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Disfunción muscular en pacientes con EPOC: Papel del estrés en el retículo endoplasmático y la alteración del potencial regenerativo.

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RESUMEN

Se ha estudiado el potencial de regeneración muscular en pacientes EPOC con y sin sarcopenia. También se ha investigado otro mecanismo de regeneración muscular mediante el análisis del estrés a nivel de retículo endoplasmático (RE) en el cuádriceps y en el diafragma de pacientes con patología respiratoria aguda (neoplasia pulmonar localizada) y crónica (EPOC).

El músculo esquelético de pacientes EPOC con sarcopenia tiene incrementado el potencial regenerativo asociado con un aumento de los inhibidores de crecimiento muscular (miostatina) y del daño muscular que produce modificaciones del fenotipo muscular (cambio de fibras lentas a rápidas, que son más pequeñas y existencia de miofibras híbridas).

En el cuádriceps de los pacientes con sarcopenia y EPOC y especialmente en los enfermos con neoplasia de pulmón hay marcadores de estrés de RE. En el diafragma de los pacientes EPOC, a pesar de presentar menor fuerza, no se detecta alteraciones en el estrés del RE.

ABSTRACT

Muscle regeneration potential has been studied in COPD patients with and without sarcopenia. Another mechanism of muscle regeneration has also been investigated by analyzing endoplasmic reticulum (ER) stress in the quadriceps and diaphragm of patients with acute (localized pulmonary neoplasia) and chronic (COPD) respiratory pathology.

The skeletal muscle of COPD patients with sarcopenia has increased regenerative potential associated with an increase in muscle growth inhibitors (myostatin) and muscle damage that produces modifications of the muscle phenotype (change from slow to fast fibers, which are smaller and existence of hybrid myofibers).

In the quadriceps of patients with sarcopenia and COPD and especially in patients with lung neoplasia there are markers of ER stress. In the diaphragm of COPD patients, despite presenting less strength, no alterations in ER stress are detected.

INDICE

PREFACIO

Comunicaciones

En los siguientes congresos se han presentado los resultados preliminares de la presente tesis doctoral

Congresos Internacionales:

- Congreso *American Thoracic Society* (ATS), Dallas, Texas 17 – 22 Mayo 2019.

- Congreso *European Respiratory Society* (ERS) Madrid, 28 Septiembre – 2 Octubre 2019.

- Congreso Barcelona-Boston *Lung Conference* Barcelona, 17 Septiembre – 18 Enero 2020.

- Congreso *European Respiratory Society (*ERS) Virtual 6 – 9 Septiembre 2020.

Congresos Nacionales:

- XXXVII Diada Pneumològica Societat Catalana de Pneumologia (SOCAP) Terrassa 4– 6 Abril 2019.

- 53º Congreso Sociedad Española de Neumología y Cirugía Torácica (SEPAR) Virtual 12 – 14 Noviembre 2020.

Publicaciones derivadas de la presente tesis

- **Sancho-Muñoz A**, Guitart M, Chiaradía DAR, Gea J, Martínez-Llorens J, Barreiro E. Deficient muscle regeneration potential in sarcopenic COPD patients: Role of satellite cells. J Cell Physiol doi:10.1002/jcp.30073. Q1

- Barreiro E, Salazar-Degracia A, **Sancho-Muñoz A**, Gea J. Endoplasmic reticulum stress and unfolded protein response profile in quadriceps of sarcopenic patients with respiratory diseases. J Cell Physiol. 2019;234(7):11315-11329. doi:10.1002/jcp.27789. Q1

- Barreiro E, Salazar-Degracia A, **Sancho-Muñoz A**, Aguiló R, Rodríguez-Fuster A, Gea J. Endoplasmic reticulum stress and unfolded protein response in diaphragm muscle dysfunction of patients with stable chronic obstructive pulmonary disease. J Appl Physiol (1985). 2019;126(6):1572-1586. doi:10.1152/japplphysiol.00670.2018. Q1

Otros estudios

- Barreiro E, **Sancho-Muñoz A**, Puig-Vilanova E, et al. Differences in micro-RNA expression profile between vastus lateralis samples and myotubes in COPD cachexia. J Appl Physiol (1985). 2019;126(2):403-412. doi:10.1152/japplphysiol.00611.2018. Q1

ABREVIATURAS

ADN: Ácido desoxiribonucleico

ARN: Ácido ribonucleico

ASK1: Quinasa reguladora de la señal de apoptosis 1

ATF 4: Factor de transcripción activador 4

ATF 6: Factor de transcripción activador 6

ATP: Adenosin trifosfato

ATS: American Thoracic Society

BAX: Regulador apoptótico X asociado a BCL2

BIP: Proteína inmunoglobulina de unión

BODE: Body mass índex, Airflow Obstruction, Dysnea and Exercise capacity

CAT: COPD Assessment Test

CHOP: Proteína homóloga C/EBP

CSA: Cross sectional area

DE: Desviación estándar

DL_{CO}: Capacidad de trasferencia de monóxido de carbono

EiF2a: Factor 2 α de iniciación de la traducción eucariótica

EPOC: Enfermedad pulmonar obstructiva crónica

ERS: European Respiratory Society

FEV_1: Volumen espiratorio forzado en el primer segundo

FFMI: Fat free Mass index

FVC: Capacidad vital forzada

GAPDH: Antigliceraldehído-3-fosfato deshidrogenasa

GOLD: Global Iniciative for Chronic Obstructive Lung Disease

HSP: Proteína de choque térmico

IMC: Índice de masa corporal

IRE 1: Enzima requeridora de inositol 1

LABA: Agonista Beta 2 de larga duración

LAMA: Anticolinérgico de larga duración

LC3: Proteína 1 de cadena ligera asociada a los microtúbulos 3

MDA: Antimalondialdehído de proteínas

MMRC: Escala modificada del Medical Research Council

MRF: Factor miogénico regulador

MyHC: Cadena pesada de la miosina

Myf-5: Factor miogénico 5

MyoD: Proteína 1 de diferenciación miogénica

P62: proteína de la nucleoporina p62

PaO$_2$: Presión parcial de Oxígeno

Pax: Paired box protein

PCR: Reacción en cadena de la polimerasa

PDIA3: Proteína disulfuro isomerasa-3

PERK: Proteína quinasa similar a la ER

PI3K: Fosfatidilinositol 3-quinasa

PKR: proteína quinasa R

PVDF: Difluoruro de polivinilideno

qRT-PCR: Reacción en cadena de la polimerasa de transcripción inversa

QMVC: Contracción isométrica máxima del miembro inferior dominante

RE: Retículo endoplasmático

RER: Relación de intercambio respiratorio

SC: Célula satélite

SpO_2: Saturación periférica de oxígeno

TRAF2: Factor 2 asociado al receptor del factor de necrosis antitumoral

UPR: Respuesta compensatoria a proteínas no plegadas/mal plegadas

VCO_2: Producción de dióxido de carbono

VE: Ventilación minuto

VL: Vasto lateral

VO_2: Consumo de oxígeno

XBP1: Proteína de unión a la caja X 1

INTRODUCCIÓN

1. **Definición de la Enfermedad Pulmonar Obstructiva Crónica**

La enfermedad pulmonar obstructiva crónica (EPOC) es una neumopatía prevenible cuyos síntomas más frecuentes incluyen disnea, tos y/o producción de esputo. La disnea es el síntoma más discapacitante de todos ellos. Aparece en estadios iniciales durante la actividad física, llegando a estar presente en reposo en fases avanzadas de la enfermedad. La tos con producción de esputo de forma regular durante un mínimo de 3 meses al año durante 2 años consecutivos constituye los denominados criterios clínicos de bronquitis crónica típicos de esta patología ("Definition and Classification of Chronic Bronchitis for Clinical and Epidemiological Purposes," 1965).

Se caracteriza por una limitación al flujo aéreo parcialmente reversible y progresiva (Agusti, 2022). Esta limitación al flujo aéreo está asociada a una respuesta inflamatoria del pulmón debida a exposición a gases o partículas nocivas, derivadas fundamentalmente del humo del tabaco. Además de las exposiciones, está influenciada por factores del huésped como el desarrollo pulmonar anormal, el envejecimiento acelerado y las anormalidades genéticas. Para el diagnóstico de la limitación al flujo aéreo que caracteriza a la EPOC, es necesaria la realización de una espirometría forzada. En ella, observamos un descenso del cociente entre el volumen espirado en el primer segundo (FEV_1) y la capacidad vital forzada (FVC), que es inferior a 70 tras la prueba broncodilatadora (Agusti, 2022).

2. **Epidemiologia de la EPOC**

La EPOC constituye un importante desafío de salud pública mundial al representar una causa importante de morbilidad y discapacidad ya que muchos pacientes que la padecen durante años mueren prematuramente debida a la

propia enfermedad o a sus complicaciones (Mathers & Loncar, 2006; Pauwels et al., 2001). La prevalencia de la EPOC es de un 10% de la población en edad adulta en países desarrollados (Buist et al., 2007;). En nuestro país asciende hasta un 12% aproximadamente (Soriano et al., 2020). Además, se prevé que la prevalencia aumente en los años próximos (Miravitlles et al., 2017; Miravitlles & Soler-Cataluña, 2017; Vogelmeier et al., 2017).

3. <u>Clasificación de la gravedad de la EPOC</u>

3.1 <u>Valoración unidimensional</u>

Inicialmente se ha clasificado el estadio de la EPOC en base a la gravedad de la limitación del flujo aéreo, es decir, en función del FEV_1 obtenido en estabilidad clínica (% del valor de referencia, teniendo en cuenta edad, peso, talla, género y etnia).

En el consenso entre *European Respiratory Society* (ERS) y *American Thoracic Society* (ATS) de 2004 se definieron los siguientes estadios de gravedad (Celli & MacNee, 2004).

GRAVEDAD de la EPOC	% FEV_1/FVC	FEV_1 (%ref)
En riesgo	>70	>80%
Leve	<70	>80%
Moderado	<70	80-50%
Grave	<70	30-50%
Muy grave	<70	<30%

Tabla 1: Clasificación de la gravedad de la EPOC según la ATS-ERS 2004

3.2 *Valoración multidimensional*

La evidencia científica ha puesto de manifiesto, que valorar la gravedad de la EPOC teniendo en cuenta únicamente el grado de obstrucción de la vía aérea mediante la medición del FEV_1, no es suficiente. Es necesaria la valoración de otros parámetros como los síntomas, el riesgo de exacerbaciones, la presencia de comorbilidades, el estado nutricional o la capacidad de ejercicio del paciente. Esto ha motivado el desarrollo de diferentes clasificaciones multidimensionales de la EPOC. A continuación, se describen las principales clasificaciones multidimensionales con implicaciones pronósticas.

3.2.1 *Índice BODE*

El índice BODE (*Body mass index, Airflow Obstruction, Dysnea and Excercise capacity*), se realiza una valoración de la EPOC en la que no solo se tiene en cuenta la esfera pulmonar. Engloba cuatro variables (Celli et al., 2004): estado nutricional evaluado por el índice de masa corporal (IMC), grado de obstrucción de vía aérea (FEV_1) escala disnea (mMRC, *modificada del Medical Research Council*) y la capacidad de esfuerzo (metros caminados en la prueba de marcha de 6 minutos).

En la siguiente tabla se ilustran las 4 variables que componen el índice BODE y los valores de las mismas:

	0	1	2	3
FEV_1 (%)	≥65	50-64	36-49	≤35
Distancia, prueba 6 minutos (m)	≥350	250-349	150-249	≤149
IMC (Kg/m^2)	<21	≤21		
Puntuación escala disnea mMRC	0-1	2	3	4

Tabla 2: Variables y valores asignados para el cálculo del índice BODE

Se realiza la suma de puntos asignados a cada una de las cuatro variables que componen el índice. De esta forma se clasifican los pacientes con EPOC en cuartiles según el valor del índice BODE (Tabla 3).

Puntuación en escala BODE	Mortalidad global al año
0-2 puntos	20%
3-4 puntos	30%
5-6 puntos	40%
7-10 puntos	80%

Tabla 3: Mortalidad según la puntuación del índice BODE

El índice BODE, es capaz de predecir la mortalidad en pacientes con EPOC, tanto por causas exclusivamente respiratorias como por todas las causas (Celli et al., 2004; JM, 2004).

3.2.2 Clasificación GOLD

La iniciativa global para la Enfermedad Pulmonar Obstructiva Crónica (GOLD) recomienda también la consideración de otros parámetros además del grado de obstrucción bronquial para una mejor valoración de la gravedad de la EPOC. Los parámetros en los que se basa son: el grado de obstrucción al flujo aéreo (FEV_1 postbroncodilatador), sintomatología y riesgo de exacerbaciones.
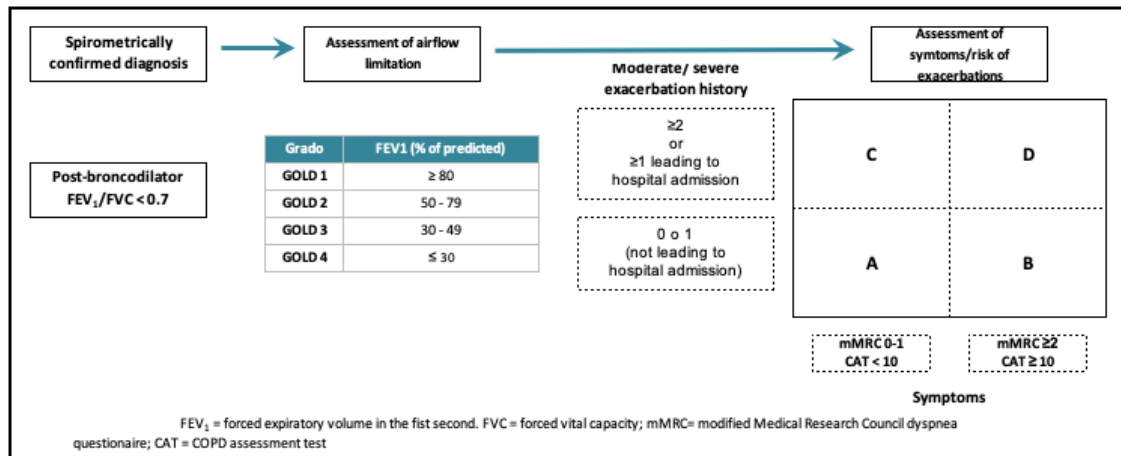
Figura 1: GOLD COPD Strategy 2020 (Singh et al., 2019)

- La valoración del grado de limitación al flujo aéreo se evalúa mediante FEV$_1$ postbroncodilatador. En base al valor obtenido se clasifica al paciente en uno de cuatro grupos: GOLD 1: FEV$_1$≥80% ref.; GOLD 2: FEV$_1$ entre el 80 y 50% ref.; GOLD 3: FEV$_1$ entre el 50 y 30% ref. y GOLD 4: FEV$_1$<30% ref.

- La evaluación de la sintomatología se realiza mediante cuestionarios validados para la EPOC (CAT – *COPD Assessment Test*) (Jones, 2013) o el cuestionario clínico para la EPOC (CCQ – *Clinical COPD Questionnaire*) (van der Molen et al., 2003). También se utiliza la escala mMRC que proporciona exclusivamente una evaluación de la disnea.

- La evaluación del riesgo de exacerbaciones se realiza mediante la recogida del número de estos episodios detectados y tratados en el año previo. Se valora la presencia de dos o más episodios moderados o uno o más graves por año. Se entiende por episodio moderado aquel que, de forma ambulatoria, es necesario tratar con corticoesteroides sistémicos y/o antibioticoterapia para su estabilización. Estos episodios no precisan de consulta a Urgencias o ingreso hospitalario. Se considera episodio grave aquel que requiere de consulta a Urgencias o ingreso en hospital para su estabilización.

Así pues, clasificamos los pacientes en 4 categorías: GOLD A, B, C o D, todos ellos con implicaciones terapéuticas (Singh et al., 2019).

3.2.3 Clasificación GesEPOC

La guía española de la EPOC (GesEPOC) propone en primer lugar: estratificar a los pacientes en alto o bajo riesgo, es decir en aquellos con mayor o menor probabilidad de presentar agudizaciones, progresión de enfermedad, complicaciones o mayor mortalidad.

Los factores que se evalúan para la estratificación del riesgo son, de igual modo que en la clasificación GOLD, el grado de obstrucción al flujo aéreo medido por el FEV_1 postbroncodilatador, el nivel de disnea medido por la escala mMRC y la presencia de agudizaciones en el último año. A mayor nivel de riesgo, mayor necesidad de intervenciones terapéuticas.

En la siguiente figura se muestran los valores de los factores a considerar para la estratificación de los pacientes en riesgo bajo o alto.
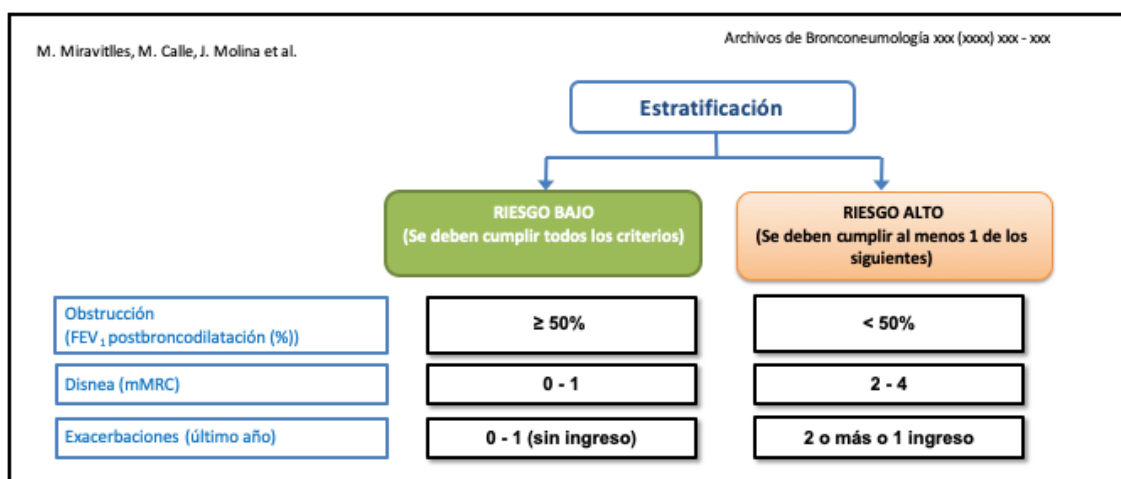

Figura 2: Estratificación del riesgo en pacientes con EPOC

En los pacientes de alto riesgo se realizan a su vez, 3 categorías llamadas fenotipos que son: no agudizador, agudizador eosinofílico, agudizador no eosinofílico (Miravitlles et al., 2021).

4. <u>Comorbilidades</u>

Con frecuencia, los pacientes con EPOC asocian enfermedades crónicas y comorbilidades. Son de elevada importancia por su relevancia clínica e implicación pronóstica. Son responsables del impacto en el número de hospitalizaciones, el gasto sanitario y la mortalidad (Barnes & Celli, 2009; Mannino et al., 2008, 2015).

Es posible que factores etiológicos como el tabaco, la contaminación o la disminución de actividad física, sean algunas de las causas de las manifestaciones extrapulmonares y comorbilidades en la EPOC como por ejemplo la osteoporosis, la caquexia y la disfunción muscular entre otras. Estos factores también podrían influir en la aparición o empeoramiento de otras enfermedades concomitantes como la afectación cardiovascular, la ansiedad/depresión y el cáncer de pulmón (Barnes & Celli, 2009; Fry et al., 2012).

Más del 75% de los pacientes con EPOC grave y muy grave presentan algún tipo de comorbilidad, y un 45% tiene más de una (Kessler et al., 2011). La hipertensión arterial sistémica es la más frecuente (42%) seguida de problemas cardiovasculares (20%), diabetes mellitus (14%), osteoporosis (11%), depresión (9%) síndrome metabólico (9%) y cáncer (6%) (Kessler et al., 2011). Además, sólo un 61% de los pacientes recibe medicación específica para el tratamiento de estas comorbilidades (Kessler et al., 2011).

Una forma de evaluar las comorbilidades o enfermedades asociadas a la EPOC es mediante los índices de comorbilidad. Uno de los más usados es el índice de *Charlson*. Se trata de un sistema de evaluación de la supervivencia a largo plazo (en concreto a diez años) en función de la edad y de las comorbilidades del sujeto (Charlson et al., 2008). La comorbilidad medida por este índice constituye un factor predictor independiente de mortalidad en los pacientes con EPOC y se ha demostrado que guarda relación con una mayor probabilidad de ingreso hospitalario tras una exacerbación, con una mayor tasa de reingreso y con una estancia hospitalaria más prolongada (Almagro et al., 2012). Entre sus inconvenientes, sin embargo, se encuentra el hecho de que no incluye las patologías frecuentemente asociadas a la EPOC como la hipertensión arterial, las arritmias, la anemia, un índice de masa corporal bajo y trastornos mentales como la ansiedad y la depresión, por lo que las comorbilidades pueden quedar infravaloradas si se utiliza el índice de Charlson como único método de cuantificación (Almagro et al., 2010). Por eso, en 2012 se propuso el índice de COTE (*Comorbidity test*), que considera específicamente la comorbilidad asociada a la EPOC en relación con la supervivencia de los pacientes. De esta forma, ayuda a evaluar el riesgo de mortalidad en estos pacientes (Divo et al., 2012). Consta de 10 ítems y su valoración se obtiene sumando la puntuación asignada a las enfermedades presentes en el paciente. Entre ellas se incluyen la arritmia (la fibrilación auricular es frecuente en pacientes con EPOC), así como la ansiedad, proporcionando a esta última patología un gran peso, por su fuerte asociación con el riesgo de mortalidad. Una puntuación igual o mayor a 4 se asocia con un aumento del riesgo de muerte en 2,3 veces (Divo et al., 2012).

Se describirán brevemente las comorbilidades asociadas a los pacientes con EPOC que se han analizado en esta tesis, ya sea por su elevada prevalencia (alteraciones nutricionales y disfunción muscular) o por su relevancia clínica (cáncer de pulmón)**.**

4.1. Cáncer de pulmón

El tabaquismo es el principal factor etiológico que tienen en común tanto la EPOC como el cáncer de pulmón. No obstante, el incremento de riesgo de cáncer de pulmón en pacientes con EPOC se produce incluso en pacientes que nunca han fumado (Turner et al., 2007). Por lo tanto el riesgo de cáncer de pulmón en los pacientes con EPOC es independiente al tabaquismo (Turner et al., 2007).

Los pacientes con EPOC tienen entre 3 y 4 veces mayor riesgo de padecer cáncer de pulmón que la población general (Wasswa-Kintu et al., 2005) siendo éste una de las causas frecuente de muerte de estos enfermos (Calverley et al., 2007). Los tipos histológicos de cáncer de pulmón asociados a EPOC son el carcinoma escamoso y el de célula pequeña (Wasswa-Kintu et al., 2005).

4.2. Alteraciones nutricionales y disfunción muscular

Las alteraciones nutricionales son frecuentes en los pacientes con EPOC. Se han descrito problemas a nivel de ingesta calórica, como de composición corporal y de metabolismo basal e intermediario (A. M. Schols & Wouters, 2000). Pérdidas de peso sin causa aparente pueden ocurrir hasta en el 50% de pacientes con EPOC grave, pero también en el 10-15% de enfermos en estadios leve-moderado (Creutzberg et al., 1998). No obstante, existe una gran variabilidad geográfica en esta pérdida de peso de los pacientes con EPOC. Mientras que en EEUU, Europa del Norte y del Este el bajo peso parece afectar al 10-30% de los pacientes con EPOC (Vermeeren et al., 2006), en España se ha descrito que afecta únicamente al 2-3% de la población con la enfermedad (Coronell et al., 2002).

Actualmente existen tres formas de identificar alteraciones nutricionales asociadas a la EPOC (Gea et al., 2014):

- El porcentaje del peso del paciente sobre su peso ideal, aceptándose como bajo peso valores inferiores al 80-85%
- Índice de masa corporal (IMC) inferior a 18,5 kg/m^2, siendo grave por debajo de 16 kg/m^2 y muy grave si está por debajo de 15 kg/m^2
- Índice de masa libre de grasa (*Fat Free Mass Index* o FFMI) inferior a 16 kg/m^2 en varones y 15 kg/m^2 en mujeres

La pérdida de peso de los pacientes con EPOC se produce fundamentalmente a expensas de la disminución de masa muscular, siendo menos relevante la pérdida de grasa y masa ósea. También puede haber alteraciones en la composición corporal en ausencia de pérdida de peso, sobre todo en mujeres (Agustí, 2005; A. M. Schols & Wouters, 2000). La pérdida de masa muscular conlleva un empeoramiento en la calidad de vida y en el pronóstico de la enfermedad (A. M. Schols et al., 1998; Swallow et al., 2007).

La pérdida de masa muscular en pacientes con EPOC probablemente se debe a un desequilibrio entre la síntesis y destrucción de proteínas o proteólisis (Fig. 3) (Gea et al., 2014).
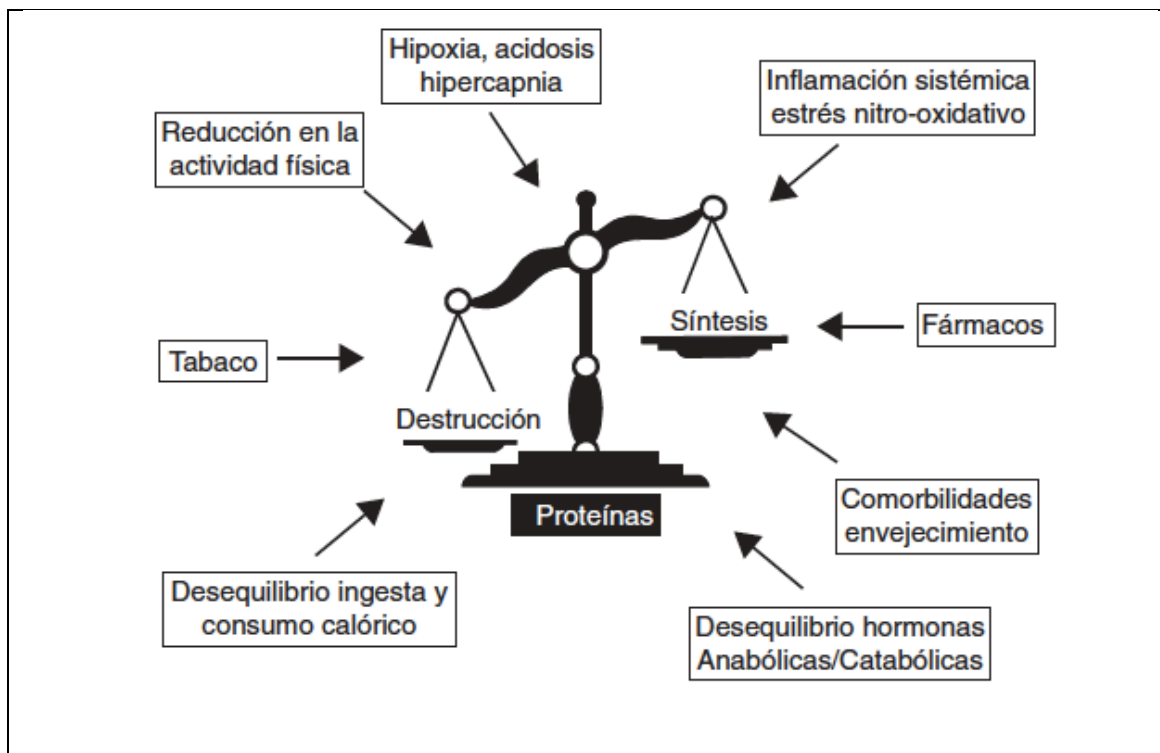
Figura 3: Factores implicados en la aparición del desequilibrio entre síntesis y degradación proteica en los pacientes con EPOC y bajo peso. Tomada de. *Gea J, Martínez-Llorens J, Barreiro E. Alteraciones nutricionales en la enfermedad pulmonar obstructiva crónica. Med Clin (Barc). 2014;143:78-84*

A continuación, se describen los diferentes mecanismos moleculares de proteólisis. El sistema más importante de destrucción proteica está ligado al **proteosoma**, que es un complejo encargado de realizar la degradación de aquellas proteínas que han sido señalizadas con anterioridad por el sistema de la ubiquitina o que han sido modificadas por el estrés oxidativo. Este mecanismo se encuentra claramente incrementado en los pacientes con EPOC y bajo peso (Fermoselle et al., 2012). En el tejido muscular de estos pacientes se ha observado también la presencia de un mayor número de núcleos con signos apoptóticos (Barreiro et al., 2011), lo que probablemente sea reflejo del proceso de recambio nuclear, con disminución de la capacidad de síntesis proteica. Otro mecanismo de proteolisis, es el de las **calpaínas** (enzimas muy presentes en el tejido muscular) que pueden llevar a una acción proteolítica

perjudicial en situaciones como la reducción de actividad física. Por el momento no existe evidencia de su papel en la EPOC. La **autofagia** (sistema autofagosoma-lisosoma) parece que se hallaría también anormalmente incrementado en los músculos de pacientes con EPOC que presentan bajo peso (Hussain & Sandri, 2013).

Las **alteraciones nutricionales** en pacientes con EPOC, pueden ocasionar alteraciones musculares mediante disminución y/o alteración en la síntesis de proteínas. Se ha descrito que las alteraciones nutricionales producen una disminución de la masa muscular con cambios en las proporciones y los tamaños de las fibras (Puig-Vilanova, Rodriguez, et al., 2015). Así también las alteraciones musculares producen una disfunción muscular (Remels et al., 2013).

La disfunción muscular se define como la incapacidad de un músculo para cumplir su cometido y se expresa como alteraciones en la fuerza, en la resistencia o en ambas (Maltais et al., 2014). La disfunción muscular en pacientes con EPOC constituye una de las comorbilidades más importantes, con repercusiones negativas en la capacidad de ejercicio, la calidad de vida y pronóstico (Gea et al., 2018, 2019; Gea & Martínez-Llorens, 2019; Gosselink et al., 1996; Kwan et al., 2019; Marquis et al., 2002; Seymour et al., 2010; Shrikrishna et al., 2012; Swallow et al., 2007). Es muy prevalente en los pacientes con EPOC, afectando a una tercera parte de estos enfermos, y aunque es más frecuente en estadios avanzados puede estar presente en fases iniciales de la enfermedad (Seymour et al., 2010).

La afectación a nivel de músculos periféricos conllevará la pérdida de fuerza en ellos (Vermeeren et al., 2006) así como una limitación en la actividad física y la capacidad de ejercicio (Gea et al., 2014). Sin embargo, la disfunción muscular

de los miembros inferiores difiere enormemente de la que se produce en los músculos respiratorios (Barreiro, 2019; Gea et al., 2019; Gea & Martínez-Llorens, 2019; Jaitovich & Barreiro, 2018). Los músculos de las extremidades inferiores suelen estar más gravemente afectados y tienen más implicaciones en sus actividades de la vida diaria (Barreiro, Bustamante, et al., 2015; Barreiro, 2017; Barreiro et al., 2018; Barreiro & Jaitovich, 2018; Jaitovich & Barreiro, 2018; Maltais et al., 2014). Mientras que en los músculos respiratorios, los pacientes EPOC con baja masa muscular pueden presentar problemas de ventilación, sobre todo durante el ejercicio y en las exacerbaciones.

La pérdida de masa muscular en pacientes con EPOC y su consiguiente pérdida de funcionalidad ocasionando la denominada sarcopenia, probablemente tiene un origen multifactorial (Gea et al., 2014; Macario et al., 2009).

A continuación, se describen los factores implicados en la pérdida de masa muscular y la consiguiente disfunción, también conocida como sarcopenia, en los pacientes con EPOC.

1. **Inflamación local y sistémica:** Pueden conducir a la activación de diferentes vías celulares produciendo atrofia y/o disfunción muscular mediante apoptosis, autofagia, estrés oxidativo y activación de sistemas catabólicos como el del proteosoma-ubiquitina (Gea et al., 2013). Los mediadores inflamatorios pueden activar diferentes procesos biológicos y vías metabólicas que favorecen las alteraciones en el estado nutricional (Gea et al., 2014). Por ejemplo, se ha demostrado que los niveles plasmáticos de TNF-α y de sus receptores I y II, así como de algunas otras citoquinas (IL-6 e IL-8) se encuentran más elevados en los pacientes con EPOC que presentan pérdida de peso que en sus controles con peso

normal (Barreiro et al., 2011; De Godoy et al., 1996; Di Francia et al., 1994; A. M. W. J. Schols et al., 1996; Swallow et al., 2007). Además el TNF-α pueden inhibir *per se* la contracción muscular (Reid et al., 2002). Sin embargo, el papel de las citoquinas a nivel local muscular no está claro. Algunos estudios sugieren que podrían participar no sólo en el daño y la disfunción muscular si no también en los mecanismo de reparación (Barreiro, 2019; Barreiro, Sznajder, et al., 2015; Donaldson et al., 2012; Gea et al., 2019; Gea & Barreiro, 2008; Gea & Martínez-Llorens, 2019; Jaitovich & Barreiro, 2018).

2. **Estrés oxidativo local y sistémico:** Cuando la presencia de radicales libres (se hallan presentes tanto a nivel sistémico como en el músculo y grasa de los pacientes con EPOC) supera la capacidad de los mecanismos antioxidantes, se producen lesiones de diferentes estructuras moleculares (proteínas, lípidos, ADN), con importantes consecuencias en la estructura y función celulares (Gea et al., 2009; Jackson & Farrell, 1993). El aumento de estrés oxidativo y nitrosativo (Barreiro et al., 2003, 2005; Fermoselle et al., 2012; Puig-Vilanova, Rodriguez, et al., 2015) y por otra parte, la reducción de la capacidad enzimática en las vías oxidativas (Natanek et al., 2013), contribuyen también a la disfunción muscular en los pacientes con EPOC.

3. **Estado nutricional:** Como ya se ha mencionado anteriormente, las alteraciones nutricionales son frecuentes en los enfermos con EPOC (A. M. Schols & Wouters, 2000). Estas alteraciones pueden ocasionar disminución de la masa muscular, cambios en las proporciones y tamaños de las fibras (Puig-Vilanova, Rodriguez, et al., 2015) y finalmente disfunción muscular (Remels et al., 2013).

