DOES OXIDATIVE STRESS MODULATE LIMB MUSCLE ATROPHY IN SEVERE COPD PATIENTS?

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Running head: redox balance and proteolysis in severe COPD

Key words: muscle protein loss; oxidative stress; quadriceps muscle dysfunction; severe COPD; signaling pathways; ubiquitin-proteasome system

Word count: 3,654
ABSTRACT

Oxidative stress may differentially regulate protein loss within peripheral muscles of severe COPD patients exhibiting different body composition.

Oxidation levels of proteins, myosin heavy chain (MyHC), and myonuclei, superoxide anion, antioxidants, actin, creatine kinase, carbonic anhydrase-3, ubiquitin proteasome system, redox-signaling pathways, inflammation, and muscle structure and damage were quantified in limb muscles of severe COPD patients with and without muscle wasting and in sedentary controls.

Compared to controls, in the quadriceps of muscle-wasted COPD patients, levels of protein carbonylation, oxidation of MyHC and myonuclei, superoxide anion production, superoxide dismutase, total protein ubiquitination, E214k, atrogin-1, FoxO-1, and p65 were higher, while content of MyHC, creatine kinase, carbonic anhydrase-3, myogenin, and fast-twitch fiber size were decreased. Importantly, in non-wasted COPD patients, whereas MyHC was more oxidized than in controls, its content was preserved. Muscle inflammation and glutathione levels did not differ between patients and controls. In all patients, muscle structure abnormalities were increased, while muscle force and exercise capacity were reduced.

In severe COPD, while muscle oxidative stress increases regardless of their body composition, protein ubiquitination and loss of MyHC were enhanced only in patients exhibiting muscle atrophy. Oxidative stress does not seem to directly modulate muscle protein loss in these patients.

**Word count:** 200
INTRODUCTION

Highly prevalent conditions such as chronic obstructive pulmonary disease (COPD) are frequently associated with muscle loss and skeletal muscle dysfunction. These systemic manifestations have a considerable impact on the exercise tolerance and quality of life of the patients, and are also associated with increased mortality(1,2). The ubiquitin-proteasome has been identified as the major proteolytic system involved in the degradation of muscle proteins of several catabolic states including cancer-induced cachexia(3,4) and COPD, both limb (5-7) and respiratory (8) muscles of patients with relatively well-preserved body composition. Despite this progress, in severe COPD, the degree of total protein ubiquitination or the upstream signal regulation of muscle protein degradation has not yet been fully explained. In line with this, the family of transcription factors forkhead box O (FoxO) has been shown to regulate atrogin-1 in skeletal muscles of COPD patients(6), in cancer cachectic muscles(9), and in the atrophying diaphragm of patients exposed to mechanical ventilation(10). It remains, however, to be elucidated whether other cellular signaling pathways such as mitogen activated protein kinases (MAPK), nuclear factor (NF)-kB, myostatin, and myogenin may also contribute to muscle atrophy in patients with severe COPD.

Among several etiological factors (11,12), redox imbalance, which has consistently been shown to be involved in the muscle dysfunction of severe COPD patients (12-21), may also contribute to signaling pathways that modulate muscle loss and atrophy (22). In this regard, increased oxidant levels within cells may act as second messengers that regulate pathological signaling leading to enhanced proteolysis and muscle atrophy. Nevertheless, whether increased oxidative stress may trigger muscle proteolysis within the peripheral muscles of severe COPD patients remains an open question. On this basis, it was hypothesized that oxidative stress may differentially regulate protein loss within the muscles of patients with severe COPD exhibiting a wide range of body composition. Therefore, in the present study, our objectives were to assess the following potentially interrelated molecular
events within the limb muscles of a population of severe COPD patients exhibiting different degrees of body composition: 1) redox balance [protein oxidation, superoxide anion content in myonuclei and muscle fiber compartments, antioxidant enzymes, and reduced glutathione (GSH)], 2) levels of redox-sensitive signaling pathways, total protein ubiquitination and markers of the ubiquitin-proteasome system, 3) inflammation, 4) the content of key contractile and functional proteins, previously shown to be consistently oxidized and prone to degradation in COPD muscles (12,15,23) such as myosin heavy chain (MyHC), actin, creatine kinase, and carbonic anhydrase-3, and 5) muscle structural abnormalities. A group of healthy sedentary control subjects was also recruited in the investigation.

METHODS

(See the online depository for additional information).

Human study subjects

Twenty-nine stable Caucasian male severe COPD patients(24) were recruited from the COPD clinics at Hospital del Mar and Hospital Clinic (Barcelona). Patients were further subdivided into those exhibiting low weight and reduced muscle mass [body mass index (BMI) ≤ 21 kg/m² and fat-free mass index (FFMI) ≤ 18 kg/m², muscle-wasted patients, n=18] and normal weight and muscle mass (BMI > 21 kg/m² and FFMI > 18 kg/m², non-wasted patients, n=11) following previously published studies(25,26). Additionally, 10 healthy male sedentary control subjects were also recruited from the general population. Approval was obtained from the institutional Ethics Committees on Human Investigation (Hospital del Mar and Hospital Clinic, Barcelona). Informed written consent was obtained from all individuals.

Anthropometrical and Functional Assessment
Anthropometrical evaluation included BMI and FFMI(17,27). Lung function and quadriceps muscle strength were evaluated in both patients and controls as formerly described(28-31).

