**ENDOPLASMIC RETICULUM STRESS AND UNFOLDED
PROTEIN RESPONSE PROFILE IN QUADRICEPS OF
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**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, FFMI 16 kg/m² and n=19, non-sarcopenic, FFMI 18 kg/m²), 13 patients with LC-cachexia (FFMI 17 kg/m²), and 19 healthy controls (FFMI, 19 kg/m²), expression markers of ER stress, UPR [protein kinase-like ER kinase (PERK), activating transcription factor (ATF)6, and inositol-requiring enzyme (IRE)1-alpha], oxidative stress, autophagy, proteolysis, and apoptosis (qRT-PCR 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-alpha 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.

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INTRODUCTION

Muscle mass loss and dysfunction (sarcopenia) are characteristic features of patients with chronic respiratory and cardiac conditions and 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 (Gosselink et al, 2000;Marquis et al, 2002;Seymour et al, 2010;Shrikrishna et al, 2012;Swallow et al, 2007;Gea et al, 2018a;Gea and Martinez-Llorens, 2018;Gea et al, 2018b). 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 et al, 2015;Barreiro, 2017;Barreiro et al, 2018;Barreiro and Jaitovich, 2018;Jaitovich and 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 et al, 2015;Barreiro, 2017;Barreiro et al, 2018;Barreiro and Jaitovich, 2018;Jaitovich and 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 et al, 2014c). 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 et al, 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 et al, 2017;Nakanishi et al, 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 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: 1) well-known markers of ER stress, 2) markers of UPR pathways [activating transcription factor (ATF)6, protein kinase-like ER kinase (PERK), and inositol-requiring enzyme (IRE)1], 3) markers of oxidative stress, autophagy, apoptosis, and proteolysis, and 4) 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.

MATERIALS AND METHODS

(See detailed methodologies in the online supporting information)

Study design and population

This was a prospective, controlled, cross-sectional study, in which 40 male patients with stable COPD (Miravittles and Soler-Cataluna, 2017; Miravittles et al, 2017; Vogelmeier et al, 2017) and 13 male patients with lung cancer 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 non-sarcopenic COPD, n=19) following the international consensus criteria on muscle wasting and sarcopenia (Cao and Morley, 2016; Muscaritoli et al, 2010) and published criteria (Puig-Vilanova et al, 2014c). Lung cancer patients had not previously received any specific treatment for 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 lung cancer following currently available international guidelines (Chansky et al, 2017). All study groups of individuals were Caucasian. In the present investigation, 9 sarcopenic COPD patients and 2 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 et al, 2014c).

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.

Anthropometrical and functional assessment

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

Muscle biopsies

Muscle samples were obtained from the quadriceps muscle (vastus lateralis, VL) of all groups of patients and control subjects using the open muscle biopsy technique, as previously described (Fermoselle et al, 2012;Puig-Vilanova et al, 2014c;Puig-Vilanova et al, 2014a;Puig-Vilanova et al, 2015;Puig-Vilanova et al, 2014b).

Biological analyses

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 –II (fast-twitch fibers) isoforms were identified using anti-MyHC-I antibody (Sigma-Aldrich, Saint Louis, MO, USA), following methodologies previously published (Fermoselle et al, 2012;Puig-Vilanova et al, 2014c;Puig-Vilanova et al, 2014a;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).

Gene expression was assessed using qRT-PCR. MicroRNA RT was performed using TaqMan® microRNA assays (Life Technologies) following the manufacturer’s instructions and previous studies (Table E1) (Livak and Schmittgen, 2001;Puig-Vilanova et al, 2015).

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 et al, 2016;Puig-Vilanova et al, 2014c;Salazar-Degracia et al, 2016;Salazar-Degracia et al, 2018). The following primary antibodies were used: anti-binding

