

Activation of Satellite Cells in the Intercostal Muscles of Patients With Chronic Obstructive Pulmonary Disease

Juana Martínez-Llorens,^{a,b} Carme Casadevall,^b Josep Lloreta,^{a,c,d} Mauricio Orozco-Levi,^{a,b,d} Esther Barreiro,^{a,b,d} Joan Broquetas,^{a,b,e} and Joaquim Gea^{a,b,d}

^aServei de Pneumologia, Hospital del Mar-IMIM, Barcelona, Spain

^bUnitat de Recerca en Múscul i Aparell Respiratori (URMAR), Hospital del Mar-IMIM, Barcelona, Spain

^cDepartament de Patologia, Hospital del Mar-IMIM, Barcelona, Spain

^dUniversitat Pompeu Fabra, CIBER de Enfermedades Respiratorias (CibeRes), Barcelona, Spain

^eUniversitat Autònoma de Barcelona, CIBER de Enfermedades Respiratorias (CibeRes), Barcelona, Spain

OBJECTIVE: The respiratory muscles of patients with chronic obstructive pulmonary disease (COPD) display evidence of structural damage in parallel with signs of adaptation. We hypothesized that this can only be explained by the simultaneous activation of satellite cells. The aim of this study was to analyze the number and activation of those cells along with the expression of markers of microstructural damage that are frequently associated with regeneration.

PATIENTS AND METHODS: The study included 8 patients with severe COPD (mean [SD] forced expiratory volume in 1 second, 33% [9%] of predicted) and 7 control subjects in whom biopsies were performed of the external intercostal muscle. The samples were analyzed by light microscopy to assess muscle fiber phenotype, electron microscopy to identify satellite cells, and real-time polymerase chain reaction to analyze the expression of the following markers: insulin-like growth factor 1, mechano growth factor, and embryonic and perinatal myosin heavy chains (MHC) as markers of microstructural damage; Pax-7 and m-cadherin as markers of the presence and activation of satellite cells, respectively; and MHC-I, IIa, and IIx as determinants of muscle fiber phenotype.

RESULTS: The patients had larger fibers than healthy subjects (54 [6] vs 42 [4] μm^2 ; $P < .01$) with a similar or slightly increased proportion of satellite cells, as measured by ultrastructural analysis (4.3% [1%] vs 3.7% [3.5%]; $P > .05$) or expression of Pax-7 (5.5 [4.1] vs 1.6 [0.8] arbitrary units [AU]; $P < .05$). In addition, there was greater activation of satellite cells in the patients, as indicated by increased expression of m-cadherin (3.8 [2.1] vs 1.0 [1.2] AU; $P = .05$). This was associated with

increased expression of markers of microstructural damage: insulin-like growth factor 1, 0.35 (0.34) vs 0.09 (0.08) AU ($P < .05$); mechano growth factor, 0.45 (0.55) vs 0.13 (0.17) AU ($P = .05$).

CONCLUSIONS: The intercostal muscles of patients with severe COPD show indirect signs of microstructural damage accompanied by satellite cell activation. This suggests the presence of ongoing cycles of lesion and repair that could partially explain the maintenance of the structural properties of the muscle.

Key words: COPD. Muscle dysfunction. Damage. Repair. Satellite cells.

Activación de células satélite en el músculo intercostal de pacientes con EPOC

OBJETIVO: Los músculos respiratorios de los pacientes con enfermedad pulmonar obstructiva crónica (EPOC) presentan lesiones estructurales, que coexisten con signos de adaptación. Nuestra hipótesis es que esto sólo puede explicarse si se produce simultáneamente la activación de sus células satélite. El propósito del presente trabajo ha sido valorar el número y la eventual activación de dichas células, así como la expresión de marcadores de microlesión estructural, ligados a la regeneración.

