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UPREGULATION OF PROINFLAMMATORY CYTOKINES IN THE INTERCOSTAL MUSCLES OF COPD PATIENTS.

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KEY WORDS. Chronic lung disease, interleukin 1, interleukin 6, muscle function, respiratory muscle, tumour necrosis factor-alpha.

RUNNING HEAD. COPD, cytokines and intercostal muscles

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ABSTRACT (Abstract word count: 198 177)

Muscle dysfunction is a characteristic feature of COPD. Recent studies suggest that cytokines may operate as local regulators of both muscle function and muscle regeneration. The **aim** of this study was to characterise the expression of different cytokines in the external intercostal muscle of COPD.

Methods: Muscle biopsies were obtained both from 25 stable COPD patients and 8 healthy controls. Local TNF-alpha, IL-1 beta, IL-6 and IL-10 expressions (*real-time* PCR and ELISA), sarcolemmal damage (immunohistochemistry), and the transcript levels of CD18 were assessed.

Results: Muscle TNF-alpha and IL-6 transcripts were significantly higher in COPD patients compared to controls, and IL-1 beta and sarcolemmal damage showed a strong tendency in the same direction. Similar results were observed at the protein level. The CD18 panleukocyte marker was similar in COPD and controls. Moderate correlations were found between either TNF-alpha or IL-6 transcripts and FEV1. Respiratory muscle function was impaired in COPD patients, and it correlated to both the severity of lung function impairment . Moreover, respiratory muscle function was also related to and the TNF-alpha muscle expression.

Conclusions: COPD is associated with the upregulation of proinflammatory cytokines in the intercostal muscles. This phenomenon might be involved in respiratory muscle dysfunction.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterised by non-fully reversible airflow obstruction. It is, however, a heterogeneous syndrome which, in addition to the classical involvement of lung parenchyma and airways, appears to be associated with a number of systemic manifestations. These include nutritional abnormalities, reduction of lean mass and muscle dysfunction. Central to this is the role that respiratory muscles play, since they represent the active component of the ventilatory pump. In COPD, respiratory muscles have to cope with increased workloads, mechanical disadvantages related to hyperinflation and, as recently demonstrated, an intrinsic reduction in their contractile properties[1,2]. All these factors contribute to the impairment of the respiratory muscle capacity detected in these patients[2]. Despite a general consensus regarding this issue and its clinical consequences, little is known about the underlying pathogenic mechanisms that lead to impairment of the intrinsic contractile properties of respiratory muscles. A growing amount of data, however, supports the involvement of cytokines and oxidative stress in the pathogenesis of muscle weakness in COPD (see an excellent review in Couillard et al.)[3]. Both loss of muscle mass and reduced strength have been attributed to increases in systemic cytokine levels such as those of TNF-alpha, IL-1 beta and IL-6. Nevertheless, the presence of differences in the severity of muscle weakness among different muscle groups in stable COPD patients[4] indicates that, in addition to systemic factors, the process is probably regulated by intrinsic factors of the muscles themselves.

Recent studies indicate that skeletal muscles are capable of synthesising a variety of cytokines[5,6] and that these cytokines could act as endogenous mediators of muscle dysfunction and/or adaptation via their autocrine /paracrine effects[7]. In the light of all of this, we hypothesised that the increased activity of respiratory muscles might induce the

upregulation of local proinflammatory cytokines in COPD, and that the latter may contribute to the pathogenesis of respiratory muscle weakness. Alternatively (or perhaps complementarily), the expression of these cytokines may play a role in the adaptive mechanisms which coexist with such functional muscle impairment. In both cases, local cytokine expression may also account for differences previously observed in the phenotype and function of different muscle groups in COPD[8-10]. Therefore, the purpose of the present study was to characterise the cytokine expression profile of a typical thoracic respiratory muscle (the external intercostal) in COPD patients.

METHODS

Subjects. Twenty-five clinically stable COPD patients and 8 healthy subjects of similar age were included. While patients were consecutively recruited from our outpatient clinics, healthy control subjects were shared with a multinational study aimed at describing the effects of healthy ageing on muscle phenotype of elderly people across Europe (PanEuropean Network for Ageing Muscle, the PENAM project, see the funding section). All COPD patients were ex-smokers (47±13 pack/year, mean period free of smoking, 9 years) whereas none of the control subjects had ever been a smoker. In order to exclude gender-related effects, and on the basis of the COPD gender distribution in our country, only males were included. Control GOLD criteria were used to define COPD[11], whereas stability was defined as the absence of exacerbations during the three months before study entry. Subjects with chronic respiratory failure, metabolic diseases, cardiovascular problems, concomitant respiratory disorders, or treatments with drugs known to modify muscle structure or function were excluded. The study was designed according to the World Medical Association guidelines for research in humans

and approved by the institutional Ethics Committee. Written informed consent was obtained from all individuals.