**Muscle biopsies**

Muscle samples were obtained from the vastus lateralis of severe COPD patients and controls using the open muscle biopsy technique(12,13,15-17).

**Muscle biology analyses**

All muscle biology analyses were conducted blind in the same laboratory by the same investigators, at Hospital del Mar-IMIM (Barcelona).

*Immunoblotting of 1D electrophoresis.* Markers of proteolysis, signaling pathways, redox balance, muscle proteins, and carbonylated MyHC were determined using immunoblotting(12-17,23,32).

*Detection of superoxide anion within myonuclei.* The presence of superoxide anion was detected using the fluorescent probe dihydroethidium (DHE) on 3-micrometer muscle paraffin-embedded sections from all study subjects following previously published methodologies (33).

*Detection of superoxide anion radicals in muscle compartments.* Superoxide anion levels were detected in cytosolic, membrane, and mitochondria compartments following previous published methodologies (23).

*Reduced glutathione (GSH) in muscles.* GSH content was measured using the Glutathione Assay (Northwest Life Sciences Specialties, Vancouver, WA, USA) following the specific manufacturer’s instructions and published methodologies (34).

*Cytokine Enzyme-linked Immunosorbent Assay (ELISA).* Protein levels of the cytokines tumor necrosis factor (TNF)-alpha, interferon-gamma, and vascular endothelial growth factor
(VEGF) were quantified in the muscles of all study subjects using specific sandwich ELISA kits (RayBiotech, Norcross, GA, USA) following previously published methodologies (16).

**Muscle structure.** On 3-micrometer muscle paraffin-embedded sections from both patients and controls, MyHC-I and –II isoforms were identified using specific antibodies as published elsewhere (12-14,16,23,32). The area fraction of normal and abnormal muscle was also evaluated following previously published methodologies(35).

**Statistical Analysis**

Results are presented as mean (SD). Comparisons of physiological and biological variables among healthy controls and either wasted or non-wasted severe COPD patients were analyzed using one-way and Tukey’s *post hoc* analysis of variance. Correlations between physiological and biological variables were explored using the Pearson’s correlation coefficient.

**RESULTS**

**Clinical and functional characteristics**

As shown in Table 1, both BMI and FFMI were significantly decreased in the muscle-wasted severe COPD patients compared to both non-wasted patients and control subjects. Lung function parameters were significantly impaired in all COPD patients compared to the controls, and muscle-wasted patients exhibited a more severe lung disease than patients with preserved body composition. Compared to control subjects, both groups of severe COPD patients exhibited reduced quadriceps muscle force and exercise capacity, being the latter dramatically impaired in the muscle-wasted patients. Importantly, airway obstruction (FEV₁) and muscle mass (FFMI) were positively correlated among COPD patients (r=0.380, p=0.014).

**Muscle redox balance**
Nuclear superoxide anion detection. Compared to healthy controls (Figure 1A and 1B), the vastus lateralis of both non-wasted and wasted COPD patients exhibited greater levels of nuclear DHE fluorescence (Figures 1C-1G).

Superoxide anion in muscle compartments. Compared to healthy subjects, levels of superoxide anion were significantly increased in the membrane and cytosolic fractions of both non-wasted and wasted COPD patients (Figure 2A and 2B), while superoxide anion from the mitochondrial compartment was significantly increased only in the vastus lateralis of the wasted patients (Figure 2C).

Protein carbonylation. Total protein carbonylation levels were significantly greater in limb muscles of both muscle-wasted and non-wasted than in the controls (Figure 3A). Among patients, protein carbonylation levels correlated with superoxide anion levels within the membrane and cytosolic muscle fractions ($r=0.691 \ p=0.039$ and $r=0.0621 \ p=0.074$, respectively). 

Antioxidants. Mn-superoxide dismutase (SOD) protein content was higher in limb muscles of muscle-wasted patients compared to both healthy controls and non-wasted patients (Figure 3B). CuZn-SOD protein levels were also significantly increased in limb muscles of muscle-wasted patients compared to control subjects (Figure 3C). Muscle protein content of the antioxidants catalase, glutathione peroxidase-I, peroxiredoxin-II, peroxiredoxin-III, and GSH did not differ between patients and controls (Table 2).

Contractile and functional muscle proteins

Protein levels of MyHC were significantly reduced in limb muscles of muscle-wasted COPD patients compared to both control subjects and non-wasted patients (Figure 3D). No differences were observed in MyHC content between non-wasted patients and healthy controls (Figure 3D). Nevertheless, levels of carbonylated MyHC were significantly increased in limb muscles of both groups of patients compared to healthy controls (Figure 3E). Levels of muscle actin were not different between severe COPD patients and control subjects (Figure
4A). Compared to controls, protein content of creatine kinase was significantly reduced in limb muscles of muscle-wasted COPD, whereas muscles of the non-wasted patients showed a tendency to be decreased (p=0.09, Figure 4B). Limb muscles of both muscle-wasted and non-wasted patients exhibited a significant decline in carbonic anhydrase-3 content compared to control subjects (Figure 4C). Among the COPD patients, no significant correlations were found between the content of any of these proteins and either physiological or molecular variables.