PACIENTES Y MÉTODOS: Se incluyó en el estudio a 8 pacientes con EPOC grave —media \pm desviación estándar del volumen espiratorio forzado en el primer segundo: un 33 \pm 9% del valor de referencia— y a 7 controles, a quienes se realizó una biopsia del músculo intercostal externo. La muestra se analizó mediante microscopía óptica (fenotipo fibrilar), electrónica (células satélite) y técnica de reacción en cadena de la polimerasa *en tiempo real* (marcadores de microlesión: factor de crecimiento similar a la insulina de tipo 1, factor de crecimiento mecánico e isoformas de cadenas pesadas de miosina [MyHC] embrionaria e isoformas de perinatal; de presencia y activación de células satélite: Pax-7 y m-cadherina, respectivamente; y condicionantes del fenotipo fibrilar: MyHC-I, IIa y IIx).

RESULTADOS: Los pacientes tuvieron unas fibras mayores que los sujetos sanos (54 \pm 6 frente a 42 \pm 4 μm^2 ; $p < .01$), con una población de células satélite conservada o ligeramente incrementada (cuantificación ultraestructural: 4,3 \pm 1% frente al 3,7 \pm 3,5%, p no significativa; Pax-7: 5,5 \pm 4,1

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Correspondence: Dr J. Gea
Servei de Pneumologia, Hospital del Mar-IMIM
Pg. Marítim, 25-27
08003 Barcelona, Spain
E-mail: jgea@imim.es

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frente a $1,6 \pm 0,8$, unidades arbitrarias [ua], respectivamente, $p < 0,05$) y una mayor activación (m-caderina: $3,8 \pm 2,1$ frente a $1,0 \pm 1,2$ ua; $p = 0,05$). Esto se asociaba a valores aumentados de marcadores de microlesión (factor de crecimiento similar a la insulina de tipo 1: $0,35 \pm 0,34$ frente a $0,09 \pm 0,08$ ua, $p < 0,05$; factor de crecimiento mecánico: $0,45 \pm 0,55$ frente a $0,13 \pm 0,17$ ua, $p = 0,05$).

CONCLUSIONES: Los músculos intercostales de pacientes con EPOC grave muestran signos indirectos de microlesión, acompañados de la activación de sus células satélite. Esto apunta a la presencia de ciclos continuados de lesión y reparación, lo que podría explicar parcialmente la conservación de sus propiedades estructurales.

Palabras clave: EPOC. Disfunción muscular. Daño. Reparación. Células satélite.

Introduction

Although chronic obstructive pulmonary disease (COPD) is mainly characterized by changes in the pulmonary parenchyma and the airways, awareness has been increasing in recent years regarding the importance of systemic aspects of the disease.^{1,2} Notable among these is skeletal muscle dysfunction, which is associated with marked symptoms and influences prognosis.¹⁻³ However, the phenotypic changes occurring in the different skeletal muscles of patients with COPD are not homogeneous.⁴ For instance, the respiratory muscles display changes that favor aerobic activity,⁵ whereas the changes in muscles of the extremities occur in the opposite direction.⁶ Nevertheless, some phenomena, such as oxidative stress^{7,8} and muscle damage,^{9,10} appear to be common to the different muscle groups.

Muscle damage is defined as any structural lesion caused by a specific insult.^{11,12} Under normal circumstances, this triggers repair and regeneration processes.¹³ The cellular element that is fundamentally involved in these phenomena is the satellite cell, which when activated fuses with the muscle fibers and donates its nucleus.¹¹ Depending on variables associated with the type of stimulus and the integrity of the repair mechanisms themselves, these processes can give rise to a muscle that is similar to the original one or is modified (remodeling). Analysis of animal models of inflammatory lung disease has revealed that the muscles of the extremities have reduced regenerative capacity,¹⁴ possibly explaining the muscle wasting phenotype observed in patients with COPD. We hypothesized that, in contrast, the respiratory muscles of patients with COPD maintain their regenerative capacity, thus facilitating phenotypic adaptation. Therefore, the aim of this study was to analyze the behavior of satellite cells in the external intercostal muscles of patients with severe COPD. As secondary objectives, we assessed changes in various molecular markers of microstructural damage and repair, along with their relationship with satellite cells and muscle phenotype.

