

# Cytokine profile in quadriceps muscles of patients with severe COPD

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## ABSTRACT

**Background:** Systemic proinflammatory cytokines and oxidative stress have been described in association with peripheral muscle wasting and weakness of patients with severe chronic obstructive pulmonary disease (COPD), but their expression in skeletal muscle is unknown. The objectives of the present study were to determine muscle protein levels of selected cytokines in patients with COPD and to study their relationships with protein carbonylation as a marker of oxidative stress, quadriceps function and exercise capacity.

**Methods:** We conducted a cross sectional study in which 36 cytokines were detected using a human antibody array in quadriceps specimens obtained from 19 patients with severe COPD and seven healthy controls. Subsequently, selected cytokines (tumour necrosis factor (TNF) $\alpha$ , TNF $\alpha$  receptors I and II, interleukin (IL) 6, interferon  $\gamma$ , transforming growth factor (TGF)  $\beta$  and vascular endothelial growth factor (VEGF)), as well as protein carbonylation (oxidative stress index) were determined using an enzyme linked immunosorbent assay (ELISA) in all muscles.

**Results:** Compared with controls, the vastus lateralis of patients with COPD showed significantly lower protein ELISA levels of TNF $\alpha$ , which positively correlated with their quadriceps function, TNF $\alpha$  receptor II and VEGF. Protein ELISA levels of IL6, interferon  $\gamma$  and TGF $\beta$  did not differ between patients and controls. Quadriceps protein carbonylation was greater in patients and inversely correlated with quadriceps strength among them.

**Conclusions:** These findings do not support the presence of a proinflammatory environment within the quadriceps muscles of clinically and weight stable patients with severe COPD, despite evidence for increased oxidative stress and the presence of muscle weakness.

Muscle dysfunction, characterised by reduced muscle strength and endurance, is one of the most important systemic manifestations of advanced chronic obstructive pulmonary disease (COPD), leading to reduced exercise tolerance, quality of life and survival.<sup>1</sup> Oxidative stress and systemic inflammation, among other factors, are proposed contributors to this process of muscle wasting and dysfunction, although their actual roles remain debatable. For instance, serum levels of the cytokine tumour necrosis factor (TNF) $\alpha$ , its soluble receptors I and II and other inflammatory cytokines such as interleukin (IL) 6 and IL8 along with acute phase reactants were shown to be increased in patients with severe COPD and muscle wasting compared with patients with identical disease severity without weight loss.<sup>2-6</sup>

To date, only two studies<sup>7,8</sup> have attempted to explore cytokine protein expression in the lower limb muscles of patients with severe COPD showing inconsistent results. Specifically, quadriceps of patients with severe COPD exhibited much greater levels of TNF $\alpha$  and inflammatory cell counts than controls in one study,<sup>7</sup> while no immunohistochemical TNF $\alpha$  expression was detected in the muscles of patients or controls in the second study.<sup>8</sup> Against this background, we hypothesised that patients with severe COPD and muscle weakness would exhibit increased levels of different cytokines, including TNF $\alpha$ , in their limb muscles. To test this hypothesis, we first sought to explore the profile of multiple proinflammatory and anti-inflammatory cytokines using a human cytokine antibody array in the quadriceps of patients with severe COPD, with a wide spectrum of body composition and weight, and in those of healthy subjects. On the basis of both our antibody array results and of those from the current literature,<sup>2-6</sup> we quantified protein levels of selected cytokines in all muscles using an enzyme linked immunosorbent assay (ELISA). We also explored muscle oxidative stress levels by measuring total protein carbonylation. Finally, we also assessed whether muscle cytokine levels and protein oxidation, as measured by ELISA, were associated with quadriceps strength and/or exercise capacity in the patients.

## METHODS

### Subjects

Nineteen male patients with stable severe COPD and seven healthy control individuals from four different European geographical areas were recruited on an outpatient basis. All individuals were Caucasian, and were simultaneously participating in the project of the European Network for Investigating the Global Mechanisms of Muscle Abnormalities (ENIGMA) in COPD, specifically designed to investigate the mechanisms involved in muscle dysfunction in COPD ([www.pul.unimaas.nl/enigma/enigma.htm](http://www.pul.unimaas.nl/enigma/enigma.htm)). A diagnosis of COPD was established on the basis of the guidelines of the Global Initiative for Chronic Obstructive Lung Disease.<sup>9</sup> The inclusion and exclusion criteria established in our study were identical in the four centres. All patients were exclusively receiving inhaled medication (long acting beta<sub>2</sub> agonists, anticholinergics and low dose inhaled corticosteroids). Patients receiving oral corticosteroid treatment were not included in the study. The sample size of both patient and control populations was calculated on the basis of formerly published

human studies, where similar physiological and biological approaches were used.<sup>7 8 10–12</sup>

### Study design

This was a cross sectional study in which patients with COPD were compared with age matched healthy control subjects, designed in accordance with the ethical standards on human experimentation in our institutions. The ethics committees at IMIM-Hospital del Mar (Barcelona, Catalonia, Spain), Maastricht University Hospital (Maastricht, The Netherlands), Royal Brompton Hospital (London, UK) and Cruces Hospital (Barakaldo, Basque Country, Spain) approved all experiments. Informed written consent was obtained from all individuals (see the online supplement for additional information).

### Nutritional and functional assessment

Nutritional evaluation included body mass index (BMI) and determination of the fat free mass index (FFMI) by bioelectrical impedance.<sup>13</sup> Forced spirometry and determination of static lung volumes, carbon monoxide transfer and arterial blood gases were performed using standard procedures, and reference values by Quanjer and colleagues<sup>14</sup> were used. Arterial blood gases were performed using standard procedures. Patients with COPD only performed a progressive incremental exercise test on a cycloergometer (Monark-Crescent 864; Varberg, Sweden).<sup>15</sup> Quadriceps strength was evaluated in both patients and controls by isometric maximum voluntary contraction (MVC) and in response to magnetic stimulation of the dominant lower limb as formerly described, respectively<sup>16 17</sup> (see the online supplement for additional information).