**Ubiquitin-proteasome system**

Total protein ubiquitination levels were greater in the vastus lateralis of the muscle-wasted patients compared to control subjects (Figure 5A). Muscle content of the 20S proteasome subunit C8 did not differ between any of the patients and control subjects (Figure 5B). However, levels of the ubiquitin-conjugating enzyme E214k and of the E3 ligase atrogin-1 (muscle atrophy F-box) were greater in the vastus lateralis of muscle-wasted COPD patients than in control muscles (Figures 5C and 5D, respectively). Limb muscles of the non-wasted patients exhibited an almost significant increase (p=0.08) in atrogin-1 protein levels compared to the controls (Figure 5D). Muscle content of the E3 ligase MURF-1 did not differ between any of the study groups (Figure 5E).

**Signaling pathways of muscle proteolysis**

*FoxO pathway*. Muscle content of FoxO-1 transcription factor was significantly higher in the vastus lateralis of the muscle-wasted patients than in control subjects (Figure 6A). Among COPD patients, muscle FoxO1 levels correlated with total muscle protein ubiquitination (r=0.570, p=0.003).

*MAPK pathway*. Muscle levels of MAPK subfamilies c-Jun terminal (JNK), extracellular regulated kinases (ERK)1/2, and p38 did not differ between COPD patients and healthy controls (Table 2).
**NF-κB pathway.** In the limb muscles, no differences were observed between patients and healthy controls regarding protein levels of the transcription factor p50 (Figure 6B). Protein content of the p65 factor, however, exhibited a significant rise in the vastus lateralis of the muscle-wasted patients compared to control subjects (Figure 6C). Levels of p65 also showed a tendency to be increased in limb muscles of the non-wasted patients compared to control muscles (Figure 6C). Among COPD patients, p65 protein content positively correlated with total muscle protein ubiquitination ($r=0.526$, $p=0.008$).

**Muscle growth and differentiation.** Muscle levels of myostatin protein did not differ between patients and healthy controls (Table 2). Nevertheless, protein content of myogenin was significantly reduced within limb muscles of both muscle-wasted and non-wasted severe COPD patients compared to healthy controls (Figure 6D). Among all COPD patients, myogenin levels were significantly associated with FFMI ($r=0.402$, $p=0.046$).

**Inflammatory cytokines.** ELISA levels of interferon-gamma, tumor necrosis factor (TNF)-alpha, and vascular endothelial growth factor (VEGF) did not significantly differ in the limb muscles between patients and controls (Table 2).

**Muscle structure**

**Fiber type composition.** Proportions of type I fibers were significantly reduced within limb muscles of muscle-wasted patients compared to healthy controls (Table 3). Additionally, the size of type II fibers was decreased (21%) in vastus lateralis of muscle-wasted patients compared to both healthy controls and non-wasted patients (Table 3 and Figure E4).

**Muscle abnormalities.** Proportions of abnormal muscle were greater in the vastus lateralis of both muscle-wasted and non-wasted patients than in healthy controls (Table 3). No significant differences were observed between the two groups of COPD patients regarding muscle structural abnormalities (Table 3).
DISCUSSION

A major novel finding in the present investigation is that levels of oxidative stress markers such as superoxide anion within the different muscle compartments and myonuclei, total protein carbonylation, and MyHC oxidation were increased within the limb muscles of all severe COPD patients regardless of their body composition, while a decline in MyHC content and atrophy of type II fibers were only seen in limb muscles of the muscle-wasted patients. Moreover, compared to the controls, the vastus lateralis of the latter patients also showed a significant rise in the levels of several markers of the proteolytic ubiquitin-proteasome system such as total protein ubiquitination and atrogin-1 and E2,14k protein content and of the redox-sensitive signaling pathways FoxO-1 and NF-kB (p65). Levels of muscle inflammatory parameters did not differ between COPD patients and healthy controls. Additionally, levels of functional muscle proteins such as creatine kinase and carbonic anhydrase-3 were decreased among the patients. On the basis of the current findings it would be possible to conclude that oxidative stress does not seem to directly modulate loss of contractile proteins, within the atrophied muscles of patients with severe COPD. Such a conclusion is partly counter to our initial hypothesis.

Exercise capacity and peripheral muscle function and structure

As previously demonstrated (12,16-21), in the current investigation, COPD patients with muscle wasting exhibited severe impairment of aerobic capacity together with moderate reduction in quadriceps muscle force. The latter parameter was similar within the two groups of patients regardless of their muscle mass. The greater proportions of type II fibers observed within the limb muscles of the muscle-wasted patients, albeit of smaller size, could be a contributing factor. Furthermore, muscle mass was shown to be directly associated with disease severity as measured by FEV1.

A novel finding in the study refers to the degree of muscle structure abnormalities, which was increased and similar in both groups of patients. These findings suggest that
factors related to COPD, rather than body composition, are more likely to account for the increase in muscle structure alterations among the patients. Furthermore, the vastus lateralis muscle of the muscle-wasted patients exhibited a less resistant phenotype (decreased proportion of slow-twitch fibers) together with signs of atrophy of the fast-twitch fibers. These findings are in agreement with earlier investigations in which limb muscles of severe COPD patients with abnormal body composition exhibited a shift towards a lower resistant phenotype (12,16,17,36). Besides, they are also in line with another investigation from some of us (32), in which the size of type II fibers was of smaller size in the gastrocnemius of rats with cancer-induced cachexia. Altogether these findings suggest that fast-twitch rather than slow-twitch fibers are likely to be a major target for enhanced muscle proteolysis, at least in the models in question.