Functional evaluations. Lung and respiratory muscle function were assessed using conventional techniques. Briefly, forced spirometry with bronchodilator response (Datospir 92, Sibel, Barcelona, Spain) as well as intrathoracic gas volume, airway resistance and carbon monoxide diffusing capacity of the lung (DLco) (Masterlab, Jaeger, Würzburg, Germany) were measured in each individual. Blood samples were obtained from the radial artery of the non-dominant arm and blood gas pressures were measured by conventional polarographic techniques (RapidLab 860 Bayer HealthCare, Berkshire, UK). The strength of respiratory muscles was determined by measuring maximal respiratory pressures generated at the mouth during forced inspiratory (MIP) and expiratory (MEP) efforts performed against an occluded airway from residual volume (RV) and total lung capacity (TLC), respectively. Reference values were those appropriate for a Mediterranean population[12-15]. Inspiratory muscle resistance was in turn assessed during two different threshold inspiratory tests[16]. The first was an incremental test, whereby patients and volunteers breathed against progressive loads (approximately, +8 cm H_2O every 2 minutes) until maximal inspiratory sustainable threshold pressure (Pth_{max}) was reached. This is considered a mixed outcome in terms of both endurance and strength components. In the second test, subjects breathed against a submaximal constant load (80% of the Pth_{max}) until exhaustion. The period that elapsed was defined as the endurance time (T_{th80}), which is considered to be more specifically reflective of muscle resistance.

Biopsies. Samples from the external intercostal muscles were taken from the anterior axillary line at the sixth intercostal space as previously described[16].

RNA isolation, Reverse Transcription and real-time PCR. Total RNA was extracted from skeletal muscle using TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA USA). Following quantification of total RNA, one μg was reverse transcribed using oligo$(dT)_{12\text{-}18}$ primer and Superscript II reverse transcriptase (Invitrogen, Life Technologies). cDNA synthesis was performed in a GeneAmp PCR system 2400 (Perkin Elmer, Richmond, CA, USA), and an aliquot was used for real-time PCR amplification. This was performed with the ABI PRISM 7900HT Sequence Detector (Applied Biosystems, Foster City, CA, USA). Predeveloped TaqMan Assays were used to quantify transcripts for different cytokines (TNF-alpha, IL-1 beta, IL-6 and IL-10) as well as for integrin CD18 (a global marker of leukocytes) (Assays-on-Demand Gene Expression Products, Applied Biosystems). Beta-actin gene was used as the endogenous control (*housekeeping*). Probe context for the studied genes appear in table 1. TaqMan [(c)] MGB probes were labelled at the 5' end with the reporter dye molecule 6-FAM (6-carboxy-fluorescein) and at the 3' end with a non-fluorescent quencher (NFQ).

Samples were always assayed in triplicate and the average value was taken (intra-assay variability coefficients, 0.3 to 3.3 %). The PCR mixture was incubated 2 min at 50° C for AmpErase uracil-N-glycosylase-mediated decontamination, followed by 10 min at 95° C to activate AmpliTaq Gold DNA polymerase. Subsequently, a total of 50 cycles were performed; these consisted of a denaturation step for 15 sec. at 95° C and a combined annealing-extension step for 1 min at 60° C. Data were analysed with the Sequence Detector software (SDS). The standardised target gene was compared with an external reference (i.e. a cDNA that was used in every assay). The relative copy number was calculated according to the comparative threshold cycle (C_T) method[17].

Protein level. The cytokine contents were determined by enzyme-linked immunosorbent assay (ELISA) using a commercial kit which includes monoclonal antibodies against human TNF-α, IL-1 beta, IL-6 and IL-10, using the standard procedure recommended in the manufacturer's instructions (Amersham Pharmacia Biotech Limited, Buckinghamshire, UK). Briefly, 200 µl of the sample were added to each well for a final dilution of 1:0.35. The microplates were then incubated for 3 h at room temperature (RT), and subsequently washed 6 times with 400 µl of the appropriate buffer. Then 200 µl of the cytokine antibody were added to each well, and incubated for 2 h at RT. Washes were then repeated and 50 µl of the substrate solution were added to each well and incubated for one h at RT. Immediately after that, 50 µl of the amplifier solution were added and the mixture was again incubated for 30 min at RT. The process was stopped using a specific solution, and the absorbance of each well was determined (Multiskan MS reader, Labsystems, Vantaa, Finland).