4. **Decondicionamiento:** incide directamente en el estado nutricional y está asociado al pronóstico de la enfermedad (Laveneziana & Palange, 2012). Hoy en día se cree que es la causa principal de las alteraciones musculares periféricas que presentan los sujetos con EPOC. La reducción de la

actividad física ya sea secundaria a la limitación ventilatoria, al estilo de vida o a la depresión reactiva que a menudo acompaña a la enfermedades son muy frecuentes en los pacientes con EPOC (Garcia-Aymerich et al., 2004; Pitta et al., 2005). Los músculos esqueléticos de las extremidades inferiores de los pacientes con EPOC tienen alteraciones que son similares a los que presentan pacientes con inmovilización o desuso de un grupo muscular, produciendo fibras de menor tamaño y aumento de la proporción de las de tipo II (Bloomfield, 1997). Sin embargo, estos cambios pueden ser reversibles, al menos parcialmente con el entrenamiento (Maltais et al., 1996; Sala et al., 1999). Es más, se ha demostrado que el incremento de la actividad física puede favorecer también la recuperación nutricional (Ferreira et al., 2008).

5. **Cambios mecánica ventilatoria:** La longitud precisa a la que deben contraerse las fibras musculares del diafragma determina en gran medida las diferencias entre la disfunción muscular respiratoria y la de las extremidades (Barreiro & Jaitovich, 2018; Gayan-Ramirez & Decramer, 2013; Gea et al., 2013; Jaitovich & Barreiro, 2018). Como tal, la limitación del flujo de aire impone mayores cargas inspiratorias que modifican la geometría del tórax y conducen a un acortamiento de la longitud del músculo del diafragma (Barreiro & Jaitovich, 2018; Gayan-Ramirez & Decramer, 2013; Gea et al., 2013; Jaitovich & Barreiro, 2018). Además, las cargas inspiratorias continuas a las que están expuestos los pacientes también inducen un efecto similar al del entrenamiento en el diafragma que puede compensar en parte los efectos nocivos de otros factores y hacer que el músculo sea más resistente a la fatiga desde el punto de vista estructural y molecular, al menos en las primeras fases de la enfermedad (Gea et al., 2013; Similowski et al., 1991).

6. **Hipoxia-hipercapnia:** La hipoxia puede influir en la producción de algunos péptidos implicados en el apetito (leptina, grelina y proteincinasa activada por AMP), puede reducir las concentraciones de determinadas hormonas

anabolizantes. También puede aumentar el nivel de inflamación sistémica y de estrés oxidativo, así como producir un desequilibrio proteico, apoptosis y alteraciones en la regeneración muscular (Brunelle & Chandel, 2002; Gea & Barreiro, 2008; Gonzalez & Wood, 2010; Kulisz et al., 2002; Yun et al., 2005). Todo ello puede conducir a una reducción en la fuerza y en la resistencia musculares. La hipercapnia por su parte puede producir también disfunción muscular (Rafferty et al., 1999), aunque en general, ésta última es producto de la acidosis secundaria, que induce un descenso en la reserva energética y desequilibrio en la síntesis y degradación proteica (England et al., 1991; Gea et al., 2014) y afecta también directamente a la contracción muscular (Rafferty et al., 1999).

7. **Hormonas anabolizantes:** La hormona del crecimiento (GH) aumenta la producción y acciones del factor de crecimiento asociado a la insulina (IGF-1), que interviene en la síntesis proteica e inhibe la degradación. Aunque los niveles de GH pueden ser normales en los pacientes con EPOC, la interacción entre GH e IGF-1 parece estar alterada (Creutzberg & Casaburi, 2003). Por otra parte, la testosterona, hormona anabolizante que aumenta la síntesis proteica muscular, podría también estar disminuida en algunos pacientes con EPOC, contribuyendo así a reducir su masa muscular (Laghi et al., 2005).

8. **Comorbilidades y envejecimiento:** Factores externos a la EPOC, pero frecuentemente asociados a ella, como posibles etiologías adicionales de la pérdida ponderal, masa muscular y disfunción muscular. Se trata de enfermedades muy prevalentes como la insuficiencia cardiaca, la diabetes mellitus o el cáncer de pulmón. Por otra parte, el envejecimiento *per se* implica una pérdida de masa muscular y atrofia de las fibras musculares (Morley, 2012; Puig-Vilanova, Rodriguez, et al., 2015; Rehn et al., 2012; Sun et al., 2008).

9. **Exacerbaciones:** Es uno de los factores que se cree contribuye de forma determinante a la disfunción muscular, tanto por su propio efecto, al aumentar la inflamación y estrés oxidativo, como por otros factores asociados a ella como son el encamamiento y los fármacos esteroideos (Gea et al., 2013; Maltais et al., 2014; Man et al., 2009). Estos fármacos utilizados de forma sistémica principalmente durante las exacerbaciones, inhiben la síntesis proteica y activan el catabolismo causando una miopatía y/o pérdida de masa muscular (Gea et al., 2014). Los fármacos agonistas β2 por su parte, pueden aumentar también el gasto energético incluso en reposo (Amoroso et al., 1993). Por otra parte, los pacientes con EPOC con mayor grado de disfunción muscular también tienen un riesgo superior de exacerbaciones (Vilaró et al., 2010).

10. **Tabaco:** El tabaco es un anorexígeno de efecto central. Se ha descrito un desequilibrio energético, con aumento del gasto energético basal que se estima en un 120% del normal. Puede también dar lugar a pérdida de masa muscular aumentando la proteólisis, la apoptosis, la autofagia y a través de mecanismos epigenéticos (Barreiro et al., 2011; Barreiro & Gea, 2014; Fermoselle et al., 2012; Guo et al., 2013). También produce un aumento de la inflamación sistémica y del estrés oxidativo (Barreiro et al., 2010). Todo ello disminuye la proporción de fibras de tipo I, lesiona las fibras y reduce la actividad mitocondrial (Barreiro & Gea, 2014).

11. **Alteraciones epigenéticas:** Diversas modificaciones epigenéticas identificadas hasta el momento, como la metilación de ADN y la acetilación y metilación de histonas, pueden contribuir a la miogénesis, así como a la respuesta frente a la inmovilización o ejercicio en los pacientes con EPOC contribuyendo a la estructura final del músculo (Puig-Vilanova, Ausin, et al., 2014; Puig-Vilanova, Martínez-Llorens, et al., 2015).

12. **Disminución capacidad regeneración de los músculos esqueléticos:** Los músculos esqueléticos tienen un potencial regenerativo tras lesionarse.

Los mioblastos progenitores se fusionan durante el desarrollo para formar los músculos esqueléticos. Las células satélite (SC) quiescentes pueden activarse, e iniciar el proceso de miogénesis. El número de células satélite se modifica en condiciones específicas como el envejecimiento (Suetta et al., 2013), la atrofia muscular por desuso (Snijders et al., 2014), el reposo prolongado en cama (Arentson-Lantz et al., 2016) y la exposición al tabaco (Chan et al., 2020). En los músculos intercostales de pacientes con EPOC grave se detectó la activación de las células satélite junto con cambios microestructurales (Martínez-Llorens et al., 2008). Sin embargo, la regeneración muscular y los recuentos de células satélite se redujeron en el vasto lateral (VL) de pacientes con EPOC con composición corporal preservada (Menon et al., 2012; Thériault et al., 2012, 2014).

Diversos factores regulan la actividad de estas células satélite, produciendo su activación como manteniéndose en situación quiescente. Los principales factores de regulación de la fase miogénica inicial de proliferación son el Six1/4, Pax3 (*Paired box protein*) y Pax7, mientras que Myf5 (factor 5 miogénico) y MyoD (proteína 1 de diferenciación miogénica) conducen las células hacia el proceso de diferenciación. Esta última fase conlleva también la fusión de los miocitos formando miotubos y se lleva a cabo a través de los factores miogenina (MyoG) y el MRF4 (factor miogénico regulador 4) entre otros (Bentzinger et al., 2012).

La miostatina es un potente regulador negativo de la masa muscular en los mamíferos. La miostatina mantiene la quiescencia de las células satélite, mientras que su ausencia desencadena la activación de las células satélite. La supresión del gen de la miostatina favorece la masa muscular y puede conducir a la hipertrofia muscular (Ohno et al., 2016). Además, el bloqueo de la miostatina provocó una mejora en el potencial regenerativo de los músculos de las extremidades de los ratones tras una lesión inducida por cardiotoxinas (Ohno et al., 2016). Recientemente, también se ha demostrado que la miostatina desempeña un papel clave en la sarcopenia y se sugirió que su bloqueo mejoraba la regeneración muscular tras una lesión (Scimeca et al., 2017). Además, la inhibición de la miostatina redujo

la atrofia muscular a través de la regulación al alza de los marcadores implicados en la regeneración muscular en los músculos de las extremidades de las ratas (Wurtzel et al., 2017).

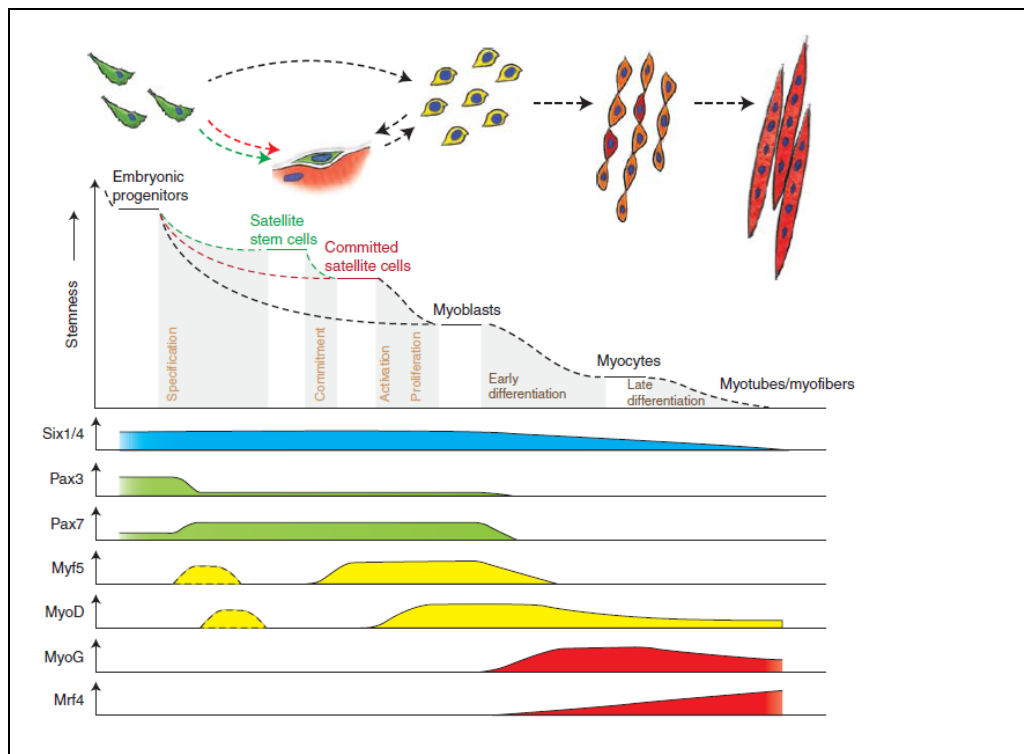Aunque actualmente se desconocen si estos factores están implicados en los pacientes con EPOC.

Figura 4: Regulación del proceso de miogénesis por los factores de trascripción. Tomada de *Bentzinger CF1, Wang YX, Rudnicki MA.Building muscle: molecular regulation of myogenesis Alteraciones nutricionales en la enfermedad pulmonar obstructiva crónica. Cold Spring Harb Perspect Biol. 2012 Feb 1;4(2).*

13. Alteraciones funcionamiento del Retículo Endoplásmico celular:

En situaciones de inmovilización en modelos animales en los que se detecta pérdida de masa y la consiguiente disfunción muscular (sarcopenia), se han descrito alteraciones en el funcionamiento del

retículo endoplásmico (RE). El retículo endoplásmico (RE) es un orgánulo intracelular que se encarga del plegado, procesamiento y tráfico de proteínas dentro de las células. Está implicado en la regulación de la masa, la función y el metabolismo del músculo esquelético (Afroze & Kumar, 2019).

La acumulación de proteínas no plegadas/mal plegadas puede ser el resultado de alteraciones en la homeostasis celular (envejecimiento, infecciones, hipoxia, desórdenes metabólicos, etc.).

Las chaperonas y las foldasas normalmente restauran el plegado de las proteínas. Sin embargo, cuando estas no pueden realizar el correcto plegado de las proteínas en el contexto de una enfermedad crónica o aguda, se conduce a la degradación de estas proteínas por el RE a través de varias vías.

El mecanismo de respuesta a proteínas no plegadas/mal plegadas (UPR) se activa al acumularse proteínas no plegadas dentro de las células eucariotas (Chakrabarti et al., 2011; Kelsen, 2016). La UPR forma parte de un programa de señalización que se ejecuta mediante la acción de tres receptores transmembrana del RE con funciones distintas (Chakrabarti et al., 2011; Kelsen, 2016). Se ha demostrado que la UPR inducida por el RE señala el desarrollo y la regeneración muscular (Bohnert et al., 2018; Nakanishi et al., 2005; Xiong et al., 2017). También se ha descrito elevada activación de los marcadores de la UPR en los músculos esqueléticos de ratones con caquexia inducida por cáncer de pulmón. Se ha inducido un fenotipo menos resistente a la fatiga en los músculos de los mismos animales bloqueando la UPR con 4-fenilbutirato (Bohnert et al., 2016). Estos resultados sugieren que la UPR desempeña

un papel clave en el mantenimiento de la masa muscular, al menos en modelos experimentales de caquexia. El estrés del RE desempeña un papel en el mantenimiento de la fisiología muscular, en el envejecimiento y en la adaptación a la actividad y el metabolismo muscular y al ejercicio (Afroze & Kumar, 2019; Almanza et al., 2019; Bohnert et al., 2016, 2018). La actividad contráctil crónica induce un aumento de la expresión de los marcadores de estrés del RE y de la UPR en animales (Memme et al., 2016) y humanos (Kim et al., 2011). El estrés del RE y la UPR también están implicados en la fisiopatología de ciertas miopatías (Afroze & Kumar, 2019). Queda por determinar si estos factores pueden desempeñar un papel en la disfunción muscular respiratoria de los pacientes con enfermedades respiratorias crónicas como la EPOC.

HIPÓTESIS

GENERAL:

En los músculos de las extremidades inferiores (cuádriceps) y también en los inspiratorios (diafragma) de los pacientes con EPOC y sarcopenia, existirán alteraciones en la regeneración muscular; y también a nivel intracelular en el procesamiento proteico por parte del retículo endoplasmático.

ESPECIFICAS:

Manuscrito 1:

El potencial de regeneración muscular estará disminuido en el vasto lateral (VL) de los pacientes con EPOC, especialmente en aquellos que, a su vez, presentan sarcopenia.

Manuscrito 2:

La expresión de los marcadores de estrés del retículo endoplasmático (RE) y de la respuesta compensatoria a proteínas no plegadas/mal plegadas (UPR) estará regulada de forma diferente en el vasto lateral (VL) de los pacientes con sarcopenia asociada a enfermedades respiratorias de carácter crónico (EPOC) o subagudo (cáncer de pulmón).

Manuscrito 3:

La disfunción muscular respiratoria asociada a la EPOC inducirá diferentes niveles de expresión de estrés a nivel de RE y UPR en el diafragma, y será variable en función de la gravedad de la obstrucción bronquial.

OBJETIVOS

Manuscrito 1:

- Analizar en el vasto lateral de pacientes EPOC grave con un amplio rango de composición corporal, incluyendo aquellos con sarcopenia, los siguientes parámetros:

  1. Cuantificación de células satélite (potencial regenerativo) y progenitores comprometidos.

  2. Marcadores de regeneración muscular y miostatina

  3. Alteraciones a nivel de estructura y fenotipo muscular

- Analizar en el vasto lateral de sujetos sanos los siguientes parámetros:

  1. Cuantificación de células satélite (potencial regenerativo) y progenitores comprometidos.

  2. Marcadores de regeneración muscular y miostatina

  3. Alteraciones a nivel de estructura y fenotipo muscular

- Comparación entre las alteraciones a nivel de regeneración del vasto lateral de los pacientes con EPOC y sujetos sanos.

Manuscrito 2:

- Explorar en muestras de vasto lateral de pacientes con EPOC con y sin sarcopenia, diferentes alteraciones a nivel de estrés del retículo endoplasmático (RE), mediante análisis de los siguientes parámetros:

  1. Marcadores de estrés de RE
  2. Marcadores de las vías UPR (ATF6, PERK, IRE1)
  3. Marcadores de estrés oxidativo, autofagia, apoptosis y proteólisis
  4. Fenotipo muscular (tipos de fibras y morfometría)

- Analizar en pacientes con cáncer de pulmón las alteraciones en el retículo endoplasmático (RE), mediante la valoración de los siguientes parámetros:

  1. Marcadores de estrés de RE
  2. Marcadores de las vías UPR (ATF6, PERK, IRE1)
  3. Marcadores de estrés oxidativo, autofagia, apoptosis y proteólisis
  4. Fenotipo muscular (tipos de fibras y morfometría)

- Comparación entre las alteraciones a nivel de retículo endoplasmático del vasto lateral de los pacientes con EPOC y cáncer de pulmón.

Manuscrito 3:

- Explorar en el diafragma de pacientes con EPOC, en función de la gravedad de la enfermedad, las alteraciones en el retículo endoplasmático (RE), mediante la valoración de los siguientes parámetros:

    1. Marcadores de estrés de RE
    2. Marcadores de las vías UPR (ATF6, PERK, IRE 1)
    3. Marcadores de apoptosis y proteólisis
    4. Estructura muscular (tipo de fibras y morfometría) y agregados de lipofucsina.

- Comparar las altercaciones a nivel de retículo endoplasmático del diafragma de los pacientes con EPOC con los sujetos control.

METODOLOGÍA Y RESULTADOS

MANUSCRITO 1

ORIGINAL RESEARCH ARTICLE

Cellular Physiology WILEY

Deficient muscle regeneration potential in sarcopenic COPD patients: Role of satellite cells

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Abstract

Sarcopenia is a major comorbidity in chronic obstructive pulmonary (COPD). Whether deficient muscle repair mechanisms and regeneration exist in the vastus lateralis (VL) of sarcopenic COPD remains debatable. In the VL of control subjects and severe COPD patients with/without sarcopenia, satellite cells (SCs) were identified (immunofluorescence, specific antibodies, anti-Pax-7, and anti-Myf-5): activated (Pax-7+/Myf-5+), quiescent/regenerative potential (Pax-7+/Myf-5-), and total SCs, nuclear activation (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling [TUNEL]), and muscle fiber type (morphometry and slow- and fast-twitch, and hybrid fibers), muscle damage (hematoxylin-eosin staining), muscle regeneration markers (Pax-7, Myf-5, myogenin, and MyoD), and myostatin levels were identified. Compared to controls, in VL of sarcopenic COPD patients, myostatin content, activated SCs, hybrid fiber proportions, TUNEL-positive cells, internal nuclei, and muscle damage significantly increased, while quadriceps muscle strength, numbers of Pax-7+ /Myf-5- and slow- and fast-twitch, and hybrid myofiber areas decreased. In the VL of sarcopenic and nonsarcopenic patients, TUNEL-positive cells were greater, whereas muscle regeneration marker expression was lower than in controls. In VL of severe COPD patients regardless of the sarcopenia level, the muscle regeneration process is triggered as identified by SC activation and increased internal nuclei. Nonetheless, a lower regenerative potential along with significant alterations in muscle phenotype and damage, and increased myostatin were prominently seen in sarcopenic COPD.

KEYWORDS
COPD, lower limb muscles, muscle regeneration markers, myostatin, satellite cells

1 | INTRODUCTION

Sarcopenia is a major comorbidity commonly associated with chronic respiratory diseases including chronic obstructive pulmonary (COPD). The reduction of muscle mass and strength of the lower limbs differs vastly from that taking place in the respiratory muscles (Esther Barreiro, 2019; Gea & Martínez-Llorens, 2019; Gea et al., 2019; Jaitovich & Barreiro, 2018). Sarcopenia limits the exercise capacity of patients, thus having a negative impact on their daily-life activities and quality of life (Kwan et al., 2019; Shrikrishna et al., 2012). Furthermore, sarcopenia predicts death for the same degree of disease severity as measured by the level of airway obstruction (Marquis et al., 2002; Swallow et al., 2007).

In the multifactorial etiology of sarcopenia associated with COPD, several clinical factors and conditions inherent to the respiratory disease, namely deconditioning, exert deleterious effects on

the limb muscles through biological events and mechanisms as previously reported in relevant investigations. Most of the investigations have focused on the elucidation of factors and mechanisms primarily involved in the process of muscle mass loss in the limb muscles of the patients (Barreiro, 2019; Barreiro et al., 2015; Gea & Martinez-Llorens, 2019; Gea et al., 2019; Jaitovich & Barreiro, 2018) and animal models (Chacon-Cabrera et al., 2014, 2016) Nonetheless, few studies have addressed the issue of the ability of the skeletal muscles to regenerate, let alone in muscles of COPD patients. The regenerative potential of a muscle will determine its capacity to regenerate following injury.

The progenitor myoblasts fuse during development to form skeletal muscles. Under normal conditions, in adult muscles, the sporadic fusion of satellite cells (SCs), defined as postnatal muscle stem cells, takes place to replace the muscle turnover related to daily life activity. As skeletal muscle has the ability to regenerate following injury, regeneration of muscle tissue is a very finely regulated process in adults. The interaction between SCs and the microenvironment is the basis of muscle regeneration. As such, SC numbers have been shown to be modified under specific conditions such as in aging (Suetta et al., 2013), disuse muscle atrophy (Snijders et al., 2014), prolonged bed rest (Arentson-Lantz et al., 2016), and exposure to cigarette smoking (Chan et al., 2020). These modifications may alter the process of muscle regeneration in those conditions, which in turn, may coincide in patients with respiratory diseases such as in COPD.

The population of SCs is not homogeneous. Indeed, it has been proposed that SCs are heterogeneous populations of stem cells and committed progenitor cells that play different roles in the process of muscle regeneration. The activity of the skeletal muscle influenced the numbers of SCs in different models. For instance, aging hampered muscle regrowth following a period of disuse muscle atrophy in healthy humans (Suetta et al., 2013). In middle-aged adults, a decline in SC counts was observed in the atrophic muscles following a 14-day period of bed rest (Arentson-Lantz et al., 2016). Nonetheless, changes in SC counts were not detected in the quadriceps muscle of healthy young subjects following a 2-week period of one-legged knee immobilization (Snijders et al., 2014).

Importantly, in the intercostal muscles of severe COPD patients, activation of SCs along with microstructural changes was detected (Martinez-Llorens et al., 2008). However, muscle regeneration and SC counts were reduced in the vastus lateralis (VL) of patients with COPD with preserved body composition (Menon et al., 2012; Thériault et al., 2012, 2014). Whether muscle regeneration events and SC counts and types may differ in the lower limb muscles of patients with a different degree of disease severity and/or body composition remains to be fully elucidated.

Myostatin is a potent negative regulator of muscle mass in mammals. Myostatin maintains the quiescence of SCs, whereas its absence triggers activation of SCs. Deletion of myostatin gene favors muscle mass and may lead to muscle hypertrophy (Ohno et al., 2016). Moreover, myostatin blockade elicited an improvement in the regenerative potential of limb muscles of mice following cardiotoxin-induced injury (Ohno et al., 2016). Recently, it has also been demonstrated that myostatin plays a key role in sarcopenia and it was suggested that its blockade improved muscle regeneration following injury (Scimeca et al., 2017). Moreover, myostatin inhibition reduced muscle atrophy through upregulation of markers involved in muscle regeneration in limb muscles of rats (Wurtzel et al., 2017).

On this basis, we hypothesized that the muscle regenerative potential as measured by the number of stem cells and other markers of muscle regeneration may be altered in the quadriceps of patients with COPD, especially in those with sarcopenia. Furthermore, protein levels of the potent negative regulator of muscle regeneration myostatin were also assessed in the VL of sarcopenic COPD patients. Accordingly, the study objectives were that in the VL of severe COPD patients with a wide range of body composition, including those with sarcopenia, the following events were analyzed: (1) identification and counts of stem cells (regenerative potential) and committed progenitors, (2) markers of muscle regeneration and myostatin, and (3) muscle structural abnormalities and phenotype. A group of control subjects was also recruited for the purpose of the investigation, in whom muscle biopsies were also obtained and analyzed accordingly.

2 | METHODS

2.1 | Study design and population

This was a prospective, controlled, cross-sectional study, in which 45 patients (25 males) with stable COPD (Miravitlles et al., 2017; Vogelmeier et al., 2017) were recruited consecutively from the COPD clinics of the Respiratory Department at Hospital del Mar (Barcelona) over the years 2018–2019. Additionally, 13 age-matched control subjects (six males) were recruited from the general population (patients' relatives or friends) at Hospital del Mar. COPD patients were further subdivided into those with and without loss of muscle mass and strength (sarcopenic COPD, $n = 26$, 11 males and nonsarcopenic COPD, $n = 19$, 14 males) following the international consensus criteria on muscle wasting and sarcopenia (Cao & Morley, 2016; Muscaritoli et al., 2010) and previously published criteria (Esther Barreiro et al., 2019; Puig-Vilanova, Martinez-Llorens, et al., 2015).

In all patients, reduced muscle mass was defined as a fat-free mass index (FFMI) $\le 18 \, kg/m^2$, a cut-off value established for a Mediterranean population in accordance with both previously published criteria (Esther Barreiro et al., 2019; Puig-Vilanova, Martinez-Llorens, et al., 2015) and the international consensus on the definition of sarcopenia (Cao & Morley, 2016; Muscaritoli et al., 2010). Moreover, muscle weakness was defined on the basis of previous investigations (approximately 25% reduction in quadriceps force compared to that observed in control subjects; Puig-Vilanova, Martinez-Llorens, et al., 2015; Seymour et al., 2010). Control subjects were never-smoker male and female sedentary control individuals recruited from the general population (patients' relatives or friends), while patients in both groups were active smokers or

ex-smokers. All patients were on inhaled bronchodilators. They were clinically stable at the time of the study, without episodes of exacerbation or oral steroid treatment in the previous 3 months. None of them presented significant comorbidities. All groups of individuals were Caucasian.

2.1.1 | Exclusion criteria

Exclusion criteria for COPD patients and control subjects included other chronic respiratory or cardiovascular disorders, acute exacerbations in the last 3 months, limiting osteoarticular condition, chronic metabolic diseases, suspected para-neoplastic or myopathic syndromes, and/or treatment with drugs known to alter muscle structure and/or function including oral corticosteroids. COPD patients and control subjects were qualified as sedentary after being specifically inquired about whether they were conducting any regular outdoor physical activity, going regularly to the gymnasium, or participating in any specific training program. Specifically, sedentarism was defined on the basis of the following criteria: (1) if subjects were not engaged in one or more of these activities: walking, running, bike riding, swimming, dancing, gardening, or weight lifting more than five times per week, (2) not performing at least 3 h/week of endurance-type physical activity, and/or (3) inactive general state in which leisure-time physical activity was minimal (Ricciardi, 2006). Moreover, the time spent in sedentary postures (lying and sitting) was also considered in the assessment of sedentarism in the study groups (Ricciardi, 2006).

2.2 | Ethics

The current investigation was designed in accordance with both the ethical standards on human experimentation in our institution and the World Medical Association guidelines (Seventh revision of the Declaration of Helsinki, Fortaleza, Brazil, 2013) (Shrestha & Dunn, 2020) for research on human beings. Approval was obtained from the institutional Ethics Committee on Human Investigation (Hospital del Mar-IMIM, Barcelona, project number 2018/7937/I). Informed written consent was obtained from both patients and control subjects.

2.3 | Anthropometric and functional assessment

Anthropometric evaluation included body mass index (BMI) and determination of FFMI using bioelectrical impedance (Esther Barreiro et al., 2019; Puig-Vilanova, Martínez-Llorens, et al., 2015). Nutritional parameters were also evaluated through conventional blood tests. Diagnostic criteria for sarcopenia were BMI < 21 kg/m^2 and FFMI < 18 kg/m^2 in all patients (Esther Barreiro et al., 2019; Coin et al., 2008; Puig-Vilanova, Martínez-Llorens, et al., 2015).

Lung function was evaluated through determination of spirometry, static lung volumes, diffusion capacity, and blood gases using standard procedures and well-established reference values (Roca et al., 2014; Roca, Burgos, Barberà, et al., 1998; Roca, Burgos, Sunyer, et al., 1998).

Quadriceps muscle strength was evaluated in both patients and controls through the determination of the isometric maximum voluntary contraction (QMVC) of the dominant lower limb as formerly described (Esther Barreiro et al., 2019; Puig-Vilanova, Martínez-Llorens, et al., 2015). Briefly, patients were seated with both trunk and thigh fixed on the rigid support of an exercise platform (Domyos HGH 050; Decathlon). The highest value from three brief reproducible maneuvers (<5% variability among them) was accepted as the QMVC.

2.3.1 | Exercise capacity

Exercise capacity was assessed through the 6-min walking distance following previous methodologies (Rodriguez et al., 2012). The test consisted of two attempts (with at least a 30-min rest between them) in a 30-meter corridor. Encouragement was given every minute and the test was interrupted if symptoms of exhaustion appeared. A modified Borg scale was used to quantify the levels of dyspnea and leg discomfort.

Maximum exercise capacity was also measured using standardized incremental on a cycle ergometer (Jones et al., 1985; Rodriguez et al., 2012). Pulmonary gas exchange and ventilatory measurements were obtained from calibrated signals derived from a rapid response gas analyzer and a mass flow sensor. Oxygen uptake (VO_2), pulmonary carbon dioxide output (VCO_2), minute ventilation (VE), respiratory exchange ratio (RER) were also registered during each respiration. Heart rate was determined using a 10-lead online electrocardiogram and oxygen saturation by pulse oximetry (SpO_2). After 1 min of breathing at rest, subjects pedaled on an electrically braked cycle ergometer (Ergoline Ergometrix 900; Uberprüfung) (Jones et al., 1985; Rodriguez et al., 2012). An integrated computer recorded cardiorespiratory variables during the test (Ultima; MedGraphics Corporation). Patients were encouraged to continue until they could no longer sustain the target pedaling load.

2.4 | Blood samples and muscle biopsy

Patients and control subjects rested for 1 h on a chair with legs half-flexed, a time at which blood samples were obtained, after an overnight fasting period, right before initiation of the surgical procedures (Esther Barreiro et al., 2019; Puig-Vilanova, Martínez-Llorens, et al., 2015). Specimens from the VL portion of the quadriceps muscle (50–100 mg average weight) were obtained from all subjects using the open biopsy technique as previously described (Esther Barreiro et al., 2019; Puig-Vilanova, Martínez-Llorens, et al., 2015). Muscle specimens were always cleaned out of any blood contamination with saline. They were immediately frozen in liquid nitrogen and stored in

the −80°C freezer (under permanent alarm control) for further analyses or immersed in an alcohol-formol bath for 2 h to be thereafter embedded in paraffin. Frozen tissues were used for messenger RNA (mRNA) expression and immunoblotting techniques, while paraffin-embedded tissues were used for the assessment of structural modifications (immunohistochemical analysis).

2.5 | Biological analyses

2.5.1 | Muscle fiber counts and morphometry

Myosin heavy chain I (MyHC-I) and MyHC-II isoforms were identified in paraffin sections (3-μm thick) from VL muscles corresponding to the three study groups. The following antibodies were used: anti-MyHC-I (ab11083; Abcam) and anti-MyHC-II (ab51263; Abcam) antibodies. Myofibers positively stained appeared in brown color. The fibers positively stained with the two antibodies simultaneously were identified as the hybrid fibers. The cross-sectional area, calculated using the mean least diameter, and the proportions of type I, type II, and of the hybrid fibers were estimated with the aid of a light microscope (Olympus BX 61; Olympus Corporation) coupled with an image-digitizing camera (Pixera Studio; version 1.04; Pixera Corporation) and a digital image processing software (ImageJ; version 2006.02.01; National Institutes of Health). In all study groups, the number of fibers measured and counted in each muscle preparation ranged between 100 and 300 (Esther Barreiro et al., 2019; Puig-Vilanova, Martínez-Llorens, et al., 2015).

2.5.2 | Muscle structure abnormalities

Three-micrometer paraffin-embedded sections from the muscle specimens of the three study groups were used to assess the proportions of muscle abnormalities (Esther Barreiro et al., 2019; Puig-Vilanova, Martínez-Llorens, et al., 2015). A grid of 63 point-intercepts (7 × 9 rectangular pattern was superimposed onto the image of the muscle cross-section at a magnification of ×400 under the light microscope (Olympus BX 61) using an image digitizing camera (Olympus DP 71; Olympus Corporation). Each point-intercept was assigned to a specific category and entered into the software. Categories for point counting were defined as follows: (1) normal muscle, (2) internal nucleus, (3) inflammatory cell, (4) lipofuscin, (5) abnormal viable, (6) inflamed/necrotic, (7) vessel, and (0) no count. The area fraction for each category was defined as the percentage of points that fell on each of these traits relative to the total number of points superimposed on all viable fields (all features except for categories 0 and 7) of each cross-section. The area fraction of normal muscle was equivalent to the proportions of points falling in category 1, while the area fraction of abnormal muscle was determined by calculation of the proportion of points included in the other categories (categories 2–6).

2.5.3 | Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

In 3-μm paraffin-embedded sections of muscle specimens, activation of the myonuclei was determined using the TUNEL assay (ApopTag Peroxidase In Situ Apoptosis Detection Kit; Merck Millipore). The manufacturer's instructions and previously published studies were followed (Barreiro et al., 2011; Salazar-Degracia et al., 2016). In brief, during nuclear activation, fragments of genomic DNA can be generated. These strand breaks in the DNA sequence can be identified by labeling 3'-OH terminal groups with modified nucleotides in an enzymatic reaction catalyzed by the terminal deoxynucleotidyl transferase (TdT) enzyme. Muscle sections were fixed, permeabilized, and immediately incubated with the TUNEL Working Strength TdT Enzyme and the anti-Digoxigenin Conjugate. TdT catalyzed the adding of digoxigenin-dNTP at 3'-OH terminal groups in single- and double-stranded DNA. After washing, the sections were incubated with an antidigoxigenin antibody conjugated with peroxidase, which resulted in brown color upon reaction. Negative control experiments, in which the TdT enzyme was not added, were also performed. Apoptotic nuclei appeared in brown color, while negative nuclei were green (methyl green counterstaining). Only nuclei located within the muscle fiber boundary were counted in the study. Positive nuclei and the total number of nuclei were counted by two trained observers (correlation coefficient 95%). Apoptotic nuclei were expressed as the percentage of the TUNEL-positive nuclei to the total number of counted nuclei (Barreiro et al., 2011; Salazar-Degracia et al., 2016). A minimum of 300 nuclei was counted in each muscle specimen for all the study groups.

