Myostatin/Smad2/Smad3 pathway define a differential clinical phenotype in COPD-associated sarcopenia

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In sarcopenic limb muscles of patients with COPD, myostatin/Smad2/Smad3 pathway was differentially activated from patients without sarcopenia and healthy controls with similar degree of severity of lung disease https://bit.ly/4g4YHdx

Cite this article as: Núñez-Robainas A, Guitart M, López-Postigo A, *et al*. Myostatin/Smad2/Smad3 pathway define a differential clinical phenotype in COPD-associated sarcopenia. *ERJ Open Res* 2025; 11: 00772-2024 [DOI: 10.1183/23120541.00772-2024].

Received: 31 July 2024
Accepted: 14 Nov 2024

Abstract

Background Sarcopenia, defined as the loss of muscle mass and function, represents one of the most relevant comorbidities in patients with COPD even at early stages. We hypothesised that sarcopenia defines a specific clinical phenotype in COPD irrespective of respiratory disease severity. Markers of myostatin/Smad2/Smad3 and IGF-1/PI3K/Akt may be differentially expressed in the vastus lateralis (VL) of patients with COPD-associated sarcopenia.

Methods In muscle specimens from VL, markers of the myostatin/Smad2/Smad3, Smad4 and IGF-1/PI3K/Akt pathways were evaluated (real-time PCR and immunoblotting) and correlations between clinical and biological variables of patients with sarcopenia (n=23), without sarcopenia (n=18) and healthy controls (n=13) were examined.

Results In the VL of sarcopenic COPD patients, expression levels of myostatin, Smad2/Smad3 and Smad4 increased compared with those in nonsarcopenic patients and healthy controls. In sarcopenic limb muscles of patients with COPD, the myostatin Smad2/Smad3 pathway was differentially activated from patients without sarcopenia and healthy controls. Among sarcopenic patients, myostatin and p-Smad3/Smad3 levels negatively correlated with fat-free mass index (r=−0.727, p=0.026 and r=−0.703, p=0.035, respectively), myostatin and Smad4 levels correlated with quadriceps strength (r=−0.886, p=0.003 and r=−0.431, p=0.040, respectively) and myostatin correlated with diffusion capacity (r=−0.781, p=0.022). Remarkable negative correlations were observed between clinical parameters related to body composition and quadriceps muscle strength and levels of the myostatin Smad2/Smad3 pathway, suggesting its implication in the process of muscle atrophy in COPD. IGF1 gene expression was also upregulated in the VL of sarcopenic patients.

Conclusion Collectively, these findings offer a potential therapeutic target in COPD-associated sarcopenia.

Introduction

COPD is a major respiratory condition that affects several organs and tissues other than the airways and lungs. Sarcopenia, defined as the loss of muscle mass and function [1, 2], represents one of the most relevant comorbidities in patients with COPD even during early stages of the disease [3, 4]. Exercise tolerance, daily life activities, quality of life and mortality are severely hindered by the presence of sarcopenia in these patients regardless of the severity of the respiratory disease [5, 6].

Multiple factors related to the clinical status of the patients and biological mechanisms intrinsic to muscle function, structure and metabolism are involved in the underlying pathophysiology of sarcopenia in

patients with COPD [7]. Sarcopenia, defined as a muscle disease characterised by poor muscle strength [8, 9], varies widely across different muscle groups. Lower limb muscles are usually more seriously affected than those of the upper limbs or the respiratory system [7]. Cellular and molecular mechanisms such as increased muscle damage, a slow-to-fast fibre type switch, systemic inflammation, oxidative stress, increased muscle catabolism and poor muscle regeneration ability are counted among the most relevant contributors to sarcopenia in COPD [7, 10].

Myostatin, a member of the transforming growth factor (TGF)-β family, is a secreted growth differentiation factor synthesised by the myofibers that inhibits muscle growth [11]. In experimental models myostatin has been shown to regulate muscle protein synthesis and catabolism [12–14]. In limb muscles of patients with COPD, discrepant results have been published as to the expression levels of myostatin [15–18] in several models and conditions. Follistatin, an activin-binding protein, is part of the inhibin–activin–follistatin axis that binds and neutralises members of the TGF-β superfamily, playing a relevant role in cellular proliferation and differentiation. Levels of follistatin gene expression were upregulated in the vastus lateralis (VL) of COPD patients with muscle weakness [19].

The transcription factors Smad2/Smad3 act downstream of myostatin to induce a program of muscle atrophy that is independent of E3 ligases of the ubiquitin–proteasome system. Inhibition of Smad2/Smad3 and Smad4 favours muscle hypertrophy independent of satellite cell activation. The pathway of insulin-like growth factor (IGF)-1/phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt) controls protein synthesis and muscle hypertrophy in adults. Different polymorphisms of IGF1 are related to respiratory muscle strength in COPD patients [20]. Whether the expression of the different molecules involved in this pathway differs in limb muscles of patients with COPD-associated sarcopenia, independently of disease severity or lung function, remains to be fully elucidated.

We hypothesised that a differential clinical phenotype of patients with COPD and sarcopenia may be defined irrespective of the status of the respiratory disease. Moreover, such a distinct clinical phenotype may correlate well with the expression of the different markers of the myostatin/Smad2/Smad3 and IGF-1/PI3K/Akt pathways in the limb muscles of COPD patients with sarcopenia. In the VL of patients with COPD-associated sarcopenia, defined as a decrease in muscle strength, compared with a group of nonsarcopenic COPD patients with a similar respiratory disease severity and healthy controls, the following objectives were formulated: 1) to analyse the myostatin/Smad2/Smad3 and Smad4 pathway; 2) to examine the levels of the IGF-1/PI3K/Akt pathway; and 3) to evaluate potential correlations between expression levels of the different molecules and parameters of the clinical phenotypes.

Methods

Study design, inclusion and exclusion criteria

Please see specific details in the online data supplement.

This was a case–control investigation, in which the STROBE guidelines were followed. 41 patients (22 males and 19 females) with stable COPD [21] and 13-age-matched control subjects (6 males and 7 females) were recruited in the Pneumology Department at Hospital del Mar (Barcelona, Spain). COPD patients were further divided into two groups: 1) those with normal muscle mass and strength (nonsarcopenic COPD, n=18, 13 males and 5 females); and 2) those with sarcopenia (reduced muscle mass and strength, sarcopenic COPD, n=23, 9 males and 14 females) following the muscle wasting and sarcopenia international consensus criteria and previous studies [8, 9]. A fragment of the muscle samples from the patients and healthy controls was also used in a previous investigation that aimed to assess the muscle regeneration potential of patients with COPD-associated sarcopenia [21].