**Redox balance and content of key muscle proteins**

We and other investigators (12-20,23,34,37) have already shown, on several occasions, that protein oxidation is increased in the muscles of patients with COPD. Indeed, oxidative stress has widely been proposed as one of the most important mechanisms involved in the etiology of peripheral muscle dysfunction in COPD. In the current study we confirm once more that lower limb muscle proteins undergo severe oxidation. Moreover, it is also being reported that both mitochondrial Mn-SOD and cytosolic CuZn-SOD protein content was greater in the vastus lateralis of the muscle-wasted COPD patients, whereas levels of catalase, glutathione peroxidase-I, peroxiredoxin-II and –III, and GSH did not differ between patients and controls. This is in agreement with previous reports(13,17), leading to the concept that superoxide anion is likely to be a major player in the oxidative cascade of limb muscles in COPD.

Importantly, compared to both sedentary controls and non-wasted patients, limb muscles of the muscle-wasted patients exhibited a 4-fold decrease in MyHC content (26% of the control levels). A second relevant finding in the study is related to the 3-fold increase in
carbonylation levels of MyHC within peripheral muscles of both muscle-wasted and non-wasted patients. These results are in line with a previous report from some of us (23), in which MyHC content was dramatically decreased in the diaphragms of severe COPD patients, while also exhibiting a substantial rise in carbonylation levels. Taken together all these findings, it could be argued that while oxidative stress is clearly involved in the pathophysiology of COPD muscle dysfunction (14,16,17), enhanced contractile protein breakdown may rely on other mechanisms not directly linked to increased oxidative stress within the myofibers, at least in peripheral muscles. Other factors such as the degree of the airway obstruction, diffusion capacity, hypoxia, and deconditioning may have also influenced enhanced MyHC protein loss in COPD patients. However, these aspects were not addressed in the current investigation and would rather be the focus of future research.

In the current investigation, an attempt to assess the content of key muscle proteins previously shown to be targeted by oxidants (12,15,23) as well as by the proteolytic systems (8,38) has been made. We found that levels of the enzymes creatine kinase and carbonic anhydrase-3, but not contractile actin, were reduced in the limb muscles of muscle-wasted and non-wasted patients. In previous studies (12,15,23), compared to healthy controls, the respiratory and limb muscles of the severe patients exhibited a significant decrease in creatine kinase content and activity, while carbonic anhydrase-3 levels did not differ between groups in any of the muscles (12,15,23). Differences in disease severity, patients were more severe in the current study than in the previous reports (12,15,23), and in body composition may account for the discrepancies observed among the three studies. Future investigations are required to specifically disentangle the factors that may contribute to alterations in the content of functional muscle proteins in severe COPD.

**Ubiquitin-proteasome system and signaling pathways**

The ubiquitin-proteasome system degrades both cytosolic and nuclear proteins, and myofibrillar proteins, which are the predominant proteins in skeletal muscles. In the current
investigation, levels of the ubiquitin-conjugating enzyme E2_{14k} and of the E3 ligase atrogin-1, but not MURF-1, were significantly greater in the vastus lateralis of the muscle-wasted COPD patients than in the healthy sedentary controls. This is partly in agreement with a previous report\((6)\) in which mRNA levels of atrogin-1 and MURF-1 were shown to be up-regulated in the limb muscles of stable, relatively well nourished, severe COPD patients. In that study \((6)\), however, the increase in atrogin-1 protein content observed in the severe COPD patients did not reach the statistical significance. More recently, limb muscle mRNA levels of atrogin-1, but not MURF-1, were also shown to be upregulated in stable non-wasted severe COPD patients\((7)\). Additionally, MURF-1 and atrogin-1 mRNA levels were shown to be upregulated in the vastus lateralis of hospitalized non-wasted patients during an acute exacerbation \((5)\). Differences in the number of patients, in their body composition, in study conditions, and in the methodologies employed in each case (mRNA expression versus protein content herein) might account for the minor discrepancies between studies. Another novel finding in the investigation is that muscle-wasted severe COPD patients exhibited greater protein ubiquitination levels in their atrophying muscles. In view of the present findings and of those previously published in limb \((5-7)\) and respiratory muscles \((8,39)\) of severe COPD patients, it could be assumed that the ubiquitin-proteasome system seems to play a relevant role in the process of muscle atrophy in severe COPD patients.

In severe COPD, the upstream signaling regulation of muscle protein degradation, especially of the ubiquitin-proteasome system, has not yet been fully elucidated. The line has been put forward that the transcription factors FoxO play crucial roles in the regulation of muscle wasting \((4,40)\). For instance, FoxO1 has been shown to regulate atrogin-1 in skeletal muscles of COPD patients \((6)\), in cancer cachectic muscles \((9)\), and in the atrophying diaphragm of patients exposed to mechanical ventilation for several days \((10)\). In the present investigation, protein levels of FoxO1, on top of being increased within the vastus lateralis of the muscle-wasted patients, also exhibited a positive correlation with levels of total muscle
protein ubiquitination among all patients. The latter finding suggests that enhanced muscle protein ubiquitination may be signaled, at least to some extent, by FoxO1 in severe COPD.

The MAPK cascade leads to the activation of protein kinases and transcription factors through phosphorylation, resulting in signal transduction, hence playing a key role in cell signaling within tissues. In the current study, protein content of different phosphorylated MAPKs was not explored. Total protein levels, however, of the best characterized MAPK subfamilies JNK, ERK1/2, and p38 were not different between COPD patients and control subjects. These results suggest that despite the relevance of MAPK signaling pathway in a variety of physiological and pathophysiological processes, it may not play a significant role in the COPD-associated muscle atrophy.