Sarcolemmal damage. The muscle section was stained with monoclonal antibodies against albumin (Research Diagnostics Inc, Flanders, NJ, USA) and the binding was visualised using the biotin-streptoaviodin-peroxidase technique (AB600, IC019, The Binding Site, Birmingham, UK), and developed with 3-3'diaminobenzidine (DAB, D5337, Sigma, St Louis, MIS, USA). Tissue was then observed under light microscopy and the image was digititalised (Pixera Studio 1.2, Visual Communication System, Pixera Corporation, Los Gatos, CA, USA). Those fibres showing immunopositivity for intrafibrillar albumin were identified and the percentage of positive cells was determined. At least 100 fibres were evaluated by two independent and well-trained observers in each case. These observers were blind with respect to sample identification. The edges of the section as well as areas with artefacts were excluded from the analysis.

Statistical analyses. Values are expressed as mean ± SD. The normality of the distribution for each variable was assessed using the Kolmogorov-Smirnov test, and analysis of differences was done with the t-test for unpaired data. The relationships between different variables were assessed using the Pearson's correlation coefficient. For the correlation analysis both the entire study population as well as the COPD group and (*post hoc* analysis) the subgroup with a very severe disease (FEV_1 < 30 % pred.) were used. The analysis of the potential interactions between lung function, respiratory muscle function, sarcolemmal damage, the leukocyte marker and cytokine expressions was assessed using multiple regression analysis. Analyses were performed using the Statistical Package for Social Sciences, version 12 for Windows© (SPSS Inc., Chicago, IL, USA). Significance was accepted at $p < 0.05$.

RESULTS

Comparison between COPD and controls. General and anthropometric characteristics, as well as lung and muscle function data in both patients and control subjects, are indicated in table 2. Group mean values for age and body mass index (BMI) were similar between COPD patients and control subjects. Whereas the COPD group showed a moderate-to-severe obstructive ventilatory defect, healthy controls presented normal pulmonary function. Inspiratory muscle strength and endurance were significantly lower in COPD with respect to the healthy subjects.

The levels of TNF-alpha and IL-6 mRNAs found in external intercostal muscles from COPD patients were significantly higher than those observed in healthy controls ($p<0.001$ and $p<0.01$, respectively) (figure 1a). IL-1 mRNA levels showed similar behaviour, although the difference did not reach statistical significance in this case

(p=0.09). Finally, no differences were observed for mRNA levels of the non-inflammatory cytokine IL-10 between COPD and controls.

Protein levels of different proinflammatory cytokines showed similar behaviour to their homonymous mRNA. More precisely, COPD patients showed significantly higher TNF-alpha and IL-1 beta protein levels than controls ($p<0.001$ and $p<0.05$, respectively), with IL-6 exhibiting a strong tendency in the same sense (figure 1b). Sarcolemmal damage in turn showed a strong tendency to be higher in COPD patients (p=0.06) (figure 2) whereas no significant differences were detected in the leukocyte marker (transcript for integrin β2 subunit CD18)[18].

Correlations between Lung and Respiratory Muscle functions. The impairment in respiratory muscle function correlated with both the level of airway obstruction (FEV_1 with either maximal inspiratory pressure (MIP), r=0.436, $p<0.05$; or with maximal sustainable pressure (Pth_{max}), r=0.457, $p<0.01$), and that of pulmonary hyperinflation (RV/TLC with MIP, r=-0.479, $p<0.01$).

Intertranscript correlations. The levels of TNF-alpha mRNA were positively correlated with the concentration of IL-1 beta mRNA (r=0.567, p=0.001). This relationship was maintained unaltered when only COPD patients were considered (r=0.565, $p<0.01$). No significant associations were found between the leukocyte marker and cytokine levels.

Correlations between mRNA and protein levels. These relationships oscillated moderately, with *r* values ranging from 0.484 ($p<0.01$) in the case of TNF-alpha to 0.583 in the case of IL-6 ($p<0.001$).

Correlations between transcript levels and lung function. A moderate but significant inverse correlation existed between the level of airway obstruction, as expressed by FEV1, and the local TNF-alpha and IL-6 gene expressions (r= -0.421 and

-0.416, respectively, $p<0.05$ both for linear relationships; and r=-0.511 and r=-0.462, $p<0.01$ both for curvilinear fits) when all subjects were considered (figures 3a and 3b). The latter relationship remained essentially unaltered when only COPD patients were considered (r= -0.366 for the linear fit). No additional significant correlations were found between cytokine gene expressions and any of the nutritional and lung function parameters in COPD patients (including static lung volumes, DLco and blood gases) analysed in this study.