Patients and Methods

Patients

The study included 8 patients with severe COPD—forced expiratory volume in 1 second (FEV_1)/forced vital capacity (FVC) $<70\%$ and $FEV_1 <50\%$ of reference¹—who were consecutively recruited in the outpatient clinic of our hospital; all were classified as stable (without changes in disease characteristics or treatment in the previous 3 months). The following exclusion criteria were applied: alcohol consumption greater than 100 g/d or drug use; presence of cancer, endocrine disorders, psychiatric conditions, or severe orthopedic disease; and associated muscle or neurologic disease. Patients were also excluded if they were unable to cooperate in the study or if they were receiving treatment that could affect the structure or function of the muscles. A control group was included that comprised 7 healthy volunteers (recruited from the general population at the same time as patient recruitment) who participated simultaneously in a parallel study. Both groups were made up exclusively of men to avoid possible interference related to sex differences. The objectives of the study were explained to all participants, who provided written consent. The study was approved by the ethics committee of the hospital.

Methods

Assessment of nutritional status and respiratory function. Biologic and anthropometric parameters were used to assess nutritional status. Conventional assessment of respiratory function was performed in all patients by spirometry (Datospir 500 spirometer, SIBEL, Barcelona, Spain), determination of static lung volumes and airway resistance (Masterlab, Jaeger, Würzburg, Germany), and analysis of carbon monoxide diffusing capacity (gas analyzer included in the Masterlab equipment). In all cases the results were compared with Mediterranean population reference values.¹⁵⁻¹⁷ Arterial blood gases were also analyzed (Rapidlab 860, Bayer, Chiron Diagnostics GmbH, Tuttlingen, Germany).

Assessment of muscle function. Respiratory muscle function was assessed by determination of maximum static mouth pressures according to a previously published standard method¹⁸ and using reference values for a Mediterranean population.¹⁹ In addition, inspiratory muscle endurance was assessed according to the method described previously by our group.¹⁸ Finally, the grip strength of the nondominant hand was determined using the standard maneuver with a specific dynamometer (Biopac Systems, Goleta, California, USA) connected to a digital polygraph (Biopac Systems). The maximum value of 3 consecutive valid and reproducible maneuvers was taken.

Muscle biopsy and sample processing. Samples of external intercostal muscle were obtained from the fifth intercostal space, randomly varying the side of the body from which the biopsy was obtained, according to the technique described by our group.¹⁸ The muscle samples were divided into various portions immediately after extraction. A fragment was fixed in formaldehyde and embedded in paraffin for immunohistochemical analysis of fiber type, according to standard protocols used in our laboratory.¹⁸ The second piece of muscle was processed using standard methods for ultrastructural analysis, according to a previously published procedure.⁹ The following criteria were applied for identification of satellite cells by electron microscopy: a) a plasma membrane separating the cell from the adjacent muscle fiber; b) a continuous basement membrane between the satellite cells and the muscle fiber; and c) a heterochromatic nucleus.²⁰ The proportion of satellite cells was expressed as a

TABLE
General, Anthropometric, Nutritional, and Functional Characteristics of the Study Groups^a

	Severe COPD	Controls	P
No. of subjects	8	7	—
Mean age, y	67 (6)	69 (6)	NS
Tobacco exposure, pack-years	62 (22)	—	—
BMI, kg/m ²	26.4 (4.5)	25.9 (2.7)	NS
Serum albumin, g/dL	4.3 (0.5)	4.7 (0.4)	NS
Serum cholesterol, mg/dL	179 (23)	191 (13)	NS
FEV ₁ , % of reference	33 (9)	96 (17)	^c
FEV ₁ /FVC, %	45 (9)	74 (3)	^c
RV, % of reference	189 (36)	104 (15)	^c
RV/TLC, %	67 (5)	42 (3)	^c
DLCO, % of reference	68 (26)	98 (16)	^b
PaO ₂ , mm Hg	67 (2)	—	—
PaCO ₂ , mm Hg	47.5 (2.8)	—	—
MIP, cm H ₂ O	-76 (18)	-95 (17)	^b
MIP, % of reference	73 (17)	93 (16)	^b
MEP, cm H ₂ O	112 (45)	142 (30)	NS
MEP, % of reference	61 (20)	82 (18)	^b
Pth _{max} , cm H ₂ O	-44 (11)	-56 (15)	NS
Tth80, min	11.7 (6.3)	15.9 (6.1)	^b
HGS, kg	18.6 (7.7)	26.8 (6.3)	^b

Abbreviations: COPD, chronic obstructive lung disease; DLCO, diffusing capacity of the lung for carbon monoxide; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; HGS, hand grip strength; BMI, body mass index; NS, not significant; MEP, maximum expiratory pressure; MIP, maximum inspiratory pressure; Pth_{max}, maximum sustainable threshold pressure; RV, residual volume; Tth80, inspiratory sustainable threshold endurance time (80% of maximum); TLC, total lung capacity.