Muscle mass loss was identified by fat-free mass index (FFMI)$\leqslant$18 kg·m^{-2}, an established cut-off value for a Mediterranean population following previously published criteria and the international definition of sarcopenia [2, 21]. Based on previous investigations, muscle weakness (reduced muscle strength) was defined as an approximate 25% reduction of the quadriceps force when compared with control subjects [22]. Control subjects recruited from the general population were never-smokers with a sedentary lifestyle, whereas patients were ex-smokers or active smokers. The BODE index (body mass index (BMI), airflow obstruction, dyspnoea and exercise) was assessed in all patients. All COPD patients were clinically stable without exacerbations at least 3 months prior to study entry. Additionally, patients were on inhaled bronchodilators and did not present any significant comorbidities. All participants in the study were white.

Exclusion criteria were: severe exacerbations in the last 3 months, chronic respiratory diseases other than COPD, chronic metabolic diseases, chronic cardiovascular disorders, osteoarticular diseases, myopathic or

paraneoplastic syndromes and/or drug treatments, including oral corticosteroids, all of which are known to affect muscle structure and/or function.

All recruited individuals were qualified as sedentary. The following criteria were used to define sedentarism: 1) level of engagement of the subjects in activities such as walking and running is no more than five times per week; 2) the execution of physical activity during free time is minimal; 3) the time dedicated to performing physical activities that build endurance is less than 3 h per week; and 4) the performance of moderate and high-intensity activity requires less than 10% of the daily energy [23]. Additionally, other behaviours, including the time spent watching television, sleeping and sitting were also considered in the assessment of the subject's sedentarism [23].

Ethics approval

The present investigation was designed for research on human beings following the ethical values on human research in our institute and the World Medical Association guidelines (Seventh Revision of the Declaration of Helsinki, Fortaleza, Brazil, 2013) [24]. The institutional Ethics Committee on Human Investigation approved this investigation (Hospital del Mar-IMIM, Barcelona, project number 2018/7937/I). All the recruited subjects signed an informed written consent prior to study entry.

Nutritional and functional parameters

Nutritional evaluation included BMI and FFMI determination by means of bioelectrical impedance [25]. The criteria used for the diagnosis of sarcopenia were: 1) BMI<21 $kg \cdot m^{-2}$ and 2) FFMI<18 $kg \cdot m^{-2}$ in all patients [15, 25]. The reference values of spirometry, diffusion capacity, static lung volumes and blood gases were used in the present study [26]. The quadriceps muscle strength of all participants was evaluated by the isometric maximum voluntary contraction (QMVC) of the dominant lower limb as previously described [21]. To do so, patients were seated on an exercise platform with both their trunk and thigh fixed on a rigid support (Domyos HGH 050; Decathlon). QMVC was defined as the highest value from three brief reproducible manoeuvres (<5% variability among them). The evaluation of nutritional parameters was also determined through conventional blood tests.

Exercise capacity and physical activity

The 6-min walking distance was used to assess the exercise capacity of all subjects as previously reported [27]. The test consisted of walking a 30-m corridor twice with at least a 30-min rest between them. During the test, encouragement was given every minute and if exhaustion signs appeared, the test was interrupted. To measure the levels of leg discomfort and dyspnoea, an adapted Borg scale was used. To assess the maximum exercise capacity, measurements with a standardised incremental cycle ergometer were collected [27, 28]. To obtain pulmonary gas exchange and ventilatory values, signals from gas analysers and mass flow sensors previously calibrated were used. Additionally, during each respiration rates of oxygen uptake (V'_{O_2}), minute ventilation (V'_E), pulmonary carbon dioxide output (V'_{CO_2}) and respiratory exchange ratio were also recorded. A 10-lead online ECG was used to measure heart rates, while oxygen saturation was determined by pulse oximetry (S_{pO_2}). After 1 min of breathing at repose, all candidates were asked to pedal on an electrically braked cycle ergometer (Ergoline Ergometrix 900; Uberprüfung) [27, 28]. During this test, cardiorespiratory variables were recorded in an integrated computer (Ultima; MedGraphics Corporation). Subjects were always motivated to remain exercising until they were unable to tolerate the pedalling goal.

Physical activity was assessed using the International Physical Activity Questionnaire (IPAQ) (table 1) [29, 30]. Accelerometers (BodyMedia, SenseWear, Display DD100, Pittsburgh, PA, USA) were worn for 7 consecutive days, including night sleep. Activity levels were established as: 1) high level: 1500–3000 metabolic equivalent of task (MET); 2) moderate level: 1500–600 MET; and 3) low level: <600 MET (table 1).

Blood and muscle specimens

Blood samples were extracted from all individuals after fasting for one night, in the previous hour to the surgery [21]. All subjects had to rest for 1 h on a chair with their legs half flexed before the biopsy. VL samples were obtained from the quadriceps muscles (50–100 mg average weight) using the open biopsy technique [21] and were rinsed with saline solution to eliminate possible blood contamination. Muscle specimens were frozen in liquid nitrogen and stored at −80°C (under controlled supervision) for further analyses. Frozen muscle samples were used for messenger RNA (mRNA) and protein expression analysis.

TABLE 1 Physical activity measurement tools

Tool	Variables	MET score
IPAQ	1) Walking (3.3 MET × min × week)	High: 1500–3000
	2) Moderate physical activity (4 MET × min × week)	Moderate: 600–1500
	3) Vigorous physical activity (8 MET × min × week)	Low: <600
	4) Total (walking+moderate+vigorous)	
	5) Sitting time, min	
Accelerometer	1) Physical activity level	
	2) Steps·day^{-1}	
	3) Activity energy expenditure, kcal	
	4) Basal metabolic rate, kcal	

IPAQ: International Physical Activity Questionnaire; MET: metabolic equivalent of task. According to the IPAQ scoring protocol, a given MET value was multiplied by the number of minutes and by the number of days the activity was performed, with a value of 3.3 for walking, 4 for moderate physical activity and 8 for vigorous physical activity.

Biological analyses

RNA isolation

A total of 50 mg of VL muscle samples were diluted in 1 mL Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and homogenised with a T10 basic Ultra-Turrax Polytron (IKA-Werke GmbH & Co. KG) following previously published methodologies [31, 32].

mRNA reverse transcription

A total of 100 ng mRNA was used to generate cDNA with an oligonucleotide (dT)12-18 primer, dithiothreitol, dNTP mix and Super-Script III reverse transcriptase following the producer's guidelines (Thermo Fisher Scientific, Waltham, MA, USA) and previously published procedures [31, 32]. In these experiments, the entire population of patients and controls were analysed.

Real-time PCR

Real-time PCR assays were carried out through the QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) following previously published procedures (table 2) [32, 33].

Immunoblotting

Protein levels of the target antigens were analysed using immunoblotting following previously published procedures [34]. For technical reasons of the 10-well gels, only 9 subjects per group were analysed.