2.5.4 | Satellite cell identification using immunofluorescence microscopy

Specific antibodies were used to detect quiescently, activated, and total SCs using immunofluorescence as previously described (Guitart et al., 2018). Briefly, 3-μm paraffin sections of the muscle specimens of all study groups were deparaffinized and rehydrated by successive immersions in xylene, ethanol 100%, ethanol 90%, ethanol 70%, and phosphate-buffered saline (PBS). A pressure cooker containing 10 mM citrate buffer (pH 6.0) was used to boil the sections for 20 min. Following a 2-h cooling period, sections were blocked with blocking solution (3% bovine serum albumin [BSA], 10% goat serum, and 0.5% triton in PBS) for 1 h. The sections were then incubated overnight at 4°C with a mixture of two different primary antibodies: mouse monoclonal anti-Pax-7 IgG1k supernatant (1:20; Developmental Studies Hybridoma Bank) and rabbit polyclonal anti-Myf-5 IgG (1:100; AVIVA Systems), prepared in blocking solution. After incubation with the primary antibodies, the sections were incubated with the corresponding secondary antibodies at room temperature for 1 h: Alexa Fluor® 488 AffiniPure goat anti-mouse IgG, Fcγ Subclass 1 Specific (1:800; Jackson Immunoresearch), and Alexa Fluor® plus 555 goat ant-irabbit IgG (H + L) (1:1000; Thermo Fisher

Scientific) respectively, which were also prepared in blocking solution along with 4′,6′-diamidino-2-phenylindole (1:1000). Finally, the sections were mounted using 70% glycerol in 30% PBS. A fluorescence microscope (Nikon Eclipse Ni; Nikon) at a magnification of ×400 coupled with a digitizing camera was used to count the number of SCs. Anti-Pax-7 antibody alone was used to detect quiescent SCs, while the combination of anti-Pax-7 and anti-Myf-5 antibodies detected committed SCs (Guitart et al., 2018). The addition of quiescent and committed SCs corresponded to the total number of SCs. Results are expressed as follows: (1) Pax-7+/Myf-5- quiescent SCs, (2) Pax-7+/Myf-5+ activated SCs, and (3) the addition of both types of cells identified as total SCs. All types of fibers were normalized by the number of myofibers counted within the ten fields analyzed in each muscle preparation. The number of counted SCs ranged from 25 to 144 (quiescent), from 0 to 38 (activated), and from 31 to 165 (total SCs) for the 10 fields counted in each muscle preparation in a similar fashion in the three study groups.

2.5.5 | RNA isolation

Snap-frozen muscle specimens were used to isolate total RNA using Trizol reagent (Life technologies). Fifty mg of muscle samples diluted in 1 ml of Trizol reagent was homogenized using a T10 basic Ultra-Turrax Polytron (IKA®-Werke GmbH & Co. KG) at maximum speed for 20 s. The homogenized samples were incubated for a short period (10 min) at room temperature to allow the nuclear proteins to dissociate. Subsequently, 0.2 ml chloroform was added to the samples, which were vigorously shaken manually for 15 s to be incubated at room temperature for 3 min. The samples were centrifuged at 12,000 g at 4°C for 15 min. The centrifugation process separated the homogenates into a lower red organic phase and white interphase, and a colorless upper aqueous phase. RNA content remained in the aqueous phase. The RNA-containing aqueous phase was transferred to a fresh tube and the RNA was precipitated by adding 0.5 ml isopropanol. The samples were then centrifuged at 12,000 g at 4°C for 10 min. The pellet was kept and washed with 1 ml 75% ethanol and the samples were mixed using a vortex, to be thereafter centrifuged at 7,500 g at 4°C for 5 min. Finally, the RNA pellet was air-dried for 10 min and dissolved in RNase-free. Total RNA concentrations were determined spectrophotometrically using the NanoDrop 1000 (Thermo Fisher Scientific).

2.5.6 | mRNA reverse transcription (RT)

Complementary DNA was generated from 100 ng mRNA using oligo (dT) 12-18 primers, dNTP mix, dithiothreitol, and the Super-Script III reverse transcriptase as indicated by the manufacturer's instructions (Life Technologies). RNA was reverse-transcribed using SuperScript III reverse transcriptase and oligo(dT) primers in a 20-μl total reaction volume at 50°C for 60 min. The RT reaction was finished by heating at 70°C for 15 min to stop the reaction. Samples were stored at −80°C until further use.

2.5.7 | Quantitative real time-polymerase chain reaction (qRT-PCR) amplification

Interestingly, qRT-PCR reactions were performed using the QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific) together with the following commercially available gene expression assays: PAX3 (Hs00240950_m1; Life Technologies), PAX7 (Hs00242962_m1; Life Technologies), MYF5 (Hs00929416_g1; Life Technologies), MYOD1 (Hs00159528_m1; Life Technologies), MYOG (Hs01072232_m1; Life Technologies), MYH7 (Hs00165276_m1; Life Technologies), MYH2 (Hs00430042_m1; Life Technologies) and MYH1 (Hs00428600_m1; Life Technologies). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1; Life Technologies) served as the endogenous control for mRNA gene expression (Table 1; Esther Barreiro et al., 2019; Guitart et al., 2018; Puig-Vilanova, Martínez-Llorens, et al., 2015). Duplicates from all samples were run and the average value was calculated for each study sample. The results obtained from the experiments were collected and analyzed using the ExpressionSuite Software version 1.1 from Applied Biosystems (Thermo Fisher Scientific), in which the comparative CT method $2^{-\Delta\Delta Ct}$ for relative quantification was used as previously reported (Guitart et al., 2018; Livak & Schmittgen, 2001).

2.5.8 | Immunoblotting

Protein levels of myostatin were analyzed using immunoblotting procedures, as previously described (Esther Barreiro et al., 2019; Guitart et al., 2018; Puig-Vilanova, Martínez-Llorens, et al., 2015). Briefly, frozen samples from all experimental groups were homogenized in lysis buffer: 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 150 mM NaCl, 100 mM NaF, 10 mM Na pyrophosphate, 5 mM EDTA, 10% glycerol, 0.5% Triton-X, 5 μg/ml aprotinin, 2 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, and 10 μg/ml pepstatin A.

Proteins were separated by electrophoresis, transferred to polyvinylidene difluoride (PVDF) membranes, blocked with BSA, and incubated overnight with the corresponding primary antibody: Myostatin (anti-GDC8; Bethyl Laboratories) and the endogenous control GAPDH (anti-GAPDH antibody; Santa Cruz Biotechnology).

Antigens from all samples were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and a chemiluminescence kit. For each of the antigens, samples from the different groups were always detected in the same picture under identical exposure times. PVDF membranes were scanned with the Molecular Imager Chemidoc XRS System (Bio-Rad Laboratories) using the software Quantity One version 4.6.5 (Bio-Rad Laboratories). Optical densities of specific proteins were quantified using the software Image Lab version 2.0.1 (Bio-Rad Laboratories). Final optical densities obtained in each specific group of subjects and muscle corresponded to the mean values of the different samples (lanes) of each of the

TABLE 1 Gene expression assays used to assess muscle regeneration marker levels

Gene symbol Muscle regeneration markers	Assay ID	Genbank accession number
PAX3	Hs00240950_m1	NM_000438.5 NM_013942.4 NM_181457.3 NM_181458.3 NM_181459.3 NM_181460.3 NM_181461.3
PAX7	Hs00242962_m1	NM_001135254.1 NM_002584.2 NM_013945.2
MYF5	Hs00929416_g1	NM_005593.2
MYOD1	Hs00159528_m1	NM_002478.4
MYOG	Hs01072232_m1	NM_002479.5
MYH7	Hs00165276_m1	NM_000257.3
MYH2	Hs00430042_m1	NM_001100112.1 NM_017534.5
MYH1	Hs00428600_m1	NM_005963.3
GAPDH	Hs99999905_m1	NM_001289746.1 NM_002046. 5

Abbreviations: ID, identification; Hs, homo sapiens; m1, multiexonic gene assay does not detect genomic DNA; g1, multiexonic gene assay may detect genomic DNA if present in the sample; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NM, mRNA RefSeq database category; MYF5, myogenic factor 5; MYOD1, myogenic differentiation 1; MYOG, myogenin; MYH7, myosin heavy chain 7; MYH2, myosin heavy chain 2; MYH1, myosin heavy chain 1; PAX3, paired box gene 3; PAX7, paired box gene 7.

antigens studied. To validate equal protein loading among various lanes, the glycolytic enzyme GAPDH was used as the protein loading controls in all the immunoblots.

2.5.9 | Myostatin identification using immunohistochemical procedures

Myostatin was identified in paraffin sections (3-μm thick) from VL muscles corresponding to the three study groups using conventional immunohistochemical procedures as previously described (Puig-Vilanova, Martínez-Llorens, et al., 2015; Puig-Vilanova, Rodríguez, et al., 2015). Following deparaffinization, a pressure cooker containing 10 mM citrate buffer (pH 6.0) was used to boil the sections for 20 min. Following a 2-h cooling period, sections were blocked with 6% hydrogen peroxide for 15 min and with a blocking solution (3% BSA, 10% goat serum, and 0.5% triton in PBS) for 1 h. The sections were then incubated overnight at 4°C with the primary antibody: Myostatin (anti-GDG8; Bethyl Laboratories). Slides were then incubated with a universal secondary antibody (polystain-1 step kit; HRP for DBA, mouse and rabbit, Neo Biotech) for 30 min, followed by incubation with the substrate diaminobenzidine (DAB kit, Neo Biotech) for 5 min. Hematoxylin counterstaining was performed, and slides were dehydrated and mounted for conventional microscopy. Images of the stained muscle sections were captured with a light microscope (Olympus BX 61; Olympus Corporation) coupled with an image-digitizing camera (Pixera Studio; version 1.0.4; Pixera Corporation).

2.6 | Statistical analysis

The normality of the study variables was checked using the Shapiro-Wilk test. All the results are expressed as mean (standard deviation). For the quantitative variables, one-way analysis of variance (ANOVA) with Tukey's posthoc analysis was used to adjust for multiple comparisons among the study groups. For the qualitative variables (smoking history), χ^2 test was used to assess differences among the three study groups. A level of significance of $p \leq .05$ was established.

3 | RESULTS

3.1 | Functional and nutritional status of the study subjects

Body composition as measured by BMI and FFMI was significantly reduced in sarcopenic COPD patients compared to nonsarcopenic controls and control subjects (Table 2). Smoking history including the number of packs-year was similar in both groups of COPD patients and significantly differed from that of the controls (never smokers; Table 2). As expected, both groups of COPD patients exhibited severe airflow limitation and a decline in diffusion capacity, especially the sarcopenic patients (Table 2). Moreover, compared to control subjects, exercise tolerance was significantly reduced in both groups of COPD patients, whereas quadriceps muscle strength was significantly lower only in the sarcopenic patients compared to both nonsarcopenic and control subjects (Table 2). Nutritional blood parameters including CRP did not significantly differ among the three study groups of subjects, while fibrinogen levels were significantly greater in both groups of COPD patients than in the controls (Table 2).

3.2 | Muscle phenotype and damage

The proportions of slow-twitch muscle fibers were significantly lower in the VL of the sarcopenic COPD patients than in the control subjects, while those of the hybrid fibers increased in the limb muscles of both groups of COPD patients (Figure 1a and Table 3). Furthermore, the proportions of hybrid fibers were significantly greater in the lower limb muscles of the sarcopenic patients than in nonsarcopenic patients (Figure 1a and Table 3). Compared to the controls, the

TABLE 2 Clinical characteristics of the COPD patients and control subjects

		COPD patients	
	Control subjects, N = 13	Nonsarcopenic, N = 19	Sarcopenic, N = 26
Anthropometry			
Age (years)	66 (5)	65 (7)	62 (8)
Body weight (kg)	72 (15)	75 (11)	52 (9)***,###
Males/females	6/7	14/5	11/15
BMI (kg/m²)	27 (4)	28 (2)	19 (3)#
FFMI (kg/m²)	17 (2)	18 (1)	14 (1)***,###
Smoking history			
Active, N (%)	0 (0)	15 (79)***	16 (62)***
Ex-smoker, N (%)	0 (0)	4 (21)***	10 (38)***
Never smoker, N (%)	13 (100)	0 (0)***	0 (0)***
Packs-year	0 (0)	61***	55***
Lung function testing			
FEV1, % predicted	97 (14)	36 (11)***	34 (11)***
FVC, % predicted	98 (12)	67 (15)***	70 (16)***
FEV₁/FVC	76 (6)	42 (10)***	38 (12)***
RV, % predicted	112 (14)	185 (43)***	224 (52)***,#
TLC, % predicted	97 (7)	108 (14)	122 (18)***,#
RV/TLC	43 (9)	61 (9)***	65 (8)***
DLco, % predicted	91 (6)	46 (16)***	35 (13)***
KCO, % predicted	93 (19)	49 (16)***	40 (14)***
PaO₂ (KPa)	NA	9.4 (2.0)	8.6 (4.0)
PaCO₂ (KPa)	NA	5.5 (0.5)	5.8 (1.0)
Exercise capacity and muscle function			
VO₂ max (% pred)	120 (32)	59 (18)***	54 (24)***
6-min walking distance (m)	523 (55)	421 (79)**	443 (64)**
QMVC (Kg)	42 (13)	40 (12)	31 (7)**,##
QMVC (Kg)/FFM (Kg)	0.9 (0.1)	0.8 (0.2)	0.8 (0.2)
Blood parameters			
Albumin (g/dL)	4.6 (0.2)	4.5 (0.3)	4.6 (0.2)
Total proteins (g/dL)	7.1 (0.3)	7.0 (0.4)	7.1 (0.6)
CRP (mg/dL)	0.3 (0.3)	0.5 (0.3)	0.3 (0.3)
Fibrinogen (mg/dL)	355 (69)	469 (87)**	418 (76)*
GSV (mm/h)	10 (7)	17 (13)	10 (11)

Note: Values are expressed as mean (SD).
Abbreviations: BMI, body mass index; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; DLco, carbon monoxide transfer; FEV₁, forced expiratory volume in 1 s; FFM, fat-free mass; FFMI, fat-free mass index; FVC, forced vital capacity; GSV, globular sedimentation velocity; KCO, Krough transfer factor; N, number of patients; PaCO₂, arterial carbon dioxide partial pressure; PaO₂, arterial oxygen partial pressure; pred, predicted; QMVC, quadriceps maximum voluntary contraction; QMVC/FFM, quadriceps maximum voluntary contraction/fat-free mass; RV, residual volume; TLC, total lung capacity; VO₂ peak, peak exercise oxygen uptake.
Statistical significance: *p < .05, **p < .01, ***p < .001 between any of the groups of COPD patients and the control subjects; #p < .05, ##p < .01, ###p < .001 between sarcopenic and nonsarcopenic COPD patients.

cross-sectional area of slow-twitch fibers was significantly reduced in the VL of both groups of COPD patients. Furthermore, the size of both fast-twitch and hybrid fibers was significantly smaller in the muscles of the sarcopenic patients than in both control subjects and nonsarcopenic patients (Figure 1a and Table 3). The proportions of muscle structural abnormalities and internal nuclei counts were significantly greater in the VL of both groups of COPD patients than in control subjects (Figure 1b). Besides this, muscle structural abnormalities were also significantly greater in the VL of sarcopenic than in nonsarcopenic COPD patients (Figure 1b). Interestingly, the number of TUNEL-positive nuclei was significantly increased in the VL of both groups of COPD patients compared to those in the control
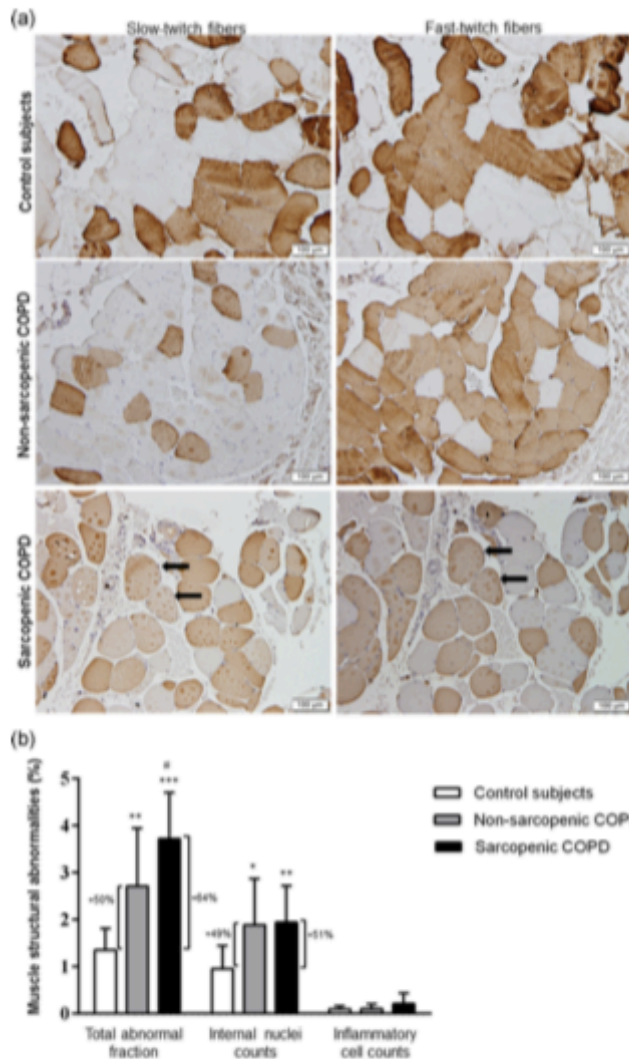
FIGURE 1 (a) Representative images of VL cross-sectional histological preparations. Myofibers positively stained for slow-twitch antibody appear in brown color in the left panels. Myofibers positively stained for fast-twitch antibody appear in brown color in the right panels. Hybrid myofibers are positively stained for slow-twitch and fast-twitch antibodies (black arrows). (b) Mean values and standard deviation of muscle abnormalities: as measured by total abnormal fraction and proportions of internal nuclei and inflammatory cells identified in the VL of control subjects (white bars) and in both nonsarcopenic (gray bars) and sarcopenic COPD patients (black bars). Statistical significance: $^*p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$ between any of the COPD patient groups and the control subjects; $^\#p \leq .05$ between sarcopenic and nonsarcopenic COPD patients. COPD, chronic obstructive pulmonary; VL, vastus lateralis

subjects (Figure 2a,b). Among all COPD patients, significant correlations were detected between muscle phenotype variables (proportions of slow- and fast-twitch fibers and CSA of type I fibers) and lung function parameters (degree of airway obstruction and diffusion capacity, respectively, Table 4A). Among the COPD patients, significant positive correlations were also detected between CSA of slow and fast-twitch fibers and hybrid fibers and FFMI (Table 4A). Furthermore, among all COPD patients as a whole, quadriceps strength significantly correlated with TUNEL-positive nuclei and muscle abnormalities (Table 4A).

3.3 | Satellite cells in muscles of COPD patients

The number of quiescent SCs as measured by Pax-7+/Myf-5- cells was significantly lower only in VL of the sarcopenic COPD patients than in the control subjects (Figure 3a,b). Counts of activated SCs (Pax-7+/Myf-5+) were significantly greater in the VL of both groups of COPD patients than in control subjects (Figure 3a,b). The numbers of total SCs did not significantly differ among the three study groups (Figure 3a,b). Besides this, among all COPD patients as a whole, the number of muscle

TABLE 3 Fiber type characteristics of the vastus lateralis in the study subjects

	Control subjects	COPD patients	
		Nonsarcopenic	Sarcopenic
Fiber Type I proportion (%)	33.0 (5.9)	28.7 (13.2)	26.6 (9.4)*
Fiber type II proportion (%)	65.2 (5.6)	64.3 (13.7)	63.0 (10.5)
Hybrid fiber proportion (%)	1.8 (1.7)	6.9 (4.1)**	10.3 (6.7)**,#
Type I Fibers CSA (μm²)	3589 (1260)	2678 (540)*	2085 (475)***
Type II Fibers CSA (μm²)	2800 (1322)	2819 (851)	1503 (395)**,##
Hybrid fibers CSA (μm²)	2777 (1534)	2608 (910)	2042 (827)***,#

Note: Mean values and standard deviation of the proportions and sizes (cross-sectional areas) in the VL of both groups of COPD patients and the control subjects.

Abbreviations: COPD, chronic obstructive pulmonary; CSA, cross-sectional area; N, sample size.

Statistical significance: *$p < .05$, **$p < .01$, and ***$p < .001$ between any of the groups of COPD patients and the control subjects; #$p < .05$, ##$p < .01$ between sarcopenic and nonsarcopenic COPD patients.

activated SCs inversely correlated with plasma levels of CRP (Table 4A). Specifically, in VL of sarcopenic COPD patients, the expression levels of PAX7 and MYOD significantly correlated with the degree of airway obstruction (Table 4B). Moreover, in the same muscles, proportions of slow-twitch fibers significantly correlated with the lung function parameters FEV$_1$/FVC and DLco (Table 4B).

3.4 | Expression of muscle regeneration and regulatory markers in COPD patients

Gene expression levels of the regeneration markers Pax-7 and Myf-5 (proliferation phase) and MyoD and MyHCI (differentiation phase) were significantly reduced in the VL of both groups of COPD patients compared to the control subjects (Figure 4a). Protein expression
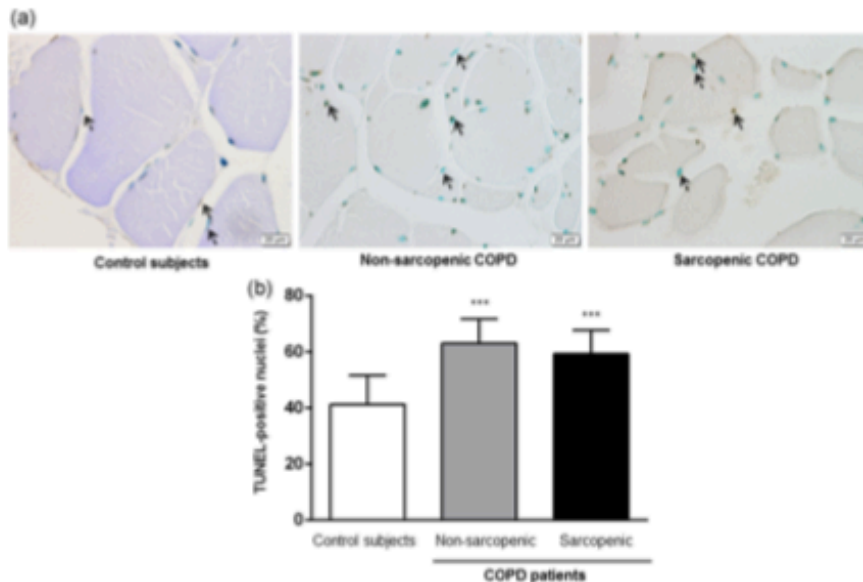

FIGURE 2 (a) Representative images of TUNEL-positively stained nuclei (brown, black arrows) and TUNEL-negative nuclei (green, dotted arrows) in the VL. (b) Mean values and standard deviation of the percentage of positively stained nuclei for the TUNEL assay in the VL of the control subjects (white bars), nonsarcopenic COPD patients (gray bars), and sarcopenic COPD patients (black bars). Statistical significance: ***$p \leq .001$ between any of the groups of COPD patients and the control subjects. COPD, chronic obstructive pulmonary; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; VL, vastus lateralis

TABLE 4A Significant correlations of the study variables among all the study COPD patients

	FEV₁/FVC	FEV₁	DL_CO	KCO	FFMI	QMVC	CRP
TUNEL						$r = 0.432$ $p = 0.019$	
Abnormal fraction						$r = -0.470$ $p = 0.010$	
Internal nuclei						$r = -0.367$ $p = 0.036$	
Fiber type I %	$r = 0.523$ $p = 0.003$	$r = 0.418$ $p = 0.021$	$r = 0.543$ $p = 0.003$				
Fiber type II %	$r = -0.428$ $p = 0.003$	$r = -0.444$ $p = 0.007$	$r = -0.467$ $p = 0.006$				
Type I fibers CSA			$r = 0.460$ $p = 0.018$	$r = 0.444$ $p = 0.020$	$r = 0.493$ $p = 0.007$		
Type II fibers CSA					$r = 0.673$ $p = 0.000$		
Hybrid fibers CSA					$r = 0.415$ $p = 0.018$		
Pax-7+/Myf-5+							$r = -0.426$ $p = 0.015$

Note: In the table, units have been omitted for the sake of clarity, as they are already described in the corresponding figures and tables.
Abbreviations: COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; CSA, cross-sectional area; DLco, carbon monoxide transfer; FEV₁, forced expiratory volume in 1 s; FFMI, fat-free mass index; FVC, forced vital capacity; KCO, Krough transfer factor; QMVC, quadriceps maximum voluntary contraction.

levels of myostatin significantly increased in the VL of the sarcopenic patients compared to both control subjects and nonsarcopenic COPD patients (Figure 4b,c). The expression of MyHCIIx was significantly upregulated only in the VL of the sarcopenic COPD patients compared to the control subjects (Figure 4a).

4 | DISCUSSION

In this investigation, the most relevant findings were that in sarcopenic COPD patients with preserved nutritional status and decreased exercise capacity, muscle strength was, indeed, significantly reduced and in the VL of these patients, a significant decline in muscle regeneration potential as measured by the number of Pax-7+/Myf-5- SCs, a decrease in slow-twitch fiber type proportions and in the size of both fast-twitch and hybrid fibers, along with a significant rise in both hybrid fibers proportions and muscle structural abnormalities were observed. Importantly, in the limb muscles of both groups of COPD patients, the numbers of internal nuclei, activated SCs (Pax-7+/Myf-5+), and TUNEL-positive nuclei were significantly greater than in the controls, while the expression of markers of early (proliferation phase, Pax-7, and Myf-5) and late (differentiation phase, MyoD, and MyHC) muscle regeneration was downregulated. Levels of the potent negative regulator myostatin increased only in the limb muscles of the sarcopenic COPD patients. Collectively, these findings suggest that markers of regenerative potential in the limb muscles of sarcopenic COPD patients are reduced and myostatin may play a significant role. Moreover, as the rise in muscle damage levels were detected to a greater extent in the VL of the sarcopenic COPD patients (64% greater than in control muscles), such structural alterations are also likely to be involved in the triggering of the muscle regeneration process (Cheung & Rando, 2013; Yin et al., 2013). The most relevant results are discussed below.

Previous investigations have shown that the number of central nuclei, a marker of muscle regeneration, and those of senescent SCs were increased in lower limb muscles of COPD patients with different degrees of body composition (Thériault et al., 2012, 2014).

Among COPD patients, significant correlations were observed between lung function parameters, especially airway obstruction and diffusion capacity, and the proportions of slow-twitch fibers, and patients with better lung function parameters were those with a higher proportion of type I fibers. Conversely, proportions and size of type II fibers inversely correlated with the degree of airway obstruction and diffusion capacity. These are relevant findings that suggest that lung function partly contributes to the slow-to-fast fiber type switch in the lower limb muscles of COPD patients as also previously implied (Esther Barreiro et al., 2018; Puig-Vilanova, Martinez-Llorens, et al., 2015; Puig-Vilanova, Rodriguez, et al., 2015). Furthermore, the size of slow- and fast-twitch fibers and that of hybrid fibers also correlated with FFMI, suggesting that lean body
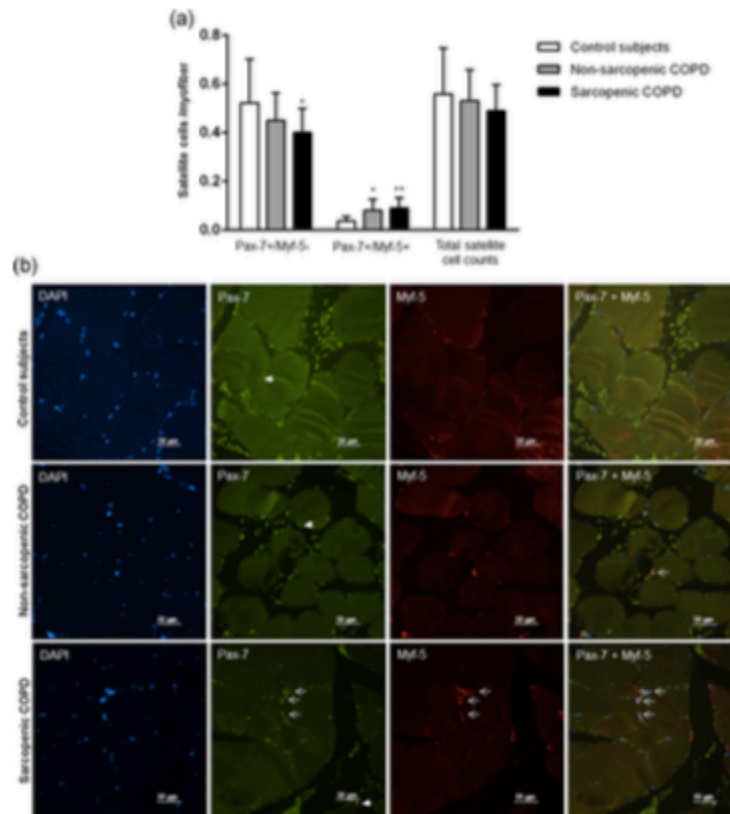
FIGURE 3 (a) Mean values and standard deviation of Pax-7+/Myf-5- (quiescent), Pax-7+/Myf-5+ (activated), and total satellite cell counts per myofiber in the VL of control subjects (white bars) and in both nonsarcopenic COPD patients (gray bars) and sarcopenic COPD patients (black bars). Statistical significance: *$p \leq$ .05, **$p \leq$ .01 between any of the groups of COPD patients and the control subjects. (b) Representative images of immunofluorescence staining of DAPI (left panels), Pax-7 (middle-left panels), Myf-5 (middle-right panels), and cells positively stained for both Pax-7 and Myf-5 markers (right panels) in muscles of control subjects and in both nonsarcopenic and sarcopenic COPD patients. Triangle arrows indicate Pax-7 positive cells (quiescent satellite cells) and thin arrows indicate double-stained nuclei for both Pax-7 and Myf-5 positive cells (activated satellite cells). COPD, chronic obstructive pulmonary; DAPI, 4′,6′-diamidino-2-phenylindole

mass was associated with a greater CSA of all the muscle fiber types among the COPD patients. In addition, muscle damage and internal nuclei counts were also inversely correlated with the isometric strength of the quadriceps muscle of all the COPD patients. Collectively, these are relevant novel findings that indicate that muscle structure and function are clearly interrelated and should be assessed on a routine basis in COPD patient clinics, especially in patients with alterations in their body composition. Another interesting finding in the study was the negative correlation found between CRP plasma levels and the number of activated SCs. These results illustrate that systemic inflammation as measured by CRP somehow influenced the process of muscle regeneration as also implied to occur in muscles of COPD patients (Vogiatzis et al, 2007).

In the current investigation, a relatively large number of sarcopenic COPD patients of a fairly "young age", with a significant decline in quadriceps muscle function, preserved nutritional status, and altered body composition were carefully recruited. With the aim of elucidating whether muscle regeneration potential may be hampered in the lower limb muscles of sarcopenic COPD patients, two different phenotypes of SCs were determined in the present study. As previously characterized (Kuang et al., 2007), sublaminar SCs that express Pax-7 but do not express Myf-5 constitute the SC reservoir of a given muscle. In that seminal investigation (Kuang et al., 2007), it was clearly demonstrated that Pax-7+/Myf-5+ SCs preferentially differentiate into muscle fibers, whereas Pax-7+/Myf-5- SCs contribute to the SC reservoir enlarging this compartment within

TABLE 4B Significant correlations of the study variables among sarcopenic COPD patients

	FEV₁/FVC	DLco
Fiber type I %	$r = .504$ $p = .047$	$r = .474$ $p = .087$
PAX7	$r = .537$ $p = .015$	
MYOD	$r = .703$ $p = .000$	

Note: For the sake of clarity in the table, units have been omitted, as they are already being shown in the corresponding figures and tables.
Abbreviations: COPD, chronic obstructive pulmonary disease; CSA, cross-sectional area; DLco, carbon monoxide transfer; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity.

skeletal muscle. The conclusions from that study (Kuang et al, 2007) were that two different subpopulations of SCs were established on the basis of their ability to express Myf-5 (Kuang et al, 2007). As such Pax-7+/Myf-5+ SCs were identified as the committed myogenic progenitors, while Pax-7+/Myf-5- SCs were defined as the actual stem cells (Kuang et al, 2007).