^aData are shown as mean (SD).

^bP<.05.

^cP<.001.

percentage of the total number of muscle fiber nuclei in a given area.²¹

The third portion of the muscle sample was immediately placed in a cryovial and frozen in liquid nitrogen. It was then stored at -70°C prior to processing for analysis of expression of messenger RNA (mRNA) for genes associated with microstructural damage, repair processes, and fiber phenotype. Real-time polymerase chain reaction (PCR) was used according to a previously described method.²² mRNA expression was analyzed for the adult isoforms (I, IIa, and IIx, all of which are directly involved in determining fiber phenotype) and nonadult isoforms (perinatal and embryonic, indicators of stress and microstructural lesion) of myosin heavy chains (MHC), markers of satellite cells (Pax 7 and m-cadherin, markers of the cell population and cell activation, respectively), and for mechano growth factor (MGF) and insulin-like growth factor-1 (IGF-1), which are also indicators of stress and microstructural damage of the muscle fibers and of the onset of muscle regeneration.

Statistical Analysis

Quantitative variables were shown as mean (SD). Since the Kolmogorov-Smirnov test revealed a nonnormal distribution of the different variables analyzed, between-groups comparisons were made with the nonparametric Mann-Whitney test and analysis of the correlation between quantitative variables was performed using the Spearman correlation coefficient (ρ). In all cases, P values less than or equal to .05 were considered statistically significant.

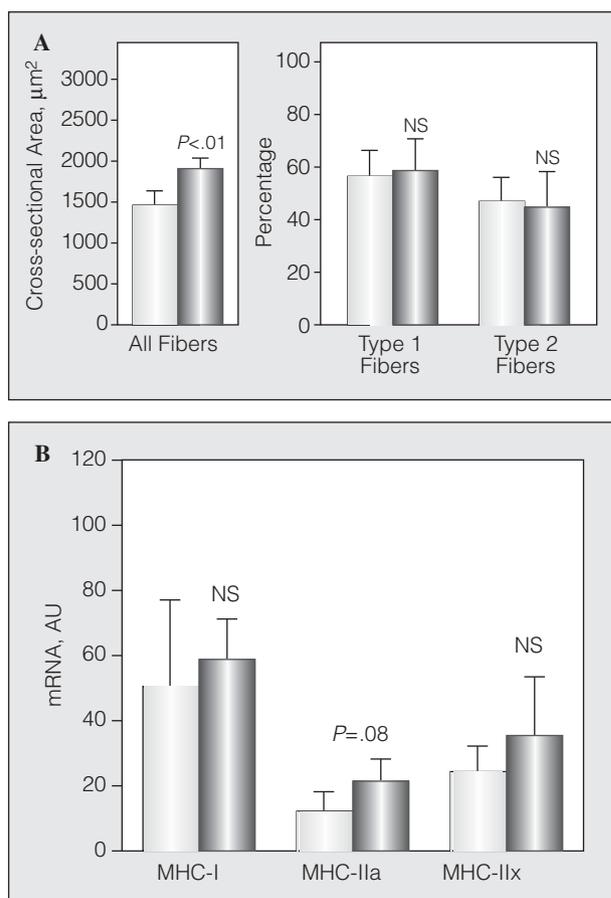


Figure 1. Fiber phenotype in the external intercostal muscle of healthy control subjects (light colored bars) and patients with severe chronic obstructive pulmonary disease (dark bars). (A) Overall size of the fibers (cross-sectional area) and percentages of type 1 and type 2 fibers. (B) Expression of messenger RNA (mRNA) for the adult isoforms (I, IIa, and IIx) of the myosin heavy chains (MHC). NS indicates not significant; AU, arbitrary units.