immunoglobulin protein (BIP; 1:1000, ab37073) antibody (Abcam, Cambridge, UK), anti-protein disulphide-isomerase-3 (PDIA3; 1:1000, ADI-SPA-725-F) antibody (Enzo life science Framingdale, NY, USA), anti-phosphatidylinositol 3-kinase (PI3K; 1:1000, 3358S) antibody (Cell signaling, Boston, MA, USA), anti-activating transcription factor 6 (ATF6; 1:100, ab37149), anti-protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK; 1:1000, ab79483), anti-phospho-PERK (1:1000, ab192591), anti-eukaryotic translation initiation factor 2alpha (eIF2a; 1:500, ab181467), anti-phospho-eIF2a (1:1000, ab32157), anti-C/EBP-homologous protein (CHOP; 1:2000, ab11419), anti-inositol-requiring enzyme 1 (IRE1; 1:1000, ab37073), anti-tumor necrosis factor receptor associated factor 2 (TRAF2; 1:1000, ab37118), anti-X-box binding protein 1 (XBP1; 1:1000, ab37152) antibodies from Abcam, anti-microtubule-associated protein 1 light chain 3 (LC3; 1:1000, #2775S) antibody (Cell signaling), anti-nucleoporin p62 (p62; 1:1000, P0067) antibody (Sigma-Aldrich St. Louis, MO, USA), anti-signal-regulating kinase 1 (ASK1; 1:500, sc-5294) antibody from Santa Cruz (Santa Cruz, CA, USA), anti-malondialdehyde-protein adducts (MDA; 1:4000, MD20A-G1b) antibody (Academy Bio-Medical Company, Houston, TX, USA), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:2000, sc-25778) antibody (Santa Cruz). To validate equal protein loading across lanes, the glycolytic enzyme GAPDH was used as the protein loading control in all immunoblots (Figures E2-E5). Standard stripping methodologies were employed 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).

Statistical analyses

Statistical power was calculated using specific software (StudySize 2.0, CreoStat HB, Frolunda, Sweden). FFMI was selected as the target variable on the basis of the T-test to estimate the statistical power between healthy controls and LC-cachexia patients. On the

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basis of a standard power statistics established at a minimum of 80% and assuming an alpha 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 (ANOVA), in which Tukey’s post-hoc analyses 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 Chi-Square test was employed 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 $P\leq0.05$ 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, USA).

RESULTS

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 non-sarcopenic patients, while no differences were observed

between the latter patients and the healthy controls (Table 1). Body weight change, expressed as kg 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 to controls (Table 1). Compared to 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).

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 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 Figure E1).

Markers of ER stress and UPR in sarcopenic muscles

ER stress. Compared to 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 binding immunoglobulin protein (BIP), HSP70, calreticulin, and protein disulfide-isomerase A (PDIA)3 were also upregulated compared to 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 BCL-2 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 (Figures 1C-1D, and E2). Protein levels of BIP were almost significantly increased ($p=0.084$) in the VL of the LC-cachexia patients compared to the control subjects, while no significant differences were observed among the COPD patients (Figures 1B and E2).

ATF6 arm. Gene expression and protein levels of activating transcription factor (ATF)6 pathway were significantly upregulated in VL of LC-cachexia, and ATF6 protein content was also increased in muscles of sarcopenic COPD patients (Figures 2A-2B and E3A).

PERK arm. C/EBP-homologous protein (CHOP) gene expression was significantly greater in muscles of LC-cachexia and in both groups of COPD patients, while that of PERK and ATF4 did not differ among the study groups (Figure 3A). Protein levels of activated (phosphorylated) PERK, eIF2 α , and CHOP were significantly higher in the VL of the LC-cachexia patients than in the controls (Figures 3B-3D and E3B). Moreover, protein levels of

CHOP were also significantly increased in the VL of COPD patients as a whole and in the sarcopenic group (Figures 3D and E3B).

IRE1 arm. Gene expression of tumor necrosis factor receptor associated factor (TRAF)2 and X-box binding protein (XBP)1, 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 non-sarcopenic COPD patients (Figure 4A). Protein levels of IRE1 did not significantly differ among the study groups (Figures 4B and E4). TRAF2 protein levels were significantly increased in the VL of both groups of COPD patients (Figures 4C and E4). Interestingly, protein levels of unspliced XBP1u and spliced XBP1s were significant greater in the VL of both LC-cachexia and COPD patients as a whole and in sarcopenic (XBP1u) than in the controls (Figures 4D-4E and E4).