Importantly, in the present investigation, a significant decline in the number of Pax-7+/Myf-5- SCs was detected only in the limb muscles of the sarcopenic COPD patients, but not in those with preserved body composition and normal quadriceps muscle function. These findings imply that the SC reservoir was hampered in the sarcopenic muscles, which may further jeopardize the process of muscle regeneration. In fact, the proportions of slow-twitch muscle fibers were reduced only in the VL of the sarcopenic COPD patients. Moreover, the size of slow-twitch, fast-twitch, and hybrid myofibers was also significantly smaller in the lower limb muscles of the sarcopenic patients, but not in those with preserved body composition. Additionally, muscle structural abnormalities were even greater in the VL of the sarcopenic COPD patients than in those with no muscle loss. These results, which are very consistent with those obtained in previous investigations (Barreiro et al, 2019; Puig-Vilanova, Martinez-Llorens, et al, 2015; Puig-Vilanova, Rodriguez, et al, 2015), maybe partly explained by the poorer regenerative potential detected in the limb muscles of the sarcopenic patients. Proportions of hybrid fibers increased in the limb muscles of both groups of COPD patients and the proportions were even greater in the VL of the sarcopenic patients than in the nonsarcopenic group, while their CSA was smaller in the former patients. These are relevant findings that imply that muscles of COPD patients, especially of the
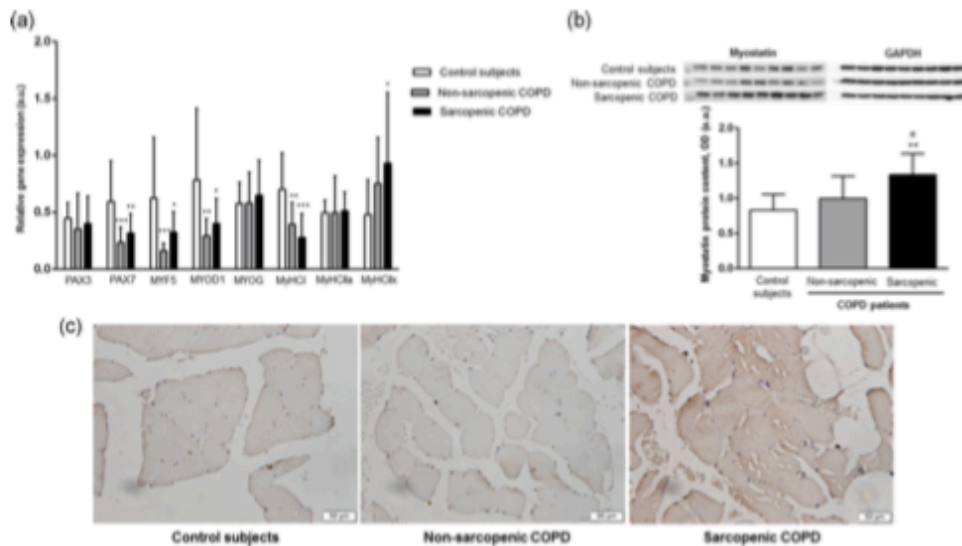

FIGURE 4 (a) Mean values and standard deviation (relative expression) of genes of muscle structure and regeneration in the VL of the control subjects (white bars), nonsarcopenic COPD (gray bars), and sarcopenic COPD patients (black bars). Statistical significance: *$p \le .05$, **$p \le .01$, ***$p \le .001$ between any of the groups of COPD patients and the control subjects. (b) Mean values and standard deviation of myostatin protein, measured in ODs using a.u. in the VL of the control subjects (white bars) and in both nonsarcopenic (gray bars) and sarcopenic COPD patients (black bars). Statistical significance: **$p \le .01$ between sarcopenic COPD patients and the control subjects; #$p \le .05$ between sarcopenic and nonsarcopenic COPD patients. (c) Representative images of VL cross-sectional histological preparations. Myostatin protein appears in brown color. a.u., arbitrary unit; COPD, chronic obstructive pulmonary; OD, optical density; VL, vastus lateralis

sarcopenic ones, are able to adapt to environmental factors such as inactivity, exercise, or aging (Medler, 2019). Collectively, these events are of paramount importance, as a correct muscle regeneration program is required to attain full recovery of muscle mass and function in response to different training modalities. Thus, the current results have potential clinical implications for the design of specific exercise training programs as patients with a defective regenerative potential may be less susceptible to improving their muscle mass and/or function, even those with preserved nutritional status.

It is worth noting that the lower limb muscles of both groups of COPD patients experienced the activation of a muscle regeneration program in a similar fashion. In line with this, the number of specific activated SCs (Pax-7+/Myf-5+), internal nuclei, and TUNEL-positive nuclei were notably and similarly increased in the VL of both groups of severe COPD patients compared to those detected in the control muscles. These findings are in line with previous results, in which the process of muscle regeneration was also triggered in the VL of severe COPD patients with a wide range of muscle mass loss (Thériault et al., 2012, 2014). However, in another study (Menon et al., 2012), the number of SCs was similar between COPD patients and healthy controls at baseline. Differences in the level of alterations in body composition compartments and/or muscle function and mass may account for discrepancies encountered among studies (Menon et al., 2012; Thériault et al., 2012, 2014).

Furthermore, gene expression levels of the early markers of muscle regeneration Pax-7 and Myf-5 were downregulated in the VL of both groups of severe COPD patients compared to the controls. Pax-7 and Myf-5 transcription factors play key roles during the proliferation phase of the muscle regeneration process (Guitart et al., 2018; Yin et al., 2013). In keeping with this, similar results were also reported in the quadriceps muscle of cachectic COPD patients (Plant et al., 2010; Thériault et al., 2012, 2014). The downregulation in gene expression of the transcription factors MyoD and Myogenin and of MyHC-I isoform as markers of late muscle differentiation during the regeneration process was another relevant finding in the investigation. These findings are also in line with those previously demonstrated in the muscles of patients with advanced COPD (Plant et al., 2010; Puig-Vilanova, Martínez-Llorens, et al., 2015; Thériault et al., 2012, 2014).

Myostatin, a member of the transforming growth factor beta family, is a negative regulator of muscle growth. Myostatin is also known to inhibit SC and myoblast proliferation through several mechanisms that lead to cell cycle withdrawal (Walsh & Celeste, 2005). Importantly, myostatin levels were significantly greater in the limb muscles of the sarcopenic patients than those detected within the nonsarcopenic patients or the control subjects. These results are line with previous studies, in which myostatin levels were consistently demonstrated to rise in the lower limb muscles of patients with advanced COPD (Harish et al., 2019; Plant et al., 2010; Puig-Vilanova, Martínez-Llorens, et al., 2015; Walsh & Celeste, 2005). In view of all these findings, it would be possible to conclude that myostatin may have interfered with the process of muscle cell proliferation early on

during the regeneration process, thus leading to poor muscle growth and development following injury. Hence, this may be another mechanism of muscle mass loss in addition to increased proteolysis and/or apoptosis as also consistently shown in previous investigations (Plant et al., 2010; Puig-Vilanova, Martínez-Llorens, et al., 2015; Vogiatzis et al., 2010). Nonetheless, elucidation of the precise role and implications of myostatin on muscle regeneration and growth in COPD sarcopenia will have to be definitively confirmed in future investigations.

On the other hand, the reported findings may also have future clinical implications as myostatin blockade using specific antibodies has been shown to partly revert the loss of muscle mass and function in several experimental models (Harish et al., 2019; Iskenderian et al., 2018; St. Andre et al., 2017) and in patients (Burch et al., 2017; Scimeca et al., 2017). Whether anti-myostatin antibodies can also be effectively used in patients with nonmuscle diseases such as in sarcopenic COPD will be a matter of research in future investigations. This will shed more light on the implications of myostatin in the muscle regenerative capacity of sarcopenic COPD patients.

5 | CONCLUSIONS

In the lower limb muscles of severe COPD patients regardless of the degree of sarcopenia, the muscle regeneration process is triggered as identified by SC activation, and a rise in internal nuclei counts. Nonetheless, regenerative potential along with significant alterations in muscle phenotype (slow-to-fast switch phenotype and smaller fast-twitch and hybrid myofibers) and muscle damage were prominently seen in the sarcopenic COPD. A rise in the muscle growth inhibitor myostatin was also detected only in the VL of the sarcopenic COPD patients, which may further aggravate loss of muscle mass and function in this specific group of patients. These findings have clinical implications as not all COPD patients may equally respond to exercise and/or muscle training modalities, and myostatin blockade should be specifically customized to patients with sarcopenia in COPD.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Study conception and design: Esther Barreiro, Antonio Sancho-Muñoz, and Juana Martínez-Llorens. *Patient assessment and recruitment and sample collection:* Antonio Sancho-Muñoz and Juana

Martinez-Llorens. _Molecular biology analyses:_ Maria Guitart. _Statistical analyses and data interpretation:_ Maria Guitart, Antonio Sancho-Muñoz, and Esther Barreiro. _Manuscript drafting and intellectual input:_ Esther Barreiro, Antonio Sancho-Muñoz, Maria Guitart, Joaquim Gea, Juana Martinez-Llorens, and Diego A. Rodríguez. _Manuscript writing final version:_ Esther Barreiro.

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MANUSCRITO 2

ORIGINAL RESEARCH ARTICLE

WILEY | Cellular Physiology

Endoplasmic reticulum stress and unfolded protein response profile in quadriceps of sarcopenic patients with respiratory diseases

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Abstract

Impaired muscle strength and mass (sarcopenia) are common in patients with respiratory cachexia, namely chronic obstructive pulmonary disease (COPD) and in lung cancer (LC)-cachexia. Misfolded/unfolded proteins in endoplasmic reticulum (ER) induce the compensatory unfolded protein response (UPR). Expression of ER stress and UPR markers may be differentially upregulated in vastus lateralis (VL) of patients with respiratory sarcopenia associated with either a chronic condition (COPD) or subacute (LC)-cachexia. In VL specimens from 40 COPD patients ($n = 21$, sarcopenic, fat-free mass index [FFMI] 16 kg/m^2 and $n = 19$, nonsarcopenic, FFMI 18 kg/m^2), 13 patients with LC-cachexia (FFMI 17 kg/m^2), and 19 healthy controls (FFMI 19 kg/m^2), expression markers of ER stress, UPR (protein kinase-like ER kinase [PERK], activating transcription factor [ATF] 6, and inositol-requiring enzyme [IRE] 1-α), oxidative stress, autophagy, proteolysis, and apoptosis (reverse transcription polymerase chain reaction and immunoblotting), and fiber atrophy (histology) were assessed. Atrophy and muscle wasting and weakness were seen in both groups of sarcopenic patients. Compared to healthy controls, in muscles of LC-cachexia patients, expression of ER stress markers and UPR (three arms) was significantly upregulated, while in sarcopenic COPD, expression of a few ER stress markers and IRE1-α arm was upregulated. ER stress and an exaggerated UPR were observed in the VL muscle of patients with respiratory sarcopenia. The three branches of UPR were similarly upregulated in muscles of cancer cachectic patients, whereas in sarcopenic COPD patients, only IRE1 was upregulated. The differential profile of muscle UPR in chronic and subacute respiratory conditions offers a niche for the design of specific novel therapeutic approaches.

KEYWORDS

chronic and subacute respiratory sarcopenic conditions, COPD, endoplasmic reticulum stress, lower limb muscles, lung cancer, unfolded protein response

1 | INTRODUCTION

Muscle mass loss and dysfunction (sarcopenia) are characteristic features of patients with chronic respiratory and cardiac conditions and also in cancer. Several studies have shown that for the same degree of airway obstruction in patients with chronic obstructive pulmonary disease (COPD), poor muscle mass, and weakness of the quadriceps negatively influenced their quality of life and prognosis (Gea & Martinez-Llorens, 2018; Gea, Pascual, et al., 2018; Gea, Sancho-Muñoz, & Chalela, 2018; Gosselink, Troosters, & Decramer, 2000; Marquis et al., 2002; Seymour et al., 2010; Shrikrishna et al., 2012; Swallow et al., 2007). In patients with lung cancer (LC), muscle wasting and cachexia also reduced their quality of life and survival (Evans et al., 2008; Fearon et al., 2011). Despite that both respiratory and limb muscle dysfunction are present in patients with COPD, muscles of the lower limbs are usually more severely affected and have greater implications in their daily life activities (Barreiro, 2017; Barreiro & Jaitovich, 2018; Barreiro et al., 2015, 2018; Jaitovich & Barreiro, 2018; Maltais et al., 2014).

In the etiology of sarcopenia, several clinical aspects and molecular mechanisms are involved. Our group and others have contributed with several studies to the demonstration that systemic inflammation, increased oxidative stress, proteolytic signaling, autophagy and apoptosis, and epigenetic events participate in the multifactorial etiology of sarcopenia in COPD as well as in LC-cachexia (Barreiro, 2017; Barreiro & Jaitovich, 2018; Barreiro et al., 2015; Barreiro et al., 2018; Jaitovich & Barreiro, 2018; Maltais et al., 2014). Indeed, similar biological and structural features were identified in the vastus lateralis (VL) of patients with respiratory cachexia (Puig-Vilanova, Rodriguez, et al., 2014). The endoplasmic reticulum (ER) organelle is in charge of folding, processing, and trafficking of proteins within the cells. Accumulation of unfolded proteins may result from alterations in cellular homeostasis (aging, infections, hypoxia, glucose and calcium imbalance, etc). When proper folding cannot be restored by chaperones and foldases, unfolded proteins are usually targeted to be degraded by ER through several pathways. Furthermore, unfolded protein response (UPR) is normally induced as a persistent accumulation of unfolded proteins within eukaryote cells (Chakrabarti, Chen, & Varner, 2011; Kelsen, 2016). Three ER transmembrane receptors with distinct functions have been shown to mediate UPR as part of a complex signaling program (Chakrabarti et al., 2011; Kelsen, 2016). Importantly, ER-induced UPR was also shown to signal muscle development and regeneration (Bohnert, McMillan, & Kumar, 2017; Nakanishi, Sudo, & Morishima, 2005; Xiong et al., 2017). Markers of UPR were also highly activated in skeletal muscles of mice with LC-induced cachexia and blockade of UPR with 4-phenylbutyrate induced a less fatigue-resistant phenotype in the muscles of the same animals (Bohnert et al., 2016). These findings suggest that UPR plays a key role in muscle mass maintenance, at least in experimental models of cachexia. Nonetheless, investigations are needed to elucidate the potential role of UPR in muscles of patients with sarcopenia associated with respiratory diseases.

Therefore, we hypothesized whether the expression of ER stress and UPR markers may be upregulated to a different extent in the VL of patients with sarcopenia associated with respiratory diseases of either a chronic condition (COPD) or a subacute disease (LC). Accordingly, the study objectives were to explore in VL specimens obtained from COPD patients with and without sarcopenia, in those with LC-induced cachexia, and in age-matched healthy control subjects: (a) Well-known markers of ER stress, (b) markers of UPR pathways [activating transcription factor [ATF] 6, protein kinase-like ER kinase (PERK), and inositol-requiring enzyme (IRE) 1], (c) markers of oxidative stress, autophagy, apoptosis, and proteolysis, and (d) muscle phenotype (fiber types and morphometry). Gene expression profile was analyzed for all the target markers along with protein levels of the markers whose expression significantly differed in the muscles of any study group of patients from those detected in the control subjects.

2 | MATERIALS AND METHODS

(See detailed methodologies in the online supporting information.)

2.1 | Study design and population

This was a prospective, controlled, cross-sectional study, in which 40 male patients with stable COPD (Miravitlles & Soler-Cataluna, 2017; Miravitlles et al., 2017; Vogelmeier et al., 2017) and 13 male patients with LC-cachexia according to the international consensus criteria (Evans et al., 2008; Fearon et al., 2011) were recruited. Additionally, 19 age-matched male healthy controls were recruited from the general population (patients' relatives or friends) at Hospital del Mar (Barcelona). COPD patients were further subdivided into those with and without loss of muscle mass and muscle strength (sarcopenic COPD, $n = 21$ and nonsarcopenic COPD, $n = 19$) following the international consensus criteria on muscle wasting and sarcopenia (Cao & Morley, 2016; Muscaritoli et al., 2010) and published criteria (Puig-Vilanova, Rodriguez, et al., 2014). LC patients had not previously received any specific treatment of the lung neoplasm: Chemotherapy, radiotherapy, or systemic corticosteroids at the time of entry into this specific investigation. Clinical staging was assessed in all cachectic patients with LC following currently available international guidelines (Chansky et al., 2017). All study groups of individuals were Caucasian. In the present investigation, nine sarcopenic COPD patients and two age-matched sedentary control subjects were also involved in another study aimed to investigate the proteolytic mechanisms involved in sarcopenic muscles of patients with severe COPD and in those with LC-cachexia (Puig-Vilanova, Rodriguez, et al., 2014).

The current investigation was designed in accordance with both the ethical standards on human experimentation in our institution and the World Medical Association guidelines (Helsinki Declaration of 2008) for research on human beings. Approval was obtained from

the institutional Ethics Committee on Human Investigation (Hospital del Mar-IMIM, Barcelona). Informed written consent was obtained from all individuals.

2.2 | Anthropometrical and functional assessment

Anthropometrical, nutritional, and lung function evaluations were conducted as previously reported (Coin et al., 2008; Steiner, Barton, Singh, & Morgan, 2002). Quadriceps muscle strength was evaluated as formerly described (Coronell et al., 2004; Swallow et al., 2007).

2.3 | Muscle biopsies

Muscle samples were obtained from the quadriceps muscle (VL) of all groups of patients and control subjects using the open muscle biopsy technique, as previously described (Fermoselle et al., 2012; Puig-Vilanova, Aguiló, et al., 2014; Puig-Vilanova, Ausin, Martinez-Llorens, Gea, & Barreiro, 2014; Puig-Vilanova, Rodriguez, et al., 2014; Puig-Vilanova et al., 2015).

2.4 | Biological analyses

2.4.1 | Muscle fiber counts and morphometry

On three micrometer muscle paraffin-embedded sections from all groups of patients and controls, MyHC-I (slow-twitch fibers) and MyHC-II (fast-twitch fibers) isoforms were identified using anti-MyHC-I antibody (Sigma-Aldrich, Saint Louis, MO), following methodologies previously published (Fermoselle et al., 2012; Puig-Vilanova, Aguiló, et al., 2014; Puig-Vilanova, Rodriguez, et al., 2014; Puig-Vilanova et al., 2015). RNA isolation. Total RNA was first isolated from snap-frozen skeletal muscle specimens as previously described (Puig-Vilanova et al., 2015).

2.4.2 | Gene expression was assessed using reverse transcription polymerase chain reaction (qRT-PCR)

MicroRNA RT was performed using TaqMan® microRNA assays (Life Technologies, Carlsbad, CA) following the manufacturer's instructions and previous studies (Supporting Information Table E1; Livak & Schmittgen, 2001; Puig-Vilanova et al., 2015).

2.4.3 | Immunoblotting

Protein levels of the different molecular markers analyzed in the study were explored by means of immunoblotting procedures as previously described (Barreiro et al., 2018; Chacon-Cabrera, Lund-Palau, Gea, & Barreiro, 2016; Puig-Vilanova, Rodriguez, et al., 2014; Salazar-Degracia et al., 2016; Salazar-Degracia et al., 2018). The following primary antibodies were used: Anti-binding immunoglobulin protein (BIP; 1:1,000, ab37073) antibody (Abcam, Cambridge, UK), anti-protein disulfide isomerase-3 (PDIA3; 1:1,000, ADI-SPA-725-F) antibody (Enzo Life Science, Farmingdale, NY), anti-phosphatidylinositol 3-kinase (PI3K; 1:1,000,

3358S) antibody (Cell Signaling, Boston, MA), anti-ATF6 (1:100, ab37149), anti-protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK; 1:1,000, ab79483), anti-phospho-PERK (1:1,000, ab192591), anti-eukaryotic translation initiation factor 2α (eiF2a; 1:500, ab181467), anti-phospho-eiF2a (1:1,000, ab32157), anti-C/EBP-homologous protein (CHOP; 1:2,000, ab11419), anti-IRE1 (1:1,000, ab37073), antitumor necrosis factor receptor-associated factor 2 (TRAF2; 1:1,000, ab37118), anti-X-box binding protein 1 (XBP1; 1:1,000, ab37152) antibodies from Abcam, anti-microtubule-associated protein 1 light chain 3 (LC3; 1:1,000, #2775S) antibody (Cell Signaling), anti-nucleoporin p62 (p62; 1:1,000, P0067) antibody (Sigma-Aldrich, St. Louis, MO), anti- apoptosis signal-regulating kinase 1 (ASK1; 1:500, sc-5294) antibody from Santa Cruz Biotechnology (Santa Cruz, CA), anti-malondialdehyde-protein adducts (MDA; 1:4,000, MD20A-G1b) antibody (Academy Bio-Medical Company, Houston, TX), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:2,000, sc-25778) antibody (Santa Cruz Biotechnology). To validate equal protein loading across lanes, the glycolytic enzyme GAPDH was used as the protein loading control in all immunoblots (Supporting Information Figures E2–E5). Standard stripping methodologies were used to detect the phosphorylated proteins (PERK and EiF2a) and the loading control GAPDH for each of the markers (BIP, PDIA3, PI3K, ATF6, PERK, EiF2a, CHOP, IRE1, TRAF2, XBP1, LC3B, p62, ASK1, and MDA).

2.5 | Statistical analyses

Statistical power was calculated using specific software (StudySize 2.0, CreoStat HB, Gothenburg, Sweden). FFMI was selected as the target variable on the basis of t test to estimate the statistical power between healthy controls and LC-cachexia patients. On the basis of a standard power statistics established at a minimum of 80% and assuming an α error of 0.05, the statistical power was sufficiently high to detect a minimum difference of two points between groups in the sample size ($N = 11$ minimum number of subjects in each group) and standard deviation. Normality of the study variables was checked using Shapiro–Wilk test. The comparisons between study groups were analyzed using one-way analysis of variance, in which Tukey's post hoc analysis was used to adjust for multiple comparisons. Comparisons were explored between healthy controls and either any group of COPD patients or LC-cachexia patients. Pearson's χ^2 test was used to assess potential differences between groups in the qualitative variables such as smoking history. Correlations between clinical and biological variables were explored using the Pearson's correlation coefficient. Variables that described the clinical characteristics of the study population are represented as mean and standard deviation, whereas all the molecular variables are represented as mean and 95% confidence interval. A level of significance of $p \leq 0.05$ was established. Statistical analyses were performed using the Statistical Package for the Social Sciences (Portable SPSS, PASW statistics 18.0 version for windows, SPSS Inc., Chicago, IL).

TABLE 1 Anthropometric characteristics and functional status of the study subjects

	Healthy controls (N = 19)	LC-cachexia patients (N = 13)	All COPD patients (N = 40)	All COPD patients	
				Nonsarcopenic COPD (N = 19)	Sarcopenic COPD (N = 21)
Anthropometry					
Age (years)	66 (7)	68 (9)	68 (5)	68 (5)	67 (5)
BMI (kg/m²)	26 (3)	23 (2)*	24 (5)**	27 (2)	21 (3)***, §§§
FFMI (kg/m²)	19 (3)	17 (1)*	17 (2)**	18 (1)	16 (2)***, §
Body weight (kg)	72 (8)	66 (7)*	65 (10)**	74 (4)	62 (10)***, §§§
Body weight change (kg/last year)	0 (0)	−5 (1.6)***	−1.9 (2.1)***	−0.2 (2.3)	−2.4 (1.8)***, §
Lung cancer staging (%)	NA	I/II/IV 30/30/40	NA	NA	NA
Smoking history					
Active (N, %)	6, 32	9, 69**	24, 60**	10, 53*	14, 67*
Ex-smoker (N, %)	9, 47	4, 31**	16, 40**	9, 47*	7, 33*
Never smoker (N, %)	4, 21	0, 0**	0, 0**	0, 0*	0, 0*
Packs-year	54 (22)	58 (12)	62 (24)	61 (28)	62 (19)
Lung function					
FEV₁ (% pred)	90 (12)	61 (22)**	40 (19)***	44 (19)***	35 (19)***, p = 0.096
FVC (% pred)	90 (12)	72 (18)**	64 (18)***	65 (19)***	62 (17)***
FEV₁/FVC (%)	74 (4)	65 (18)**	47 (13)***	48 (12)***	46 (13)***
RV (% pred)	104 (20)	96 (46)	180 (70)***	178 (78)***	182 (71)***
TLC (% pred)	97 (13)	85 (20)	104 (22)	101 (26)	107 (18)
RV/TLC	43 (5)	51 (13)	61 (12)***	58 (13)***	64 (12)***
DLco (% pred)	88 (19)	75 (18)	55 (27)***	56 (24)***	53 (30)***
Kco (% pred)	86 (17)	94 (23)	64 (22)***	60 (20)***	70 (24)*
PaO₂ (kPa)	11.3 (1.3)	10.5 (1.0)	9.7 (1.5)***	9.7 (1.4)**	9.9 (1.7)*
PaCO₂ (kPa)	5.3 (0.5)	5.2 (0.5)	5.5 (0.7)	5.3 (0.7)	5.6 (0.8)
Exercise capacity and muscle function					
VO₂ peak (% pred)	83 (15)	57 (10)**	53 (21)***	57 (20)***	48 (22)***
WR peak (% pred)	74 (15)	53 (10)*	44 (20)***	49 (21)*	38 (17)***
6-min walking test (m)	500 (76)	422 (48)**	425 (121)**	425 (112)	425 (132)
QMVC (kg)	38 (2)	34 (2)**	30 (1)***	31 (3)***	29 (3)***
Blood parameters					
Albumin (g/dl)	4.3 (0.4)	3.5 (0.7)***	4.3 (0.5)	4.3 (0.3)	4.3 (0.6)
Total proteins (g/dl)	7.1 (0.7)	6.8 (0.8)	7.2 (0.6)	7.2 (0.6)	7.2 (0.6)
CRP (mg/dl)	0.4 (0.3)	4.2 (2.7)*	1.1 (1.7)*	0.6 (0.5)	1.6 (2.4)*
Fibrinogen (mg/dl)	391 (113)	570 (169)**	453 (91)*	444 (85)	464 (99)
GSV (mm/h)	9 (9)	49 (36)*	20 (16)**	18 (11)	21 (20)*

Note. BMI: body mass index; COPD: chronic obstructive pulmonary disease; CRP: C-reactive protein; DLco: carbon monoxide transfer; FEV₁: forced expiratory volume in one second; FFMI: fat-free mass index; FVC: forced vital capacity; GSV: globular sedimentation velocity; Kco: Krogh transfer factor; LC: lung cancer; N: number of patients; NA: not applicable; PaCO₂: arterial carbon dioxide partial pressure; PaO₂: arterial oxygen partial pressure; pred: predicted; QMVC: quadriceps maximal velocity contraction; RV: residual volume; TLC: total lung capacity; VO₂ peak: peak exercise oxygen uptake; WR peak: peak work rate.

Values are expressed as mean (standard deviation).

*$p \leq 0.05$,

**$p \leq 0.01$, and

***$p \leq 0.001$ between either LC-cachexia patients or any group of COPD patients (as a whole and separately, nonsarcopenic and sarcopenic COPD patients) and the healthy control subjects. § $p \leq 0.05$ and §§§ $p \leq 0.001$ between the nonsarcopenic and the sarcopenic COPD patients.

3 | RESULTS

3.1 | Clinical characteristics

Clinical and functional variables of control subjects, COPD patients and LC-cachexia patients are illustrated in Table 1. Age did not significantly differ among the study subjects. Body composition as measured by body mass index (BMI) and fat-free mass index (FFMI) was significantly reduced in both COPD and LC-cachexia patients (Table 1). Additionally, body weight, BMI, and FFMI were also significantly lower in sarcopenic COPD patients than in either healthy controls or nonsarcopenic patients, while no differences were observed between the latter patients and the healthy controls (Table 1). Body weight change, expressed as kilogram lost in the previous year, was also significantly lower in both LC-cachexia and COPD patients, especially in sarcopenic COPD patients, than control subjects (Table 1). In all groups of patients, the proportions of active smokers were similar and were

significantly greater than those observed in the healthy controls (Table 1). The number of packs-year, however, was similar among all study groups (Table 1). No significant correlations were detected between smoking history and any of the study biological variables among any study group. All COPD patients exhibited severe airflow limitation and moderate functional signs of emphysema (diffusion capacity status), whereas LC-cachexia patients showed a mild-to-moderate airflow obstruction and no functional signs of emphysema. COPD patients exhibited very mild hypoxemia with no hypercapnia compared with controls (Table 1). Compared with healthy controls, in LC-cachexia and COPD patients, exercise capacity and quadriceps strength were significantly reduced (Table 1). Levels of C-reactive protein, fibrinogen,

and globular sedimentation velocity were also significantly greater in COPD and LC-cachexia patients, especially in the latter patients, than the controls (Table 1).

3.2 | Muscle structural features

As expected, the proportions of slow-twitch fibers were significantly lower in the VL of both groups of patients than in healthy subjects (Table 2 and Supporting Information Figure E1). In VL, the cross-sectional area of fast-twitch fibers was significantly smaller in LC-cachexia and COPD patients, particularly in sarcopenic patients, than in control subjects, while that of slow-twitch fibers was only reduced in COPD patients (Table 2 and Supporting Figure E1).

TABLE 2 Main clinical characteristics and functional variables of the study subjects

		Severe COPD	
	Controls (N = 10)	Noncachectic (N = 14)	Cachectic (N = 15)
Subjects			
Female/male	4/6	3/11	2/13
Anthropometry			
Age (years)	62 (6)	67 (10)	65 (9)
BMI (kg/m²)	27 (2)	26 (3)	18 (2)*** §§§
FFMI (kg/m²)	19 (2)	18 (2)	15 (1)*** §§§
Smoking history			
Active (N, %)	0, 0	6, 43***	6, 40***
Ex-smoker (N, %)	5, 50	8, 57***	9, 60***
Never smoker (N, %)	5, 50	0, 0***	0, 0***
Pack-years	62 (16)	69 (18)	66 (31)
Lung function			
FEV₁ (% pred)	94 (9)	35 (11)***	31 (12)***
FVC (% pred)	92 (8)	54 (16)***	58 (18)***
FEV₁/FVC (%)	75 (3)	45 (10)***	40 (11)***
RV (% pred)	115 (18)	195 (53)**	210 (69)***
TLC (% pred)	104 (10)	115 (20)	119 (17)
RV/TLC (%)	43 (3)	66 (9)***	69 (11)***
DLco (% pred)	102 (16)	49 (21)***	42 (18)***
Kco (% pred)	94 (14)	58 (21)***	57 (21)***
PaO₂ (mmHg)	85 (2.7)	68 (9.5)***	65 (8.8)***
PaCO₂ (mmHg)	42 (4.2)	46 (4.5)	45 (5.3)
Exercise capacity and muscle force			
QMVC (kg)	44 (11)	32 (8)*	23 (11)*** §
6-min walking distance (m)	526 (47)	408 (111)*	380 (113)**
Blood parameters			
Albumin (g/dl)	4.5 (0.4)	4.2 (0.4)	4.6 (0.9)
Total proteins (g/dl)	6.3 (1.5)	6.9 (0.7)	6.7 (1.2)
CRP (mg/dl)	0.9 (0.6)	1.7 (0.5)*	6.0 (5.6)** §§
Fibrinogen (mg/dl)	384 (108)	416 (90)*	535 (127)* §
GSV (mm/h)	15 (7)	29 (13)*	28 (13)*

Note. BMI: body mass index; COPD: chronic obstructive pulmonary disease; CRP: C-reactive protein; DLco: carbon monoxide transfer; FEV₁: forced expiratory volume in one second; FFMI: fat-free mass index; FVC: forced vital capacity; GSV: globular sedimentation velocity; Kco: Krogh transfer factor for diffusing capacity; N: number of patients; PaCO₂: arterial carbon dioxide partial pressure; PaO₂: arterial oxygen partial pressure; pred: predicted; QMVC: quadriceps maximal velocity contraction; RV: residual volume; TLC: total lung capacity.

Values are expressed as mean (standard deviation).

*p ≤ 0.05,

**p ≤ 0.01, and

***p ≤ 0.001 between any group of COPD patients and the control subjects.

§p ≤ 0.05,

§§p ≤ 0.01, and

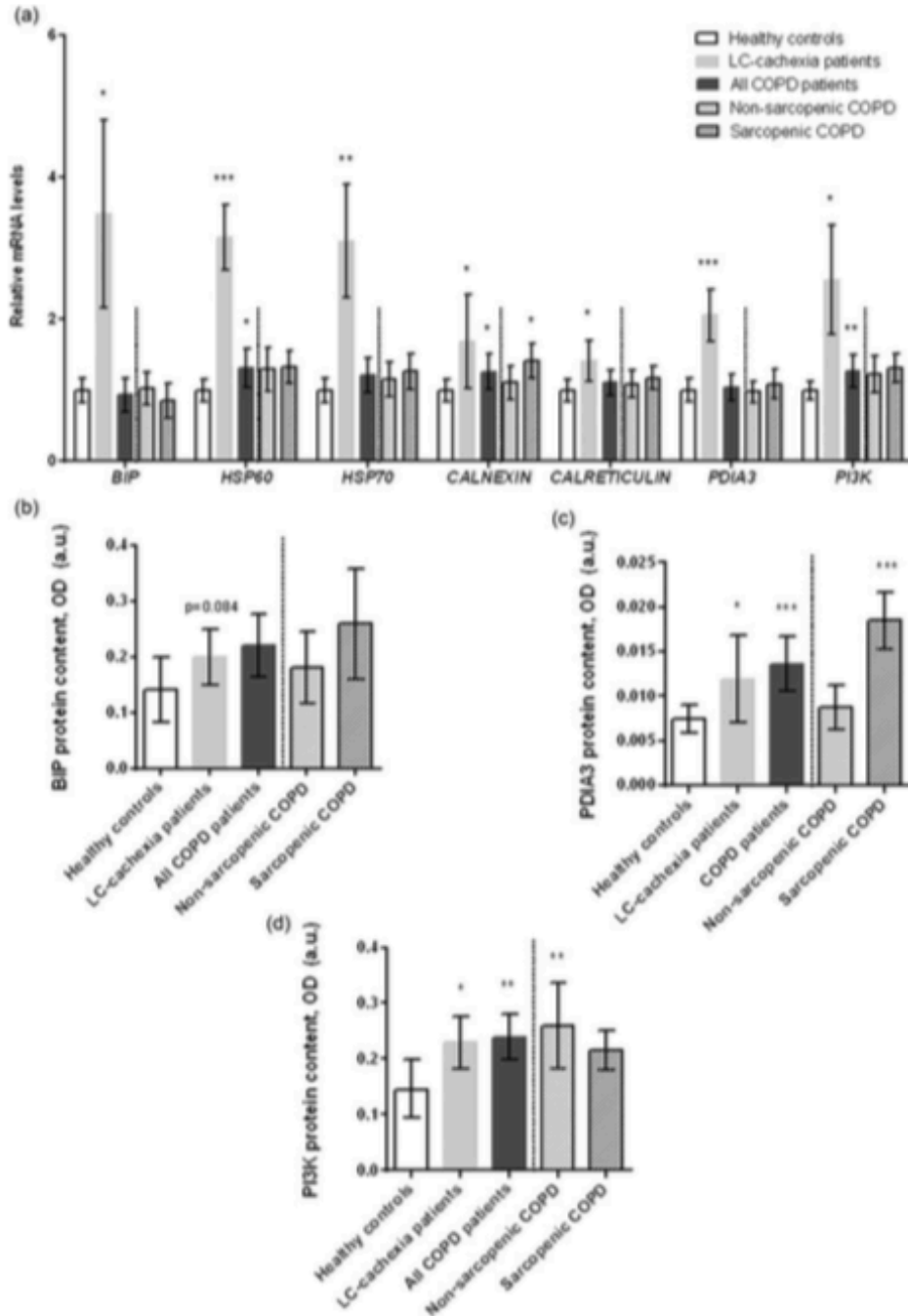§§§p ≤ 0.001 between noncachectic and cachectic patients.
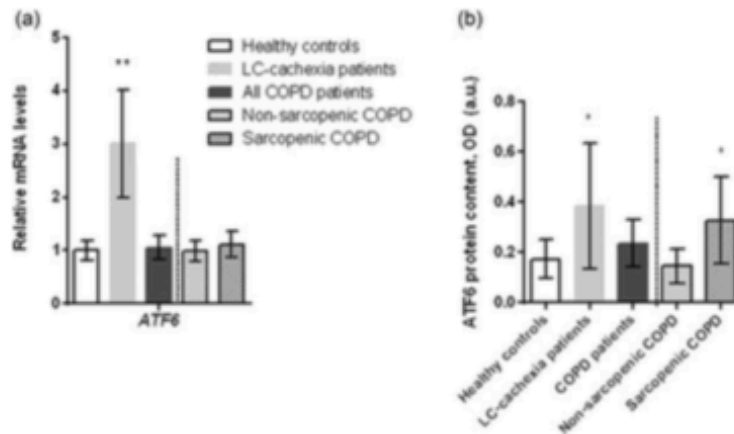
FIGURE 1 Continued.