Results

General, Anthropometric, and Functional Data

The Table shows the main general, anthropometric, nutritional, and functional data of the 2 study groups. There were no significant differences between patients with severe COPD and healthy control subjects in terms of analytical variables relating to nutrition or body mass index, but the patients had obstructive ventilatory abnormalities (a criterion for inclusion in the study), air trapping, reduced diffusing capacity of the lung for carbon monoxide, and hypoxemia. Respiratory and peripheral muscle strength was slightly reduced. Inspiratory muscle endurance was also reduced in patients with COPD and was correlated with FEV₁ ($\rho=0.700$, $P<.05$).

Fiber Phenotype, Markers of Microstructural Damage, and Satellite Cells

Figure 1 shows the characteristics of the external intercostal muscle fibers. The fibers were up to 24%

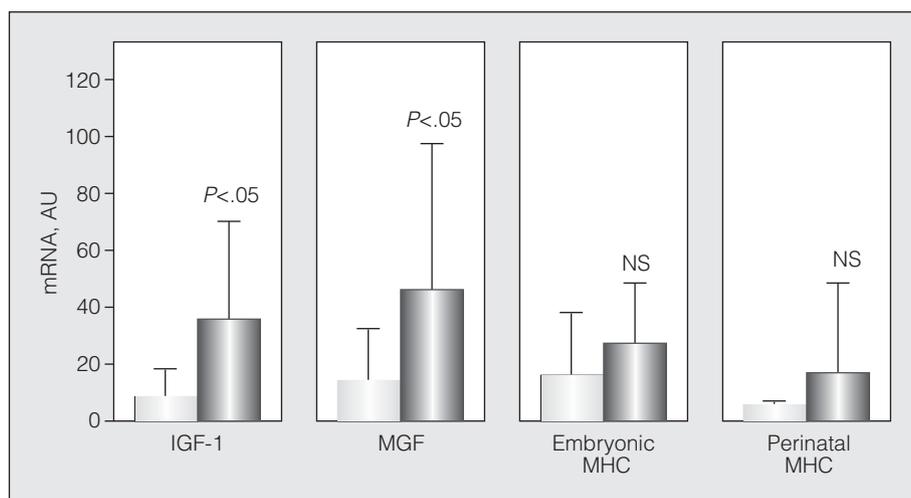


Figure 2. Messenger RNA expression of markers for microstructural damage/cell stress and initiation of regeneration: insulin-like growth factor 1 (IGF-1), mechano growth factor (MGF), and non-adult (embryonic and fetal) isoforms of myosin heavy chains (MHC). Light colored bars indicate healthy control subjects and dark bars patients with chronic obstructive pulmonary disease. NS indicates not significant; AU, arbitrary units.

larger in patients with severe COPD than in healthy controls. The percentages of the different fiber types were similar in patients and healthy controls, with a predominance of type 1 fibers (Figure 1A). Analysis of mRNA expression for genes that determine fiber phenotype revealed a marked tendency towards overexpression of MHC-IIa in patients with COPD, without apparent differences in mRNA expression for other adult isoforms (Figure 1B).

Increased expression of mRNA for MGF and IGF-1 was indicative of muscle stress and microstructural damage. Although embryonic and perinatal isoforms of MHC showed a similar tendency toward increased mRNA expression, the differences were not statistically significant (Figure 2). The size of the satellite cell population was found to be unaltered (ultrastructural analysis) or slightly increased (expression of Pax-7 mRNA) in patients with severe COPD, and mRNA expression of the marker of activation (m-cadherin) was upregulated (Figures 3A and 3B).

Relationship Between Structural and Functional Variables

Some of the markers of microstructural damage displayed a direct relationship with the degree of functional impairment in patients with COPD. Thus, mRNA expression for IGF-1 and MGF were correlated or displayed a clear trend towards correlation with residual volume ($\rho=0.943$ and $\rho=0.921$, respectively; $P < .01$ in both cases) and FEV_1 ($\rho=-0.771$ [$P=.07$] and $\rho=-0.724$ [$P=.08$], respectively). In addition, the population of satellite cells, represented by the variables used for ultrastructural analysis, also showed a significant relationship with lung function (Figure 4). Finally, body mass index was inversely correlated with markers of microstructural damage and displayed a trend towards direct correlation with markers of repair (IGF-1 mRNA, $\rho=-0.943$; MGF mRNA, $\rho=-0.921$; $P < .01$ in both cases; m-cadherin mRNA, $\rho=0.714$ [$P=.09$]).