### Biopsies

Muscle samples from patients with COPD and controls were obtained from the quadriceps (vastus lateralis) by open muscle biopsy, as described previously,<sup>10 11</sup> at both IMIM-Hospital del

Mar and Cruces Hospital (11 patients and five controls), while the needle biopsy technique<sup>18</sup> was used at Maastricht University and Royal Brompton Hospitals (eight patients and two controls). Samples were 20–30 mg in size, on average. Muscle specimens were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for further analysis or immersed in an alcohol-formol bath for 2 h to be thereafter embedded in paraffin (see the online supplement for additional information).

### Muscle biopsy analyses

All muscle biology analyses were conducted in the same laboratory (IMIM-Hospital del Mar).

### Human cytokine antibody arrays

The expression of 36 cytokines was detected in the quadriceps muscles of both COPD patients and healthy controls using a specific human cytokine antibody array<sup>19</sup> (Panomics, Inc. Redwood City, California, USA) following the manufacturer's instructions (see the online supplement for additional information).

### Cytokine ELISA

After a careful analysis of the microarray cytokine profile in the quadriceps of both COPD patients and controls and on the basis of former studies,<sup>2–6</sup> protein expression of the cytokines TNF $\alpha$ , TNF $\alpha$  receptors I and II, IL6, interferon  $\gamma$ , transforming growth factor (TGF)  $\beta$  and vascular endothelial growth factor (VEGF) was quantified in all muscles using specific sandwich ELISA kits (Biosource Europe, Nivelles, Belgium) following the manufacturer's instructions for each cytokine (see the online supplement for additional information).

### Protein carbonyl ELISA

Total levels of those highly reactive carbonyl groups in the protein side chains were detected by reaction (derivatisation) with 2,4-dinitrophenylhydrazine using the protein carbonyl

**Table 1** Main characteristics and functional variables of the study subjects

	Control subjects (n = 7) Mean (SD) [95% CI]	COPD patients (n = 19) Mean (SD) [95% CI]	Statistical data Mean difference [95% CI], p value
Age (y)	63 (8) [52 to 74]	64 (8) [61 to 68]	-1 [-12 to +9], p = 0.742
BMI (kg/m <sup>2</sup> )	28 (1.7) [25.8 to 30]	22.5 (5.2) [20 to 25]	+5.5 [+2.5 to +8.4], p = 0.001
FFMI (kg/m <sup>2</sup> )	19.1 (1.2) [17.6 to 20.6]	16 (2.7) [14.6 to 17.4]	+3.1 [+1.3 to +4.9], p = 0.002
FEV <sub>1</sub> (% pred)	110 (18) [87 to 134]	33 (13) [27 to 40]	+77 [+54 to +100], p < 0.0001
FVC (% pred)	100 (12) [85 to 114]	69 (19) [60 to 78]	+31 [+16 to +46], p = 0.001
RV (%)	112 (1) [109 to 115]	202 (50) [178 to 226]	-89 [-113 to -65], p < 0.0001
TLC (% pred)	104 (4) [92 to 115]	120 (15) [113 to 127]	-17 [-26 to -7], p = 0.002
DLco (% pred)	93 (2) [88 to 98]	47 (27) [32 to 61]	+46 [+32 to +61], p < 0.0001
KCO (% pred)	85 (5) [72 to 98]	57 (29) [41 to 72]	+28 [+12 to +45], p = 0.002
Pao <sub>2</sub> (kPa)	NA	9.2 (1.4) [9.8 to 8.5]	
Paco <sub>2</sub> (kPa)	NA	5.5 (0.9) [5.9 to 5.0]	
Vo <sub>2</sub> max (% pred)	NA	55 (7) [36 to 74]	
W <sub>r</sub> max (% pred)	NA	45 (8) [22 to 67]	
QMVC (kg)	40.4 (6.2) [32.7 to 48]	29.9 (8.9) [23.9 to 35.9]	+10.5 [+1.9 to +19], p = 0.02
Q twitch tension (kg)	10.2 (2.4) [7.2 to 13.2]	8.5 (2.7) [6.7 to 10.2]	+1.7 [-1.4 to +4.8], p = 0.238
Type I fibres (%)	43 (10.6) [26 to 60]	29 (7.4) [24 to 33]	+14 [-1 to +30], p = 0.03
Type II fibres (%)	57 (10.6) [40 to 74]	71 (7.3) [66 to 76]	-14 [-30 to +2], p = 0.03

Data are presented as mean (SD) and [95% CI].

BMI, body mass index; COPD, chronic obstructive pulmonary disease; DLco, carbon monoxide transfer; FEV<sub>1</sub>, forced expiratory volume in 1 s; FFMI, fat free mass index; FVC, forced vital capacity; KCO, Krogh transfer factor; MVC, maximal velocity contraction; NA, not available; Pao<sub>2</sub>, arterial oxygen partial pressure; Paco<sub>2</sub>, arterial carbon dioxide partial pressure; pred, predicted; Q, quadriceps; RV, residual volume; TLC, total lung capacity; Vo<sub>2</sub>max, peak exercise oxygen uptake; W<sub>r</sub>max, maximal mechanical power output.