NF-κB is one of the most relevant signaling pathways leading to skeletal muscle loss. It is composed by a family of five members (p65, Rel-B, c-Rel, p50, and p52), which are all expressed within skeletal muscles. The NF-κB pathway was recently shown to be upregulated in the diaphragm of severe COPD patients (39). In the muscle-wasted patients of the current study, protein expression of p65, but not p50, was greater in their vastus lateralis, and also showed a positive correlation with levels of total muscle protein ubiquitination. Altogether, the present findings suggest that FoxO1 and NF-κB, but probably not MAPK, are likely to play a major role in the regulation of muscle contractile protein loss through enhanced ubiquitination in severe COPD.

Myostatin, which is a member of the transforming growth factor (TGF)-beta superfamily, is almost exclusively expressed in skeletal muscles and is a potent negative regulator of muscle mass. It has been lately shown that myostatin expression was increased in the vastus lateralis (7,41) and diaphragm (39) of severe COPD patients. It has also been suggested that resistance training reduces myostatin levels in the limb muscles of non-wasted COPD patients (42,43), eventually contributing to enhanced muscle mass in these patients. In the present investigation, protein levels of myostatin did not differ between any of the study
groups. Differences in the study design, in the degree of muscle wasting and the number of patients, together with the lack of a control group of healthy subjects in some of the studies (42, 43) could account for discrepancies among investigations.

Another important myogenic transcription factor required for muscle development during embryonic and fetal life is myogenin. Myogenin also plays a key role in skeletal muscle differentiation, maintenance and repair, regulating muscle metabolism and energy utilization. In the present study, myogenin protein levels were decreased in both groups of severe COPD patients. These findings lead to the conclusion that the repair mechanisms are probably defective within the limb muscles of severe COPD patients, irrespective of their nutritional status. Actually, muscle structural abnormalities were also encountered in both groups of patients.

**Study limitations**

(See the online depository)

**Conclusions**

In contrast to our initial hypothesis, in severe COPD patients, while muscle protein oxidation is increased regardless of their body composition, loss of contractile MyHC and total protein ubiquitination were enhanced only in patients exhibiting muscle atrophy. This process appears to be signaled by FoxO and NF-kB pathways. Oxidative stress, however, does not seem to directly modulate muscle protein loss in these patients. Other factors such as disease and emphysema severity, hypoxia, and deconditioning, may also influence muscle atrophy in severe COPD.
ACKNOWLEDGMENTS

The authors are thankful to Mrs. Marina Sabate, and Mrs. Monica Vila-Ubach for their assistance with part of the laboratory experiments. This study has been supported by FIS 06/1043; FIS 11/02029; CIBERES; SAF 2007-62719; 2005-SGR01060; 2009-SGR-393; SEPAR 2008; SEPAR 2010; FUCAP 2008; FUCAP 2011; and Marató TV3 (MTV3-07-1010) (Spain); and BIO-BRIDGE (LSHG-CT-2006-037939) projects (E.U.). Dr. Esther Barreiro was a recipient of the ERS COPD Research Award 2008.
Reference List


FIGURE LEGENDS

Figure 1:

Superoxide anion detection with the fluorescent probe DHE (red staining, top panels) and DAPI (blue staining, bottom panels) in the vastus lateralis of a healthy control subject (panels A and B, 200 x), a non-wasted severe COPD patient (panels C and D, 200 x), and a wasted COPD patient (panels E and F, 200 x). Yellow stars indicate nuclei that were positively stained for both DHE and DAPI. The percentage of positively stained nuclei (mean and standard deviation) for both DHE and DAPI was significantly higher in the vastus lateralis of non-wasted and wasted severe COPD patients than in the control muscles (panel G).
Figure 2:

A) Mean values and standard deviation of superoxide anion production (nmol/micrograms) in the membrane fraction of the vastus lateralis in both non-wasted and wasted severe COPD patients were significantly greater than in control subjects (*: p<0.05).

B) Mean values and standard deviation of superoxide anion production (nmol/micrograms) in the cytosol compartment of the vastus lateralis in both non-wasted and wasted severe COPD patients were significantly greater than in control subjects (*: p<0.05 and **: p<0.01).

C) Mean values and standard deviation of superoxide anion production (nmol/micrograms) in the mitochondrial fraction of the vastus lateralis in wasted severe COPD patients were significantly greater than in control subjects (*: p<0.05). Muscle levels of mitochondrial superoxide anion, however, did not significantly differ between healthy controls and non-wasted severe COPD patients (ns: non significant).

Figure 3:

A) Mean values and standard deviation [optical densities (OD) expressed in arbitrary units (a.u.)] of total carbonylated protein levels were significantly greater (*:p<0.05) in the limb muscles of both non-wasted and muscle-wasted COPD patients than in the healthy sedentary controls.
B) Representative immunoblots of Mn-superoxide dismutase protein content in healthy controls, non-wasted and wasted severe COPD patients, respectively. Mean values and standard deviation [optical densities (OD) expressed in arbitrary units (a.u.)] of protein content of Mn-superoxide dismutase were significantly higher in the vastus lateralis of the muscle-wasted COPD patients than in both healthy sedentary controls (**:p<0.001) and non-wasted patients (†††:p<0.001). Note that noncontiguous gel lanes are being demarcated by black lines.