Correlations between mRNA levels and respiratory muscle function. No significant relationships were observed between local cytokine gene expressions and those variables representing respiratory muscle function in the overall study population. However, if only COPD patients were considered, TNF-alpha transcript showed an inverse relationship with Pth_{max} (r= -0.433, $p< 0.05$) in COPD patients. This relationship was even stronger (r= -0.666, $p< 0.05$) when only individuals with a very severe disease ($FEV_1< 30\%$) were analysed (*post hoc* analysis). Moreover, the multiple regression analysis, which included both lung volumes (as represented by RV/TLC) and the level of the TNF-alpha transcript, significantly increased the accuracy of the prediction for Pth_{max} in this population (r=0.743, $p<0.01$).

Finally, sarcolemmal damage did not correlate with cytokine gene expression or with functional variables.

DISCUSSION

The main finding of the present study is the upregulation of TNF-alpha and IL-6 gene expressions observed in the external intercostal muscles of COPD patients, along with a similar tendency evidenced in IL-1 beta. Protein levels followed a similar pattern.

In addition, an inverse relationship was found between respiratory muscle function and the local muscle transcript of TNF-alpha in COPD patients.

Respiratory muscle dysfunction is commonly observed in COPD, and is believed to be the result of the combination of different deleterious factors[2-4,]. Some are specific to these muscles, such as those changes occurring in the geometry of the thorax as a result of pulmonary hyperinflation. Other are general and therefore, can be shared by respiratory and peripheral muscles: systemic inflammation, drugs and nutritional abnormalities, among others. However, molecular mechanisms occurring within different muscles are much less understood. Although the diaphragm is the main respiratory muscle at rest, other muscles such as the external intercostals and parasternals also participate actively in ventilation[19]. In addition, when there is an increase in ventilatory demands, the intercostal muscles progressively play a more important role[20,21].

Recent reports indicate that myocytes are capable of synthesising a variety of cytokines[5] which may act as regulators of skeletal muscle function and structure through an autocrine/paracrine mechanism[22]. Moreover, in a recent and significant paper, Vassilakopoulos et al. demonstrated that an increase in ventilatory loads induces the upregulation of different cytokines in the ventilatory muscles of rats[6]. Based on these observations, we hypothesised both that local expression of proinflammatory cytokines is upregulated in the intercostals of COPD patients, and that this upregulation may be contributing to the development of respiratory muscle weakness. Although the enhancement of the corresponding gene expressions has been confirmed by the present study, significant correlations with those variables reflecting muscle function were only found with the TNF-alpha transcript. TNF-alpha has long been associated with muscle catabolism in different diseases including COPD[23]. In addition, it is commonly accepted

that loss of muscle mass is one of the main factors contributing to general muscle weakness in such patients[2]. Previous studies indicate that muscle function may be compromised by chronic elevation of circulating TNF-alpha via the development of oxidative stress[24], a phenomenon which we have recently reported within the respiratory muscles of COPD patients[25]. In addition to the action of systemic TNF-alpha on muscle, the present study also suggests that the local expression of this cytokine might have a role in respiratory muscle dysfunction, but probably in the context of a multifactorial scenario (as suggested by the multiple regression analysis). However, our findings should be taken cautiously since they do not demonstrate a cause-effect relationship. Further studies should be designed to address this point.

In the present study, COPD patients also showed an upregulation of the IL-6 gene expression in the intercostal muscle. Moreover, this upregulation was related with the degree of airway obstruction. Recent studies demonstrated that IL-6 expression is induced by muscle contractions. Since inspiratory muscles of COPD patients face increased workloads, our findings could be interpreted as an adaptive response to these higher contractile demands.

The third proinflammatory cytokine included in the study, IL-1, is considered a catabolic substance which, like TNF-alpha, has been shown to be able to induce contractile dysfunction through the presence of oxidative stress[26]. In the present study the increase in IL-1 beta transcript bordered on statistical significance but the corresponding protein was clearly augmented. In addition, a strong positive correlation was detected between the level of this cytokine mRNA and that of TNF-alpha. These findings suggest the existence of a synergistic action between both cytokines, which would provide a mechanism for increasing the inflammatory response within the respiratory muscles of COPD patients.