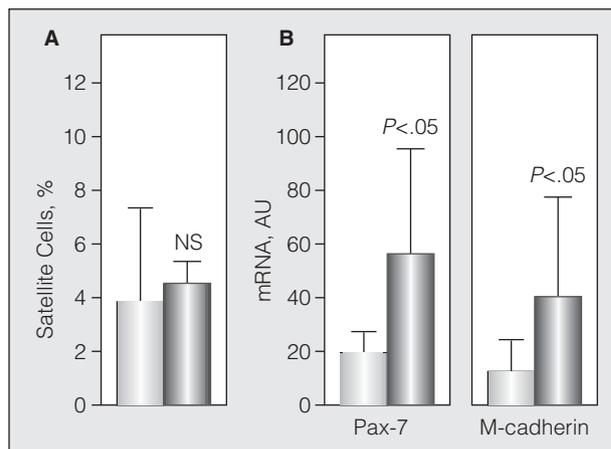


Figure 3. Data on satellite cells. (A) Results of electron microscopy; the proportion of satellite cells is expressed as a percentage of the total number of nuclei identifiable in the muscle. (B) Messenger RNA expression of markers of cell type (Pax-7) and activation (m-cadherin) of satellite cells. NS indicates not significant; AU, arbitrary units.

Discussion

In this study, we found that the intercostal muscles of patients with severe COPD display signs of microstructural damage along with activation of satellite cells, the numbers of which were conserved or slightly increased. These phenomena appear to be linked to the degree of functional impairment of the patients and may explain the presence of a fiber phenotype that was not suggestive of wasting.

The respiratory muscles of patients with COPD display increased structural damage. This appears to be at least partly due to overexertion during breathing,^{9,22} although other factors may also be involved, such as oxidative stress and local inflammation.^{7,22} However, the respiratory muscles also display a modified phenotype that facilitates their ventilatory function.^{5,19} Animal models appear to show that both phenotypes, damage and remodeling, are directly linked via mechanisms of muscle repair.^{13,23,24} Satellite

cells are an essential element in that process, although to date we are not aware of them having been analyzed in patients with COPD or in animal models of the disease.

In this study, we observed increases in molecular markers that are highly indicative of the presence of microstructural damage in the intercostal muscles of the patients. The use of those markers allows detection of more subtle phenomena that could pass unnoticed with conventional morphometric techniques. Thus, both nonadult isoforms of MHC and certain growth factors are expressed in situations of extreme muscle stress and microstructural damage.^{13,25,26} Our results also show that the expression of some of these molecules is associated with the degree of impairment of respiratory function. These findings are consistent with previous studies using complementary techniques that revealed evidence of structural damage to the intercostal muscles in patients with COPD.²² In addition, IGF-1 and its genetic variant MGF also intervene directly in the initial stages of muscle regeneration by stimulating the differentiation and proliferation of satellite cells.²⁷

This study also showed that the population of satellite cells is conserved or slightly increased (depending on the variable analyzed) in the intercostal muscles of patients with COPD, with a degree of activation that is significantly higher than in healthy subjects. The satellite cells are located between the basement membrane and the sarcolemma of the muscle fibers,^{28,29} and they are responsible for the growth, repair, and maintenance of the skeletal muscle.²⁹ Their numbers remain relatively stable throughout adult life,³⁰ due to their capacity for self-renewal.^{29,30} With aging, however, the percentage decreases, leading to a reduced regenerative capacity of the muscle in elderly individuals.³¹ When damage occurs, the satellite cells are activated before beginning to proliferate, differentiate, and fuse with the muscle fibers. The vast majority of satellite cells are activated within 24 hours of damage occurring,²⁰ but this period of activation is relatively short (around 120 hours),³² making detection of activated cells difficult except under experimental conditions or conditions in which the stimulus is ongoing (as is probably the case in our patients). The finding that deterioration of lung function was associated with the density of the satellite cells in our study is indicative of a modulatory role for mechanical overload.

