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ATROPHY AND HYPERTROPHY SIGNALING IN THE DIAPHRAGM OF PATIENTS WITH COPD

DRIES TESTELMANS[+(1)], TIM CRUL[+], KAREN MAES[+], ANOUK AGTEN[+], MARK CROMBACH[+(1)], MARC DECRAMER[+] AND GHISLAINE GAYAN-RAMIREZ[+]

[+]Respiratory Muscle Research Unit, Laboratory of Pneumology and Respiratory Division, Katholieke Universiteit Leuven, Leuven, Belgium

[(1)] Fellow of the "Fonds voor Wetenschappelijk Onderzoek – Vlaanderen"

To whom correspondence should be addressed: Ghislaine Gayan-Ramirez

Labo Ademspieren, O&N 1 bus 706

Herestraat 49

B-3000 Leuven

Belgium

Tel: +32 (16) 33 01 93

Fax: +32 (16) 34 71 26

e-mail: ghislaine.gayanramirez@med.kuleuven.be

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ABSTRACT

We investigated whether atrophy and hypertrophy signaling was altered in the diaphragm of COPD patients.

Diaphragm fiber dimensions and proportion, expression of markers of the ubiquitin-proteasome, the NF-κB pathways, the muscle regulatory factors and myostatin were studied in diaphragm biopsies from 19 patients with severe COPD and 13 patients without COPD.

Type I proportion was significantly increased in the diaphragm of COPD patients while type II proportion was decreased. Cross-sectional area of all fiber types was reduced in the COPD patients. In addition, MAFbx mRNA was higher in the diaphragm of COPD patients while Nedd4 mRNA decreased. Cytoplasmatic levels of IκBα and IκBβ were decreased in the COPD patients as was the nuclear NF-κB p50 DNA-binding activity. MyoD mRNA and its nuclear protein content were decreased in the diaphragm of COPD patients and myogenin mRNA and protein levels remained unchanged. Myostatin mRNA was decreased but its protein levels in the nuclear and cytoplasmic fraction were significantly increased in the COPD patients.

These data showed that the ubiquitin-proteasome pathway, the NF-κB pathway and myostatin protein were upregulated in the diaphragm of COPD patients while MyoD expression was reduced. These alterations may contribute to diaphragm remodeling in COPD.

Word count: 196

Key words: diaphragm, muscle, myostatin, proteasome pathway, transcription factors

INTRODUCTION

Inspiratory muscle weakness in patients with chronic obstructive pulmonary disease (COPD) is clinically relevant, since maximum inspiratory pressure is correlated with survival in these patients. Adaptation of the diaphragm revealed a greater proportion of type I fibers with a decreased proportion of type II fibers in COPD patients[1, 2]. This shift towards a slower, more fatigue-resistant profile, also consistent with the adaptation observed in the expression of the SERCA pumps[3], has been linked to chronic increased activity of the diaphragm in COPD patients[4]. Reduced force generation of single diaphragm fibers with decreased myosin content was also found in response to COPD[5]. Moreover, these fibers had decreased calcium sensitivity that could contribute to muscle weakness at submaximal activation[5]. These changes were already present in patients with mild to moderate COPD. Impaired cross-bridge cycling kinetics, fiber atrophy and sarcomeric injury were also observed[2, 5]. Structural changes in the titin molecule may weaken the stability of the muscle filaments, resulting in fiber damage[2, 5]. Finally, protein oxidation, increased oxidative capacity and mitochondrial function occur in line with the progression of the disease[5].

Whether atrophy and hypertrophy signaling pathways are altered in the diaphragm of COPD patients has been poorly investigated. However, this area is of particular interest knowing that COPD is the most common respiratory disease and the diaphragm the most important inspiratory muscle. The potential role of the ubiquitin-proteasome pathway, the NF-κB pathway and the muscle regulatory factors (MRF's) and myostatin was investigated in the present study.

The ubiquitin-proteasome pathway is one of the major proteolytic systems involved in muscle protein breakdown. Before degradation by the proteasome, proteins need to be ubiquitinated, a process requiring an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin-ligating enzyme. The latter is important as it determines which proteins are targeted for degradation. Among the E3 ligases, MuRF1 and

MAFbx are rapidly upregulated during muscle atrophy and are therefore used as markers of the ubiquitin-proteasome pathway activation. Another E3-ligase, Nedd4, was shown to be upregulated in situations of disuse but not in catabolic states[6].

The classical NF-κB pathway is activated during inflammatory processes. After degradation of the inhibitory IκB proteins, the p65/p50 heterodimers are moving to the nucleus where they target genes of inflammatory pathways[7]. Interestingly, disuse muscle atrophy caused by unloading was shown to activate an alternative NF-κB pathway involving p50 and Bcl-3 but not p65[7].

The MRFs, MyoD and myogenin, belong to the basic helix-loop-helix transcription factors. In mature skeletal muscles, MyoD is mainly expressed in fast-twitch muscles and myogenin mainly in slow-twitch muscles[8]. Changes in MyoD and myogenin have been reported after muscle disuse caused by denervation, immobilization or hindlimb suspension[8]. Alterations in MyoD and myogenin have been suggested to drive muscle phenotype adaptations[8].

Finally, myostatin, an inhibitor of skeletal muscle mass, has been shown to increase during atrophy caused by hindlimb unloading or microgravity as well as in conditions of glucocorticoid-induced muscle atrophy[9]. Myostatin is believed to suppress proliferation and differentiation of muscle precursor cells and to upregulate the ubiquitin-proteasome system[9] .

The implication of these pathways in diaphragm remodeling in response to COPD is still unknown. Therefore, the expression of markers of atrophy signaling MuRF1, MAFbx, Nedd4 for the ubiquitin-proteasome pathway, IκBα, IκBβ, p50 and p65 subunits for the NF-κB pathway, as well as of hypertrophy signaling MyoD, myogenin and myostatin were measured in the diaphragm of COPD patients in comparison to control subjects.

MATERIALS AND METHODS

Population

Diaphragm muscle biopsies were obtained from 19 patients with severe COPD and from 13 patients without COPD (controls). Biopsies were obtained during thoracotomy for lung transplantation in the COPD patients and during oesophagectomy or lobectomy for oesophageal and lung cancer respectively (stage $T_{1-2}N_{0-1}M_0$) in the patients without COPD. Informed consent was obtained from each participant. The study was approved by the Ethics Committee of the Universitaire Ziekenhuizen, Leuven.

Before surgery, pulmonary function testing was carried out. Patient characteristics are shown in Table 1. Twelve out of the 19 COPD patients were taking corticosteroids (methylprednisolone 4mg/day for 8 patients, 8mg/day for 4 patients).