Local expression of TNF-alpha and other proinflammatory cytokines has been reported previously in human locomotor muscles[5,27] and appears to be related in part to the level of muscle activity. However, the absence of significant correlations between lung function and local cytokine expressions in our COPD patients does not support a significant regulatory role for this factor, at least in the intercostal muscles. Therefore, the significant relationships observed in our study between the level of airway obstruction and intercostal muscle cytokine expressions in the overall population might reflect the effects of this regulatory mechanism. Although in the current study, this linear relationship does not persist when considering the COPD group alone, this could be attributable to the fact that most of the patients included (92%) were severe or very severe (FEV1<50% pred.). Further research will provide more conclusive evidence in the respiratory muscle overload in local proinflammatory cytokine overexpression.

Another One complementary factor that could partly account for changes in the expression of proinflammatory cytokines is fibre damage, since these substances play an important role in mediating muscle regeneration and healing[28]. In keeping with this, inspiratory loading has been reported to be associated with respiratory muscle damage[29] and subsequent remodelling[30]. In the present study proinflammatory cytokine upregulation was associated with a marked tendency to show higher levels of sarcolemmal damage in muscles of COPD patients. Once again, although a cause-effect relationship cannot be inferred from our data, we have ventured to speculate concerning a possible link between both findings. However, it is clear that further research will be necessary to identify the factors that lead to the overexpression of local cytokines observed in the intercostal muscles in the present study.

Potential limitations of the study

The possibility of obtaining valid specimens of the main respiratory muscle, the diaphragm, from human beings *in vivo* is very limited due to the need for very invasive surgical procedures and the potential bias derived from intrinsic comorbidity. By contrast, external intercostal muscles, whose main function is to assist inspiration when the diaphragm's function deteriorates or is insufficient to cope with the current workload, are moreaccessible[16]. In addition, the model used in the present study allows the possibility of a careful selection of both patients and controls, avoiding the problems resulting from comorbidity.

The second limitation of our study is that it has been performed only in male patients. However, gender distribution of COPD is still markedly biased towards men in our environment. The alternative of mixing both genders in a common group with a net predominance of men was discarded since the mechanisms conditioning and modulating muscle phenotype are very liable to be influenced by gender.

The third relative limitation of the study is of a methodological nature since our biological techniques are quantitative rather than topographic. In other words, they permit very precise quantification of the cytokines, but their cellular source cannot be identified. Muscle biopsies, in addition to myocytes, may contain inflammatory and epithelial cells, which also may be a source of cytokines. However, the absence of differences for CD 18 (a panleukocyte surface marker) transcript between COPD and controls suggests that inflammatory cell population was not increased in the former. This is in keeping with data published by Gosker et al. who were unable to find an increase in inflammatory cells in skeletal muscles of COPD[31]. In addition, the lack of a relationship between the mRNA content for either TNF-alpha or IL-6 and CD18 in our study suggests that upregulation of these cytokines is not likely to be accounted for by

the resident macrophages and infiltrated leukocytes in the intercostal muscles of COPD patients. Moreover, previous studies using *in situ* hybridisation and immunohistochemical techniques have demonstrated that fibres are a major source of different proinflammatory cytokines in muscle[5].

To sum up, our study demonstrates that different proinflammatory cytokine genes are upregulated in the external intercostal muscles of COPD patients. In addition, some evidence has been found to support the hypothesis that local expression of TNF-alpha might play a role in respiratory muscle dysfunction.

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REFERENCES

1. Levine S, Nguyen T, Kaiser LR, Rubinstein NA, Maislin G, Gregory C, Rome LC, Dudley GA, Sieck GC, Shrager JB. Human diaphragm remodeling associated with chronic obstructive pulmonary disease: clinical implications. Am J Respir Crit Care Med 2003; 168:706-713.

2. American Thoracic Society and European Respiratory Society. Skeletal muscle dysfunction in chronic obstructive pulmonary disease. Am J Resp Crit Care Med 1999; 159 (suppl):S1-S40.

3. Couillard A, Prefaut C. From muscle disuse to myopathy in COPD: potential contribution of oxidative stress. Eur Respir J 2005;26:703-719.

4. Gosselink R, Troosters T, Decramer M. Distribution of muscle weakness in patients with stable chronic obstructive pulmonary disease. J Cardiopulm Rehabil 2000; 20:353-360.

5. Greiwe JS, Cheng B, Rubin DC, Yarasheski KE, Semenkovich CF. Resistance exercise decreases skeletal muscle tumor necrosis factor alpha in frail elderly humans. FASEB J 2001; 15:475-482.

