Skeletal muscle molecular responses to resistance training and dietary supplementation in COPD

Online Supplement
Materials and Methods

Subjects
Patients with COPD were recruited from outpatient clinics and from those referred for pulmonary rehabilitation at Glenfield Hospital in Leicester (UK). Stable outpatients who met clinical and spirometric criteria for moderate to severe COPD (FEV$_1$/FVC ratio <70%, FEV$_1$<50% predicted) with significant self-reported exercise limitation (MRC Grade III, IV or V) were included in the study. Exclusion criteria were based on the use of oral corticosteroid, oral anticoagulant or long term oxygen therapy. Patients with co-morbid conditions, i.e. cardiovascular complications contributing to exercise limitation or preventing exercise training were also excluded. Healthy controls were recruited from the local population. Subjects were not taking part in regular exercise training programs and COPD patients who underwent pulmonary rehabilitation in the last 12 months were excluded. Approval was obtained from the Leicestershire and Rutland Research Ethics Committee (UK) and all participants provided informed written consent. The study was registered with the UK National Research Register [(NRR) reference: N0123192026].

Study design
All participants undertook fully supervised maximal intensity resistance exercise training for 8 weeks as described below. Patients with COPD were randomly allocated in double blind fashion to receive a dietary protein-carbohydrate supplement or a non-nutritive placebo at the time of training (see below). Healthy control subjects all received placebo. Randomisation (random varying blocks of 2, 4 and 6 volunteers) was performed by the Nottingham University Clinical Trials Unit using a web-based system. Randomisation was stratified for gender and muscle mass to ensure the treatment groups were matched at baseline. Outcome assessments were performed at baseline, 24 hrs after the first training session (muscle biopsy and plasma insulin only), 4 wks, and at the end of the training intervention (8 wks). The trial design and flow of patients is summarised in the diagram shown in the online supplement (Fig. 1S).

Interventions
Resistance training
All participants underwent 8 weeks of voluntary maximal intensity, bilateral, lower-limb resistance training on an isokinetic dynamometer (Cybex II Norm, Stoughton, MA, USA). Training was fully supervised and consisted of 3 sessions per week. Subjects performed 5 sets of 30 maximal knee extensions at a pre-set angular velocity of 180°/sec, with 1 min rest between each set. Maximal knee extension effort was chosen in an attempt to ensure a high proportion of muscle fibre recruitment and volunteers were verbally encouraged at all times to elicit maximal effort. During training, peak torque (Newton-metres; Nm) and total work done (Joules; J) was recorded for each contraction. This training programme was chosen because it does not result in the rapid fatigue associated with slower velocities of contraction (thereby stimulating training adaptation), involves recruitment of fast and slow muscle fibres,(1) and has been demonstrated to completely restore lower limb muscle mass and
increase isometric strength above baseline following 2 weeks immobilisation induced muscle wasting in young, healthy volunteers.(2)

**Dietary supplementation**

COPD patients were randomly allocated to receive a dietary protein-carbohydrate supplement or placebo throughout training. The supplement contained 19 g protein (Whey protein concentrate and milk protein isolate) and 49 g glucose polymer carbohydrate (Vitargo Gainers Gold, Swecarb, Sweden) made up to 500 ml of water. This is sufficient protein to saturate post-exercise muscle protein synthesis(3;4) and increase insulin above a concentration known to inhibit muscle protein breakdown in healthy, young volunteers.(5) The placebo was an identical volume non-nutritive and non-caloric drink that contained flavourings in an attempt to match the taste of the protein-carbohydrate supplement. Supplementation was provided in an unmarked package and supervised by the research team who ensured the supplement was ingested immediately after each training session. Both participants and researchers were blinded to the nutritional intervention. Healthy volunteers received only the placebo intervention.

**Outcome Measurements**

At baseline, spirometry was measured in the seated position (Model R; Vitalograph, Buckingham, UK) according to standards set out by the European Respiratory Society(6); the best of three attempts was taken and was recorded. Body mass index was calculated from height [measured using a wall-mounted stadiometer (SECA, Birmingham, UK to the nearest 0.1 cm] and weight [measured in light clothing to the nearest 0.1 kg] (SECA, Birmingham, UK). Physical activity was assessed at baseline using the adapted physical activity (PA) questionnaire for the elderly.(7) This questionnaire is not disease-specific and was chosen to allow comparison between patients with COPD and healthy controls. The questionnaire has previously been used to describe patients with COPD.(8;9) The questionnaire is interviewer-led and asks about household sporting and leisure activities within the last year, to produce an overall activity score of 0-35. A higher score indicates a greater level of PA.