Diaphragm biopsies

Full-thickness biopsy specimens were obtained from the costal diaphragm. Part of the biopsy was frozen in isopentane for histological examination. The other part was frozen in liquid nitrogen and stored at - 80°C to determine the role of markers of atrophy or hypertrophy signaling.

Histochemistry

Serial sections of the diaphragm were stained with Hematoxylin and Eosin to determine histological changes and for myofibrillar adenosine triphosphatase to measure fiber cross-sectional area and proportions.

RNA extraction and real-time quantitative PCR

Total RNA was isolated using the Trizol method. The reverse transcription was performed with the Superscript III First-Strand Synthesis System (Invitrogen, Merelbeke, Belgium) according to the manufacturer's protocol.

Quantitative RT-PCR assay was performed on an ABI Prism 7700 Sequence Detection System, (Applied Biosystems, Lennik, Belgium) using the Platinum Sybr Green qPCR Supermix UDG kit (Invitrogen, Merelbeke, Belgium), according to the manufacturer's protocol. The primers used for this study are shown in table 2. Expression of the 18S gene was used to standardize the quantification of target cDNA.

Protein extraction

Approximately 40 mg of diaphragm were used for dual extractions of nuclear and cytoplasmic proteins with the NE-PER kit according to the company protocol (Pierce Biotechnology, Erembodegem, Belgium). Protein concentration was determined using the Bradford method (Biorad, Nazareth, Belgium).

Gel electrophoresis and immunoblotting

Muscle proteins were separated on 12% SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride (PVDF) membrane. Membranes were incubated with the appropriate primary and secondary antibodies (see online supplement) to detect either IκBα, IκBβ, MyoD, myogenin, myostatin or MAFbx. Proteins were visualized with the ECL Plus detection kit (GE Healthcare, Diegem, Belgium). Proper separation of nuclear and cytoplasmatic fractions was assessed while measuring Histone H3 (Figure E2) and α-tubulin, respectively.

NF-κB activity

Activation of NF-κB p50 and p65 was detected using a microtiter plate assay by Pierce Biotechnology. Nuclear proteins were incubated in wells coated with double-stranded NF-κB consensus oligonucleotides. Bound p50 or p65 was identified with antibodies for the respective protein and detected with a HRP-coupled secondary antibody followed by ECL reagent, as recommended by the manufacturer. Luminescence signal was read in a luminometer (kindly provided by F. Claessens, Labo Legendo).

Statistics

An unpaired *t* test was used (data were normally distributed) to compare patients characteristics, mRNA and protein expression between the two groups. Relationships were assessed with the Pearson correlation analysis. A p value of 0.05 or less was considered significant. Statistics were performed using the GraphPad software (V 4.01).

RESULTS

Patient Characteristics

Anthropometric characteristics and pulmonary function data are provided in table 1. The COPD patients did not differ from the controls with respect to height and age. BMI was significantly lower in the COPD patients. COPD patients had severe (GOLD III) or very severe (GOLD IV) COPD.

Diaphragm histochemistry

As shown in figure 1, cross-sectional area of both type I and type II fibers was significantly reduced with 45 and 30 %, respectively in patients with COPD compared to controls. Moreover, there was a significantly higher proportion of type I fibers in the diaphragm of COPD patients in comparison with the control group (73% vs. 48%) while proportion of type II was decreased.

Muscle regulatory factors

MyoD mRNA (-63%) (Figure 2A) and its protein content (-24%) (Figure 2B) were significantly decreased in the diaphragm of COPD patients, while there was no significant difference in myogenin mRNA and protein expression. An inverse relationship was found between diaphragmatic MyoD protein levels and diaphragm type I fiber proportion (r= -0.65, p=0.04).

Ubiquitin-proteasome pathway

MAFbx mRNA was significantly higher in the diaphragm of COPD patients (Figure 3A) while MuRF1 mRNA levels remained unchanged. MAFbx protein levels tended to be increased in the diaphragm of COPD patients (controls: 100±61%, COPD: 160±51%, p=0.08). Nedd4 mRNA was significantly decreased in the diaphragm of patients with COPD (Figure 3B).

NF-κB pathway

Nuclear NF-κB p50 DNA-binding activity was decreased by 62 % in the diaphragm of COPD patients (Figure 4A) while NF-κB p65 DNA-binding activity did not change. Cytoplasmatic levels of IκBα (-24%) (Figure 4B) and IκBβ (-58%) (Figure 4C) were decreased in the diaphragm of COPD patients.

Myostatin

In the diaphragm of COPD patients, myostatin mRNA was significantly decreased (-86%) in comparison with the control group (Figure 5A). Myostatin protein levels, both in the nuclear and cytoplasmatic fraction of the diaphragm, were significantly increased with 169 and 74% respectively in the COPD patients (Figure 5B and 5C).

DISCUSSION

The present study shows that atrophy and hypertrophy signaling is altered in the diaphragm of severe COPD patients. In particular, the NF-κB pathway, the ubiquitin-proteasome pathway and myostatin were activated while MyoD signaling was depressed. Diaphragm atrophy and fiber adaptation towards a slower profile was also observed.

Diaphragm fiber shift and atrophy

In agreement with previous studies[5], the proportion of type I fibers was higher in the diaphragm of the COPD patients in the present study. This adaptation has been regarded as beneficial expecting the diaphragm of COPD to be more resistant to fatigue[1]. This shift towards a slower profile has also been observed at the level of the SERCA pumps[3]. It has also been shown that resting energy expenditure as measured by diaphragm tension-time index, and the neural drive to the diaphragm are increased in severe COPD at rest[4, 10]. These adaptations have been suggested to result from continuous overload of the diaphragm due to COPD. The diaphragm of severe COPD patients seems to undergo a long-term moderate continuous endurance training[5]. In limb muscles, endurance training results in a shift from fast to slow fiber type[11]. A major difference between the diaphragm of COPD patients and limb muscle studies is the long-term and continuous training underwent by the diaphragm and the age of the population since severe COPD affects elderly individuals. But also in lifelong trained elderly, endurance training results in a greater proportion of type I fiber in vastus lateralis muscle[11].

In the present study, cross-sectional area of type I and type II fibers was decreased in the diaphragm of the COPD patients as previously described[5]. These data indicate that despite continuous overload resulting in increased diaphragm activity, diaphragm atrophy develops in COPD patients.

The muscle regulatory factors: MyoD and myogenin

The data of the present study suggest that MyoD is probably implicated in the fiber shift seen in the diaphragm of COPD patients. In skeletal muscle, the alterations in MyoD and myogenin are suggested to be a drive for fiber shifting under conditions such as denervation and unloading[8]. In this regard a decrease in MyoD will lead to a shift towards a slower, more oxidative profile[8]. Our study shows decreased MyoD mRNA and protein levels in the diaphragm of the COPD patients which is compatible with the increased proportion of type I fibers. The inverse relationship between MyoD protein levels and the type I proportion found in the present study further supports a role for MyoD in fiber shifting. A decrease in diaphragmatic MyoD protein levels has been recently shown in a hamster model of emphysema[12].