6. Vassilakopoulos T, Divangahi M, Rallis G, Kishta O, Petrof B, Comtois A, Hussain SNA. Differential cytokine gene expression in the diaphragm in response to strenuous resistive breathing. Am J Respir Crit Care Med 2004; 170:154-161.

7. Reid MB and Li Y.-P. Cytokines and oxidative signalling in skeletal muscle. Acta Physiol Scand 2001; 171:225-232.

8. Levine S, Kaiser L, Leferovich J, Tikunov B. Cellular adaptations in the diaphragm in chronic obstructive pulmonary disease. N Engl J Med 1997; 337:1799-1806.

9. Whittom F, Jobin J, Simard PM, LeBlanc P, Simard C, Bernard S, Belleau R, Maltais F. Histochemical and morphological characteristics of the vastus lateralis muscle in COPD patients. Med Sci Sports Exerc 1998; 30:1467-1474.

10. Gea J, Pasto M, Carmona MA, Orozco-Levi M, Palomeque J, Broquetas J. Metabolic characteristics of the deltoid muscle in patients with chronic obstructive pulmonary disease. Eur Respir J 2001; 17:939-945.

11. Global Initiative for Chronic Obstructive Lung Disease. Global Strategy for the Diagnosis, Management and Prevention of Chronic Obstructive Pulmonary Disease. NHLBI/WHO workshop report. Bethesda, National Heart, Lung and Blood Institute. Update of the Management Sections, GOLD website (www.goldcopd.com).

12. Roca J, Sanchis J, Agusti-Vidal A, Segarra F, Navajas D, Rodríguez-Roisín R, Casan P, Sans S. Spirometric reference values from a Mediterranean population. Bull Eur Physiopathol Respir 1986; 22:217-224.

13. Roca J, Burgos F, Barbera JA, Sunyer J, Rodríguez-Roisín R, Castellsagué J, Sanchis J, Antoo JM, Casan P, Clausen JL. Prediction equations for plethysmographic lung volumes. Respir Med 1998; 92:454-460.

14. Roca J, Rodriguez-Roisin R, Cobo and, Burgos F, Perez J, Clausen JL. Single-breath coal monoxide diffusing capacity prediction equations from a Mediterranean population. Am Rev Respir Dis 1990; 141:1026-1032.

15. Morales P, Sanchis J, Lamb PJ, Diez JL. Maximum static respiratory pressures in adults. The reference values for a Mediterranean Caucasian population. Arch Bronconeumol 1997; 33:213-219.

16. Ramirez-Sarmiento A, Orozco-Levi M, Guell R, Barreiro E, Hernandez N, Mota S, Sangenis M, Broquetas JM, Casan P, Gea J. Inspiratory muscle training in patients

with chronic obstructive pulmonary disease: structural adaptation and physiologic outcomes. Am J Respir Crit Care Med 2002; 166:1491-1497.

17. Livak K, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ method. Methods 2001; 25:402-408.

18. Harris ES, McIntyre TM, Prescott SM, Zimmerman GA. The leukocyte integrins. J Biol Chem 2000; 275:23409-23412.

19. DiMarco A, Romaniuk JR, Supinski GS. Paraesternal and external intercostal shortening during eupneic breathing. J Appl Physiol 1990; 60:2222-2226.

20. De Troyer A. The electro-mechanical response of canine inspiratory intercostal muscles to increased resistance: the caudal rib-cage. J Appl Physiol 1992; 451:463-476.

21. DiMarco A, Romaniuk JR, Supinski GS. Paraesternal and external intercostal responses to various respiratory maneuvrers. J Appl Physiol 1992; 73:979-986.

22. Steensberg A, Keller C, Starkie RL, Osada T, Febbraio MA, Pedersen BK. IL-6 and TNF-alpha expression in, and release from, contracting human skeletal muscle. Am J Physiol Endocrinol Metab. 2002; 283:E1272-E1278.

23. Di Francia M, Barbier D, Mege JL, Orehek J. Tumor necrosis factor-alpha levels and weight loss in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1994; 150:1453-1455.

24. Li X, Moody MR, Engel D, Walker S, Clubb FJ, Sivasubramanian N et al. Cardiacspecific overexpression of tumor necrosis factor-a causes oxidative stress and contractile dysfunction in mouse diaphragm. Circulation 2000; 102:1690-1696.

25. Barreiro E, de la Puente B, Minguella J, Corominas JM, Serrano S, Hussain SN, Gea J. Oxidative Stress and Respiratory Muscle Dysfunction in Severe Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med 2005; 171:1116-1124.