FIGURE 2 (a) Mean values and 95% confidence intervals of expression levels of ATF6 marker expressed as relative messenger RNA levels in the vastus lateralis muscle of the study groups: Healthy controls, LC-cachexia, and COPD patients. (b) Mean values and 95% confidence intervals of ATF6 protein content in the vastus lateralis muscle as measured by optical densities in arbitrary units (OD, a.u.). ATF: activating transcription factor; COPD: chronic obstructive pulmonary disease; LC: lung cancer. $*p \leq 0.05$ and $**p \leq 0.01$ between either the LC-cachexia patients or any of COPD patient groups (nonsarcopenic and sarcopenic patients) and the healthy controls

3.3 | Markers of ER stress and UPR in sarcopenic muscles

3.3.1 | ER stress

Compared with healthy controls, gene expression levels of heat shock protein (HSP) 60, calnexin, and PI3K were significantly upregulated in the VL of COPD patients as a whole and LC-cachexia patients (Figure 1a). Furthermore, in VL of LC-cachexia patients, gene expression levels of BIP, HSP70, calreticulin, and protein disulfide isomerase-A (PDIA) 3 were also upregulated compared with the controls (Figure 1a). Among LC-cachexia patients, significant correlations were found between gene expression levels of the ER stress markers HSP60 and calnexin and weight loss ($r = 0.844$ and $p = 0.035$ and $r = 0.813$ and $p = 0.049$, respectively). In the same group of patients, correlations were detected between gene expression of the ER stress marker PDIA3 and the clinical variables FFMI and weight loss ($r = 0.669$ and $p = 0.101$ (almost significant) and $r = 0.754$ and $p = 0.03$, respectively). Muscle gene expression levels of PDIA3 also significantly correlated with the proportions of type I (positively) and type II (negatively) fibers ($r = 0.740$, $r = -0.740$, and $p = 0.036$, respectively) among LC-cachexia patients. In VL of the same patients, gene expression levels of BAX and BCL2 also significantly correlated with the proportions

of type I and type II fibers ($r = 0.731$ and $p = 0.014$ and $r = 0.598$ and $p = 0.042$, respectively).

Interestingly, protein levels of PDIA3 and PI3K were significantly greater in the VL of both COPD as a group and LC-cachexia patients than in the control subjects (Figure 1c,d and Supporting Information Figure E2). Protein levels of BIP were almost significantly increased ($p = 0.084$) in the VL of the LC-cachexia patients compared with the control subjects, while no significant differences were observed among the COPD patients (Figure 1b and Supporting Information Figure E2).

3.3.2 | ATF6 arm

Gene expression and protein levels of ATF6 pathway were significantly upregulated in VL of LC-cachexia, and ATF6 protein content was also increased in muscles of sarcopenic COPD patients (Figure 2a,b and Supporting Information Figure E3a).

3.3.3 | PERK arm

CHOP gene expression was significantly greater in muscles of LC-cachexia and in both groups of COPD patients, while that of PERK and

FIGURE 1 (a) Mean values and 95% confidence intervals of expression levels of the following markers: BIP, HSP60, HSP70, calnexin, calreticulin, PDIA3, and PI3K expressed as relative messenger RNA levels in the vastus lateralis muscle of the study groups: Healthy controls, LC-cachexia, and COPD patients. (b) Mean values and 95% confidence intervals of BIP protein content in the vastus lateralis muscle as measured by optical densities in arbitrary units (OD, a.u.). (c) Mean values and 95% confidence intervals of PDIA3 protein content in the vastus lateralis muscle as measured by optical densities in arbitrary units (OD, a.u.). (d) Mean values and 95% confidence intervals of PI3K protein content in the vastus lateralis muscle as measured by optical densities in arbitrary units (OD, a.u.). BIP: binding immunoglobulin protein; HSP: heat shock protein; COPD: chronic obstructive pulmonary disease; LC: lung cancer; PDIA3: protein disulfide isomerase family A member 3; PI3K: phosphatidylinositol 3-kinase. $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$ between either the LC-cachexia patients or any of COPD patient groups (nonsarcopenic and sarcopenic patients) and the healthy controls

ATF4 did not differ among the study groups (Figure 3a). Protein levels of activated (phosphorylated) PERK, EiF2a, and CHOP were significantly higher in the VL of the LC-cachexia patients than in the controls (Figure 3b–d and Supporting Information Figure E3b). Moreover, protein levels of CHOP were also significantly increased in the VL of COPD patients as a whole and in the sarcopenic group (Figure 3d and Supporting Information Figure E3b).

3.3.4 | IRE1 arm

Gene expression of TRAF2 and XBP1, as markers of IRE1 pathway, was upregulated in VL of COPD patients and the latter marker also in LC-cachexia group (Figure 4a). Moreover, TRAF2 gene expression was upregulated in muscles of both sarcopenic and nonsarcopenic COPD

patients (Figure 4a). Protein levels of IRE1 did not significantly differ among the study groups (Figure 4b and Supporting Information Figure E4). TRAF2 protein levels were significantly increased in the VL of both groups of COPD patients (Figure 4c and Supporting Information Figure E4). Interestingly, protein levels of unspliced XBP1u and spliced XBP1s were significantly greater in the VL of both LC-cachexia and COPD patients as a whole and in sarcopenic (XBP1u) than in the controls (Figure 4d–e and Supporting Information Figure E4).

3.4 | Markers of oxidative stress, autophagy, proteolysis, and apoptosis in muscles

Gene expression of atrogin-1 and MuRF-1 was only significantly upregulated in VL of LC-cachexia compared with healthy controls
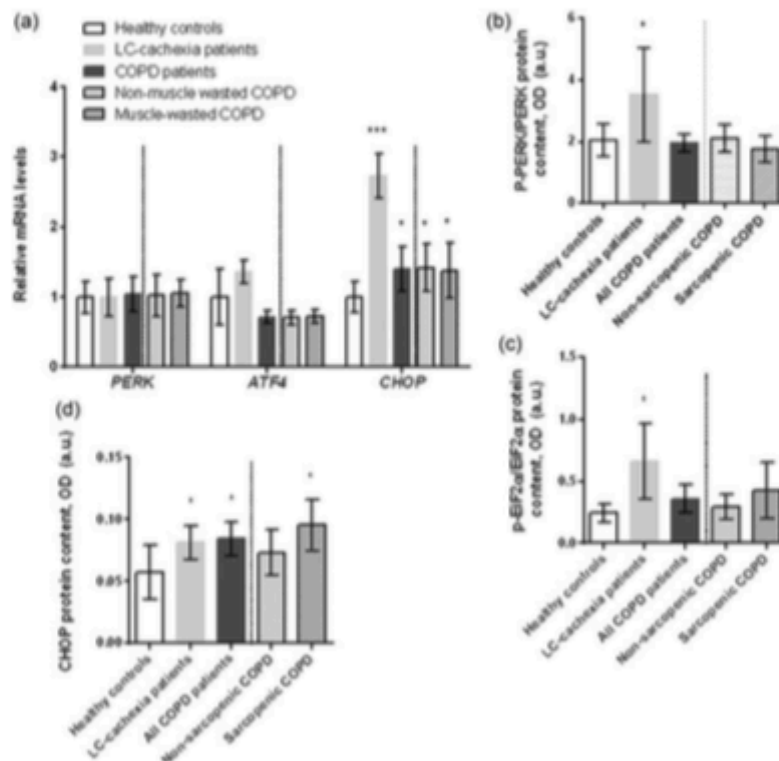

FIGURE 3 (a) Mean values and 95% confidence intervals of expression levels of the following markers: PERK, ATF4, and CHOP expressed as relative messenger RNA levels in the vastus lateralis muscle of the study groups: Healthy controls, LC-cachexia, and COPD patients. (b) Mean values and 95% confidence intervals of activated PERK protein content in the vastus lateralis muscle as measured by optical densities in arbitrary units (OD, a.u.). (c) Mean values and 95% confidence intervals of activated EiF2a protein content in the vastus lateralis muscle as measured by optical densities in arbitrary units (OD, a.u.). (d) Mean values and 95% confidence intervals of CHOP protein content in the vastus lateralis muscle as measured by optical densities in arbitrary units (OD, a.u.). ATF: activating transcription factor; CHOP: C/EBP-homologous protein; COPD: chronic obstructive pulmonary disease; EiF2a: eukaryotic translation initiation factor 2a; LC: lung cancer; PERK: protein kinase R (PKR)-like endoplasmic reticulum kinase. *$p \leq 0.05$ and ***$p \leq 0.001$ between either the LC-cachexia patients or any of COPD patient groups (nonsarcopenic and sarcopenic patients) and the healthy controls
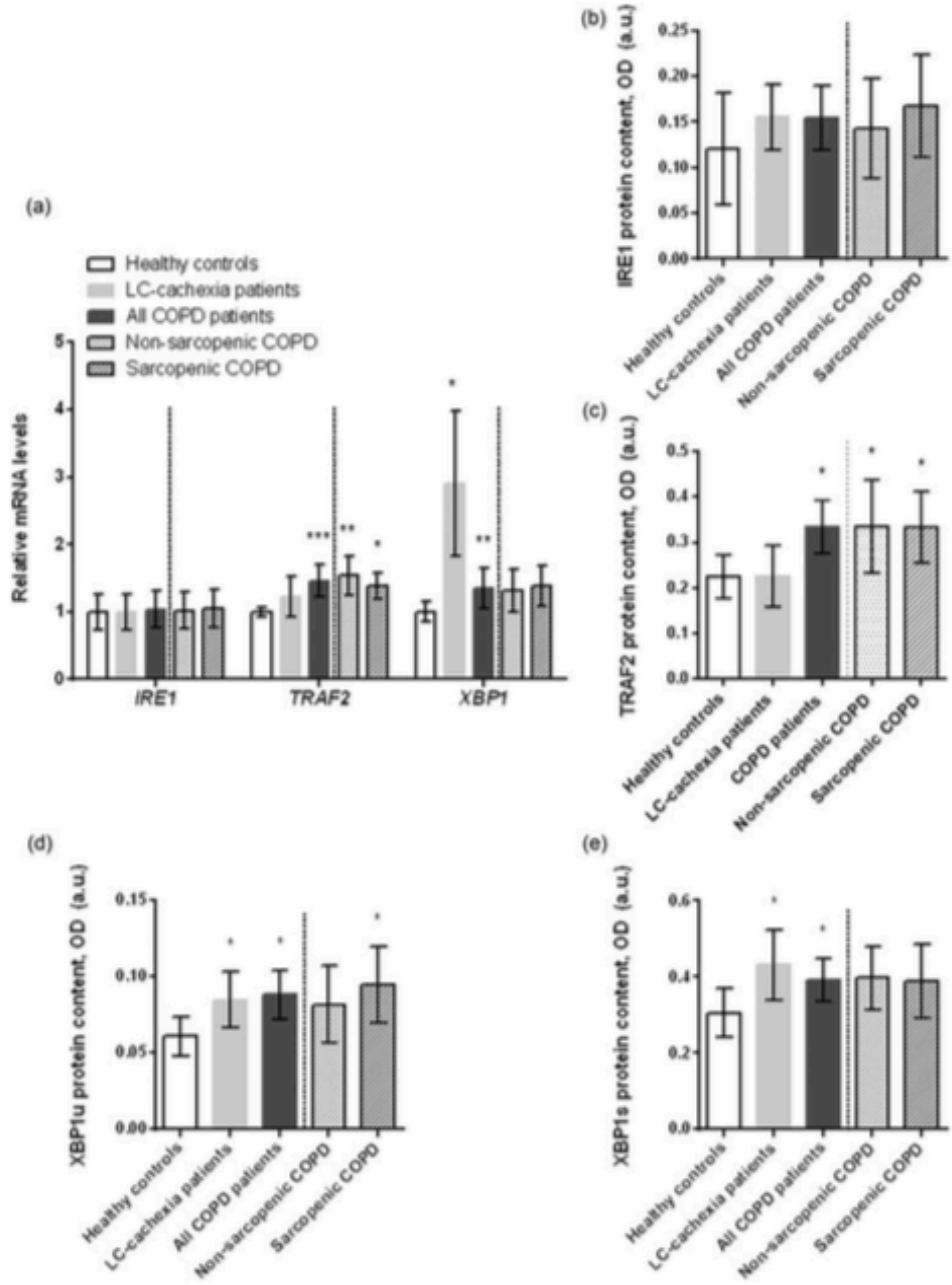
FIGURE 4 Continued.

(Figure 5a). Protein levels of microtubule-associated proteins 1A/1B light chain 3B (LC3B) were only significantly greater in the VL of all COPD patients as a whole and in the sarcopenic patients than in control subjects (Figure 5b and Supporting Information Figure E5), while protein levels of the autophagy marker p62 did not significantly differ among the study groups (Figure 5c and Supporting Information Figure E5). Protein oxidation levels as measured by MDA-protein adduct levels were significantly higher in the VL of all study groups of patients than in healthy controls (Figure 5d and Supporting Information Figure E5).

Gene expression of apoptosis markers such as caspase-7, caspase-9, and ASK were significantly upregulated in VL of LC-cachexia patients compared with the controls (Figure 6a). Moreover, ASK gene expression was also upregulated in muscles of both sarcopenic and nonsarcopenic COPD patients and as a whole compared with healthy controls (Figure 6a). Protein levels of ASK were also significantly increased in the VL of LC-cachexia and COPD patients as a whole, especially in the sarcopenic COPD patients (an almost significant increase; Figure 6b and Supporting Information Figure E5). Significant correlations were found between caspase-7 gene expression and the proportions of type I and type II fibers ($r = 0.874$ and $r = -0.874$, $p = 0.005$, respectively) in the muscles of the LC-cachexia patients.

4 | DISCUSSION

The current investigation has addressed a novel relevant question in well-characterized patients with respiratory sarcopenia of two different etiologies, chronic (COPD) versus subacute (LC) conditions. Sarcopenic COPD patients had a more severe airway obstruction but diffusion capacity was similar to that seen in patients with normal body composition. Despite that PaO_2 levels were significantly lower in all groups of COPD patients compared with healthy controls, such a reduction was very mild even in the sarcopenic patients. As expected, in the VL of both LC-cachexia patients and sarcopenic COPD patients, muscle phenotype was characterized by a rise in the proportions of fast-twitch fibers, while its size was significantly smaller as an indication of muscle atrophy. These findings are similar to those previously reported in limb muscles from cachectic COPD patients and in those with LC (Barreiro et al., 2018; Fermoselle et al., 2012; Puig-Vilanova, Rodriguez, et al., 2014). Moreover, in the sarcopenic COPD patients, slow-twitch cross-sectional area was also reduced compared to that seen in the controls. The expression profile of markers of ER stress and UPR differs

to some extent in sarcopenic muscles of patients with COPD from those with LC-cachexia. These are novel findings that prompt UPR as a potential signaling driver of muscle atrophy in patients with respiratory diseases.

Correct folding, processing, and trafficking of proteins take place in the ER, which also plays a central role in protein synthesis and cell calcium homeostasis (Almanza et al., 2018). ER stress results from the accumulation of misfolded proteins in response to injurious conditions that disrupt cell homeostasis. Intrinsic ER perturbations such as those occurring in cancer (genetic instability and mutations) and in neurodegenerative disease may result in ER stress (Almanza et al., 2018). Moreover, extrinsic perturbations derived from microenvironmental stress (depletion of nutrients and oxygen and acidosis) and increased reactive oxygen species production also lead to ER stress (Almanza et al., 2018).

Despite that UPR has been recently shown to underlie the pathophysiology of several acute (critical illness) and chronic lung conditions (cystic fibrosis, pulmonary fibrosis, and COPD; Baek et al., 2012; Bartoszewski et al., 2008; Hassan et al., 2014; Lawson et al., 2011), its role in skeletal muscles is not well known, let alone in specific conditions characterized by severe muscle mass loss. Activation of ER stress and UPR may also take place in skeletal muscles during exercise and aging (Bohnert et al., 2017). As protein folding in the ER is calcium-, redox-, and energy-dependent, ER stress may be a relevant mechanism leading to muscle wasting in chronic diseases. In fact, perturbations in those homeostatic processes have been reported in the lower limb muscles of COPD patients with muscle dysfunction and wasting (Barreiro et al., 2010; Fermoselle et al., 2012; Puig-Vilanova, Ausin, et al., 2014; Puig-Vilanova, Rodriguez, et al., 2014; Puig-Vilanova et al., 2015). In the current study, we sought to explore whether ER stress and UPR may be upregulated in sarcopenic muscles with two different respiratory diseases: chronic versus subacute disease.

ER stress activates the UPR that is mediated by three ER transmembrane sensors identified as ATF6, PERK, and IRE1. UPR reverses ER stress by slowing the flow of new proteins into ER through a series of transcriptional, translational, and posttranslational processes that increase ER capacity for protein folding and processing, enhance elimination of misfolded proteins, and expand the size of ER (Kelsen, 2016). Importantly, UPR may also induce apoptosis and protein degradation if ER stress cannot be reversed (Chakrabarti et al., 2011;

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FIGURE 4 (a) Mean values and 95% confidence intervals of expression levels of the following markers: *IRE1*, *TRAF2*, and *XBP1* expressed as relative messenger RNA levels in the vastus lateralis muscle of the study groups: Healthy controls, LC-cachexia, and COPD patients. (b) Mean values and 95% confidence intervals of IRE1 protein content in the vastus lateralis muscle as measured by optical densities in arbitrary units (OD, a.u.). (c) Mean values and 95% confidence intervals of TRAF2 protein content in the vastus lateralis muscle as measured by optical densities in arbitrary units (OD, a.u.). (d) Mean values and 95% confidence intervals of unspliced XBP1u protein content in the vastus lateralis muscle as measured by optical densities in arbitrary units (OD, a.u.). (e) Mean values and 95% confidence intervals of spliced XBP1s protein content in the vastus lateralis muscle as measured by optical densities in arbitrary units (OD, a.u.). COPD: chronic obstructive pulmonary disease; IRE1: endoplasmic reticulum to nucleus signaling 1; LC: lung cancer; TRAF2: TNF receptor-associated factor 2; XBP1: X-box binding protein 1. *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ between either the LC-cachexia patients or any of COPD patient groups (nonsarcopenic and sarcopenic patients) and the healthy controls
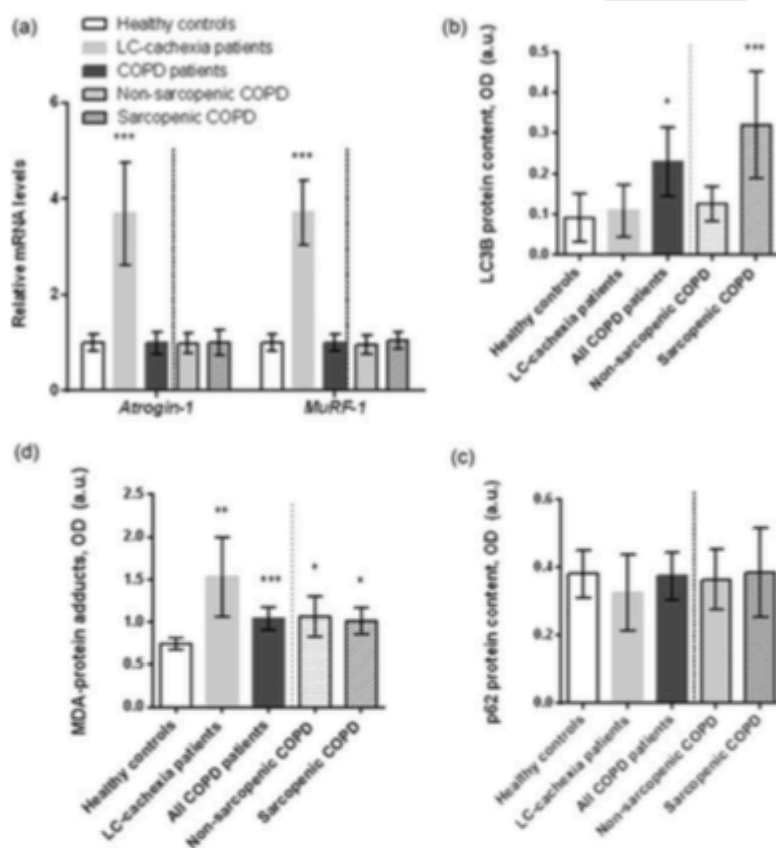
FIGURE 5 (a) Mean values and 95% confidence intervals of expression levels of the following markers: *Atrogin-1* and *MuRF-1* expressed as relative messenger RNA levels in the vastus lateralis muscle of the study groups: Healthy controls, LC-cachexia, and COPD patients. (b) Mean values and 95% confidence intervals of LC3B protein content in the vastus lateralis muscle as measured by optical densities in arbitrary units (OD, a.u.). (c) Mean values and 95% confidence intervals of p62 protein content in the vastus lateralis muscle as measured by optical densities in arbitrary units (OD, a.u.). (d) Mean values and 95% confidence intervals of MDA-protein adducts in the vastus lateralis muscle as measured by optical densities in arbitrary units (OD, a.u.). COPD: chronic obstructive pulmonary disease; LC: lung cancer; LC3B: microtubule-associated protein 1 light chain 3; MDA: malondialdehyde; MuRF-1: muscle ring finger protein-1; p62: nucleoporin p62. $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$ between either the LC-cachexia patients or any of COPD patient groups (nonsarcopenic and sarcopenic patients) and the healthy controls

Kelsen, 2016). Indeed, restoration of cell homeostasis and survival or apoptosis is also determined upon activation of the UPR signaling pathways (Chakrabarti et al., 2011; Kelsen, 2016).

Posttranslational processing of all membrane and secretory proteins in the ER comprises a variety of resident chaperones, foldases, oxidoreductases, and disulfide isomerases (Schroder & Kaufman, 2005). These chaperones promote disulfide bonds and glycosylation of proteins in the ER under a process of quality control before proteins exit this organelle. Molecular chaperones such as calnexin, calreticulin, BIP, and other HSPs specifically bind unfolded or misfolded proteins in

the ER to correct folding. In the present study, expression levels of the chaperones HSP60, calnexin, and PI3K were upregulated in the VL of both sarcopenic COPD and LC-induced cachexia patients. Additionally, in the lower limb muscles of the latter patients, expression levels of BIP, HSP70, calnexin, calreticulin, and PDIA3 were also significantly upregulated. These findings imply that ER stress is present in the peripheral muscles of patients with respiratory sarcopenia, and particularly in those with LC. In keeping with, an upregulation of ER stress markers was also seen in the hindlimb muscles of cachectic mice bearing the Lewis lung carcinoma (Bohnert et al., 2016). However, as far
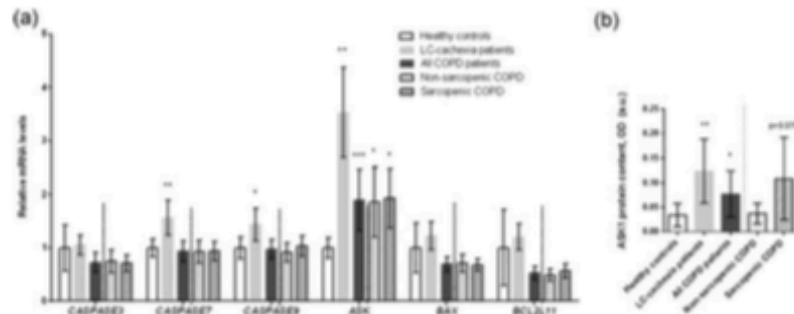
FIGURE 6 (a) Mean values and 95% confidence intervals of expression levels of the following markers: CASPASE3, CASPASE7, CASPASE9, ASK, BAX, and BCL2L11 expressed as relative messenger RNA levels in the vastus lateralis muscle of the study groups: Healthy controls, LC-cachexia, and COPD patients. (b) Mean values and 95% confidence intervals of ASK1 protein content in the vastus lateralis muscle as measured by optical densities in arbitrary units (OD, a.u.). ASK1: apoptosis signal-regulating kinase 1; BAX: BCL2-associated X apoptotic regulator; BCL2L11: BCL2-like 11 apoptosis regulator Bcl-2; COPD: chronic obstructive pulmonary disease; LC: lung cancer. *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ between either the LC-cachexia patients or any of COPD patient groups (nonsarcopenic and sarcopenic patients) and the healthy controls

as we are concerned the current investigation is the first to report the presence of ER stress fingerprints in the skeletal muscles of actual patients with respiratory sarcopenia of two different etiologies and time-frame: Chronic versus subacute conditions.

In response to unfolded or misfolded proteins, ATF6 pathway is activated through a series of translocation and irreversible proteolytic processing steps leading to the upregulation of prosurvival transcriptional programs (Chakrabarti et al., 2011). Gene expression levels of ATF6 were only significantly upregulated in the VL of patients with LC-induced cachexia, while protein levels of this marker were also significantly increased in the limb muscles of the sarcopenic COPD patients. Interestingly, ATF6 may also interact with protein degradation pathways such as the ubiquitin–proteasome system to enhance proteolysis and autophagy. Atrogin-1 and MuRF-1 gene expression levels were significantly upregulated in the VL of patients with cancer cachexia. Nonetheless, protein levels of the autophagy marker LC3B were only significantly greater in the muscles of the sarcopenic COPD patients, thus suggesting that ATF6 rather triggers the ubiquitin–proteasome pathway in muscles of cancer cachectic patients than autophagy. Previous studies also demonstrated a rise in autophagy markers in the VL of cachectic COPD but not in those with LC (Guo et al., 2013; Puig-Vilanova, Rodriguez, et al., 2014).

Both prosurvival and proapoptotic programs are signaled by the PERK arm of UPR following the accumulation of unfolded or misfolded proteins. The transmembrane protein PERK consists of a cytosolic protein kinase domain and an ER luminal stress sensor. In the study, protein levels of active PERK which phosphorylates eIF2a, were significantly greater in the VL of the LC-cachexia patients. During ER stress phosphorylation of eIF2a by PERK downregulates protein synthesis by blocking translation (Almanza et al., 2018; Rowlands, Panniers, & Henshaw, 1988). Consistently, protein levels

of p-EIF2a were also significantly higher in the muscles of cancer cachectic patients, thus implying that PERK arm was indeed activated in the lower limb muscles of these patients.

Gene expression of CHOP was upregulated in the quadriceps of patients with LC-cachexia and in those with COPD to a lower extent. Moreover, protein levels of CHOP were also significantly increased in the lower limb muscles of both LC-cachexia and sarcopenic COPD patients. ATF6 can also induce the expression of XBP1 and CHOP to enhance UPR signaling (Almanza et al., 2018). In line with this, gene expression and protein levels of CHOP and XBP1 were also significantly greater in the VL of LC-cachexia and in COPD patients, especially in sarcopenic (CHOP and XBP1u proteins), thus implying that these three pathways may enhance UPR signaling cascades and downstream mechanisms in muscles of those patients. Importantly, increased expression of CHOP may result in oxidative stress, ATP depletion, and cell death (Almanza et al., 2018; Chakrabarti et al., 2011; Hiramatsu et al., 2014). In the present study, oxidative stress levels were significantly greater in the VL of both cancer cachectic and all groups of COPD patients, as also consistently demonstrated in previous studies (Barreiro et al., 2010, 2018; Puig-Vilanova, Rodriguez, et al., 2014). Expression levels of markers of apoptosis such as caspases 7 and 9 and ASK were also upregulated in the VL of patients with cancer cachexia. Despite that no correlations were found between oxidative stress and autophagy or apoptosis markers, these observations suggest that apoptosis may preferentially mediate muscle mass loss in patients with LC-induced cachexia and UPR can be a powerful signaling mechanism. Indeed, previous investigations have also shown that apoptosis rather than proteolysis was the major mechanism leading to muscle wasting in animal models of cancer cachexia (Salazar-Degracia et al., 2016) and to a lower degree in COPD sarcopenia (Agusti et al., 2002).

IRE1 signals both prosurvival and proapoptotic programs in response to misfolded and unfolded proteins (Chakrabarti et al., 2011). IRE1 has endoribonuclease and serine–threonine kinase domains that exert different actions. Cytosolic IRE1 dimers also interact with adaptors such as TRAF2 to drive ASK, kinases, and nuclear factor-κB signaling pathways (Hu, Han, Couvillon, Kaufman, & Exton, 2006; Nguyen et al., 2004). Importantly, gene and protein expression levels of TRAF2 were significantly upregulated in the lower limb muscles of both groups of COPD patients. Gene and protein expression levels of ASK were also increased in the VL of cancer cachectic and in COPD patients. Collectively, these findings suggest that TRAF2 UPR marker may signal the initiation of apoptosis leading to muscle atrophy in both conditions of respiratory sarcopenia. In line with this, IRE branch of UPR was also upregulated in the hindlimb muscles of LC cachectic mice (Bohnert et al., 2016). Protein synthesis and the load of new proteins entering the ER are reduced by the action of IRE1 pathway such as XBP1. Interestingly, in the VL of patients with LC-cachexia and in COPD, gene expression levels of XBP1 were upregulated compared with levels in the controls. Furthermore, protein levels of the spliced and unspliced isoforms, XBP1s and XBP1u, respectively, were also significantly increased in the muscles of both cancer cachectic and sarcopenic COPD patients.

4.1 | Study critique

In the current investigation, upregulation of the expression of the three branches of UPR and ER stress markers in the lower limb muscles of patients with respiratory sarcopenia was a relevant observation. Of note are the significantly greater levels of expression and number of UPR markers that were upregulated in the quadriceps of the cancer cachectic patients with respect to levels encountered in the muscles of the COPD patients, even those with sarcopenia. Indeed, significant correlations between nutritional status (FFMI), body weight loss, and muscle fiber types with biological variables were only seen among patients with LC-cachexia. It is very likely that the faster rate of muscle mass loss observed in cancer cachexia as opposed to chronic disease (COPD) may account for the significantly stronger UPR and ER stress responses observed in the muscles of patients with LC-cachexia. Indeed, as the main purpose of UPR is to restore ER function to improve proper folding under stress conditions, a more exaggerated response in muscles of LC-cachexia patients may help prevent additional loss. Nonetheless, the current cross-sectional study design did not allow us to ascertain whether ER stress and UPR may be a trigger of muscle loss or may protect muscles from undergoing further protein loss. Intervention studies may help elucidate this relevant question in the near future. Despite these concerns, the current investigation is the first to demonstrate the presence of ER stress and UPR activation in the lower limb muscles of patients with two major respiratory conditions.

It should also be mentioned that differences in gene expression and protein levels between patients and control subjects were not exactly consistent for all the study markers. Methodological issues directly related to either RT-PCR or immunoblotting analyses may account for those differences. For some of the markers, the magnitude of the differences between LC-cachexia patients and healthy controls were higher than those detected between COPD patients and the controls, especially for gene expression. Finally, it should be mentioned that despite the interest in assessing potential differences in expression levels of the study markers between nonsarcopenic and sarcopenic COPD patients, the investigation was not specifically targeted to explore such a question. Importantly, the degree of lung function, exercise capacity, and muscle strength impairment was similar in both groups of COPD patients in contrast to previous investigations, in which cachectic COPD patients were more severely affected than nonsarcopenic patients (Barreiro et al., 2008, 2009; Fermoselle et al., 2012; Puig-Vilanova, Rodriguez, et al., 2014). This may partly account for discrepancies in expression levels of some of the study markers.