**Muscle Biopsies**

Biopsies were obtained from the vastus lateralis muscle of all participants using a microbiopsy technique as previously described.(10) Several passes with the microbiopsy needle were made to harvest sufficient material, which was very well tolerated due to the less invasive nature of the procedure compared to the Bergstrom method.

Samples were taken at baseline, 24 hrs after the 1st training session, at mid point (4 wks) and at the end of the resistance training programme (8 wks). The biopsies were taken after a fast of at least 4 hrs and (apart from baseline samples) 24 hours after the previous training session. Samples were immediately snap-frozen, stored in liquid nitrogen and later analysed as described below.

**Blood sampling**
Venous blood was drawn in the fasted state at the same 4 time-point muscle biopsy samples were obtained. Samples were immediately added to preservative, briefly left on ice and following centrifugation plasma samples were stored at -80 °C until analysed for insulin concentration, using a Human Insulin specific RIA kit (Millipore, Billerica, MA, USA), according to the manufacturer’s protocol.

**Isometric Quadriceps Strength**

Before the first outcome assessments, subjects attended a familiarisation session for muscle strength determination. Isometric strength of the quadriceps muscle group was determined during maximal voluntary contraction of the knee extensors, with the knee fixed at an angle of 70° (Cybex II Norm, CSMi, Stoughton, USA). Subjects underwent three attempts of the manoeuvre, each separated by 30s, on 2 consecutive occasions. The highest value obtained was recorded as isometric strength.

**Isokinetic Muscle Function**

Isokinetic torque during knee extensor exercise was recorded at an angular velocity of 60°/s to maximise motor unit recruitment. Subjects performed 2 bouts of 5 repetitions of isokinetic knee extension, with each bout being separated by 1 minute. Isokinetic peak torque and work output were recorded during each contraction. Positioning and stabilisation of the subject in the upright, seated position were standardised according to manufacturer guidelines. The chair monorail and back translation were adjusted so that the centre of the dynamometer head was in line with the subjects’ knee joint line. A seatbelt, thigh strap and contra-lateral limb stabiliser were used to ensure that movement of other body parts was limited. The knee/hip adaptor pad was then strapped to the distal part of the tibia five cm above the lateral malleolus. The subjects’ leg was held out so that the knee was straight (0 degrees) and range of movement was set between 10-80° flexion. The weight of the limb was measured to allow the computer system to correct for gravity in its calculations. The measurements recorded were peak torque (Newton-metres: Nm) and total isokinetic work (cumulative over a set) (Joules: J) for each of the sets.

**Lean Mass**

Whole body and thigh (hip-to-mid patella) lean mass (LM) was measured using dual Energy X-ray Absorptiometry (DEXA) (Lunar Prodigy Advance, GE Healthcare, UK). To determine the composition of the dominant thigh, a region of interest (ROI) was traced using custom analysis software. The upper limit of this ROI was the lowest point of the ischial tuberosity, and the lower limit was the knee joint line. The pubic symphysis and the most lateral part of the thigh were used as the medial and lateral limits.(11) Whole body fat free mass (FFM) was calculated as lean mass + bone mineral mass. Fat free mass index (FFMI) was calculated from height and total body fat free mass. Patients were deemed to have muscle wasting if FFMI < 16kg/m² in men or < 15kg/m² in women. These criteria have been used previously to define muscle wasting in COPD and are predictive of poorer functional performance and quality of life in this population.(12-14)
Whole Body Exercise Performance

After a familiarisation test at baseline, subjects performed a symptom limited, exhaustive, incremental cycle ergometer test (at baseline and after completion of training only). Exercise work rate was increased by 10 W per min in the COPD group and 20 W per min in the healthy control group using a ramp protocol. Peak workrate and breath-by-breath measurements of gas exchange and ventilation were recorded (Zan-600 ErgoTest, Meßgeräte GmbH, Oberthulba, Germany).