C) Representative immunoblots of CuZn-superoxide dismutase protein content in healthy controls, non-wasted and wasted severe COPD patients, respectively. Mean values and standard deviation [optical densities (OD) expressed in arbitrary units (a.u.)] of protein content of CuZn-superoxide dismutase were significantly higher in the vastus lateralis of the muscle-wasted COPD patients than in the healthy sedentary controls (*:p<0.05). Protein levels of CuZn-superoxide dismutase did not significantly (n.s.) differ between non-wasted COPD patients and healthy controls. Note that noncontiguous gel lanes are being demarcated by black lines.

D) Representative immunoblots of MyHC protein content in healthy controls, non-wasted and wasted severe COPD patients, respectively. Mean values and standard deviation [optical densities (OD) expressed in arbitrary units (a.u.)] of protein content of contractile MyHC were significantly lower in the vastus lateralis of the muscle-wasted
COPD patients than in both healthy sedentary controls (*:p<0.05) and non-wasted patients (†:p<0.05). Note that noncontiguous gel lanes are being demarcated by black lines.

E) Representative immunoblots of carbonylated MyHC protein in healthy controls, non-wasted and wasted severe COPD patients, respectively. Mean values and standard deviation [optical densities (OD) expressed in arbitrary units (a.u.)] of carbonylated MyHC were significantly greater in the limb muscles of both non-wasted (*:p<0.05) and muscle-wasted (**:p<0.01) COPD patients than in the healthy sedentary controls. Note that noncontiguous gel lanes are being demarcated by black lines.

Figure 4:

A) Representative immunoblots of actin protein content in healthy controls, non-wasted and wasted severe COPD patients, respectively. Mean values and standard deviation [optical densities (OD) expressed in arbitrary units (a.u.)] of contractile actin did not significantly differ (n.s.) among healthy controls and any of the groups of COPD patients. Note that noncontiguous gel lanes are being demarcated by black lines.
B) Representative immunoblots of creatine kinase protein content in healthy controls, non-wasted and wasted severe COPD patients, respectively. Mean values and standard deviation [optical densities (OD) expressed in arbitrary units (a.u.)] of creatine kinase content were significantly lower in the limb muscles of the muscle-wasted COPD patients (*:p<0.05) than in the healthy sedentary controls. Protein levels of creatine kinase showed a strong tendency to be decreased (p=0.09) in the vastus lateralis of the non-wasted patients compared to the controls. Note that noncontiguous gel lanes are being demarcated by black lines.

C) Representative immunoblots of carbonic anhydrase-3 protein content in healthy controls, non-wasted and wasted severe COPD patients, respectively. Mean values and standard deviation [optical densities (OD) expressed in arbitrary units (a.u.)] of carbonic anhydrase-3 content were significantly lower in the limb muscles of both muscle-wasted and non-wasted COPD patients (*:p<0.05) than in the healthy sedentary controls. Note that noncontiguous gel lanes are being demarcated by black lines.
Figure 5:

A) Representative immunoblots of ubiquitinated proteins in healthy controls, non-wasted and wasted severe COPD patients, respectively. Mean values and standard deviation [optical densities (OD) expressed in arbitrary units (a.u.)] of total ubiquitinated protein levels were significantly higher in the quadriceps of the wasted COPD patients (*:p<0.05) than in the healthy sedentary control subjects. Levels of total ubiquitinated proteins did not significantly (n.s.) differ between non-wasted COPD patients and healthy controls. Note that noncontiguous gel lanes are being demarcated by black lines.

B) Representative immunoblots of 20S proteasome C8 subunit content in healthy controls, non-wasted and wasted severe COPD patients, respectively. Mean values and standard deviation [optical densities (OD) expressed in arbitrary units (a.u.)] of the 20S proteasome C8 subunit did not significantly differ (n.s.) among healthy controls and any of the groups of COPD patients. Note that noncontiguous gel lanes are being demarcated by black lines.

C) Representative immunoblots of E2_{14k} protein content in healthy controls, non-wasted and wasted severe COPD patients, respectively. Mean values and standard deviation [optical densities (OD) expressed in arbitrary units (a.u.)] of the ubiquitin-conjugating enzyme E2_{14k} were significantly greater in the quadriceps of both non-wasted and wasted COPD patients (*:p<0.05) than in the healthy sedentary control subjects. Note that noncontiguous gel lanes are being demarcated by black lines.

D) Representative immunoblots of atrogin-1 protein content in healthy controls, non-wasted and wasted severe COPD patients, respectively. Mean values and standard deviation [optical densities (OD) expressed in arbitrary units (a.u.)] of the ubiquitin-ligase enzyme atrogin-1 were significantly greater in the limb muscles of the muscle-wasted COPD patients (*:p<0.05) than in the healthy sedentary control subjects.
Protein levels of atrogin-1 showed a strong tendency to be increased (p=0.08) in the vastus lateralis of the non-wasted patients compared to the controls. Note that noncontiguous gel lanes are being demarcated by black lines.

E) Representative immunoblots of MURF-1 protein content in healthy controls, non-wasted and wasted severe COPD patients, respectively. Mean values and standard deviation [optical densities (OD) expressed in arbitrary units (a.u.)] of the ubiquitin-ligase enzyme MURF-1 did not significantly differ (n.s.) among healthy controls and any of the groups of COPD patients. Note that noncontiguous gel lanes are being demarcated by black lines.