**Table 2** Microarray expression of the different cytokines in the quadriceps of the COPD patients with respect to the control subjects

Cytokine	Control subjects	COPD patients	p Value
CTLA (au)	0.0281 (0.0047)	0.0192 (0.0122)	0.075
IL2 (au)	0.0143 (0.0001)	0.0129 (0.0067)	0.120
IL6R (au)	0.0125 (0.0009)	0.0143 (0.0048)	0.731
IL7 (au)	0.0105 (0.0002)	0.0108 (0.0036)	0.629
IL15 (au)	0.0204 (0.0029)	0.0205 (0.0131)	0.891
MIP-1 $\alpha$ (au)	0.0127 (0.0001)	0.0095 (0.0025)	0.005
TGF $\beta$ (au)	0.0078 (0.0002)	0.0044 (0.0012)	0.005
VCAMP-1 (au)	0.0142 (0.0029)	0.0128 (0.0043)	0.075
VEGF (au)	0.0102 (0.0058)	0.0081 (0.0051)	0.036
ApoI/Fas (au)	0.0193 (0.0025)	0.0159 (0.0098)	0.120
GM-CSF (au)	0.0149 (0.0006)	0.0155 (0.0053)	0.235
IL3 (au)	0.0137 (0.0010)	0.0120 (0.0030)	0.005
IL6 (au)	0.0088 (0.0051)	0.0120 (0.0050)	0.945
IL10 (au)	0.0110 (0.0030)	0.0111 (0.0022)	0.331
IL17 (au)	0.0121 (0.0012)	0.0114 (0.0052)	0.019
MIP-1 $\beta$ (au)	0.0119 (0.0021)	0.0109 (0.0028)	0.044
MIP-4 (au)	0.0193 (0.0044)	0.0184 (0.0072)	0.183
IFN $\gamma$ (au)	0.0096 (0.0017)	0.0045 (0.0056)	0.036
RANTES (au)	0.0144 (0.0033)	0.0106 (0.0071)	0.160
Leptin (au)	0.0208 (0.0022)	0.0195 (0.0096)	0.235
IL5 (au)	0.0037 (0.0067)	0.0040 (0.0073)	0.367
IL12 (au)	0.0414 (0.0102)	0.0393 (0.0175)	0.783
MIP-5 (au)	0.0118 (0.0023)	0.0140 (0.0049)	0.367
TNF $\alpha$ (au)	0.0189 (0.0019)	0.0113 (0.0055)	<0.0001
TNF $\alpha$ R I (au)	0.0164 (0.0004)	0.0184 (0.0051)	0.891
TNF $\alpha$ R II (au)	0.0189 (0.0022)	0.0236 (0.0092)	0.075
IL1 $\alpha$ (au)	0.0015 (0.0015)	0.0030 (0.0028)	0.265
IL1 $\beta$ (au)	0.0089 (0.0036)	0.0086 (0.0051)	0.103
IL1R $\alpha$ (au)	0.0137 (0.0062)	0.0166 (0.0049)	0.731
IL4 (au)	0.0222 (0.0051)	0.0226 (0.0098)	0.999
IL8 (au)	0.0129 (0.0024)	0.0134 (0.0016)	0.139
Eotaxin (au)	0.0208 (0.0020)	0.0202 (0.0064)	0.075
EGF (au)	0.0231 (0.0005)	0.0209 (0.0048)	0.019
MMP3 (au)	0.0166 (0.0014)	0.0228 (0.0101)	0.120
ICAMP-1 (au)	0.0124 (0.0039)	0.0135 (0.0059)	0.731
IP-10 (au)	0.0122 (0.0051)	0.0166 (0.0078)	0.367

Data are presented as median and (interquartile range).

au, arbitrary units; COPD, chronic obstructive pulmonary disease; CTLA, cytotoxic T lymphocyte antigen; EGF, epidermal growth factor; GM-CSF, granulocyte-macrophage-colony stimulating factor; ICAM, intercellular adhesion molecule; IFN, interferon; IL, interleukin; IP-10, interferon  $\gamma$  inducible protein 10; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinases; R, receptor; RANTES, regulated upon activation normal T cell expressed and secreted; TGF, transforming growth factor; TNF, tumour necrosis factor; VCAMP; vascular cell adhesion molecule; VEGF, vascular endothelial growth factor.

enzyme immunoassay kit (Zenith Technologies Corp. Ltd, Dunedin, New Zealand) following the manufacturer's instructions (see the online supplement for additional information).

### Immunohistochemistry

Muscle morphometry in both patients and controls was assessed as previously described.<sup>10–12</sup> Monoclonal anti-MyHC-I (clone MHC; Biogenesis Inc., UK, 1/20 dilution) and monoclonal anti-MyHC-II (clone MY-32; Sigma, St Louis, Missouri, USA, 1/100 dilution) antibodies were used to identify type I and type II muscle fibres, respectively. At least 100 fibres were counted in each specimen (see the online supplement for additional information).

### Statistical analysis

Data are presented as mean (SD) with 95% confidence intervals (CI) for parametric data (table 1) and as median (interquartile range) for non-parametric data (table 2). The unpaired t test or the Mann-Whitney non-parametric test was used for