Also the calcineurin-NFAT (nuclear factor activate T-cells) pathway, known to specifically induce the slow-twitch muscle program, may have played a role in the diaphragm fiber shift observed in the COPD patients. Overload of the diaphragm in these patients may have activated calcineurin while increasing intracellular calcium. It is, indeed, well known that calcineurin acts as a sensor of sustained elevated intracellular calcium concentration. Although not investigated in the present study, the role of the calcineurin-NFAT should not be neglected.

Myostatin

In the present study, myostatin mRNA levels in the diaphragm of COPD patients were decreased while its protein expression was increased. Differences in myostatin mRNA and protein expression levels have been previously reported in skeletal muscle atrophy[13, 14]. Discrepancy is probably related to the auto-regulation mechanism of myostatin based on a negative feedback loop with increasing levels of active myostatin protein resulting in downregulation of myostatin mRNA expression[15].The data of the present study are in agreement with these findings.

The lower myostatin mRNA levels found in the diaphragm of the COPD patients is compatible with the increased proportion of type I fibers since myostatin is mainly expressed in

fast-type fibers[16]. Myostatin could play a role in modulating gene expression controlling muscle fiber type. Moreover, the myostatin gene has been reported to be a target gene of MyoD as expression of myostatin can be activated by MyoD[17]. The lower levels of diaphragmatic MyoD in our COPD patients could contribute to the downregulation of myostatin mRNA.

Surprisingly, mRNA levels of myostatin, known as a negative regulator of muscle growth were decreased in the diaphragm of COPD patients despite the presence of atrophy. In fact, previous studies on peripheral muscles are consistent with our observation[18, 19]. It has even been suggested that the role of myostatin would rather be the inhibition of hypertrophy than the induction of atrophy, since increased myostatin concentration was not sufficient for muscle mass loss.

However, in the present study, myostatin protein levels in the nuclear and in the cytoplasmic fraction were upregulated in the diaphragm of the COPD patients, and may possibly have contributed to the observed diaphragm atrophy. Several studies have, indeed, reported enhanced myostatin levels in animals after disuse[9] and also in patients with chronic muscle atrophy[9].

Finally, the presence of myostatin protein in the nuclear fraction is intriguing but is not new[13, 16]. Indeed, myostatin protein was found to be essentially located in the nucleus of the myotubes[16] while in rat muscle, it was clearly more expressed in the nuclear pellet than in the cytoplasmic fraction or the total homogenate[13]. Further experiments are needed to determine the implication of nuclear myostatin.

NF-κB pathway

In the present study, decreased content of the cytoplasmatic IκBα and IκBβ was observed in the diaphragm of the COPD patients, suggesting enhanced NF-κB signaling. IκBα content was less decreased than IκBβ. In fact, IκBα has a κB recognition sequence in its promoter region and hence resynthesis of IκBα can be induced by NF-κB, while this is not the

case for IκBβ[20]. NF-κB p50 DNA-binding activity was reduced in the diaphragm of COPD patients in the present study, while p65 DNA-binding activity remained unchanged. However, the p50 subunit is able to dimerize with another p50 subunit, and these homodimers inhibit gene transcription[21]. Similar findings were reported in the vastus lateralis of patients with diabetes type 2 and low-grade chronic inflammation[22]. Interestingly, suppression of MyoD mRNA via NF-κB has been described[23]. Hence the increased NF-κB activity could contribute to the decreased diaphragmatic MyoD protein levels in the COPD patients. Activation of NF-κB also decreased MyoD protein stability and hence could promote MyoD degradation[24]. Increased NF-κB activity has been reported in the quadriceps of COPD patients with low body weight, suggesting a role for NF-κB in skeletal muscle atrophy in these patients[25].

The ubiquitin-proteasome pathway

Upregulation of MAFbx mRNA was found in the diaphragm of COPD patients while MuRF1 mRNA was unchanged. These data are in line with the findings previously reported in mild to moderate COPD patients and are also compatible with the increased protein ubiquitination reported in the diaphragm of COPD patients[5]. Interestingly, MAFbx has been associated with specific degradation of myosin[26]. Therefore upregulation of MAFbx in the diaphragm of COPD patients is in line with the reduction of diaphragm myosin content reported in these patients[27]. On the other hand, MuRF1 has been found to target specific subset of myofibrillar proteins such as titin[28]. Preserved diaphragm titin content in COPD patients[27] is in agreement with unaltered MuRF1 mRNA expression.

In the present study, Nedd4 mRNA was significantly downregulated in the diaphragm of the COPD patients. Nedd4 is a HECT (homologous to E6-AP carboxyl terminus) domain containing E3 ubiquitin-protein ligase which has been shown to be upregulated in atrophy caused by reduced muscle tension, but not in cachexia atrophy[6]. Decreased passive diaphragm tension is present in COPD patients[27]. Reduced muscle tension may possibly involve integrin

signaling or titin mechanotransduction. In COPD patients, alternative splicing of the titin gene leading to a modulation of the titin's spring segment has been proposed as a potential contributor to the decreased passive tension seen in stretched diaphragm fiber[27]. Hence, it seems that this reduction in titin stretching does not induce an upregulation of Nedd4 in the present study. These data suggest that the decrease in passive force generation reported in the diaphragm of COPD patients, will not induce activation of the ubiquitin-proteasome pathway by upregulation of Nedd4.

Potential triggers

There are several factors related to COPD that might have affected the different pathways investigated in the present study. First hypoxia, a common feature in COPD patients is known to inhibit the expression of muscle regulatory factors[29] and to activate the NF-κB pathway[30]. Second, diaphragm shortening caused by pulmonary hyperinflation may have affected some of these pathways. Indeed, in chronic hyperinflation, the resting sarcomere length in human diaphragm muscle fibers decreases according to the degree of pulmonary hyperinflation[31]. Increased proteolysis may be involved in sarcomere deletion. As such, diaphragm shortening may have indirectly contributed to the activation of the ubiquitin proteasome pathway. In addition, increased mechanical load caused by pulmonary hyperinflation may have also contributed to changes in MyoD and/or myostatin expression while mimicking the effect of endurance training. There are, however, no clear data in the literature supporting this concept. Also corticosteroid known to upregulate MAFbx and MuRF1[32] and to selectively decrease MyoD expression[33] and upregulate myostatin gene expression[9] may have contributed to the alterations reported in the present study. Obviously, chronic inflammation, as occurring in COPD patients, may have altered several of these pathways too. In particular, circulating cytokines are known to stimulate proteolysis through the ubiquitin proteasome pathway[34] and to destabilize MyoD[24].