Although only based on cross-sectional observations, the outcome of microstructural damage and repair observed in this study appears to be consistent with the conserved fiber phenotype of the intercostal muscles of the patients. Earlier studies in larger groups of patients have occasionally shown an increase in the proportion of type 2 fibers (particularly type 2a),³³ an observation that could be related to the overexpression of the corresponding gene shown in the present study. Activation of this gene could respond to periodic high-intensity demands on the muscle (effort, exacerbations) that would mimic aerobic training.³³

However, from a functional point of view, conservation of muscle phenotype coexists with alteration of strength and endurance of the respiratory muscles. This would probably be more associated with mechanical factors (as would apparently be indicated by the relationship between respiratory muscle function and lung function) or systemic

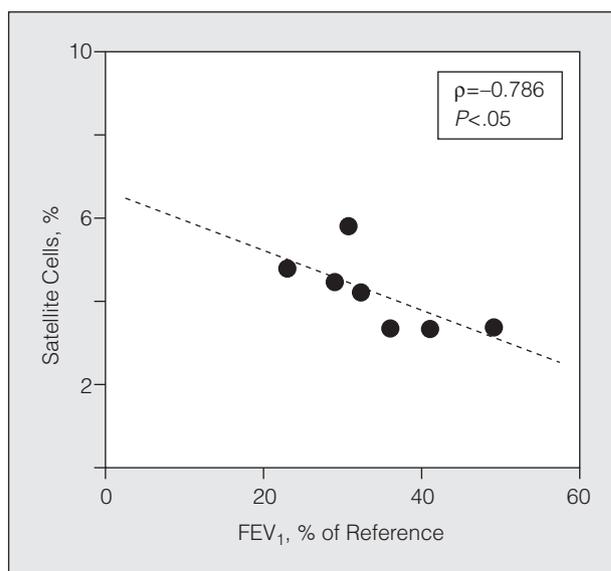


Figure 4. Correlation between the degree of obstruction—shown as forced expiratory volume in 1 second (FEV₁)—and the size of the satellite cell population.

factors (as also indicated by dysfunction of both respiratory muscles and the muscles of the hand).

Another interesting association revealed by this study is that observed between body mass index and markers of microstructural damage and repair in patients with COPD. Such an association suggests that these phenomena could underlie the amount of muscle mass in a patient. This hypothesis should be addressed in future studies.

The clinical interest in this study lies in its potential implications for rehabilitation therapy. Although the intercostal muscles appear to be easily damaged, if the reparative capacity is maintained in patients with COPD, high-intensity breathing exercises could be considered to improve the phenotype of those muscles. It will now be necessary to obtain data on the process of regeneration in the limb muscles, the phenotype of which displays characteristics of wasting and for which the response to intense physical exercise is not clear.

The main respiratory muscle in healthy subjects is the diaphragm. However, obtaining valid samples has been limited by the small number of candidates and the tendency for relevant comorbidity to be present. The choice of the external intercostal muscles in this study appears valid for a number of reasons. Firstly, it allows selection of the study subjects to exclude any relevant comorbidity. Secondly, the external intercostal muscles in patients with COPD are used for breathing even at rest,³⁴ and they participate progressively in ventilatory effort with increasing load on the system.^{34,35} Thus, these muscles would not only reflect the effect of baseline ventilatory activity in patients with COPD but, as mentioned, would also reflect periodic increases in that activity.

In the present study, we decided to complement traditional morphometric evaluations²⁸ with analysis of molecular markers specifically expressed in satellite cells rather than myonuclei or the muscle fibers themselves. Thus, Pax-7, a regulator of myogenesis which must be

activated in order to generate satellite cells, is a good marker of this cell population.³⁶ In addition, it distinguishes between satellite cells and pluripotent stem cells, which do not express it.³⁶ In turn, m-cadherin is an adhesion molecule whose expression increases in activated satellite cells, and as such is considered a good marker for this phenomenon.³⁶

In conclusion, this study has shown that the intercostal muscles of patients with severe COPD maintain a stable population of satellite cells, whose activity is found to be increased. This activation and the parallel expression of markers of regeneration probably respond to the presence of microstructural damage. Given that these events are accompanied by a conserved fiber phenotype, our results are suggestive of successful repair processes in the intercostal muscles of these patients.

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