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 to healthy controls (Figure 5A). Protein levels of microtubule-associated proteins 1A/1B light chain 3B (LC3)B were only significantly greater in the VL of all COPD patients as a whole and in the sarcopenic patients than in control subjects (Figures 5B and E5), while protein levels of the autophagy marker p62 did not significantly differ among the study groups (Figures 5C and 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 (Figures 5D and E5).

Gene expression of apoptosis markers such as caspase-7, caspase-9, and apoptosis signal-regulating kinase (ASK) were significantly upregulated in VL of LC-cachexia patients compared to the controls (Figure 6A). Moreover, ASK gene expression was also upregulated in muscles of both sarcopenic and non-sarcopenic COPD patients and as a whole compared to

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) (Figures 6B and 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.

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 to 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 et al, 2014c). 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; Feroselle et al, 2012; Puig-Vilanova et al, 2014c; Puig-Vilanova et al, 2014b; 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 post-translational 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;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, oxido-reductases, and disulfide isomerases(Schroder and 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 heat shock proteins (HSP) specifically bind unfolded or misfolded proteins in the ER in order 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 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 pro-survival 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 et al, 2014c).

Both pro-survival and pro-apoptotic 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 eIF2 α , were significantly greater in the VL of the LC-cachexia patients. During ER stress phosphorylation of eIF2 α by PERK downregulates protein synthesis by blocking translation (Almanza et al, 2018;Rowlands et al, 1988). Consistently, protein levels of p-eIF2 α 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 C/EBP homologous protein (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

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3 implying that these three pathways may enhance UPR signaling cascades and downstream
4 mechanisms in muscles of those patients. Importantly, increased expression of CHOP may
5 result in oxidative stress, ATP depletion, and cell death (Almanza et al, 2018;Chakrabarti et
6 al, 2011;Hiramatsu et al, 2014). In the present study, oxidative stress levels were significantly
7 greater in the VL of both cancer cachectic and all groups of COPD patients, as also
8 consistently demonstrated in previous studies (Barreiro et al, 2010;Barreiro et al, 2018;Puig-
9 Vilanova et al, 2014c). Expression levels of markers of apoptosis such as caspase 7 and 9 and
10 ASK were also upregulated in the VL of patients with cancer cachexia. Despite that no
11 correlations were found between oxidative stress and autophagy or apoptosis markers, these
12 observations suggest that apoptosis may preferentially mediate muscle mass loss in patients
13 with LC-induced cachexia and UPR can be a powerful signaling mechanism. Indeed, previous
14 investigations have also shown that apoptosis rather than proteolysis was the major
15 mechanism leading to muscle wasting in animal models of cancer cachexia (Salazar-Degracia
16 et al, 2016) and to a lower degree in COPD sarcopenia (Agusti et al, 2002).

17 IRE1 signals both pro-survival and pro-apoptotic programs in response to misfolded
18 and unfolded proteins (Chakrabarti et al, 2011). IRE1 has endoribonuclease and serine-
19 threonine kinase domains that exert different actions. Cytosolic IRE1 dimers also interact
20 with adaptors such as TRAF2 to drive ASK, kinases, and nuclear factor (NF)- κ B signaling
21 pathways (Hu et al, 2006;Nguyen et al, 2004). Importantly, gene and protein expression
22 levels of TRAF2 were significantly upregulated in the lower limb muscles of both groups of
23 COPD patients. Gene and protein expression levels of ASK were also increased in the VL of
24 cancer cachectic and in COPD patients. Collectively, these findings suggest that TRAF2 UPR
25 marker may signal the initiation of apoptosis leading to muscle atrophy in both conditions of
26 respiratory sarcopenia. In line with this, IRE branch of UPR was also upregulated in the
27 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 to 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.

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 in order 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 non-sarcopenic 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 non-sarcopenic patients (Barreiro et al, 2008; Barreiro et al, 2009; Fermoselle et al, 2012; Puig-Vilanova et al, 2014c). This may partly account for discrepancies in expression levels of some of the study markers.

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, 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.