Statistical analysis

Sample size was calculated using the software G*Power 3.1.9.4 (HHU, Düsseldorf, Germany) [35] with both myostatin protein content and gene expression as the outcome variables, accepting an α risk of 0.05 and a β risk of 0.2 (80% power). A minimum of 9 and 12 patients in each group, respectively, was required to achieve a statistical power of 91% and 89% (effect size f=1.597457 and f=1.228391, respectively). Specifically, in this study, a final statistical power of 99% was achieved.

The Shapiro–Wilk test was used to assess the normality of the distribution of the study variables. Results are expressed as mean±SD. To analyse differences in quantitative variables between study groups, an ANOVA with Tukey's *post hoc* analysis was carried out or a t-test for comparisons between two groups. To assess differences in qualitative variables, a chi-squared test was performed. A multivariate linear regression analysis was used to examine potential associations between clinical and biological variables using the Pearson's correlation coefficient. Correlations were assessed only among the sarcopenic COPD patients. Correlations are displayed in graphical correlation matrices, obtained from the R package corrplot (https://cran.r-project.org/web/packages/corrplot/index.html), in two different colours: blue for positive correlations and red for negative. The correlation coefficients are depicted in the ruler located in the right-hand side of the corrplot. Furthermore, significant correlations are also represented individually in scatter-plots in the online supplement. Specific p-values are stated in all figures and the statistical significance was considered when $p \leqslant 0.05$.

TABLE 2 Gene names and ID used for real-time PCR analysis

Gene symbol	Assay ID	GenBank accession number
MSTN	Hs00976237_m1	NM_005259.2
INHBA	Hs01081598_m1	NM_002192.2
ACVR2A	Hs00155658_m1	NM_001278579.1
		NM_001278580.1
		NM_001616.4
ACVR2B	Hs00609603_m1	NM_001106.3
SMAD2	Hs00183425_m1	NM_001003652.3
		NM_001135937.2
		NM_005901.5
SMAD3	Hs00969210_m1	NM_001145102.1
		NM_001145103.1
		NM_001145104.1
		NM_005902.3
SMAD4	Hs00929647_m1	NM_005359.5
AKT1	Hs00178289_m1	NM_001014431.1
		NM_001014432.1
		NM_005163.2
PI3KR1	Hs00933163_m1	NM_001242466.1
		NM_181504.3
		NM_181523.2
		NM_181524.1
IGF1	Hs01547656_m1	NM_000618.4
		NM_001111283.2
		NM_001111284.1
FST	Hs00246256_m1	NM_006350.3
		NM_013409.2
GAPDH	Hs99999905_m1	NM_001289746.1
		NM_002046.5

ID: identification; Hs: *Homo sapiens*; m1: multi-exonic gene assay does not detect genomic DNA; NM: mRNA reference sequence category; MSTN: myostatin; INHBA: activin β-A chain; ACVR2A: activin A receptor type 2A; ACVR2B: activin A receptor type 2B; SMAD2: Smad family member 2; SMAD3: Smad family member 3; SMAD4: Smad family member 4; AKT1: Akt serine/threonine kinase 1; PI3KR1: phosphoinositide-3-kinase regulatory subunit 1; IGF1: insulin-like growth factor 1; FST: follistatin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Results

Clinical features of COPD patients and healthy controls

As expected, in sarcopenic COPD patients, body composition parameters such as BMI, fat-free mass (FFM) and FFMI were significantly reduced compared with healthy controls and nonsarcopenic COPD patients (table 3). Smoking history (pack-years) was similar for both groups of patients and the healthy controls were all never-smokers (table 3). Compared with controls, all patients exhibited a severe airway obstruction and decreased diffusion capacity, and air trapping was significantly higher in the sarcopenic than in nonsarcopenic patients and healthy controls (table 3). Patients did not show hypoxaemia or hypercapnia (table 3). BODE scores were similar between the two groups of patients (table 3). In both groups of COPD patients, exercise tolerance and physical activity (physical activity level and steps·d^{-1}) significantly declined compared with healthy controls (table 3). Baseline metabolic rates significantly decreased in sarcopenic COPD patients compared with both control subjects and nonsarcopenic COPD patients (table 3). Quadriceps muscle strength, but not handgrip strength, significantly decreased in the sarcopenic COPD patients compared with healthy subjects and nonsarcopenic patients (table 3). Nutritional blood parameters did not significantly vary across groups (table 3). The inflammatory parameter fibrinogen was significantly greater in both groups of patients than in healthy controls (table 3).

Myostatin upregulation in muscles of COPD patients

Gene expression levels were upregulated in the VL of both groups of COPD patients, whereas protein levels only increased in the sarcopenic patients compared with healthy controls and nonsarcopenic patients (figure 1a,b). Among sarcopenic patients, myostatin protein levels were negatively associated with FFMI, lung (carbon monoxide diffusion capacity) and quadriceps muscle functions (figure 2 and supplementary figure S1).

TABLE 3 Clinical characteristics of the study subjects

	Control subjects, n=13	COPD patients	
		Nonsarcopenic, n=18	Sarcopenic, n=23
Anthropometry			
Age, years	66±6	64±6	62±7
Males/females	6/7	13/5	9/14
BMI, kg·m^{-2}	27±4	28±2	19±3***, ###
FFM, kg	47±12	48±7	37±4**, ###
FFMI, kg·m^{-2}	17±2	18±1	14±1***, ###
Smoking history			
Active	0 (0)	14 (78)	15 (65)
Ex-smoker	0 (0)	4 (22)	8 (35)
Never smoker	13 (100)	0 (0)	0 (0)
Pack-years	0±0	60±29***	55±24***
Lung function			
FEV_1, % predicted	97±14	36±11***	34±11***
FVC, % predicted	98±12	67±16***	69±15***
FEV_1/FVC	76±6	42±11***	38±12***
RV, % predicted	112±14	185±43***	222±53***, #
TLC, % predicted	97±7	108±14	121±19***, #
RV/TLC	43±9	61±9***	65±8***
D_{LCO}, % predicted	92±6	46±16***	34±14***
K_{CO}, % predicted	92±19	48±16***	39±14***
P_{aO_2}, kPa	NA	9.6±2.1	9.3±1.7
P_{aCO_2}, kPa	NA	5.6±0.5	5.7±0.9
BODE index			
Mild (0–2)	NA	4 (22)	0 (0)
Moderate (3–4)	NA	6 (33)	9 (39)
Severe (5–6)	NA	5 (28)	10 (43)
Very severe (7–10)	NA	3 (17)	4 (17)
Physical activity			
Walking, MET	NA	1523±2295	1734±1708
Moderate, MET	NA	637±1825	265±442
Vigorous, MET	NA	1182±3981	435±1143
Total	NA	3342±5749	2434±2481
Sitting time, min	NA	315±157	426±197
Physical activity level	1.72±0.21	1.48±0.16**	1.41±0.21***
Steps·d^{-1}	10 248±3907	5409±4056**	4325±2866***
AEE, kcal	819±264	738±593	794±819
BMR, kcal	1462±262	1478±149	1231±75**, ###
Exercise capacity			
V'_{O_2} max, % predicted	120±32	59±18***	55±23**
6MWT, m	523±55	424±79**	442±67***
6MWT, % predicted	106±15	87±15	82±14
Muscle function			
ND-HGS, kg	29±3.1	29±3.2	25±1.8
ND-QMVC, kg	42±13	40±12	31±8*, #
QMVC, kg/FFM, kg	0.9±0.1	0.8±0.2	0.8±0.2
Blood parameters			
Albumin, g·dL^{-1}	4.6±0.2	4.5±0.3	4.6±0.2
Total proteins, g·dL^{-1}	7.1±0.3	7.0±0.5	7.0±0.6
CRP, mg·dL^{-1}	0.3±0.3	0.5±0.3	0.3±0.3
Fibrinogen, mg·dL^{-1}	355±69	475±91***	423±77*
ESR, mm·h^{-1}	10±7	16±13	10±12