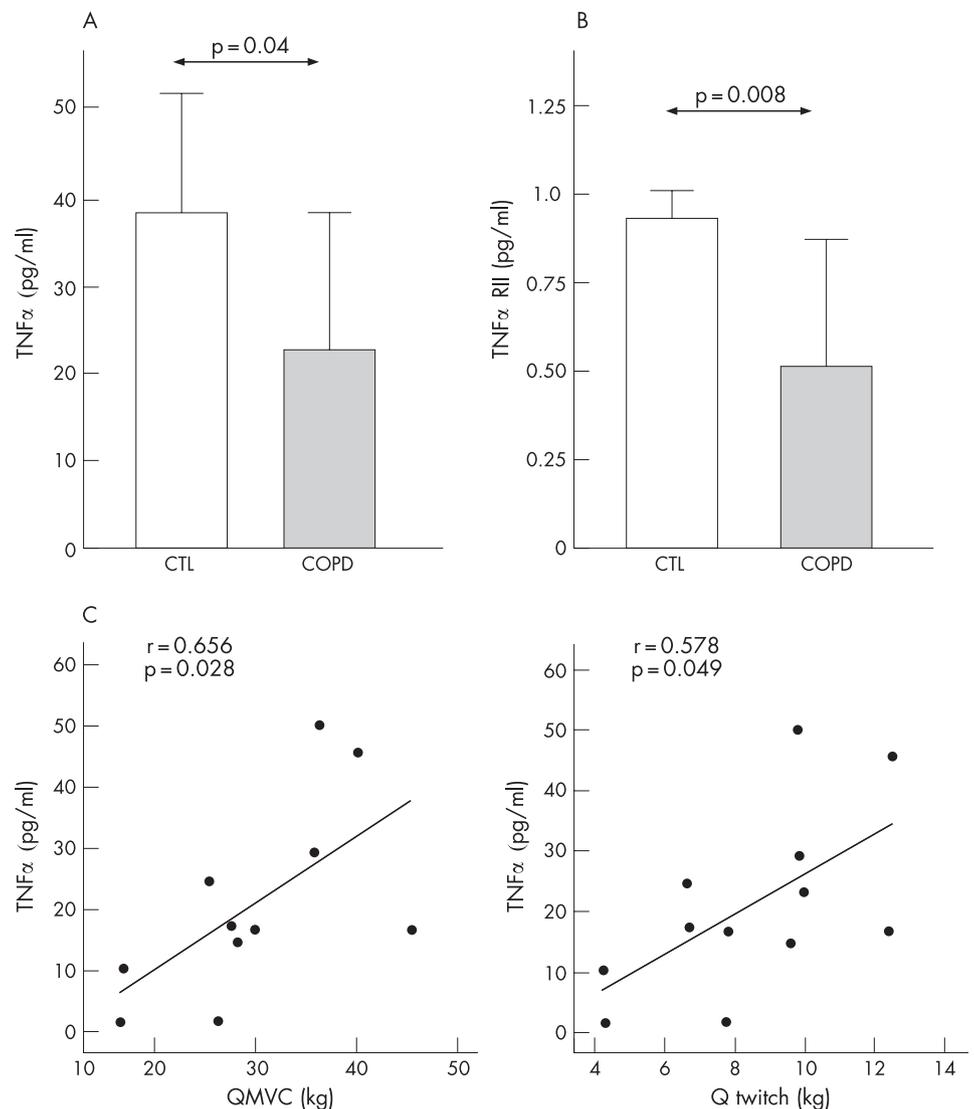
comparisons between controls and COPD patients. While 95% CI, mean differences and p values were indicated for the parametric t test, only exact p values were noted for the Mann-Whitney non-parametric test. Spearman's correlation coefficient was used to assess relationships among different variables within all COPD patients. The sample size was based on assumptions of 80% power to detect an improvement of more than 20% in measured outcomes at a level of significance of  $p \leq 0.05$ .

## RESULTS

### Characteristics of the study subjects

Table 1 shows the main characteristics of the study subjects. As expected, no significant differences were found in age between controls and patients with COPD, and pulmonary function testing confirmed the presence of advanced disease among the patient group. BMI and FFMI were significantly lower in patients compared with controls. Muscle strength, as evaluated by quadriceps MVC, was significantly reduced in patients

**Figure 1** (A) Mean (SD) values for tumour necrosis factor (TNF)  $\alpha$ , as measured by ELISA, were significantly lower in the quadriceps of patients with severe chronic obstructive pulmonary disease (COPD) compared with control (CTL) muscles (21.9 (15.2) pg/ml (95% CI 14.0 to 29.7) vs 37.0 (16.5) pg/ml (95% CI 16.5 to 57.5), mean difference +15.1 (95% CI -4.9 to +35.2)). (B) Mean (SD) values for TNF $\alpha$  receptor II, as measured by ELISA, were significantly lower in the muscles of the patients with COPD compared with controls (0.5 (0.3) pg/ml (95% CI 0.3 to 0.74) vs 0.9 (0.1) pg/ml (95% CI 0.7 to 1.1), mean difference +0.4 (95% CI +0.1 to +0.6)). (C) Among all COPD patients, muscle TNF $\alpha$  levels significantly and positively correlated with both maximum voluntary contraction (MVC) (left) and twitch force of the quadriceps (Q) (right). Note that only 11 and 12 COPD patients are depicted in the corresponding graphs, respectively, as these physiological measurements could not be obtained in all patients or gave error values in a few.



compared with controls (mean (SD) MVC 40.4 (6.2) kg vs 29.9 (8.9);  $p = 0.02$ ), although this did not achieve significance for twitch tension. The proportions of type I fibres were significantly lower while those of type II fibres were higher in the quadriceps of patients with COPD compared with controls ( $p = 0.03$ ).

#### Microarray expression of the different cytokines

Table 2 shows the cytokine levels (optical densities expressed as arbitrary units) detected using the specific human cytokine antibody arrays (see the online supplement for additional information).