In conclusion, this study showed that diaphragm fiber atrophy and fiber adaptation towards a slower profile are present in the diaphragm of severe COPD patients. In addition, several signaling pathways are altered in the diaphragm of COPD patients. These include upregulation of the NF-κB pathway, the ubiquitin proteasome pathway and myostatin and downregulation of MyoD. All together, these alterations may contribute to diaphragm dysfunction in COPD patients.

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LEGENDS TO THE FIGURES

Figure 1: Cross-sectional area (CSA) of type I and type II fibers in the diaphragm of controls (solid bars, n=6) and patients with severe COPD (open bars, n=6). **Values represent means and standard deviation. *p<0.05**

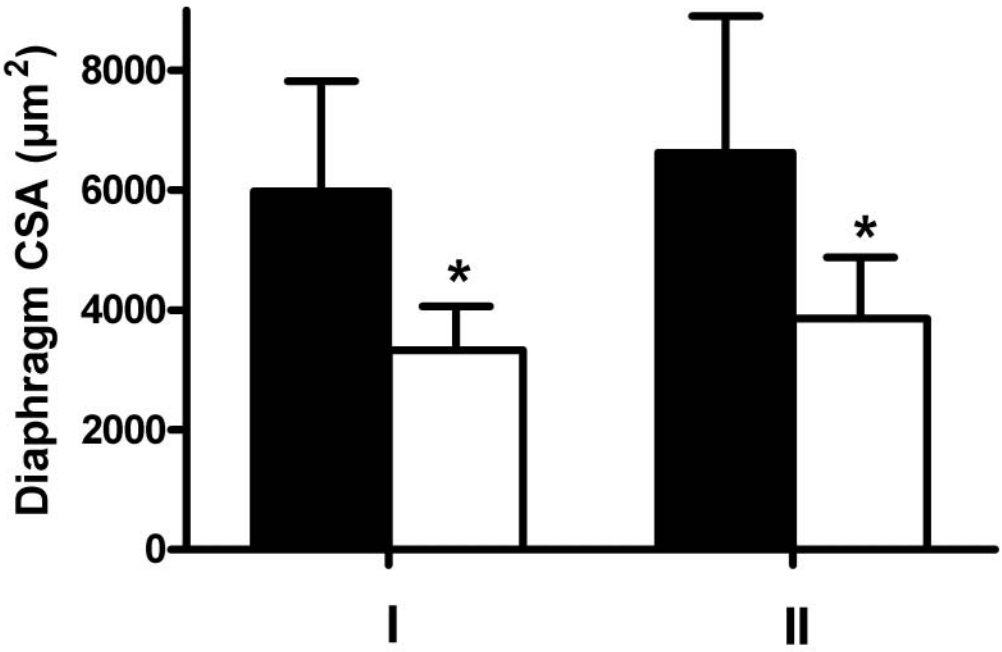


Figure 2: (A) MyoD mRNA (expressed per 10^6 18s copies) and (B) protein (relative to controls, means and standard deviation) expression in the diaphragm of controls and patients with COPD. For mRNA, each point represents an individual patient. Horizontal line represents the mean value. For MyoD mRNA expression: n=10 controls and n=11 COPD. For MyoD protein expression: n=9 controls and n=15 COPD. *p<0.05, **p<0.005

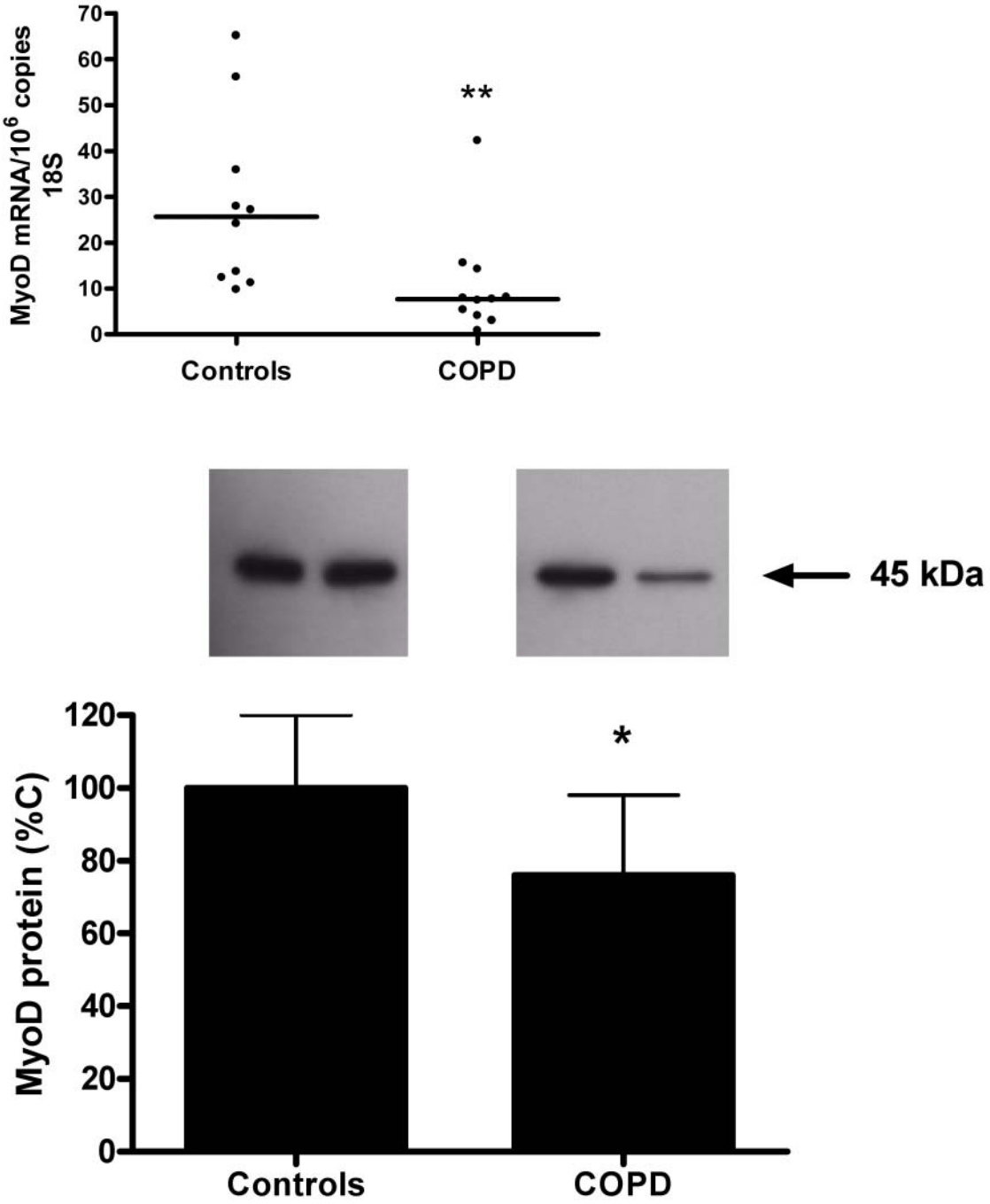


Figure 3: MAFbx mRNA expression (A) and Nedd4 mRNA expression (B) in the diaphragm of controls (n=10) and patients with COPD (n=11). mRNA is expressed per 10^6 18s copies. Each point represents an individual patient. Horizontal line represents the mean value. *p<0.05, **p<0.01

