Supplementary materials

Study Group

Twenty stable patients with COPD, ten with low FFMI ($COPD_L$) and ten with normal FFMI ($COPD_N$), and tenage, gender and smoking status-matched healthy subjects with normal FFMI were included in the present study.TheCOPD patients had a history compatible with the disease, at least 10 pack/years of smoking and evidence of chronic airflow limitation (post bronchodilator FEV_1 / FVC < 0.7).[1] Subjects were considered ex-smokers if they had not smoked for at least 6 month.

All patients were treated with short- and long-acting bronchodilators and inhaled corticosteroids. They were clinically stable at the time of the study, with no exacerbation or oral steroid treatment in the previous six weeks. None of the patients had significant co-morbidities.

Patient characterization included: a) Clinical assessment, b) Smoking status and cumulative smoking history, c) Spirometry and blood gases, d) Body composition by bioimpedance analysis (BIA), e) Maximal isometric quadriceps maximal voluntary contraction (QMVC), e) exercise tolerance-6 minute walking distance, f) Health related quality of life (HRQoL) (St George's Respiratory Questionnaire), g) number of exacerbations in the previous year, h) Activities of daily living (London Chest Activity of Daily Living Scale [LCADL]) questionnaire; and i) Physical activity questionnaire (Voorrips).

MEASUREMENTS

Lung function

Spirometry was measured (Alpha Spirometer; Vitalograph, Buckingham, UK)

according to American Thoracic Society/European Respiratory Society standards in all subjects [2] before and after the administration of 2.5 mg of nebulised salbutamol. Arterial blood gases were measured (Ciba Corning 800, USA).

Body composition

Body composition was estimated by a leg-to-leg bioelectric impedance device (TBF-300M, TANITA Corporation, Tokyo, Japan) while subjects were in the standing position. Fat free mass index was obtained by dividing FFM in Kg by height^2. Low FFMI was defined as <16 kg.m^{-2} for male and < 15 kg.m^{-2} for female COPD patients.[3]

Exercise tolerance and muscle strength

As a measure of exercise tolerance all 30 participants in the study performed a 6MWT according to ATS guidelines.[4] As a measure of muscle function,[5] muscle strength was assessed as the maximal isometric quadriceps voluntary contraction (QMVC) using a strain gauge dynamometer (Chatillon® K-MSC 500, Ametek, Florida). Subjects were asked to sit in a purpose-built chair with an inextensible strap connecting the ankle to a strain gauge; knees were flexed to 90°; the strain gauge and couplings were all aligned to ensure that the contraction was isometric. After a previous muscle warm up QMVC was performed 3 to 4 times with vigorous encouragement and rests between contractions; the biggest effort recorded was used for analysis.

Health related quality of life (HRQoL) and physical activity level

HRQoL was assessed using the St. George's Respiratory Questionnaire.[6] Physical activity level was assessed using the Voorrips physical activity questionnaire in all subjects participating in the study.[7] In addition, COPD patients' activities of daily living were assessed by the London Chest Activity of Daily Living Scale (LCADL).[8]

***Vastus Lateralis* muscle protein extraction**

Total protein was extracted and purified from ~0.1 g *vastus lateralis* muscle by homogenisation (Tissue Lyser, Qiagen Ltd. West Sussex, UK) of tissue in RIPA (1× PBS, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS with protease inhibitors). Total protein was measured by the bicinchoninic acid method (BCA protein assay kit, Bio-Rad Laboratories, Hercules, CA).

Skeletal muscle fibre composition

Paraffin-embedded tissue sections (5μm) were de-waxed and re-hydrated through graded ethanol using standard procedures.[9] Antigen retrieval was performed using Novocastra retrieval buffer (pH8)in a de-cloaking chamber (Biocare Medical, USA), tissue sections were washed, placed on Leica Vision Biosystems Bond max immmunostaining robot. After Peroxidease activity was removed by incubation with 3% H_2O_2, slides were washed in TBS-0.1%Tween 20, blocked with 20% normal goat serum (NGS)in TBST and incubated with mouse anti human myosin Type I antibody in blocking solution for 3h in RT (1:2000, DAKO,Sweden) dilution. Slides were washed in TBST and incubated