#### Muscle specific protein expression (ELISA measurements)

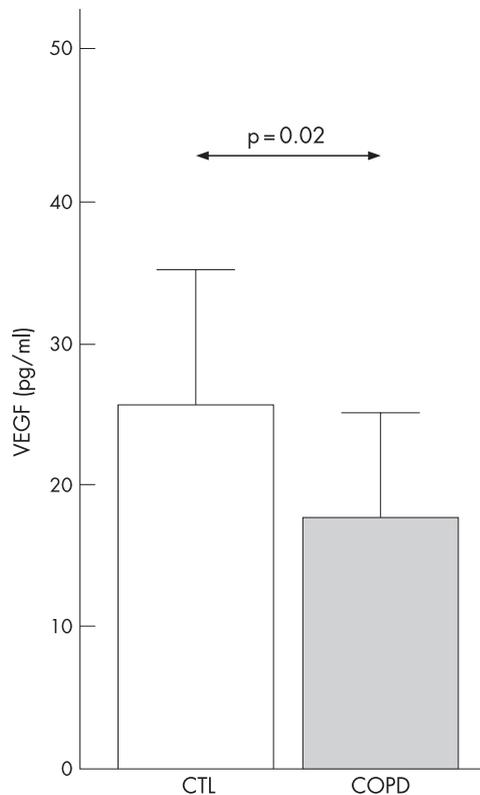
##### Expression of TNF $\alpha$ and TNF $\alpha$ receptors I and II

As shown in fig 1A, protein expression (ELISA measurements) of the cytokine TNF $\alpha$  was lower in the muscles of the patients with COPD compared with controls. No significant differences were observed in the levels of TNF $\alpha$  receptor I in the muscles of the COPD patients compared with those in controls (0.20 (0.051) pg/ml (95% CI 0.17 to 0.23) vs 0.21 (0.04) pg/ml (95% CI 0.157 to 0.256), mean difference +0.003 (95% CI -0.047 to +0.054)). However, ELISA levels of the TNF $\alpha$  receptor II were

significantly reduced in the quadriceps of patients with COPD compared with controls (fig 1B). When the COPD patients were analysed as a group, muscle TNF $\alpha$  ELISA levels significantly and positively correlated with both MVC and the involuntary twitch force of the quadriceps (fig 1C). Moreover, TNF $\alpha$  levels were also positively correlated with those of the TNF $\alpha$  receptor II ( $r = 0.932$ ,  $p < 0.001$ ). No other relationships were found between TNF $\alpha$  or TNF $\alpha$  receptors I and II and lung function, exercise tolerance parameters or fibre type distribution among all patients with COPD.

##### Expression of IL6, Interferon $\gamma$ and TGF $\beta$

Mean (SD) values for IL6, as measured by ELISA, did not differ between patients with COPD and controls (18.9 (3.1) pg/ml (95% CI 17.4 to 20.4) vs 19.5 (2.2) pg/ml (95% CI 16.8 to 22.2), mean difference +0.6 (95% CI -2.1 to +3.3)). Likewise, muscle protein levels of interferon  $\gamma$  did not show any significant difference between patients and controls (10.4 (1.7) IU/ml (95% CI 9.3 to 11.5) vs 11.9 (2.9) IU/ml (95% CI 8.2 to 15.6), mean difference +1.5 (95% CI -2.1 to +5.1)). Muscle levels of the cytokine TGF $\beta$  were also similar between patients with COPD and controls (23.2 (6.9) pg/ml (95% CI 19.9 to 26.5) vs 23.0 (2.5) pg/ml (95% CI 19.9 to 26.1), mean difference -0.1 (95% CI -4.2 to +3.9)).



**Figure 2** Mean (SD) values for vascular endothelial growth factor (VEGF), as measured by ELISA, were significantly lower in the muscles of patients with chronic obstructive pulmonary disease (COPD) compared with control (CTL) muscles (16.8 (6.9) pg/ml (95% CI 13.4 to 20.1) vs 25.2 (9.7) pg/ml (95% CI 9.7 to 40.7), mean difference +8.5 (95% CI -6.3 to +23.2)).

#### Expression of VEGF

Protein levels of the cytokine VEGF (ELISA measurements) were significantly reduced in the quadriceps of COPD patients compared with control muscles (fig 2). No relationships were found between muscle levels of VEGF, lung or muscle functions, exercise tolerance parameters or fibre type distribution among all patients with COPD (see the online supplement for additional information).

#### Muscle protein carbonylation (ELISA measurements)

Muscle protein carbonylation levels were significantly higher in the quadriceps of COPD patients than in controls (fig 3A). When considering all COPD patients as a group, muscle protein carbonylation levels were significantly and negatively correlated with both MVC and the involuntary twitch force of the quadriceps (fig 3B), as well as with muscle levels of TGF $\beta$  (fig 3C). There was a trend towards an inverse correlation between TNF $\alpha$  levels and the magnitude of protein carbonylation in the muscles of all patients with COPD ( $r = -0.451$ ,  $p = 0.08$ ). No other relationships were found between muscle protein carbonylation, lung function, exercise tolerance parameters or fibre type distribution among all COPD patients.

#### DISCUSSION

The main findings in this study were obtained as a result of a prospective exploratory approach using antibody microarrays followed by specific quantification of selected cytokines in the muscles. Hence, in patients with COPD compared with control subjects, we found that: (1) BMI, FFMI, quadriceps force and

type I fibres were significantly reduced, (2) muscle TNF $\alpha$  levels were significantly reduced and positively correlated with quadriceps strength, (3) muscle levels of the TNF $\alpha$  receptor II were also significantly reduced and were related to those of TNF $\alpha$ , (4) muscle levels of the cytokine VEGF were lower and (5) muscle protein oxidation, as measured by total protein carbonylation, was significantly increased and negatively correlated with quadriceps muscle strength among all patients.

#### Study limitations

The main limitations of our study are related to the small size of the muscle specimens and the relatively small number of both patients and controls recruited. As the study involved the use of relatively “invasive” procedures and many different tests were performed in most of the study subjects, we were compelled to obtain rather small muscle specimens. In addition, the volunteer healthy control individuals were recruited on an outpatient basis and had not been hospitalised for any reason in the previous months before study entry as opposed to former studies where subjects underwent surgical procedures. Moreover, very restrictive inclusion and exclusion criteria were used to carefully select the study population. Another limitation encountered in our study was directly related to the methodology. As we chose quite a comprehensive exploratory approach to the study of levels of several cytokines in the quadriceps of our population, we were compelled to use those types of laboratory techniques that require relatively little amounts of sample because of their high sensitivity. It should also be mentioned that the results obtained using the antibody microarray on the one hand, and the ELISA on the other, showed some discrepancies with regard to protein expression of the cytokines interferon  $\gamma$  and TGF $\beta$ . The fact that the former is basically a qualitative exploratory technique whereas the latter is an accurate method to specifically quantify protein levels probably account for such discrepancies. Moreover, the ELISA results were confirmed and verified by repetition of some of the experiments as well as by the use of triplicates in all cases.