Muscle Biopsy Analysis

A wide range of genes (Quantitative RT-PCR) and proteins (Western blotting with infrared detection) representing muscle protein breakdown, anabolic signalling, myogenesis, and transcription were measured. All have previously been associated with molecular regulation of muscle mass in cell, and less frequently, in animal based research. As far as we are aware, this is the first time such a comprehensive battery of measurements has been made over the course of an intervention aimed at increasing muscle mass in COPD patients. For the aid of clarity, genes and proteins have been grouped into the following subclasses:

Protein breakdown
Target genes and proteins included several members of the ubiquitin-proteasome pathway, namely the 20S proteasome, MAFbx, MuRF1 and ZNF216, along with calpain-3 a muscle specific member of the calpain family of calcium activated proteases.

Protein synthesis
Target genes and proteins thought to regulate translation initiation of muscle protein synthesis, namely Akt, p70s6 kinase, GSK3α, GSK3β, 4EBP1 and Redd1.

Myogenesis
Target genes and proteins comprising of MyoD, myogenin and myostatin.

Transcription factors
Transcription factors were targeted based on their reported involvement in muscle mass regulation, autophagy and insulin resistance and comprised members of the family of Forkhead transcription factors, FOXO1 and FOXO3, and RUNX1.

Pro-inflammatory cytokines
Tumour necrosis factor (TNF)-α and IL-6 mRNA expression.

Quantitative RT-PCR
RNA was extracted from frozen muscle biopsies using TRI Reagent (Ambion, Huntingdon, UK), according to the manufacturers protocol. First strand cDNA was then synthesised from 1 µg RNA
using random primers (Promega) and Superscript III (Invitrogen). Additional reactions were performed, in which the reverse transcriptase was omitted to allow for assessment of genomic DNA contamination. All reactions were performed in the ABI 7900HT Fast Sequence Detection System (Applied Biosystems, Foster City, CA). Each well contained 2 µl of cDNA, 18 µM of each primer, 5 µM probe, and Universal Taqman 2X PCR Mastermix for fast reaction (Applied Biosystems) in a 25 µl final volume. Each sample was run in duplicate. Primers and MGB TaqMan probes (Applied Biosystems, Foster City, CA, USA) were designed such that probes spanned over exon-exon boundaries to avoid genomic amplification. Hydroxymethylbilane synthase (HMBS) was used as internal control, and all genes of interest were labelled with the fluorescent reporter FAM. Ct values of the target gene were normalized to Ct values of the house-keeping gene in COPD patients and healthy control volunteers, and the final results were calculated according to the $2^{\Delta\Delta Ct}$ method. The baseline for each subject was used as the calibrator and was set at 1.

**Western Blotting**

Cytosolic and nuclear lysates were prepared from each muscle biopsy sample, and target protein expression was determined using Western blotting as described by Constantin et al (12). Total protein concentration was measured using the Bradford assay (Bradford, MM, Analytical Biochemistry, 1976). Antibodies to determine phosphorylated Akt1 (serine$^{473}$; PAkt1) and total Akt1(60 kDa), phosphorylated eukaryotic translation factor 4E-binding protein 1 (4E-BP1) (threonine$^{37/46}$; P4E-BP1) and total 4E-BP1(15-20 kDa), phosphorylated p70 ribosomal S6 kinase (threonine$^{389}$ Pp70S6K) (Pp70S6K) and total p70S6K (70 kDa), phosphorylated GSK3α (serine$^{21}$; PGSK3α) and total GSK3α (51 kDa), phosphorylated GSK3β (serine$^{9}$; PGSK3β) and total GSK3β (46 kDa), phosphorylated FOXO1 (serine$^{256}$; PFOXO1; 82 kDa) and total FOXO1 (78-82 kDa) and phosphorylated FOXO3 (serine$^{252}$; PFOXO3; 97 kDa) and total FOXO3 (82-97 kDa) and Redd1 (28 kDa) were obtained from Cell Signaling Technology (Danvers, MA, USA). MuRF1 (42 kDa) and MAFbx (42 kDa) antibodies, produced in-house by Pfizer Inc (USA), were provided to us as gifts. Myostatin antibody (MW 45 kDa) was obtained from Novus Biologicals (Littleton, CO, USA). PDK4 (46 kDa), calpain-3 (94 kDa), Myogenin (34 kDa) and MyoD antibodies (35 kDa) were obtained from Insight Biotechnology (Insight Biotechnology Ltd, Middlesex, UK). 20 S proteasome antibody (29kDa) was purchased from Biomol. All proteins were visualized by developing with either an IRDye 800 labelled secondary anti-rabbit antibody or an IRDye 680 labelled secondary anti-mouse antibody (used in multiplex detection) and were further quantified using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). The infrared signals have a greater dynamic linear range compared to chemiluminescence.