26. Cheng XS, Shimokawa H, Momii H, Oyama J, Fukuyama N, Egashira K, Nakazawa H, Takeshita A. Role of superoxide anion in the pathogenesis of cytokine-induced myocardial dysfunction in dogs in vivo. Cardiovasc Res 1999; 42:651-659.

27. Rabinovich R, Figueras M, Ardite E, Carbo N, Troosters T, Filella X, Barberà JA, Fernandez-Checa JC, Argilés JM, Roca J. Increased tumour necrosis factor-alpha plasma levels during moderate-intensity exercise in COPD patients. Eur Respir J 2003; 21:789-794.

28. Kuru S, Inukai A, Kato T, Lieng Y, Kimura S, Sobue G. Expression of tumor necrosis factor-alpha in regenerating muscle fibers in inflammatory and non-inflammatory myopathies. Acta Neuropathol 2003; 105:217-224.

29. Zhu E, Petrof BJ, Gea J, Comtois N, Grassino AE. Diaphragm muscle fiber injury after inspiratory resistive breathing. Am J Respir Crit Care Med 1997; 155:1110-1116.

30. Gea J, Hamid Q, Czaika G, Zhu E, Mohan-Ram V, Goldspink G, Grassino A. Expression of myosin heavy-chain isoforms in the respiratory muscles following inspiratory resistive breathing. Am J Respir Crit Care Med 2000; 161:1274-1278.

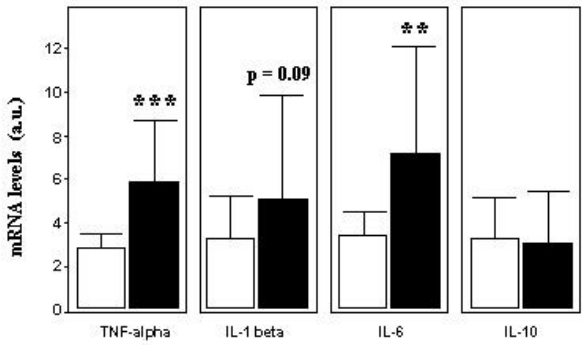
31. Gosker HR, Kubat B, Schaart G, van der Vusse GJ, Wouters EF, Schols AM. Myopathological features in skeletal muscle of patients with chronic obstructive pulmonary disease. Eur Respir J 2003; 22:280-285.

Figure Legends.

Figure 1.

(a) Relative cytokine mRNA levels in external intercostal muscles of COPD patients and control subjects. (b) Protein levels of the corresponding cytokines in muscles from both groups. Values are represented by means ± SD. White bars represent controls whereas black bars correspond to COPD patients. *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$).

C. Casadevall et al. figure 1a



C. Casadevall et al. figure 1b

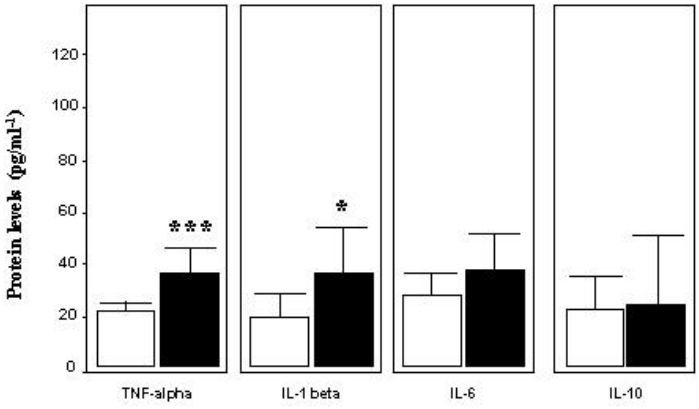


Figure 2. Sarcolemmal damage (as assessed by the percentage of albumin positive fibres) in the intercostal muscles of COPD and controls.