5 | CONCLUSIONS

ER stress and an exaggerated UPR were observed in the VL muscle of patients with respiratory sarcopenia and cachexia, particularly in those with LC. The three branches of UPR were similarly upregulated in muscles of cancer cachectic patients, whereas in the sarcopenic COPD patients, the IRE1 arm was mostly upregulated in their VL. The differential expression profile of ER stress and UPR markers observed in chronic and acute respiratory diseases offers a niche for the design of novel customized therapeutic approaches which may encompass exercise training along with pharmacological strategies purported to boost ER function. Eventually, these strategies will have implications in the clinical management of the patients.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

Study conception and design: E. B; patient assessment and recruitment and sample collection: A. S.-M; molecular biology analyses: A. S.-D.; statistical analyses and data interpretation: E. B., A. S.-D., J. G.; manuscript drafting and intellectual input: E. B, A. S.-D., A. S.-M., J. G.; manuscript writing final version: E. B.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

RESEARCH ARTICLE

Endoplasmic reticulum stress and unfolded protein response in diaphragm muscle dysfunction of patients with stable chronic obstructive pulmonary disease

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Barreiro E, Salazar-Degracia A, Sancho-Muñoz A, Aguiló R, Rodríguez-Fuster A, Gea J. Endoplasmic reticulum stress and unfolded protein response in diaphragm muscle dysfunction of patients with stable chronic obstructive pulmonary disease. *J Appl Physiol* 126: 1572–1586, 2019. First published April 18, 2019; doi:10.1152/japplphysiol.00670.2018.—Respiratory muscle dysfunction is common in patients with chronic obstructive pulmonary disease (COPD). Chronic contractile activity induces endoplasmic reticulum (ER) stress and unfolded protein response (UPR) in animals (animals and humans). We hypothesized that the respiratory muscle dysfunction associated with COPD may upregulate ER stress and UPR expression in diaphragm of stable patients with different degrees of airway obstruction and normal body composition. In diaphragm muscle specimens of patients with mild and moderate-to-severe COPD with preserved body composition and non-COPD controls (thoracotomy because of lung localized neoplasms), expression of protein misfolding (ER stress) and UPR markers, proteolysis and apoptosis (qRT-PCR and immunoblotting), and protein aggregates (lipofuscin, histology) were quantified. All patients and non-COPD controls were also clinically evaluated: lung and muscle functions and exercise capacity. Compared with non-COPD controls, patients exhibited mild and moderate-to-severe airflow limitation and diffusion capacity and impaired exercise tolerance and diaphragm strength. Moreover, compared with the controls, in the diaphragm of the COPD patients, slow-twitch fiber proportions increased, gene expression but not protein levels of protein disulfide isomerase family A member 3 and phosphatidylinositol 3-kinase catalytic subunit type 3 were upregulated, and no significant differences were found in markers of UPR transmembrane receptor pathways (activating transcription factor-6, inositol-requiring enzyme-1α, and protein kinase-like ER kinase), lipofuscin aggregates, proteolysis, or apoptosis. In stable COPD patients with a wide range of disease severity, reduced diaphragm force of contraction, and normal body composition, ER stress and UPR signaling were not induced in the main respiratory muscle. These findings imply that ER stress and UPR are probably not involved in the documented diaphragm muscle dysfunction (reduced strength) observed in all the study patients, even in those with severe airflow limitation. Hence, in stable COPD patients with normal body composition, therapeutic strategies targeted to treat diaphragm muscle dysfunction should not include UPR modulators, even in those with a more advanced disease.

NEW & NOTEWORTHY In stable chronic obstructive pulmonary disease patients with a wide range of disease severity, diaphragm muscle weakness, and normal body composition, endoplasmic reticulum stress and unfolded protein response (UPR) signaling were not induced in the main respiratory muscle. These findings imply that endoplasmic reticulum stress and UPR are not involved in the documented diaphragm muscle dysfunction observed in the study patients, even in those with severe airflow limitation. In stable chronic obstructive pulmonary disease patients with normal body composition, therapeutic strategies should not include UPR modulators.

COPD; diaphragm; endoplasmic reticulum stress; respiratory muscle dysfunction; unfolded protein response

INTRODUCTION

The prevalence of chronic obstructive pulmonary disease (COPD) is projected to increase in the next decades and is nowadays a major cause of mortality worldwide (32, 33, 44). Skeletal muscle dysfunction with and without muscle wasting is a very relevant systemic manifestation that leads to increased morbidity and mortality. In patients with COPD, the lower limb muscles are usually more severely affected than the respiratory muscles, which must remain active throughout their existence (3, 4, 9, 24, 28, 44). However, respiratory muscle dysfunction has also been well documented in several studies (4, 9, 18, 24, 44), and it has a negative impact on the patients' prognosis because of its implications in hypercapnic respiratory failure, acute exacerbations, and exercise limitation (18, 24, 43).

In COPD, the pathophysiological factors involved in the respiratory muscle dysfunction differ from those identified in the peripheral muscles (9, 18, 24). The precise length at which the diaphragm muscle fibers need to contract determines to a great extent the differences between respiratory and limb muscle dysfunction (9, 18, 20, 24). As such, airflow limitation imposes increased inspiratory loads that modify thorax geometry leading to shortening of the diaphragm muscle length (9, 18, 20, 24). Furthermore, the continuous inspiratory loads to

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which the patients are exposed to also induce a training-like effect in the diaphragm that may partly offset the deleterious effects of other factors and may render the muscle more fatigue resistant from structural and molecular standpoints, at least in early stages of the disease (20, 42). Several biological mechanisms have also been shown to contribute to the reported ventilatory muscle dysfunction in COPD (4, 9, 19, 22–24, 28).

Stress of the endoplasmic reticulum (ER) organelle, which is responsible for folding, processing, and trafficking of proteins in the cells, is involved in the regulation of skeletal muscle mass, function, and metabolism (1). Perturbations of cellular homeostasis during the course of infections, aging, and metabolic derangements induce an accumulation of unfolded proteins that lead to further imbalance of intracellular homeostasis and functional alterations (15, 25). Chaperones and foldases normally restore folding of proteins. However, a failure to fold proteins in the context of chronic or acute disease leads to degradation of these proteins by the ER through several pathways. Accumulation of unfolded proteins triggers the unfolded protein response (UPR) in eukaryote cells (15, 25). UPR is part of a sophisticated signaling program that is executed through the action of three ER transmembrane receptors with very distinct functions (15, 25).

ER stress plays a role in the maintenance of muscle physiology, in aging, and in the adaptation to muscle activity and metabolism, and exercise (1, 2, 13, 14). Interestingly, chronic contractile activity induced a rise in the expression of ER stress markers and UPR in animals (31) and humans (26). ER stress and UPR are also involved in the pathophysiology of certain myopathies (1). Whether these factors may play a role in the respiratory muscle dysfunction of patients with chronic respiratory diseases such as COPD remains to be identified.

On this basis, we hypothesized that the respiratory muscle dysfunction associated with COPD induces differential expression levels of ER stress and UPR in the main respiratory muscle, the diaphragm, in stable patients with different degrees of airway obstruction with no nutritional alterations, compared with a control group of non-COPD individuals. Accordingly, the study objectives were to explore in diaphragm muscle specimens obtained from COPD patients with a wide range of airway obstruction and in age-matched non-COPD control subjects: 1) surrogate markers of ER stress, 2) markers of the ER transmembrane receptor pathways [activating transcription factor (ATF) 6, protein kinase-like ER kinase (PERK), and inositol-requiring enzyme (IRE) 1], 3) markers of apoptosis and proteolysis, and 4) muscle structure (fiber types and morphometry) and lipofuscin aggregates. A group of non-COPD control subjects who also underwent thoracotomy was recruited for the purpose of the current investigation.

MATERIALS AND METHODS

Study Subjects

This was a prospective controlled cross-sectional study in which 20 patients with stable COPD and normal body composition and 8 age-matched non-COPD controls were recruited. COPD patients were further subdivided into the following two groups: mild COPD patients ($n = 11$) and moderate-to-severe COPD patients ($n = 9$). Diaphragm muscle specimens were obtained from all subjects (both patients and controls) who underwent thoracotomy for a localized lung neoplasm. Smoking history was similar between patients and healthy controls. All patients were on bronchodilators. They were clinically stable at the time of the study, without episodes of exacerbation or oral steroid treatment in the previous four months. None of them presented significant comorbidities. All groups of individuals were Caucasian.

Exclusion criteria. Exclusion criteria for COPD patients and the control subjects included other chronic respiratory or cardiovascular disorders, acute exacerbation in the last 3 mo, limiting osteoarticular condition, chronic metabolic diseases, suspected paraneoplastic or myopathic syndromes, severe muscle wasting, and/or treatment with drugs known to alter muscle structure and/or function, including oral corticosteroids. COPD patients and the non-COPD controls were qualified as sedentary after being specifically inquired about whether they were conducting any regular outdoor physical activity, going regularly to the gymnasium, or participating in any specific training program.

The current investigation was designed in accordance with both the ethical standards on human experimentation in our institutions and the World Medical Association guidelines (Helsinki Declaration of 2008) for research on human beings. Approval was obtained from the institutional Ethics Committee on Human Investigation (Hospital del Mar, Barcelona, Spain). Informed written consent was obtained from all individuals.

Anthropometrical and Functional Assessment of Patients and Non-COPD Controls

Anthropometrical evaluation included body mass index (BMI) and determination of the fat-free mass index (FFMI) by bioelectrical impedance (16). Nutritional parameters were also evaluated through conventional blood tests.

Lung function was evaluated through determination of spirometric values, static lung volumes, diffusion capacity, and blood gases using standard procedures and reference values by Roca et al. (38–40).

Inspiratory muscle strength was assessed through determination of maximal inspiratory pressure at the mouth (Sibelmed-163; Sibel, Barcelona, Spain) during an occluded maneuver from residual volume. Both COPD patients and control subjects underwent maximal transdiaphragmatic pressure measurements, which were calculated from the difference between both maximal gastric and esophageal pressures obtained during a sniff maneuver from forced residual capacity (30). Two balloon catheters were positioned in the midesophagus and gastric cavity and then coupled to pressure transducers (Transpac II; Abbot, Chicago, IL) connected to a digital recorder (BIOPAC Systems, Santa Barbara, CA) (5, 34).

Exercise capacity was assessed through the 6-min walking distance following current guidelines (11, 36, 37). The test consisted of two attempts (with at least a 30-min rest between them) in a 30-meter corridor. Encouragement was given every minute, and the test was interrupted if symptoms of exhaustion appeared. Patients and the non-COPD controls also performed a progressive incremental exercise test performed on a cycloergometer as previously described (41).

Muscle Biopsies and Blood Samples

Diaphragm biopsies. During thoracotomy for localized lung lesions, diaphragm biopsy specimens were obtained from the anterior costal diaphragm lateral to the insertion of the phrenic nerve (5, 30). The localized lesions were always either peripheral solitary nodes or small lung neoplasms not showing major airway obstruction in any case, as assessed by fiber optic bronchoscopy. Muscle samples were 30–50 mg size in average.

Muscle sample specimens were always cleaned out of any blood contamination with saline. They were immediately frozen in liquid nitrogen and stored in the −80°C freezer (under permanent alarm control) for further analyses or immersed in an alcohol-formol bath for 2 h to be thereafter embedded in paraffin. Frozen tissues were used for immunoblotting techniques, whereas paraffin-embedded tissues were used for the assessment of myosin heavy chain isoforms (immunohistochemical analysis). All subjects were prevented from doing

any potentially exhausting physical exercise 10–14 days before coming to the hospital to undergo the surgical procedures.

Blood samples. In both patients and non-COPD controls, blood samples were drawn at 8:00 a.m. following an overnight fasting period to determine nutritional status and inflammatory parameters.

Biological Analyses

All biology analyses were conducted blind in the same laboratory by the same investigators, at Hospital del Mar-Institut Hospital del Mar d'Investigacions Mèdiques (Barcelona). A description of the different techniques and target biomarkers follows.

RNA isolation. Total RNA was first isolated from snap-frozen skeletal muscles using Trizol reagent following the manufacturer's protocol (Life Technologies, Carlsbad, CA). Total RNA concentrations were determined photometrically using the NanoDrop 1000 (Thermo Scientific, Waltham, MA).

Gene Expression Levels of the Study Markers

mRNA reverse transcription. Reverse transcription was performed using TaqMan RNA assays (Life Technologies) following the manufacturer's instructions. First-strand cDNA was generated from mRNA using oligo(dT)$_{12-18}$ primers and the Super-Script III reverse transcriptase following the manufacturer's instructions (Life Technologies).

Real time-PCR amplification. TaqMan-based qPCR reactions were performed using the ABI PRISM 7900HT Sequence Detector System (Applied BioSystems, Foster City, CA) together with commercially available predesigned primers, and probes of the genes of all the study biomarkers as shown in Table 1. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control for mRNA gene expression. Subsequently, mRNA data were collected and subsequently analyzed using SDS Relative Quantification Software version 2.1 (Applied BioSystems) in which the comparative C_T method ($2^{-\Delta\Delta C_T}$) for relative quantification was employed (27). Samples were always run in triplicates, and their corresponding expression was calculated as the mean value of the three measurements. Results in Figs. 1–8 are expressed as the expression of fold change relative to the mean value of the control group, which was equal to 1.

Protein levels using immunoblotting of 1D electrophoresis. Protein levels of the different molecular markers analyzed in the study were explored by means of immunoblotting procedures as previously described (5, 7, 8, 10, 12, 16, 17, 29, 30). Briefly, frozen muscle samples from the diaphragm muscles of both patients and control subjects were homogenized in a buffer containing 50 mM HEPES, 150 mM NaCl, 100 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA, 0.5% Triton X, 2 µg/ml leupeptin, 100 µg/ml PMSF, 2 µg/ml aprotinin, and 10 µg/ml pepstatin A. The entire procedures were always conducted at 4°C. Protein levels in crude homogenates were spectrophotometrically determined with the Bradford method using triplicates in each case and bovine serum albumin as the standard (Bio-Rad protein reagent; Bio-Rad, Hercules, CA). The final protein concentration in each sample was calculated from at least two Bradford measurements that were almost identical. Equal amounts of total protein (ranging from 20 to 100 µg, depending on the antigen and antibody) from crude muscle homogenates were always loaded on the gels, as well as identical sample volumes/lanes. For the purpose of comparisons among the different groups of COPD patients and controls, muscle sample specimens were always run together and kept in the same order.

Three fresh 10-well minigels were always simultaneously loaded for each of the antigens and run together in the same minicell box. Experiments were confirmed at least two times for all of the antigens analyzed in the investigation. Fresh gels were specifically loaded for each of the antigens in most cases. However, in a few cases, antigens were identified from stripped membranes.

Proteins were then separated by electrophoresis, transferred to polyvinylidene difluoride (PVDF) membranes, blocked with 1% bovine serum albumin or 5% nonfat milk, depending on the primary antibody, and incubated overnight with the corresponding selective primary antibodies. In the study, the most relevant markers of UPR pathways and ER stress were evaluated using specific primary antibodies as described below.

Markers of ER stress. Anti-protein disulfide isomerase family A member 3 (PDIA3) antibody from Enzolife (Farmingdale, NY), anti-phosphatidylinositol 3-kinase catalytic subunit type 3 (PI3K) antibody from Cell Signaling (Boston, MA), and anti-binding immunoglobin protein (BIP) antibody from Abcam (Cambridge, UK) were used.

Markers of ATF6 pathway. Anti-ATF6 from Abcam antibody was used.

Markers of PERK pathway. Anti-PERK and anti-C/EBP-homologous protein (CHOP) antibodies from Abcam were used.

Markers of IRE1 pathway. Anti-endoplasmic reticulum to nucleus signaling 1 (IRE1), anti-TNF receptor associated factor 2 (TRAF2), and anti-X-box-binding protein 1 (XBP1) from Abcam antibodies were used.

Markers of proteolysis (muscle-specific E3 ligases). Anti-atrogin-1 antibody from Acris (Herford, Germany) and anti-muscle ring finger protein-1 (MuRF-1; Santa Cruz, Santa Cruz, CA) antibody were used.

Finally, anti-GAPDH antibody from Santa Cruz was also used to identify the loading control in all the immunoblots and antibodies conducted in the study.

Antigens from all samples were detected with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) and a chemiluminescence kit (Thermo Scientific, Rockford, IL). For each of the antigens, samples from the different groups were always detected in the same image under identical exposure times. The specificity of the different antibodies was confirmed by omission of the primary antibody and incubation of the membranes only with secondary antibodies. PVDF membranes were scanned with the Molecular Imager Chemidoc XRS System (Bio-Rad Laboratories) using the software Quantity One version 4.6.5 (Bio-Rad Laboratories). Optical densities of specific proteins were quantified using the software Image Laboratory version 2.0.1 (Bio-Rad Laboratories). Final optical densities obtained in each specific group of subjects corresponded to the mean values of the different samples (lanes) of each of the study antigens. To validate equal protein loading across lanes, the glycolytic enzyme GAPDH was used as the protein-loading controls in all of the immunoblots (Figs. 3–7).

Standard stripping methodologies were employed to detect the loading control GAPDH for each of the analyzed markers (BIP, PDIA3, PI3K, ATF6, PERK, CHOP, IRE1, TRAF2, XBP1, MuRF-1, and atrogin-1). Briefly, membranes were stripped of primary and secondary antibodies through one 30-min wash with a stripping solution (25 mM glycine, pH 2.0, and 1% SDS) followed by two consecutive 10-min washes containing phosphate-buffered saline with Tween at room temperature. Membranes were blocked with bovine serum albumin and reincubated with primary and secondary antibodies following the procedures described above.

Muscle structural analyses. Fiber counts and morphometry were assessed on 3-µm muscle paraffin-embedded sections from all groups of patients and controls. MyHC-I (slow-twitch fibers) and -II (fast-twitch fibers) isoforms were counted by identification of the proportions of the latter fibers using specific anti-MyHC-II antibody (Sigma-Aldrich, St. Louis, MO), following methodologies previously published (34–37). The nonstained fibers were identified as the MyHC-I myofibers. Fast-twitch fibers were positively stained with the corresponding antibody (brown), whereas nonstained fibers were slow-twitch fibers. The cross-sectional area, mean least diameter, and proportions of type I and type II fibers were determined using a light microscope (Olympus BX 61; Olympus, Tokyo, Japan), which was coupled with an image-digitizing camera (Olympus DP 71; Olympus Corporation), and the Image J software (National Institute of Health,

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Table 1. *Probes used for the quantitative analyses of the target genes using qRT-PCR.*

	Target Gene	Gene Symbol	Assay ID	GeneBank Accession No.
Markers of protein misfolding	BiP	HSPA5	Hs99999174_m1	NM_005347.4
	HSP60	HSPD-1	Hs01036753_g1	NM_002156.4
	HSP70	HSPA1A	Hs00359163_s1	NM_005345.5
	CNX	CANX	Hs01558409_m1	NM_001024649.1
				NM_001746.3
	CRT	CALR	Hs00189032_m1	NM_004343.3
	PDIA3	PDIA3	Hs00607126_m1	NM_005313.4
	PI3K	PIK3C3	Hs00176908_m1	NM_001308020.1
				NM_002647.3
ATF6 pathway	ATF6	ATF6	Hs00232586_m1	NM_007348.3
PERK pathway	PERK	EIF2AK3	Hs00984006_m1	NM_001313915.1
				NM_004836.6
	ATF4	ATF4	Hs00909569_g1	NM_001675.4
				NM_182810.2
	CHOP	DDIT3	Hs00358796_g1	NM_001195053.1
				NM_001195054.1
				NM_001195055.1
				NM_001195056.1
				NM_001195057.1
				NM_004083.5
IRE1 pathway	IRE1	ERN1	Hs00176385_m1	NM_001433.3
	TRAF2	TRAF2	Hs00184192_m1	NM_021138.3
	XBP1	XBP1	Hs00231936_m1	NM_001079539.1
				NM_005080.3
Markers of proteolysis	Atrogin-1	FBXO32	Hs01041408_m1	NM_001242463.1
				NM_058229.3
				NM_148177.2
	MuRF-1	TRIM63	Hs00261590_m1	NM_032588.3
Markers of apoptosis	CASP3	CASP3	Hs00234387_m1	NM_004346.3
				NM_032991.2
	CASP7	CASP7	Hs00169152_m1	NM_001227.4
				NM_001267056.1
				NM_001267057.1
				NM_001267058.1
				NM_001320911.1
				NM_033338.5
				NM_033339.4
				NM_033340.3
	CASP9	CASP9	Hs00609647_m1	NM_001229.4
				NM_001278054.1
				NM_032996.3
	ASK	MAP3K5	Hs01039896_m1	NM_005923.3
	BAX	BAX	Hs00180269_m1	NM_001291428.1
				NM_001291429.1
				NM_001291430.1
				NM_001291431.1
				NM_004324.3
				NM_138761.3
				NM_138763.3
				NM_138764.4
	BCL2L11	BCL2L11	Hs01076940_m1	NM_001204106.1
Loading control	GADPH	GAPDH	Hs99999905_m1	NM_002046.5

ID, identification; Hs, *Homo sapiens*; NM, mRNA RefSeq database category; BiP, binding immunoglobin protein; HSPA 5, heat shock protein family A (Hsp70) member 5; HSP, heat shock protein; HSPD-1, heat shock protein family D (Hsp60) member 1; HSPA1A, heat shock protein family A (Hsp70) member 1A; CNX, CANX, calnexin; CRT, calreticulin; PDIA3, protein disulfide isomerase family A member 3; PI3K, PIK3C3, phosphatidylinositol 3-kinase catalytic subunit type 3; ATF, activating transcription factor; PERK, protein kinase R (PKR)-like endoplasmic reticulum kinase; EIF2AK3, eukaryotic translation initiation factor 2 α kinase 3; CHOP, C/EBP-homologous protein; DDIT3, DNA damage-inducible transcript 3; IRE1, ERN1, endoplasmic reticulum to nucleus signaling 1; TRAF2, TNF receptor-associated factor 2; XBP1, X-box-binding protein 1; FBXO32, F-box protein 32; MuRF-1, muscle ring finger protein-1; TRIM63, tripartite motif containing 63; CASP, caspase; ASK1, apoptosis signal-regulating kinase 1; MAP3K5, mitogen-activated protein kinase kinase kinase 5; BAX, B cell lymphoma 2 (BCL2)-associated X apoptotic regulator; BCL2L11, BCL2-like 11 apoptosis regulator Bcl-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

available at http://rsb.info.nih.gov/ij/) (Fig. 1). At least 100 fibers were measured and counted in the muscles from all study groups of patients and controls.

Lipofuscin aggregates. Formation of lipofuscin was measured through identification of aggregates in the diaphragms of both study groups of subjects. Lipofuscin aggregates were identified on 3-μm paraffin-embedded muscle cross sections using hematoxylin-eosin staining. Images of the lipofuscin areas were taken at ×400 under a light microscope (Olympus BX 61), which was coupled with an image-digitizing camera (Olympus DP 71). To quantify the area of lipofuscin aggregates in all images of the study muscles, a digital pen and the Image J software (National Institute of Health, available at
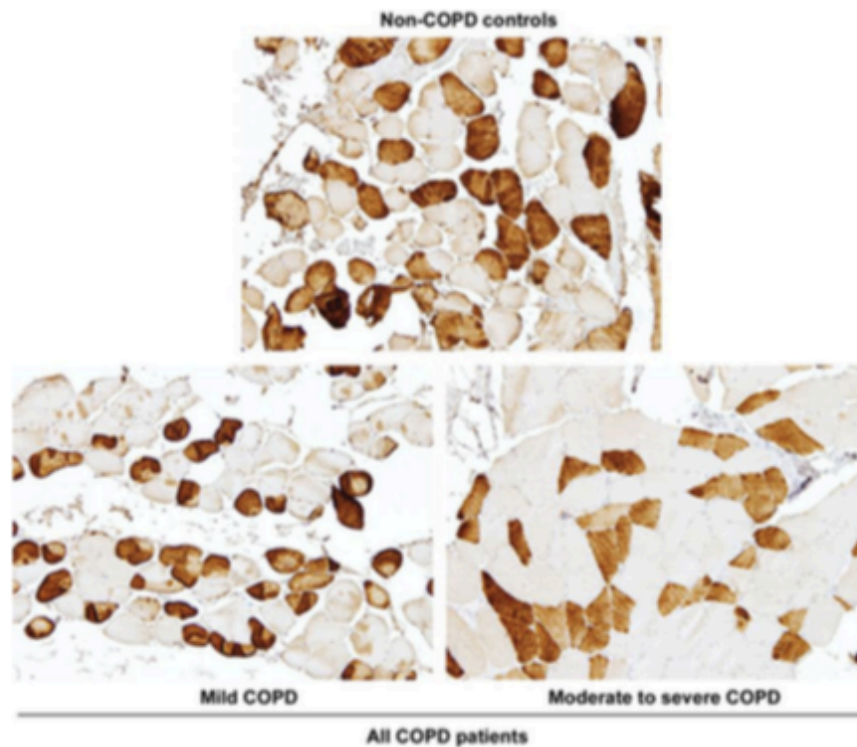
Fig. 1. Representative examples of stained muscle fibers (×100) within diaphragm muscles of non-chronic obstructive pulmonary disease (COPD) controls (top), mild COPD (bottom right), and moderate-to-severe COPD patients (bottom left). Fast-twitch fibers were positively stained with the corresponding antibody (brown), whereas nonstained fibers were slow-twitch fibers.

http://rsb.info.nih.gov/ij/) were used (Fig. 2). Data are expressed as the percentage of the ratio of the measured lipofuscin area to the total area of muscle cross section in each sample (both patients and controls).

Statistical Analyses

Statistical power was calculated using specific software (StudySize 2.0; CreoStat HB, Frolunda, Sweden). Forced expiratory volume in 1 s (FEV_1) was selected as the target variable on the basis of the *t*-test to estimate the statistical power between healthy controls and COPD patients as a whole. On the basis of standard power statistics established at a minimum of 80% and assuming an α-error of 0.05, the statistical power was sufficiently high to detect a minimum difference of 22 points between groups in the sample size ($N = 8$ minimum number of subjects in each group) and SD. Additionally, the sample size in the study was similar to that used in previous investigations (5, 11, 34–37) in the field. Normality of the study variables was checked using Shapiro-Wilk test. One-way analysis of variance, in which Tukey's post hoc analyses were used to adjust for multiple comparisons, was employed to test potential differences among the study groups. Pearson's Chi-Square test was employed to assess potential differences between groups in the qualitative variables such as smoking history. Variables that described the clinical characteristics of the study population are represented as means and SD, whereas the

molecular variables are represented as means and 95% confidence interval. A level of significance of $P \leq 0.05$ was established. Statistical analyses were performed using the Statistical Package for the Social Sciences (Portable SPSS, PASW statistics 18.0 version for windows; SPSS, Chicago, IL).

RESULTS

Clinical Characteristics

Clinical and functional variables of all the study subjects are shown in Table 2. Age did not significantly differ among the study subjects. Body composition as measured by body mass index (BMI) and fat-free mass index (FFMI) did not significantly differ among the study groups (Table 2). Smoking history was similar among the study groups (Table 2). All COPD patients exhibited mild and moderate-to-severe airflow limitation and mild airway trapping (Table 2). Compared with the controls, in moderate-to-severe COPD patients, exercise capacity and diaphragm muscle strength were significantly reduced (Table 2). Levels of C-reactive protein, fibrinogen, and globular sedimentation velocity were significantly greater in
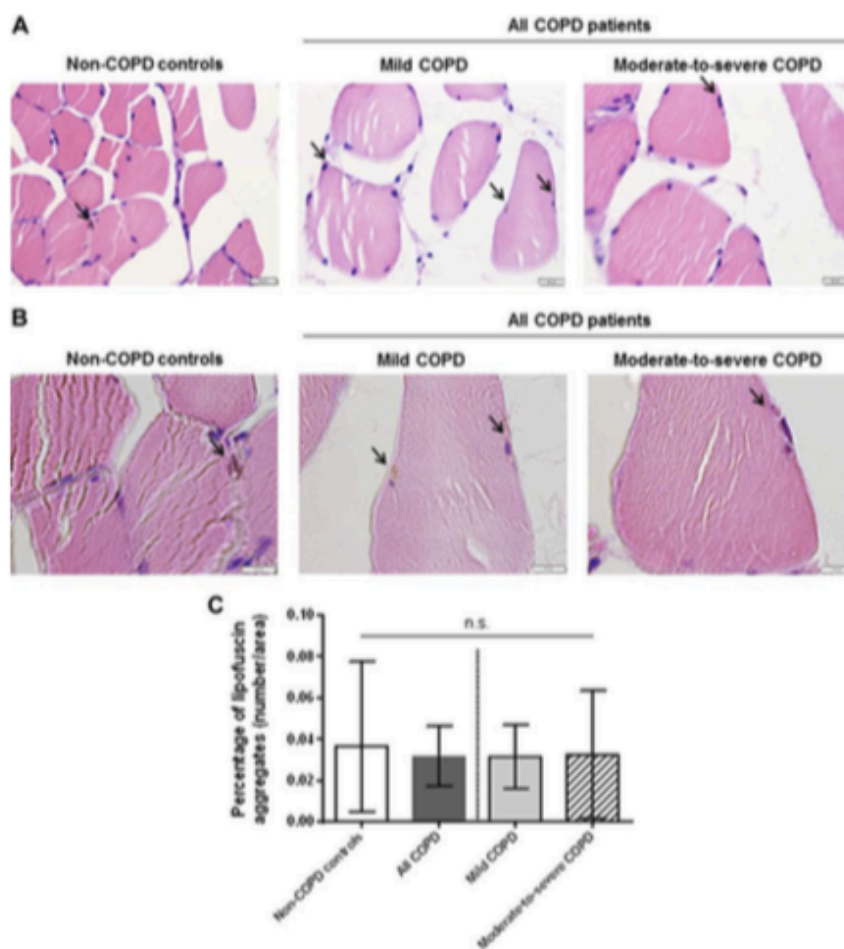
Fig. 2. *A*: representative examples of lipofuscin aggregates (×400, black arrows) within diaphragm muscle samples of non-chronic obstructive pulmonary disease (COPD) controls, mild COPD, and moderate-to-severe COPD patients. *B*: representative examples of lipofuscin aggregates at a higher magnification (×1,000, black arrows) within diaphragm muscle samples of non-COPD controls, mild COPD, and moderate-to-severe COPD patients. *C*: mean values and 95% confidence intervals of lipofuscin aggregates identified in diaphragm muscle specimens of non-COPD controls, mild COPD, and moderate-to-severe COPD patients. Lipofuscin content is expressed as the percentage of lipofuscin aggregates with respect to the total area of the measured muscle samples in each group of study subjects.

moderate-to-severe COPD patients than in the non-COPD control subjects or the mild COPD (Table 2).

Muscle Structural Features

As expected, the proportions of slow-twitch fibers were significantly higher in the diaphragm of all COPD patients, especially in the moderate-to-severe patients compared with those observed in the controls (Table 3 and Fig. 1). The cross-sectional area of either slow- or fast-twitch muscle fibers did significantly differ among the study groups (Table 3 and

Fig. 1). Levels of lipofuscin aggregates did not significantly differ among the study groups of patients (Fig. 2, *A–C*).

Markers of ER Stress and UPR in the Diaphragm Muscle

In the diaphragm of patients with COPD as a whole, gene expression levels of PI3K and PDIA3 were upregulated compared with those seen in the controls (Fig. 3*A*). Gene expression of the latter marker was also upregulated in the diaphragms of the moderate-to-severe COPD patients (Fig. 3*A*). Nonetheless, gene expression levels in the diaphragm
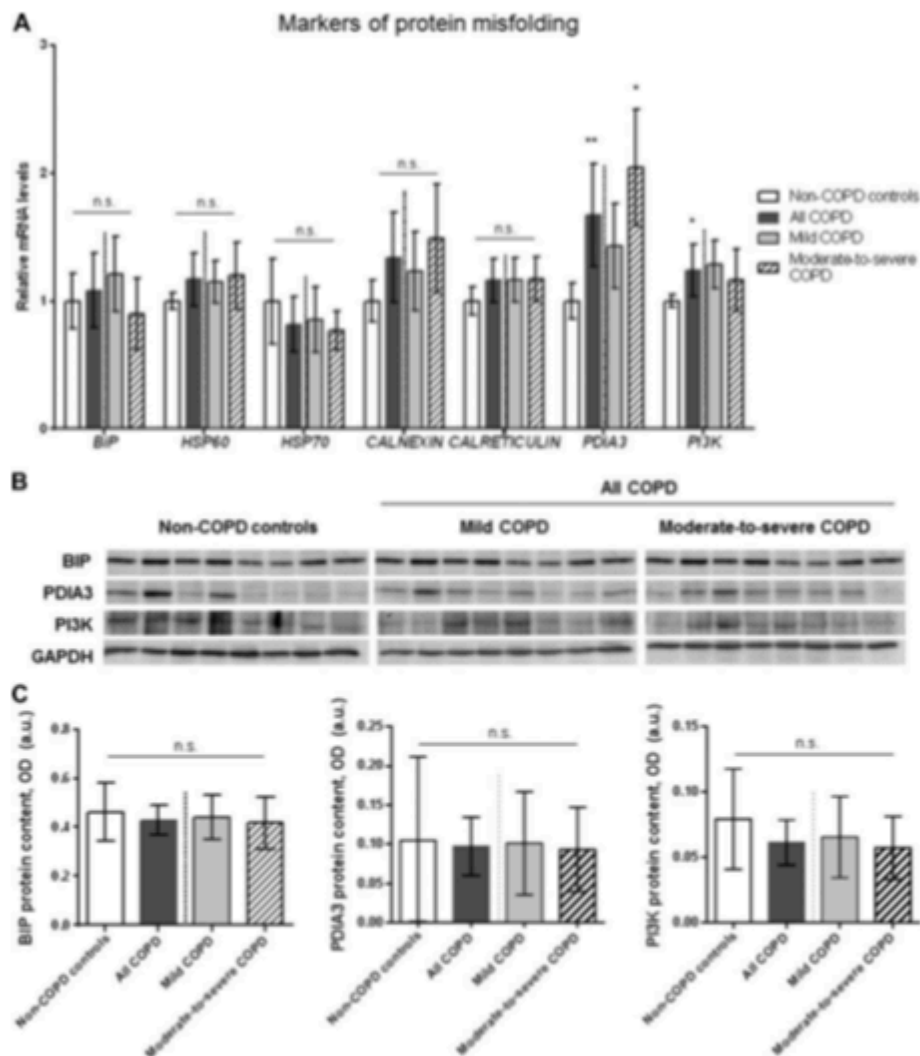
Fig. 3. *A*: mean values and 95% confidence intervals of the levels of mRNA expression in the diaphragm muscle samples for non-chronic obstructive pulmonary disease (COPD) controls, mild COPD, and moderate-to-severe COPD patients expressed as relative mRNA levels of the following markers: binding immunoglobin protein (*BIP*), heat shock protein (*HSP*) 60, *HSP70*, calnexin, calreticulin, protein disulfide-isomerase (*PDIA3*), and phosphatidylinositol 3-kinase catalytic subunit type 3 (*PI3K*). Representative immunoblots (*B*) and mean values and 95% confidence intervals of protein content as measured by optical densities (OD) in arbitrary units (au) (*C*) of the following markers: BIP, PDIA3, and PI3K in the diaphragm muscles of non-COPD controls, mild COPD, and moderate-to-severe COPD patients. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. *$P \leq 0.05$ and **$P \leq 0.01$ between any of the COPD patients (mild and moderate-to-severe COPD patients) or as a whole group and the non-COPD controls.

of the ER stress markers BIP, heat shock protein 60, heat shock protein 70, calnexin, or calreticulin did not significantly differ among the study groups of patients (Fig. 3*A*). Protein levels of the markers PI3K, PDIA3, and BIP were assessed in the diaphragms of the study groups. No significant differences were observed in muscle protein levels of these ER stress markers among the study groups of subjects (Fig. 3, *B* and *C*). Gene and protein expression levels of ATF6 pathway in the respiratory muscle did not significantly differ between COPD patients and the controls (Fig.
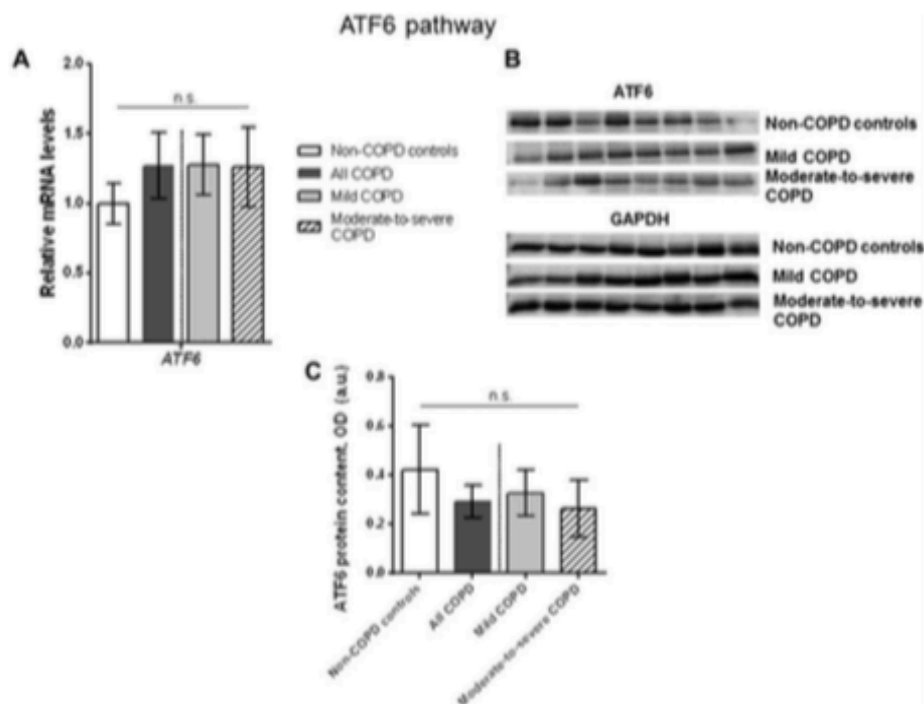
Fig. 4. A: mean values and 95% confidence intervals of the levels of mRNA expression (A), representative immunoblots (B), and mean values and 95% confidence intervals of protein content as measured by optical densities (OD) in arbitrary units (au) (C) of the activating transcription factor (ATF) 6 in the diaphragm muscles of non-chronic obstructive pulmonary disease (COPD) controls, mild COPD, and moderate-to-severe COPD patients. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

4, A–C). Gene and protein expression levels of the PERK pathway (PERK, ATF4, and CHOP) did not significantly differ in the muscles between patients and the non-COPD controls (Fig. 5, A and B). Gene and protein levels of the IRE1 pathway (IRE1, TRAF2, and XBP1) in the diaphragm muscle were not significantly different in the patients from those seen in the controls (Fig. 6, A–C).

