Vitamin D and skeletal muscle strength and endurance in chronic obstructive pulmonary disease

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SUBJECTS AND METHODS

Stable COPD patients were recruited opportunistically as they attended hospital outpatient clinics and also those on the department's clinical database were invited to participate. Control subjects were recruited by advertisement and through local community groups. Subjects were excluded if they had significant co-morbidities for example unstable cardiovascular disease, malignancy or limiting musculoskeletal disorders. Study participants were sampled throughout the year, and there was no difference in the time of year measured between the patient and control groups. All subjects provided informed written consent and the study was approved by the Ethics Committee of The Royal Brompton Hospital.

A systematic history including exacerbation rate and average daily dose (ADD) of oral corticosteroids in the preceding year was obtained. Vitamin D intake was assessed with a standardised recall questionnaire developed by ourselves, based on known dietary sources of vitamin D.[1] We did not exclude patients on vitamin D supplements so that our study group would represent a normal, unselected clinic population. The Yale physical activity survey (YPAS) [2] and the St George's Respiratory Questionnaire (SGRQ) were completed.

Fat Free Mass (FFM) was measured with a Bodystat 1500 bioelectrical impedance device using a disease specific regression equation in COPD patients [3] and the device's internal algorithm for controls. Spirometry, plethysmographic lung volumes and gas transfer were obtained in COPD patients using a CompactLab system (Jaeger, Würzburg, Germany). Only spirometry was measured in control subjects.

Muscle strength and endurance

Maximum isometric quadriceps force (QMVC) and handgrip strength were measured as previously described.[4] Unpotentiated twitch quadriceps force (TwQ) was assessed by

magnetic femoral nerve stimulation, using two Magstim 200 monopulse units discharged simultaneously through a 70mm branding iron coil.[5] A stimulus response curve was generated to ensure supramaximality of stimulation and the mean of at least 5 stimulations at 100% stimulator output was taken.

Quadriceps endurance was measured using repetitive magnetic stimulation with a Magstim Rapid 2 stimulator and a flexible mat coil placed over the body of the quadriceps.[6] Stimuli were delivered at a frequency of 30 Hz with a duty cycle of 0.4 (2s on, 3s off) for 60 trains. The stimulator intensity was adjusted for each subject initially to generate 20% of their supine QMVC. Decay in force produced by consecutive trains was used as an index of endurance. Endurance curves were compared between COPD and control groups, and vitamin D sufficient and insufficient subjects within groups. Vitamin D insufficiency was defined as 25(OH)D levels of <75nmol/l.

Sniff nasal inspiratory pressure (SNIP) was measured through a plug occluding one nostril during a maximal sniff performed through the contralateral nostril.[7]

Serum measurements

Serum was stored and batch analysis of calcidiol - 25(OH)D, calcitriol - $1,25(OH)_2D$, hsCRP, IL6, electrolytes and albumin was performed following study completion. Serum 25(OH)D was measured by radioimmunoassay procedure after acetonitrile extraction (25-hydroxyvitaminD RIA; Immunodiagnostic Systems, Boldon, Tyne and Wear). $1,25(OH)_2D$ was measured by enzyme immunoassay after immunoextraction (1,25-DihydroxyVitaminD EIA; Immunodiagnostic systems, Boldon, Tyne and Wear).

Muscle biopsy

Muscle biopsy samples were taken from the vastus lateralis of the dominant leg.[6] 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 sample. Tissue was snap frozen in liquid nitrogen and subsequently stored at -80^{o}C for later analysis.

For real-time quantitative PCR (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 MHC1, 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 ºC for 10 minutes, then 40 cycles of 95 ºC for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds. The PCR products were run on a 2% agarose gel to confirm the size of the product. Messenger RNA levels for all genes of interest were normalised to Human RPLPO mRNA levels and values were log transformed to obtain a normal distribution. Primer sequences are shown in Table 1.

Statistical Analysis

Descriptive statistics are reported as mean (SD) 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 factors influencing muscle strength. Disease severity was considered in terms of percent predicted; airflow obstruction (FEV_1), gas transfer (TLco) and gas trapping (RV/TLC). 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. Analysis was performed using SPSS for windows version 16.0 and STATA release 10.1.

Vitamin D and lung function in patients and controls

In the COPD group, 25(OH)D was associated with FVC (%pred) (r=0.21, p=0.04) but no other measure of lung function, whilst $1,25(OH)_2D$ 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 FEV_1 (%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, 25(OH)D, $1,25(OH)_2D$, PTH or albumin.

In the control group, 25(OH)D was correlated with FEV_1 (l) (r=0.27, p=0.006) and FVC (l) (r=0.31, p=0.002). $1,25(OH)_2D$ was correlated with FEV_1 (l) (r=0.41, p<0.001) and FVC(l) (r=0.38, p<0.001) as well as FEV_1(% pred) (r=0.25, p=0.02). After stepwise multivariate analysis; weight, pack years and $1,25(OH)_2D$ remained independently associated with FEV_1(%pred) (r^2=0.22), and weight and $1,25(OH)_2D$ remained independently associated with FVC (%pred) (r^2=0.22). Other factors not retained in the models were daily vitamin D intake, season measured, 25(OH)D, PTH or albumin.