with goat anti-mouse HRP conjugated antibody (1:500) in NGS-Tween20, washed and incubated with Tyramide Cy5 (Blue) (Perkin Elmer). Antibody elution using HIER [9] sections for Type II co-localisation were retrieved for 10 min in Bond ER2 epitope retrieval solution followed by protein digestion using trypsin (0.5mg/ml, Sigma) in Tris/$CaCl_2$ buffer at 37°C for 15 min. Sections were incubated with 3% H_2O_2, washed and blocked with 20% Normal goat serum in TBST, followed by the incubation with mouse anti human myosin Type II antibody with blocking solution (1:5000) for 2 h in RT. 30 min incubation with secondary goat anti-mouse antibody HRP labelled in NGS-Tween 20 (1:500) were performed, followed by washes and incubation with Tyramide Cy3 (Red) (Perkin Elmer) for 10 min and counterstained with DAPI. Tiled images of the entire section were acquired using a Zeiss 710 confocal microscope. Five images per patient were included in the analysis. A total of 959.6±146.4; 715.0±89.2 and 918.6±95.2 fibres in Control subjects, $COPD_N$ and $COPD_L$ respectively (p=ns) were assessed. Type I, Type II and hybrid (identified by the two antibodies) fibres were counted using a manual tag protocol using Media Cybernetics Image pro Plus (Image-Pro Plus, Media Cybernetics, Inc. Bethesda, MD. USA) and expressed as a proportion of total fibres assessed.

Lipid infiltration

To assess the lipid infiltration a western blot was performed using a polyclonal rabbit anti-perilipin A antibody (1/1000 dilution; P1998-200UL, Sigma) as previously described.[10] To validate equal protein loading among various lanes, polyvinylidenedifluoride (PVDF) membrane was stripped and reprobed with a

monoclonal anti-GAPDH antibody (1/5000 dilution, Santa Cruz Biotechnology, Inc, USA)**(Figure1)**.

Markers of ageing

CDKN2A/p16and Sirtuins

Markers of aging p16, sirtuin1 and sirtuin6 were determined using immunoblotting. 20 μg protein, as determined by the BCA protein micro assay (BioRAD, Hercules, CA), were resolved by sodium-dodecyl sulfate-polyacrylamide gel electrophoresis on 10% polyacrylamide gels. Proteins were transferred to Immobilon-P PVDF membranes (Millipore, Billerica, MA), blocked with 5% dry milk (Bio-Rad, München, Germany) in TBS (Sigma) for 1 hour and probed an overnight at 4oC with primary antibodies against p16 (1/1000 dilution, ab 81278, Abcam, UK), sirtuin1 (1/1000 dilution, ab 32441, Abcam, UK) and sirtuin6 (1/1000 dilution, ABE102, Millipore, UK) the membranes were then washed with TBS/Tween 0.05% (Sigma) three times for 10 minutes each and probed with the secondary antibody (goat anti-rabbit IgG-HRP: #sc-2004, Santa Cruz Biotechnology, Inc, USA) during 1 h at room temperature. Proteins were then visualized using the ECL Detection System (Pierce, Rockford, IL) as per the manufacturer's instructions.

Relative telomere length measurement

DNA was isolated from *vastus lateralis* biopsies using Maxwell®16 machine (Promega, UK) according to the manufacturers' instructions. DNA concentration and integrity were assessed by NanoDrop ND-1000 (NanoDrop, Wilmington,

DE, USA). Relative telomere length was measured by quantitative PCR, following the method described by Cawthon.[11] Each sample was analysed in triplicate using primer sets specific for telomere length and a single-copy gene amplicon*36B4* (acidic ribosomal phosphor protein). The relative T/S ratio (repeat copy number to single copy gene number) for each experimental sample was determined in relation to the control DNA sample. The inter-assay variation was assessed by comparing the relative telomere estimates (T/S ratio) across assays for the positive controls, which were assayed on every assay plate. The average inter-assay coefficient of variance was 0.3% for telomere and 0.1% for 36B4 plates.

Muscle oxidative stress markers

4-Hydroxy-2-nonenal (HNE) levels were assessed by incubating the membrane with polyclonal anti-HNE antibody (1/3000 dilution, AB5605, Millipore, UK). Specific proteins were detected with horseradish peroxidise (HRP)-conjugated secondary antibodies and a chemiluminescence kit (NEL103E001EA, PerkinElmer, Inc, USA).[12] Experiment where membrane was incubated with the secondary antibody only has served as negative control. Blot was scanned with an imaging densitometer and optical densities (OD) of specific proteins were quantified using ImagePro®Plus (Media Cybernetics, Inc. Bethesda, MD. USA). Total HNE OD values were calculated for each sample by adding OD of individual positive protein bands and normalized by GAPDH optical density (1/5000 dilution, Santa Cruz biotechnology, Inc, USA).