Data are presented as mean±SD or n (%). BMI: body mass index; FFM: fat-free mass; FFMI: fat-free mass index; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; RV: residual volume; TLC: total lung capacity; D_{LCO}: carbon monoxide diffusion capacity; K_{CO}: transfer coefficient of the lung for carbon monoxide; P_{aO_2}: arterial oxygen partial pressure; P_{aCO_2}: arterial carbon dioxide partial pressure; MET: metabolic equivalent of task; AEE: activity energy expenditure; BMR: baseline metabolic rate; V'_{O_2} max: peak exercise oxygen uptake; 6MWT: 6-min walk test; ND: nondominant; HGS: handgrip strength; QMVC: quadriceps maximum voluntary contraction; BODE: body mass index, airflow obstruction, dyspnoea and exercise; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; NA: not applicable. *: p<0.05; **: p<0.01; ***: p<0.001 between any of the groups of COPD patients and the healthy controls; #: p<0.05; ###: p<0.001 between sarcopenic and nonsarcopenic COPD patients.

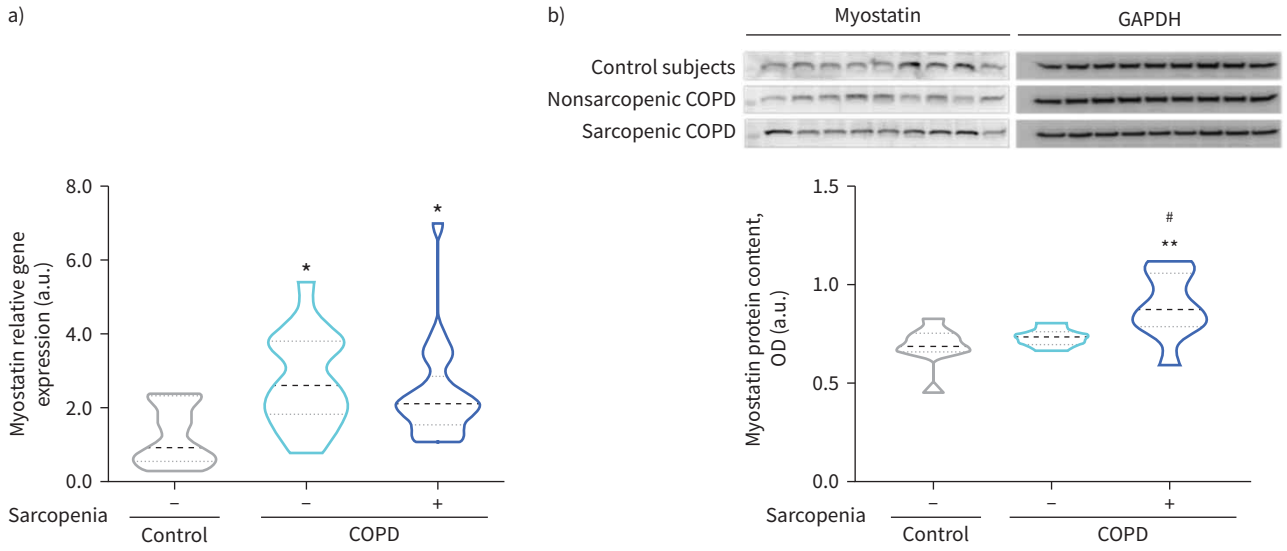

FIGURE 1 a) Violin plots with median (dashed line), first and third quartiles (dotted lines), and maximum and minimum values of myostatin relative gene expression (a.u.) in the vastus lateralis (VL) muscle of all study groups (n=13 control, n=18 nonsarcopenic COPD, n=23 sarcopenic COPD). b) Immunoblots and violin plots with median (dashed line), first and third quartiles (dotted lines) and maximum and minimum values of myostatin protein content (optical density (OD), a.u.) in the VL muscle of all study groups (n=9 per group). a.u.: arbitrary units; GAPDH: glyceraldehyde-3-phosphate dehydrogenase. *: p⩽0.05, **: p⩽0.01 for comparisons between either of the two groups of patients and the control subjects; #: p⩽0.05 for comparisons between nonsarcopenic and sarcopenic COPD patients.

Smad2/Smad3 and Smad4 transcription factors in muscles of COPD patients

Muscle Smad2 gene or protein expression did not significantly differ between patients and controls (figure 3a,b). However, a significant rise in levels of p-Smad2/Smad2 were seen in limb muscles of sarcopenic COPD patients compared with healthy subjects and nonsarcopenic patients (figure 3b). Compared with controls, Smad3 gene expression was downregulated in the VL of both nonsarcopenic and sarcopenic COPD patients, whereas no significant differences were observed in Smad3 protein expression (figure 3c,d). Protein levels of p-Smad3/Smad3 significantly increased only in muscles of the sarcopenic patients (figure 3d). Inverse correlations were observed between p-Smad2/Smad2 and p-Smad3/Smad3 ratio levels in muscles of sarcopenic patients and the clinical parameters K_{CO} (carbon monoxide transfer coefficient) and FFMI (figure 2 and supplementary figure S2, respectively), and between Smad2 gene expression level and both total lung capacity (TLC) and quadriceps muscle strength (figure 2 and supplementary figure S3, respectively). Moreover, p-Smad2 protein levels positively correlated with BODE index and residual volume (RV)/TLC, whereas it was negatively associated with forced expiratory volume in 1 s (FEV_1) (figure 2 and supplementary figure S4). Smad4 gene expression levels did not significantly differ across study groups (figure 4a). Nonetheless, Smad4 protein levels significantly increased in limb muscles of sarcopenic COPD patients compared with healthy controls (figure 4b). In patients, protein levels of Smad4 inversely correlated with muscle function parameters (figure 2).