Markers of Proteolysis and Apoptosis in Muscles

Muscle gene and protein expression levels of E3 ligases atrogin-1 and MuRF-1 did not significantly differ between patients and the controls (Fig. 7, A–C). Finally, gene expression levels of the apoptotic markers caspase-3, caspase-7, signal-regulating kinase, B cell lymphoma 2-associated X apoptotic regulator, and B cell lymphoma 2 were not different in the diaphragms of the patients from those seen in the non-COPD controls (Fig. 8).

DISCUSSION

Summary of the Main Findings

First of all, it should highlighted that gene expression levels of the protein misfolding (ER stress) markers PDIA3 and PI3K were upregulated in the diaphragm of the COPD

patients of a wide range of airway obstruction and normal body composition compared with the control subjects. Moreover, gene expression levels of PDIA3 were significantly upregulated in the diaphragm of the patients with a more severe airway obstruction. Nonetheless, no significant differences were detected in the diaphragm among the study groups when protein levels were analyzed using immunoblotting. In line with this, counts of the ER stress marker lipofuscin aggregates did not significantly differ in the diaphragm between any of the patient groups and the non-COPD controls.

On the other hand, muscle gene expression and protein levels of the three ER transmembrane receptor pathways (ATF6, PERK, and IRE1) did not significantly differ between patients and the non-COPD control subjects. Correspondingly, gene and protein expression levels of markers of the downstream pathways analyzed in the study, namely proteolysis and apoptosis, did not significantly differ between patients and the control subjects.

To our knowledge, the current investigation is the first to show evidence on the status of ER stress and UPR in the human diaphragm of patients with COPD along with those without this condition who were the control subjects.
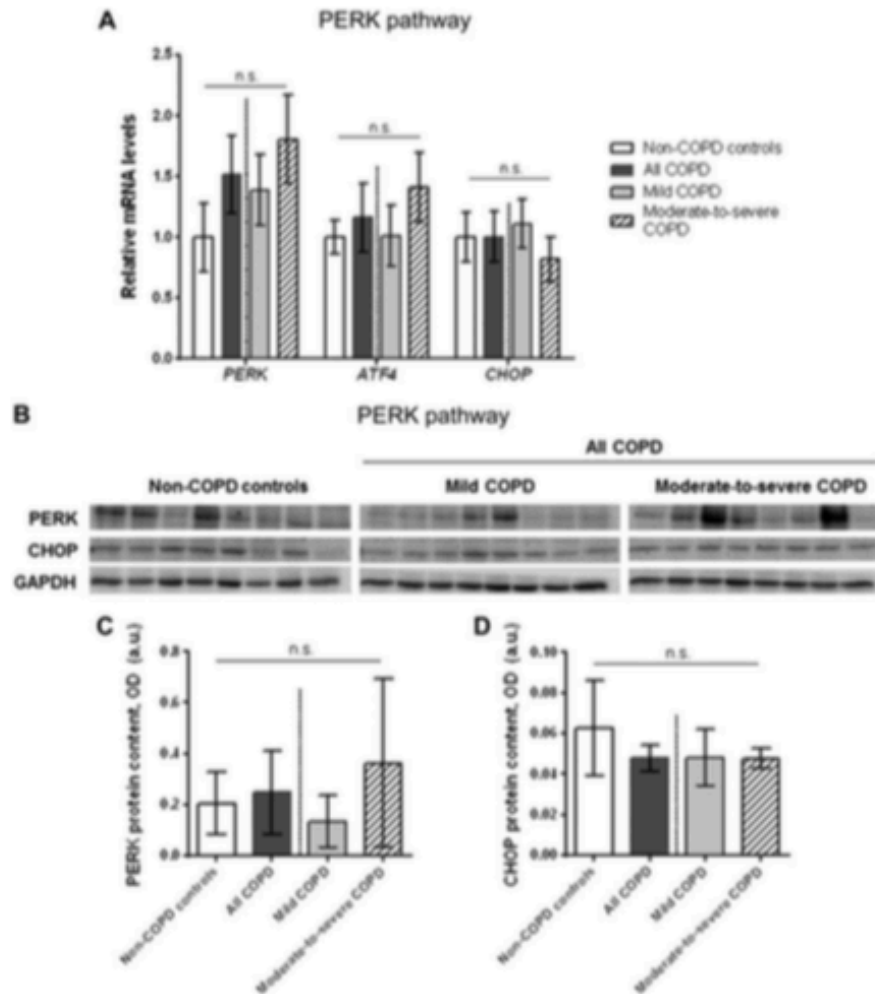
Fig. 5. A: mean values and 95% confidence intervals of the levels of mRNA expression in the diaphragm muscle samples for non-chronic obstructive pulmonary disease (COPD) controls, mild COPD, and moderate-to-severe COPD patients expressed as relative mRNA levels of the following markers: protein kinase-like ER kinase (PERK), activating transcription factor (ATF) 4, and C/EBP-homologous protein (CHOP). Representative immunoblots (B) and mean values and 95% confidence intervals of protein content as measured by optical densities (OD) in arbitrary units (au) of the markers PERK (C) and CHOP (D) in the diaphragm muscles of non-COPD controls, mild COPD, and moderate-to-severe COPD patients. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

ER Stress and UPR in the Diaphragm Muscle of Patients with COPD

In the ER, physiological folding, processing, and trafficking of proteins occur in all cells. ER is also involved in calcium homeostasis. Accumulation of misfolded proteins in response to several injuries that disrupt cell homeostasis leads to ER stress. Unpaired cysteine residues, exposed hydrophobic regions, and aggregation of proteins are counted among the most relevant markers of unfolded or misfolded proteins. Interestingly, three main ER transducers (ATF6, PERK, and IRE1α) compose UPR in response to ER stress. These ER transmembrane sensors slow the flow of new proteins into ER through several biochemical processes (transcriptional, translational, and posttranslational modifications), which enhance ER activity for protein folding and processing while favoring the elimination of misfolded proteins and increasing the size of ER. When ER stress cannot be reversed by UPR, apoptosis and increased protein catabolism are induced within the cells (1, 15, 25). In fact, UPR signaling pathways may
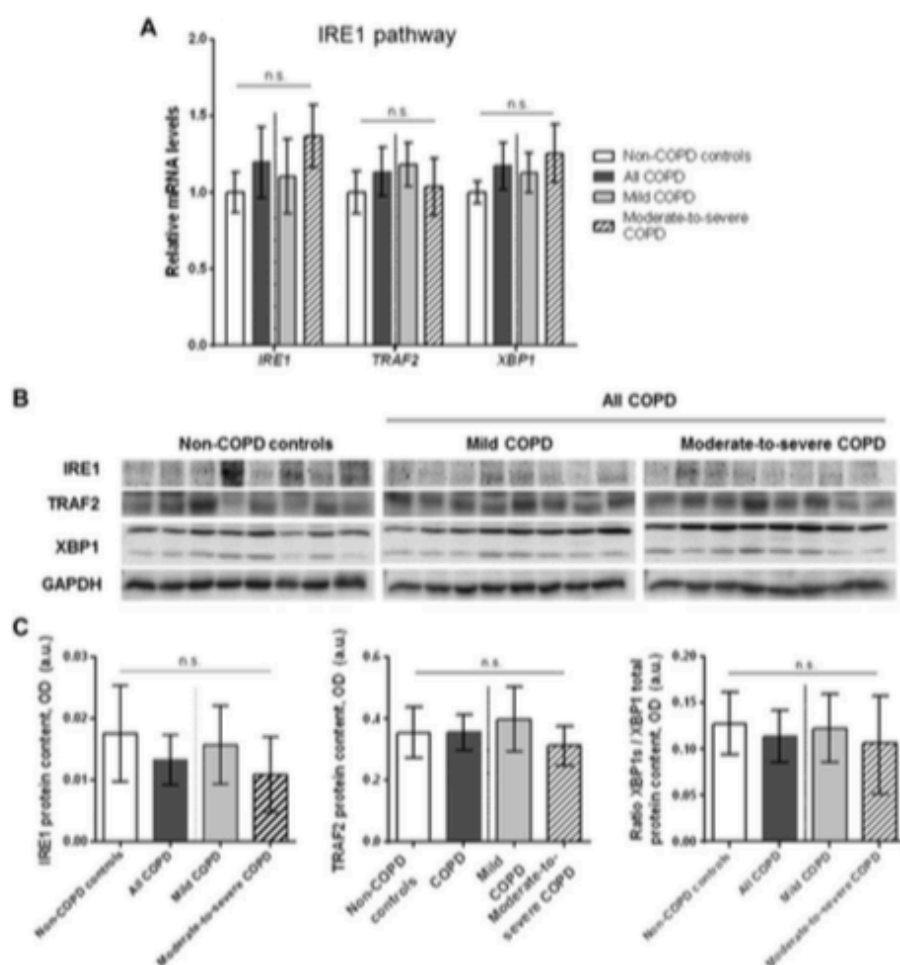
Fig. 6. *A*: mean values and 95% confidence intervals of the levels of mRNA expression. Representative immunoblots (*B*) and mean values and 95% confidence intervals of protein content as measured by optical densities (OD) in arbitrary units (au) (*C*) of the following markers: inositol-requiring enzyme (IRE)-1, TNF receptor-associated factor 2 (TRAF2), and X-box-binding protein 1 (XBP1) in the diaphragm muscles of non-chronic obstructive pulmonary disease (COPD) controls, mild COPD, and moderate-to-severe COPD patients. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

determine restoration of cell survival or apoptosis (1, 15, 25). Hence, ER stress and UPR are very relevant signaling pathways that may determine cell fate in chronic and acute conditions (1, 15, 25).

Despite that ER stress and UPR were demonstrated to be involved in the pathophysiology of critical illness and in response to increased muscle activation (1, 15, 25), its potential role in the skeletal muscle dysfunction of chronic respiratory diseases such as COPD has not yet been defined. Activation of UPR in muscles may take place as a result of fluctuations in calcium levels within the ER during muscle contractions and

exercise (1, 45). Because COPD patients are continuously exposed to the increased inspiratory loads, we reasoned that ER stress and UPR markers would be upregulated in their diaphragm muscle, especially in those with reduced diaphragm force of contraction. In the present study, only the expression of the ER stress markers PDIA3 and PI3K was upregulated in the diaphragm of patients with COPD, especially in those with a more severe disease (greater airway obstruction and inspiratory loads). However, protein levels of these markers did not significantly differ in the respiratory muscle between patients and the non-COPD controls. These findings suggest that post-
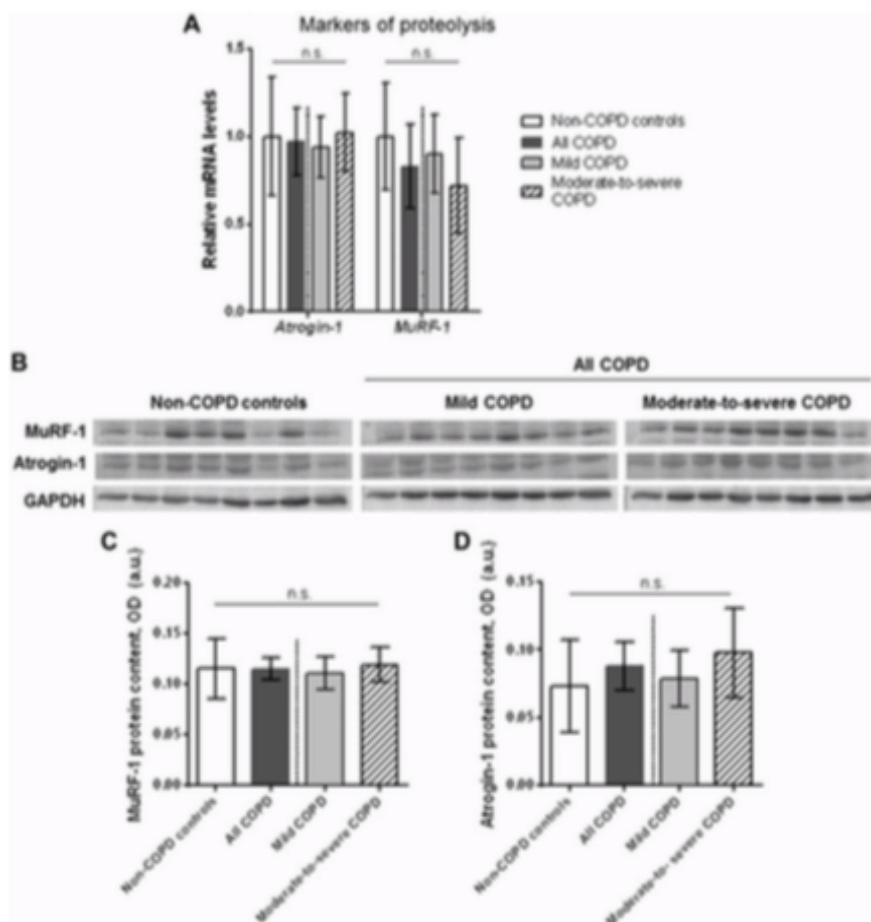
Fig. 7. A: mean values and 95% confidence intervals of the levels of mRNA expression. Representative immunoblots (B) and mean values and 95% confidence intervals of protein content as measured by optical densities (OD) in arbitrary units (au) of the markers muscle ring finger protein-1 (MuRF-1, C) and atrogin-1 (C) in the diaphragm muscles of non-chronic obstructive pulmonary disease (COPD) controls, mild COPD, and moderate-to-severe COPD patients. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

transcriptional regulation of the ER stress markers may have taken place in the diaphragm, thus precluding potential differences in protein levels of those markers between COPD and the controls (1, 15, 25). These findings also imply that stronger muscle contractions such as those taking place during exacerbations might have induced a more powerful stimulus to trigger ER stress and UPR. In line with this, bouts of running induced a significant rise in ER stress and UPR in muscles of humans (26) and rats (31). Nonetheless, exacerbation-induced strong muscle contractions of the diaphragm would not possibly be studied in clinical settings. On the other hand, aging may modulate ER stress and UPR (13). Because the non-COPD controls were age matched, aging was not a factor with a potential influence in the response to ER stress in this study.

Other factors such as medication or increased levels of acute-phase reactants that were seen among the COPD patients were probably not involved in the induction of ER stress or UPR in the main respiratory muscle, since no differences were observed between patients and the non-COPD controls.

Importantly, it should also be mentioned that gene expression and protein levels of proteolytic markers and apoptosis did not differ in the diaphragm of the COPD patients from those detected in the non-COPD controls. These findings are in agreement with the lack of significant modifications encountered in the levels of ER stress and UPR in the diaphragm muscle of patients with stable COPD. Future investigations conducted on animal models of COPD should
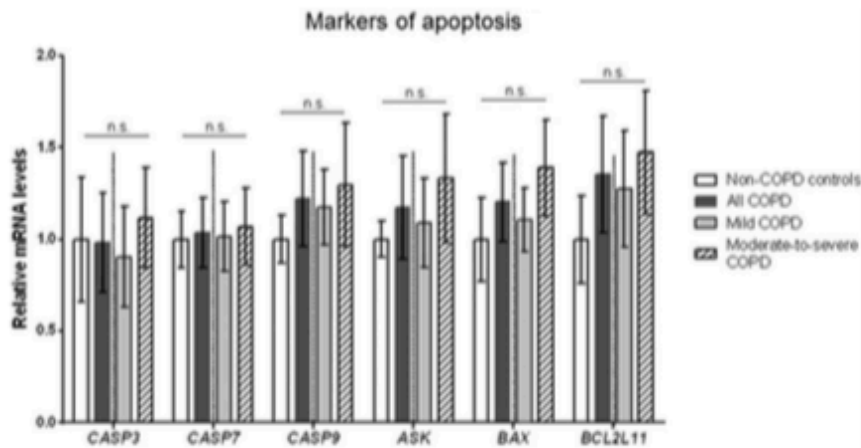
Markers of apoptosis

Fig. 8. Mean values and 95% confidence intervals of expression levels of the following markers: caspase (*CASP*) *3, CASP7, CASP9*, apoptosis signal-regulating kinase (*ASK*), B cell lymphoma 2 (BCL2)-associated X (*BAX*), and BCL2-like 11 apoptosis regulator Bcl-2 (*BCL2L11*) expressed as relative mRNA levels in the diaphragm muscle of non-chronic obstructive pulmonary disease (COPD) controls, mild COPD, and moderate-to-severe COPD patients.

explore whether greater muscle contractions as a result of exacerbations induce ER stress and UPR in the diaphragm.

Lung and Respiratory Muscle Functions and Structure in COPD Patients

In the study, two separate groups of COPD patients were studied with the aim to explore whether disease severity might have influenced the expression of markers of either ER stress or UPR. To test this objective, COPD patients of a wide range of airway obstruction and normal body composition and diaphragm muscle dysfunction were recruited for the purpose of the investigation. In line with this, a first group of patients exhibited a mild airway obstruction, whereas the second group showed moderate-to-severe airway obstruction (FEV$_1$ 72 and 50%, respectively). In general, all of the patients exhibited a moderate reduction in total lung diffusion capacity and exercise tolerance. Body composition as measured by BMI and FFMI did not differ among the study subjects. Signs of systemic manifestations were also seen among the COPD patients, especially in the more severe ones, since a significant rise in blood C-reactive protein, fibrinogen, and globular sedimentation velocity blood markers was detected compared with the non-COPD controls. These findings are consistent with previous investigations from our group in which patients with COPD exhibited similar clinical and pathophysiological features (5, 34).

Importantly, the previously reported (5, 34) switch toward a more resistant phenotype has also been observed in the current investigation. These findings were especially seen in the diaphragm of the patients with a more severe airway obstruction. Diaphragm function as measured by transdiaphragmatic pressure was significantly reduced in the COPD patients, especially in the moderate-to-severe patients, compared with the non-COPD controls. Such a reduction in diaphragm strength may be the result of a decline in muscle fiber contractility and/or the increase in residual lung volume experienced by the patients,

especially those with severe airflow limitation. Because no signs of muscle atrophy, as also previously reported (4, 5, 9, 20, 24), were detected in the diaphragm muscle of any of the patient groups, it is likely that the greater values of residual volume, which modifies the position of this muscle to properly contract, may have partly contributed to the reduced transdiaphragmatic pressure observed in the patients with more severe airflow limitation. In this model of human disease, in which biopsies were obtained from thoracic surgery, in vitro contractility experiments could not be made because of logistics constraints. Future investigations should aim to decipher the specific contribution of those factors to the reported decline in diaphragm strength. Finally, the switch toward a more resistant phenotype was probably the result of the increased inspiratory loads that patients are exposed to, which may mimic a "training-like effect" of the diaphragm myofibers (4, 5, 9, 20, 21, 24).

Study Limitations

Diaphragm muscle biopsies from the study subjects were obtained during thoracotomy because of localized lung lesions, the gold standard technique to obtain diaphragm specimens from different populations. Although lung volume reduction surgery also makes it possible to obtain diaphragm specimens, only very severe COPD patients undergo that type of surgery, thus making the study of mild and moderate patients or non-COPD control subjects impossible. Therefore, diagnostic-therapeutic thoracotomy is the only approach available for studying moderate and mild COPD and normal lung function subjects. Accordingly, subjects recruited for the purpose of the study share a common morbidity: the presence of a small and localized lung neoplasm. Nevertheless, we do not believe that this condition has made any significant contribution to the results obtained in the analyses of the diaphragm muscles, since extremely restrictive criteria were employed to properly select the population, and subjects showing either nutritional abnormalities or paraneoplastic syndromes were systematically

Table 2. *Anthropometric characteristics and functional status of the study subjects.*

	Non-COPD Controls	All COPD Patients	COPD Patients	
			Mild COPD	Moderate-to-severe COPD
N	8	20	11	9
Anthropometry				
Age, yr	66 (5)	69 (7)	70 (6)	68 (7)
BMI, kg/m^2	24 (3)	26 (3)	25 (3)	28 (3)
FFMI, kg/m^2	18 (1)	18 (1)	18 (1)	18 (1)
Smoking history				
Active, N, %	4, 50	10, 50	5, 45	5, 56
Ex-smoker, N, %	3, 38	10, 50	6, 55	4, 44
Never smoker, N, %	1, 12	0, 0	0, 0	0, 0
Packs-year	46 (15)	58 (29)	52 (22)	65 (36)
Lung function				
FEV$_1$, %predicted	86 (7)	62 (13)***	72 (4)***	50 (6)***§§§
FVC, %predicted	85 (12)	78 (11)	83 (8)	72 (11)*
FEV$_1$/FVC, %	72 (3)	58 (8)***	63 (5)*	53 (9)***§§
RV, %predicted	104 (24)	134 (44) $P = 0.090$	123 (48)	146 (38)
TLC, %predicted	97 (11)	100 (15)	98 (13)	104 (17)
RV/TLC	47 (7)	50 (10)	47 (11)	53 (8)
DL$_{CO}$, %predicted	87 (18)	72 (14)*	72 (15)	73 (14)
K$_{CO}$, %predicted	87 (18)	73 (18) $P = 0.084$	79 (17)	67 (18) $P = 0.069$
Pa$_{O_2}$, kPa	11 (2)	11 (1)	11 (1)	10 (1)
Pa$_{CO_2}$, kPa	6 (0.3)	5 (1)	5 (1)	5 (1)
Exercise capacity				
Vo$_{2peak}$, %predicted	85 (12)	66 (19)*	66 (21) $P = 0.072$	65 (17) $P = 0.064$
WR$_{peak}$, %predicted	62 (7)	48 (13)**	50 (11) $P = 0.081$	47 (16)*
6-min walking test, m	500 (75)	447 (95)	501 (65)	362 (65)**§§§
Muscle function				
MIP, cmH$_2$O	85 (4)	83 (17)	88 (15)	76 (16)
Pdi$_{max}$, cmH$_2$O	133 (25)	105 (17)**	117 (11) $P = 0.095$	91 (11)***§§
Blood parameters				
Albumin, g/dl	4 (0.3)	4 (0.4)	4 (0.5)	4 (0.2)
Total proteins, g/dl	7 (1)	7 (1)	7 (1)	7 (0.3)
CRP, mg/dl	0.3 (0.2)	0.6 (0.3)*	0.5 (0.2)	0.7 (0.4)**
Fibrinogen, mg/dl	338 (60)	394 (108)	341 (61)	463 (120)*§
GSV, mm/h	6 (4)	15 (9)***	10 (8)	20 (6)***§§

Values are expressed as means (SD); n, no. of patients. COPD, chronic obstructive pulmonary disease; BMI, body mass index; FFMI, fat-free mass index; FEV$_1$, forced expiratory volume in 1 s; pred, predicted; FVC, forced vital capacity; RV, residual volume; TLC, total lung capacity; DL$_{CO}$, carbon monoxide transfer; K$_{CO}$, Krough transfer factor; Pa$_{O_2}$, arterial oxygen partial pressure; Pa$_{CO_2}$, arterial carbon dioxide partial pressure; Vo$_{2peak}$, peak exercise oxygen uptake; WR$_{peak}$, peak work rate; MIP, maximal inspiratory pressure; Pdi$_{max}$, maximal transdiaphragmatic pressure; CRP, C-reactive protein; GSV, globular sedimentation velocity. Statistical significance: *$P \le 0.05$, **$P \le 0.01$ and ***$P \le 0.001$ between any of the COPD patients and the non-COPD controls; §$P \le 0.05$, §§$P \le 0.01$, and §§§$P \le 0.001$ between mild COPD patients and moderate-to-severe COPD patients. Moreover, actual P values below $P \le 0.01$ are indicated for the same type of comparisons.

excluded. Therefore, we consider all the findings reported in the study to be rather associated with COPD. Furthermore, it should also be acknowledged that the current experimental approaches were not suited to conduct in vitro contractility analyses.

In conclusion, in stable COPD patients with a wide range of disease severity, reduced diaphragm force of contraction, and normal body composition, ER stress and UPR signaling were not induced in the main respiratory muscle. These findings imply that ER stress and UPR are probably not involved in the documented diaphragm muscle dysfunction (reduced strength) observed in all of the study patients, even in those with severe airflow limitation. Hence, in stable COPD patients with normal body composition, therapeutic strategies targeted to treat diaphragm muscle dysfunction should not include UPR modulators, even in those with a more advanced disease.

Table 3. *Fiber type composition in the diaphragm of the study subjects.*

Muscle Fiber Type Composition	Non-COPD Controls	All COPD Patients	COPD Patients	
			Mild COPD	Moderate-to-severe COPD
N	8	20	11	9
Type I fibers, %	47 (5)	52 (6)*	50 (6)	55 (6)*§
Type II fibers, %	53 (5)	48 (6)*	50 (6)	45 (6)*§
Type I fiber CSA, μm^2	2,204 (677)	2,556 (737)	2,553 (796)	2,560 (706)
Type II fiber CSA, μm^2	2,532 (621)	2,476 (990)	2,463 (1,035)	2,493 (992)

Values are expressed as mean (SD). COPD, chronic obstructive pulmonary disease; CSA, cross-sectional area. *$P \le 0.05$ between any of the COPD patients and the non-COPD controls; §$P \le 0.05$ between mild COPD patients and moderate-to-severe COPD patients.

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DISCUSIÓN

MANUSCRITO 1: **Deficient muscle regeneration potential in sarcopenic COPD patients: Role of satellite cells.**

En el primer trabajo de investigación, los hallazgos más relevantes fueron que los pacientes con EPOC, tanto con sarcopenia como con estado nutricional conservado, presentaban una disminución en la capacidad de ejercicio y en la fuerza muscular. Sin embargo, el principal hallazgo fue que, en el músculo vasto lateral del cuádriceps (VL) de ambos grupos de pacientes con EPOC, el número de núcleos internos, células satélite activadas (Pax-7+/Myf-5+) y núcleos TUNEL-positivos fue significativamente mayor que en los controles, mientras que la expresión de los marcadores de regeneración muscular temprana (fase de proliferación, Pax-7 y Myf-5) y tardía (fase de diferenciación, MyoD y MyHCI) se redujo. Los niveles del potente regulador negativo miostatina sólo aumentaron en los músculos de las extremidades de los pacientes EPOC con sarcopenia.

En conjunto, estos resultados sugieren que los marcadores del potencial regenerativo en los músculos de las extremidades de los pacientes EPOC, y fundamentalmente en los que tienen sarcopenia, están reducidos y que la miostatina puede desempeñar un papel importante. Además, como el aumento de los niveles de daño muscular se detectó en mayor medida en el VL de los pacientes EPOC y sarcopenia (un 64% mayor que en los músculos de los sujetos control), es probable que dichas alteraciones estructurales también estén implicadas en el desencadenamiento del proceso de regeneración muscular (Cheung & Rando, 2013; Yin et al., 2013).

En los pacientes con EPOC independientemente del grado de composición corporal, el número de núcleos centrales (marcador de la regeneración muscular) y los de las células satélite senescentes están aumentados en los músculos de las extremidades inferiores (Thériault et al., 2012, 2014).

En los pacientes con EPOC, se observaron correlaciones positivas entre los parámetros de la función pulmonar, especialmente la obstrucción de las vías respiratorias y la capacidad de difusión, y las proporciones de fibras de contracción lenta. Los pacientes con mejores parámetros de función pulmonar tienen una mayor proporción de fibras de tipo I. Por el contrario, las proporciones y el tamaño de las fibras de tipo II se correlacionaron de forma inversa con el grado de obstrucción de las vías respiratorias y la capacidad de difusión. Se trata de hallazgos relevantes que sugieren que la función pulmonar contribuye en parte al cambio de tipo de fibra lenta a rápida en los músculos de las extremidades inferiores de los pacientes con EPOC (Barreiro et al., 2018; Puig-Vilanova, Martínez-Llorens, et al., 2015; Puig-Vilanova, Rodriguez, et al., 2015). Además, el tamaño de las fibras de contracción lenta y rápida y el de las fibras híbridas también se correlacionaron con el FFMI, lo que sugiere que la masa corporal magra se asocia con una mayor área de todos los tipos de fibras musculares. Además, el daño muscular y el recuento de núcleos internos también se correlacionaron inversamente con la fuerza isométrica del músculo cuádriceps. En conjunto, se trata de hallazgos novedosos y relevantes que indican que la estructura y la función muscular están claramente interrelacionadas y deberían evaluarse de forma rutinaria asistencialmente a los pacientes con EPOC, especialmente en pacientes con alteraciones en su composición corporal.

Otro hallazgo interesante del estudio fue la correlación negativa encontrada entre los niveles plasmáticos de la proteína C reactiva (PCR) y el número de células satélite activadas. Estos resultados muestran que la inflamación sistémica, medida por la PCR, influye de alguna manera en el proceso de regeneración muscular.

En la presente investigación, se reclutó cuidadosamente a un número relativamente grande de pacientes con EPOC y sarcopenia de una edad bastante "joven", con un declive significativo de la función muscular del

cuádriceps, un estado nutricional preservado y una composición corporal alterada. Con el objetivo de dilucidar si el potencial de regeneración muscular puede ser obstaculizado en los músculos de las extremidades inferiores de los pacientes con EPOC y sarcopenia, se determinaron dos fenotipos diferentes de células satélite en el presente estudio. Como se caracterizó anteriormente (Kuang et al., 2007), las células satélite sublaminares, que expresan Pax-7 pero no expresan Myf-5, constituyen el reservorio de células satélite de un músculo determinado. En esa investigación (Kuang et al., 2007), se demostró que las células satélite Pax-7+/Myf-5+ se diferencian preferentemente en fibras musculares, mientras que las células satélite Pax-7+/Myf-5- contribuyen al reservorio de células satélite ampliando este compartimento dentro del músculo esquelético. Las conclusiones de ese estudio (Kuang et al., 2007) fueron que se establecieron dos subpoblaciones diferentes de células satélite sobre la base de su capacidad de expresar Myf-5 (Kuang et al., 2007). Así, las células satélite Pax-7+/Myf-5+ se identificaron como los progenitores miogénicos comprometidos, mientras que las células satélite Pax-7+/Myf-5- se definieron como las células madre propiamente dichas (Kuang et al., 2007).

Es importante destacar que, en nuestra investigación, se detectó una disminución significativa del número de células satélite Pax-7+/Myf-5- (células madre) sólo en los músculos de las extremidades de los pacientes EPOC con sarcopenia, pero no en los que tenían una composición corporal conservada y una función muscular normal del cuádriceps. Estos hallazgos implican que el depósito de células satélite se vio obstaculizado en los músculos esqueléticos de los pacientes con EPOC y sarcopenia, lo que podría poner en peligro el proceso de regeneración muscular. De hecho, las proporciones de fibras musculares de contracción lenta se redujeron sólo en el VL de los pacientes EPOC con sarcopenia. Además, el tamaño de las miofibras de contracción lenta, rápida e híbrida también era significativamente menor en los músculos de los pacientes con EPOC y sarcopenia, pero no así en los que tenían una composición corporal conservada. Además, las anormalidades estructurales

musculares fueron incluso mayores en el VL de los pacientes con EPOC y sarcopenia que en los que no tenían pérdida muscular. Estos resultados son muy consistentes con los obtenidos en investigaciones previas (Barreiro et al., 2019; Puig-Vilanova, Martínez-Llorens, et al., 2015; Puig-Vilanova, Rodriguez, et al., 2015), quizá se expliquen en parte por el peor potencial regenerativo detectado en los músculos de las extremidades de los pacientes con EPOC y sarcopenia.