F)

**Figure 6:**

A) Representative immunoblots of FoxO1 protein content in healthy controls, non-wasted and wasted severe COPD patients, respectively. Mean values and standard deviation [optical densities (OD) expressed in arbitrary units (a.u.)] of transcription factor FoxO1 were significantly greater in the limb muscles of the wasted COPD patients (*:p<0.05) than in the healthy sedentary controls. Protein levels of FoxO1 did not significantly (n.s.) differ between non-wasted COPD patients and healthy controls. Note that noncontiguous gel lanes are being demarcated by black lines.

B) Representative immunoblots of p50 protein content in healthy controls, non-wasted and wasted severe COPD patients, respectively. Mean values and standard deviation
[optical densities (OD) expressed in arbitrary units (a.u.)] of the NF-kB pathway, as measured by the transcription factor p50, did not significantly differ (n.s.) among healthy controls and any of the groups of COPD patients. Note that noncontiguous gel lanes are being demarcated by black lines.

C) Representative immunoblots of p65 protein content in healthy controls, non-wasted and wasted severe COPD patients, respectively. Mean values and standard deviation [optical densities (OD) expressed in arbitrary units (a.u.)] of the transcription factor p65 were significantly greater in the limb muscles of the muscle-wasted COPD patients (*:p<0.05) than in the healthy sedentary controls. Protein levels of p65 showed a strong tendency to be increased (p=0.08) in the vastus lateralis of the non-wasted patients compared to the controls. Note that noncontiguous gel lanes are being demarcated by black lines.

D) Representative immunoblots of myogenin protein content in healthy controls, non-wasted and wasted severe COPD patients, respectively. Mean values and standard deviation [optical densities (OD) expressed in arbitrary units (a.u.)] of the myogenic transcription factor myogenin were significantly lower in the vastus lateralis of both non-wasted and muscle-wasted COPD patients (*:p<0.05) than in the healthy sedentary controls. Note that noncontiguous gel lanes are being demarcated by black lines.
C Femose et al. Figure 6

D

E)
Table 1. Anthropometric characteristics and functional status of severe COPD patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Control subjects n=10</th>
<th>Non-wasted severe COPD patients, n=11</th>
<th>Muscle-wasted severe COPD patients, n=18</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years</strong></td>
<td>65 (9)</td>
<td>67 (5)</td>
<td>63 (11)</td>
</tr>
<tr>
<td><strong>BMI, Kg/m²</strong></td>
<td>28.2 (4.4)</td>
<td>28.5 (4.6)</td>
<td>18.9 (2.6) ***, †††</td>
</tr>
<tr>
<td><strong>FFMI, Kg/m²</strong></td>
<td>22.5 (2.5)</td>
<td>22.7 (2.4)</td>
<td>15.9 (1.7) ***, †††</td>
</tr>
<tr>
<td><strong>FEV₁, % pred</strong></td>
<td>95 (22)</td>
<td>45 (19) ***</td>
<td>32 (14) ***, †</td>
</tr>
<tr>
<td><strong>FVC, % pred</strong></td>
<td>95 (14)</td>
<td>78 (18) **</td>
<td>55 (12) ***, ††</td>
</tr>
<tr>
<td><strong>FEV₁/FVC</strong></td>
<td>75 (11)</td>
<td>44 (11) ***</td>
<td>42 (11) ***</td>
</tr>
<tr>
<td><strong>RV, %</strong></td>
<td>110 (3)</td>
<td>169 (46) **</td>
<td>208 (62) ***</td>
</tr>
<tr>
<td><strong>TLC, % pred</strong></td>
<td>113 (18)</td>
<td>109 (12)</td>
<td>116 (17) **</td>
</tr>
<tr>
<td><strong>RV/TLC</strong></td>
<td>43 (2)</td>
<td>54 (10) **</td>
<td>67 (10) ***, ††</td>
</tr>
<tr>
<td><strong>DLco, % pred</strong></td>
<td>106 (18)</td>
<td>58 (20) ***</td>
<td>34 (12) ***, ††</td>
</tr>
<tr>
<td><strong>Kco, % pred</strong></td>
<td>97 (21)</td>
<td>39 (26) ***</td>
<td>38 (21) ***</td>
</tr>
<tr>
<td><strong>PaO₂, kPa</strong></td>
<td>12.6 (1.7)</td>
<td>9.6 (1.8) *</td>
<td>9.7 (0.9) *</td>
</tr>
<tr>
<td><strong>PaCO₂, kPa</strong></td>
<td>4.9 (0.5)</td>
<td>5.8 (0.7) *</td>
<td>5.8 (1.1) *</td>
</tr>
<tr>
<td><strong>VO₂peak, % pred</strong></td>
<td>98 (13)</td>
<td>77 (18) *</td>
<td>29 (7) ***, †††</td>
</tr>
<tr>
<td><strong>WRpeak, % pred</strong></td>
<td>95 (15)</td>
<td>66 (18) **</td>
<td>23 (11) ***, †††</td>
</tr>
<tr>
<td><strong>QMVC, kg</strong></td>
<td>39.3 (3.7)</td>
<td>30.7 (3.2) ***</td>
<td>28.4 (2.4) ***</td>
</tr>
</tbody>
</table>

Data are presented as mean (SD).