Myogenic factors studied - background

We chose to look at myogenic regulatory factors as they have been shown to be influenced by $1,25(OH)_2D$ in vitro, and have persistent up-regulation in VDR knockout mice.[8] Mrfs have been extensively studied in embryogenesis and in cell culture models where 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.[9] 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.[10] MyoD and myogenin mRNA has been shown to increase after resistance exercise training in both young and old people, whilst myf5 mRNA increased only in the young.[11]

Whilst they clearly have an ongoing role in adult muscle which has yet to be clearly elucidated, the results of our study suggest that mrf4 and myf5 may be important regulators of muscle fibre type. One study in adult rats has demonstrated high levels of myogenin mRNA in muscles that predominantly consist 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. [12] 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. [13]

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Table 1: Primer Sequences

Gene	Forward	Reverse
RPLPO	TCTACAACCCTGAAGTGCTTGATATC	GCAGACAGACACTGGCAACATT
MHC1	CCCTGGAGACTTTGTCTCATTAGG	AGCTGATGACCAACTTGCGC
MyHCIIa	TCACTTATGACTTTTGTGTGAACCT	CAATCTAGCTAAATTCCGCAAGC
MyHCIIx	TGACCTGGGACTCAGCAATG	GGAGGAACAATCCAACGTCAA
myogenin	GCTGTATGAGACATCCCCCTACTT	CGTAGCCTGGTGGTTCGAA
mrf4	CCCCTTCAGCTACAGACCCAA	CCCCCTGGAATGATCGGAAAC
myf5	GATGTAGCGGATGGCATTCC	AGGTCAACCAGGCTTTCGAA

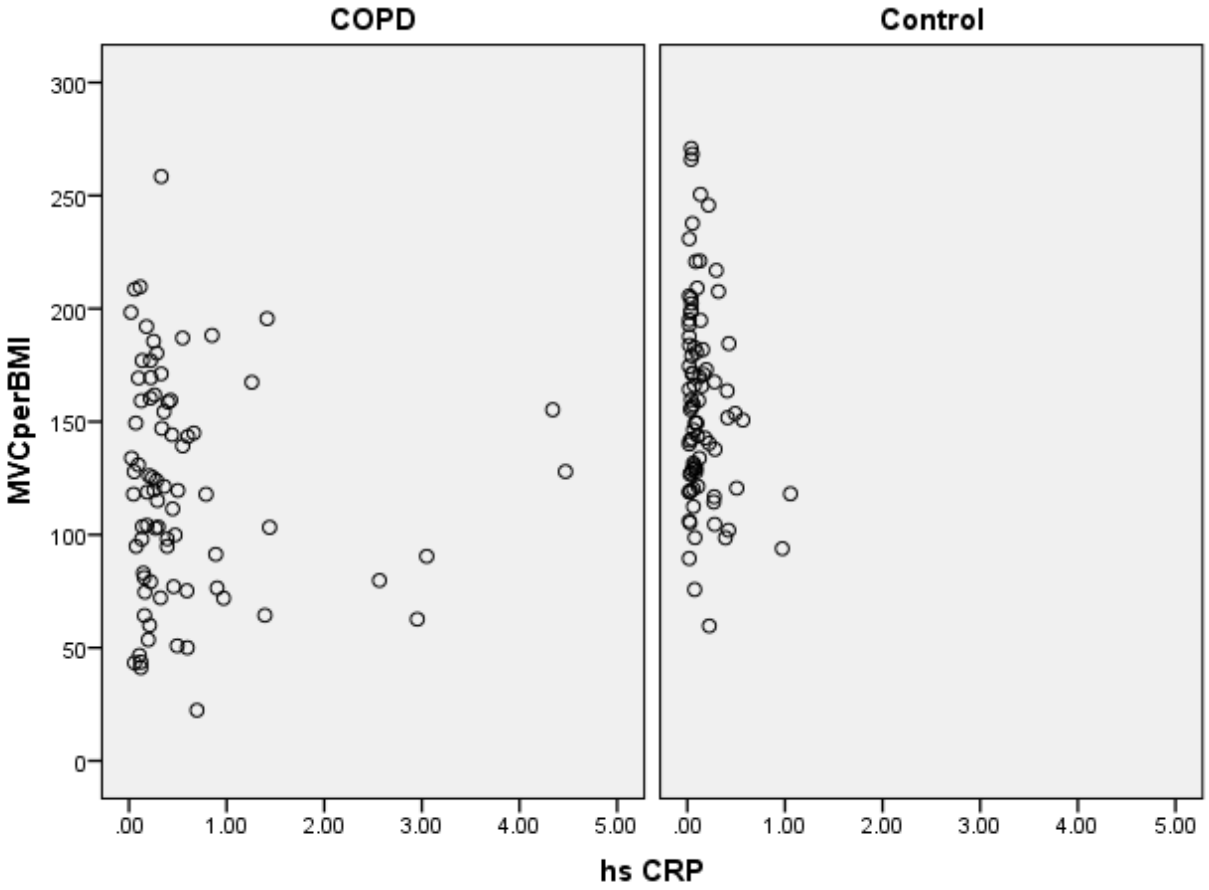

Figure E1 There was no relationship between CRP levels and quadriceps strength in either COPD patients (n=104) or controls (n=100).