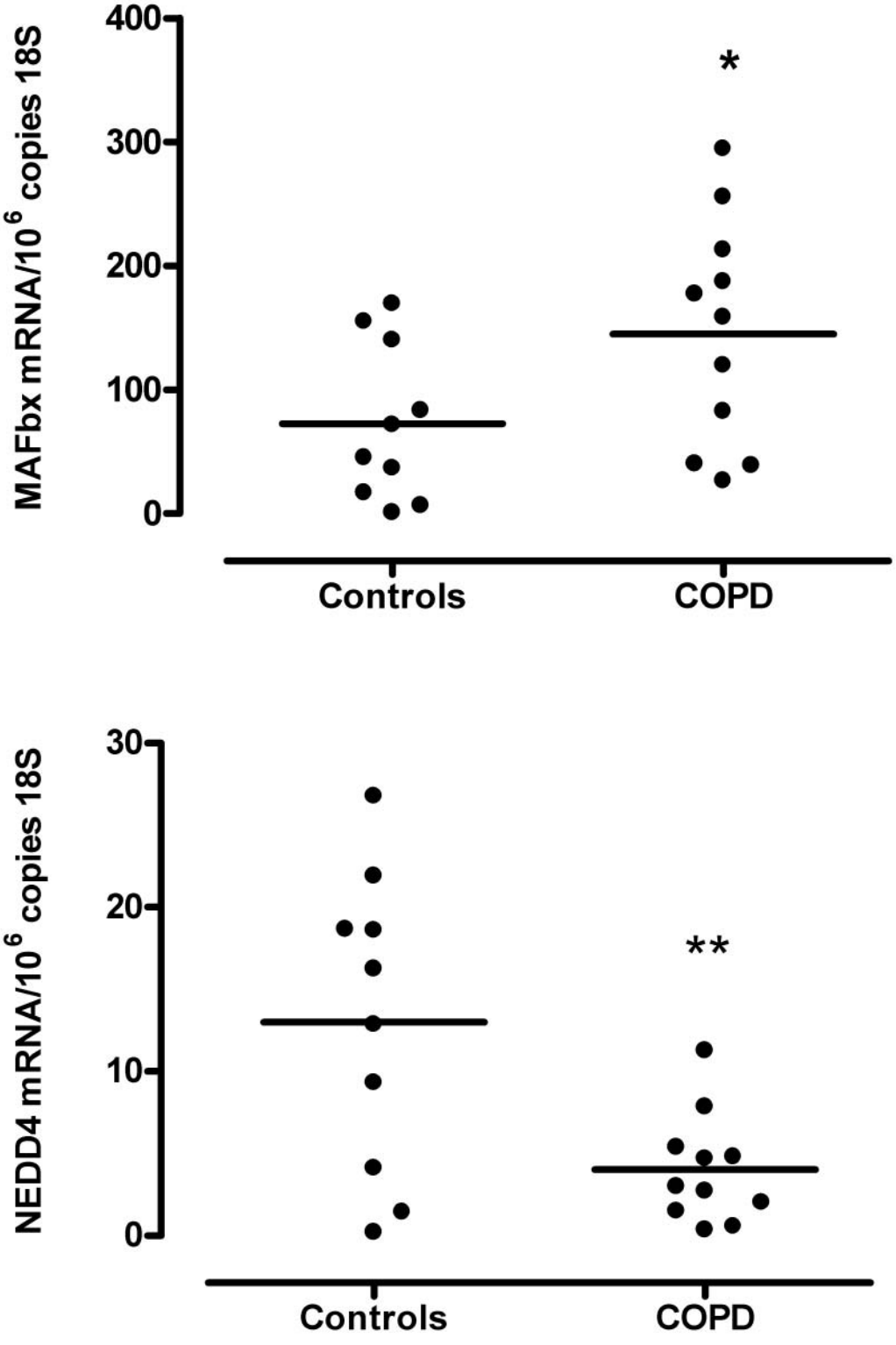
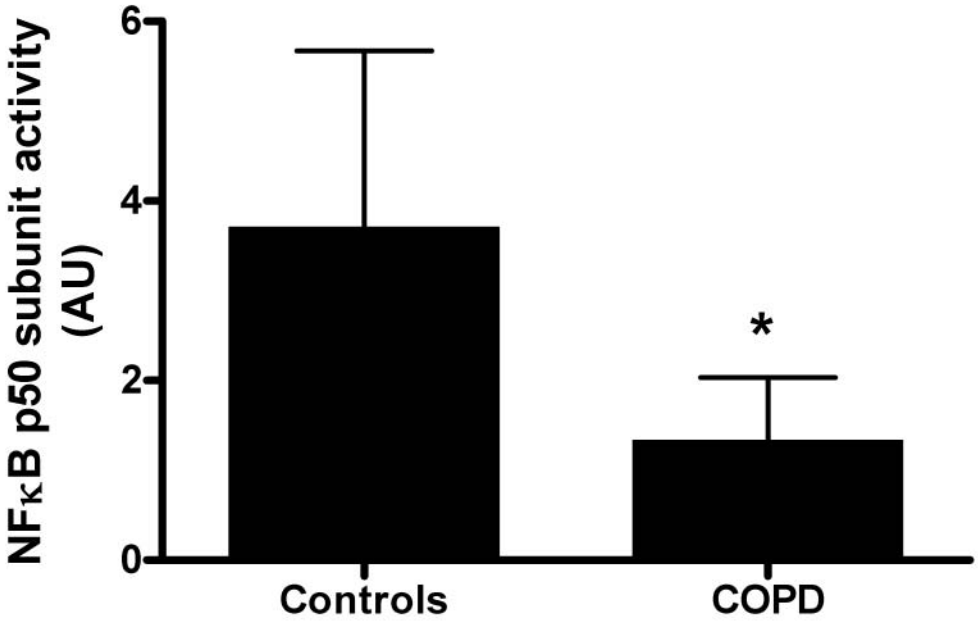
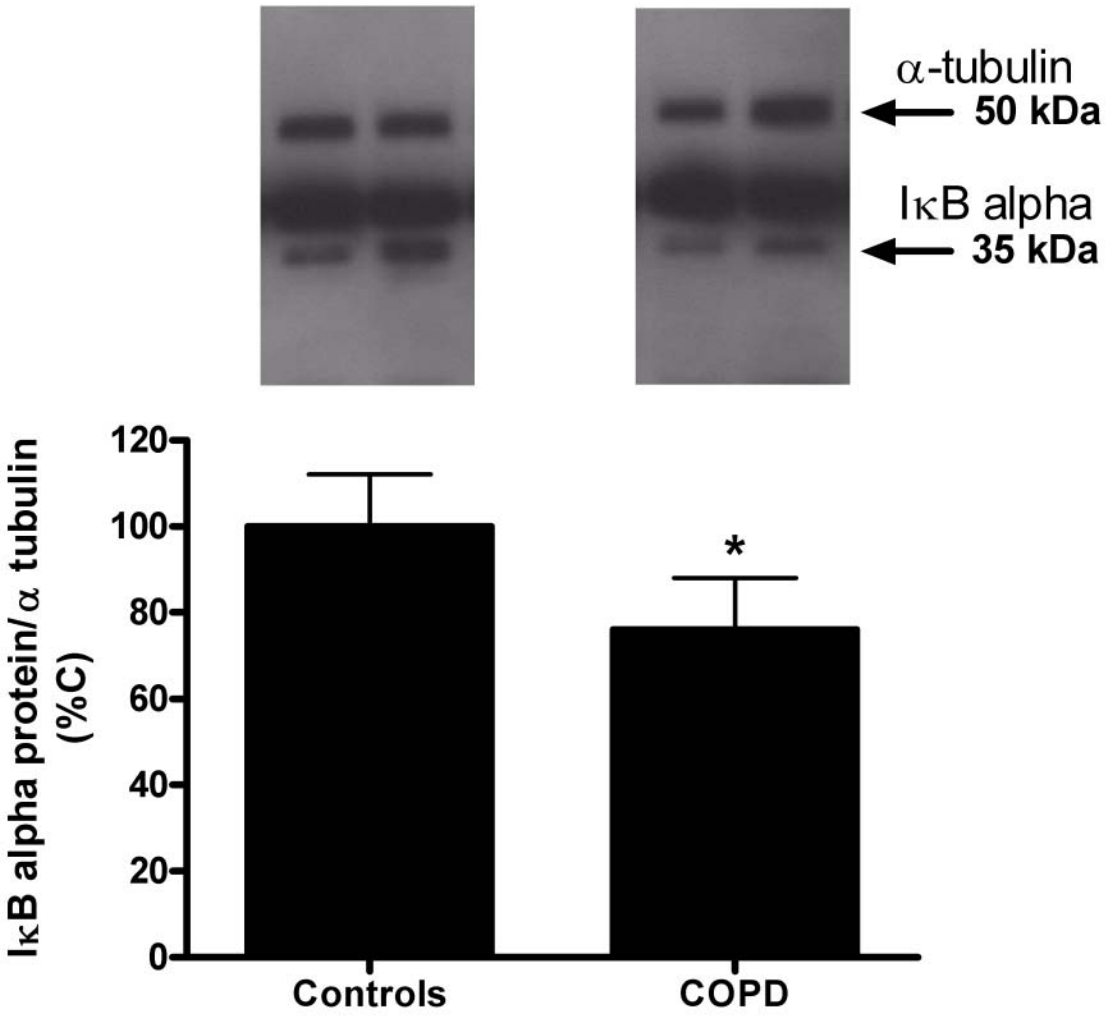