**Data Analysis**

The principle objective of the study was the investigation of the molecular signalling responses to resistance training and training combined with feeding in relation to gains in muscle function that would be clinically significant in patients with COPD. Meaningful changes in mRNA and protein
expression across the range of planned targets are difficult to quantify but most human studies have enrolled in the region of 10 subjects. Our objective was to relate changes in gene and protein expression to gains in muscle function that would be clinically significant in patients with COPD. Based on previous data from our pulmonary rehabilitation programme we estimated a 20% increase in muscle strength following training would be clinically and physiologically significant. To detect this strength difference (80% power, \( \alpha = 0.05 \)) we required 25 patients to complete training in each group. To account for predicted dropouts in the COPD group a larger number of subjects was recruited. Our a priori aim was to recruit sufficient numbers of patients with low muscle mass (according to accepted criteria) to allow a subgroup analysis comparing the responses between patients with low and preserved muscle mass. However, the demanding nature of the study with the requirement for intensive physical training and repeated muscle sampling meant we could not recruit sufficient patients with low muscle mass and we therefore present data for the whole cohort.

Because observation of molecular signalling changes over time was the primary objective of the study, we included only subjects who provided at least a baseline and 24 hour time-point muscle biopsy (see Fig 1S). An independent Student t-test was used to compare baseline clinical and functional data between controls and COPD patients. Two-way repeated measures ANOVA, and when appropriate a Least Significant Difference (LSD) post-hoc test, was used to compare within group changes over time and differences between treatment groups (control vs COPD patients receiving placebo vs COPD patients receiving supplement). Training intensity progression was analysed using repeated measures ANOVA with Bonferroni corrections for multiple comparisons.

Because of the non-ordinate nature of the molecular data, comparison of two independent groups was performed using Mann-Whitney's non-parametric analysis, and comparison of more than two time related groups was performed using Friedman's non-parametric analysis. Data in Tables and Figures are expressed as mean ± SEM. Significance was set at the p<0.05 level.
Results

Seventy-one patients with COPD and 22 HC volunteers were enrolled to the study. Fig. 1S shows the flow through the study and the number of viable muscle samples available for analysis at each time point in each group.

Baseline (pre-training)

Physical Characteristics
Baseline physical characteristics are shown in Table 1. There were no significant differences at baseline, between the COPD (P) and COPD (S) groups. Compared with HC, patients had a significantly longer smoking history and worse lung function. Quadriceps muscle function and whole body exercise performance were lower in patients than HC. Thigh fat free mass was lower in patients compared with HC, but this difference was not statistically significant. Seven and six subjects in the COPD (P) and COPD (S) respectively were deemed muscle wasted according to our criteria.

Molecular data
Baseline protein and mRNA expression levels in COPD patients as a whole and in HC are shown in Tables 2 and 1S respectively. At baseline, MAFbx and MuRF1 protein expression, phosphorylation of p70s6kinase, Redd1 protein expression, myogenin and MyoD protein expression and nuclear FOXO1 protein expression were significantly greater in COPD patients than HC. Baseline myostatin mRNA expression was greater in COPD patients but there were no other significant differences in protein expression between the groups.

Training induced changes

Functional data
Thigh lean mass increased significantly relative to baseline in the HC (4.1(0.8)% and 5.4(0.9)% at 4 and 8 wks of RT, respectively) and COPD (P) (4.6(0.9)% and 6.2(1.7)% and COPD (S) (3.9(1.3)% and 4.0(1.1)% groups (Fig. 1A). Isometric quadriceps strength also increased relative to baseline in all groups at 4 and 8 weeks RT (HC: 10.0(4.1)% and 12.4(4.2)% and COPD (P) 16.9(4.3) and 17.7(3.7)% and COPD (S) 14.6(2.8)% and 18.0(3.4)%; Fig. 1B.
There were no significant differences in the training induced gains in muscle mass or strength between the HC group and the COPD (P) and COPD (S) groups. Similarly, the absolute training induced increase in thigh lean mass was not different between the groups (HC: 232(40)g, COPD (P): 215(49)g and COPD (S): 148(43)g).
Whole body cycling exercise performance increased significantly relative to baseline in the HC (Δ peak VO₂ 22.5(7.1)%; p<0.01; Δ peak workload 12.2(2.8)%; p<0.001,) and COPD (P) (Δ peak VO₂ 21.2(8.7)%; p<0.05; Δ peak workload 24.0 (8.6)%; p<0.05) groups, but changes in the COPD(S) group
were not statistically significant (Δ peak VO2 16.7(17.2)%, p=0.3; Δ peak workload 19.7(10.4)%, p=0.07).