**Definition of abbreviations**: BMI, body mass index; Kg, kilograms; FEV₁, forced expiratory volume in one second; pred, predicted; FVC, forced vital capacity; TLC, total lung capacity; RV, residual volume; DLco, carbon monoxide transfer; Kco, Krogh transfer factor; PaO₂, arterial oxygen partial pressure; PaCO₂, arterial carbon dioxide partial pressure; VO₂peak, peak exercise oxygen uptake; WRpeak, peak work-rate; QMVC, quadriceps isometric maximum voluntary contraction.

**Statistical significance is expressed as follows**: * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, between any of the groups of severe COPD patients and controls; † p ≤ 0.05, †† p ≤ 0.01, ††† p ≤ 0.001 between non-wasted and wasted severe COPD patients.
Table 2. Biological markers in the vastus lateralis of severe COPD patients and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>Control subjects</th>
<th>Non-wasted severe COPD patients</th>
<th>Muscle-wasted severe COPD patients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antioxidants</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catalase, OD, a.u.</td>
<td>0.148 (0.063)</td>
<td>0.177 (0.032)</td>
<td>0.165 (0.018)</td>
</tr>
<tr>
<td>GPx-1, OD, a.u.</td>
<td>0.635 (0.376)</td>
<td>0.741 (0.280)</td>
<td>0.609 (0.344)</td>
</tr>
<tr>
<td>Prx-II, OD, a.u.</td>
<td>0.304 (0.149)</td>
<td>0.294 (0.118)</td>
<td>0.272 (0.063)</td>
</tr>
<tr>
<td>Prx-III, OD, a.u.</td>
<td>0.107 (0.039)</td>
<td>0.129 (0.040)</td>
<td>0.117 (0.031)</td>
</tr>
<tr>
<td>GSH, microM</td>
<td>17.631 (7.288)</td>
<td>21.090 (8.691)</td>
<td>23.137 (11.701)</td>
</tr>
<tr>
<td><strong>Signaling Pathways</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JNK, OD, a.u.</td>
<td>0.110 (0.062)</td>
<td>0.120 (0.070)</td>
<td>0.109 (0.054)</td>
</tr>
<tr>
<td>ERK-1/2, OD, a.u.</td>
<td>0.508 (0.240)</td>
<td>0.483 (0.126)</td>
<td>0.473 (0.187)</td>
</tr>
<tr>
<td>p38, OD, a.u.</td>
<td>0.228 (0.103)</td>
<td>0.189 (0.092)</td>
<td>0.219 (0.119)</td>
</tr>
<tr>
<td>Myostatin, OD, a.u.</td>
<td>0.330 (0.063)</td>
<td>0.327 (0.064)</td>
<td>0.326 (0.064)</td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-gamma, pg/mL</td>
<td>50.190 (15.140)</td>
<td>40.480 (22.630)</td>
<td>55.850 (25.004)</td>
</tr>
<tr>
<td>TNF-alpha, pg/mL</td>
<td>28.25 (4.830)</td>
<td>26.990 (5.600)</td>
<td>26.320 (6.550)</td>
</tr>
<tr>
<td>VEGF, pg/mL</td>
<td>10.660 (4.740)</td>
<td>8.280 (3.580)</td>
<td>8.820 (5.062)</td>
</tr>
</tbody>
</table>

Data are presented as mean (SD).

*Definition of abbreviations: COPD, chronic obstructive pulmonary disease; OD, optical densities; a.u., arbitrary units; GPx, glutathione peroxidase; Prx, peroxiredoxin; GSH, reduced glutathione; microM, micromolar; JNK, c-Jun N-terminal kinase; ERK 1/2, extracellular-signal-regulated kinase; p38, p38 mitogen-activated protein kinase; pg, picograms; mL, milliliter; IFN, interferon; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.*
Table 3. Structural characteristics of the *vastus lateralis* muscle in severe COPD patients and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>Control subjects</th>
<th>Non-wasted severe COPD patients</th>
<th>Muscle-wasted severe COPD patients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VASTUS LATERALIS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I fibers, %</td>
<td>30 (5)</td>
<td>29 (7)</td>
<td>23 (6) *, †</td>
</tr>
<tr>
<td>Type II fibers, %</td>
<td>70 (5)</td>
<td>71 (7)</td>
<td>77 (6) *, †</td>
</tr>
<tr>
<td>Cross sectional area,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>type I fibers, µm²</td>
<td>2581 (278)</td>
<td>2452 (425)</td>
<td>2243 (514)</td>
</tr>
<tr>
<td>Cross sectional area,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>type II fibers, µm²</td>
<td>2909 (540)</td>
<td>2880 (361)</td>
<td>2306 (533) *, †</td>
</tr>
<tr>
<td>Normal muscle, %</td>
<td>98.5 (0.4)</td>
<td>97.5 (0.7) **</td>
<td>97.5 (0.9) *</td>
</tr>
<tr>
<td>Abnormal muscle, %</td>
<td>1.5 (0.4)</td>
<td>2.5 (0.7) **</td>
<td>2.5 (0.9) *</td>
</tr>
</tbody>
</table>

Data are presented as mean (SD).
Statistical significance is expressed as follows: * p ≤ 0.05, ** p ≤ 0.01, between any of the groups of severe COPD patients and controls; † p ≤ 0.05 between muscle-wasted and non-wasted COPD patients.