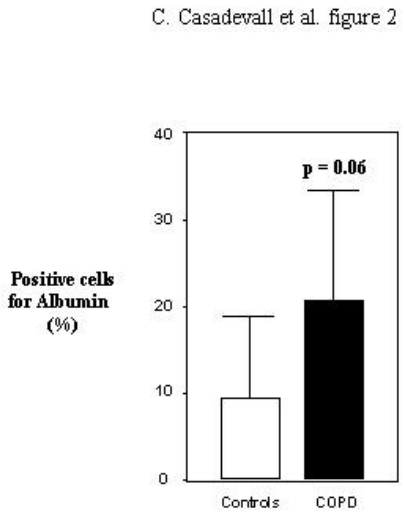
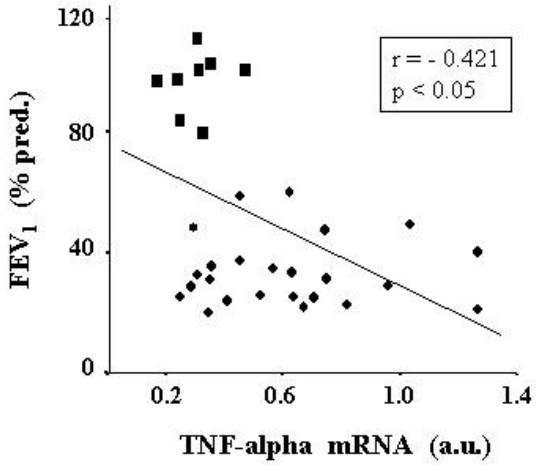


Figure 3. Relationships between cytokine expression and airway obstruction: (a) TNF-alpha mRNA vs. FEV1; (b) IL-6 mRNA vs. FEV1. Controls are represented as squares.

C. Casadevall et al, figure 3a



C. Casadevall et al, figure 3b

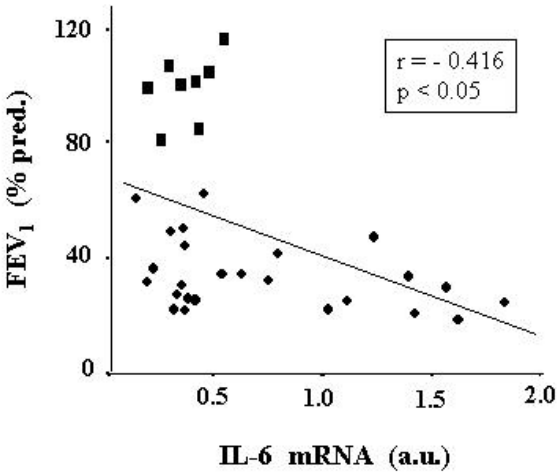


Table 1. Probes used for quantitative real-time PCR of the studied genes.

Genes	Assay ID	Nucleotide sequence (5'-3')	Genebank Accession #
TNF-alpha	Hs00174128_m1	ATGTTGTAGCAAACCCTCAAGCTGA	NM_000594
IL-1 beta	Hs00174097_m1	TATGGAGCAACAAGTGGTGTTCTCC	NM_000576
IL-6	Hs00174131_m1	ATTCAATGAGGAGACTTGCCTGGTG	NM_000600
IL-10	Hs00174086_m1	CTACGGCGCTGTCATCGATTTCTTC	NM_000572
ACTB (*Housekeeping*)	Hs99999903_m1	TCGCCTTTGCCGATCCGCCGCCCGT	NM_001101

All TaqMan® MGB (minor groove binder) probes are dual-labelled with a reporter dye (6-FAM) at the 5' end and a non-fluorescent quencher (NFQ) at the 3' end. TNF-alpha, Tumor Necrosis Factor alpha; ACTB, Actin beta.

Table 2. Subject characteristics

	Control (n = 8)	COPD (n = 25)
Age, yr	69 (6)	66 (5)
BMI, kg/m^2	26.0 (2.5)	27.6 (3.9)
Lung function		
FEV_1, % pred	100 (13)	34 (12) ***
FEV_1/FVC, %	72 (3)	46 (11) ***
RV, % pred	108 (13)	176 (39) ***
TLC, % pred	103 (5)	105 (17)
RV/TLC, %	40 (3)	63 (8) ***
PaO_2, mm Hg	97 (3)	65 (3) ***
Respiratory Muscle function		
MIP, % pred	85 (20)	65 (22) *
Pth_{max}, cm H_2O	-66 (22)	-40 (19) *
Tth_{80}, min	15.8 (5.6)	10.7 (5.8)
MEP, % pred	93 (23)	70 (19)**

Values are represented by means (± SD). **Abbreviations:** BMI, body mass index; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; RV, residual volume; MIP, maximum inspiratory pressure; Pth_{max}, maximum inspiratory sustainable pressure (obtained during progressive inspiratory threshold loading); Tth_{80}, endurance time under constant submaximal inspiratory loads (80% Pth_{max}). MEP, maximum expiratory pressure. (*), (**) and (***) express $p < 0.05$, 0.01 and 0.001, respectively, between control subjects and COPD patients.