Las proporciones de fibras híbridas aumentaron en los músculos de las extremidades de ambos grupos de pacientes con EPOC y fueron incluso mayores en el grupo de sarcopenia. Sin embargo, el área de las fibras musculares fue menor en el grupo de pacientes con EPOC y sarcopenia. Éstos son hallazgos relevantes que implican que los músculos de los pacientes con EPOC, especialmente los que tienen sarcopenia, son capaces de adaptarse a los factores ambientales como la inactividad, el ejercicio o el envejecimiento (Medler, 2019).

En conjunto, estos hechos son de suma importancia, ya que se requiere un correcto programa de regeneración muscular para lograr la plena recuperación de la masa y la función muscular en respuesta a diferentes modalidades de entrenamiento. Por lo tanto, los resultados actuales tienen posibles implicaciones clínicas para el diseño de programas de entrenamiento específicos, ya que los pacientes con un potencial regenerativo defectuoso pueden ser menos susceptibles de mejorar su masa y/o función muscular, incluso aquellos con un estado nutricional preservado.

Cabe destacar que los músculos de las extremidades inferiores de ambos grupos de pacientes con EPOC experimentaron la activación de un programa de regeneración muscular de forma similar. En consonancia con esto, el

número de células satélite específicas activadas (Pax-7+/Myf-5+), los núcleos internos y los núcleos positivos para TUNEL aumentaron notablemente y de forma similar en el VL de ambos grupos de pacientes con EPOC grave en comparación con los detectados en los músculos de los sujetos control. Estos hallazgos están en consonancia con resultados anteriores, en los que el proceso de regeneración muscular también se desencadenó en el VL de pacientes con EPOC grave con un amplio rango de pérdida de masa muscular (Thériault et al., 2012, 2014). Sin embargo, en otro estudio (Menon et al., 2012), el número de células satélite fue similar entre los pacientes con EPOC y los controles sanos. Las diferencias en el nivel de alteración de los compartimentos de la composición corporal y/o la función y la masa muscular pueden explicar las discrepancias encontradas entre los estudios (Menon et al., 2012; Thériault et al., 2012, 2014).

Además, los niveles de expresión génica de los marcadores tempranos de regeneración muscular Pax-7 y Myf-5 estaban regulados a la baja en el musculo de ambos grupos de pacientes con EPOC grave en comparación con los controles. Los factores de transcripción Pax-7 y Myf-5 desempeñan papeles clave durante la fase de proliferación del proceso de regeneración muscular (Guitart et al., 2018; Yin et al., 2013). En consonancia con esto, también se han descrito resultados similares en el cuádriceps de pacientes con EPOC y caquexia (Plant et al., 2010; Thériault et al., 2012, 2014).

La disminución de la expresión génica de los factores de transcripción MyoD y Myogenina y de la isoforma MyHC-I como marcadores de diferenciación muscular tardía durante el proceso de regeneración fue otro hallazgo relevante de esta investigación. Estos hallazgos también están en línea con los demostrados previamente en los músculos de pacientes con EPOC avanzada (Plant et al., 2010; Puig-Vilanova, Martínez-Llorens, et al., 2015; Thériault et al., 2012, 2014).

La miostatina, un miembro de la familia del factor de crecimiento transformante beta, es un regulador negativo del crecimiento muscular. También se sabe que la miostatina inhibe la proliferación de células satélite y mioblastos a través de varios mecanismos que conducen a la retirada del ciclo celular (Walsh & Celeste, 2005). Es importante destacar que los niveles de miostatina fueron significativamente mayores en los músculos de las extremidades de los pacientes sarcopénicos que los detectados en los pacientes sin sarcopenia o en los sujetos control. Estos resultados están en consonancia con estudios anteriores, en los que se demostró que los niveles de miostatina aumentaban sistemáticamente en los músculos de las extremidades inferiores de los pacientes con EPOC avanzada (Harish et al., 2019; Plant et al., 2010; Puig-Vilanova, Martínez-Llorens, et al., 2015; Walsh & Celeste, 2005). A la vista de todos estos hallazgos, sería posible concluir que la miostatina puede haber interferido en el proceso de proliferación de las células musculares en una fase temprana del proceso de regeneración, lo que ha conducido a un crecimiento y desarrollo muscular deficientes tras la lesión. Por lo tanto, este puede ser otro mecanismo de pérdida de masa muscular, además del aumento de la proteólisis y/o apoptosis, como también se ha demostrado en investigaciones anteriores (Plant et al., 2010; Puig-Vilanova, Martínez-Llorens, et al., 2015; Vogiatzis et al., 2010). No obstante, la elucidación del papel preciso y las implicaciones de la miostatina en la regeneración y el crecimiento muscular en la sarcopenia de los pacientes EPOC tendrá que confirmarse definitivamente en futuras investigaciones. Por otro lado, los hallazgos descritos también pueden tener futuras implicaciones clínicas, ya que se ha demostrado que el bloqueo de la miostatina mediante anticuerpos específicos revierte parcialmente la pérdida de masa y función muscular en varios modelos experimentales (Harish et al., 2019; Iskenderian et al., 2018; St. Andre et al., 2017) y en pacientes (Burch et al., 2017; Scimeca et al., 2017). Si los anticuerpos antimiostatina también pueden utilizarse eficazmente en pacientes con enfermedades no musculares, como en la EPOC con sarcopenia, será un tema de investigación futura.

MANUSCRITO 2: Endoplasmic reticulum stress and unfolded protein response profile in quadriceps of sarcopenic patients with respiratory diseases.

En el segundo manuscrito se ha abordado una cuestión relevante y novedosa en pacientes bien caracterizados con sarcopenia respiratoria de dos etiologías diferentes, condición crónica (EPOC) frente a subaguda (Cáncer de Pulmón). Como se esperaba, en el vasto lateral del cuádriceps (VL) tanto de los pacientes con caquexia por cáncer de pulmón como en los enfermos con EPOC y sarcopenia, el fenotipo muscular se caracterizaba por un aumento en las proporciones de fibras de contracción rápida, mientras que su tamaño era significativamente menor, como indicación de atrofia muscular. Estos hallazgos son similares a los descritos previamente en los músculos de las extremidades de los pacientes caquécticos con EPOC y en aquellos con cáncer de pulmón (Barreiro et al., 2018; Fermoselle et al., 2012; Puig-Vilanova, Rodriguez, et al., 2015). Además, en los pacientes con EPOC y sarcopenia, el área transversal de las fibras de contracción lenta también se redujo en comparación con la observada en los controles.

El perfil de expresión de los marcadores de estrés del retículo endoplásmico (RE) y de la UPR difiere en cierta medida en los músculos sarcopénicos de los pacientes con EPOC de aquellos con caquexia por cáncer de pulmón. Estos son hallazgos novedosos que impulsan a la UPR como un potencial impulsor de la señalización de la atrofia muscular en pacientes con enfermedades respiratorias.

El correcto plegamiento, procesamiento y tráfico de proteínas tiene lugar en el RE, que también desempeña un papel central en la síntesis de proteínas y la homeostasis del calcio celular (Almanza et al., 2019). El estrés del RE se

produce por acumulación de proteínas mal plegadas en respuesta a condiciones perjudiciales que alteran la homeostasis celular. Las perturbaciones intrínsecas del RE, como las que se producen en el cáncer (inestabilidad genética y mutaciones) y en enfermedades neurodegenerativas, pueden dar lugar al estrés del RE (Almanza et al., 2019). Además, las perturbaciones extrínsecas derivadas del estrés microambiental (agotamiento de nutrientes y oxígeno y acidosis) y el aumento de la producción de especies reactivas de oxígeno también conducen al estrés del RE (Almanza et al., 2019).

A pesar de que recientemente se ha demostrado que la UPR subyace en la fisiopatología de varias afecciones pulmonares agudas (enfermedades críticas) y crónicas (fibrosis quística, fibrosis pulmonar y EPOC; (Baek et al., 2012; Bartoszewski et al., 2008; Hassan et al., 2014), su papel en los músculos esqueléticos no es bien conocido, y mucho menos en afecciones específicas caracterizadas por una pérdida grave de masa muscular. La activación del estrés del RE y la UPR también puede tener lugar en los músculos esqueléticos durante el ejercicio y el envejecimiento (Bohnert et al., 2018). Dado que el plegado de proteínas en el RE depende del calcio, del redox y de la energía, el estrés del RE puede ser un mecanismo relevante que conduzca al desgaste muscular en las enfermedades crónicas. De hecho, se han reportado perturbaciones en esos procesos homeostáticos en los músculos de las extremidades inferiores de pacientes con EPOC con disfunción y desgaste muscular (Barreiro et al., 2010; Fermoselle et al., 2012; Puig-Vilanova, Ausin, et al., 2014; Puig-Vilanova, Martínez-Llorens, et al., 2015; Puig-Vilanova, Rodriguez, et al., 2015). En el presente estudio, se ha buscado explorar si el estrés del RE y la UPR pueden estar regulados en los músculos sarcopénicos con dos enfermedades respiratorias diferentes: la enfermedad crónica frente a la subaguda.

El estrés del RE activa la UPR que está mediada por tres sensores transmembrana del RE identificados como ATF6, PERK e IRE1. El UPR revierte el estrés del RE frenando el flujo de nuevas proteínas hacia el RE a través de una serie de procesos transcripcionales, traslacionales y postraduccionales que aumentan la capacidad del RE para el plegamiento y procesamiento de proteínas, mejoran la eliminación de proteínas mal plegadas y amplían el tamaño del RE (Kelsen, 2016). Es importante destacar que la UPR también puede inducir la apoptosis y la degradación de proteínas si el estrés del RE no se puede revertir (Chakrabarti et al., 2011; Kelsen, 2016). De hecho, el restablecimiento de la homeostasis celular y la supervivencia o la apoptosis también están determinados por la activación de las vías de señalización de la UPR (Chakrabarti et al., 2011; Kelsen, 2016).

El procesamiento postraduccional de todas las proteínas de membrana y secretoras en el RE comprende una variedad de chaperonas residentes, foldasas, oxidorreductasas y disulfuro isomerasas (Schröder & Kaufman, 2005). Estas chaperonas promueven los enlaces disulfuro y la glicosilación de las proteínas en el RE en un proceso de control de calidad antes de que las proteínas salgan de este orgánulo. Las chaperonas moleculares, como la calnexina, la calreticulina, la BIP y otras HSP, se unen específicamente a las proteínas desplegadas o mal plegadas en el RE para corregir su plegamiento.

En el presente estudio, los niveles de expresión de las chaperonas HSP60, la calnexina y la PI3K estaban regulados al alza en el VL de los pacientes con EPOC y sarcopenia y con caquexia inducida por cáncer de pulmón. Además, en los músculos de las extremidades inferiores de estos últimos pacientes, los niveles de expresión de BIP, HSP70, calnexina, calreticulina y PDIA3 también estaban significativamente regulados. Estos hallazgos implican que el estrés de RE está presente en los músculos periféricos de los pacientes con sarcopenia respiratoria, y particularmente en aquellos con cáncer de pulmón. En

consonancia, también se observó una regulación al alza de los marcadores de estrés de RE en los músculos de las extremidades posteriores de ratones caquécticos portadores del carcinoma pulmonar de Lewis (Bohnert et al., 2016). Sin embargo, en lo que a nosotros respecta, la presente investigación es la primera que informa de la presencia de huellas de estrés de RE en los músculos esqueléticos de pacientes reales con sarcopenia respiratoria de dos etiologías y plazos diferentes: crónica versus subaguda.

En respuesta a las proteínas desplegadas o mal plegadas, la vía de ATF6 se activa a través de una serie de pasos de translocación y procesamiento proteolítico irreversible que conducen a la regulación de los programas transcripcionales de prosupervivencia (Chakrabarti et al., 2011). Los niveles de expresión génica de ATF6 sólo se incrementaron significativamente en el VL de los pacientes con caquexia inducida por cáncer de pulmón, mientras que los niveles de proteína de este marcador también se incrementaron significativamente en los músculos de las extremidades de los pacientes sarcopénicos con EPOC. Curiosamente, ATF6 también puede interactuar con las vías de degradación de proteínas, como el sistema del proteosoma de ubiquitina, para potenciar la proteólisis y la autofagia. Los niveles de expresión de los genes Atrogin-1 y MuRF-1 estaban significativamente regulados en el VL de los pacientes con caquexia por cáncer de pulmón. Sin embargo, los niveles de proteína del marcador de autofagia LC3B sólo fueron significativamente mayores en los músculos de los pacientes con EPOC sarcopénico, lo que sugiere que el ATF6 desencadena más bien la vía del proteosoma de ubiquitina en los músculos de los pacientes con caquexia por cáncer que la autofagia. Estudios anteriores también demostraron un aumento de los marcadores de autofagia en el VL de los EPOC sarcopénicos, pero no en aquellos con cáncer pulmón (Guo et al., 2013; Puig-Vilanova, Rodriguez, et al., 2015).

Tanto los programas de prosupervivencia como los proapoptóticos son señalados por el brazo PERK de la UPR tras la acumulación de proteínas desplegadas o mal plegadas. La proteína transmembrana PERK consta de un dominio de proteína quinasa citosólica y un sensor de estrés luminal del RE. En el estudio, los niveles de proteína PERK activa, que fosforila eIF2a, fueron significativamente mayores en el VL de los pacientes con caquexia por cáncer de pulmón. Durante el estrés del RE la fosforilación de eIF2α por PERK regula a la baja la síntesis de proteínas bloqueando la traducción (Almanza et al., 2019; Rowlands et al., 1988). Consistentemente, los niveles de proteína de p-EIF2α también fueron significativamente más altos en los músculos de los pacientes caquécticos por cáncer de pulmón, lo que implica que el brazo de PERK estaba efectivamente activado en los músculos de las extremidades inferiores de estos pacientes.

La expresión génica de CHOP estaba regulada al alza en los cuádriceps de los pacientes con caquexia por cáncer de pulmón y en los de EPOC en menor medida. Además, los niveles de proteína CHOP también aumentaron significativamente en los músculos de las extremidades inferiores de los pacientes con caquexia por cáncer y EPOC sarcopénica. ATF6 también puede inducir la expresión de XBP1 y CHOP para potenciar la señalización UPR (Almanza et al., 2019). En consonancia con esto, la expresión génica y los niveles de proteína CHOP y XBP1 también fueron significativamente mayores en el VL de los pacientes con caquexia por cáncer de pulmón y en los pacientes con EPOC, especialmente en los sarcopénicos (proteínas CHOP y XBP1u), lo que implica que estas tres vías pueden potenciar las cascadas de señalización UPR y los mecanismos descendentes en los músculos de esos pacientes.

Es importante destacar que el aumento de la expresión de CHOP puede provocar estrés oxidativo, agotamiento de ATP y muerte celular (Almanza et al.,

2019; Chakrabarti et al., 2011; Hiramatsu et al., 2014). En el presente estudio, los niveles de estrés oxidativo fueron significativamente mayores en el VL tanto de los pacientes caquécticos por cáncer de pulmón como de todos los grupos de pacientes con EPOC (MSA-protein adducts), como también se ha demostrado consistentemente en estudios anteriores (Barreiro et al., 2010, 2018; Puig-Vilanova, Rodriguez, et al., 2015). Los niveles de expresión de los marcadores de apoptosis como las caspasas 7 y 9 y ASK también fueron regulados al alza en el VL de los pacientes con caquexia por cáncer de pulmón. A pesar de que no se encontraron correlaciones entre el estrés oxidativo y los marcadores de autofagia o apoptosis, estas observaciones sugieren que la apoptosis puede mediar preferentemente en la pérdida de masa muscular en pacientes con caquexia inducida por cáncer y la UPR puede ser un poderoso mecanismo de señalización. De hecho, en investigaciones anteriores también se ha demostrado que la apoptosis, más que la proteólisis, era el principal mecanismo que conducía al desgaste muscular en modelos animales de caquexia por cáncer (Salazar-Degracia et al., 2016) y en menor grado en la sarcopenia por EPOC (Agustí et al., 2002).

IRE1 señala tanto programas prosupervivencia como proapoptóticos en respuesta a proteínas mal plegadas y desplegadas (Chakrabarti et al., 2011). IRE1 tiene dominios de endorribonucleasa y serinetreonina quinasa que ejercen diferentes acciones. Los dímeros citosólicos de IRE1 también interactúan con adaptadores como TRAF2 para impulsar las vías de señalización ASK, quinasas y del factor nuclear-κB (Hu et al., 2006; Nguyen et al., 2004). Es importante destacar que los niveles de expresión génica y proteica de TRAF2 estaban significativamente regulados al alza en los músculos de las extremidades inferiores de ambos grupos de pacientes con EPOC. Los niveles de expresión génica y proteica de ASK también aumentaron en el VL de los enfermos caquécticos con cáncer de pulmón y en los pacientes con EPOC. En conjunto, estos resultados sugieren que el marcador TRAF2 UPR puede señalar el inicio de la apoptosis que conduce a la atrofia muscular

en ambas condiciones de sarcopenia respiratoria. En línea con esto, la rama IRE de la UPR también fue regulada al alza en los músculos de las extremidades posteriores de los ratones caquécticos por cáncer de pulmón (Bohnert et al., 2016). La síntesis de proteínas y la carga de nuevas proteínas que entran en el RE se reducen por la acción de la vía IRE1 como XBP1. Curiosamente, en el VL de los pacientes con caquexia por cáncer de pulmón y en la EPOC, los niveles de expresión génica de XBP1 estaban regulados al alza en comparación con los niveles de los controles. Además, los niveles de proteína de las isoformas empalmadas y no empalmadas, XBP1s y XBP1u, respectivamente, también aumentaron significativamente en los músculos de los pacientes con caquexia por cáncer de pulmón y con EPOC sarcopénica.

Crítica del estudio

En la presente investigación, la regulación al alza de la expresión de las tres ramas de marcadores de estrés UPR en los músculos de las extremidades inferiores de los pacientes con sarcopenia respiratoria fue una observación relevante. Cabe destacar los niveles significativamente mayores de expresión y el número de marcadores de la UPR que fueron regulados al alza en los cuádriceps de los pacientes caquécticos por cáncer con respecto a los niveles encontrados en los músculos de los pacientes con EPOC, incluso en aquellos con sarcopenia. De hecho, sólo se observaron correlaciones significativas entre el estado nutricional (FFMI), la pérdida de peso corporal y los tipos de fibras musculares con las variables biológicas entre los pacientes con caquexia por cáncer de pulmón. Es muy probable que la tasa más rápida de pérdida de masa muscular observada en la caquexia por cáncer de pulmón, en contraposición a la enfermedad crónica (EPOC), pueda explicar las respuestas significativamente más fuertes de la UPR y del estrés del RE observadas en los músculos de los pacientes con caquexia por cáncer de pulmón. De hecho, como el objetivo principal de la UPR es restaurar la función del RE para mejorar el plegamiento adecuado en condiciones de estrés, una respuesta más exagerada en los músculos de los pacientes con caquexia por cáncer de

pulmón puede ayudar a prevenir la pérdida adicional. No obstante, el diseño actual del estudio transversal no nos permitió determinar si el estrés del RE y la UPR pueden ser un desencadenante de la pérdida muscular o pueden proteger a los músculos de una mayor pérdida de proteínas. Los estudios de intervención podrían ayudar a dilucidar esta relevante cuestión en un futuro próximo. A pesar de estas preocupaciones, la presente investigación es la primera que demuestra la presencia de estrés del RE y la activación de la UPR en los músculos de las extremidades inferiores de los pacientes con dos importantes afecciones respiratorias.

También hay que mencionar que las diferencias en la expresión génica y los niveles de proteínas entre los pacientes y los sujetos de control no fueron exactamente consistentes para todos los marcadores del estudio. Las cuestiones metodológicas directamente relacionadas con los análisis de RT-PCR o de inmunoblotting pueden explicar esas diferencias. Para algunos de los marcadores, la magnitud de las diferencias entre los pacientes con caquexia por cáncer de pulmón y los controles sanos fue mayor que las detectadas entre los pacientes con EPOC y los controles, especialmente para la expresión génica. Por último, cabe mencionar que, a pesar del interés por evaluar las posibles diferencias en los niveles de expresión de los marcadores del estudio entre los pacientes con EPOC no sarcopénicos y los sarcopénicos, la investigación no se dirigió específicamente a explorar dicha cuestión. Es importante destacar que el grado de deterioro de la función pulmonar, la capacidad de ejercicio y la fuerza muscular fue similar en ambos grupos de pacientes con EPOC, en contraste con investigaciones anteriores, en las que los pacientes caquécticos con EPOC estaban más afectados que los no sarcopénicos (Barreiro et al., 2008, 2009; Fermoselle et al., 2012; Puig-Vilanova, Rodriguez, et al., 2015). Esto puede explicar en parte las discrepancias en los niveles de expresión de algunos de los marcadores del estudio.

MANUSCRITO 3: Endoplasmic reticulum stress and unfolded protein response in diaphragm muscle dysfunction of patients with stable chronic obstructive pulmonary disease.

En primer lugar, cabe destacar que los niveles de expresión génica de los marcadores de mal plegamiento de proteínas (estrés de RE) PDIA3 y PI3K estaban regulados al alza en el diafragma de los pacientes con EPOC con un amplio rango de obstrucción en las vías respiratorias y composición corporal normal en comparación con los sujetos de control. Además, los niveles de expresión génica de PDIA3 aumentaron significativamente en el diafragma de los pacientes con una obstrucción más grave de las vías respiratorias. No obstante, no se detectaron diferencias significativas en el diafragma entre los grupos del estudio cuando se analizaron los niveles de proteínas mediante inmunotransferencia. En consonancia con esto, los recuentos de agregados de lipofuscina, marcador de estrés del RE, no difirieron significativamente en el diafragma entre ninguno de los grupos de pacientes y los controles sin EPOC.

Por otra parte, la expresión de genes musculares y los niveles de proteínas de las tres vías de receptores transmembrana del RE (ATF6, PERK e IRE1) no difirieron significativamente entre los pacientes y los sujetos control sin EPOC. Del mismo modo, los niveles de expresión génica y proteica de los marcadores de las vías descendentes analizadas en el estudio, a saber, la proteólisis y la apoptosis, no difirieron significativamente entre los pacientes y los sujetos control. Hasta donde sabemos, la presente investigación es la primera que muestra pruebas sobre el estado del estrés del RE y la UPR en el diafragma humano de los pacientes con EPOC junto con los que no padecen esta enfermedad, que fueron los sujetos de control.

En el RE de todas las células se produce el plegamiento fisiológico, el procesamiento y el tráfico de proteínas. El RE también participa en la homeostasis del calcio. La acumulación de proteínas mal plegadas en respuesta a varias lesiones que alteran la homeostasis celular conduce al estrés del RE. Los residuos de cisteína no apareados, las regiones hidrofóbicas expuestas y la agregación de proteínas se encuentran entre los marcadores más relevantes de las proteínas desplegadas o mal plegadas. Curiosamente, tres transductores principales del RE (ATF6, PERK e IRE1) componen la UPR en respuesta al estrés del RE. Estos sensores transmembrana del RE ralentizan el flujo de nuevas proteínas hacia el RE a través de varios procesos bioquímicos (modificaciones transcripcionales, traslacionales y postraduccionales), que potencian la actividad del RE para el plegamiento y procesamiento de proteínas, al tiempo que favorecen la eliminación de las proteínas mal plegadas y aumentan el tamaño del RE. Cuando el estrés del RE no puede ser revertido por la UPR, se induce la apoptosis y el aumento del catabolismo proteico en las células (Afroze & Kumar, 2019; Chakrabarti et al., 2011; Kelsen, 2016). De hecho, las vías de señalización de la UPR pueden determinar la restauración de la supervivencia celular o la apoptosis (Afroze & Kumar, 2019; Chakrabarti et al., 2011; Kelsen, 2016). Por lo tanto, el estrés del RE y la UPR son vías de señalización muy relevantes que pueden determinar el destino de las células en condiciones crónicas y agudas (Afroze & Kumar, 2019; Chakrabarti et al., 2011; Kelsen, 2016).

A pesar de que se ha demostrado que el estrés del RE y la UPR están implicados en la fisiopatología de las enfermedades críticas y en respuesta al aumento de la activación muscular (Afroze & Kumar, 2019; Chakrabarti et al., 2011; Kelsen, 2016), aún no se ha definido su posible papel en la disfunción del músculo respiratorio de las enfermedades pulmonares crónicas como la EPOC. La activación de la UPR en los músculos puede tener lugar como resultado de las fluctuaciones en los niveles de calcio dentro del RE durante las contracciones musculares y el ejercicio (Afroze & Kumar, 2019; Wu et al.,

2011). Dado que los pacientes con EPOC están continuamente expuestos a mayores cargas inspiratorias, razonamos que el estrés del RE y los marcadores de la UPR estarían regulados al alza en su músculo diafragma, especialmente en aquellos con una fuerza de contracción diafragmática reducida. En el presente estudio, sólo la expresión de los marcadores de estrés de RE PDIA3 y PI3K estaba regulada al alza en el diafragma de los pacientes con EPOC, especialmente en aquellos con una enfermedad más grave (mayor obstrucción en las vías respiratorias y cargas inspiratorias). Sin embargo, los niveles de proteína de estos marcadores no difirieron significativamente en el músculo respiratorio entre los pacientes y los controles sin EPOC. Estos hallazgos sugieren que la regulación postranscripcional de los marcadores de estrés de RE puede haber tenido lugar en el diafragma, excluyendo así las posibles diferencias en los niveles proteicos de dichos marcadores entre la EPOC y los controles (Afroze & Kumar, 2019; Chakrabarti et al., 2011; Kelsen, 2016). Estos resultados también implican que las contracciones musculares más fuertes, como las que tienen lugar durante las exacerbaciones, podrían haber inducido un estímulo más potente para desencadenar el estrés del RE y la UPR. En consonancia con esto, las sesiones de carrera indujeron un aumento significativo del estrés del RE y de la UPR en los músculos de los seres humanos (Kim et al., 2011) y de las ratas (Memme et al., 2016). No obstante, las fuertes contracciones musculares del diafragma inducidas por la exacerbación no podrían ser estudiadas en entornos clínicos. Por otra parte, el envejecimiento puede modular el estrés del RE y la UPR (Bohnert et al., 2016). Dado que los controles sin EPOC estaban emparejados por edad, el envejecimiento no fue un factor con una posible influencia en la respuesta al estrés del RE en este estudio.

Otros factores, como la medicación o el aumento de los niveles de reactantes de fase aguda que se observaron entre los pacientes con EPOC, probablemente no intervinieron en la inducción del estrés del RE o la UPR en el

músculo respiratorio principal, ya que no se observaron diferencias entre los pacientes EPOC y los sujetos control.

Es importante mencionar también que la expresión génica y los niveles proteicos de los marcadores proteolíticos y de apoptosis no difieren en el diafragma de los pacientes con EPOC de los detectados en los controles sin EPOC. Estos hallazgos concuerdan con la falta de modificaciones significativas encontradas en los niveles de estrés de RE y UPR en el músculo del diafragma de los pacientes con EPOC estable. Futuras investigaciones realizadas en modelos animales de EPOC deberían explorar si las mayores contracciones musculares como resultado de las exacerbaciones inducen el estrés del RE y la UPR en el diafragma.

En el estudio, se analizaron dos grupos distintos de pacientes con EPOC con el objetivo de explorar si la gravedad de la enfermedad podría haber influido en la expresión de los marcadores de estrés del RE o de la UPR. Para comprobar este objetivo, se reclutaron pacientes con EPOC con un amplio rango de obstrucción en las vías respiratorias y con una composición corporal normal y una disfunción del diafragma. De acuerdo con esto, un primer grupo de pacientes presentaba una obstrucción leve en las vías respiratorias, mientras que el segundo grupo mostraba una obstrucción moderada (FEV$_1$ 72% y 50%, respectivamente). En general, todos los pacientes presentaban una reducción moderada de la capacidad de difusión pulmonar y de la tolerancia al ejercicio. La composición corporal medida por el IMC y el FFMI no difería entre los sujetos del estudio. También se observaron signos de manifestaciones sistémicas entre los pacientes con EPOC, especialmente en los más graves, ya que se detectó un aumento significativo de los marcadores sanguíneos de proteína C reactiva, fibrinógeno y velocidad de sedimentación globular en comparación con los sujetos control. Estos resultados coinciden con investigaciones anteriores en las que los pacientes con EPOC presentaban

características clínicas y fisiopatológicas similares (Barreiro et al., 2005; Puig-Vilanova, Aguiló, et al., 2014).

Es importante destacar que el cambio hacia un fenotipo más resistente del que se informó anteriormente (Barreiro et al., 2005; Puig-Vilanova, Aguiló, et al., 2014) también se ha observado en la presente investigación. Estos hallazgos se observaron especialmente en el diafragma de los pacientes con una obstrucción más grave en las vías respiratorias. La función del diafragma, medida por la presión transdiafragmática, se redujo significativamente en los pacientes con EPOC, especialmente en los pacientes con obstrucción moderada, en comparación con los sujetos control. Esta reducción de la fuerza del diafragma puede ser el resultado de una disminución de la contractilidad de las fibras musculares y/o del aumento del volumen pulmonar residual experimentado por los pacientes, especialmente en aquellos con limitación grave del flujo aéreo. Dado que no se detectaron signos de atrofia muscular, como también se informó anteriormente (Barreiro et al., 2005; Barreiro, Bustamante, et al., 2015; Barreiro & Jaitovich, 2018; Gea et al., 2013; Jaitovich & Barreiro, 2018), en el músculo del diafragma de ninguno de los grupos de pacientes, es probable que los mayores valores de volumen residual, que modifican la posición de este músculo para contraerse adecuadamente, puedan haber contribuido en parte a la menor presión transdiafragmática observada en los pacientes con una limitación del flujo aéreo más grave. En este modelo de enfermedad humana, en el que las biopsias se obtuvieron a partir de la cirugía torácica, no se pudieron realizar experimentos de contractilidad in vitro por limitaciones logísticas. Las investigaciones futuras deberían tener como objetivo descifrar la contribución específica de estos factores a la disminución de la fuerza del diafragma que se ha descrito. Por último, el cambio hacia un fenotipo más resistente fue probablemente el resultado de las mayores cargas inspiratorias a las que están expuestos los pacientes, que pueden imitar un "efecto similar al del entrenamiento" de las miofibras del diafragma (Barreiro et al., 2005; Barreiro, Bustamante, et al.,

2015; Barreiro & Jaitovich, 2018; Gea et al., 2000, 2013; Jaitovich & Barreiro, 2018).

Limitaciones del estudio

Las biopsias del músculo del diafragma de los sujetos del estudio se obtuvieron durante una toracotomía debido a las lesiones pulmonares localizadas, que es la técnica de referencia para obtener muestras de diafragma de diferentes poblaciones. Aunque la cirugía de reducción del volumen pulmonar también permite obtener muestras del diafragma, sólo los pacientes con EPOC muy grave se someten a ese tipo de cirugía, lo que hace imposible el estudio de los pacientes leves y moderados o de los sujetos control. Por lo tanto, la toracotomía terapéutica diagnóstica es el único método disponible para estudiar a los sujetos con EPOC moderada y leve y con función pulmonar normal. En consecuencia, los sujetos reclutados para el estudio comparten una morbilidad común: la presencia de una neoplasia pulmonar pequeña y localizada. Sin embargo, no creemos que esta condición haya contribuido significativamente a los resultados obtenidos en los análisis de los músculos del diafragma, ya que se emplearon criterios extremadamente restrictivos para seleccionar adecuadamente la población, y se excluyeron sistemáticamente los sujetos que presentaban anomalías nutricionales o síndromes paraneoplásicos. Por lo tanto, consideramos que todos los hallazgos comunicados en el estudio están más bien asociados a la EPOC. Además, también hay que reconocer que los enfoques experimentales actuales no eran adecuados para realizar análisis de contractilidad in vitro.

CONCLUSIONES

- En los músculos de las extremidades inferiores de los pacientes graves con EPOC, independientemente del grado de sarcopenia, se desencadena el proceso de regeneración muscular identificado por la activación de células satélite y el aumento de los recuentos de núcleos internos.

- En los pacientes con EPOC y sarcopenia se observa de forma destacada el potencial regenerativo junto con alteraciones significativas en el fenotipo muscular (fenotipo de cambio de lento a rápido y miofibras híbridas y de contracción rápida más pequeñas) y el daño muscular.

- Se detecta un aumento del inhibidor del crecimiento muscular miostatina sólo en el VL de los pacientes con EPOC y sarcopenia, lo que puede agravar aún más la pérdida de masa y función muscular en este grupo específico de pacientes.

- Los hallazgos en la alteración de la capacidad de regeneración del VL de los pacientes con EPOC podrían condicionar la respuesta al ejercicio y/o a las modalidades de entrenamiento muscular.

- Se observa estrés de RE y UPR elevadas en el VL de pacientes con sarcopenia y caquexia respiratoria, particularmente en aquellos con cáncer de pulmón.

- Las tres ramas de la UPR están reguladas de forma similar en los músculos de los pacientes caquécticos con cáncer de pulmón, mientras que en los enfermos con EPOC y sarcopenia, la rama IRE1 está mayormente regulada en su VL.

- El perfil de expresión diferencial de los marcadores de estrés del RE y de la UPR observado en las enfermedades respiratorias crónicas (EPOC) y agudas (cáncer de pulmón) ofrece un nicho para el diseño de nuevos enfoques terapéuticos personalizados que pueden abarcar el entrenamiento del ejercicio junto con estrategias farmacológicas que supuestamente impulsan la función del retículo endoplasmático.

- Los pacientes con EPOC estable presentan una fuerza de contracción del diafragma reducida.

- En el diafragma de los pacientes con EPOC no existen alteraciones en el estrés del RE ni en la señalización de la UPR.

- El estrés del RE y la UPR probablemente no están implicados en la disfunción documentada del músculo del diafragma (fuerza reducida) observada en todos los pacientes del estudio, incluso en aquellos con limitación grave del flujo aéreo. Por lo tanto, las estrategias terapéuticas dirigidas a tratar la disfunción del músculo del diafragma no deberían incluir moduladores de la UPR, ni siquiera en aquellos con una enfermedad más avanzada.

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