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LIST OF ABBREVIATIONS

ANOVA: one-way analysis of variance

ASK: apoptosis signal-regulating kinase

ATF: activating transcription factor

BMI: body mass index

BIP: binding immunoglobulin protein

BSA: bovine serum albumin

CHOP: C/EBP-homologous protein

COPD: chronic obstructive pulmonary disease

ER: endoplasmic reticulum

LC: lung cancer

LC3: microtubule-associated protein 1 light chain 3

EiF2 α : eukaryotic translation initiation factor 2 alpha

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

HSP: heat shock protein

IRE: inositol-requiring enzyme

MDA: malondialdehyde

MyHC: myosin heavy chain

NF: nuclear factor

P62: nucleoporin p62

PDIA: protein disulfide-isomerase

PERK: protein kinase-like ER kinase

QMVC: quadriceps maximal velocity contraction

SDS: sodium dodecyl sulfate

TRAF2: tumor necrosis factor receptor associated factor

UPR: unfolded protein response

VL: vastus lateralis

XBP1: X-box binding protein

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FIGURE LEGENDS

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 mRNA 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.). *Definition of abbreviations:* BIP, binding immunoglobulin protein; HSP, heat shock protein; PDIA3, protein disulfide isomerase family A member 3; PI3K, phosphatidylinositol 3-kinase. *Statistical significance:* * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ between either the LC-cachexia patients or any of COPD patient groups (non-sarcopenic and sarcopenic patients) and the healthy controls; §§§ $p \leq 0.001$ between the non-sarcopenic and the sarcopenic COPD patients.

Figure 2: (A) Mean values and 95% confidence intervals of expression levels of ATF6 marker expressed as relative mRNA 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.). *Definition of abbreviations:* ATF, activating transcription factor; *Statistical significance:* * $p \leq 0.05$ and ** $p \leq 0.01$ between either the LC-cachexia patients or any of COPD patient groups (non-sarcopenic and sarcopenic patients) and the 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 mRNA 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 Eif2 α 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.). *Definition of abbreviations:* PERK, protein kinase R (PKR)-like endoplasmic reticulum kinase; ATF, activating transcription factor; CHOP, C/EBP-homologous protein; Eif2 α , eukaryotic translation initiation factor 2 alpha. *Statistical significance:* * $p \leq 0.05$ and *** $p \leq 0.001$ between either the LC-cachexia patients or any of COPD patient groups (non-sarcopenic and sarcopenic patients) and the healthy controls.

Figure 4: (A) Mean values and 95% confidence intervals of expression levels of the following markers: *IRE1*, *TRAF2* and *XBPI* expressed as relative mRNA 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.). *Definition of abbreviations:* IRE1, endoplasmic

reticulum to nucleus signaling 1; TRAF2, TNF receptor associated factor 2; XBP1, X-box binding protein 1. *Statistical significance:* * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ between either the LC-cachexia patients or any of COPD patient groups (non-sarcopenic 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 mRNA 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.). *Definition of abbreviations:* MuRF-1, muscle ring finger protein-1; LC3B, microtubule-associated protein 1 light chain 3; p62, nucleoporin p62; MDA, malondialdehyde. *Statistical significance:* * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ between either the LC-cachexia patients or any of COPD patient groups (non-sarcopenic and sarcopenic patients) and the healthy controls; §§ $p \leq 0.01$ between the non-sarcopenic and the sarcopenic COPD patients.

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 mRNA 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.). *Definition of abbreviations:* ASK1, apoptosis signal-regulating kinase 1; BAX, BCL2 associated X apoptotic regulator; BCL2L11, BCL2 like 11 apoptosis regulator

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Table 1. Anthropometric characteristics and functional status of the study subjects

	Healthy controls	LC-cachexia patients	All COPD patients	All COPD patients	
	N= 19	N= 13	N= 40	Non-sarcopenic COPD	Sarcopenic COPD
	N=19	N= 21			
Anthropometry					
Age (years)	66 (7)	68 (9)	68 (5)	68 (5)	67 (5)
BMI (kg/m ²)	26 (3)	23 (2)*	24 (5)**	27 (2)	21 (3)***, §§§
FFMI (kg/m ²)	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/III/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					
FEV ₁ (% 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)***
FEV ₁ /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)*
PaO ₂ (kPa)	11.3 (1.3)	10.5 (1.0)	9.7 (1.5)***	9.7 (1.4)**	9.9 (1.7)*
PaCO ₂ (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					
V _O ₂ 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)*

Values are expressed as mean (standard deviation).

