

**Vitamin D and Skeletal Muscle
Function in Chronic Obstructive
Pulmonary Disease: Clinical
Associations, Molecular Mechanisms
and Genetic Influences**

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degree of MD(Res)**

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Abstract

This thesis investigates the role that serum 25(OH)D and serum 1,25(OH)₂D concentration play in skeletal muscle dysfunction in patients with Chronic Obstructive Pulmonary Disease (COPD) with reference to clinical impact, potential molecular mechanisms and genetic influences.

In a large cohort of COPD patients, neither serum 25(OH)D or serum 1,25(OH)₂D concentration were found to be associated with any volitional or non-volitional measures of peripheral or respiratory muscle strength. In a group of age and sex matched healthy control subjects serum 1,25(OH)₂D concentration was associated with muscle strength measures even after correction for potential confounding factors. Vitamin D status was not associated with quadriceps endurance in either COPD or control subjects when measured by repetitive magnetic stimulation.

A sub-set of patients underwent a quadriceps muscle biopsy and mRNA levels of muscle fibre type and myogenic regulatory factors were measured. Serum 25(OH)D concentration was associated with *MyHCIIa* mRNA expression in control, but not COPD, subjects. *Myf5* mRNA expression was strongly associated with *MHCI* mRNA expression whilst *mrf4* mRNA expression was strongly associated with *MyHCIIa* mRNA expression in control, but not COPD, subjects.

Genotyping for all subjects included in the study was carried out for certain polymorphisms that were chosen because they have previously been reported to have an influence on the renin-angiotensin system in normal subjects, and may thus have an influence on skeletal muscle strength, or were already associated with muscle strength. In combination, the ACE I/D polymorphism, the AGT Met235Thr and the ATR1 A1166C polymorphisms had a significant influence on muscle strength in the COPD group. In normal subjects, the ACE I/D polymorphism was significantly associated with serum 25(OH)D concentration, and this association was stronger after correcting for confounding factors.

Abbreviations Used

1,25(OH) ₂ D	1,25 di-hydroxyvitamin D
25(OH)D	25 hydroxyvitamin D
ABG	Arterial blood gases
ACE	Angiotensin converting enzyme
ADD	Average daily dose
ATP	Adenosine triphosphate
ATR1	Angiotensin II receptor type I
ATS	American Thoracic Society
BK ₂ R	Bradykinin 2 receptor
BMD	Bone mineral density
BMI	Body mass index
BTS	British Thoracic Society
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CYP27B1	1 α -hydroxylase
D allele	Deletion allele of ACE gene polymorphism
DEXA	Dual energy x-ray absorptiometry
DBP	Vitamin D binding protein
EIA	Enzyme immunoassay
ERK	Extracellular signal-regulated kinases
FEV ₁	Forced expiratory volume in one second
FFM	Fat free mass
FFMI	Fat free mass index
FVC	Forced vital capacity
GOLD	Global initiative for chronic obstructive lung disease
hsCRP	High sensitivity C reactive protein
I allele	Insertion allele of ACE gene polymorphism
IFN γ	Interferon γ
IGF-1 etc	Insulin like growth factor
IL1 etc	Interleukin
IP3	Inositol 1,4,5 triphosphate
MAPK	Mitogen activated protein kinase
MHC I etc.	Myosin heavy chain
MRFs	Myogenic regulatory factors
MuRF-1	Muscle ring finger protein 1
NHANES	National health and nutrition examination survey
ODN	oligodeoxynucleotide
PaCO ₂	Arterial carbon dioxide tension

PaO ₂	Arterial oxygen tension
PCR	Polymerase chain reaction
PI3K	phosphatidylinositol-3-kinase
PPAR	Peroxisome proliferator activated receptor
PTH	Parathyroid hormone
QMVC	Quadriceps maximum voluntary contraction
QOL	Quality of life
qPCR	Quantitative polymerase chain reaction
RAS	Renin angiotensin system
RCT	Randomised Controlled Trial
REE	Resting energy expenditure
RIA	Radioimmunoassay
RV	Residual volume
SD	Standard deviation
SGRQ	St George's Respiratory Questionnaire
SNiP	Sniff nasal pressure
SNP	Single nucleotide polymorphism
TGFβ	Transforming growth factor β
TLC	Total lung capacity
TLCO	Carbon monoxide transfer factor
TNFα	Tumour necrosis factor alpha
Tregs	T regulatory cells
TRPC3	transient receptor potential cation channel, subfamily C, member 3
TwQu	Twitch quadriceps force
URTI	Upper respiratory tract infection
US	Ultrasound
VDR	Vitamin D receptor
VDRKO	Vitamin D receptor knockout
VO _{2 max}	Maximum oxygen consumption
WBC	White blood cells
YPAS	Yale physical activity survey

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Publications arising from the thesis

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Table of Contents

Chapter 1: Introduction	18
1.1: Chronic Obstructive Pulmonary Disease	18
1.2: Pathophysiology of COPD	18
1.3: Weight loss in COPD	20
1.4: Loss of muscle in COPD	20
1.5: Skeletal Muscle Physiology	21
1.6 Muscle Fibre Type	22
1.7: Muscle function in COPD	23
1.8: Potential mechanisms of muscle weakness in COPD	26
1.8.1: Systemic versus local factors	27
1.8.2: Role of exacerbations	28
1.8.3: Inflammation	29
1.8.4: Ageing	30
1.8.5: Resting energy expenditure	30
1.8.6: Corticosteroid therapy	30
1.8.7: Genetic susceptibility	31
1.8.8: Nutritional factors	32
1.9: Vitamin D	33
1.10: Vitamin D metabolism	33
1.11: Vitamin D and skeletal muscle function	36
1.12: Cellular actions of Vitamin D in Skeletal Muscle	36
1.13: Animal Models	39
1.14: Human studies in Vitamin D and muscle function	39

1.14.1: Cross-sectional studies	39
1.14.2: Supplementation studies	40
1.14.3: Biopsy studies	42
1.15: Vitamin D status and lung function	43
1.16: Serum 25(OH)D concentration in COPD	43
1.17: Evidence for a link between Vitamin D status and skeletal muscle dysfunction in COPD	45
1.18: Osteoporosis and Osteopenia	46
1.19: Other connections between Vitamin D status and COPD	48
1.19.1: Inflammation	48
1.19.2: Cardiovascular disease	50
1.19.3: Cancer risk	50
1.19.4: Ageing	51
1.20: Genetic influences on skeletal muscle strength	51
1.21: Research Questions	52
Chapter 2: Description of Methods	54
2.1: Ethical approval	54
2.2: Power Calculations	54
2.3: Statistical analysis	54
2.4: Study subjects	55
2.5: Dietary Vitamin D intake	57
2.6: Health-related quality of life	58
2.7: Yale Physical Activity Survey	59
2.8: Body Composition	61

2.9: Respiratory muscle strength	63
2.10: Handgrip strength	63
2.11: Quadriceps strength	64
2.12: Quadriceps endurance	66
2.13: Pulmonary function testing	68
2.14: Serum analysis	69
2.14.1: 25(OH)D	69
2.14.2: 1,25(OH) ₂ D	70
2.14.3: PTH, albumin, electrolytes and inflammatory markers	71
2.15: Genotype analysis	72
2.15.1: DNA whole blood extraction and quantification	73
2.15.2: DNA extraction reagents	73
2.15.3: DNA quantification and robot standardisation of DNA arrays	74
2.15.4: TaqMan SNP genotyping	74
2.15.5: MADGE gel ACE I/D genotyping	75
2.16: Muscle biopsy	76
2.17: mRNA expression	76
2.18: VDR protein measurement	78
Chapter 3: Skeletal Muscle Strength and Endurance in COPD	80
3.1: Introduction	80
3.2: Results	81
3.2.1: Subject demographics	81
3.2.2: Serum measurements	84

3.2.3: Factors affecting serum 25(OH)D concentration	85
3.2.4: Muscle Strength	87
3.2.5: Quadriceps endurance	93
3.2.6: Serum 25(OH)D concentration and lung function	94
3.2.7: Inflammatory mediators	95
3.3: Discussion	95
3.3.1: Study limitations	99
3.3.2: Conclusion	100
Chapter 4: Muscle biopsy sub-study	101
4.1: Introduction	101
4.2: Results	104
4.2.1: Demographic features, muscle strength and serum measurements in the sub-study group	104
4.2.2: mRNA analysis	106
4.2.3: VDR protein measurement	111
4.3: Discussion	113
Chapter 5: Genetic influences on muscle strength and Vitamin D in COPD	117
5.1: Introduction	117
5.2: Results	120
5.2.1: Allele frequencies	120
5.2.2: ACE I/D polymorphism	121
5.2.3: ACE I/D polymorphism and serum 25(OH)D concentration	123
5.2.4: AGT Met235Thr polymorphism	125

5.2.5: ATR1 A1166C polymorphism	126
5.2.6: Influence of combined genotype on muscle strength	127
5.2.7: DBP rs7041 SNP	128
5.2.8: DBP rs4588 SNP	130
5.3: Discussion	133
Chapter 6: Conclusions	137
References	139

Tables

Table 2.1:	Specificity of 1,25(OH) ₂ D RIA	71
Table 2.2:	Genetic polymorphisms	72
Table 2.3:	mRNA primer sequences	77
Table 3.1:	Demographics of COPD and control subjects in the study	82
Table 3.2:	Comparison of serum Vitamin D metabolites, Ca ²⁺ , Po ⁴⁻ , Mg ²⁺ , PTH and inflammatory markers between COPD and control groups	85
Table 3.3:	Comparison of muscle strength measurements between COPD and control groups	88
Table 3.4:	Results of univariate analysis of individual factors and their association with QMVC (kg) in the COPD and control populations	90
Table 3.5:	Factors which remained associated with QMVC after stepwise multivariate analysis in the COPD population	91
Table 3.6:	Factors which remained associated with QMVC after stepwise multivariate analysis in the Control population	92
Table 3.7:	Factors which remained associated with handgrip strength after stepwise multivariate analysis in the Control population	92
Table 4.1:	Comparison of demographic features, muscle strength and serum measurements between groups in participants of the muscle biopsy substudy	105
Table 4.2:	Comparison of mRNA expression between groups	106
Table 4.3:	Correlations between serum 25(OH)D concentration and mRNA fibre type expression, and mRNA expression of myogenic regulatory factors between groups	107
Table 4.4:	Correlations for <i>mrf4</i> and fibre type mRNA expression	109

Table 4.5:	Correlations for <i>myf5</i> and fibre type mRNA expression	110
Table 4.6:	Correlations for <i>myogenin</i> and fibre type mRNA expression	111
Table 5.1:	Allele frequencies in COPD and Control Groups	121
Table 5.2:	Characteristics of COPD subjects according to ACE I/D genotype	122
Table 5.3:	Characteristics of Control subjects according to ACE I/D polymorphism genotype	123
Table 5.4:	Characteristics of COPD subjects according to AGT genotype	125
Table 5.5:	Characteristics of Control Subjects according to AGT genotype	126
Table 5.6:	Characteristics of COPD subjects according to ATR1 genotype	127
Table 5.7:	Characteristics of Control Subjects according to ATR1 genotype	127
Table 5.8:	Characteristics of COPD subjects according to DBP rs7041 genotype	129
Table 5.9:	Characteristics of Control Subjects according to DBP rs7041 genotype	129
Table 5.10:	Characteristics of COPD subjects according to DBP rs4588 genotype	131
Table 5.11:	Characteristics of Control Subjects according to DBP rs4588 genotype	132

Figures

Figure 1.1: Mortality in COPD subjects with demonstrated quadriceps weakness	24
Figure 1.2: Atrophy hypertrophy signalling pathways in skeletal muscle	26
Figure 1.3: Actions of PTH, 25(OH)D and 1,25(OH) ₂ D in response to low serum calcium	34
Figure 1.4: An example of a family with Ricketts, late 19 th century	35
Figure 2.1: Advertisement used to recruit healthy control subjects	56
Figure 2.2: Dietary Vitamin D assessment	58
Figure 2.3: Measurement of bioelectrical impedance	62
Figure 2.4: Non-volitional assessment of quadriceps strength	66
Figure 2.5: Quadriceps endurance measurement	67
Figure 3.1: Pattern of Vitamin D supplementation in COPD and control groups	83
Figure 3.2: Distribution of COPD severity in study participants	84
Figure 3.3: Variation in serum 25(OH)D concentration in COPD and control groups according to time of year measured	87
Figure 3.4: Correlations between serum 1,25(OH) ₂ D concentration and measures of muscle strength in the COPD and control groups	89
Figure 3.5: The comparison between force decline during the endurance protocol in COPD patients and control subjects	94
Figure 4.1: Correlation between serum 25(OH)D concentration and <i>MyHCIIa</i> mRNA expression in COPD and control groups	108
Figure 4.2: Graph showing association between <i>mrf4</i> and <i>MyHCIIa</i> mRNA expression	109

Figure 4.3: Graph showing correlation between <i>myf5</i> and <i>MHCI</i> mRNA expression	110
Figure 4.4: Result of Western Blot for VDR after incubation with primary and secondary antibodies	112
Figure 5.1: The Renin-angiotensin system	118
Figure 5.2: Serum 25(OH)D concentration according to ACE I/D polymorphism genotype in COPD and control groups	124
Figure 5.3: FEV ₁ (%pred) according to DBP rs7041 genotype in COPD and Control Groups	130
Figure 5.4: FFM according to DBP rs4588 genotype in COPD and control groups	133

Chapter 1: Introduction

1.1: Chronic Obstructive Pulmonary Disease

The word emphysema is Greek meaning to inflate, and is derived from the word physa meaning breath. The term was originally used to describe air in the tissues i.e. subcutaneous emphysema. In 1721, Ruysch provided the first description of emphysema in the human lung. However, it was not until the 19th century that further light was shed on the disease by the French physician Laennec (Laennec 1834). He recognized that the disease was associated with chronic bronchitis and described ‘marked variations in the size of the air vesicles, which might be smaller than a millet seed or as large as a cherry stone or haricot’. Emphysema and chronic bronchitis are now included under the term Chronic Obstructive Pulmonary Disease (COPD) which is defined as a chronic lung disease where there is progressive damage to airways and lung parenchyma causing airflow obstruction, usually as a result of inhalation of cigarette smoke. It is estimated that worldwide, 210 million people suffer from COPD and it is predicted that it will be the fourth leading cause of death by 2030 (Bousquet, Dahl et al. 2007).

1.2: Pathophysiology of COPD

The inhalation of cigarette smoke, potential occupational exposures (Blanc, Eisner et al. 2004), and in the developing world the indoor exposure to smoke from biomass fuel (Dossing, Khan et al. 1994; Ozbay, Uzun et al. 2001), contribute to the

development of parenchymal destruction and narrowing of the airway lumen through smooth muscle hypertrophy, airway inflammation and loss of elastic recoil. The resulting loss of alveolar surface area, ventilation perfusion mismatch, airflow limitation, and gas trapping all contribute to the dyspnoea and fatigue that patients experience. The severity of disease is classified through the FEV₁ which is a measure of the degree of airflow limitation; subjects are unable to achieve faster flow rates despite maximum efforts.

The pathological mechanisms of COPD are complex and poorly understood. There is evidence for alteration of the protease / antiprotease balance mediated by an increase in neutrophils and macrophages (Djekic, Gaggar et al. 2009), increased oxidative stress (MacNee 2005), autoimmune dysfunction (Cosio, Saetta et al. 2009) and dysregulation of lung development pathways including retinoic acid, notch and hedgehog signalling (Shi, Chen et al. 2009). Although cigarette smoking is the predominant cause of COPD, not all of those who smoke develop the disease. Genetic influences are therefore likely to play an important role.

Although classified as a disease of the respiratory system, there are a number of systemic complications which are well described in COPD, some of which are more closely linked to mortality than the degree of impairment in lung function. These include weight loss, loss of muscle mass and muscle weakness, systemic inflammation, increased prevalence of osteopenia and osteoporosis and increased prevalence of cardiovascular disease and certain types of cancer.

1.3: Weight loss in COPD

Weight loss in relation to COPD was first recognized in 1898 by Fowler and Godlee (Fowler 1898). However, it was not until half a century later that its importance as a prognostic factor in COPD was first demonstrated by Boushy et al (Boushy, Adhikari et al. 1964). A few years later, Vandenberg et al reported a 5 year mortality of 50% in patients with weight loss versus 5 year mortality of 20% in those with stable weight (Vandenberg, Van de Woestijne et al. 1967). More recent studies have confirmed this association between weight loss and poor prognosis in COPD. The Copenhagen City Heart Study found that weight loss in all patients (Prescott, Almdal et al. 2002), and body mass index (BMI) in patients with an FEV₁ less than 50% predicted were independent predictors of mortality (Landbo, Prescott et al. 1999). In the National Institutes of Health intermittent positive pressure breathing trial, weight as a proportion of ideal body weight was a predictor of mortality independent of FEV₁ (Wilson, Rogers et al. 1989). An incidence of weight loss of 25% in moderate to severe COPD has been reported and 10-15% in mild COPD (Wilson, Rogers et al. 1989; Schols, Soeters et al. 1993; Engelen, Schols et al. 1994). It has also been shown that patients who gain weight after a program of nutritional support have an improved prognosis, whilst those who fail to gain weight have an increased mortality (Schols, Slangen et al. 1998).

1.4: Loss of muscle in COPD

Weight gain or loss may be due to changes in lean body weight (fat free mass) or fat mass. Loss of fat free mass (FFM) has been demonstrated in patients with COPD,

more commonly in those who are hypoxaemic or have a reduced gas transfer capacity (Schols, Mostert et al. 1991), Schols et al have described different patterns of body composition in COPD (Schols, Soeters et al. 1993). Whilst some patients have a low BMI and a low FFM (cachexia), others have a low FFM whilst maintaining their BMI (muscle atrophy). Both of these groups have decreased exercise capacity and increased mortality compared to those with a low BMI who have maintained their FFM (semi starvation).

1.5: Skeletal Muscle Physiology

Skeletal muscle contracts to generate shortening or tension. When a nerve impulse reaches the neuromuscular junction, acetylcholine is released from the presynaptic membrane, which binds to receptors on the motor end plate causing depolarisation. This depolarisation is propagated through the sarcoplasmic reticulum and causes intracellular calcium release and activation of the contractile mechanism.

The molecular mechanism of muscle contraction is based on the sliding filament model proposed by Huxley in 1971 (Huxley and Simmons 1971). Each muscle fibre is made up of hundreds or thousands of myofibrils, which form the contractile mechanism. Myofibrils are themselves composed of long strands of smaller units, sarcomeres, which are made up of two types of contractile protein filaments, actin and myosin. Actin fibres make up the thin filament and are anchored to the Z line, a sheet of α -actinin, which also connects adjacent myofibrils. Actin filaments are associated with another protein, tropomyosin that regulates binding to myosin itself in a complex with three troponin subunits (Troponin I, C and T). Between and parallel to the actin

filaments are myosin filaments. Myosin is composed of two identical 'heavy' chains arranged in an α -helix and two additional light chains forming a globular head that has enzymatic activity and can interact with the actin filament.

At rest the tropomyosin blocks the binding of the myosin head to the actin filament. Above a critical level of intracellular calcium there is a conformational change in the troponin subunits, which causes the tropomyosin complex to move exposing the binding site for the myosin heads. This binding produces cross bridges which undergo conformational change to work in a manner analogous to the oars of a boat. The formation of cross bridges is energy dependent, requiring hydrolysis of ATP to ADP and inorganic phosphate.

1.6: Muscle fibre type

Adult mammalian skeletal muscle is composed of four myosin heavy chain isoforms, with varying parameters of chemomechanical transduction mediated through their ATP hydrolysis rate and shortening velocity. These isoforms determine fibre type which can be Type I, IIa, IIb and IIx (Schiaffino and Reggiani 1996). Type I fibres have a slower twitch and develop relatively less tension but have relative fatigue resistance because of their aerobic metabolism. By contrast type IIb/x fibres have a fast high-tension twitch but are fatigable because of their anaerobic/glycolytic metabolism. Type IIa fibres have intermediate properties (Harridge, Bottinelli et al. 1996). In humans it is likely that only IIx and not IIb isoforms are expressed (Pereira, Andrikopoulos et al. 1997; Pereira Sant'Ana, Ennion et al. 1997) and the MHC IIb gene has not been found to be expressed in human muscle fibres (Bottinelli and

Reggiani 2000)

1.7: Muscle function in COPD

Skeletal muscle strength is defined as ‘the capacity of the muscle to develop maximal force’, whilst skeletal muscle endurance is defined as ‘the capacity of the muscle to maintain a certain force and to resist fatigue’. If skeletal muscle depletion is an important factor in COPD, it is logical to conclude that skeletal muscle function may also be affected; FFM has been shown to correlate strongly with peripheral and respiratory muscle strength in COPD (Engelen, Schols et al. 1994; Engelen, Schols et al. 2000).

Work has focused on the quadriceps muscle as it is one of the main muscles of locomotion, is easily accessible and it is possible to carry out supramaximal nerve stimulation to obtain non-volitional measures of strength. This is important because the presence of both quadriceps and adductor pollicis weakness have been suggested in COPD using volitional techniques (Bernard, LeBlanc et al. 1998) whereas when measured using magnetic nerve stimulation, only quadriceps weakness has been confirmed (Man, Soliman et al. 2003). The incidence of quadriceps weakness in moderate to severe COPD is 30% (Swallow, Reyes et al. 2007) and when compared with healthy age matched controls, the mean reduction in quadriceps strength is approximately 30% (Man, Soliman et al. 2003). Quadriceps weakness has been shown to be related to impaired quality of life (Simpson, Killian et al. 1992), exercise limitation (Gosselink, Troosters et al. 1996) and increased health care utilization (Decramer, Gosselink et al. 1997), as well as being a more powerful prognostic

indicator than FFM or forced expiratory volume in one second (FEV₁) (Swallow, Reyes et al. 2007) (figure 1.1). A reduction in quadriceps endurance has also been demonstrated in COPD (Coronell, Orozco-Levi et al. 2004; Swallow, Gosker et al. 2007).

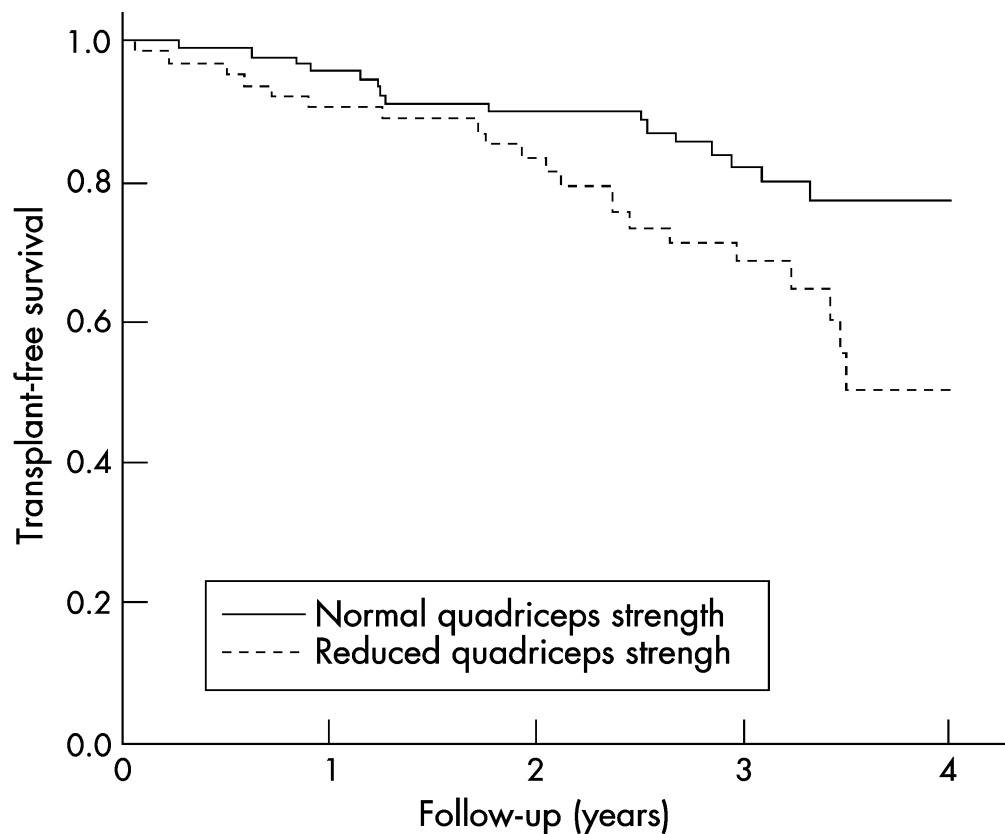


Figure 1.1: Mortality in COPD subjects with demonstrated quadriceps weakness (Swallow et al 2007)

Biopsy studies of the quadriceps in COPD have shown reduced capillarity (Jobin, Maltais et al. 1998) and oxidative capacity (Jakobsson, Jorfeldt et al. 1990) as well as muscle atrophy, with a reduction in fibre cross sectional area. A consistent finding is a reduction in the proportion of Type I compared to Type II muscle fibres (Jakobsson, Jorfeldt et al. 1990). There appears to be a decrease in the number of Type I fibres, whilst Type Iix fibres have a reduced cross-sectional area consistent with a fibre type

shift from type I to type IIx fibres and then a subsequent atrophy of Type IIx fibres (Gosker, Kubat et al. 2003).