The weekly progression of total isokinetic work performed during training (weekly average) by each group is shown in Fig. 1C. Mean absolute work performed by controls was significantly greater at all stages of training than in both COPD groups, but there was no significant difference in the rate of progression of work during training between the HC and COPD groups and between the COPD (P) and COPD (S) groups.

Molecular data
Figures and data depicting training induced changes in protein expression are given in the main manuscript. Data and figures depicting training induced changes in mRNA expression are given here.

Expression of genes (mRNA) involved in muscle protein breakdown
Changes in the expression of catabolic genes from baseline are shown in Fig. 2S. In HC, expression of the majority of catabolic genes increased at 4 and 8 weeks of exercise although (with the exception of calpain 3) this was not statistically significant (Fig. 2S A). The COPD patients showed up regulation in gene expression at 24 hours, 4 and 8 weeks for MuRF1, 20S proteasome and ZNF216 although statistical significance was variable (Fig. 2S B and C). There were no significant differences in the pattern of response to training between the COPD (P) and COPD (S) groups.

Expression of genes (mRNA) involved in muscle protein synthesis
Changes in mRNA expression of genes involved in the regulation of muscle protein synthesis are shown in Fig. 3S. In HC, there were increases in mRNA abundance although most were not statistically significant (Fig. 3S A). The pattern of change in expression with training was similar for the COPD groups (although statistical significance was variable) with the exception of Akt1, which was up-regulated to a greater degree in the COPD groups (Fig. 3S B and C).

There was a significant difference between the 2 COPD groups at 4 weeks (p<0.01) for p70s6kinase.

Expression of genes (mRNA) involved in myogenesis
In all three groups myostatin mRNA expression was significantly reduced at 24 hours but had returned to baseline levels at 4 and 8 weeks (Fig. 4S A,B,C). MyoD and myogenin mRNA expression increased during training in all groups, although statistical significance was variable.

There was also a significant treatment difference in the expression of MyoD mRNA after 4 weeks of exercise (p<0.05) between COPD (P) and COPD (S) groups.

Expression of muscle transcription factor mRNA.
The pattern of change in mRNA expression of FOXO1 and FOXO3 was broadly similar between the HC and COPD groups (Fig. 5S). RUNX1 was significantly up regulated after 24h exercise in all groups, decreasing significantly following 4 and 8 weeks training (Fig. 5S).
Pro-inflammatory genes
TNF-α mRNA expression increased significantly in all 3 groups after 24h exercise (Fig. 6S) In all groups however, expression declined with further training but remained significantly different from baseline at 8 wks in HC and COPD (S) groups. The pattern of change in muscle IL-6 mRNA expression was similar showing significant increases at 24 hrs and a subsequent decline after 4 and 8 weeks of training. There were no significant differences in training induced expression of inflammatory genes between the COPD groups.

Fasting plasma insulin concentration
There was no within or between group difference in fasting plasma insulin concentration at baseline or at any time point during the course of the study (Table 2S).

Associations between the molecular and functional responses to RT
Changes in protein expression, protein phosphorylation and mRNA expression were not correlated with changes in muscle mass or strength after RT.
Supplement References


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<th>COPD (n = 59)</th>
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<td>IL6</td>
<td>2.61 (0.92)</td>
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Table 1S. Gene expression from muscle samples taken at baseline.

Figures refer to mean (SEM) mRNA expression relative to endogenous HMBS used as calibrator. HC = healthy controls.
* p < 0.05 significantly different from control
Table 2S Plasma Insulin

Figures refer to mean (SEM) values expressed in mU/L.  
HC = healthy controls; COPD (P) = COPD patients receiving placebo; COPD (S) = COPD patients receiving supplement

<table>
<thead>
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<th>Plasma insulin (mU/L)</th>
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<th>COPD (P)</th>
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<td>4 Weeks</td>
<td>18.7 (2.4)</td>
<td>30.1 (6.3)</td>
<td>20.0 (2.7)</td>
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<td>8 Weeks</td>
<td>20.1 (3.3)</td>
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Supplement Figure Legends