Figure 4: NF-κB p50 DNA-binding activity (A), protein expression of IκBα (B) and IκBβ (C) (normalized to α-tubulin and expressed relative to controls) in the diaphragm of controls (n=9)

and patients with COPD (n=15). Values represent mean and standard deviation. *p<0.01, ***p<0.001




α-tubulin
50 kDa
IκB alpha
35 kDa
IκB alpha protein/α tubulin (%C)
120
100
80
60
40
20
0
*
Controls
COPD

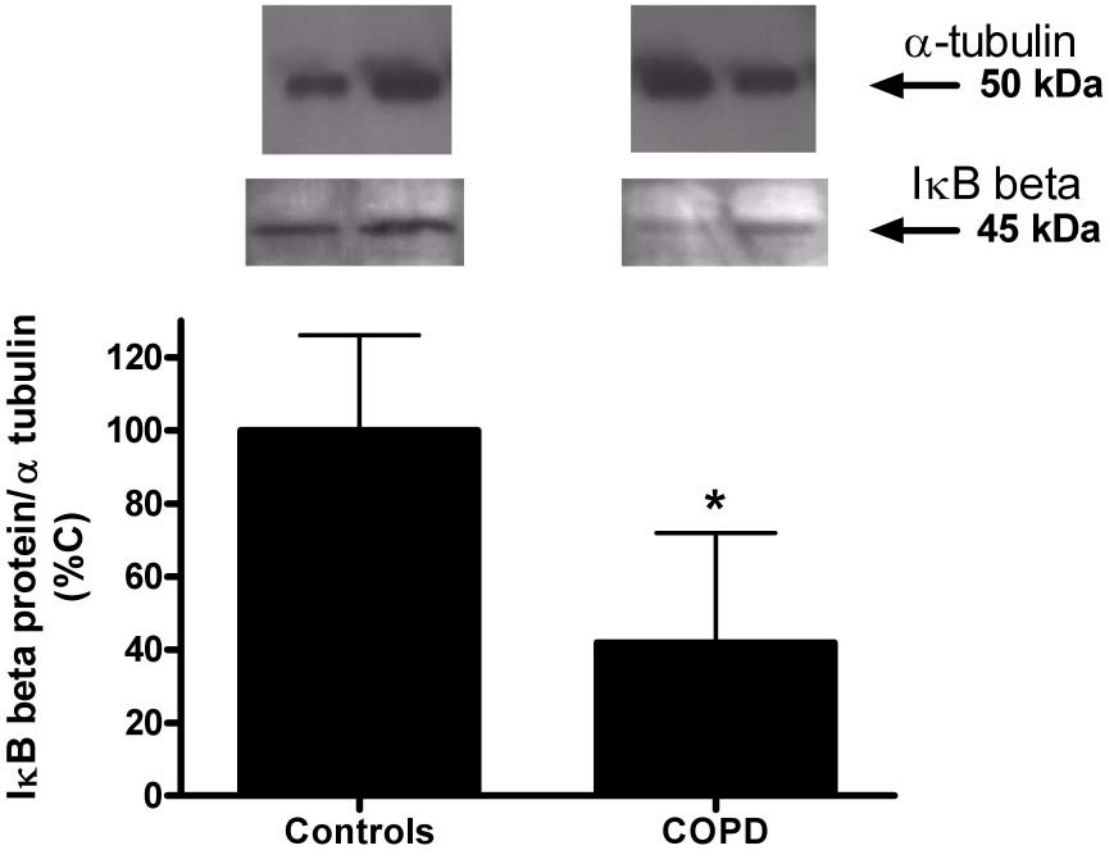
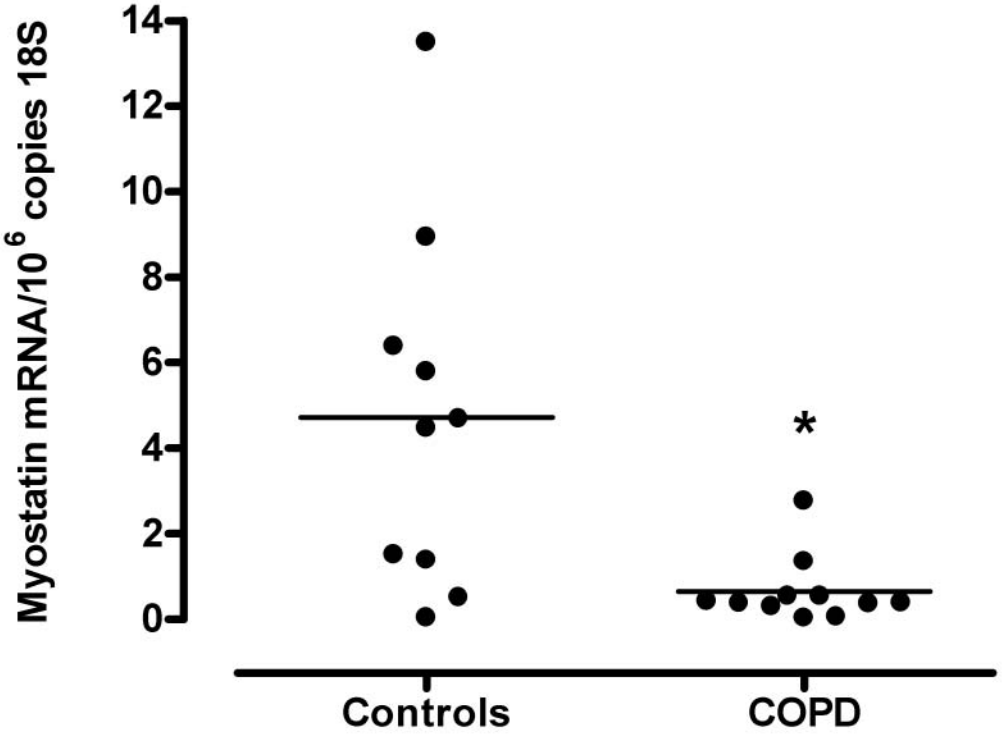
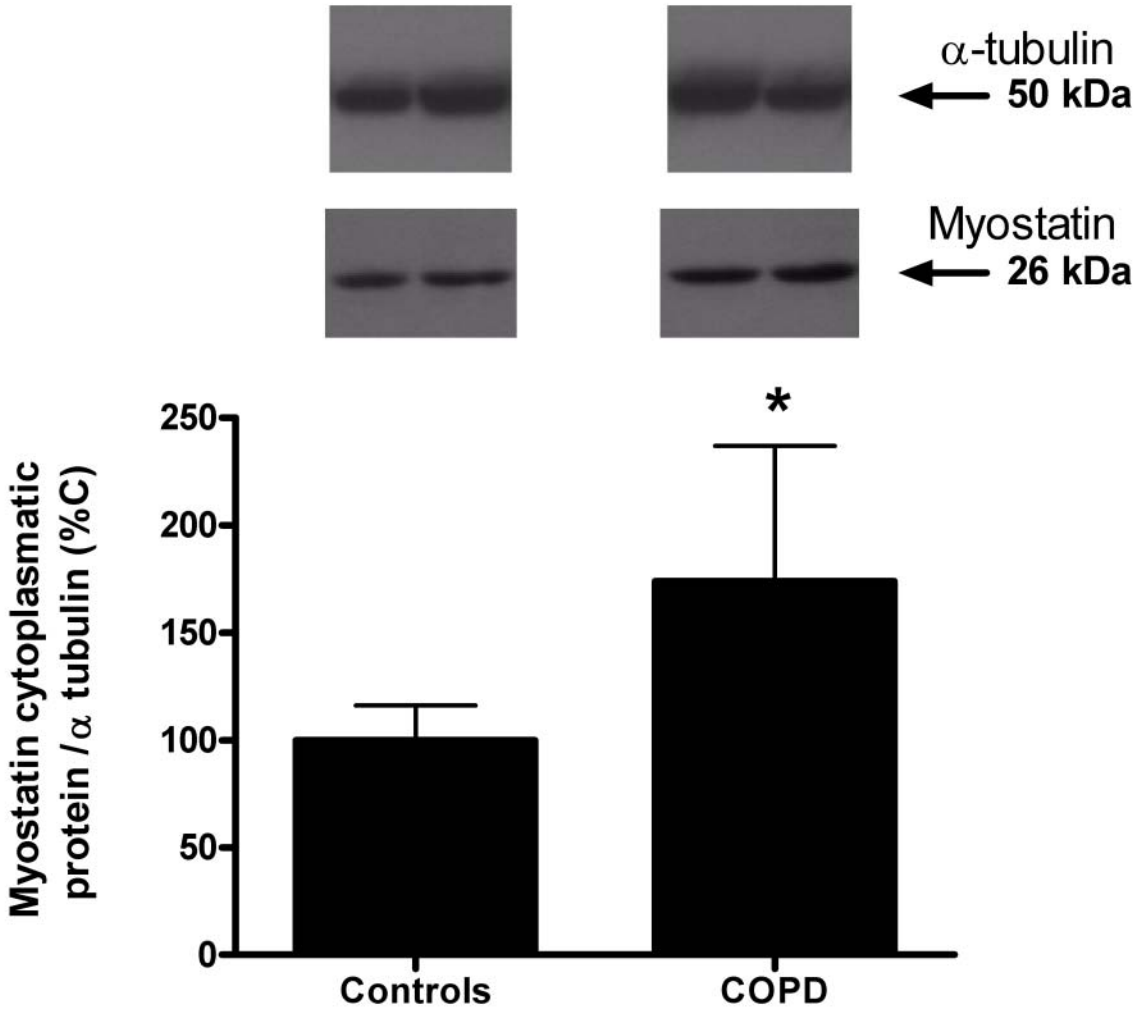