It is not clear whether loss of muscle strength is due solely to decreased muscle bulk or whether a myopathy of the skeletal muscle develops. During exercise, there is early lactate production in patients with COPD despite similar oxygen delivery (Maltais, Jobin et al. 1998) as well as reduced overall mechanical efficiency (Richardson, Leek et al. 2004) consistent with intrinsic muscular abnormalities. On the other hand, force per unit cross-sectional area is maintained. Bernard et al found that strength normalised for muscle cross sectional area for patients with COPD was the same as in control subjects (Bernard, LeBlanc et al. 1998), and Engelen et al also found that strength per kilogram of muscle in COPD patients was similar to controls (Engelen, Schols et al. 2000). These findings support the hypothesis that skeletal muscle weakness in COPD can be predominantly explained by a reduction in muscle bulk rather a reduction in specific force due to a myopathic process.

At a molecular level, there are mechanisms for atrophy and hypertrophy which are not completely understood (figure 1.2). During conditions of muscle atrophy, there is induction of genes for protein degradation (known as atrogenes), whilst expression of genes related to growth is suppressed. Atrophy involves activation of the ubiquitin–proteasome system which causes breakdown of muscle proteins. Early on in this pathway there is induction of ubiquitin ligases such as *atrogin-1* and *MuRF-1*, and it is regulated by a family of transcription factors known as FOXO's (Sandri, Lin et al. 2006). Muscle hypertrophy on the other hand has a signalling pathway which involves IGF-I, insulin, phosphatidylinositol-3 kinase (PI3K) and Akt (Sandri, Lin et

al. 2006). Importantly, Akt has been shown to have an inhibitory effect on FOXO transcription factors (Sandri, Sandri et al. 2004). Transcription factors involved in the hypertrophy pathway include the peroxisome proliferator activated receptor (PPAR) which has been shown to cause an increase in the proportion of type I fibres in mice (Yong-Xu Wang 2004), and it is possible that a deficiency of PPAR or its associated cofactors may be relevant to the increase in proportion of Type II fibres seen in COPD. These hypertrophy / atrophy pathways are a final common pathway in all mechanisms of skeletal muscle loss.

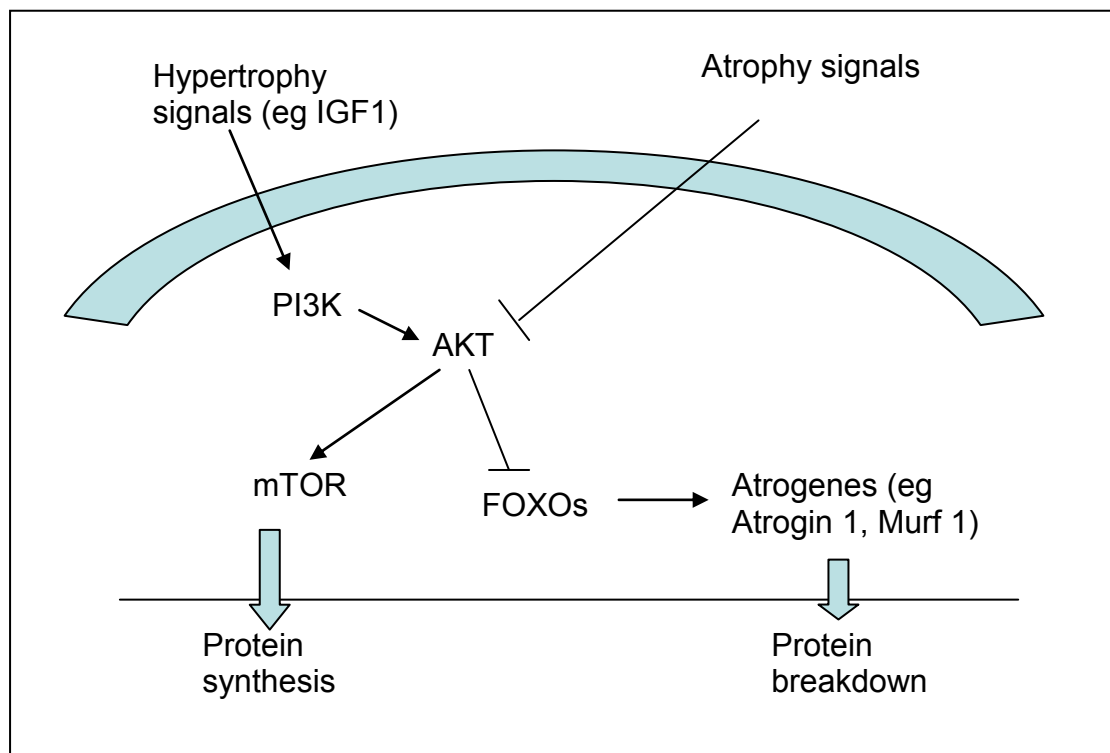


Figure 1.2: Atrophy / Hypertrophy signalling pathways in skeletal muscle

1.8: Potential mechanisms of muscle weakness in COPD

A number of different potential mechanisms for loss of body mass and muscle strength in COPD have been proposed which include aging, deconditioning, systemic

inflammation, oxidative stress, increased resting energy expenditure, nutrition, hypoxia, hypercapnia, hormonal factors, corticosteroid treatment and genetic susceptibility (American Thoracic Society/European Respiratory Society 1999) (1999; Hopkinson, Nickol et al. 2004).

It is well established that reduction in physical activity can lead to deconditioning (Appell 1990) with a loss of muscle mass, reduced oxidative capacity, decreased capillarity and mitochondrial density and a reduction in proportion of type I fibres, consistent with changes that are seen in the muscles of patients with COPD. Interestingly, these changes appear to occur relatively quickly. In healthy elderly people, ten days bed rest produces a 16% fall in quadriceps strength (Kortebein, Ferrando et al. 2007).

1.8.1: Systemic versus local factors

Debate is ongoing as to whether muscle weakness in COPD is a generalised, systemically determined phenomenon or one that predominantly is the result of disuse. The ‘compartment theory’ is based on the premise that changes in muscle function depend on the demands placed upon the muscle in question (Gea, Orozco-Levi et al. 2001). It is argued that patients walk less because of dyspnoea, which leads to disuse atrophy and quadriceps weakness. Upper limb strength is relatively well maintained because there is preservation of upper body activity and the shoulder girdle muscles are also accessory muscles of respiration. This theory is supported by a number of studies which have shown evidence of quadriceps weakness but not loss of handgrip strength in COPD (Gea, Orozco-Levi et al. 2001). Gosselink et al have

shown that proximal upper limb weakness as well as quadriceps and respiratory muscle weakness does occur in COPD whilst handgrip strength and accessory respiratory muscle strength is maintained (Gosselink, Troosters et al. 2000). They argue that patients continue to use their hands in generalised day to day activity, but avoid raising arms and walking.

A more recent study also confirms that reduced physical activity must play a significant role in the development of muscle weakness. Ultrasound (US) was used to measure the rectus femoris cross-sectional area and demonstrated that quadriceps wasting occurs early in subjects with COPD and is independently associated with physical activity (Shrikrishna, Patel et al. 2012).

1.8.2: Role of exacerbations

Short term studies have shown that exacerbations of COPD are associated with increased inflammatory mediators and acute and partially reversible reductions in both quadriceps (Spruit, Gosselink et al. 2003) and handgrip strength (Saudny-Unterberger, Martin et al. 1997). Since exacerbations are also associated with immobility (Pitta, Troosters et al. 2006), negative nitrogen balance (Saudny-Unterberger, Martin et al. 1997), and the administration of corticosteroids it seems reasonable to hypothesize that the development of skeletal muscle depletion over time would be associated with exacerbation frequency. When patients are hospitalised for an exacerbation, quadriceps strength falls by a further 5%, and recovery of baseline strength is not seen in all patients (Jakobsson, Jorfeldt et al. 1990). Reduced FFM is associated with exacerbation frequency both in a cross sectional study (Hopkinson,

Nickol et al. 2004) and with decline in FFM prospectively (Hopkinson, Tennant et al. 2007).

1.8.3: Inflammation

Patients with COPD have evidence of systemic as well as airway inflammation with elevation of White blood cell count (WBC), C-reactive protein (CRP), Interleukin 6 (IL-6), Interleukin 8 (IL-8), tumour necrosis factor alpha (TNF α) and fibrinogen demonstrated in COPD compared to control subjects (Gan, Man et al. 2004). These have been shown to increase with COPD exacerbations (Wedzicha, Seemungal et al. 2000) and persistently raised systemic markers of inflammation over one year have been associated with increased mortality in COPD patients (Agusti, Edwards et al.). Interestingly in the latter study, TNF α and IL-8 appear to be markers of smoking status, either past or present, rather than COPD itself.

TNF α induces protein loss through activation of the ubiquitin-proteasome pathway. Direct application of TNF α reduces single fibre force in vitro (Reid, Lannergren et al. 2002). A number of studies have linked TNF α with weight loss and depletion of FFM in COPD (Eid, Ionescu et al. 2001) and increased circulating TNF α levels are associated with a poor response to nutritional intervention in cachectic patients with COPD (Schols, Creutzberg et al. 1999). However, increased TNF α has not been found on muscle biopsy in patients with COPD (Barreiro, Schols et al. 2008).

1.8.4: Ageing

Ageing is associated with a relative decrease in fat free mass and an increase in the proportion of fat mass, known as sarcopenia. There is a reduction in type II fibres which can be attenuated to some extent by exercise (Larsson 1978). There is accumulating evidence that COPD may be a disease of accelerated ageing. This includes shortened telomere length in peripheral blood leukocytes (Savale, Chaouat et al. 2009) which has been shown to be smoking dose dependent, increased oxidative stress in the lungs (Drost, Skwarski et al. 2005) in human clinical studies, and the development of accelerated ageing and emphysema in klotho knockout mouse models (Funada, Nishimura et al. 2004). Klotho is an ‘antiageing’ molecule that is a regulator of oxidative stress and cell senescence.

1.8.5: Resting energy expenditure

Nutritional depletion will occur if energy expenditure exceeds energy intake. Total daily expenditure is the sum of resting energy expenditure (REE), diet induced thermogenesis and physical activity. REE was found to be elevated in 25% of a group of COPD patients (Creutzberg, Schols et al. 1998). This may be due to an increase in the work of breathing or inflammation.

1.8.6: Corticosteroid therapy

Myopathy is a well recognised complication of high doses of corticosteroid (Viïres, Pavlovic et al. 1990). It classically affects the proximal muscles and muscle biopsies

show atrophy of type IIb fibres. Although steroid induced myopathy certainly can occur in COPD (Decramer, de Bock et al. 1996) interpretation is difficult because of confounding by the effects of frequent exacerbations themselves which are the usual indications for oral steroid therapy. A two week course of prednisolone had no effect on skeletal muscle parameters in stable COPD patients (Hopkinson, Man et al. 2004) and cross-sectional studies have not found an association between steroid use and muscle depletion in COPD (Schols, Soeters et al. 1993; Hopkinson, Nickol et al. 2004).

1.8.7: Genetic susceptibility

Genetic susceptibility to loss of muscle mass and muscle weakness in COPD may explain why not all patients are affected equally. Genetic polymorphisms affecting muscle strength in normal subjects have been described for the Angiotensin Converting enzyme (ACE) (Pescatello, Kostek et al. 2006), PPAR α receptor (Ahmetov, Mozhayskaya et al. 2006), Insulin Growth Factor-1 (IGF-1) (Kostek, Delmonico et al. 2005) and alpha actinin-3 (ACTN-3) (Clarkson, Devaney et al. 2005) genes. The deletion allele of the ACE gene polymorphism is associated with greater quadriceps strength in COPD patients independent of FFM (Hopkinson, Nickol et al. 2004). Variants in the Vitamin D Receptor (VDR) gene are also associated with muscle strength in normal and COPD subjects (Hopkinson, Li et al. 2008), and variants in the bradykinin receptor gene (BDKR) are associated with reduction in FFM (Hopkinson, Eleftheriou et al. 2006).

1.8.8: Nutritional Factors

Malnutrition affects approximately a third of patients with COPD and appears to worsen with more severe disease (Vermeeren, Creutzberg et al. 2006). It has been linked to skeletal muscle strength in normal subjects as well as those with COPD (Vermeeren, Creutzberg et al. 2006), but supplementation studies have shown conflicting results with regard to improvements in peripheral and respiratory muscle strength (Weekes, Emery et al. 2009) and patients have difficulty in increasing their caloric intake sufficiently due to symptoms of bloating, satiety and dyspnoea.

Three small studies have shown an increase in respiratory muscle strength with nutritional supplementation (Efthimiou, Fleming et al. 1988; Whittaker, Ryan et al. 1990; Rogers, Donahoe et al. 1992). However, other studies have failed to replicate this. A recent Cochrane review has looked at randomised controlled trials giving at least two weeks of any caloric supplementation, and found significant improvements in anthropometric measures, and exercise capacity in malnourished patients only (Ferreira, Brooks et al. 2012).

Creatine is a nutritional supplement which rapidly undergoes reversible phosphorylation in skeletal muscle to phosphocreatine and provides a source of high energy phosphate. Creatine supplementation has been found to enhance exercise performance in healthy populations (Branch 2003). One study has looked at creatine supplementation in patients with COPD and found that 12 weeks of supplementation combined with a rehabilitation programme showed improvements in FFM, quadriceps

strength and endurance compared to controls. However no improvement in exercise performance was seen (Fuld, Kilduff et al. 2005).

1.9: Vitamin D

Vitamin D is a pleiotropic micronutrient which has recently been the focus of a lot of research in many areas. It's well known effects are on bone turnover in its role of maintaining serum calcium levels in the body but it has also been recognised to have a role in skeletal muscle function in normal subjects. It also has important roles in the immune system, as well as being linked to lung function, and vitamin D insufficiency has been associated with osteoporosis and the incidence of some cancers, problems which develop in or are associated with COPD. This makes it an obvious target for research in this area.

1.10: Vitamin D metabolism

Cholecalciferol is produced by the action of sunlight on the skin, although it can also be included in the diet. Cholecalciferol is metabolised in the liver to 25 hydroxyvitamin D (25(OH)D), which is transported and stored in the blood bound to vitamin D binding protein (DBP). When required, 25(OH)D is metabolised to the active form, 1,25 di-hydroxyvitamin D (1,25(OH)₂D), by the enzyme 1 α -hydroxylase which is produced predominantly in the kidney, although it has now been identified in a number of other target organs where 1,25(OH)₂D is thought to work in a paracrine fashion. In the kidney, it has been demonstrated that 25(OH)D bound to DBP is delivered to the site of the enzyme 1 α -hydroxylase by megalin / cubilin mediated

receptor endocytosis, although it is not clear whether this process occurs in all tissues (Nykjaer, Fyfe et al. 2001).

1,25(OH)₂D has a classically recognised role in serum calcium regulation under the tight control of parathyroid hormone (PTH) (figure 1.3). It acts to increase calcium absorption from the gut, reduce calcium loss by increasing resorption from the kidneys, and to release calcium from available stores ie bone by stimulating osteoclasts to resorb calcium.

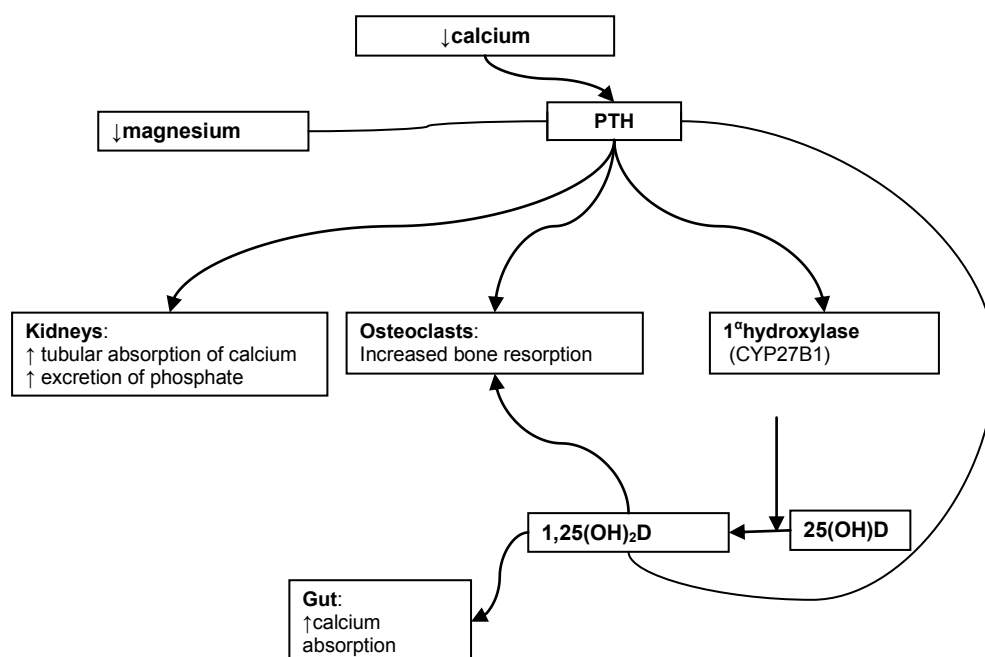


Figure 1.3: Actions of serum PTH, 25(OH)D and 1,25(OH)₂D in response to low serum calcium

Vitamin D deficiency prevents absorption of calcium from the gut, and hence ongoing resorption of calcium from bone occurs to maintain serum calcium levels leading to

osteomalacia in adults, or the development of rickets in childhood (figure 1.4). During growth, mineralisation of bone does not occur and subsequent deformity develops as the bone does not weight bear effectively. After growth has ceased, vitamin D deficiency still causes resorption of bone osteoid and pains and deformity can still occur in severe cases. Rickets became extremely common in Europe and particularly England in the mid 20th Century due to the industrialisation process. Cities became overcrowded and this in combination with pollution prevented children obtaining adequate sunlight exposure.



Figure 1.4: An example of a family with Ricketts, late 19th Century

It wasn't until after the First World War that Harriette Chick and Elsie Dalyell working in a children's hospital in Vienna demonstrated that rickets was due to lack

of sunlight and could be cured by sunlight exposure, by irradiation from a mercury quartz lamp, or by taking cod liver oil which is now known to contain vitamin D₃ (Carpenter 2008). The incidence of rickets in the UK decreased dramatically in the 1950's with the introduction of the clean air act, food fortification and increased public awareness of the problem. However, more recently Vitamin D deficiency appears to be a re-emerging problem in the UK particularly amongst immigrant populations with pigmented skin, lack of sunlight exposure and prolonged periods of breast feeding (Prentice).

1.11: Vitamin D status and skeletal muscle function

Although less well recognised than its classic effects on bone, vitamin D has long been known to have an important role in skeletal muscle function. Patients with osteomalacia develop a myopathy which is usually proximal, and skeletal muscle biopsies in these patients have shown a reduction in size and number of type II muscle fibres (Yoshikawa, Nakamura et al. 1979). VDR knockout mice have reduced type I and type II fibre diameter, and abnormal expression of myogenic regulatory factors which is corrected by the addition of 1,25(OH)₂D in vitro (Endo, Inoue et al. 2003).

1.12: Cellular actions of Vitamin D in Skeletal Muscle

1,25(OH)₂D exerts its actions by binding to the VDR which has been demonstrated to have genomic effects resulting in gene transcription, as well as more rapid non-genomic effects through VDRs situated in the cytoplasm. The receptors have been demonstrated to be identical in chick intestinal cells (Huhtakangas, Olivera et al.

2004). VDRs have been demonstrated in skeletal muscle (Boland, de Boland et al. 1995) and interestingly the amount of receptors has been shown to decrease with age (Bischoff-Ferrari, Borchers et al. 2004). However there is some controversy over the demonstration of VDR in skeletal muscle depending on the technique used. A more recent study using the D6 VDR antibody which does not show any background protein in VDR knockout mice, was not able to demonstrate the VDR protein in human or rat skeletal muscle (Wang and DeLuca 2011).

There is no doubt however that $1,25(\text{OH})_2\text{D}$ has significant actions on skeletal muscle. Cell studies have shown that $1,25(\text{OH})_2\text{D}$ stimulates initial rapid raised intracellular calcium levels through inositol 1,4,5 triphosphate (IP3) mediated release of calcium from the sarcoplasmic reticulum (Vazquez, de Boland et al. 2000). This is followed by stimulation of extra-cellular calcium influx through both store operated and voltage dependent calcium channels (Bauman, Valinietse et al. 1984; Boland 1986), an effect which is blocked by the administration of anti-VDR antisense oligodeoxynucleotides (ODN's) and is mediated by TRPC3 protein (Santillan, Katz et al. 2004). This effect has been demonstrated in both immature myoblasts and differentiated myotubes (Boland 1986)

Both in vivo and in vitro studies have shown that 25OHD , but not $1,25(\text{OH})_2\text{D}$ stimulates phosphate uptake in cells which is necessary for adenosine triphosphate (ATP) synthesis (de Boland, Albornoz et al. 1983; De Boland, Gallego et al. 1983). One study in $25(\text{OH})\text{D}$ and phosphate deficient rats has shown an increase in in vitro concentration of phosphate in muscle cells in vitro after the administration of

25(OH)D, followed by stimulation of phosphate dependent metabolic processes including ATP synthesis (Birge and Haddad 1975)

Other effects of 1,25(OH)₂D demonstrated at a cellular level are those on muscle cell growth and differentiation. Genomic and non-genomic affects have been demonstrated in C2C12 myoblast cultures, both of which pathways tend to result in cell growth, but the pathways are complex and the role of 1,25(OH)₂D not completely understood.

1,25(OH)₂D has been shown to rapidly activate components of the MAPK family, namely MAPK kinase and p38. The former leads to the activation of ERK1/2 and subsequent phosphorylation of a range of proteins and transcription factors involved in cell proliferation and differentiation such as c-myc and c-fos, and cAMP response element binding protein (Morelli, Buitrago et al. 2001; Ronda, Buitrago et al. 2007). p38 has been demonstrated to activate heat shock protein 27 which has an important role in its actions on the skeletal muscle cytoskeleton (An, Fabry et al. 2004; Buitrago, Ronda et al. 2006)

Demonstrated genomic effects of 1,25(OH)₂D include promotion of factors involved in myogenesis (desmin, myogenin and IGF2) and inhibition of factors that negatively regulate muscle mass (myostatin, proliferating cell nuclear antigen) with an overall effect of increased muscle fibre size and diameter in C2C12 myoblasts (Garcia, King et al.). European Sea Bass treated with varying doses of cholecalciferol show a dose dependant increase in white muscle fibre size and amount (Alami-Durante, Cluzeaud et al. 2011).

1.13: Animal models

VDR knockout mice have a 20% reduction in size of all fibre types compared to wild type controls, and show persistent upregulation of myogenic regulatory factors which are involved in growth and differentiation of myocytes and are normally down-regulated in mature muscle (Endo, Inoue et al. 2003). These mice have also been shown to have abnormal swim behaviour when compared to wild type mice with slower recovery (Kalueff, Lou et al. 2004; Burne, Johnston et al. 2006; Minasyan, Keisala et al. 2009). Other tests of motor co-ordination also show impairment in VDR knockout mice. However it is difficult to pinpoint the cause of their disabilities as they systemically lack the VDR receptor and may have neurological and cardiac dysfunction as well as skeletal muscle issues.

1.14: Human studies of Vitamin D status and Muscle Function

1.14.1: Cross-sectional Studies

A number of studies have supported a link between vitamin D status and skeletal muscle function in the elderly (Dhesi, Bearne et al. 2002; Zamboni, Zoico et al. 2002; Bischoff-Ferrari, Dietrich et al. 2004; Stewart, Alekel et al. 2009; Houston, Tooze et al. 2011) but there are also a number of negative studies published (Stein, Wark et al. 1999; Annweiler, Beauchet et al. 2009). There is a wide variation in cut off values used for serum 25(OH)D concentration, as well as variation in outcome measures

which include the 'sit to stand test', physical performance score, falls, handgrip and quadriceps strength which may explain the conflicting results found.

1.14.2: Supplementation Studies

Interventional studies looking at potential benefits of Vitamin D supplementation on muscle strength have also been extremely variable with regards to dose and duration of vitamin D supplementation given and techniques used for assessing muscle function and have therefore unsurprisingly shown conflicting results. Meta-analyses have also been difficult to carry out and interpret for the same reasons.

Only a small number of intervention studies published have used established measures of muscle strength. Dhese et al randomised 139 patients with a history of falls and low serum 25(OH)D concentration ($<12\mu\text{g/l}$) to receive 600,000 iu of ergocalciferol im or placebo and used QMVC as one of their outcome measures. No difference was seen after 6 months in QMVC between placebo and control although changes were seen in other measures of physical performance (Dhese, Jackson et al. 2004). Zhu et al again recruited those with a history of falls and low serum vitamin D concentration ($<20\text{ng/ml}$) to have 1,000 IU ergocalciferol or placebo with calcium for 1 year. They demonstrated an improvement in hip muscle strength in those with lower baseline serum 25(OH)D concentration (Zhu, Austin et al. 2010). A Chilean study recruited community dwelling older subjects with serum 25(OH)D concentration $<16\text{ng/ml}$ to have 400iu cholecalciferol with calcium daily vs. calcium only in an exercise intervention group vs. a non-exercise group. They measured QMVC and handgrip strength and found improvement with both training groups, but

not with vitamin D supplementation either with or without training. However they did find an improvement in other functional measures as well as bone mineral density scores (Bunout, Barrera et al. 2006). Another German study recruited 242 elderly subjects with serum 25(OH)D concentration < 78nmol/l and randomised them to receive 800iu of cholecalciferol + calcium per day vs calcium alone for 1 year and carried out their follow up assessments 8 months later. They found a significant decrease in the number of falls and also an improvement in quadriceps strength in the Vitamin D supplementation group (Pfeifer, Begerow et al. 2009).

A recent meta-analysis of 13 randomised controlled trials (RCTs) involving elderly subjects who were vitamin D deficient or insufficient concluded that improvements in muscle strength were seen in studies with daily doses of 800-1000i u. An improvement in balance was also found, but no effect on gait (Muir and Montero-Odasso 2011).