Figure 1S. Study Flow Diagram.
93 subjects were recruited to the study (71 COPD and 22 HC) but only those with at least a viable baseline and 24 hour biopsy are included in the analysis (21 HC and 59 COPD). Non-viable biopsies at baseline (10): damaged samples/ defrosted.
Reasons for patient withdrawal at each stage, in all groups, are as follows:
COPD (S) = 1 unable to tolerate biopsy (after baseline/ before 24 hour biopsy), 2 exacerbation/ 1 back pain/ 1 family bereavement/ 1 too busy (weeks 1-4), 1 exacerbation/ 1 advised to stop by GP (weeks 4-8).
COPD (P) = 1 unable to tolerate biopsy (after baseline/ before 24 hour biopsy), 1 knee pain (weeks 1-4), 1 unable to re-start training post-operatively (weeks 4-8).
Healthy Control = 1 spouse of a COPD patient wanting to withdraw at the same time.

![Study Flow Diagram](image-url)
Figure 2S. Expression of target genes regulating muscle protein breakdown in response to training.

Gene expression is represented as relative changes from basal. Values are expressed as $2^{\Delta\Delta Ct}$ normalized to endogenous HMBS. Data represent mean ± SEM.

A: HC = Healthy Control subjects; B: COPD (P) = COPD patients receiving placebo; C: COPD (S) = COPD patients receiving supplement

* Significantly different from baseline (p < 0.05)
** Significantly different from baseline (p < 0.01)
*** Significantly different from baseline (p < 0.001)
# Significantly different from 24 hours (p < 0.05)
## Significantly different from 24 hours (p < 0.01)
### Significantly different from 24 hours (p < 0.001)
Figure 3S. Expression of target genes regulating muscle protein synthesis in response to training

Gene expression is represented as relative changes from basal. Values are expressed as $2^{\triangle\triangle\text{Ct}}$ normalized to endogenous HMBS. Data represent mean ± SEM.

A: HC = Healthy Control subjects; B: COPD (P) = COPD patients receiving placebo; C: COPD (S) = COPD patients receiving supplement.

* Significantly different from baseline ($p < 0.05$)
** Significantly different from baseline ($p < 0.01$)
# Significantly different from 24 hours ($p < 0.05$)
## Significantly different from 24 hours ($p < 0.01$)
Figure 4S. Expression of target genes regulating myogenesis in response to training
Gene expression is represented as relative changes from basal. Values are expressed as $2^{-\Delta\Delta Ct}$ normalized to endogenous HMBS. Data represent mean ± SEM.

A: HC = Healthy Control subjects; B: COPD (P) = COPD patients receiving placebo; C: COPD (S) = COPD patients receiving supplement.

* Significantly different from baseline (p < 0.05)
** Significantly different from baseline (p < 0.01)
# Significantly different from 24 hours (p < 0.05)
## Significantly different from 24 hours (p < 0.01)
### Significantly different from 24 hours (p < 0.001)
Figure 5S. Gene expression of transcription factors in response to training.
Gene expression is represented as relative changes from basal. Values are expressed as $2^{\Delta\Delta\text{Ct}}$ normalized to endogenous HMBS. Data represent mean ± SEM.
A: HC = Healthy Control subjects; B: COPD (P) = COPD patients receiving placebo; C: COPD (S) = COPD patients receiving supplement.
* Significantly different from baseline (p < 0.05)
** Significantly different from baseline (p < 0.01)
*** Significantly different from baseline (p < 0.001)
# Significantly different from 24 hours (p < 0.05)
## Significantly different from 24 hours (p < 0.01)
### Significantly different from 24 hours (p < 0.001)
Figure 6S. Gene expression of inflammatory cytokines in response to training

Gene expression is represented as relative changes from basal. Values are expressed as $2^{\Delta \Delta \text{Ct}}$ normalized to endogenous HMBS. Data represent mean ± SEM.

A: HC = Healthy Control subjects; B: COPD (P) = COPD patients receiving placebo; C: COPD (S) = COPD patients receiving supplement.

* Significantly different from baseline ($p < 0.05$)

*** Significantly different from baseline ($p < 0.001$)

# Significantly different from 24 hours ($p < 0.05$)

## Significantly different from 24 hours ($p < 0.01$)

### Significantly different from 24 hours ($p < 0.001$)