsu 2002] but Chao et al using liver tissue from rat subjects did not show a decline in PPARα with age [Chao 2002]. Mazatti et al using mice models were able to detect upregulation of PPARδ during periods of muscle unloading; and downregulation of the other PPAR isoforms. Although this was transient in nature with levels back to baseline at day 12, it points towards a metabolic response to maintain metabolic flexibility and fuel utilization [Mazzatti 2008]. This may explain the ageing upregulation of PPARs in the older subjects noticed in the present study.

Limitations

Vitamin D deficiency was prevalent within our young participant cohort. As described earlier, there is existing literature that has shown that low vitamin D levels are prevalent within a young adult cohort. Besides that, there were gender differences between the groups as well. We were only successful in recruiting young male volunteers; and female older volunteers. The invasive procedure, i.e. muscle biopsy that needed to be

performed as part of this study has most likely deterred young female participants to the study. As we were recruiting older participants in a bone health/osteoporosis clinic, where it is predominantly made up of female patients, meant that we were unsuccessful in recruiting more men. Gender variation of vitamin D levels has not been conclusive as vitamin D level is strongly influenced by factors such as geographical location/latitude and skin pigmentation [Hagenau 2009]. One UK epidemiological study did described hypovitaminosis D to be more prevalent in community living women than men [Hirani 2005]. However, there is a paucity of data in the literature on how gender itself may influence human skeletal VDR expression. Therefore it is difficult to conclude whether the imbalance in gender recruitment in the present study may have had an effect on this study's findings.

Another limitation within this study was the unsuccessful effort in protein determination of VDR despite using 2 different VDR antibodies. We were unable to get a good signal on western blot. As described earlier, additional studies were performed where protein was directly extracted from the skeletal muscle and VDR was detected. As there was limited tissue specimen and time constraints brought on by the need to submit this thesis, this part of the study was not re-attempted. However, the detection of VDR mRNA and evidence of VDR protein being present in

skeletal muscle from previous experiments done locally (unpublished observation) and existing literature [Bischoff 2001] supports the presence of VDR in human skeletal muscle. Girgis et al in their review highlighted the challenges in VDR detection in skeletal muscle due to the difference in experimental conditions; possibility of tight protein binding of VDR to DNA; low levels of VDR may be in itself enough for significant muscle function; and that VDR expression may be different in difference species and its expression varies depending on the varying stage of muscle growth [Girgis 2013].

CONCLUSION

The findings from the present study support the presence of the VDR gene in human skeletal muscle and extend this finding by showing no significant association between its level of expression and circulating levels of 25-OH-D_3. Indeed, similar VDR, sirt1, myostatin and PPARs mRNA levels were observed in skeletal muscle from older vitamin D sufficient and insufficient subjects. Interestingly, higher expression of VDR and PPARδ mRNA was observed in both older sufficient and insufficient groups when compared with the younger group. There was also higher Sirt1 mRNA expression in the older sufficient group when compared to the younger group. When both the young group (that was predominantly vitamin D deficient) was compared with older participants that were also deficient, skeletal muscle VDR gene expression was still higher in the latter group. This suggests that the ageing process per se may be influencing (by upregulation) the expression of the VDR and PPARδ genes at least under conditions of vitamin D insufficiency. Further research is required to directly compare young and old vitamin D sufficient individuals. Hence, our findings will further add to the debate of vitamin D-VDR relationship in skeletal muscle and the ageing process.

For future research, robust screening of young participants will need to be performed due to the high prevalence of vitamin D deficiency. As VDR is central to mediating the genomic and non-genomic functions of vitamin D, establishing this relationship is paramount and represents the initial step in developing a better understanding of how we can best use vitamin D in improving skeletal muscle function. It is with this understanding that further research to translate laboratory findings into clinical research can be done, such as changes in VDR expression in human skeletal muscle in those treated with vitamin D supplementation and how it correlates with clinical outcomes.