Two relatively recent studies have looked at Vitamin D supplementation in patients with COPD and potential benefits on muscle strength. Hornikx et al retrospectively analysed a subgroup of patients who took part in a trial looking at Vitamin D supplementation and exacerbation rate in COPD. This subgroup of patients underwent pulmonary rehabilitation and 50% were given high dose vitamin D supplementation and 50% received a placebo. The serum 25(OH)D concentration was increased in those receiving supplements, and significant improvements were seen in inspiratory muscle strength and maximal oxygen uptake. Improvements were also seen in QMVC and walking distance but these did not reach statistical significance (Hornikx, Van Remoortel et al. 2012). Bjerk et al carried out a pilot

study on 36 subjects with GOLD stage III and IV COPD, giving 2000iu of cholecalciferol vs placebo for 6 weeks only. They found no significant difference in the Short Physical Performance Battery Test or improvement in the St George's Respiratory Questionnaire (SGRQ) despite a demonstrated increase in 25(OH)D serum concentration (Bjerk, Edgington et al. 2013). Taken together, these results suggest that some improvements may be seen with vitamin D supplementation in subjects with COPD who are vitamin D deficient, if given high dose supplementation for a prolonged period of time.

1.14.3: Biopsy studies

A number of biopsy studies in the 1970's of subjects with renal failure or those documented as having osteomalacia have shown relatively consistent findings of either 'non-specific' or type II muscle fibre atrophy (Floyd, Ayyar et al. 1974; Dastur, Gagrath et al. 1975; Irani 1976; Yoshikawa, Nakamura et al. 1979). Two supplementation studies in different subsets of patients (bone loss of ageing and osteomalacia) have shown an increase in the proportion and size of Type IIa muscle fibres with vitamin D supplementation although serum 25(OH)D concentration was not measured (Dastur, Gagrath et al. 1975; Sorensen, Lund et al. 1979).

Only one randomised controlled trial of vitamin D supplementation, which included muscle biopsies, has been carried out on post stroke hemiplegic patients with severe vitamin D deficiency, which showed an increase in type II fibre proportion and size after 2 years of vitamin D supplementation in the treatment group, but not the placebo (Sato, Iwamoto et al. 2005).

1.15: Vitamin D status and lung function

The 3rd National Health and Nutrition survey (NHANES) was a large US population based study involving over 14,000 people. It demonstrated convincing evidence that serum 25(OH)D concentration was independently associated with both FEV₁ and FVC (Black and Scragg 2005). Between the lowest and highest quartiles of serum 25(OH)D concentration, there was a difference of 126mls in FEV₁ and 172mls in forced vital capacity (FVC), after correcting for confounding factors.

However not all studies have confirmed this association: Shaheen et al failed to demonstrate an association between adult lung function and serum 25(OH)D in a large cross sectional study (Shaheen, Jameson et al. 2011)

1.16: Serum 25(OH)D concentration in COPD

Over half of the elderly population in the UK and USA have vitamin D deficiency depending on the definition used (Holick 2007; Hypponen and Power 2007). Current debate is still ongoing about the optimal definition of vitamin D deficiency, which could be based on levels required to suppress PTH, levels required for optimal calcium absorption in the intestine and levels required to maintain bone mineral density (BMD) and prevent falls and fractures. Current consensus is that a serum 25(OH)D concentration greater than 75nmol/l is required for optimal bone health (Dawson-Hughes, Heaney et al. 2005). Factors associated with serum 25(OH)D concentration in normal subjects are sunlight exposure, latitude, season, skin type,

dietary intake of vitamin D, BMI and genetic influences, in particular polymorphisms in the vitamin D binding protein (Hypponen and Power 2007)(Lauridsen, Vestergaard et al. 2005; Taes, Goemaere et al. 2006; Ahn, Yu et al. 2010).

In the United Kingdom, sunlight is only sufficiently strong enough to produce cholecalciferol in the skin between April and November (Webb and Engelsen 2006). A recent study in city dwelling subjects in the UK suggests that current recommendations for brief episodes of sun exposure in the summer months will place most people in the 'sufficient' range of serum 25(OH)D concentration, but not the optimal range above 75nmol/l. COPD patients are less active (Pitta, Troosters et al. 2005) and spend less time outdoors (Donaldson, Wilkinson et al. 2005; Baghai-Ravary, Quint et al. 2009) compared to healthy elderly subjects due to breathlessness and leg fatigue. They are therefore less likely to have adequate sunlight exposure although this has not been measured directly. They can also have poor nutritional intake, particularly with severe disease. One study in Spain showed that only 4% of COPD subjects consumed the recommended daily intake of 10µg of vitamin D (de Batlle, Romieu et al. 2009). These factors combined put them at increased risk of having vitamin D deficiency.

One published cross-sectional study has looked at serum 25(OH)D concentration specifically in COPD patients compared to a control population (Janssens, Bouillon et al. 2010). They found that COPD patients had significantly lower serum concentration of 25(OH)D, and that 25(OH)D concentration decreased with increasing GOLD (Global initiative for chronic obstructive lung disease) Stage. The control population consisted of smokers and ex smokers who were matched for age

and sex, and no subjects were taking any vitamin D supplementation. This evidence is supported by an earlier study looking at serum 25(OH)D concentration in patients with severe lung disease who were referred for lung transplant, of whom approximately 50% had a diagnosis of COPD (Forli, Bjortuft et al. 2009). Although there was no control group, subjects had particularly low levels of 25(OH)D (38nmol/l).

1.17: Evidence for a link between vitamin D status and skeletal muscle dysfunction in COPD

No studies published prior to this research being carried out looked at serum 25(OH)D concentration in relation to muscle function in COPD. One study looked at polymorphisms in the VDR and found an association between the fokI polymorphism and muscle strength in COPD patients and control subjects. People with the C allele, which produces a shorter protein product, had a reduced quadriceps strength when compared to those with one or more T alleles, and this supports previous findings in healthy elderly men (Roth, Zmuda et al. 2004). Interestingly, the shorter allele has been associated with an increased risk of type I diabetes, and human monocytes homozygous for the short VDR protein product express higher levels of IL-12 protein and mRNA (van Etten, Verlinden et al. 2007). Thus it is possible that the reduced muscle strength associated with the C allele is due to an exaggerated response to inflammatory stimuli in muscle.

1.18: Osteoporosis and Osteopenia

Recent studies have shown a consistently high prevalence of osteoporosis and osteopenia in patients with COPD. A systematic review showed that osteoporosis occurred in 9 to 69% of patients, and osteopenia in 27 to 67%, with an overall mean prevalence of osteoporosis from 13 studies, involving 772 patients, of 35.1% (Graat-Verboom, Wouters et al. 2009). Four studies included in this review compared COPD patients and age matched healthy control subjects and there was a significant difference in prevalence: 32.5% in COPD vs. 11.4% in healthy controls. Interestingly, the prevalence of osteoporosis in COPD appears to be higher than in some other chronic lung diseases, including those involving chronic corticosteroid use, such as idiopathic pulmonary fibrosis (Aris, Neuringer et al. 1996; Katsura and Kida 2002; Tschopp, Boehler et al. 2002).

Factors which are consistently related to osteoporosis in multivariate analyses are age, sex, BMI and other anthropometric measurements, lung function and corticosteroid use (Scanlon, Connett et al. 2004; Kjensli, Mowinckel et al. 2007; Vrieze, de Greef et al. 2007).

As well as the morbidity and mortality associated with hip and wrist fractures, of particular relevance in COPD patients are vertebral compression fractures (VCFs). These cause kyphosis which has a detrimental effect on lung function (Leech, Dulberg et al. 1990; Schlaich, Minne et al. 1998). A prospective study of 245 COPD patients in Canada looked at the prevalence of VCFs seen on chest x-ray (CXR) and

found it to be 9% (Majumdar, Villa-Roel et al. 2010). BMI was the only variable independently associated with incidence of VCF.

Low serum 25(OH)D contributes to the development of osteopenia and osteoporosis as there is reduced calcium absorption from the gut, and both PTH and 1,25(OH)D₂ increase bone resorption to maintain calcium levels (Suda, Ueno et al. 2003). Vitamin D supplementation in elderly people, when given with calcium, has been shown to improve BMD and reduce the risk of falls and fracture (Chapuy, Pamphile et al. 2002; Boonen, Lips et al. 2007; Abrahamson, Masud et al. 2010), although one study giving annual high dose cholecalciferol showed an increased risk of falls compared to placebo (Sanders, Stuart et al. 2010) suggesting that dosing regimen is an important factor. The reduction in fracture risk may be due to a combination of increases in BMD and indirectly through increases in muscle strength and a reduction in falls.

It is possible therefore that Vitamin D deficiency may contribute to the increased prevalence of osteoporosis and osteopenia seen in COPD. Only one study has looked at the effect of serum 25(OH)D concentration on BMD in COPD patients and found no relationship between them. They compared BMD in 49 COPD and 40 healthy control subjects and found it to be significantly lower in COPD patients in the lumbar spine, femoral neck and total femur. However other parameters were only measured in COPD patients. FEV₁ (l) and weight were independently associated with BMD in the multivariate model which included corticosteroid use and serum 25(OH)D concentration (Franco, Paz-Filho et al. 2009).

1.19: Other connections between Vitamin D status and COPD

1.19.1: Inflammation

1,25(OH)₂D is an important modulator of the innate and adaptive immune systems. The VDR is expressed in most cells of the immune system including macrophages, dendritic cells, neutrophils, B cells and activated CD4⁺ and CD8⁺ T cells (Baeke, Takiishi et al. 2010). There are two important roles that 1,25(OH)₂D has in the regulation of the immune system which are likely to have an impact on the development and progression of COPD.

In the innate immune system, 1,25(OH)₂D stimulates the production of cathelicidin. This is an anti-microbial peptide which has broad spectrum activity against bacterial and viral pathogens. It acts as a chemo attractant for various inflammatory cell types, and is involved in epithelial proliferation and repair, and angiogenesis (Hiemstra 2007). 1,25(OH)₂D has activity against *Mycobacterium tuberculosis*, an action which has now been shown to be mediated by cathelicidin (Martineau, Wilkinson et al. 2007). More importantly, for subjects with COPD, cathelicidin has also been shown to have activity against *Pseudomonas aeruginosa* and *Escherichia coli* (Wang, Nestel et al. 2004)

Another important role of 1,25(OH)₂D in the immune system is that of T cell regulation. 1,25(OH)₂D has been identified to play an important role in maintaining T cell balance. It alters transcription of IL-2, interferon gamma (IFN γ) and IL-4 and has

been shown to directly influence CD4⁺ cells to suppress Th1 and promote Th2 differentiation (Alroy, Towers et al. 1995; Boonstra, Barrat et al. 2001). More recently it has been demonstrated that 1,25(OH)₂D suppresses production of IFN γ , IL-17 and IL-21 by CD4⁺ cells, and following 1,25(OH)₂D treatment, T cells adopted functional and phenotypic properties of regulatory T cells (Tregs), expressing high levels of FoxP3 (Jeffery, Burke et al. 2009). These latter actions have important implications on auto immune disorders such as diabetes and inflammatory bowel disease and may be relevant to autoimmune processes in COPD. The lungs of subjects who smoke but have normal lung function have been shown to have higher levels of Tregs than those who have never smoked, and compared to smokers who have developed COPD (Barcelo, Pons et al. 2008). Studies in mice have demonstrated an inflammatory airway response mediated by Th17 cells and characterised by increased neutrophilic inflammation and B cell influx, which was suppressed by the co- transfer of Tregs (Jaffar, Ferrini et al. 2009). Taken together, it appears that functional regulatory T cells may protect against the development of COPD by controlling the T cell response to immune stimuli.

The actions of 1,25(OH)₂D on the immune system are likely to be of relevance to the occurrence of respiratory infection and exacerbations in COPD. However two recently published studies found no association between serum 25(OH)D concentration at baseline and time to first exacerbation or exacerbation frequency (Kunisaki, Niewoehner et al. 2012; Quint, Donaldson et al. 2012), and one supplemental study found only a reduction in exacerbations with supplementation in those with baseline 25(OH)D <10ng/ml (Lehouck, Mathieu et al.).

1.19.2: Cardiovascular Disease

25(OH)D and 1,25(OH)₂D are also implicated in the development of cardiovascular disease. In 1 α -hydroxylase knockout mice, increased blood pressure, activation of the renin-angiotensin system, myocardial hypertrophy and decreased cardiac function are seen which are prevented by the administration of 1,25(OH)₂D (Goltzman 2010).

In human studies, low levels of serum 25(OH)D have been associated with increased risk of hypertension (Forman, Curhan et al. 2008) and cardiovascular disease (Kendrick, Targher et al. 2009), and 8 weeks of vitamin D and calcium supplementation have been shown to reduce systolic blood pressure compared to calcium alone (Pfeifer, Begerow et al. 2001). A number of large supplemental trials of Vitamin D are currently underway to try and clarify potential benefits of Vitamin D supplementation on cardiovascular outcomes.

1.19.3: Cancer Risk

Several epidemiological studies have associated low serum 25(OH)D concentration with increased risk of colorectal, breast and prostate cancer risk (Giovannucci 2005). However its effects in lung cancer have not been clearly demonstrated. VDR polymorphisms have been associated with the incidence of lung cancer (Dogan, Onen et al. 2009) and in vitro cell studies have shown that 1,25(OH)₂D has anti cancer effects such as suppression of angiogenesis and cell proliferation (Giovannucci 2005). Mouse studies have shown a protective effect of 1,25(OH)₂D against metastases in lung cancer (Nakagawa, Kawaura et al. 2004). However epidemiological data in

human studies has not shown an association between low serum 25(OH)D concentration and lung cancer (Kilkinen, Knekt et al. 2008).

1.19.4: Ageing

COPD has been hypothesised to be a disease of ageing (Ito and Barnes 2009) and evidence which supports this hypothesis is discussed above. 1,25(OH)₂D also controls many genes involved in aging (Haussler, Haussler et al. 2010) and in human studies, higher serum 25(OH)D concentrations have been associated with longer telomere length even after adjustment for age and other confounding factors (Liu, Prescott et al.; Richards, Valdes et al. 2007). Biopsy studies in humans have shown that VDR concentrations in skeletal muscle (Bischoff-Ferrari, Borchers et al. 2004) and duodenum (Ebeling, Sandgren et al. 1992) decrease with age and it is possible that decreased concentrations may be present in COPD patients causing a relative resistance to 1,25(OH)₂D which may be the link to accelerated aging in COPD.

1.20: Genetic influences on skeletal Muscle strength

As well as on lung function itself, genetic influences also appear to be important with regards to skeletal muscle function in COPD. The VDR FokI polymorphism has been associated with muscle strength in both COPD and healthy subjects, whilst the VDR Bsm polymorphism is associated with strength only in a COPD population (Hopkinson, Li et al. 2008). The ACE I/D polymorphism is also associated with strength in COPD subjects (Hopkinson, Nickol et al. 2004).

1.21: Research Questions

In summary, 1,25(OH)₂D has important actions in skeletal muscle, and subjects with COPD are known to develop skeletal muscle dysfunction which appears to be multifactorial although reduced physical activity plays a significant role. COPD subjects have been demonstrated to have a lower serum 25(OH)D concentration, Hopkinson et al demonstrated an effect of polymorphisms in the VDR and skeletal muscle strength in both COPD and healthy subjects and there are a significant number of co morbidities in COPD that are linked to Vitamin D status.

This research was therefore carried out to try and answer the following questions:

- (1) Is Vitamin D status a contributing factor to skeletal muscle dysfunction in COPD?
- (2) What are the potential molecular pathways through which 25(OH)D and 1,25(OH)₂D may influence skeletal muscle strength?
- (3) Do polymorphisms in genes involved in Vitamin D metabolism and the Angiotensin pathway influence serum 25(OH)D concentration and / or skeletal muscle strength in COPD?

A cross sectional clinical study was undertaken which compared serum 25(OH)D and 1,25(OH)₂D concentrations, and skeletal muscle strength in COPD subjects with an age and sex matched control population and this is described in Chapter 3.

A smaller sub-study was carried out in subjects who underwent a muscle biopsy to investigate whether associations between serum 25(OH)D and 1,25(OH)₂D concentrations, myogenic regulatory factor mRNA expression and muscle fibre type mRNA expression at a molecular level were similar in COPD and control subjects, and also looked at VDR protein concentrations in skeletal muscle. This is described in Chapter 4.

Genetic influences of polymorphisms in genes affecting Vitamin D metabolism and the renin-angiotensin system on serum 25(OH)D concentration and skeletal muscle strength were investigated and these are described in Chapter 5.

Chapter 2: Description of Methods

2.1: Ethical Approval

All subjects included in this work provided informed written consent and the study was approved by the Ethics Committee of The Royal Brompton Hospital. The study was registered on the UKCRN clinical trials register, number 6551.

2.2: Power Calculations

For the cross sectional clinical study, a power calculation was performed by Michael Roughton at Imperial College, which suggested that a sample size of 100 was required to give an 80% power of detecting an r^2 correlation of 0.37 assuming a SD of 12.5 kg for quadriceps maximum voluntary contraction strength and of 17iu for vitamin D level.

2.3: Statistical Analysis

Statistics were analysed using SPSS for windows version 20.0 and STATA Release 10.1. Descriptive statistics are reported as mean (standard deviation) for parametric data, and median (range) for non-parametric data. T tests were used to compare means for parametric data and the Mann-Whitney test for non-parametric data. The Spearman rank test was used for correlations. Stepwise logistic regression was used to establish clinical factors influencing muscle strength. Regression with robust variances was used to compare endurance curves between COPD and control groups,

and to compare vitamin D sufficient and insufficient subjects within groups. A p value <0.05 was considered significant.

For the work looking at genetic polymorphisms, stepwise logistic regression was used to look for factors influencing muscle strength and serum 25(OH)D levels. To look for gene - gene interactions, a hierarchy of models was used. A p value of <0.01 was considered significant.

2.4: Study Subjects

Stable COPD patients were recruited from outpatient clinics and from the department's clinical database.

Inclusion criteria were as follows:

- 1) $FEV_1/FVC < 70\%$
- 2) Significant smoking history

Exclusion criteria were as follows:

- 1) exacerbation within the preceding 3 months
- 2) uncontrolled heart failure
- 3) NIDDM
- 4) Primary musculoskeletal problem eg previous polio
- 5) malignancy

Control subjects were recruited by advertisement (figure 2.1), through a local community group for the elderly, and through collaboration with other research

groups.

Healthy over 50's We are looking for healthy men to help with a study looking at factors influencing muscle strength and aging

Here at The Royal Brompton Hospital we are interested in finding out what factors effect exercise capacity in Chronic Obstructive Pulmonary Disease (COPD) (also known as chronic bronchitis or emphysema). We are looking for healthy men to act as controls. We would like to make some measurements of the strength of your breathing and leg muscles and to take a blood sample. To take part you would be required to come to the Royal Brompton Hospital in South Kensington for about 2 hours. We would like to repeat the tests each year to see how they change over time. If you are interested or would like more details, please call Dr Abigail Jackson on 020 7351 8029, at the Muscle Laboratory at the Royal Brompton Hospital, Fulham Rd.

Expenses paid

This study has been approved by the Royal Brompton and Harefield Research Ethics Committee (07/Q0404/17)

Figure 2.1: Advertisement used to recruit healthy control subjects

Inclusion criteria:

- 1) $FEV_1/FVC > 70\%$
- 2) $FVC > 70\%$ predicted

Exclusion criteria were as follows:

- 1) uncontrolled heart failure
- 2) NIDDM

- 3) Primary musculoskeletal problem eg previous polio
- 4) malignancy

Subjects were screened via a telephone call and if suitable, invited to attend the skeletal muscle laboratory for further assessment. 95% of control subjects who were screened were able to take part in the study. A small number of these (n=6) were diagnosed with GOLD stage I COPD and therefore included in the COPD arm of the study. 90% of COPD subjects screened via telephone took part in the study.

Study participants were sampled throughout the year, and there was no difference in the time of year measured between the patient and control groups. A systematic history including exacerbation rate and average daily dose (ADD) of oral corticosteroids in the preceding year was performed.

2.5: Dietary Vitamin D Intake

We included subjects who were taking Vitamin D supplementation which could include prescribed Vitamin D3 supplementation or cod liver oil. We assessed total weekly Vitamin D intake using a recall questionnaire (figure 2.2) developed by ourselves based on known dietary sources of vitamin D (Holick 2007). There is currently no standardised way of assessing vitamin D dietary intake, which may be due to the fact that our main source of Vitamin D is through the action of sunlight on the skin. We could not directly measure sunlight exposure but it is known that COPD subjects spend less time out of doors and dietary sources of Vitamin D are likely to be more relevant for them.

Food	IU's per serving	Servings per week	IU's per week
Cod liver oil, 1 tablespoon	1,360		
Salmon, cooked, 3.5 ounces	360		
Mackerel, cooked, 3.5 ounces	345		
Tuna, canned in oil, 3 ounces	200		
Sardines, canned in oil, 1.75 ounces	250		
Margarine, fortified, 1 tablespoon	60		
Cereal, fortified 10% DV	40		
Egg (whole)	20		
Liver, beef, cooked 3.5 ounces	15		
Cheese, 1 ounce	12		
Total (per week)			

Table 2.2: Dietary Vitamin D assessment

2.6: Health-Related Quality of Life

Health-related quality of life is a tool for assessing patients own perception of their disease and how their symptoms affect them. It complements clinical assessment and other measures of physical function. A number of questionnaires have been developed and validated to assess health-related quality of life, some of which are disease specific.

In this thesis we used the St George's Respiratory Questionnaire to assess health-related quality of life in COPD subjects. The SGRQ is a 76-item supervised self-administered questionnaire. Patients complete it without conferring with anyone else, but the investigator is available to clarify the meaning of questions. Standard answers to potential patient queries are given in an accompanying manual. Three component scores are calculated: symptoms, activity, and impacts (on daily life), and a total

score. These four scores can range from 0 to 100 with a high score representing worse quality of life (QOL). It has been validated in COPD in terms of repeatability and correlation with appropriate clinical measures including MRC (medical research council) dyspnoea score, presence of wheeze, six minute walk distance, anxiety-depression and cough. (Jones, Quirk et al. 1992) It is strongly associated with arterial oxygen tension (PaO₂) (Okubadejo, Jones et al. 1996) and FEV₁ (Ferrer, Alonso et al. 1997; Jones 2001), as well as hospital readmission rates (Osman, Godden et al. 1997), and has been shown to deteriorate with exacerbations (Seemungal, Donaldson et al. 1998) More importantly, it has been shown to be a predictor of mortality in COPD independent of lung function and age, with the SGRQ activities score being most strongly associated with mortality in a 5 year follow up study with a relative risk of 1.038 (p=0.0001) (Oga, Nishimura et al. 2003).

2.7: Yale Physical Activity Survey (YPAS)

Daily physical activity can be assessed either directly with a variety of activity monitors or indirectly through questionnaires. The former gives a more accurate assessment of daily activity but involves a prolonged period of monitoring and at least 2 study visits for a subject which was not practical for this cross sectional study. The YPAS was therefore used to assess daily physical activity in both patient and control groups.

The YPAS is an interviewer administered survey that asks the individual to estimate time spent in a list of 25 activities in a typical week during the last month. These activities are categorised into work, yard work, care taking, exercise and recreational

activities. Time spent in each activity is multiplied by an intensity code ($\text{kcal} \cdot \text{min}^{-1}$) and then summed across all activities to create an index of weekly energy expenditure ($\text{kcal} \cdot \text{wk}^{-1}$). In addition, time spent in each activity is summed to provide a total time index ($\text{h} \cdot \text{wk}^{-1}$). Individuals are also asked to estimate the number of hours spent in five distinct physical activity dimensions. Specifically, they are asked to categorise the frequency and duration of vigorous activity, leisurely walking, moving, standing and sitting. Weights are assigned to each category (vigorous activity: 5; leisurely walking: 4; moving: 3; standing: 2; sitting: 1). The frequency score and duration score are multiplied together and then multiplied again by each dimension's weighting factor to calculate an index for each dimension. A summary index is the sum of the five individual indices.

This questionnaire was designed and validated in 1993 to assess physical activity in older people. In the validation study ($n=25$), the YPAS index of vigorous activity correlated positively with estimated maximal oxygen consumption (VO_2max) ($r=0.60$; $P=0.003$), whilst the weekly energy expenditure ($r=-0.47$; $P=0.01$) and daily hours spent sitting ($r=0.53$; $P=0.01$) correlated with resting diastolic blood pressure (Dipietro, Caspersen et al. 1993). Similar findings were reported in a subsequent larger validation study ($n=69$) which again showed a correlation of various YPAS measures with VO_2max (summary, moving and standing indices) (Young, Jee et al. 2001). The YPAS is a useful tool in comparing physical activity between populations (Lindamer, McKibbin et al. 2008) and has been previously used to compare COPD and control populations (Swallow, Gosker et al. 2007).

2.8: Body Composition

Height was measured to the nearest cm using a wall mounted height bar. Subjects were not wearing shoes. Weight was measured using Tanita TBF-305 single-point load cell electronic scales (Tanita Corporation, Illinois, USA). Subjects were lightly dressed. BMI was also calculated (weight/height²).

FFM was determined using bioelectrical impedance analysis. This technique uses the electrical impedance of body tissues to determine an estimate of total body water since electricity is conducted by dissolved ions. A two-compartment model is used, which assumes that adipose tissue contains no water and that the fat free mass is of particular percentage water, from which fat free mass can be derived. A number of equations have been produced based on the relationship that fat free mass is proportional to height²/resistance. Weight, height, gender, age and habitual physical activity have all been found to influence this relationship.