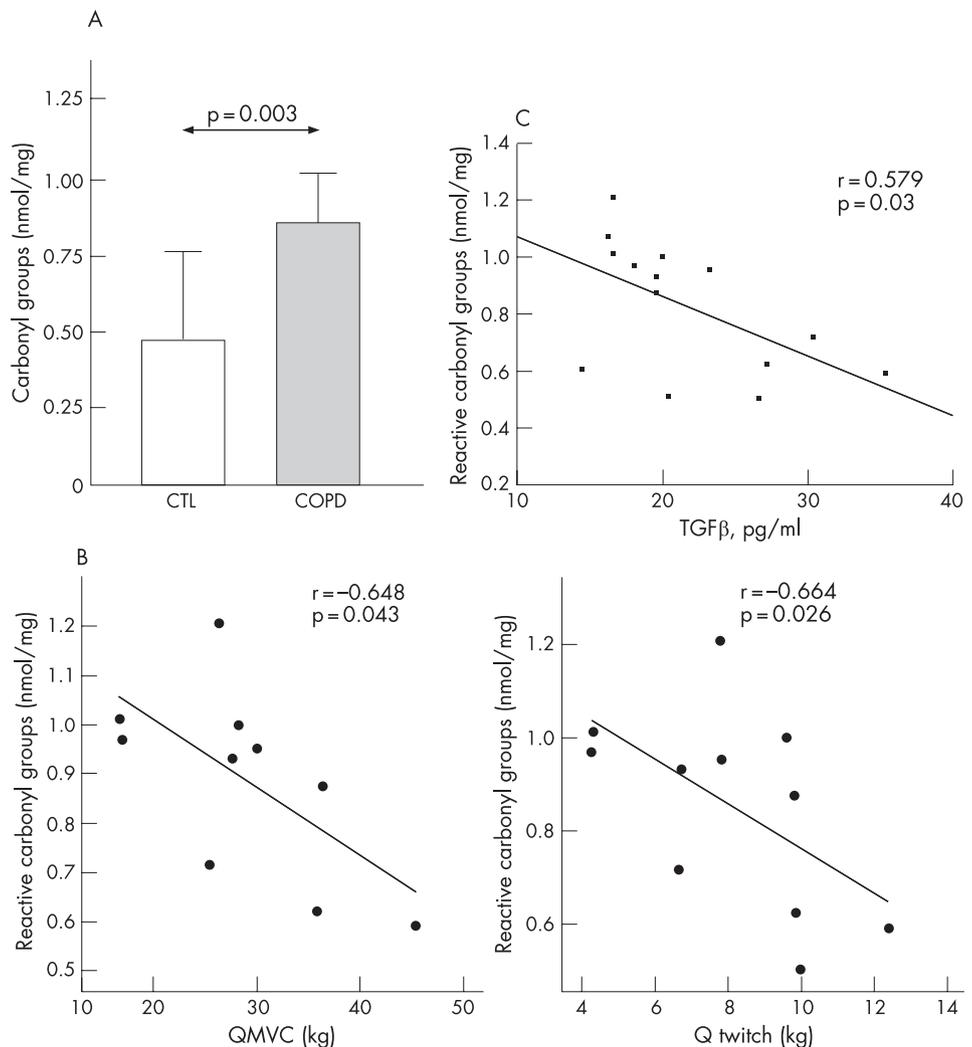
#### Quadriceps muscle structure and function

Quadriceps muscle dysfunction has been well established in the past decade<sup>16–22</sup> Among several mechanisms, decreased proportions of type I fibres have been observed in association with quadriceps muscle dysfunction of patients with COPD.<sup>22</sup> In the present study, compared with controls, both quadriceps strength and proportions of type I fibres were reduced in patients. These findings are indeed consistent with the current literature<sup>20–24</sup> and suggest that our cohort was typical of patients with advanced disease.

#### Cytokines in the vastus lateralis

TNF $\alpha$  is a pleiotropic cytokine that has long been considered to be involved in the muscle loss of patients with chronic wasting conditions, such as cancer and COPD. In fact, it has been shown that systemic inflammatory cytokines such as TNF $\alpha$ , its soluble receptors I and II, IL6 and IL8, along with acute phase reactants, are increased in the blood of patients with severe COPD and muscle wasting compared with healthy controls.<sup>2–6 25</sup> In line with this, other studies have also demonstrated an increase in various blood inflammatory cells, which might be involved in the release of systemic inflammatory cytokines in COPD patients.<sup>6 26 27</sup> Furthermore, systemic TNF $\alpha$  has been proposed to exert catabolic actions in muscles as well as to induce contractile dysfunction in chronic inflammatory conditions,

**Figure 3** (A) Mean (SD) values of total protein carbonylation, as measured by ELISA, were higher in the quadriceps of patients with chronic obstructive pulmonary disease (COPD) compared with controls (CTL) (0.83 (0.22) pg/ml (95% CI 0.69 to 0.96) vs 0.46 (0.32) pg/ml (95% CI 0.06 to 0.86), mean difference  $-0.37$  (95% CI  $-0.76$  to  $+0.02$ )). (B) Among all COPD patients, muscle protein oxidation (protein carbonylation) significantly and negatively correlated with both maximum voluntary contraction (MVC) (left) and twitch force of the quadriceps (Q) (right). Note that only 10 and 11 COPD patients are depicted in the corresponding graphs, respectively, as these physiological measurements could not be obtained in all patients, and one patient showed error values in protein carbonylation levels. (C) Among all COPD patients, quadriceps protein carbonylation levels significantly and negatively correlated with those of transforming growth factor (TGF)  $\beta$ . Note that only 14 patients are depicted in the graph, as quantification of either TGF $\beta$  or protein carbonylation levels were either not available or gave error values in a few patients.