Activin and follistatin expression in muscles of COPD patients

Compared with healthy controls, gene expression levels of activin A were downregulated in the VL of nonsarcopenic and sarcopenic patients (figure 5a). Gene expression levels of follistatin, however, were upregulated in the latter group compared with healthy subjects and nonsarcopenic patients (figure 5b). No significant differences were detected in the gene expression of either activin receptors 2A or 2B in muscles across the study groups (figure 5c,d).

Protein synthesis in muscles of COPD patients

IGF1 gene expression was upregulated in the limb muscles of sarcopenic patients compared with both nonsarcopenic COPD and control subjects (figure 6a). Gene expression of PI3KR1 or Akt did not vary across the study groups (figure 6b,c).

Discussion

In the current investigation, smoking history, disease severity (BODE), exercise capacity and lung function were significantly impaired in both groups of COPD patients, particularly in the sarcopenic patients (air

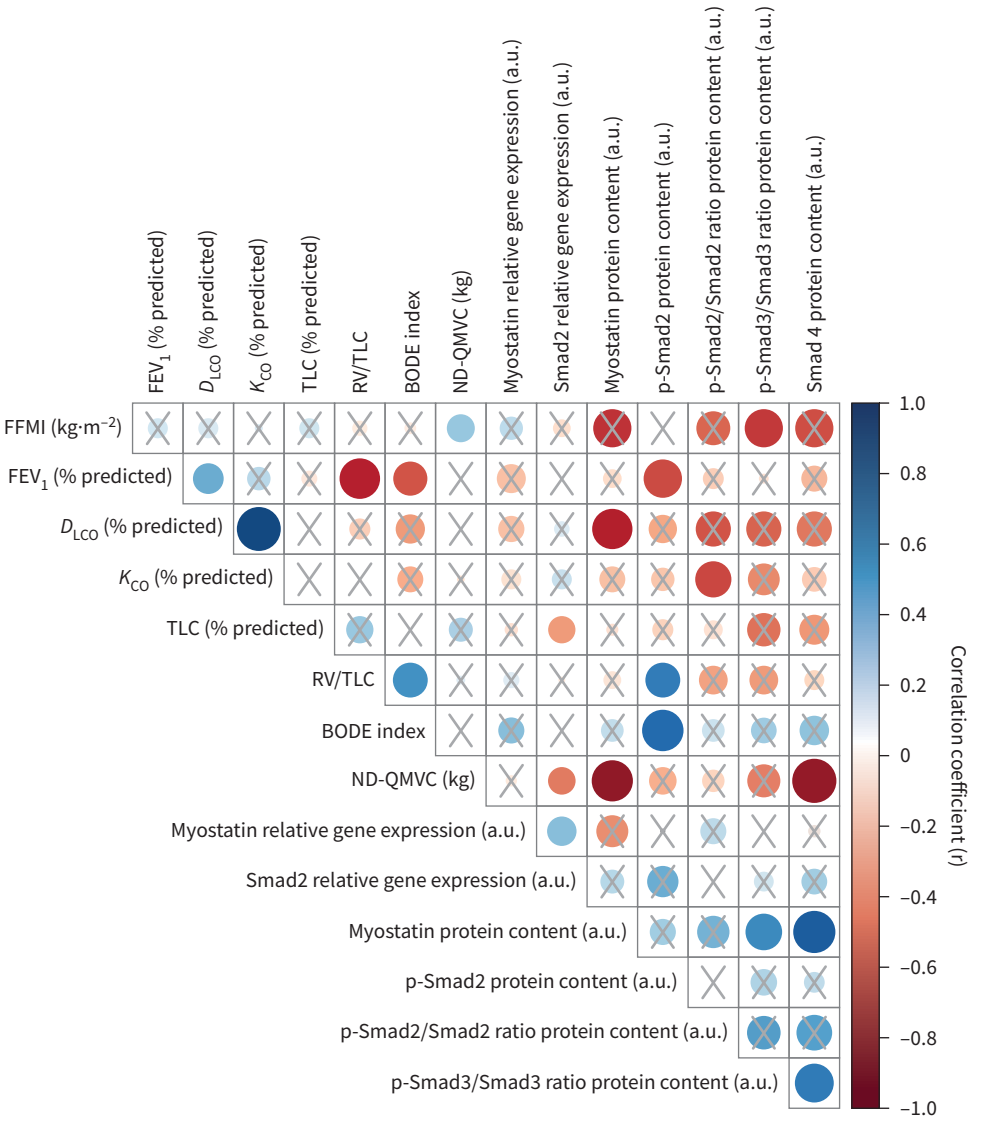

FIGURE 2 Correlation matrix of significant correlations between clinical and biological parameters in sarcopenic COPD patients. FFMI: fat-free mass index; FEV_1: forced expiratory volume in 1 s; RV: residual volume; TLC: total lung capacity; BODE: body mass index, airflow obstruction, dyspnoea and exercise; ND: nondominant; QMVC: quadriceps maximum voluntary contraction; Smad2: Smad family member 2; p-Smad2: phosphorylated Smad family member 2; Smad3: Smad family member 3; p-Smad3: phosphorylated Smad family member 3; Smad4: Smad family member 4; a.u.: arbitrary units. Significant correlations are described in the Results section. Moreover, correlations with no clinical or biological meaning such as carbon monoxide diffusion capacity (D_{LCO}) and carbon monoxide transfer coefficient (K_{CO}) were also encountered. In the matrix, blue represents positive correlations, while red represents negative correlations. Non-statistically significant correlations ($p>0.05$) are indicated by crosses within circles. The correlation coefficient is in the y axis of the graph, and is proportional to the colour intensity and size of the circle.

trapping). Nonetheless, a significant decline was observed in body composition and quadriceps muscle strength in sarcopenic patients, defining a very specific clinical phenotype. In the limb muscles of sarcopenic patients, several relevant biological events were differentially expressed. Protein levels of the growth differentiation factor myostatin significantly increased in the VL of sarcopenic COPD patients compared with both nonsarcopenic and control subjects. These findings are aligned with those disclosed previously [21, 36], whereas other reports showed no significant differences in myostatin levels in muscles of COPD patients [16, 37]. Several aspects of the clinical phenotypes or study design may account for the discrepant results among studies [16, 37–39]. Gene expression levels were also greater in the VL of