In COPD, equations validated in healthy populations tend to over-estimate FFM. Disease specific equations have been validated against other techniques for assessing body composition including deuterium dilution dual energy x-ray absorptiometry (DEXA), hydrodensitometry and skin fold anthropometry (Schols, Wouters et al. 1991).

The equations for calculating FFM used in this thesis were those of Steiner *et al* (Steiner, Barton et al. 2002) which have been validated against DEXA and incorporate weight, height and gender.

Males: FFM (kg) = $8.383 + ((0.465 * \text{Height}^2 \text{ (cm)} / \text{resistance (ohm)}) + (0.213 * \text{Weight (kg)}))$

Females: FFM (kg) = $7.610 + ((0.474 * \text{Height}^2 \text{ (cm)} / \text{resistance (ohm)}) + (0.184 * \text{Weight (kg)}))$

Fat free mass index (FFMI) was calculated by dividing FFM by height in metres squared. By convention, a value of <15 kg/m² for women or <16 kg/m² for men is considered to represent nutritional depletion.



Figure 2.3: Measurement of bioelectrical impedance

Bioelectrical impedance was measured using a Bodystat 1500 device (Bodystat, Isle of Man, UK). Subjects were supine, with arms and legs abducted so that they were not touching the subject's body or each other. Biotab Ag/AgCl electrodes (Maersk Medical, Stonehouse, UK) were placed on the hand behind the knuckle of the middle finger and on the wrist next to the ulnar head, as well as on the foot behind the 2nd toe and on the inter-malleolar line on the dominant side (figure 2.3). The device was calibrated regularly using a calibration unit of known impedance provided by the

manufacturer.

2.9: Respiratory Muscle Strength

Sniff nasal inspiratory pressure (SNiP) is a volitional non-invasive technique for assessing respiratory muscle strength. A plug is inserted into one nostril which is attached to a pressure transducer via a polyethylene tube. Subjects are asked to perform a maximal sniff manoeuvre through the contra-lateral nostril and the pressure is recorded. Measurements are repeated until reliable repeated values are seen which may take up to 10 repeats.

This technique was developed as an alternative to invasive measures of respiratory muscle function and has been validated against sniff oesophageal pressures in normal subjects as well as those with respiratory muscle weakness (Heritier, Rahm et al. 1994).

2.10: Handgrip Strength

Handgrip strength was measured using a Jamar handgrip dynamometer (Sammons Preston Rolyan, Bolingbrook, IL). In accordance with the American Society of Hand Therapy recommendations, subjects were seated with their shoulders in 00 abduction and neutral rotation, their elbow in 90° of flexion, and their forearms in neutral pronation / supination (Fess 1992). Subjects performed 6 maximal contractions alternating hands with a rest of 30 seconds in between. The maximal value of the dominant hand was used. This technique has been shown to be a reliable

measurement in older community dwelling adults (Bohannon and Schaubert 2005).

2.11: Quadriceps Strength

Quadriceps strength can be measured using the volitional technique of Edwards *et al* (Edwards, Young et al. 1977), or by using a magnetic impulse to supra-maximally stimulate the femoral nerve (Polkey, Kyroussis et al. 1996). Both techniques were used in this thesis and are described below.

For the measurement of Quadriceps maximum voluntary contraction, (QMVC), subjects sat in a modified chair with an inextensible strap connecting the ankle of their dominant leg to a strain gauge (Staininstall Ltd, Cowes, UK). The signal was amplified and passed to a computer running LabView4 software (National Instruments, Austin, Texas). The linearity of the strain gauge is factory certified from 0-100kg. The equipment was calibrated using a suspended weight for each subject. To ensure that the contraction was isometric, the subject's knees were aligned at 90 degrees, and the strain gauge and couplings were also aligned. Subjects performed a minimum of 3 sustained maximal isometric quadriceps contractions of 5-10 seconds duration. The force produced was visible online to both subject and investigator to allow positive feedback and vigorous encouragement was given. A gap of at least 30 seconds was left between contractions to allow time to recover. The maximum strength (kg) sustained for 1 second was measured for each contraction and the average of 3 contractions with a variability of less than 5% was used for each subject.

Unpotentiated twitch quadriceps force (TwQu) was assessed by magnetic femoral nerve stimulation according to the technique described by Polkey et al (figure 2.4). Subjects were seated in the same modified chair described above with the ankle of their dominant foot in a strap attached to the strain gauge in an isometric position. However they were now in a supine position and their leg was rested for 20 minutes to allow the muscle to depotentiate. Stimulation was performed using two Magstim 200 monopulse units discharged simultaneously through a 70mm branding iron coil. This combination delivers an output approximately equivalent to 120% of the output of a single unit. The coil is pressed firmly over the femoral nerve high in the femoral triangle and discharged. The twitch force produced is recorded using the equipment described above. A 30 second gap between potentiations is required to avoid 'twitch on twitch' potentiation. The position was initially adjusted and then a stimulus response curve was generated to ensure supramaximality of stimulation with 3 stimuli at 80, 85, 90, 95 and 100% of stimulator output in random order. The mean of at least 5 stimulations at 100% stimulator output was taken. Supramaximality using this technique has been demonstrated previously (Polkey, Kyroussis et al. 1996; Harris, Polkey et al. 2001; Schonhofer, Zimmermann et al. 2003)



Figure 2.4: Non-volitional assessment of quadriceps strength

2.12: Quadriceps Endurance

Quadriceps Endurance was measured using the technique described by Swallow *et al* (Swallow, Gosker et al. 2007) (figure 2.5). The subject is seated on the same modified apparatus as detailed above in a reclining position, with the ankle of their dominant leg attached to the strain gauge in an isometric position with the ankle strap. A Magstim Rapid flat oval and flexible magnetic coil was wrapped around the quadriceps muscle and fastened into place. The coil consists of an elliptical shaped coil with nine concentric insulated copper rings encased in silicone. A space is left

between the exterior of the insulation and the silicone to allow pumping of a cooling fluid, 3-ethoxy-1,1,1,2,3,4,4,5,5,6,6,6-dodecafluoro-2-trifluoromethyl-hexane. The stimulator was set at a frequency of 30 Hz, a duty cycle of 0.4 (2 s on, 3 s off), and for 50 trains (250 s). The stimulator intensity was adjusted for each subject so as to initially generate 20% of their supine QMVC. The quadriceps force produced with each stimulation was recorded with the same equipment as described previously. The maximum force produced during each train was measured manually. Endurance was then calculated as the time taken for the force produced to decline to 70% of baseline force.



Figure 2.5: Quadriceps endurance measurement

Other methods of measuring quadriceps endurance involve repetitive maximal voluntary contractions which can be difficult to maintain and depend on patient effort. Electrical stimulation has also been used but is less well tolerated by patients than magnetic stimulation (Han, Shin et al. 2006). The above technique was developed with patient tolerability in mind and has been shown to be repeatable (Swallow, Gosker et al. 2007). Repetitive magnetic stimulation has also now been trialled as a method for training the quadriceps muscle in subjects with severe COPD limited by dyspnoea with positive results on muscle strength, 6 minute walk distance and quality of life (Bustamante, Lopez de Santa Maria et al.)

2.13: Pulmonary Function Testing

COPD subjects had full lung function tests performed by technicians working in the Lung Function Department of the Royal Brompton Hospital. Spirometry was obtained using a heated pneumotachograph with flow integration, lung volumes by whole body plethysmography and gas transfer with a single breath technique (Compact Master lab system, Jaeger, Germany). Predicted values used are those of the European Coal and Steel Community (Quanjer, Tammeling et al. 1993). The equipment is regularly calibrated and the tests were performed in accordance with the British Thoracic Society Guidelines (1994).

Control Subjects had spirometry measured according to British Thoracic Society Guidelines (1994)) using a handheld spirometer (MicroLab 3500 Mk 8, Micromedical Ltd, Warwick, UK).

2.14: Serum Analysis

Whole blood was collected in lithium heparin and immediately spun in a centrifuge at 800 RPM for 20 minutes. The supernatant was removed into a new sterile container and stored at -80°C . Batch analysis was then performed for 25(OH)D, 1,25(OH)₂D, high sensitivity c-reactive protein (hsCRP), IL6, Magnesium, PTH, Calcium, Albumin, Phosphate and Magnesium. Analysis was performed by the department of Clinical Chemistry at The Royal Brompton Hospital except for analysis of 1,25(OH)₂D which was carried out by the Department of Clinical chemistry at West Park Hospital, Epsom.

2.14.1: 25(OH)D

Serum 25(OH)D concentration was measured by radioimmunoassay (RIA) after acetonitrile extraction (25-hydroxyvitaminD RIA; Immunodiagnostic Systems, Boldon, Tyne and Wear). Sodium hydroxide solution (1%) and acetonitrile were added to samples which caused precipitation of serum proteins. Following centrifugation, portions of the supernatant were incubated with 125I-labelled 25-OHD and a highly-specific sheep antibody to 25-OHD. Separation of antibody-bound tracer from free was achieved by a short incubation with Sac-Cel® (antisheep IgG cellulose) followed by centrifugation and decanting. Bound radioactivity was inversely proportional to the concentration of 25-OH D.

This assay measures 25(OH)D₃ and has a 75% cross reactivity with 25(OH)D₂. It correlates well with measurements made by high performance liquid chromatography

($r^2 = 0.89$) and the other available RIA, Diasorin ($r^2 = 0.92$) (Zerwekh 2004). However it can underestimate the total quantity of serum 25(OH)D because it does not react quantitatively with 25(OH)D₂ (Hollis 2000).

2.14.2: 1,25(OH)₂D

Serum 1,25(OH)₂D was measured by enzyme immunoassay (EIA) after immunoextraction (1,25-DihydroxyVitaminD EIA; Immunodiagnostic systems, Boldon, Tyne and Wear).

Patient samples were delipidated and 1,25(OH)₂D extracted from potential cross-reactants by incubation for 90 minutes with a highly specific solid phase monoclonal anti-1,25(OH)₂D. The immunoextraction gel was then washed and purified 1,25(OH)₂D eluted directly into glass assay tubes. Reconstituted eluates and calibrators were incubated overnight with a highly specific sheep anti-1,25(OH)₂D. A portion of this was then incubated for 90 minutes whilst shaking in microplate wells coated with a specific anti-sheep antibody. 1,25(OH)₂D linked to biotin was then added and the plate shaken for a further 60 minutes before aspiration and washing. Enzyme (horseradish peroxidase) labelled avidin was added which binds selectively to complexed biotin and, following a further wash step, colour was developed using a chromogenic substrate (TMB). The absorbance of the stopped reaction mixtures were read in a microtitre plate reader, colour intensity developed being inversely proportional to the concentration of 1,25(OH)₂D.

This sensitivity of this assay (defined as the concentration corresponding to the mean

minus 2 standard deviations of 20 replicates of the zero calibrator) is 6 pmol/L. The specificity for 1,25(OH)₂D and its metabolites is shown in table 2.1.

Table 2.1: Specificity of 1,25(OH)₂D RIA, IDS, Tyne and Wear

Analyte	Cross-reactivity
1,25-Dihydroxyvitamin D3	100%
1,25-Dihydroxyvitamin D2	39%
24,25-Dihydroxyvitamin D3	0.056%
25-Hydroxyvitamin D3	0.009 %

In view of the lack of cross-reactivity with 1,25(OH)₂D₂, this assay can again underestimate the total serum 1,25(OH)₂D. Because of its presence in extremely low concentrations in the serum combined with the presence of other interfering substances and low ionising properties, techniques for measuring serum 1,25(OH)₂D concentration have been more difficult to develop than those for measuring serum 25(OH)D concentration. However in recent years, purification procedures and liquid chromatography techniques have been developed (van den Ouweland, Vogeser et al. 2013)

2.14.3: PTH, albumin, electrolytes and inflammatory markers

Calcium, phosphate, albumin and hsCRP were all run on the Beckman DxC600 autoanalyser., and PTH and IL6 were run on the Beckman Access 2 Immunoassay

analyser (Beckman Coulter, High Wycombe, UK) according to the manufacturers instructions.

2.15: Genotype Analysis

For genotype analysis, a 5ml EDTA blood sample was taken. Samples were frozen immediately and stored at –80 degrees centigrade and analysed in a batch by Dr James Skipworth at The Department of Cardiovascular Genetics, Rayne Institute, University College, London. DNA was extracted by salting out. The ACE I/D and Bradykinin +9/-9 polymorphisms were run on a Microplate array diagonal gel electrophoresis (MADGE) gel whilst all other polymorphisms were genotyped using TaqMan (table 2.1).

Table 2.2: Genetic polymorphisms

Gene	Name	rs number	DNA change	AA change
ATR1	A1166C	rs5186	A→C	-
AGT	Met235Thr	rs699	C→T	Met→Thre
ACE	I/D	rs4646994		Alu repeat
GC		rs7041	G→T	Asp→Glut
GC		rs4588	C→A	Thre→Lys
CYP2R1		rs10741657	C→A	

2.15.1: DNA whole blood extraction and quantification

DNA extraction was carried out using the Miller *et al.* 'salting-out' method outlined in *A simple salting out procedure for extracting DNA from human nucleated cells* (Miller, Dykes et al. 1988). This included a whole-blood cellular lysis step using 'reagent A' and centrifugation at 10,000rpm for 10min, which was repeated once, followed by a nuclear lysis step using 'reagent B'. The pellets were resuspended following each centrifugation. 5M sodium per chlorate was added to the lysed nuclear pellets the tubes were left to shake for 20min. Chloroform was then added and the tubes were centrifuged at 3000rpm for 3min. The aqueous phase was transferred to a clean tube for precipitation with -20°C 100% ethanol. The woolly DNA was collected, washed with 70% ethanol and stored at room temperature in TE buffer for 2 weeks to allow the viscous DNA to dissolve

2.15.2: DNA Extraction Reagents

Reagent A (Cellular Lysis):

0.32M Sucrose	109.54g
5mM MgCl ₂	5ml of 1M solution
10mM Tris-HCl pH7.5	10ml of 1M solution
1% Triton-X-100	10ml

Made up to 1L with deionised water and stored at 4°C (discarded after 3 weeks)

Reagent B (Nuclear Lysis):

10mM Tris-HCl pH8.2	10ml of 1M solution
0.4M NaCl	23.4g

2mM Na₂EDTA pH8.0 4ml of 0.5M solution

Made up to 900ml with deionised water and autoclaved. After autoclaving 100ml

10% SDS was added. Stored at room temperature

TE Buffer:

10mM Tris 1.21g

1mM EDTA 0.37g

Make up to 1L with deionised water pH with concentrated HCl. Autoclaved

2.15.3: DNA quantification and robot standardisation of DNA arrays

Following extraction, DNA concentration of the samples were measured and standardised using a Nanodrop® 8000 spectrophotometer and Beckman Coulter Biomek® 2000 respectively, to a concentration of 15ng/μl as stock in 96-well array plates. These stocks were further diluted to 5ng/μl working stocks which were used to run ACE-SNP MADGE gels. 60μl of 1.25ng/μl DNA stocks were made up for the 8x384-well plates, by using the Beckman Coulter Biomek® 2000 again, to make up a 5ng DNA weight per sample for the running of the TaqMan assays.

2.15.4: TaqMan SNP genotyping

Genotyping of the DNA samples was carried out using TaqMan® SNP genotyping assay kits which were custom prepared to include TaqMan® Universal PCR Master Mix and SNP genotyping mix (containing two polymorphism-specific probes labelled with VIC® and FAM™ dyes to detect two forms of the alleles and sequence-specific primers surrounding the region of interest). 2μl of the reaction mix was pipetted into

each well of the 384-well plates. The polymerase chain reaction (PCR) process was conditioned to carry out an initial 10min polymerase activation step at 95°C followed by 40 thermal amplification cycles involving a 15sec denaturing step at 92°C and 1min at 60°C to allow the DNA to anneal and extend. The allele-specific annealing probes containing the fluorophores are associated with quenchers. On release during PCR (at the base-by-base displacement/elongation stage), the fluorophores fluoresce depending on which were initially bound. The Applied Biosystems™ 7900HT Fast Real-Time PCR System was used to detect optical densities at the two wavelengths and allelic discrimination data was derived indicating the allele(s) present in each sample. Since testing for these single nucleotide polymorphisms (SNPs) is well established in other studies, the primers did not have to be custom-designed and could be ordered directly. Each 384 well TaqMan assay plate contained at least 4 wells which served as negative controls eliminating the possibility of false positive results. These wells contained H₂O and were called NTCs (no template controls).

2.15.5: MADGE gel ACE I/D genotyping

MADGE was used to identify the ACE allelic composition of the DNA. ACE insertion/deletion polymorphisms were amplified by PCR and identified through DNA size differences using 7.5% MADGE. The PCR was run with three primers, to prevent mistyping (Shanmugam, Sell et al. 1993), giving products of 65bp and 85bp in length corresponding to the insertion (I) and deletion (D) alleles respectively. Genotype determination was based on the presence of one or both of these bands, representing the homozygotes (DD/II) – clearly differentiated by distance of migration – or the heterozygotes (DI) – where two bands were visible. The PCR conditions under which the 96-well plates were run involved an initial 5 min at 95°C

followed by a 35-repetition cycle of 45sec at 95°C, 45sec at 54°C and 30sec at 72°C with a final 5min at 72°C step. The 96-well plates were covered with a 20µl layer of mineral oil to reduce risk of sample evaporation. The gels were run at 110V for 1hr 10min to produce the clearly separated bands.

2.16: Muscle Biopsy

Muscle biopsy samples were taken from the vastus lateralis of the dominant leg. Informed consent was obtained. Local anaesthetic was infiltrated and a 1cm incision was made in the skin. A Bergstrom needle was then used to obtain the muscle samples. Tissue was snap frozen in liquid nitrogen and subsequently stored at -80°C for later analysis.

2.17: mRNA Expression

For real-time quantitative polymerase chain reaction (RT-qPCR), RNA was extracted from muscle biopsies using trizol (Sigma, UK) as per the manufacturer's recommendations. The concentration of RNA was quantified using a spectrophotometer (Nanodrop ND1000, Wilmington, USA). First strand cDNA was generated using Superscript[®] II Reverse Transcriptase (Invitrogen). RT-qPCR analysis was carried out in duplicate on each cDNA sample for *MHCI*, *MHCIIa*, *MHCIIx*, the myogenic regulatory factors *myogenin*, *mrf4* and *myf5*, and for the reference housekeeping gene human *RPLPO* (large ribosomal protein), using a 20 µl reaction of SYBR[®] Green Quantitative PCR Kit (Sigma Aldrich, UK) and the primer pair (4pmol) in 96 well plates (MicroAmp, Fast optical 96 well reaction plate (0.1 ml)

(Applied Biosystems, UK.). The qPCR reactions were run on the 7500 Fast Real-time PCR System (Applied Biosystems, UK.), with the following cycle program: 95 °C for 10 minutes, then 40 cycles of 95 °C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds. For each gene studied, the average of the 2 samples for each subject was taken and expressed to the power of 2. This figure was then divided by the result for the control gene (RPLPO) and then log transformed to obtain a normal distribution. The PCR products were run on a 2% agarose gel to confirm the size of the product.

For *myogenin* and *myf5*, the primer sequences as shown in table 2.2 were selected based on the work of Plant et al (Plant, Brooks et al. 2010). For *mrf4* the primer sequence chosen was used by McKay et al (McKay, O'Reilly et al. 2008).

Table 2.2: mRNA primer sequences

Gene	Forward	Reverse
<i>RPLPO</i>	TCTACAACCCTGAAGTGCTTGATATC	GCAGACAGACACTGGCAACATT
<i>MHC1</i>	CCCTGGAGACTTTGTCTCATTAGG	AGCTGATGACCAACTTGCGC
<i>MyHCIIa</i>	TCACCTATGACTTTTGTGTGAACCT	CAATCTAGCTAAATTCCGCAAGC
<i>MyHCIIx</i>	TGACCTGGGACTCAGCAATG	GGAGGAACAATCCAACGTCAA
<i>myogenin</i>	GCTGTATGAGACATCCCCCTACTT	CGTAGCCTGGTGGTTCGAA
<i>mrf4</i>	CCCCTTCAGCTACAGACCCAA	CCCCCTGGAATGATCGGAAAC
<i>myf5</i>	GATGTAGCGGATGGCATTCC	AGGTCAACCAGGCTTTCGAA

2.18: VDR Protein Measurement

Muscle samples were powdered, mixed with NP40 to extract protein and homogenised at 3000 RPM for 1 min. The supernatant was transferred into a fresh tube, diluted and quantified using the Bradford method (Bradford 1976):

Protein samples were diluted to a concentration of 1 in 50. 90µl of Bradfords solution which contains coomassie brilliant blue was added to 10µl of each sample and incubated for 30 mins. Bovine serum albumin (BSA) was used to generate a standard curve. Absorbance was then measured at 595nm and the protein concentration of samples were calculated using the following equation: emission-intercept / gradient. The samples were then diluted to give a 1mg/ml concentration of protein.

100µg of protein samples were loaded onto a 4-20% SDS PAGE gel. Proteins were separated and transferred onto a nitrocellulose membrane. The blot was incubated with 5% bovine serum albumin (BSA) in Tris-HCl buffered saline solution containing 0.1% Tween 20 (TBS-T) for 1 hour at room temperature and then with the primary VDR antibody solution (D6 monoclonal; Insight Biotech Ltd, 0.2 µg/ml) overnight. The blot was washed with PBS-Tween on a rocker for 3 lots of 10 minutes before the secondary antibody was added (rabbit antimouse) and incubated for a further 1 hour. ECL reagent was then added before exposure to kodak film.

The VDR D6 antibody was chosen as it has been shown to be more specific and sensitive than a number of other VDR antibodies (Wang, Becklund et al. 2010). 100µg protein were used based on other studies (Tishkoff, Nibbelink et al. 2008;

Wang, Becklund et al. 2010) and our own trials in the laboratory.

Chapter 3: Skeletal Muscle Strength and Endurance in COPD

3.1: Introduction

This chapter describes a clinical study comparing serum 25(OH)D and 1,25(OH)₂D concentrations and properties of skeletal muscle in COPD subjects with an age and sex matched control population.

What is known about the role that Vitamin D status plays in healthy older people is described in detail in chapter 1. The limited evidence available has demonstrated that COPD patients have a lower serum 25(OH)D concentration and this is in keeping with their reduced physical activity and poor nutritional state.

COPD patients have reduced type II muscle fibre diameter as well as a reduction in proportion and size of type I muscle fibres. Vitamin D status appears to predominantly affect Type II muscle fibres and hence we would still expect to see a link between vitamin D status and skeletal muscle strength in COPD subjects.

Skeletal muscle endurance has also been demonstrated to be reduced in COPD patients (Swallow, Gosker et al. 2007). Endurance properties of muscle depend on the number and proportion of Type I fibres and hence the findings in COPD patients are not surprising. Studies in VDR knockout mice however show reduction in both Type I and Type II fibres so it is possible that Vitamin D status is important for both fibre types and may be related to endurance properties in muscle.

The aims of this clinical study are as follows:

- 1) To establish whether serum 25(OH)D concentrations in a COPD population are lower than an age and sex matched control population.
- 2) To establish whether there is an independent relationship between serum 25(OH)D or 1,25(OH)₂D concentrations and both voluntary and involuntary measures of skeletal muscle strength in subjects with COPD.
- 3) To establish whether serum 25(OH)D or 1,25(OH)₂D concentrations are related to skeletal muscle endurance in normal or COPD subjects

3.2: Results

3.2.1: Subject demographics

104 patients with COPD and 100 control subjects were included in this study. Patient demographics are shown in Table 3.1.

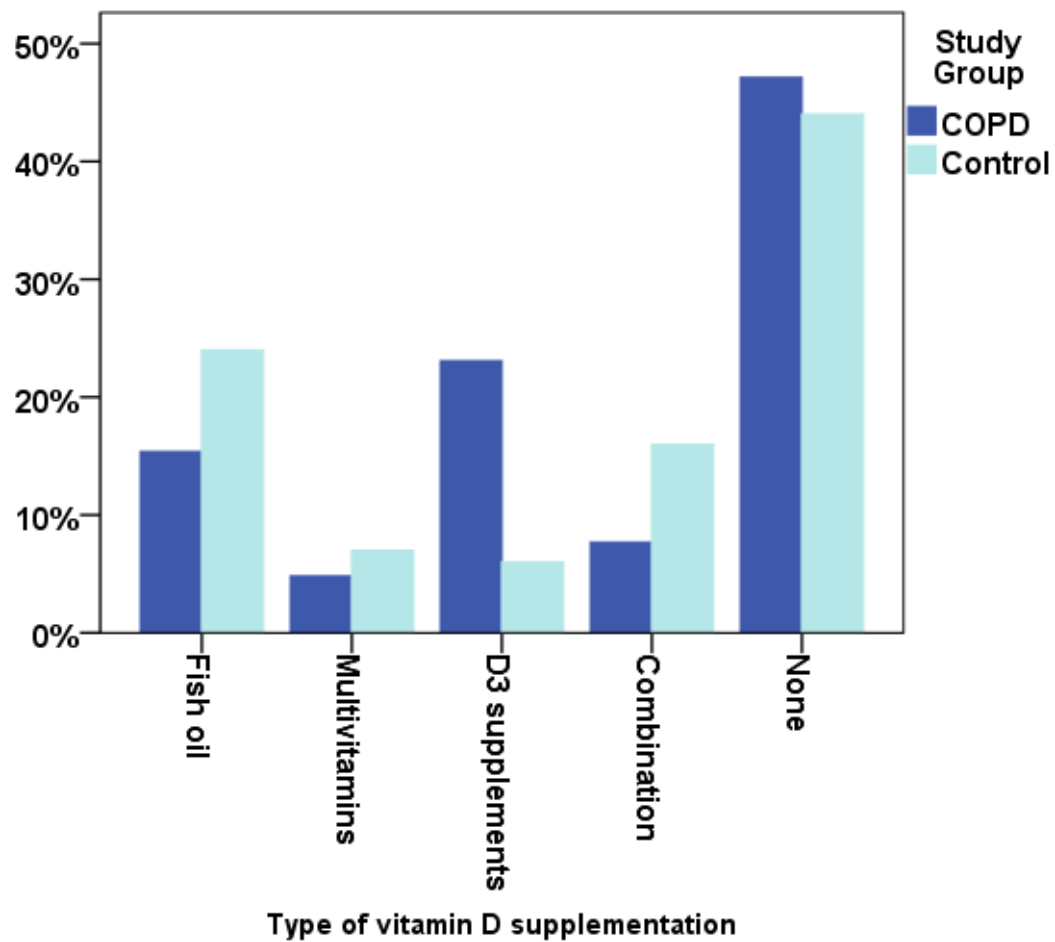
Table 3.1: Demographics of COPD and control subjects in the Study

	COPD patients (n=104)	Control subjects (n=100)	p value
Age (years)	65 (56-74)	63 (54-72)	0.18
Gender (M/F)	60 / 44	59 / 41	0.85
BMI (kg/m ²)	24.1 ± 4.3	25.9 ± 4.7	0.04
FFMI	15.8 ± 2.0	17.6 ± 3.3	<0.001
smoking pack years*	42 (30-60)	2 (0-14)	<0.001
daily vitamin D intake* (iu)	199 (60-906)	254 (89-1458)	0.10
Yale physical activity score	47.7 ± 25.9	70.9 ± 26.4	<0.001
Yale energy expenditure (kcal/week)	5176 ± 3874	7919 ± 4381	<0.001
FEV ₁ (% predicted)	44 ± 22	102 ± 16	<0.001
FVC (% predicted)	85 ± 22	105 ± 17	<0.001
TLco (% predicted)	39 ± 16	-	
pO ₂ (KPa) (n=56)	9.4 ± 1.4	-	
pCO ₂ (KPa) (n=56)	5.2 ± 0.7	-	

Values are expressed as numbers for categorical variables and mean ± standard deviation for normally distributed continuous variables. For variables that were not normally distributed, values are shown as median (range) (indicated by *).