Figure 5: (A) Myostatin mRNA (expressed per 10^6 18S copies) and myostatin protein levels in nuclear (B) and cytoplasmic (C) fraction in the diaphragm of controls and patients with COPD. For mRNA (n=10 controls and n=11 COPD), each point represents an individual patient. Horizontal line represents the mean value. For protein (n=9 controls and n=15 COPD), values normalized to α-tubulin (cytoplasmic fraction) expressed relative to controls represent mean and standard deviation. *p<0.05


Myostatin mRNA/10^6 copies 18S
14
12
10
8
6
4
2
0
*
Controls
COPD


α-tubulin
50 kDa
Myostatin
26 kDa
Myostatin cytoplasmatic protein /α tubulin (%C)
250
200
150
100
50
0
*
Controls
COPD

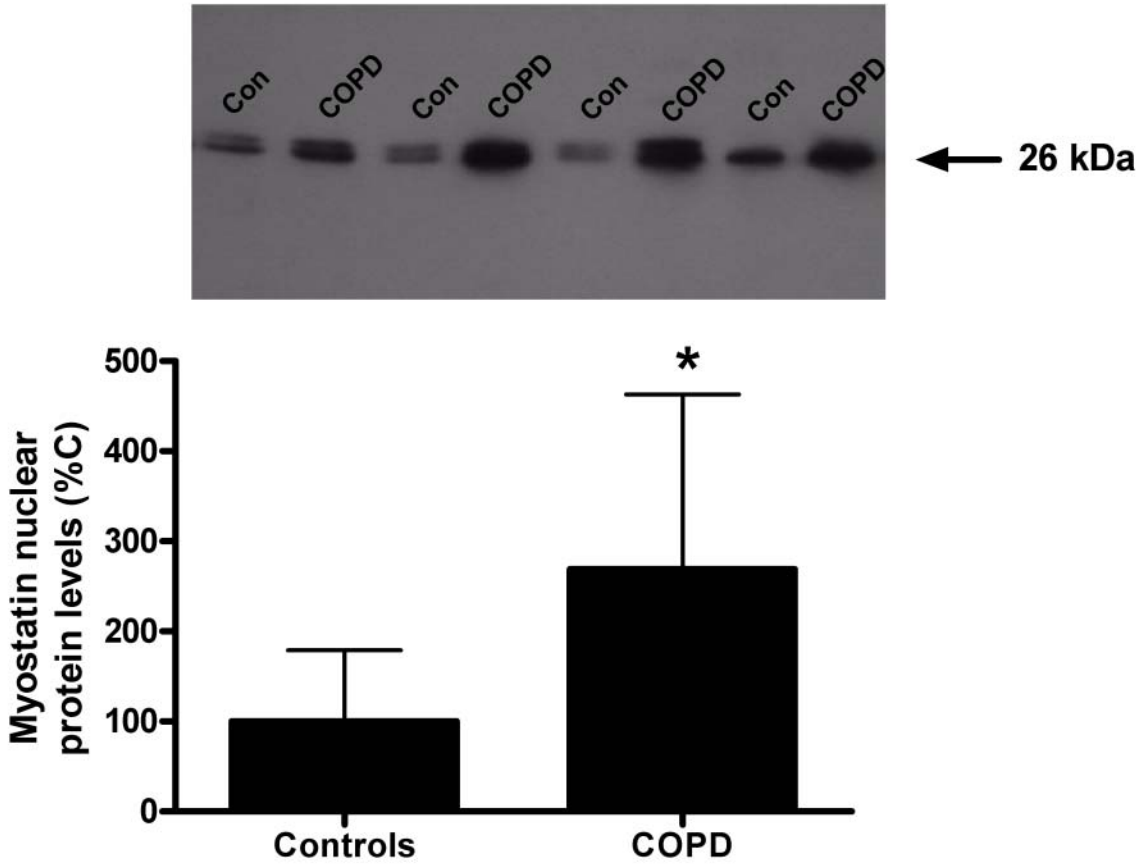

Con
COPD
Con
COPD
Con
COPD
Con
COPD
26 kDa
Myostatin nuclear protein levels (%C)
500
400
300
200
100
0
*
Controls
COPD

Table 1 Anthropometric characteristics and pulmonary function tests in the controls and in the patients with COPD

	Controls (n=13)	COPD (n=19)
Male/Female	**11/2**	**8/11**
Age (y)	**64 ± 3**	**58 ± 1**
Weight (kg)	**71 ± 3**	**58 ± 2***
BMI (kg/m²)	**25 ± 1**	**21 ± 1***
FEV_1 (L)	**2.67 ± 0.21**	**0.71 ± 0.05§**
FEV_1 (% predicted)	**98 ± 5**	**26 ± 2§**
FEV_1/FVC (%)	**76 ± 2**	**33 ± 2§**
TLC (% predicted)	**95 ± 6**	**126 ± 5+**
RV (% predicted)	**107 ± 9**	**227 ± 12++**
DL_{CO} (% predicted)	**83 ± 5**	**29 ± 2§**
K_{CO} (% predicted)	**105 ± 6**	**44 ± 3§**

BMI= body mass index; FEV_1 = forced expiratory volume in 1 sec; FVC = forced vital capacity; TLC = total lung capacity; RV = residual volume; DL_{CO} = diffusing capacity, K_{CO} = diffusion capacity corrected for alveolar ventilation. Values are mean ± SEM. *p<0.01, +p<0.001, ++p<0.00005, §p<0.0001

Table 2: Primer sequences used for real-time PCR

MAFbx	**Forward: 5' GTG GTA CTG AAA GTC CTT GAA GAC 3'** **Reverse: 5' TTA ATG TTC CCG ACC AGC A 3'**
MuRF1	**Forward: 5' GAA TAA CTG TAT CTC CAT GCT GG 3'** **Reverse: 5' GGC ATA CAA CGT GTC AAA CTT 3'**
MyoD	**Forward: 5' GAC GGC ATG ATG GAC TAC AG 3'** **Reverse: 5' AGG CAG TCT AGG CTC GAC AC 3'**
Myogenin	**Forward: 5' AGC GAA TGC AGC TCT CAC AG 3'** **Reverse: 5' AGG TTG TGG GCA TCT GTA GG 3'**
Nedd4	**Forward: 5' TTT GGA AAT TCA GCC GTG AG 3'** **Reverse: 5' CCT GGT GGT AAT CCA GAT GAA 3'**
Myostatin	**Forward: 5' TGT AAC CTT CCC AGG ACC AG 3'** **Reverse: 5' GGT AAC GAC AGC ATC GTG ATT 3'**