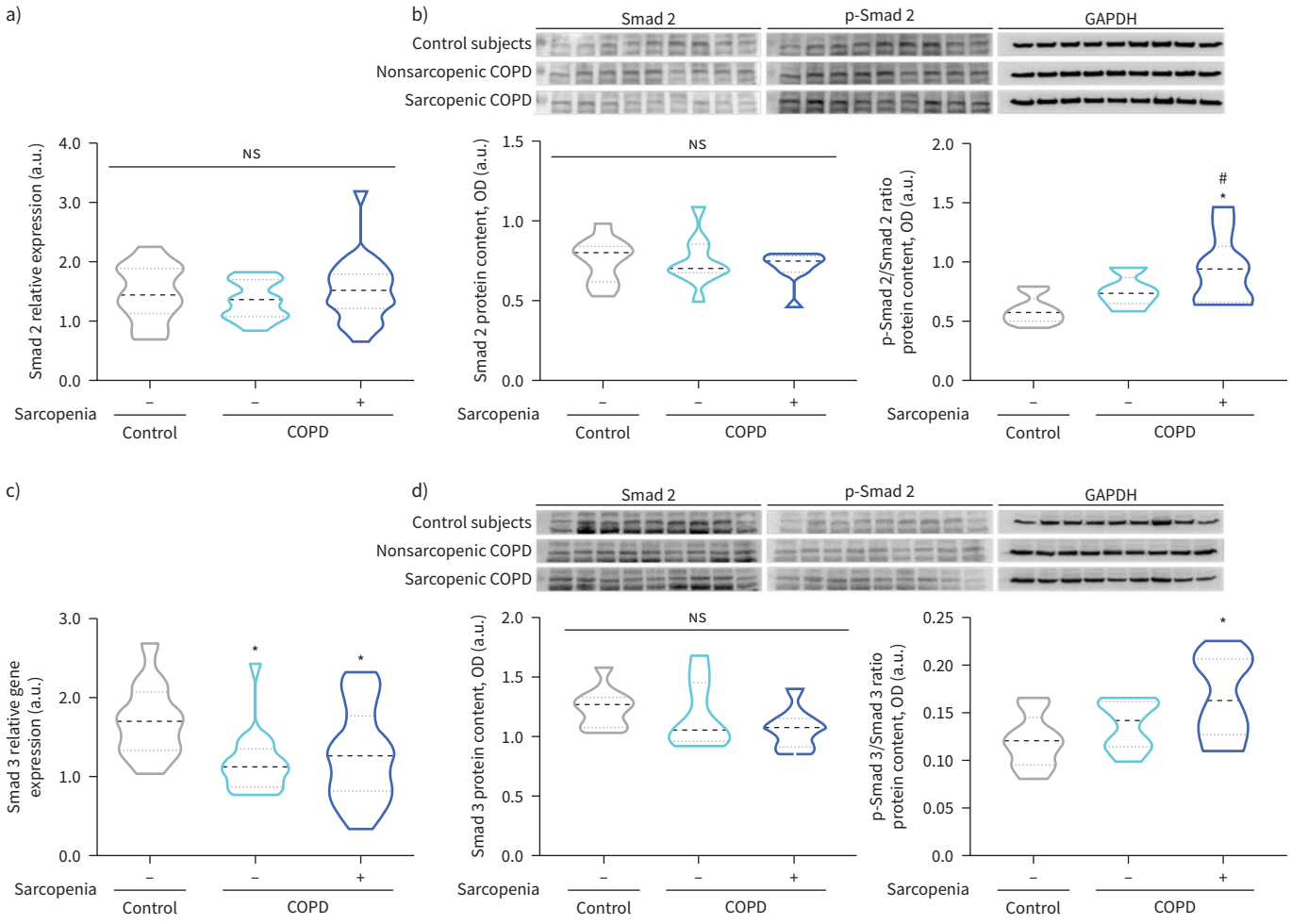

FIGURE 3 a) Violin plots with median (dashed line), first and third quartiles (dotted lines), and maximum and minimum values of Smad2 relative gene expression (a.u.) in the vastus lateralis (VL) muscle of all study groups (n=13 control, n=18 nonsarcopenic COPD and n=23 sarcopenic COPD). b) Immunoblots and violin plots with median (dashed line), first and third quartiles (dotted lines), and maximum and minimum values of Smad2 and p-Smad2/Smad2 ratio protein content (optical density (OD), a.u.) in the VL muscle of all study groups (n=9 per group). c) Violin plots with median (dashed line), first and third quartiles (dotted lines), and maximum and minimum values of Smad3 relative gene expression (a.u.) in the VL muscle of all study groups (n=13 control, n=18 nonsarcopenic COPD and n=23 sarcopenic COPD). d) Immunoblots and violin plots with median (dashed line), first and third quartiles (dotted lines), and maximum and minimum values of Smad3 and p-Smad3/Smad3 ratio protein content (OD, a.u.) in the VL muscle of all study groups (n=9 per group). Smad2: Smad family member 2; p-Smad2: phosphorylated Smad family member 2; Smad3: Smad family member 3; p-Smad3: phosphorylated Smad family member 3; a.u.: arbitrary units; GAPDH: glyceraldehyde-3-phosphate dehydrogenase. NS: not significant; *: $p \leqslant 0.05$ for comparisons between either of the two groups of patients and the control subjects; #: $p \leqslant 0.05$ for comparisons between nonsarcopenic and sarcopenic COPD patients.

nonsarcopenic patients compared with healthy subjects. Post-transcriptional regulation of the myostatin gene may explain the lack of increased protein expression in these muscles [40]. Myostatin protein levels were negatively associated with relevant clinical parameters such as FFMI, lung (diffusion capacity) and quadriceps muscle functions. These results suggest that myostatin plays a substantial role in the process of sarcopenia in COPD.

Smad2 and Smad3 are the transcription factors downstream of myostatin, which cause muscle atrophy. Conversely, inhibition of these transcription factors favours muscle hypertrophy independently of satellite cells [41]. In the study, protein levels of p-Smad2 and p-Smad3 rose significantly in the VL of the sarcopenic patients compared with control subjects and nonsarcopenic patients (p-Smad2). Interestingly, inverse correlations were also observed between p-Smad2/3 levels in the muscles of sarcopenic patients and the clinical parameters FFMI, lung function (K_{CO}) and both TLC and quadriceps strength (Smad2 gene expression). In sarcopenic patients, muscle levels of p-Smad2 protein positively correlated with