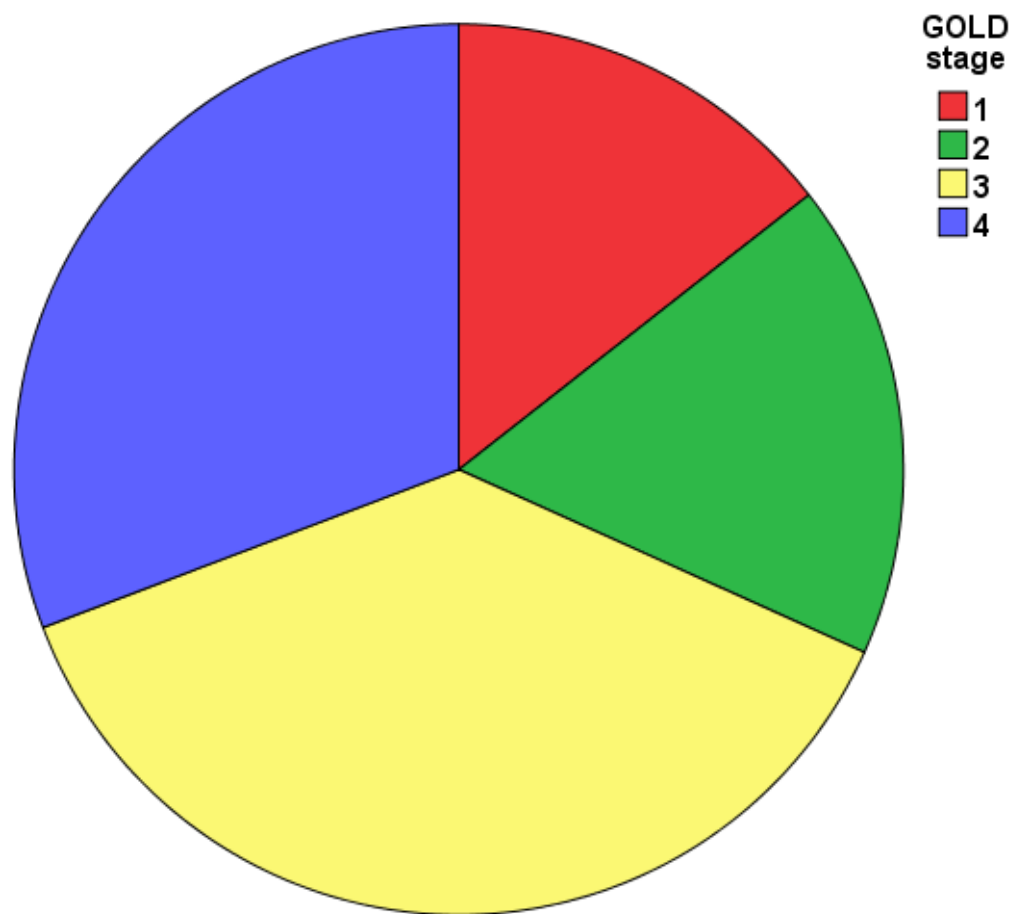
The groups are well matched for age and sex but COPD patients had a lower BMI and fat free mass, as well as reporting lower levels of physical activity. Vitamin D intake was similar in both groups. The pattern of Vitamin D supplementation was slightly different between groups: more COPD subjects were taking prescribed D3 supplements than controls, whilst more control subjects were taking fish oil supplements or a combination of supplements (figure 3.1). A similar proportion of subjects (just under 50%) in both groups were not taking any vitamin D supplementation.

Figure 3.1: Pattern of Vitamin D supplementation in COPD and control Groups



15 patients had GOLD stage 1 disease, 18 GOLD stage 2, 39 GOLD stage 3, and 32 GOLD stage 4 (figure 3.2).

Figure 3.2: Distribution of COPD severity in study participants



The median ADD of prednisone, consumed mostly as short burst treatment for exacerbations, was 2mg. Seven patients were taking regular low dose (<10mg/day) oral prednisolone. 27% of patients had no exacerbations in the preceding year, 22% had 1, 14% had 2, 11% had 3 and 26% had 4 or more exacerbations.

3.2.2: Serum measurements

Serum 25(OH)D and 1,25(OH)₂D concentrations did not differ significantly between groups, but serum PTH levels were higher in the COPD group, and the ratio of serum

25(OH)D concentration to serum PTH concentration was significantly lower in the COPD group.

No significant difference was seen between serum calcium, phosphate, magnesium or albumin levels between groups. Serum markers of inflammation (IL6 and hs CRP) were significantly higher in the COPD group (table 3.2).

Table 3.2: Comparison of serum Vitamin D metabolites, Ca^{2+} , Po^{4-} , Mg^{2+} , PTH and inflammatory markers between COPD and control groups.

Serum measurements	COPD patients (n=104)	Control subjects (n=100)	p value
25(OH)D (nmol/l)	48.5 ± 25.5	55.4 ± 28.3	0.07
1,25(OH) ₂ D (pmol/l)	81.2 ± 32.4	82.1 ± 30.1	0.82
PTH (pmol/ml)	5.2 ± 2.3	4.4 ± 2.0	0.01
25(OH)D/PTH*	9.1 (68.4)	12.4 (103.8)	0.008
Adjusted Ca^{2+} (mmol/l)	2.4 ± 0.1	2.4 ± 0.1	0.10
Phosphate (mmol/l)	1.1 ± 0.2	1.1 ± 0.2	0.27
Magnesium (mmol/l)	0.9 ± 0.8 (n=52)	0.9 ± 0.7 (n=52)	0.79
Albumin (g/l)	37.6 ± 5.7	39.0 ± 5.1	0.06
hsCRP (mg/l)*	0.30 (761.0)	0.08 (362.0)	<0.001
IL6 (pg/ml)*	2.05 (20.9)	1.18 (26.9)	<0.001

Values are expressed as numbers for categorical variables and mean ± standard deviation for normally distributed continuous variables. For variables that were not normally distributed, values are shown as median (range) (indicated by *).

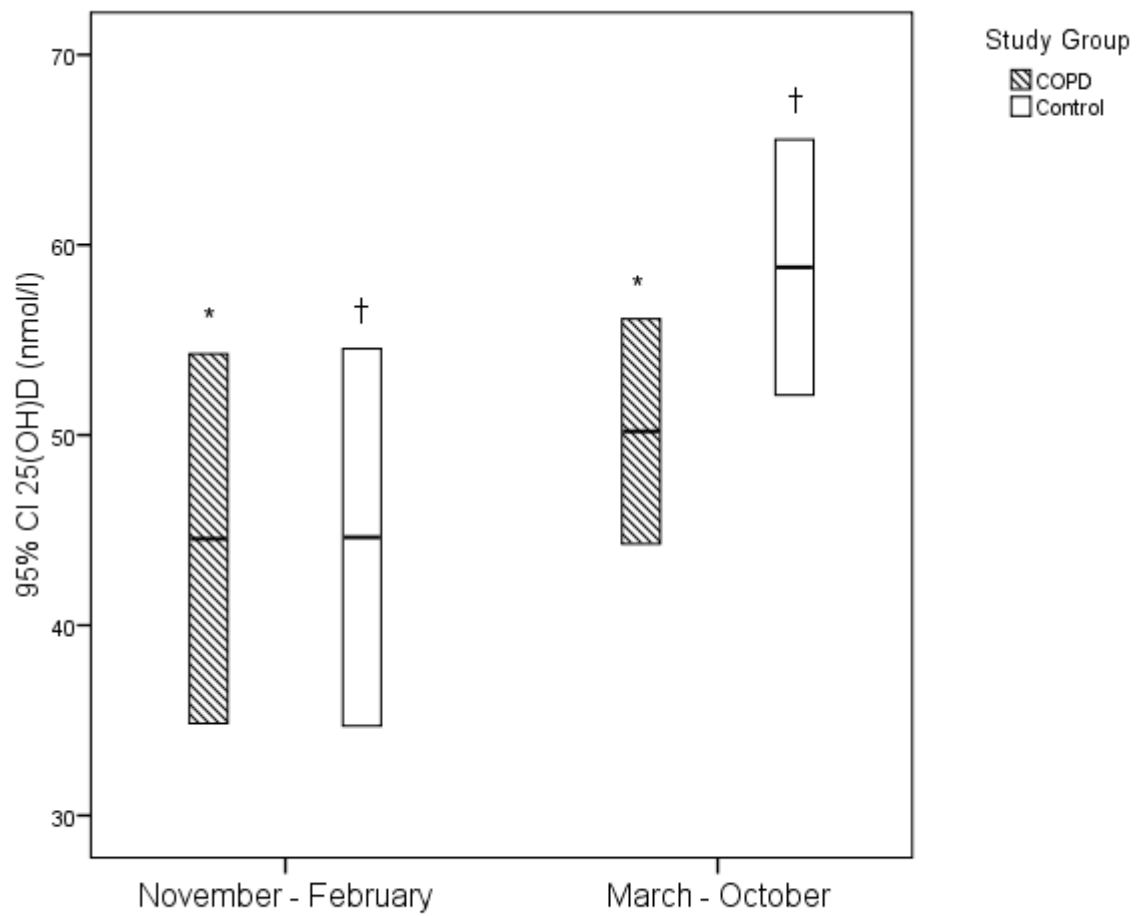
3.2.3: Factors affecting serum 25(OH)D concentration

In a stepwise multivariate regression model involving all study subjects, ethnicity, age and daily vitamin D intake were independently associated with serum 25(OH)D concentration ($r^2=0.11$). Other factors not retained in the model were study group, number of pack years, sex, BMI, time of year measured, albumin, IL6 or hsCRP.

A separate analysis of those subjects not taking vitamin D supplements showed a significantly lower dietary Vitamin D intake in COPD compared to control subjects ($982\text{iu} \pm 611$ in COPD vs. $1287\text{iu} \pm 658$ in controls, $p=0.01$), and significantly lower serum 25(OH)D concentration (COPD $41.5\text{nmol/l} \pm 24.6$; Controls $54.8\text{nmol/l} \pm 32.9$, $p=0.03$).

Analysis of serum 25(OH)D concentration according to the time of year measured showed a different pattern in the COPD and control groups. Whilst levels in subjects measured between November and February were similar in both groups (COPD: $44.6 (26.5)\text{nmol/l}$; Control: $44.6 (23.5) \text{ nmol/l}$), levels in subjects measured between March and October were significantly higher in the control group but not in the patients (COPD $50.2 (25.0) \text{ nmol/l}$, mean difference $-5.6[-16.5-5.2]$; Control: $58.8 (29.0) \text{ nmol/l}$, mean difference $-14.2 [-27.2--1.2]$) (figure 3.3).

Figure 3.3: Variation in 25(OH)D level in COPD and control groups according to time of year measured.



* mean difference -5.6[-16.5 - 5.2], $p=0.31$, † mean difference -14.2[-27.2 - -1.2], $p=0.03$

3.2.4: Muscle strength

A significant difference was seen between QMVC, handgrip strength and SNiP measurements between groups but not in TwQu (table3.3). Only 71 COPD subjects and 68 control subjects were able to tolerate the protocol for TwQu measurement due to discomfort.

Table 3.3: Comparison of muscle strength measurements between COPD and control groups.

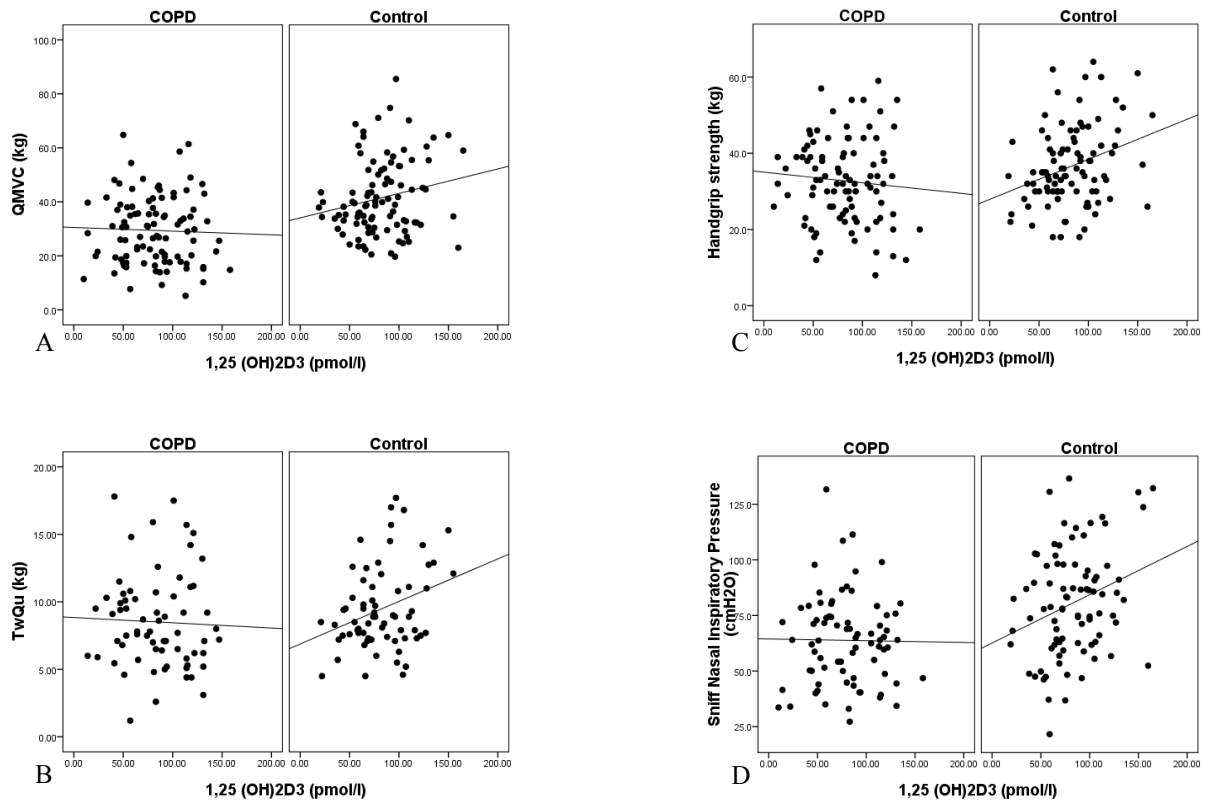
	COPD patients (n=104)	Control subjects (n=100)	<i>p value</i>
Muscle measurements			
QMVC (kg)	29.3 ± 12.5	41.2 ± 13.9	<0.001
TwQu (kg)	8.5 ± 3.4 (n=71)	9.5 ± 3.1 (n=68)	0.09
Handgrip (kg)	32.7 ± 11.0	36.5 ± 10.6	0.02
SNiP (cmH ₂ O)	64.0 ± 20.0	80.5 ± 24.0	<0.001

Values are expressed as numbers for categorical variables and mean ± standard deviation for normally distributed continuous variables. For variables that were not normally distributed, values are shown as median (range) (indicated by *).

In the COPD patients, neither serum 25(OH)D nor serum 1,25(OH)₂D concentration were associated with any measure of muscle strength (25(OH)D and QMVC: 0.005[-0.17-0.19], p=0.96; 25(OH)D and handgrip: 0.03[-0.16-0.21], p= 0.75; 25(OH)D and TwQu: -0.1[-0.34-0.15], p=5.22; 25(OH)D and SNiP: 0.04[-0.22-0.25], p=0.79; 1,25(OH)₂D and QMVC: -0.04[-0.25-0.18, p=0.73; 1,25(OH)₂D and handgrip: -0.07[-0.28-0.14], p=0.56; 1,25(OH)₂D and TxQu: -0.10[-0.34-0.15], p=0.52; 1,25(OH)₂D and SNiP: -0.07[-0.33-0.22], p=0.66) (figure 3.4) either independently or in stepwise analysis including potential confounding factors. QMVC was independently associated with sex, TLco (carbon monoxide transfer) (%pred) and albumin.

Dominant hand grip strength was associated with sex and age. SNiP was associated with sex and RV/TLC (residual volume / total lung capacity) (table 3.4).

Figure 3.4: Correlations between 1,25(OH)₂D and measures of muscle strength in the COPD and control groups.



A QMVC: COPD $r=-0.04$, $p=0.73$; Control $r=0.2$, $p=0.05$; B TwQu: COPD $r=-0.04$, $p=0.76$; Control $r=0.30$, $p=0.01$; C Handgrip strength: COPD $r=-0.08$, $p=0.44$; Control $r=0.31$, $p=0.003$; D SNiP: COPD $r=-0.01$, $p=0.91$; Control $r=0.28$, $p=0.01$

In the control group, serum 25(OH)D concentration was significantly associated with TwQu ($r=0.29[-0.02-0.48]$, $p=0.02$), SNiP ($r=0.22-0.27-0.43]$, $p=0.04$) and handgrip strength ($r=0.20[0.07-0.38]$, $p=0.05$), but not with QMVC ($r=0.18$, $p=0.07$). |Serum 1,25(OH)₂D concentration was associated with QMVC ($r=0.20[0.002-0.39]$, $p=0.05$), TwQu ($r=0.29[0.07-0.49]$, $p=0.03$), SNiP ($r=0.28[0.04-0.50]$, $p=0.03$) and handgrip ($r=0.31[0.12-0.49]$, $p=0.003$) (figure 3.4).

Univariate analysis was carried out for individual factors thought to affect skeletal muscle strength, the results of which are shown in Table 3.4.

Table 3.4: Results of univariate analysis of individual factors and their association with QMVC(kg) in COPD and Control populations

	COPD	Control
Age	-0.24 [-0.5 – 0.03]) p=0.07	-0.60 [-0.89 - -0.31] p=0.00
Sex	-16.61 [-20.34 - -12.88] p=0.00	-17.45 [-21.87 - -13.03] p=0.00
FFMI	3.6 [2.62 – 4.58] p=0.00	2.82 [2.18 – 3.46] p=0.00
Yale physical activity score	0.06 [-0.07 – 0.10] p=0.19	0.15 [0.05 – 0.25] p=0.005
FEV1 (% pred)	0.14 [0.03 – 0.25] p=0.02	-0.09 [-0.26 – 0.08] p=0.29
exacerbation rate	-1.68 [-3.26 - -0.11] p=0.04	N/A
1,25(OH)₂D (nmol/l)	-0.01 [-0.09 – 0.07] p=0.73	0.09 [-0.001 – 0.18] p=0.05
Log IL6	-0.98 [-7.09 – 5.13] p=0.73	0.48 [-5.58 – 6.55] p=0.87
Albumin (g/l)	0.59 [0.17 – 1.02] p=0.007	0.41 [-0.13 – 0.95] p=0.13

Values shown are B [95% CI] N/A = not applicable

Factors that were significant in the above univariate analysis were then included in a stepwise multivariate regression model. For COPD therefore, this model included sex, FFMI, FEV₁(% pred), exacerbation rate and albumin. Factors that remained significant in stepwise multivariate regression were sex, FEV₁(%pred) and Albumin (Table 3.5)

Table 3.5: Factors which remained associated with QMVC after stepwise multivariate analysis in COPD patients.

	B [95% confidence interval]	p value
sex	-15.94 [-14.49 - -12.29]	p = 0.00
FEV ₁ (% pred)	0.15 [0.11 – 0.74]	p = 0.001
Albumin (g/l)	0.42 [0.11 – 0.74]	p = 0.009

Factors which did not remain significant in the model were FFMI and exacerbation rate.

For the Control group the model included age, sex, FFMI, YPA score, and 1,25(OH)₂D. When included in a multivariate regression model, factors which remained significant were FFMI, YPA score, Age and sex (table 3.6)

Table 3.6: Factors which remained associated with QMVC after stepwise multivariate analysis in control subjects.

	B [95% confidence interval]	p value
FFMI	1.32 [0.36 – 2.28]	p = 0.008
YPA score	0.10 [0.03 – 0.18]	p = 0.008
Age	-0.39 [-0.07 - -0.14]	p = 0.003
Sex	-8.91 [-15.02 - -2.8]	p = 0.005

Factors which did not remain significant in the model were 1,25(OH)₂D.

When the same analysis was carried out in normal subjects for handgrip strength rather than QMVC, 1,25(OH)₂D remained significant in the model, rather than YPA score (table 3.7)

Table 3.7: Factors which remained associated with handgrip strength after stepwise multivariate analysis in control subjects.

	B [95% confidence interval]	p value
FFMI	0.90 [0.26 – 1.55]	p = 0.007
1,25(OH) ₂ D	0.07 [0.02 – 0.11]	p = 0.003
Age	-0.34 [-0.52 - -0.17]	p = 0.000
Sex	-8.43 [-12.57 - -4.29]	p = 0.000

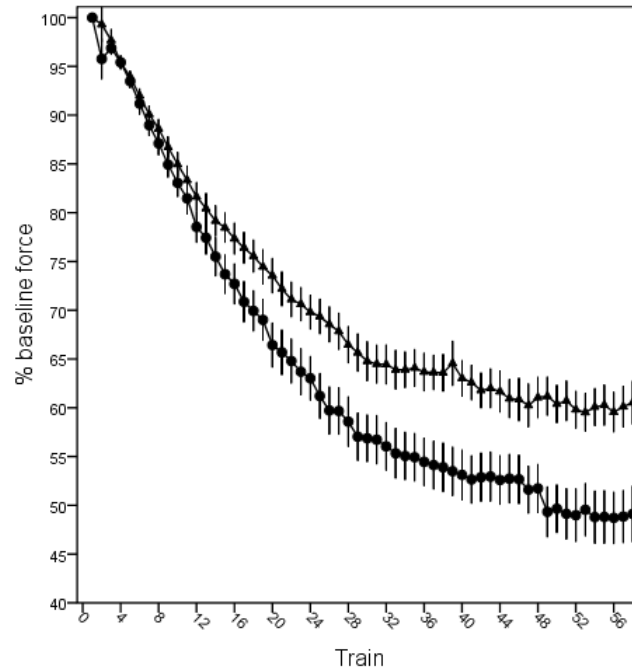
Factors which did not remain significant in the model were YPA score.

An interaction analysis was performed which showed an independent effect of COPD on QMVC ($F=20.63$, $p=0.00$) and of having serum 25(OH)D of less than 30nmol/l ($F=8.05$, $p=0.000$) but no combined effect of COPD and low 25(OH)D.

3.2.5: Quadriceps endurance

Quadriceps endurance was measured in 35 subjects in each group. A significant difference in force decay was seen between COPD and control groups ($Z=6.7\%$ [95%CI: 2.1%-11.3%], $p=0.004$) (figure 3.5). No difference was seen in force decay between vitamin D insufficient and vitamin D sufficient subjects in either study group.

Figure 3.5: The comparison between force decline during the endurance protocol in COPD patients (circles) and control subjects (triangles).



B=6.5 [95%CI 1.9-11.1], p=0.006. Error bars represent the standard error of the mean.

3.2.6: Serum 25(OH)D and 1,25(OH)₂D concentration and lung function

In the COPD group, serum 25(OH)D concentration was associated with FVC (%pred) ($r=0.21$, $p=0.04$) but no other measure of lung function, whilst serum 1,25(OH)₂D concentration was not associated with any measures of lung function. After stepwise multivariate analysis, number of exacerbations per year, pack years and weight remained independently associated with FEV₁ (%pred) ($r^2 = 0.32$), whilst number of pack years was independently associated with FVC (%pred) ($r^2=0.15$). Other factors

not retained in the models were daily vitamin D intake, season measured, serum 25(OH)D, 1,25(OH)₂D, PTH or albumin.