Abbreviations: LC, lung cancer; COPD, chronic obstructive pulmonary disease; N, number of patients; m, meters; kg, kilograms; BMI, body mass index; FFMI, fat-free mass index; NA, not applicable; FEV1, forced expiratory volume in one second; pred, predicted; FVC, forced vital capacity; RV, residual volume; TLC, total lung capacity; DLco, carbon monoxide transfer; KCO, Krough transfer factor; PaO₂, arterial oxygen partial pressure; kPa, kilopascals; PaCO₂, arterial carbon dioxide partial pressure; VO₂ peak, peak exercise oxygen uptake; WR peak, peak work rate; QMVC, quadriceps maximal velocity contraction; g, grams; dL, deciliter; CRP, C-reactive protein; mg, milligrams; GSV, globular sedimentation velocity; mm, millimeters; h, hour. *Statistical significance:* * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ between either LC-cachexia patients or any group of COPD patients (as a whole and separately, non-sarcopenic and sarcopenic COPD patients) and the healthy control subjects; § $p \leq 0.05$ and §§§ $p \leq 0.001$ between the non-sarcopenic and the sarcopenic COPD patients.

Table 2. Main clinical characteristics and functional variables of the study subjects

	Controls	Severe COPD	
		Non-cachectic	Cachectic
Subjects	N = 10	N = 14	N = 15
Female/male	4/6	3/11	2/13
Anthropometry			
Age (years)	62 (6)	67 (10)	65 (9)
BMI (kg/m ²)	27 (2)	26 (3)	18 (2) ***, \$\$\$
FFMI (kg/m ²)	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			
FEV ₁ (% pred)	94 (9)	35 (11) ***	31 (12) ***
FVC (% pred)	92 (8)	54 (16) ***	58 (18) ***
FEV ₁ /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) ***
K _{CO} (% pred)	94 (14)	58 (21) ***	57 (21) ***
PaO ₂ (mmHg)	85 (2.7)	68 (9.5) ***	65 (8.8) ***
PaCO ₂ (mmHg)	42 (4.2)	46 (4.5)	45 (5.3)
Exercise capacity & muscle force			
QMVC (kg)	44 (11)	32 (8) *	23 (11) ***, §
Six-minute 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) *

Values are expressed as mean (standard deviation).

Abbreviations: COPD, chronic obstructive pulmonary disease; N, number of patients; kg, kilograms; m, meters; BMI, body mass index; FFMI, fat-free mass index; FEV₁, forced expiratory volume in one second; pred, predicted; FVC, forced vital capacity; RV, residual volume; TLC, total lung capacity; DLco, carbon monoxide transfer; K_{CO}, Krogh transfer factor for diffusing capacity; PaO₂, arterial oxygen partial pressure; mmHg, millimeter of mercuri; PaCO₂, arterial carbon dioxide partial pressure; QMVC, quadriceps maximal velocity contraction; g, grams; dL, deciliter; CRP, C-reactive protein; mg, milligrams; GSV, globular sedimentation velocity; mm, millimeters; h, hour.

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3 *Statistical significance:* *, $p \leq 0.05$, **, $p \leq 0.01$, ***, $p \leq 0.001$ between any group of COPD patients and the control
4 subjects; §, $p \leq 0.05$, §§, $p \leq 0.01$, §§§, $p \leq 0.001$ between non-cachectic and cachectic patients.
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For Peer Review

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7 Barreiro et al. Figure 1
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Figure 1A

254x190mm (300 x 300 DPI)

Barreiro et al. Figure 1

Figure 1B-1D

254x190mm (96 x 96 DPI)

Barreiro et al. Figure 2

Figure 2

254x190mm (300 x 300 DPI)

Barreiro et al. Figure 3

Figure 3A-3C

254x190mm (300 x 300 DPI)

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Figure 3D

254x190mm (300 x 300 DPI)

Barreiro et al. Figure 4

Figure 4A-4C

254x190mm (300 x 300 DPI)

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31 Figure 4D-4E
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Barreiro et al. Figure 5

Figure 5A-5C

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Figure 5D
254x190mm (300 x 300 DPI)

Barreiro et al. Figure 6

Figure 6A

254x190mm (300 x 300 DPI)

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Figure 6B
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