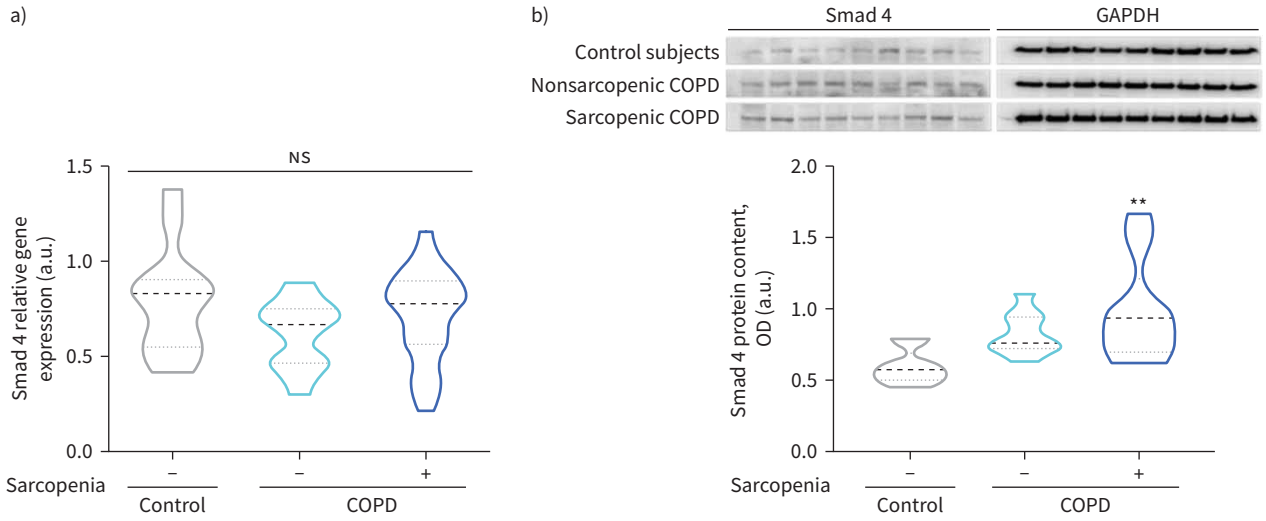

FIGURE 4 a) Violin plots with median (dashed line), first and third quartiles (dotted lines) and maximum and minimum values of Smad4 relative gene expression (a.u.) in the vastus lateralis (VL) muscle of all study groups (n=13 control, n=18 nonsarcopenic COPD and n=23 sarcopenic COPD). b) Immunoblots and violin plots with median (dashed line), first and third quartiles (dotted lines), and maximum and minimum values of Smad4 protein content (optical density (OD), a.u.) in the VL muscle of all study groups (n=9 per group). Smad4: Smad family member 4; a.u.: arbitrary units; OD: optical density; GAPDH: glyceraldehyde-3-phosphate dehydrogenase. NS: not significant; **: p⩽0.01 between either of the two groups of patients and the control subjects.

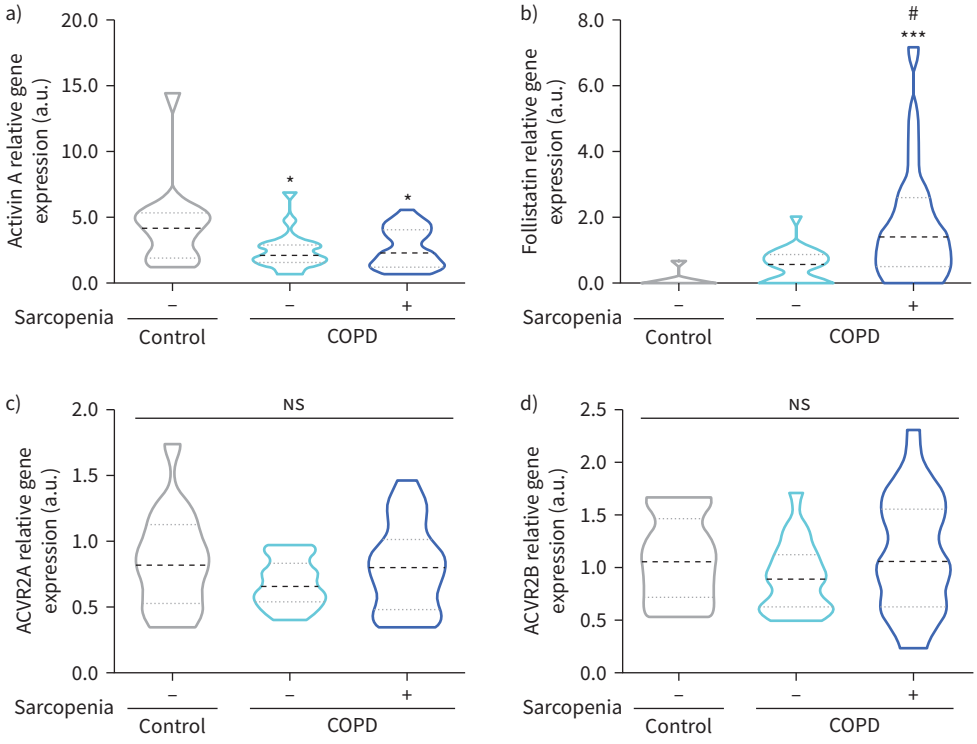

FIGURE 5 Violin plots with median (dashed line), first and third quartiles (dotted lines) and maximum and minimum values of relative gene expression of a) activin A, b) follistatin, c) ACVR2A and d) ACVR2B markers in the vastus lateralis (VL) of all study groups (n=13 control, n=18 nonsarcopenic COPD and n=23 sarcopenic COPD). a.u.: arbitrary units; ACVR2A: activin A receptor type 2A; ACVR2B: activin A receptor type 2B. NS: not significant; *: p⩽0.05; ***: p⩽0.001 between either of the two groups of patients and the control subjects; #: p⩽0.05 for comparisons between nonsarcopenic and sarcopenic COPD patients.

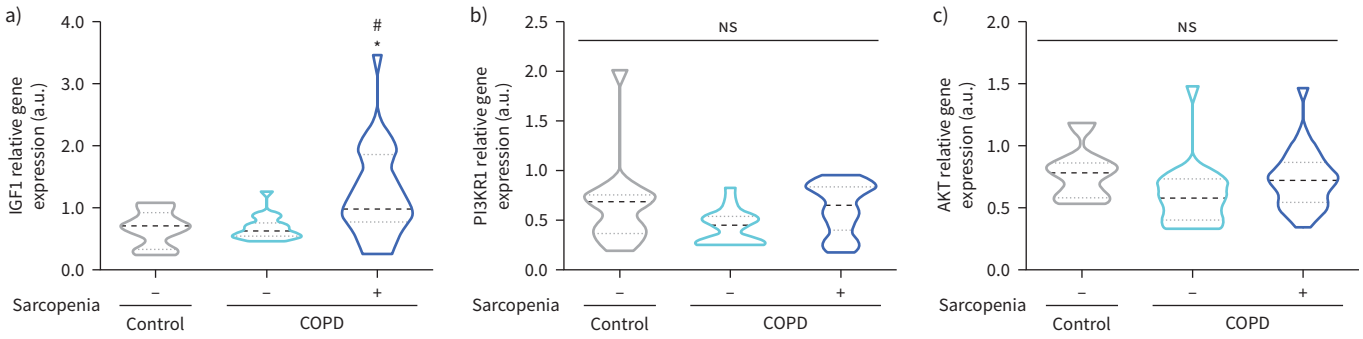

FIGURE 6 Violin plots with median (dashed line), first and third quartiles (dotted lines), and maximum and minimum values of relative gene expression of a) insulin-like growth factor 1 (IGF1), b) PI3KR1 and c) Akt in the vastus lateralis (VL) of all study groups (n=13 control, n=18 nonsarcopenic COPD and n=23 sarcopenic COPD). a.u.: arbitrary units; PI3KR1: phosphoinositide-3-kinase regulatory subunit 1; AKT1: Akt serine/threonine kinase 1. NS: not significant; *: $p \leqslant 0.05$ between either of the two groups of patients and the control subjects; #: $p \leqslant 0.01$ for comparisons between nonsarcopenic and sarcopenic COPD patients.