In the control group, serum 25(OH)D concentration was correlated with FEV₁ (l) ($r=0.27$, $p=0.006$) and FVC(l) ($r=0.31$, $p=0.002$). Serum 1,25(OH)₂D concentration was correlated with FEV₁(l) ($r=0.41$, $p<0.001$) and FVC(l) ($r=0.38$, $p<0.001$) as well as FEV₁(% pred) ($r=0.25$, $p=0.02$). After stepwise multivariate analysis; weight, pack years and serum 1,25(OH)₂D concentration remained independently associated with FEV₁(%pred) ($r^2=0.22$), and weight and serum 1,25(OH)₂D concentration remained independently associated with FVC (%pred) ($r^2=0.22$). Other factors not retained in the models were daily vitamin D intake, season measured, serum 25(OH)D, PTH or albumin.

3.2.7: Inflammatory mediators

Serum IL6 and high sensitivity CRP concentrations were both elevated in COPD patients compared to controls (table 3.2). There was no correlation between inflammatory markers and serum 25(OH)D and 1,25(OH)₂D concentrations and no relationship between inflammatory markers and any measure of skeletal muscle function.

3.3: Discussion

The main findings of this study are as follows:

- 1) No relationship was demonstrated between either serum 25(OH)D and 1,25(OH)₂D concentrations and measures of muscle strength in subjects with COPD although in the control group, serum 1,25(OH)₂D concentration was independently associated with measures of muscle strength.
- 2) Quadriceps endurance was not associated with serum 25(OH)D concentration in either study group.
- 3) Similar serum 25(OH)D and 1,25(OH)₂D concentrations were seen in both study groups. However the serum 25(OH)D/PTH ratio was significantly lower in the COPD group

Vitamin D status appears particularly to influence type II fibres and the quadriceps in COPD shows an increase in the proportion of type IIa fibres and a reduction in their cross-sectional area. Therefore, the complete lack of association between serum 25(OH)D and 1,25(OH)₂D concentrations and muscle strength in the COPD population is unexpected. Quadriceps endurance was significantly reduced in COPD patients, but in contrast to strength, which was associated with serum 25(OH)D in control subjects, there was no effect of serum 25(OH)D concentration on endurance observed in either group. This may be because the predominant effect of 1,25(OH)₂D is on type II fibres although studies in VDR knockout mice suggest otherwise.(Endo, Inoue et al. 2003)

Only one cross sectional study has looked at serum 25(OH)D concentration and handgrip strength in patients with advanced lung disease referred for lung transplant, of whom approximately 50% had COPD. This found no association between the two

parameters but in COPD muscle weakness occurs predominantly in the muscles of locomotion (Forli, Bjortuft et al. 2009).

The finding that there is no significant difference between serum 25(OH)D concentration in the study groups is unexpected as the one published study looking at this found otherwise (Janssens, Bouillon et al.). However in this study we did not exclude subjects who were taking Vitamin D supplements which was the case with the Belgian study. A similar proportion of subjects in each group were taking supplements although the type of supplementation varied as outlined above. A separate analysis of serum 25(OH)D concentration in those not taking supplements did demonstrate a significant difference in 25(OH)D levels in this subgroup. This is consistent with the reduced vitamin D intake demonstrated and probable lack of outdoor exposure in the summer months in COPD subjects. Supporting this hypothesis is that the levels of serum 25(OH)D in the normal study population were higher in those measured between March and October, but not in COPD subjects. It appears that vitamin D supplementation in COPD subjects is successful in boosting serum 25(OH)D levels.

Despite the similar concentration of serum 25(OH)D and serum 1,25(OH)₂D in both study groups, serum PTH concentration was significantly higher and the 25(OH)D/PTH ratio significantly lower in COPD patients which was an unexpected finding. PTH maintains serum calcium in the normal range through actions on the kidneys, bone and CYP27B1 which metabolises 25(OH)D to 1,25(OH)₂D, and is tightly regulated by 1,25(OH)₂D itself. A possible explanation for the difference seen in serum PTH concentration between the two groups could be the presence of

magnesium deficiency, as this can cause a blunted PTH response to low 25(OH)D levels (Sahota, Munday et al. 2006) with subsequent reduced bone turnover. Although magnesium is predominantly an intracellular ion, serum concentrations did not differ between groups making this unlikely to be a significant factor.

One explanation for this lack of effect of serum 25(OH)D and 1,25(OH)₂D concentrations in COPD is that it is masked by other processes, or simply that the magnitude of effect is small in comparison with other phenotypic modulators of muscle strength. However, even when adjusted for factors thought to drive muscle atrophy including physical activity level, disease severity and exacerbation rate, there was still no relationship observed.

An alternative hypothesis is that there is resistance to the actions of 1,25(OH)₂D in COPD patients. Although no significant difference was seen between the serum concentrations of 25(OH)D or 1,25(OH)₂D in our study groups, serum PTH was significantly higher and the 25(OH)D/PTH ratio significantly lower in COPD patients. PTH maintains serum calcium in the normal range through actions on the kidneys, bone and 25(OH)D, and is tightly regulated by serum 1,25(OH)₂D concentration. PTH levels increase with age. This is thought to reflect decreased calcium absorption and it has been hypothesised that postmenopausal women have resistance to 1,25(OH)₂D action in the gut but this has not been confirmed (Ebeling, Yergey et al. 1994). The raised serum PTH concentration in our COPD group suggests that there may also be increased resistance to 1,25(OH)₂D action in the gut with less calcium absorption, despite similar serum levels of 25(OH)D. This process

could drive increased bone resorption and may explain why COPD patients have a high risk of osteoporosis.

There are a number of potential mechanisms through which $1,25(\text{OH})_2\text{D}$ resistance could occur. 1α -hydroxylase (CYP27B1) converts $25(\text{OH})\text{D}$ to $1,25(\text{OH})_2\text{D}$ which binds to VDRs present in the target organ. The VDR then forms a heterodimer with retinoic acid receptor before it can bind to DNA and effect a response via gene transcription. The VDR is also present in the cytoplasm and has been shown to have non-genomic effects. Decreased activity of CYP27B1, or decreased expression or inappropriate activity of the VDR could result in resistance. The levels of $1,25(\text{OH})_2\text{D}$ were similar in both groups which suggests that any mechanism of resistance would involve the VDR and its interactions. Potential influences on the vitamin D pathway in skeletal muscle in COPD include the presence of inflammation, oxidative stress, reduced capillarity, muscle hypoxia and physical inactivity. Of note, no relationship between systemic inflammation and serum $25(\text{OH})\text{D}$ concentration (IL6: $p=0.82$; hsCRP: $p=0.78$) or between systemic inflammation and QMVC (IL6: $p=0.14$; hsCRP: $p=0.74$) was seen.

3.3.1: Study limitations

This is a large cross-sectional study with a well-phenotyped cohort of COPD patients and a control group matched for age and sex. However, as with any cross-sectional study, it is only possible to look for significant associations and we are not able to establish cause and effect relationships. The population was predominantly Caucasian living in the United Kingdom and results must be applied with caution to

other populations. One limitation of the study may be the use of the YPAS as a measure of physical activity rather than direct measurement. This is a recall questionnaire and therefore has inherent problems associated with it. However it is a useful tool for comparing populations, it has been validated in older people, the physical activity score has been shown to correlate with $VO_2\text{max}$, and discriminated between patients and controls in the present study (Dipietro, Caspersen et al. 1993). In our study, the physical activity score correlated with disease severity in the COPD group ($r=0.30$, $p=0.004$)

3.3.2: Conclusion

The relationship between serum 25(OH)D and 1,25(OH)₂D concentration and skeletal muscle strength observed in normal subjects was not present in COPD patients despite a similar range of serum 25(OH)D levels. These results are surprising and raise the possibility of 1,25(OH)₂D resistance in COPD.

Chapter 4: Muscle Biopsy Sub-study

4.1: Introduction

This chapter describes the findings on skeletal muscle biopsy of a sub-group of patients from the previously described clinical study with the aim of investigating potential mechanisms of 1,25(OH)₂D action in skeletal muscle.

From the evidence presented in the preceding chapter, 25(OH)D does not appear to exert its expected clinical effects on muscle in COPD subjects and may therefore be contributing to their skeletal muscle dysfunction. Therefore we wished to investigate what was happening at a molecular level. No power calculation was performed prior to this study as only small numbers of patients could potentially be recruited for muscle biopsies, and the original main outcome measure had not been investigated in COPD subjects previously (VDR protein levels in skeletal muscle).

As discussed previously, muscle biopsy studies looking at the effects of 25(OH)D deficiency have shown abnormalities in type II or IIa fibres. We firstly therefore chose to look at fibre type mRNA expression to confirm this relationship in healthy elderly subjects and to see if the relationship was maintained in COPD subjects.

As mentioned in chapter 1, 1,25(OH)₂D has been shown in vitro to have an effect on myogenic regulatory factors and we therefore selected these for investigation in skeletal muscle. The myogenic regulatory factors (MRFs) consist of myoD, myogenin, mrf4 and myf5. They have been extensively studied in embryogenesis and

in cell culture models where they have been shown to play an important role in skeletal muscle development. *MyoD* and *myf5* commit stem cells to a myogenic lineage whilst *myogenin* and *mrf4* stimulate the transition to multinucleated myofibres (Yokoyama and Asahara). However, less is known of their function in adult muscle. *Mrf4* expression does persist into adult life, whilst the other MRFs decrease shortly after birth and subsequently increase with ageing and in disease states (Stewart and Rittweger 2006). *MyoD* and *myogenin* mRNA have been shown to increase after resistance exercise training in both young and old people, whilst *myf5* mRNA increased only in the young (Kosek, Kim et al. 2006).

A potential mechanism for vitamin D resistance could involve the VDR. This has previously been demonstrated to be present in skeletal muscle (Bischoff, Borchers et al. 2001) and has been shown to be reduced in skeletal muscle with increasing age (Bischoff-Ferrari, Borchers et al. 2004). Investigations into the VDR in the duodenum have also shown an age related decrease in quantity (Ebeling, Sandgren et al. 1992). As discussed in chapter one, COPD shows some evidence of an accelerated ageing process, and if a reduced VDR concentration in skeletal muscle was shown in COPD subjects, compared to age and sex matched healthy controls, this may help to explain some of the muscle fibre changes seen in this disease

In summary, muscle fibre type and distribution is altered in COPD subjects with a shift from Type I to type IIa fibres and subsequent reduction in type IIa fibre size, the mechanism for which is not clear. Serum 25(OH)D has been shown to be related to a reduction in size of Type IIa muscle fibres on muscle biopsy and supplementation with vitamin D increases the size of Type IIa fibres (Sato, Iwamoto et al. 2005),

whilst VDRKO mice studies as well as cell studies support a role for 1,25(OH)₂D in muscle growth and differentiation via actions on myogenic regulatory factors (Endo, Inoue et al. 2003; Garcia, King et al. 2011; Girgis, Clifton-Bligh et al. 2014). 25(OH)D may therefore effect muscle fibre type through actions on the myogenic regulatory factors.

The aims of this study are as follows:

- 1) To confirm a relationship between serum 25(OH)D and 1,25(OH)₂D concentrations and skeletal muscle *MyHCIIa* mRNA expression and to look for a difference in this relationship between COPD and control subjects.
- 2) To establish whether there is a negative association between serum 25(OH)D or 1,25(OH)₂D concentration and mRNA expression of the myogenic regulatory factors *mrf 4*, *myf5* and *myogenin* in skeletal muscle in either COPD or Control subjects.
- 3) To establish whether myogenic regulatory factor mRNA expression is related to muscle fibre type expression in skeletal muscle in both COPD and Control subjects as a potential mechanism for fibre type alterations in COPD.
- 4) To compare the amount of VDR present in skeletal muscle in COPD and Control subjects

4.2: Results

4.2.1: Demographic features, muscle strength and serum measurements in the sub-study Group

26 COPD subjects and 13 control subjects agreed to take part in the muscle biopsy sub-study. Their demographic features were in general similar to those of the original study groups, although there are some important differences to highlight (Table 1):

The number of females in the control group were low (n=2) and the gender difference between the two groups did reach borderline statistical significance.

The Yale Physical Activity score between the groups did not reach statistical significance.

Handgrip strength between the groups did not reach statistical significance although other measures of muscle strength did.

There was no significant difference in serum PTH concentration or in the 25(OH)D/PTH ratio seen between the two groups unlike the total clinical study groups.

Serum hsCRP concentration, but not serum IL6 concentration, was higher in the COPD study group.

In the COPD group, there were 6 subjects with GOLD stage I disease, 7 with GOLD Stage II, 9 with GOLD Stage III, and 4 with GOLD Stage IV disease.

Table 4.1: Comparison of demographic features, muscle strength and serum measurements between groups in participants of the muscle biopsy sub-study

	COPD Group	Control Group	p value
Age (years)	63 (10)	64 (9)	0.87
Sex (M/F)	15/11	11/2	0.05
BMI	23.8 (4.1)	27.9 (5.4)	0.01
FFMI	16.0 (2.1)	19.6 (3.0)	< 0.001
smoking pack years*	43.5 (107)	10 (56)	<0.001
Yale Physical Activity Score	52.2 (32.2)	73.9 (34.1)	0.07
daily vitamin D intake (iu)*	431 (1773)	1405 (2793)	0.31
FEV₁ (% pred)	52 (26)	98 (8)	<0.001
QMVC (kg)	31.7 (11.7)	43.3 (11.8)	0.006
TwQu (kg)	8.4 (3.7)	10.7 (2.9)	0.008
dominant handgrip (kg)	33.5 (11.0)	38.4 (7.0)	0.16
SNIP (cmH₂O)	66.7 (18.9)	88.0 (18.2)	0.004
25(OH)D (nmol/l)	48.8 (28.9)	60.0 (33.9)	0.30
1,25(OH)₂D (pmol/l)	73.8 (37.4)	88.0 (30.7)	0.25
PTH (pmol/ml)	5.4 (2.6)	4.1 (2.4)	0.13
25(OH)D/PTH	48.8 (28.9)	60.0 (33.8)	0.16
IL6 (pg/ml)*	1.8 (9.9)	1.0 (2.7)	0.13
hsCRP (mg/l)*	0.34 (1.37)	0.06 (0.26)	<0.001

Values are expressed as numbers for categorical variables and mean (standard deviation) for normally distributed continuous variables. For variables that were not normally distributed, values are shown as median (range) (indicated by *).

4.2.2: mRNA analysis

MHCI mRNA expression was lower in the COPD group compared to the control group (4.19 ± 0.41 vs 3.88 ± 0.42 , $p=0.04$), whilst expression of *MyHCIIa* (COPD: 2.98 ± 0.67 , control: 2.97 ± 0.43 , $p=1.00$) and *MyHCIIx* (COPD: 3.82 ± 0.59 , control: 3.78 ± 0.52 , $p=0.82$) was similar in both groups. No difference was seen in mRNA expression for any of the MRFs between the COPD and control groups.

Table 4.2: Comparison of mRNA expression between COPD and control Groups

	COPD	Control	mean difference	p-value
Log <i>MHCI</i>	3.88 (0.42) (<i>n</i> =26)	4.19 (0.41) (<i>n</i> =12)	-0.31 [-0.61 - -0.02]	0.04
Log <i>MyHCIIa</i>	2.98 (0.67) (<i>n</i> =25)	2.97 (0.43) (<i>n</i> =13)	0.00 [-0.42 – 0.42]	1.0
Log <i>MyHCIIx</i>	3.82 (0.59) (<i>n</i> =25)	3.78 (0.52) (<i>n</i> =12)	0.04 [-0.36 – 0.45]	0.82
Log <i>mrf4</i>	3.32 (0.77) (<i>n</i> =22)	3.39 (0.54) (<i>n</i> =12)	0.07 [-0.88 – 0.44]	0.78
Log <i>myf5</i>	1.5 (0.60) (<i>n</i> =21)	1.5 (0.35) (<i>n</i> =9)	0.98 [-0.44 – 0.43]	0.98
Log <i>myogenin</i>	2.3 (0.40) (<i>n</i> =25)	2.3 (0.44) (<i>n</i> =12)	0.03 [-0.26 – 0.33]	0.83

Values are expressed as mean (standard deviation) and mean difference [95% confidence intervals].

Values shown are calculated as described in the methods section (chapter 2).

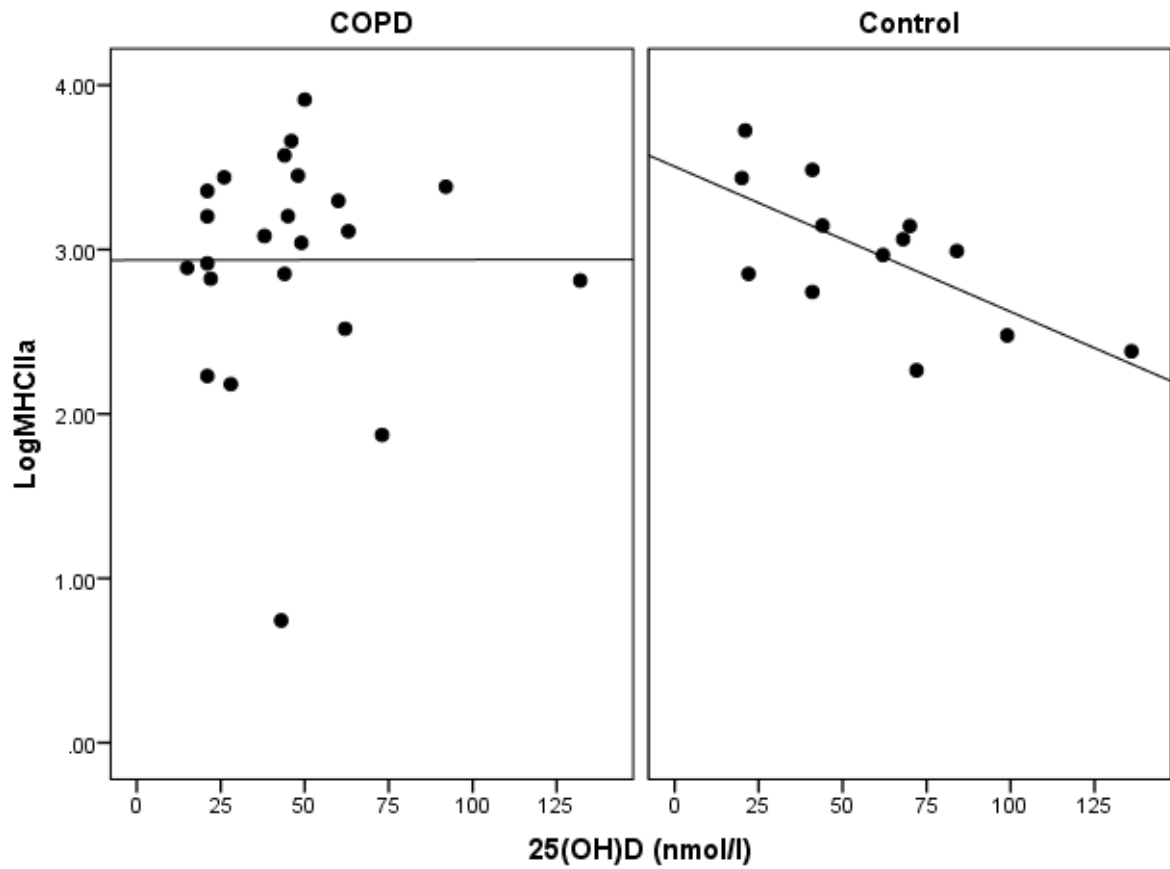
In the control group, *MyHCIIa* was associated with both 25(OH)D (figure 4.1) and 1,25(OH)₂D ($r^2=0.47$, $p=0.01$ and $r^2=0.35$, $p=0.03$ respectively). In the COPD group, no association between *MyHCIIa* and 25(OH)D ($r^2=0.00$, $p=1.0$) or 1,25(OH)₂D ($r^2=0.08$, $p=0.20$) was seen..

No association was seen between mRNA expression of MRFs and either serum 25(OH)D concentration in the COPD group (*mrf4*: $r^2=0.16$, $p=0.62$; *myf5*: $r^2=0.16$, $p=0.08$, *myogenin*: $r^2=0.01$, $p=0.72$). In the control group, there was a trend for an association between 25(OH)D and the mrf's as follows: *myf 5* ($r^2=0.38$, $p=0.08$); *mrf 4* ($r^2=0.32$, $p=0.06$); *myogenin* ($r^2=0.30$, $p=0.07$).

Table 4.3: Correlations between serum 25(OH)D concentration and mRNA fibre type expression, and mRNA expression of myogenic regulatory factors between groups.

		<i>MHCI</i>	<i>MyHCIIa</i>	<i>MyHCIIx</i>	<i>mrf4</i>	<i>myf5</i>	<i>myogenin</i>
COPD	25(OH)D	$r^2=0.04$ $p=0.35$	$r^2=0.00$ $p=1.0$	$r^2=0.02$ $p=0.57$	$r^2=0.001$ $p=0.88$	$r^2=0.16$ $p=0.08$	$r^2=0.01$ $p=0.72$
Control	25(OH)D	$r^2=0.22$ $p=0.13$	$r^2=0.47$ $p=0.01$	$r^2=0.22$ $p=0.13$	$r^2=-0.32$ $p=0.06$	$r^2=-0.38$ $p=0.08$	$r^2=0.30$ $p=0.07$

Figure 4.1: Correlation between serum 25(OH)D concentration and *MyHCIIa* mRNA expression in COPD and Control Groups



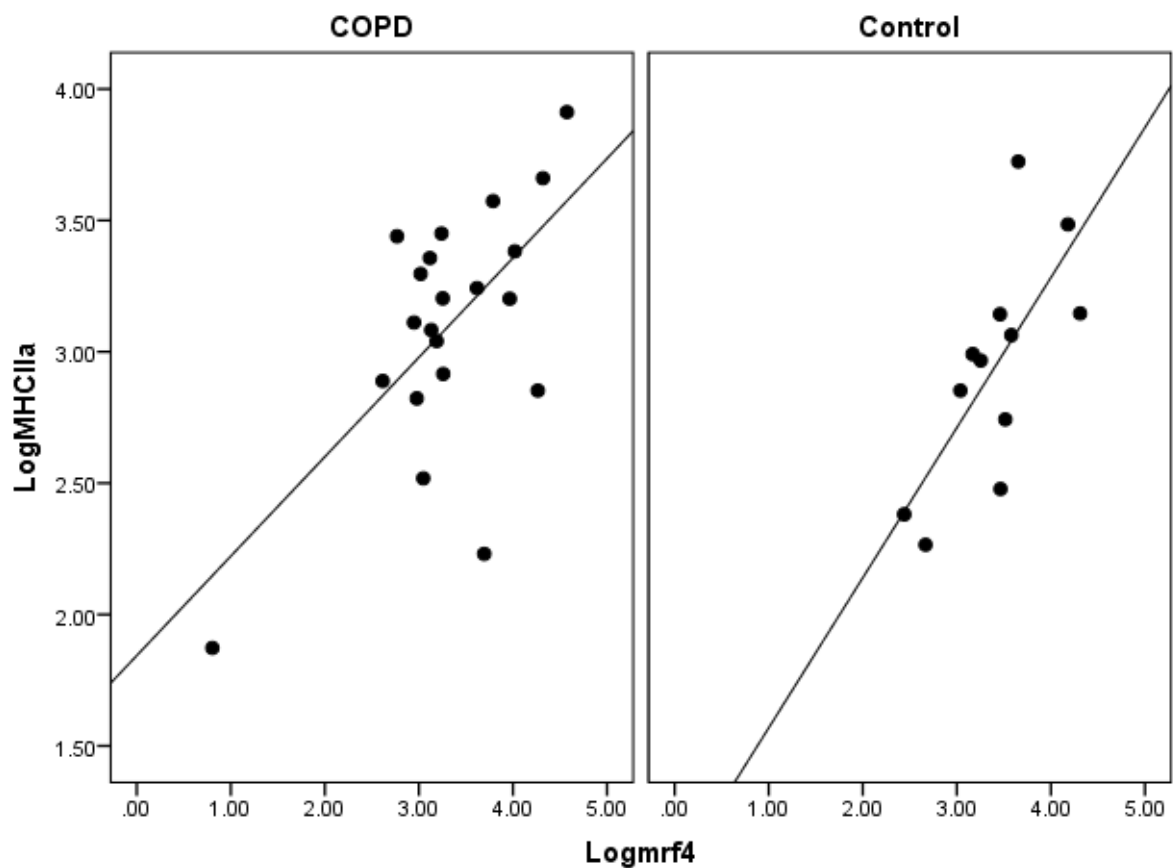
COPD: $r^2=0.00$, $p=1.0$; Control: $r^2=0.47$, $p=0.01$

In the control group a number of associations were found between MRFs and fibre type mRNA expression: *mrf4* was most strongly associated with *MyHCIIa* ($r^2=0.52$, $p=0.009$) (figure 4.2) whilst *myf5* was most strongly associated with *MHCI* ($r^2=0.72$, $p=0.004$) (figure 4.3). In the COPD group, an association was found between *mrf4* and *MyHCIIa* ($r^2=0.4x$, $p=0.02$) (figure 4.2), and *MyHCIIx* ($r^2=0.34$, $p=0.005$), but not with *MyHCIIa* and *myf5* ($r^2=0.009$, $p=0.68$) (figure 4.2) or *myogenin* (Tables 4,5&6).