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APPENDIX

Appendix 1

Protein Isolation and Solubilisation Method from Trizol Prep

After removing the clear supernatant that contains the RNA following the separation of RNA from DNA and proteins during extraction with Trizol:

- Remove and discard the interphase (containing DNA) so that just the organic phase (lower pink phase that contains the proteins) remains.
- Add 1.5ml Isopropanol per ml of Trizol originally used.
- Mix, then leave at room temperature for at least 10 mins to allow protein to precipitate.
- Spin tubes 12,000g for 10 mins.
- Add 2ml wash solution (see below) and mix on daisy wheel for 20 mins at room temperature.
- Centrifuge at 7,500g for 5 mins at 4^0 C.
- Spin tubes at 12,000g for 10 mins and add 2ml wash. Mix on daisy wheel for 20 mins at room temperature. Do this twice (i.e. 3 washes in total)
- After the final wash, vortex pellet in 2ml 100% EtOH (You can stop at this point, store your samples at -20^0 C and complete the procedure later) and leave for 20 mins at room temperature. (Removes remaining phenol and dye)
- Centrifuge at 7,500g for 5 mins at 4^0 C

- Discard supernatant and resuspend pellet by adding 400µl Protein Resuspension Solution per sample (see below).
- Sonicate on ice to redissolve.

Wash Solution

0.3M Guanidine HCl in 95% EtOH

(mW=95.53)

∴1M = 95.54g in 1000ml

∴1M = 47.765g in 500ml

∴**0.3M = 14.33g in 500ml of 95% EtOH**

Protein Resuspension Solution

50mM Tris

4% SDS

9M Urea (deionised using Amberlite)

For 100ml:

Tris	0.6055g (605mg)
SDS	4g
deionised Urea	54.05g

1. Dissolve 54.05g Urea in 80ml HPLC water.
2. Split into 2 x 50ml falcon tubes, add 2g Amberlite MB50/MB1 to each tube.
3. Leave on roller for 1hr to deionise.
4. Pool deionised urea and add Tris and SDS.
5. Make up to 100ml with HPLC water.

9M Urea	**50mM Tris**
(mW=60.06)	(mW=121.1)
∴9M = 540.54g in 1000ml	∴1M = 121.1g in 1000ml
	∴1M = 12.11g in 100ml
∴9M = 54.054g in 100ml	**∴50mM (0.05M) = 0.6055g in 100ml**

Appendix 2

Real-time PCR analysis of RT products

Procedure

1. Take 20 µl of cDNA and add 140 µl of RNase free water (8-fold dilution). Vortex and spin (make a series of further 2-fold dilutions up to 128-fold and use them as a standard curve for every plate)
2. Dilute prove and primers* 10-fold (ie take 30 µl of each primer and add 270 µl of RNAse free water). Vortex and spin
3. Place 20 µl of Master (reaction) mix onto 96-well plate.
4. Pipette 5 µl of each diluted cDNA in triplicate onto 96-well plate
6. Read on Taqman (~2 h each run)

Reagents required

2x Taqman Universal PCR Mastermix
[(contains ampliTaq gold enzyme, nucleotides, internal reference dye ROX and buffer) from ABI Biosystems cat No.4304437 (store at 4^{o}C)]

Primers: Forward and Reverse each at 10 pmol/µl (10µM)
Keep master stocks at 100 pmol/µl (100µM) (store at -20^{o}C)

Probe: dual labelled probe (standard labelling 5'FAM and 3'TAMRA)
Working stock at 10 pmol/µl (10µM) (store at -20^{o}C)
Keep master stocks at 100 pmol/µl (100µM)

Template: First strand cDNA made from total RNA.

Reactions must be done in tubes with optical clear lids. The machine can take individual 0.2 ml tubes, strips or 96 well plates.

Reaction set up

Each reaction is in a final volume of 25µl (see below).

		final concentration
2x Taqman Universal PCR Mastermix	12.5µl	x1
Forward primer (10 pmol/µl)	0.75µl	0.3µM
Reverse primer (10 pmol/µl)	0.75µl	0.3µM
Probe (dual labelled) (10 pmol/µl)	0.5µl	0.2µM
cDNA (equivalent to 5ng total RNA/µl)	5µl	10ng RNA equiv.
Water (molecular grade)	5.5µl	
Total volume	**25 µl**	