BODE and RV/TLC, whereas they were negatively associated with airway obstruction (FEV_1). These findings confirm that the myostatin Smad2/3 pathway is likely involved in the process of muscle wasting in COPD.

The transcription factor Smad4, which is a mediator of the myostatin/TGF-β and bone morphogenic protein (BMP) signalling pathways, is well known for playing key roles in cell differentiation, tissue regeneration, embryonic development, tumour progression and regulation of the immune system [42]. Smad4 plays critical roles in muscle development by exerting opposite effects to those of myostatin [43]. As such, Smad4 was shown to be key in cardiogenesis and cardiomyocyte proliferation and differentiation through the BMP signalling pathway [43, 44]. In skeletal muscles, Smad4 regulates myoblast differentiation and muscle regeneration [45]. Smad4 is recruited to the BMP pathway to promote hypertrophy as a result of a decline in p-Smad2/3 levels. Conversely, muscle atrophy takes place as a result of increased myostatin activity [46]. Smad3 and Smad4 expression levels were also differentially expressed in lung tumours of patients with underlying COPD [47]. Loss of Smad4 function was shown to accelerate lung cancer aggressiveness [48] and in patients with COPD, Smad4 levels were significantly downregulated in lung tumours compared with those without COPD [47]. Collectively, these results place Smad4 in a relevant position to be therapeutically targeted.

In this study, protein levels of the intracellular mediator Smad4 significantly increased in the limb muscles of the sarcopenic patients compared with controls. Smad4 protein levels, however, did not differ between nonsarcopenic patients and healthy subjects. Protein levels of Smad4 were also inversely correlated with muscle function parameters, suggesting its implications in the process of muscle atrophy in COPD.

Activin A, a member of the TGF-β superfamily, is a negative regulator of muscle mass. In this study, gene expression levels of activin A were downregulated in the VL of both groups of patients. Activins signal through the type II receptors IIA and IIB. Expression levels of these receptors did not vary across the study groups. The ubiquitous secretory pro-peptide follistatin acts as a potent inhibitor of the myostatin pathway, thus promoting muscle growth [41]. COPD patients with sarcopenia showed a significant rise in follistatin gene expression in their VL compared with healthy subjects and nonsarcopenic patients. Similar results were observed in a previous investigation in COPD patients with severe muscle weakness [15]. The existence of a negative-feedback loop to prevent sarcopenic muscles from experiencing further muscle wasting is a plausible mechanism in these patients. In fact, treatment of the compound bimagrumab, an ACVRII blocker, increased skeletal muscle mass with no significant effect on the functional capacity of the patients in a clinical trial [49]. Further studies should aim at defining the most suitable phenotype of sarcopenic COPD patients to receive this type of therapies. Protein synthesis and muscle hypertrophy are under the control of the axis IGF-1/PI3 K/Akt/mammalian target of rapamycin (mTOR) in adults. IGF-1 is the main anabolic signal that leads to increased protein synthesis [50]. Gene expression of IGF1 was upregulated in the sarcopenic muscles compared with nonsarcopenic patients and controls. The expression of PI3K and Akt, however, did not differ across the study groups. Collectively, these findings suggest that the rise in IGF1 expression may act as a compensatory mechanism to counterbalance the program of muscle atrophy detected in sarcopenic COPD patients.

Study limitations

The study focused on the assessment of gene and protein expression of the myostatin/Smad2/Smad3 and Smad4 pathway. Gene expression determination of activin, its receptors, follistatin and the IGF-1/PI3K/Akt pathway was prioritised due to restrictions in muscle sample availability across the patients. Assessment of the post-transcriptional regulation of IGF1 and protein content in muscles of COPD sarcopenic patients will be the subject of research in future investigations.

Conclusions

In the sarcopenic limb muscles of patients with COPD, the myostatin Smad2/Smad3 pathway was differentially activated from patients without sarcopenia and healthy controls with a similar degree of severity of lung disease. Remarkable negative correlations were observed between clinical parameters related to body composition and quadriceps muscle strength and levels of the myostatin Smad2/Smad3 pathway, suggesting its implications in the process of muscle atrophy in COPD. IGF1 gene expression was also upregulated in the VL of sarcopenic patients. Collectively, these findings offer a potential therapeutic target in COPD-associated sarcopenia.

Acknowledgements: The authors gratefully acknowledge the help provided by Mireia Admetlló, Ana Balañá and Anna Rodó with patient recruitment and clinical evaluation.

Provenance: Submitted article, peer reviewed.

Ethics statement: The present investigation was designed for research on humans following the ethical values on human research at our institute and the World Medical Association guidelines (Seventh Revision of the Declaration of Helsinki; Fortaleza, Brazil, 2013) [21]. The institutional Ethics Committee on Human Investigation approved this investigation (Hospital del Mar–IMIM, Barcelona; project number 2018/7937/I). All the recruited subjects signed an informed written consent before the beginning of the research study.

Author contributions: Study conception and design: E. Barreiro and A. Sancho-Muñoz; patient recruitment and assessment of functional parameters: A. Sancho-Muñoz; molecular biology analyses: A. Núñez-Robainas, M. Guitart and A. López-Postigo; statistical analyses and data interpretation: E. Barreiro, A. Núñez-Robainas and A. López-Postigo; manuscript drafting and intellectual input: E. Barreiro and A. Núñez-Robainas; manuscript writing – final version: E. Barreiro.

Conflict of interest: E. Barreiro was the Chief Editor of *ERJ Open Research* at the time that this article was submitted. The other authors have nothing to disclose.

Support statement: The research conducted in the study has been funded by Instituto de Salud Carlos III FIS projects 18/00075 and 21/00215; and cofunded by the European Union; Consorcio Centro de Investigación Biomédica en Red (CIBERES); the Spanish Ministry of Science and Innovation; intensifications INT19/00002 and INT22/00008, 2021-SGR-00100 of the Catalan Government; Spanish Respiratory Society grant SEPAR 2018; and Laboratorios Menarini (Spain), which did not have any role in the study design or the statistical analysis. Funding information for this article has been deposited with the Crossref Funder Registry.

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