Table 4.4: Correlations for *mrf4* mRNA and fibre type mRNA expression

		<i>MHC1</i>	<i>MyHCIIa</i>	<i>MyHCIIx</i>
COPD	<i>mrf4</i>	$r^2=0.07$, $p=0.24$	$r^2=0.40$, $p=0.02$	$r^2=0.34$, $p=0.005$
Control	<i>mrf4</i>	$r^2=0.48$, $p=0.02$	$r^2=0.52$, $p=0.009$	$r^2=0.11$, $p=0.32$

Figure 4.2: Graph showing association between *mrf4* and *MyHCIIa* mRNA expression

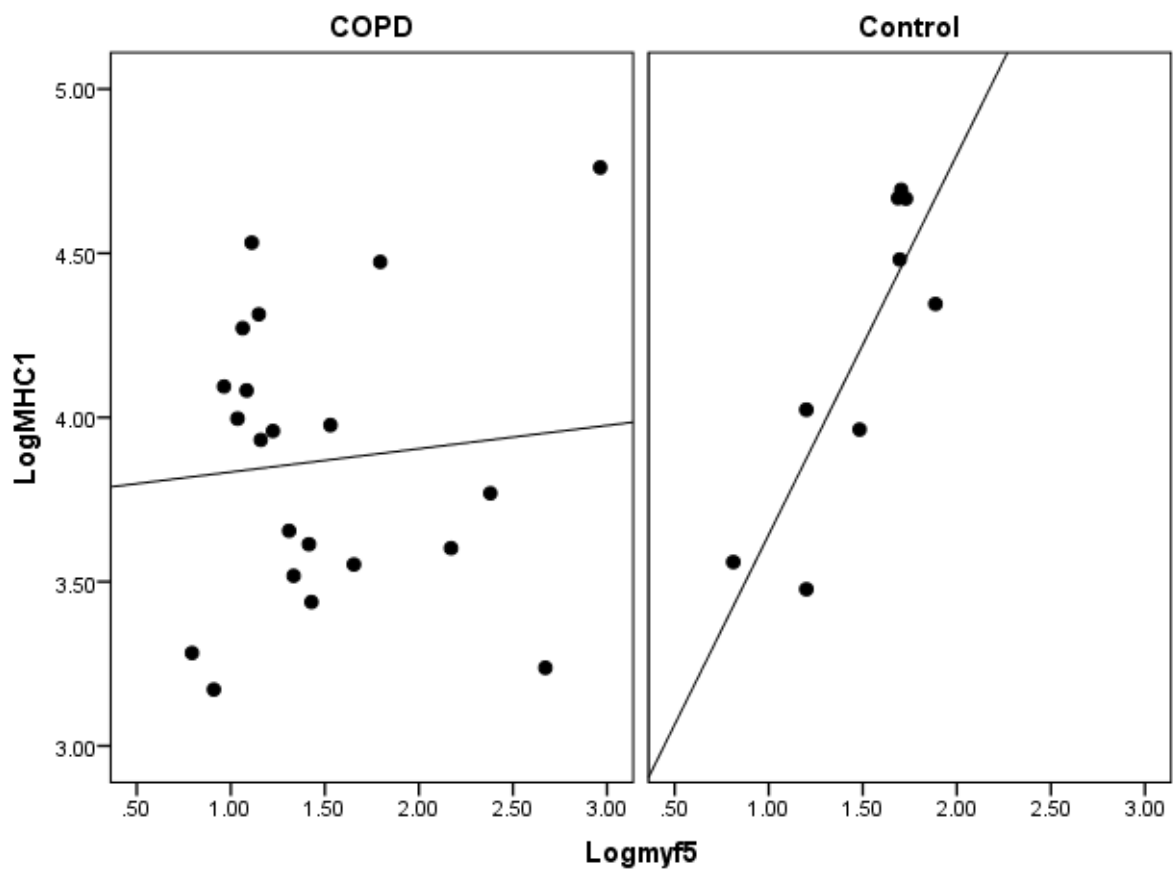


COPD: $r^2=0.40$, $p=0.02$; Control: $r^2=0.52$, $p=0.009$

Table 4.5: Correlations between *myf5* and fibre type mRNA expression.

		<i>MHCI</i>	<i>MyHCIIa</i>	<i>MyHCIIx</i>
COPD	<i>myf 5</i>	$r^2=0.009$, $p=0.68$	$r^2=0.05$, $p=0.33$	$r^2=0.05$, $p=0.32$
Control	<i>myf 5</i>	$r^2=0.72$, $p=0.004$	$r^2=0.55$, $p=0.02$	$r^2=0.41$, $p=0.07$

Figure 4.3: Graph showing correlation between *myf 5* and *MHCI* mRNA expression



COPD: $r^2=0.009$, $p=0.68$; Control: $r^2=0.72$, $p=0.004$

Table 4.6: Correlations between *myogenin* and fibre type mRNA expression.

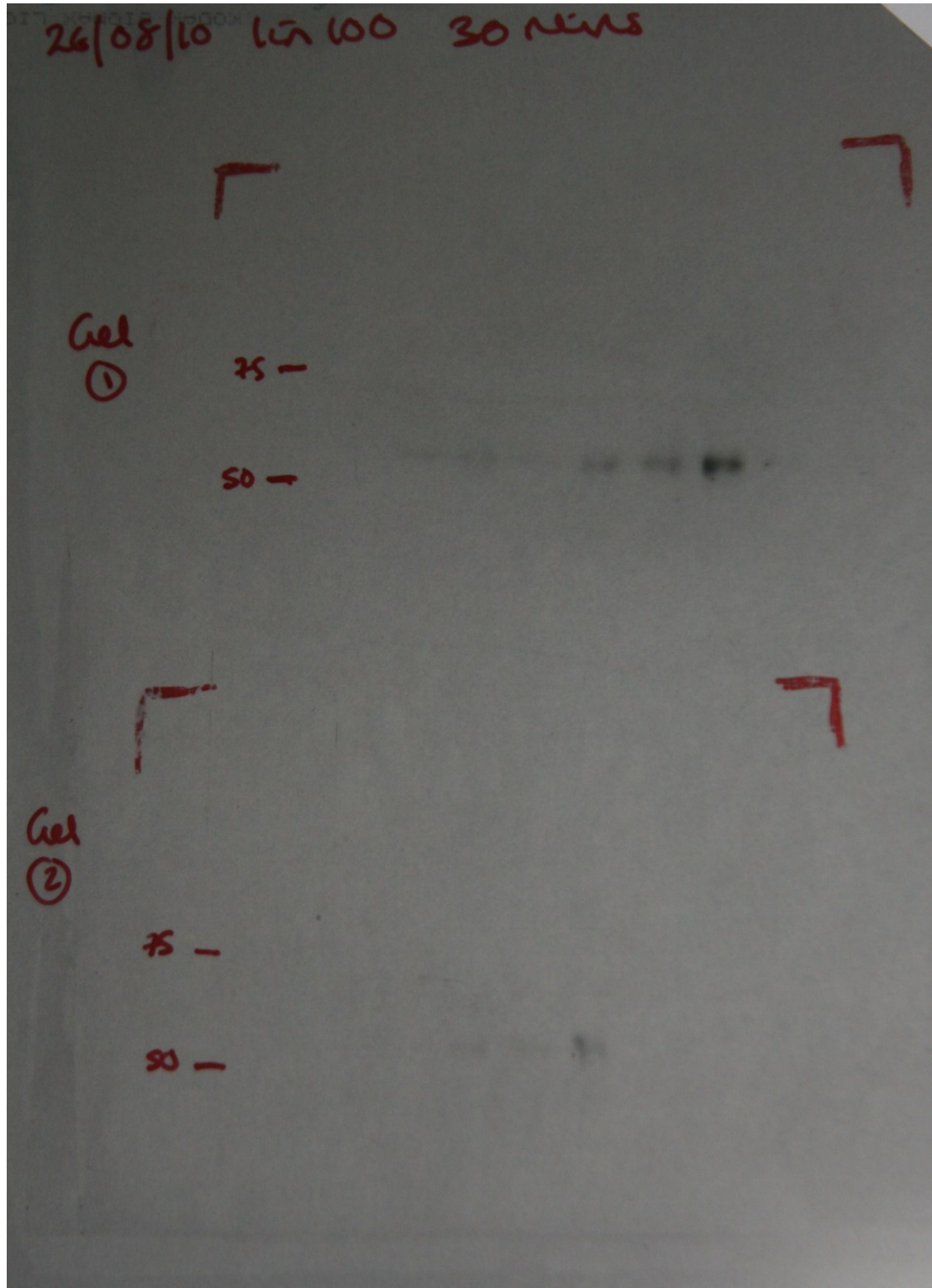
		<i>MHCI</i>	<i>MyHCIIa</i>	<i>MyHCIIx</i>
COPD	<i>myogenin</i>	$r^2=0.04$, $p=0.33$	$r^2=0.13$, $p=0.08$	$r^2=0.15$, $p=0.07$
Control	<i>myogenin</i>	$r^2=0.35$, $p=0.06$	$r^2=0.27$, $p=0.08$	$r^2=0.02$, $p=0.72$

As in the main study group, an association between serum 25(OH)D concentration and muscle strength in control subjects was confirmed in this subset of patients (COPD: $r^2=0.07$, $p=0.22$, control: $r^2=0.40$, $p=0.02$ for handgrip strength). As expected, *MyHCIIa* expression (but not *MHCI* or *MyHCIIx* expression) was associated with quadriceps strength in normal, but not COPD, subjects (COPD: $r^2=0.03$, $p=0.45$; control: $r^2=0.36$, $p=0.03$).

4.2.3: VDR Protein measurements

Despite running a number of western blots with varied protein concentrations and varied exposure times, we were unable to achieve a meaningful result for the VDR from which the amount of VDR receptor present could be quantified. A typical example of this is shown in figure 4.4.

Figure 4.4: Result of western blot for VDR after incubation with primary (VDR D6) and secondary (mouse ISF1) antibodies, and subsequent exposure for 30 mins after addition of ECL



Gel 1 from left to right: ladder; (subject identification)VDAS; KW03; GS06; VW09; HD11; CH36; FH02; VDSB. Gel 2: ladder; VDAS; VDDH; KVB; DK17; PS05; GM21; ACESB; VDBGH; VDYL. Superimposed red markings and numbers represent the ladder showing 50kDa and 75 kDa. Faint bands show for individual subjects just above the 50kDa mark which are assumed to represent the VDR (54kDa)

4.3: Discussion

The main findings of this sub study are as follows:

- 1) There was a significant association between serum 25(OH)D concentration and *MyHCIIa* mRNA expression in the control group ,but not the COPD group.
- 2) A significant association was also seen between serum 25(OH)D concentration and *mr4* mRNA expression in the control, but not the COPD group.
- 3) A significant strong correlation was found between *mrf4* and *MyHCIIa* mRNA expression and between *myf5* and *MHCI* mRNA expression again in the control, but not the COPD group.

These results are consistent with the findings in the clinical study in that both clinical associations and those associations seen at a molecular level in healthy elderly subjects do not appear to be present in those with COPD. However numbers in this study are small and it may be underpowered in order to detect associations in COPD. The signalling pathways of 25(OH)D and 1,25(OH)₂D in muscle are complex and

not completely understood and evidence that is available is outlined in chapter 1. The results of this study would suggest that they may influence muscle fibre type and or growth through actions on the myogenic regulatory factors, in particular *mrf4*.

Endo et al showed persistent up regulation of *myf5*, *myogenin* and early *MHC* isoforms in the skeletal muscle of VDRKO mice (Endo, Inoue et al. 2003). Addition of 1,25(OH)₂D to muscle cells in vitro showed down regulation of these factors. Tsuji et al demonstrated in an osteoblast cell line that 1,25(OH)₂D enhanced the expression of *Imfa*, a known inhibitor of myogenic regulatory factors (Tsuji, Kraut et al. 2001). A further study in C2C12 myocytes demonstrated increased expression of the VDR with increased translocation to the nucleus, decreased expression of markers of cell proliferation, and promotion of myocytes differentiation with increased *IGF1* and *follistatin*, and decreased *myostatin* expression, all with the addition of 1,25(OH)₂D (Garcia, King et al. 2011). They also showed variation in expression of mrfs at different time points after addition of 1,25(OH)₂D.

In our study we found a negative relationship between serum 25(OH)D and 1,25(OH)₂D concentrations and *MyHCIIa* mRNA expression: low serum 25(OH)D levels were associated with high mRNA expression of *MyHCIIa*. There was also a trend towards a negative relationship between serum 25(OH)D concentration and *mrf4* mRNA expression (R=-0.56, 0=0.06), and *myogenin* mRNA expression (R=-0.55, p=0.07). These findings are consistent with the above studies showing that 1,25(OH)₂D suppresses expression of mrfs and promotes muscle cell differentiation rather than proliferation.

The strongest associations found in this study were those between certain mrfs and MHC mRNA expression in the control group, and this suggests that different mrfs may be important in proliferation of different fibre types in adult muscle. One study in adult rats has demonstrated high levels of *myogenin* mRNA in skeletal muscle that predominantly consists of slow fibres with high levels of *myoD* mRNA in those that consist predominantly of fast fibres. However, levels of *mrf4* mRNA did not differ between muscles, whilst *myf5* mRNA was virtually undetectable (Hughes, Taylor et al. 1993). Our findings are supported by another study looking at promoter/reporter gene constructs of *mrf4* which has shown a region which promotes *mrf4* specifically in fast muscle fibres (Pin and Konieczny 2002).

We were not able to quantify the VDR in skeletal muscle in this study. A faint protein band was demonstrated in some subjects with a weight consistent with that of the VDR, but this was not enough to enable accurate measurement. This may have been due to technique as I have not previously carried out western blot analysis before, although I was supported by scientists in the laboratory proficient in doing this. A number of repeat samples were run by myself with differing protein concentrations and differing exposure times with no large difference in results seen.

Alternatively, there may not have been large amounts of the VDR present in these muscle samples. There is some debate currently about the presence or absence of VDRs in skeletal muscle and the antibodies used to detect them. A recent study by Wang et al used the same D6 antibody used in this study and could not demonstrate the presence of the VDR in either mouse or human muscle extracts (Wang, Becklund et al. 2010). They also studied other commercially available antibodies and found

that these antibodies, but not the D6 antibody were positive in samples of VDR knockout mice suggesting binding to other proteins occurred. Only one other study on human muscle extracts is published demonstrating evidence of VDR in skeletal muscle and this used a different antibody (VDR 9A7). It is therefore possible that the VDR is not present in adult skeletal muscle and 1,25(OH)₂D may act indirectly on skeletal muscle but further studies are required to investigate this further.

The limitations of this sub-study are that only a small number of subjects were studied and the study was not sufficiently powered to detect differences in some of the measurements studied. However, muscle biopsy data is limited because it is an invasive technique which people do not often want to undergo. It is also difficult to draw conclusions regarding cause and effect relationships with a cross-sectional study design. Despite this, some strong associations were found which are consistent with previous cell studies looking at the mechanisms of 25(OH)D and 1,25(OH)₂D in muscle, and this is the first reported study looking at molecular and clinical associations in human skeletal muscle in both healthy elderly subjects and COPD subjects.

In conclusion, this sub-study shows associations between serum 25(OH)D concentration and MHC mRNA expression in healthy elderly subjects which may be related to actions on myogenic regulatory factors. Consistent with the findings of the clinical study, these associations were not present in COPD subjects and this lends weight to the argument that skeletal muscle in COPD subjects is resistant to the actions of 1,25(OH)₂D, and this resistance would appear to occur at a molecular level.

Chapter 5: Genetic Influences on Muscle Strength and Vitamin D in COPD

5.1: Introduction

Potential genetic influences of the Vitamin D meatabolic pathway include polymorphisms in the vitamin D binding protein, the VDR and in genes encoding enzymes for the conversion or breakdown of 25(OH)D and 1,25(OH)₂D. The influence of certain polymorphisms in the VDR on muscle strength are outlined in Chapter 1.

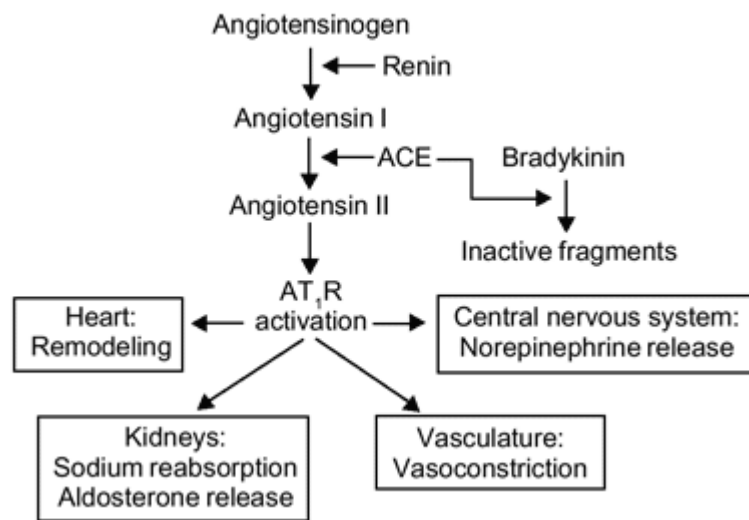
Two variants in the DBP gene (rs7041 and rs4588) have been shown to be related to serum 25(OH)D concentrations (McGrath, Saha et al. 2010), and one of these (rs7041) has been related to serum 25(OH)D levels in COPD (Janssens, Bouillon et al. 2010). However there are no published studies looking at potential influences of these DBP polymorphisms on skeletal muscle strength.

The renin-angiotensin system (RAS) has widely known systemic effects and, like the vitamin D pathway, has more recently been shown to have local effects on differing organs which include skeletal muscle.

Angiotensin II is produced by the initial conversion of angiotensinogen to angiotensin I by the action of renin, and the subsequent conversion of angiotensin I to angiotensin II by Angiotensin Converting Enzyme (ACE) (figure 5.1). Angiotensin II activates the ATR1 Receptor which stimulates aldosterone release from the adrenal cortex and

noradrenaline release from sympathetic nerve terminals resulting in vasoconstriction and sodium reabsorption. These effects are counteracted by local activation of ATR2 receptors. ACE also converts bradykinin to inactive fragments.

Figure 5.1: The Renin-angiotensin system



Whilst expression of the ATR1 receptor is widespread, ATR2 receptor expression is restricted to the adrenal glands, uterus, ovaries, lung, heart and brain and does not appear to be present in skeletal muscle (Malendowicz, Ennezat et al. 2000). Increased angiotensin II levels are associated with weight loss in subjects with cardiovascular disease (Anker, Negassa et al. 2003) and ESRF, and ATR1 receptor blockade has been shown to prevent cachexia in a rat model of congestive heart failure (Dalla Libera, Ravara et al. 2001).

Serum ACE activity has been negatively associated with skeletal muscle strength, Angiotensin II appears to exert these effects on skeletal muscle through varying pathways which include IGF-1 suppression (Song, Li et al. 2005), stimulation of NF- κ -B (Russell, Wyke et al. 2006), PPAR δ down regulation (Zoll, Monassier et al.

2006) and transforming growth factor beta (TGF β) activation (Cohn, van Erp et al. 2007) with a tendency to promote cachexia, muscle protein degradation and inflammation.

The ACE gene has a polymorphism which results in the insertion / deletion of a 287 base pair fragment. The deletion allele results in higher tissue and circulating ACE activity with consequent higher angiotensin II and reduced bradykinin levels (McCauley, Mastana et al. 2009). The DD allele has been associated with better power performance in healthy subjects (Nazarov, Woods et al. 2001; Woods, Hickman et al. 2001) and has been shown to provide a better response to isometric muscle training with an increase in quadriceps strength (Montgomery, Clarkson et al. 1999). In contrast the I allele appears to be associated with better endurance performance (Myerson, Hemingway et al. 1999) and has been associated with an increased proportion of Type I fibres in skeletal muscle (Zhang, Tanaka et al. 2003).

In a previous cross-sectional study of COPD subjects and age and sex matched controls, COPD subjects who were homozygote for the deletion allele had greater quadriceps strength despite a similar FFM (Hopkinson, Nickol et al. 2004). However in this study, no relationship was found in the control subject group.

The mechanism for this effect on skeletal muscle may be due to the increased RAS activity and there are other genetic polymorphisms in the angiotensin pathway that have been associated with varying RAS activity. One of these is the ATR1 A1166C polymorphism where the C allele has been associated with increased angiotensin II activity (Miller, Thai et al. 1999; van Geel, Pinto et al. 2000).

Although it has not been demonstrated to be associated with increased RAS, the C allele of the Angiotensinogen (AGT) Met235Thr polymorphism has been linked with muscle function. It appears to be more frequent in power athletes compared to endurance athletes or a control group (OR: 1.681 (1.176-2.401)) (Gomez-Gallego, Santiago et al. 2009). These three polymorphisms in combination (ACE I/D polymorphism, ATR1 A1166C and AGT Met235Thr) have been shown to have a combined influence on the development of diabetic nephropathy (Ahluwalia, Ahuja et al. 2009) which in itself is thought to be linked with RAS activity (St Peter, Odum et al.).

The aims of this chapter are to establish whether the ACE I/D polymorphism, the AGT Met235Thr polymorphism and the ATR1 A1166C polymorphism are associated with QMVC, either individually or in combination, in COPD subjects compared to healthy control subjects.

5.2: Results

5.2.1: Allele frequencies

Genotype distributions for COPD and Control Groups for all 3 polymorphisms are shown in table 5.1. There was a significant difference in genotype frequencies for the ACE I/D polymorphism between the COPD and Control groups ($\chi^2=6.0$, $p=0.049$). In the control group, genotype distribution was similar to that previously reported in the UK population consistent with Hardy Weinberg equilibrium {O'Dell, 1995}.

However, in the COPD group, the DD genotype frequency was higher than expected. The allele frequency for each group was as follows: COPD: I = 0.41, D = 0.59; Control: I = 0.54, D = 0.46.

A significant difference was also seen in the genotype frequencies between COPD and control groups for the AGT polymorphism ($\chi^2=6.6$, $p=0.04$). A higher frequency of the T allele was seen in the COPD group (table 5.1).

Table 5.1: Allele frequencies in COPD and Control Groups

		ACE			AGT			ATR1		
		II	ID	DD	CC	CT	TT	AA	AC	CC
Control	number	33	40	25	23	49	25	48	42	7
	frequency	0.34	0.41	0.25	0.24	0.5	0.26	0.49	0.43	0.08
COPD	number	22	39	41	11	49	37	53	40	6
	frequency	0.22	0.38	0.40	0.11	0.50	0.38	0.54	0.40	0.06

5.2.2: ACE I/D polymorphism

Characteristics of COPD subjects according to ACE I/D polymorphism are shown in Table 5.2. There was a significant difference in FEV₁ (% pred) between genotypes although this was not related to either allele; the lowest FEV₁ was seen in the ID group. No difference was seen other parameters measured.

Table 5.2: Characteristics of COPD subjects according to ACE I/D genotype

	II	ID	DD	
	(n=22)	(n=49)	(n=31)	
Age, yr	64.6 (9.4)	66.5 (7.1)	62.6 (10.2)	
BMI, kg/m ²	24.4 (3.7)	23.6 (3.9)	24.3 (5.0)	
FFM, kg	44.8 (9.1)	43.4 (7.7)	45.5 (9.7)	
FEV ₁ % predicted*	42.8 (19.1)	35.9 (15.3)	49.0 (24.3)	F=4.2 p=0.02
25(OH)D, nmol/l	55.6 (26.0)	50.6 (26.1)	42.5 (24.5)	
QMVC, kg	28.7 (14.0)	27.5 (11.4)	30.6 (12.6)	

Characteristics of the control group according to ACE genotype are shown in table 5.3. There was a significant difference seen in FFM across genotype with The D allele being associated with the highest FFM (table 5.3). QMVC was also higher in the DD group but this did not reach significance.

There was a significant difference in serum 25(OH)D concentration between allele groups. Those who were homozygote for the deletion allele had the highest serum 25(OH)D levels whilst those who were homozygote for the insertion allele had the lowest serum 25(OH)D levels (I/I: 43.3 ± 24.1 ; I/D: 56.4 ± 26.0 ; D/D: 67.8 ± 31.8 ; F=6.0, p=0.004) (figure 5.2).

Table 5.3: Characteristics of Control subjects according to ACE I/D polymorphism genotype

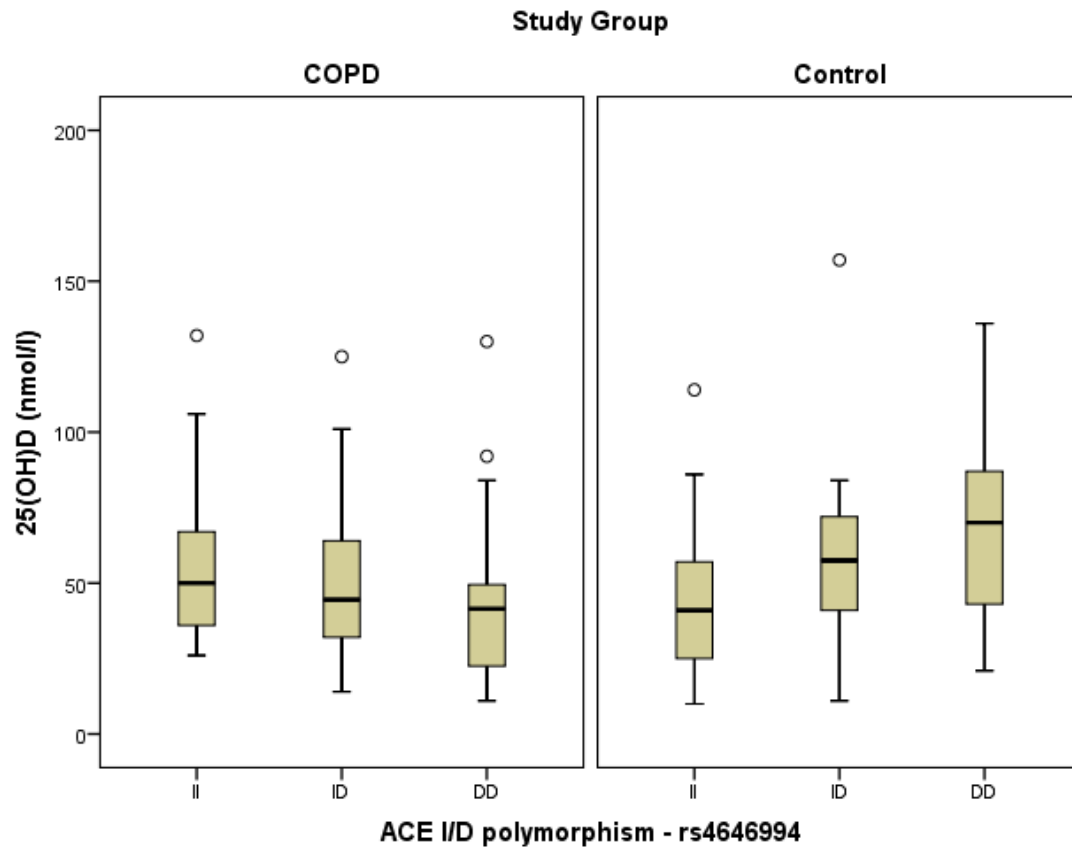
	II	ID	DD	
	(n=33)	(n=40)	(n=25)	
Age, yr	64.4 (8.1)	62.7 (10.0)	62.4 (7.8)	
BMI, kg/m ²	25.0 (3.7)	25.6 (4.7)	27.7 (5.0)	
FFM, kg [*]	49.7 (13.5)	50.5 (13.2)	58.2 (14.7)	F=3.2 p=0.045
FEV ₁ % predicted	105.6 (17.8)	100.3 (15.1)	97.2 (14.7)	
25(OH)D, nmol/l [*]	43.3 (24.1)	56.4 (26.0)	67.8 (31.8)	F=6.0 p=0.004
QMVC, kg	39.3 (13.5)	41.2 (13.1)	44.9 (15.5)	

A univariate linear model was used to look for factors influencing muscle strength.