Inflammatory Markers

Muscle cytokine levels were measured using Cytometric Bead Array (CBA) (BD Bioscience, San Jose, CA, USA) for simultaneous detection of ten cytokines (TNFα, soluble receptor $TNFR_I$, soluble receptor $TNFR_{II}$, IFNγ, IL1β, IL5, IL-6, IL8, IL10 and IL12p70) in *vastus lateralis* homogenate. The CBA technique utilizes micro particles or beads labelled with discrete fluorescence intensity. The maximum emission of capture beads is at 650 nm on RED parameter. Cytokine specific capture antibody is covalently attached to beads. The captured cytokines are detected using specific antibodies with phycoerythrin (PE) fluorochrome, which emits at 585 nm on yellow parameter. The intensity of fluorescence of yellow parameter is proportional to the amount of cytokine present in test samples. Cytokines were determined in the test samplesaccording to the manufacturer instructions. Briefly, test samples (25 µl) and PE detection antibody were incubated with capture bead reagent for 2 h in the dark at room temperature. All unbound antibodies are washed (150 µl wash buffer), re-suspended in 150 µl before acquisition on FACSArray from Becton Dickinson (BD Bioscience, San Jose, CA, USA). All ten cytokines exhibited single, well separated, peaks. Ten individual cytokine standard curves (range 20–5000 pg/ml) were run in each assay. The software used to analyse the data was BD CBA Excel Software. The range of detection was between 3 and 5000 pg/ml calculated from curve estimation for an average of five assays using power fit and $R^2 > 0.99$ for all cytokines. Inter and intra-assay coefficients of

variation for all cytokines were described by the manufacturer in the instruction manual.

Myogenesis

A western blot was performed using the anti-MyoD1 (1/1000 dilution; Abcam, UK) to assess the protein level of myogenic regulatory factor MyoD in all the groups.[13]

Markers of apoptosis

Immunoblotting

A western bolt was performed using Cleaved Caspase-3 (Asp175) antibody (1/1000 dilution, 9661S,Cell Signaling Technology, USA).to evaluate the protein levels of cleaved caspase-3 in *vastus lateralis* muscle of COPD patients and healthy controls.[14] The optical density of the specific protein band (molecular weight 17 kDa) was quantified using a densitometer and optical densities (OD) were quantified using ImagePro®Plus (Media Cybernetics, Inc. Bethesda, MD. USA). To validate equal protein loading among various samples, PVDF membrane was stripped and reprobed with a monoclonal anti-GAPDH antibody (1/5000 dilution, Santa Cruz biotechnology, Inc, USA).

TUNELassay

Apoptotic nuclei were assessed using the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling assay (TUNEL) (In Situ Cell Detection Kit, TMR red, 12 156 792 910, Roche Applied Science, UK)in paraffin-embedded

sections of *vastus lateralis* specimens following the manufacturer's instructions method which has been used in several studies before.[14] Basically, the method identifies generated small double stranded and big single stranded DNA fragments subsequent to genomic DNA fragmentation during apoptosis by labelling 3'-OH free termini with a specific enzymatic reaction and quantified by fluorescence microscopy. For all samples, tiled images of the entire section at magnification 20X were acquired using a Zeiss 710 confocal microscope. Three images per patient or healthy control were included in the analysis.TUNEL-positive nuclei were those located within the muscle fibre boundary in each section and stained in red and blue at the same time. In each muscle cross section, the TUNEL-positive nuclei (red stain) and the total number of nuclei (DAPI, blue stain) were blind counted. On this basis, in each paraffin section, apoptotic nuclei were expressed as the percentage of the TUNEL positive nuclei from the total number of counted nuclei. 200 nuclei was the minimum amount counted in each paraffin-embeddedsection Negative control experiments, in which the TUNEL reaction mixture was replaced by the label solution, an a positive control using the DNase I recombinant (04716728001,Roche, UK) to induce DNA strand breaks prior to labelling procedures as in the manufacture protocol were also conducted.

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Figure1

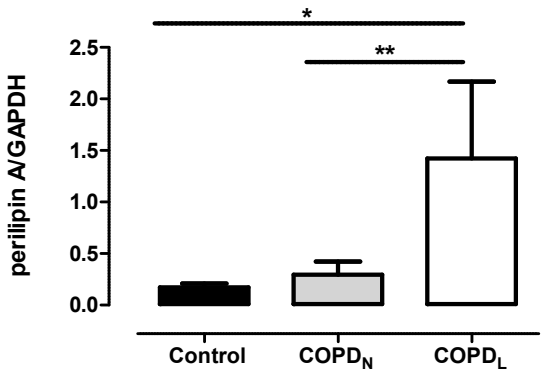

Figure legend

Figure1: Assessment of lipid infiltration; protein levels of perilipin A in *vastus lateralis* muscle of COPD patients with low fat free mass ($COPD_L$), normal fat free mass ($COPD_N$) and healthy controls. Data presented as mean ± SEM, *p<0.05, ** p<<0.05.