including COPD.<sup>28</sup> In keeping with this, TNF $\alpha$  has been shown to inhibit myogenesis through several mechanisms in in-vitro studies.<sup>29–30</sup> However, the argument has been put forward that TNF $\alpha$  may also promote skeletal muscle regeneration and remodelling, as demonstrated in animal and in-vitro studies.<sup>31–32</sup>

In the present study, refuting our initial hypothesis, ELISA protein levels of both TNF $\alpha$  and its receptor II were reduced by approximately 45% in the quadriceps of our COPD patients compared with levels detected in controls. Furthermore, among all patients, significant positive correlations were found between quadriceps force and muscle TNF $\alpha$  levels, suggesting that this cytokine is somehow involved in the muscle contractile performance of these patients. Recent observations from some of us,<sup>33–34</sup> however, have shown increased mRNA and protein levels of TNF $\alpha$  in the diaphragms and external intercostals of patients with COPD compared with controls. This is also consistent with former studies where expression of TNF $\alpha$ , IL6, IL1 $\beta$  and interferon  $\gamma$  was increased in the diaphragms of rats exposed to resistive breathing<sup>35</sup> as well as in the myocardium of rodents subjected to mechanical loading.<sup>36</sup> Collectively, these results raise the hypothesis that the expression of proinflammatory cytokines within muscle fibres is likely to be differentially regulated on the basis of the activity of each muscle.

In line with our findings, a recent study has also shown the absence of immunohistochemical TNF $\alpha$  expression as well as very low levels of neutrophils in the quadriceps of patients with

severe COPD with and without hypoxaemia either at rest or after local exercise.<sup>8</sup> Montes de Oca *et al*,<sup>7</sup> however, have recently shown a large increase in TNF $\alpha$  levels and other inflammatory parameters, as well as in leucocyte counts in the quadriceps of patients with severe COPD with normal and low BMI. To explain the discrepancy, we note that patients in our study were more severely malnourished, that body composition, as assessed by the FFMI, was not explored in the study of Montes de Oca *et al* and that inflammatory cell counts were only performed in four patients,<sup>7</sup> and not in the whole population of their study. Clearly, the design of future studies is required in order to unravel these important questions. We suggest that examining levels of inflammatory cells, which we now predict are probably very low in the limb muscles of our patients, would be worthwhile as well as prestratification of COPD patients by muscle atrophy profile. Data thus far suggest an absence of inflammatory cells in the quadriceps of patients with severe COPD compared with control muscles.<sup>24</sup> On the other hand, the possible contribution of increased circulating blood cytokines to muscle dysfunction in COPD patients cannot be ruled out in our study as no blood analyses were performed. We believe this factor should also be taken into consideration in future studies.

Another factor that might be implicated in the regulation of the expression of muscle cytokines in severe COPD is hypoxia. We do not believe, however, that chronic hypoxia has taken

place in the limb muscles of our COPD patients because their hypoxaemia was relatively mild (mean PaO<sub>2</sub> 9.2 kPa) and we saw no correlation between TNF $\alpha$  and PaO<sub>2</sub>. On the other hand, quadriceps muscle vascular remodelling and angiogenesis have been suggested to be reduced in COPD.<sup>23 37 38</sup> Hence, the decreased VEGF protein expression observed in the quadriceps of our severe COPD patients could partially account for the proposed reduced remodelling of these muscles.

### Muscle oxidative stress and its relationships with muscle cytokines

We and other investigators<sup>6 8 10-12 39-43</sup> have already shown that protein oxidation is increased in the muscles of patients with COPD. Indeed, oxidative stress has widely been proposed as one of the most important mechanisms involved in the aetiology of peripheral muscle dysfunction in COPD. In the current study, we confirmed once more that lower limb muscle proteins undergo severe oxidation. Moreover, two novel findings are being reported: among all COPD patients, protein carbonylation was negatively associated with both quadriceps muscle function and TGF $\beta$  levels in these muscles. These results support the concept that oxidation of target muscle proteins likely to be involved in muscle force generation contributes to peripheral muscle dysfunction in severe COPD. In line with this, we have recently demonstrated<sup>11</sup> that creatine kinase is more oxidised in the quadriceps of patients with severe COPD and that the activity of such an enzyme was inversely related to its levels of oxidation.

In our study, increased protein oxidation was inversely related to cytokine expression in the quadriceps of patients with very severe COPD and relative muscle loss. The molecular sources of oxidants in the quadriceps of COPD patients remain to be elucidated. On the basis of studies conducted on models of disuse and atrophy, it could be speculated that xanthine oxidase, reactive iron and probably NADPH oxidase would produce reactive oxygen species that may further react with nitric oxide to form the highly reactive species peroxynitrite, which, in turn, would lead to the formation of 3-nitrotyrosine. On the other hand, growing evidence shows that nitric oxide may also inhibit cytokine production in skeletal muscles.<sup>44</sup> Therefore, it would be possible to conclude that excessive reactive nitrogen species production, as shown to occur in the quadriceps of patients with COPD,<sup>10</sup> might partly account for the reduced cytokine protein levels encountered in the current study. Clearly, the design of future studies targeted to explore the specific roles of oxidative stress, molecular sources of reactive oxygen species and cytokine expression as well as the potential links among them in the COPD associated peripheral muscle dysfunction is of paramount importance.