For COPD subjects the model included the following factors: age; sex; FFM; exacerbation rate; daily steroid dose; FEV₁ (% pred); YPAS score; serum 25(OH)D and ACE genotype. For control subjects the model did not include exacerbation rate or daily steroid dose. ACE genotype did not remain significant in the model for either COPD or control subjects.

5.2.3: ACE I/D polymorphism and serum 25(OH)D concentration

Figure 5.2: Serum 25(OH)D concentration according to ACE I/D polymorphism genotype in COPD and Control Groups



Serum 25(OH)D concentration (nmol/l): COPD: I/I: 55.6 ± 26.0 ; I/D: 50.6 ± 26.1 ; D/D: 42.5 ± 24.5 ;

Control: I/I: 43.3 ± 24.1 ; I/D: 56.4 ± 26.0 ; D/D: 67.8 ± 31.8

When season measured, daily vitamin D intake and ethnicity were compared between genotype groups, a significant difference was seen only in ethnicity ($\chi^2=17.1$, $p=0.03$). Distribution of Caucasian subjects was similar between groups but 6 Indian subjects were included in the II genotype group. Stepwise linear regression was performed using the same variables as detailed above for COPD subjects. The ACE I/D genotype was the only factor that remained significant in the model ($B=12.3$, $p=0.001$).

5.2.4: AGT Met235Thr polymorphism

Characteristics according to AGT genotype are shown in tables 5.4 and 5.5 for COPD and Control Groups respectively. In the COPD group, there was a significant difference in both FFM and QMVC with CT heterozygote's having the lowest FFM and being weaker, whilst CC homozygotes were strongest with the highest FFM (table 5.4). No difference was seen between genotype for any parameters measured in the control group.

Table 5.4: Characteristics of COPD subjects according to AGT genotype

	CC	CT	TT		
	(n=11)	(n=49)	(n=37)		
Age, yr	62.0 (7.1)	63.9 (9.8)	66.0 (8.6)		
FFM, kg	49.7 (10.4)	42.2 (7.4)	46.1 (9.1)	F=4.4	p=0.02
QMVC, kg	36.1 (18.5)	26.5 (9.9)	30.8 (12.4)	F=3.2	p=0.04
FEV ₁ , % pred	38.7 (23.0)	41.7 (20.8)	44.6 (20.2)		
25(OH)D, nmol/l	45.6 (31.4)	45.3 (26.8)	53.5 (23.1)		

Table 5.5: Characteristics of Control Subjects according to AGT genotype

	CC	CT	TT
	(n=23)	(n=49)	(n=25)
Age, yr	61.2 (9.0)	63.0 (9.1)	65.0 (8.0)
FFM, kg	53.9 (15.0)	51.7 (14.3)	52.0 (13.1)
QMVC, kg	42.8 (14.6)	40.5 (15.0)	42.4 (11.4)
FEV ₁ , % pred	99.3 (16.1)	102.1 (17.1)	100.5 (14.1)
25(OH)D, nmol/l	58.8 (28.5)	54.7 (23.8)	51.2 (33.2)

A univariate model was used to look for factors influencing muscle strength using the same factors as detailed above. AGT genotype did not remain significant in the model for COPD nor Control subjects.

5.2.5: ATR1 A1166C polymorphism

Characteristics of COPD and Control groups according to ATR1 genotype are shown in Tables 5.6 and 5.7. No significant difference was seen between genotype groups for any parameters studied in either COPD or control groups.

Table 5.6: Characteristics of COPD subjects according to ATR1 genotype

	AA	AC	CC
	(n=53)	(n=40)	(n=6)
Age, yr	64.3 (7.9)	65.0 (10.3)	63.8 (10.5)
FFM, kg	43.1 (7.5)	44.7 (10.0)	50.4 (4.5)
QMVC, kg	27.6 (11.3)	29.2 (13.9)	38.7 (7.7)
FEV ₁ , % pred	39.3 (19.7)	46.7 (21.9)	49 (24.2)
25(OH)D, nmol/l	48.2 (28.7)	50.3 (23.5)	42.5 (15.7)

Table 5.7: Characteristics of Control Subjects according to ATR1 genotype

	AA	AC	CC
	(n=48)	(n=42)	(n=7)
Age, yr	64.6 (9.3)	62.7 (8.0)	57.9 (8.6)
FFM, kg	50.7 (15.1)	52.8 (12.0)	58.8 (18.6)
QMVC, kg	38.9 (15.0)	43.1 (12.0)	47.1 (13.3)
FEV ₁ , % pred	100.4 (17.8)	102.0 (14.8)	100.9 (13.9)
25(OH)D, nmol/l	53.3 (31.7)	53.7 (21.6)	68.7 (38.4)

When a univariate linear model was used to look for factors influencing muscle strength, ATR1 genotype was not found to be significant.

5.2.6: Influence of combined genotype on muscle strength

When all 3 genes were included in the univariate linear model looking at factors influencing QMVC in the COPD group, the following factors were found to be

significant: Age (F=9.6, p=0.003); FFM (F=9.4, p=0.003); ATR1 (F=5.9, p=0.001); ACE and AGT (F=5.1, p=0.001); ATR1 and AGT (F=12.0, p<0.001).

In the control group, the following factors were significant: age (F=4.1, p=0.048); FFM (F=15.1, p<0.001). ACE, ATR1 and AGT combined gave an F value of 2.39, p=0.059.

5.2.7: DBP rs7041 SNP

Allele frequencies were similar in both COPD (MAF 0.41) and Control (MAF 0.42) groups and were similar to those reported in the NCBI SNP database (0.39).

In the COPD group, a significant difference was found between genotype groups for FEV₁ (% predicted) with the highest FEV₁ in those homozygous for the T allele (51.3% ± 22.3) and the lowest in those who were homozygote ie GT (37.1% ± 16.6) (table 5.8 and figure 5.3). .

In the Control group, no significant difference was seen between genotype groups for any characteristics measured (table 5.9).

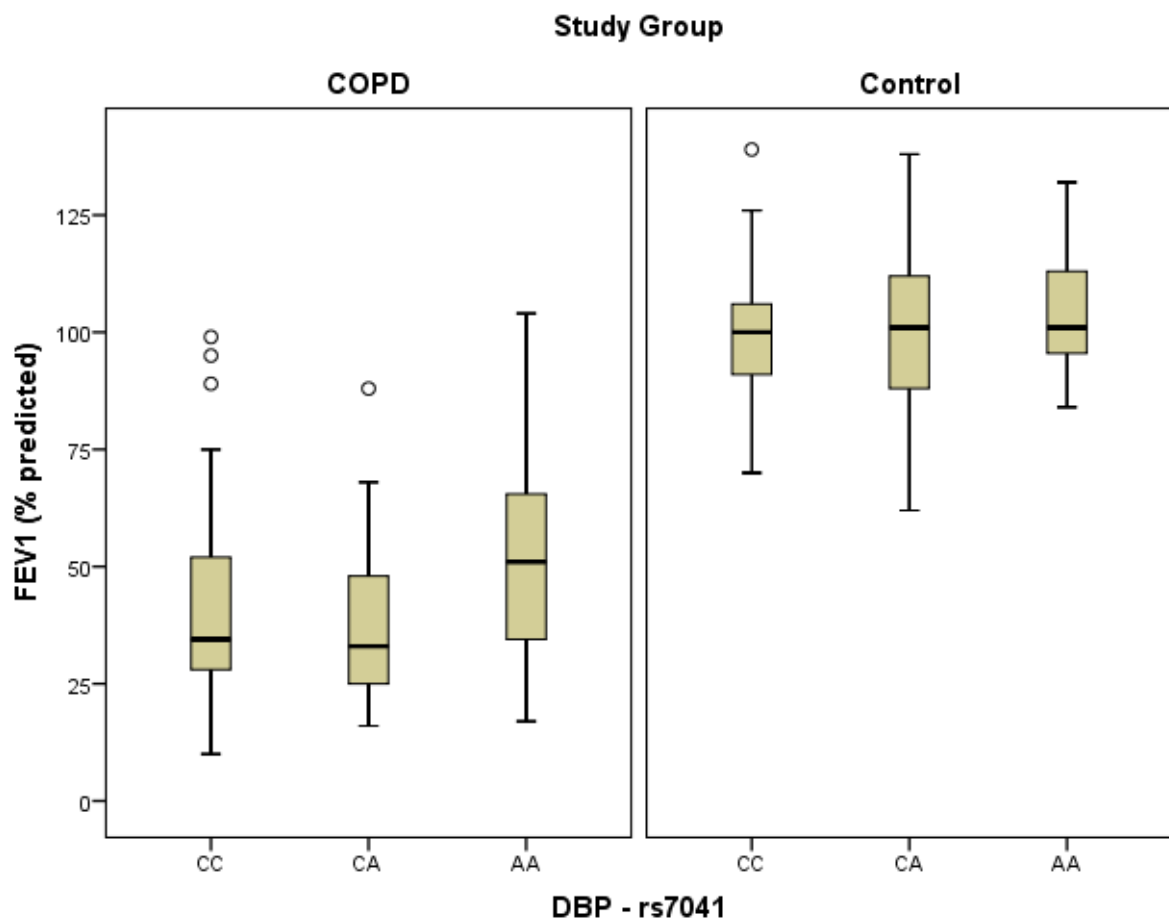
Table 5.8: Characteristics of COPD group according to DBP rs7041 phenotype

	GG	GT	TT	
	(n=42)	(n=35)	(n=23)	
Age, yr	63.6 (8.5)	67.0 (8.9)	63.0 (9.7)	
BMI, kg/m ²	24.0 (4.3)	23.6 (4.2)	24.7 (4.8)	
FFM, kg	44.5 (9.1)	43.2 (7.6)	45.6 (9.6)	
FEV ₁ % predicted*	42.4 (22.3)	37.1 (16.6)	51.3 (22.3)	F=3.4, p=0.04
5(OH)D, nmol/l	51.9 (28.7)	52.1 (26.7)	39.1 (15.1)	
1,25(OH) ₂ D, pmol/l	87.6 (32.1)	82.2 (32.2)	70.0 (31.7)	
QMVC, kg	28.2 (12.0)	27.7 (11.8)	30.9 (13.6)	

Table 5.9: Characteristics of Control Group according to DBP rs7041 phenotype

	GG	GT	TT	
	(n=33)	(n=45)	(n=17)	
Age, yr	63.7 (8.2)	63.0 (8.3)	63.4 (11.2)	
BMI, kg/m ²	27.1 (5.0)	25.6 (4.7)	24.9 (4.1)	
FFM, kg	56.2 (13.3)	50.8 (14.4)	48.5 (13.5)	
FEV ₁ % predicted	99.8 (16.1)	101.1 (17.0)	103.0 (13.0)	
25(OH)D, nmol/l	56.5 (28.8)	55.0 (30.2)	51.4 (26.5)	
1,25(OH) ₂ D, pmol/l	79.8 (32.7)	83.2 (32.7)	83.3 (18.6)	
QMVC, kg	44.9 (14.0)	41.5 (14.3)	35.1 (9.0)	

Figure 5.3: FEV₁ (% predicted) according to DBP rs 7041 phenotype in COPD and Control Groups



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5.2.8: DBP rs4588 SNP

Allele frequencies were similar in both COPD (MAF 0.26) and Control (MAF 0.30) groups and were similar to those reported in the NCBI SNP database (0.22).

In the COPD group, a significant difference was seen between genotypes for FFM(kg): CC 44.2 ± 8.6 ; CA 42.4 ± 8.2 ; AA 52.3 ± 7.3 , $F=5.0$, $p=0.009$, QMVC (kg): CC 28.3 ± 12.4 ; CA 26.5 ± 10.8 ; AA 39.1 ± 12.8 , $F=4.3$, $p=0.02$, and Handgrip

(kg): CC 31.3 ± 11.3 ; CA 32.7 ± 9.4 ; AA 41.7 ± 12.0 , $F=3.6$, $p=0.03$ (figure 5.4).

When QMVC and handgrip were corrected for FFM, there was no significant difference found (table 5.10).

Table 5.10: Characteristics of COPD Group according to DBP rs4588 phenotype

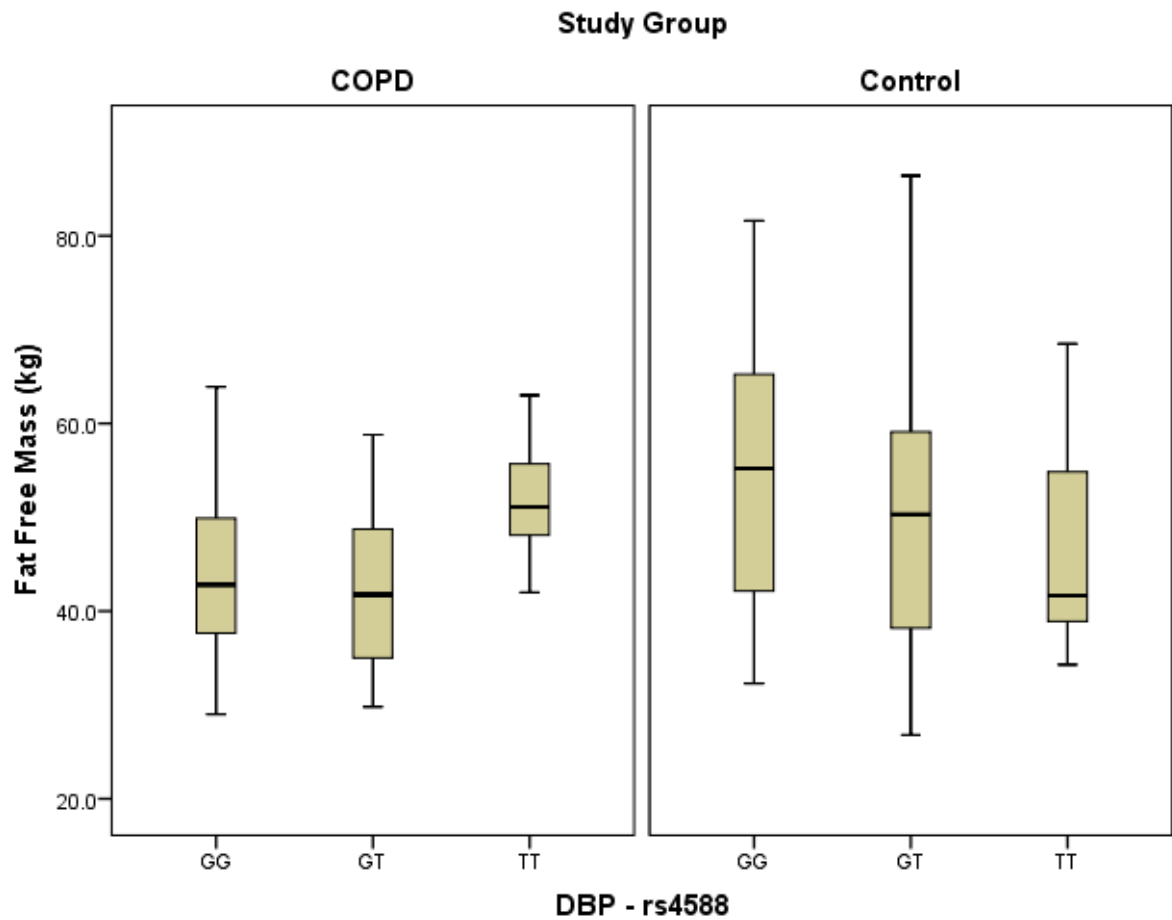
	CC	CA	AA		
	(n=59)	(n=32)	(n=10)		
Age, yr	64.1 (8.5)	65.7 (10.3)	64.7 (7.9)		
BMI, kg/m ^{2*}	24.1 (4.2)	22.9 (4.2)	27.0 (4.6)	$F=3.6$	$p=0.03$
FFM, kg [*]	44.2 (8.6)	42.4 (8.2)	52.3 (7.3)	$F=5.0$	$p=0.009$
FEV ₁ % predicted	41.3 (22.5)	42.2 (17.3)	53.0 (20.9)		
25(OH)D, nmol/l	50.8 (27.8)	46.2 (25.2)	44.8 (13.3)		
QMVC, kg [*]	28.3 (12.4)	26.5 (10.8)	39.1 (12.8)	$F=4.3$	$p=0.02$
handgrip, kg [*]	31.3 (11.3)	32.7 (9.4)	41.7 (12.0)	$F=3.6$	$p=0.03$
SNiP, cmH ₂ O	63.3 (17.5)	62.0 (21.7)	72.6 (32.1)		
QMVC / FFM	0.6 (0.2)	0.6 (0.2)	0.8 (0.2)		
handgrip / FFM	0.7 (0.2)	0.8 (0.2)	0.8 (0.2)		

In the control group, a significant difference was found only for SNiP between genotypes as follows CC 87.7 ± 23.5 ; CA 76.1 ± 21.2 ; AA 63.9 ± 29.2 , $F=4.5$, $p=0.01$ (table 5.11).

Table 5.11: Characteristics of Control Group according to DBP rs4588**phenotype**

	CC	CA	AA	
	(n=47)	(n=42)	(n=8)	
Age, yr	62.9 (8.5)	64.3 (9.5)	59.6 (6.9)	
BMI, kg/m ²	26.4 (4.5)	25.6 (5.0)	24.2 (2.7)	
FFM, kg	54.9 (13.7)	49.8 (14.2)	46.7 (12.8)	
FEV ₁ % predicted	100.2 (15.8)	103.1 (17.2)	98.9 (14.6)	
25(OH)D, nmol/l	60.6 (31.8)	50.8 (25.5)	42.2 (15.2)	
QMVC, kg	44.4 (15.4)	39.6 (12.6)	34.7 (7.3)	
Handgrip, kg	38.3 (10.7)	35.6 (11.4)	32.1 (5.1)	
SNiP, cmH ₂ O*	87.7 (23.5)	76.1 (21.2)	63.9 (29.2)	F=4.5 p=0.01
QMVC / FFM	0.8 (0.2)	0.8 (0.2)	0.8 (0.2)	
Handgrip / FFM	0.7 (0.1)	0.7 (0.1)	0.7 (0.1)	

Figure 5.4: FFM according to rs4588 genotype for COPD and Control Groups



5.3: Discussion

The significant findings of this genotyping study in a predominantly Caucasian COPD population, and age and sex matched healthy elderly control group are as follows:

- 1) The ACE I/D polymorphism, the ATR1 A1166C polymorphism and the AGT Met235Thr polymorphism have a combined influence on skeletal muscle strength in the COPD group.

- 2) The ACE I/D polymorphism is associated with serum 25(OH)D concentration in a healthy elderly population
- 3) The D allele of the ACE I/D polymorphism and the T allele of the AGT Met235Thr polymorphism have a higher frequency in the COPD group compared to the control group.
- 4) The AA genotype of the rs4588 DBP polymorphism is associated with higher FFM and measures of muscle strength in the COPD group.

The main finding of this study shows that genetic polymorphisms which influence the renin-angiotensin system have a combined influence on skeletal muscle function in COPD subjects. Polymorphisms which have evidence of increased RAS activity are associated with increased QMVC in these subjects contributing to the growing evidence that the RAS has effects on skeletal muscle.

The surprising finding was the relationship between the ACE I/D polymorphism and serum 25(OH)D concentration which remained significant when corrected for other confounding variables. A relationship between 1,25(OH)₂D and the renin-angiotensin system has been previously documented. 1,25(OH)₂D has been shown to have an inverse relationship with blood pressure (Kristal-Boneh, Froom et al. 1997) and plasma renin (Burgess, Hawkins et al. 1990). VDR KO mice have been shown to have increased renin expression and plasma angiotensin II production, with subsequent hypertension, cardiac hypertrophy and increased water intake (Li, Kong et al. 2002). However the mechanism of action of 1,25(OH)₂D on the renin angiotensin system is not clear and recent studies with VDR agonists have not shown effects on the RAS (Bernini, Carrara et al.; Atchison, Harding et al.).

The findings of a stronger measures of skeletal muscle strength with the rs 4588 DBP polymorphism are likely to be related to an influence on FFM. This may have been a chance finding, but when an interaction analysis was performed, a combined effect of COPD and the rs4588 polymorphism was significant ($F=3.41$, $p=0.35$). It would therefore appear that in COPD subjects, the AA genotype of the rs4588 DBP polymorphism indirectly confers an increase in skeletal muscle strength through an increase in FFM. These effects are unrelated to serum 25(OH)D concentrations.

The findings in this study may have been through chance but the effects of the ACE genotype on serum 25(OH)D concentration was very significant and became more so when corrected for confounding factors ($p<0.001$). Serum 25(OH)D concentration increased with the DD genotype which has been related to power performance in sport and it is possible that this effect on muscle performance is mediated through 25(OH)D which has been shown to effect type II muscle fibres in humans. The II genotype being related to proportion of Type I fibres in skeletal muscle and endurance performance in sport is also consistent with the lower 25(OH)D levels found in this group. Taking into account the findings of the muscle biopsy sub study, we can postulate that the vitamin D pathway has an important role in controlling muscle fibre type.

COPD patients are an example of the opposite end of the spectrum of environmental influences on skeletal muscle to elite athletes and this perhaps explains why the genetic influences on muscle function are more pronounced. In both circumstances skeletal muscle is forced to function under more extreme conditions and genetic

influences may be more exaggerated. This could explain why no relationship between genotype and muscle function was found in the control group in this study, and in the study by Hopkinson et al. This does not explain the increased D allele frequency seen in the COPD group in our study which may be a chance finding. A recent meta-analysis of studies looking at allele frequencies of the ACE I/D polymorphism found only a significant increased risk of COPD with the D allele in Asian populations (Li, Lan et al.). COPD subjects with quadriceps weakness have been shown to have an increased mortality (Swallow, Reyes et al. 2007) and it is therefore possible that the D allele confers a survival benefit in COPD through its influence on muscle strength.

The potential for multiple comparisons is a limitation of this study. However the genes studied were carefully selected for their known or potential effect on skeletal muscle or the renin-angiotensin system. A p value of <0.01 was considered significant. Another limitation is the inclusion of a small number of non-Caucasian subjects. However the numbers of these were small and when excluded from the analysis, no difference in results was seen.

In summary, this study shows a combined influence of genes in the renin-angiotensin pathway on skeletal muscle function in COPD subjects, as well as further evidence for a link between the Vitamin D pathway and the renin-angiotensin system. Effects of the DBP rs4588 polymorphism on FFM in COPD subjects have also been demonstrated. Further studies to investigate the role of the RAS in skeletal muscle and its interaction with 25(OH)D and 1,25(OH)₂D are required.

Chapter 6: Conclusions

This work aimed to try and unravel some of the actions of a pleiotropic hormone on an adaptable organ in a multisystem disease. It has been to a small extent successful in adding some knowledge to the field but a large amount of work remains to be done in order to unravel the complexities involved.

The clinical chapter threw up the initial surprising finding that there was no apparent relationship between serum 25(OH)D concentration and skeletal muscle function in COPD patients. This raises the possibility of resistance to 1,25(OH)₂D in skeletal muscle which is supported by the findings of the muscle biopsy study where strong associations in the healthy elderly control groups were not replicated in the COPD group, suggesting that a failure of communication may occur. Subsequent studies looking at vitamin D supplementation in COPD have failed to find an improvement in muscle function which adds weight to this argument.

The influences of genetic polymorphisms in the ACE pathway appear to be more profound in COPD where the muscle is subjected to an 'extreme' environment. These results contribute to the emerging evidence that the renin-angiotensin system is involved in skeletal muscle function and in fact appears to be linked to the Vitamin D pathway.

Further research leading on from this work would focus on the role of 1,25(OH)₂D and fibre type in skeletal muscle, the presence or absence of the VDR in the skeletal muscle of COPD subjects and the potential link between the RAS and Vitamin D in

skeletal muscle. A trial of VDR agonists in COPD subjects may also go some way towards relieving the burden of this chronic debilitating disease if found to be of benefit.

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