### CONCLUSIONS

Our findings do not support the presence of a proinflammatory environment within the quadriceps muscles of patients with severe COPD, despite evidence for increased oxidative stress and the presence of muscle weakness.

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**Competing interests:** None.

**Ethics approval:** yes.

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## Lung alert

### Role for Major Vault Protein in the innate immunity of respiratory epithelium

Major Vault Protein (MVP) is thought to be important for innate immunity and is found in antigen-presenting cells and epithelia throughout the respiratory system. This study investigated the role of MVP in human respiratory epithelium, particularly in response to infection with *Pseudomonas aeruginosa*.

As *P aeruginosa* initiates an innate immune response by forming lipid rafts on contact with lung epithelial cells, the authors analysed proteins recruited to lipid rafts and compared them with lipid rafts from uninfected cells. Using mass spectrometry, MVP was shown to be present in high concentrations within rafts. Immunofluorescence staining of *P aeruginosa* infected cells confirmed co-localisation of bacteria with MVP.

MVP recruitment into lipid rafts by wild type-CF transmembrane conductance regulator (WT-CFTR) gene-expressing cells was compared with cells expressing the  $\Delta$ F508-CFTR gene. Cells expressing  $\Delta$ F508-CFTR formed only 30% of the amount of MVP produced by WT-CFTR cells in the first 15 min of infection, although overall MVP expression was similar for both. Experimentally truncating the lipopolysaccharide outer core of *P aeruginosa* impaired CFTR binding and eliminated MVP formation entirely. Lungs harvested from MVP knockout mice infected with *P aeruginosa* showed 55% less epithelial internalisation of the bacteria with a subsequent 3.5-fold increase in bacterial burden and increased mortality rate.

This paper supports a role for MVP in the innate immune response. Lack of MVP may increase susceptibility to infection. This work suggests that CFTR binding of bacterial lipopolysaccharide promotes the recruitment of MVP into lipid rafts which, in turn, enhances the epithelial internalisation of *P aeruginosa*. The means by which MVP achieves has yet to be revealed and further studies are needed.

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**Online Data Supplement****CYTOKINE PROFILE IN QUADRICEPS MUSCLES OF PATIENTS WITH SEVERE  
CHRONIC OBSTRUCTIVE PULMONARY DISEASE**

Esther Barreiro, Annemie Schols MWJ, Michael I Polkey, Juan B. Galdiz, Harry R Gosker, Elisabeth B Swallow, Carlos Coronell, and Joaquim Gea, on behalf of the ENIGMA in COPD project

## **METHODS**

### **Subjects**

Nineteen male patients with stable severe COPD and 7 healthy control individuals from four different European geographical areas were recruited on an out-patient basis. All individuals were Caucasian, and were simultaneously participating in the project of the European Network for Investigating the Global Mechanisms of Muscle Abnormalities (ENIGMA) in COPD, specifically designed to investigate the mechanisms involved in muscle dysfunction in COPD ([www.pul.unimaas.nl/enigma/enigma.htm](http://www.pul.unimaas.nl/enigma/enigma.htm)). COPD diagnosis was established on the basis of the Global Initiative for Chronic Obstructive Lung Disease guidelines.[1] The inclusion and exclusion criteria established in our study were identical in the four centers. All the patients were exclusively on inhaled medication (long acting-beta<sub>2</sub> agonists, anticholinergics, and low dose inhaled corticosteroids). Patients receiving oral corticosteroid treatment were not included in the study. Exclusion criteria included chronic respiratory failure, treatment with oral steroids, bronchial asthma, cardiovascular disease, chronic metabolic diseases, suspected para-neoplastic or myopathic syndromes, and/or treatment with drugs known to alter muscle structure and/or function. The sample size of both patient and control populations was calculated on the basis of formerly published studies by our group and other investigators, where similar physiological and biological approaches were used in both patients and control subjects.[2-6]

### **Study Design**

This is a cross-sectional study in which COPD patients were compared to age-matched healthy control subjects designed in accordance with both the ethical standards on human experimentation in our institutions and the World Medical Association guidelines for research on human beings. The Ethics Committees on Human Investigation at Hospital del Mar-IMIM (Barcelona, Catalonia, Spain), Maastricht University Hospital (Maastricht,

Holland), Royal Brompton Hospital (London, England, United Kingdom), and Cruces Hospital (Barakaldo, Basque Country, Spain) approved all experiments. Informed written consent was obtained from all individuals.

### **Nutritional and Functional Assessment**

Nutritional evaluation included body mass index (BMI) and determination of the fat-free mass index (FFMI) by bioelectrical impedance [7]. Forced spirometry and determination of static lung volumes, carbon monoxide transfer, and arterial blood gases were performed using standard procedures, and reference values by Quanjer *et al* [8] were used. All these tests were performed in all the study subjects.

Both arterial blood gases and exercise capacity were only assessed in COPD patients. Arterial blood gases were performed using standard procedures. Patients performed a progressive incremental exercise test on a cycloergometer (Monark-Crescent 864; Varberg, Sweden) in order to determine maximal mechanical power output and oxygen uptake and reference values by Jones *et al* [9] were employed.

Quadriceps strength was evaluated in both patients and controls by isometric maximum voluntary contraction (MVC) of the dominant lower limb as formerly described.[10] Patients were seated with both trunk and thigh fixed on a rigid support of an exercise platform (Domyos HGH 050, Decathlon, Lille, France). The highest value from three brief reproducible maneuvers (<5% variability among them) was accepted as the MVC. Twitch quadriceps force in response to magnetic stimulation was also measured in both COPD patients and controls as previously described.[11]

### **Biopsies**

Muscle samples of both COPD patients and controls were obtained from the quadriceps (*vastus lateralis*) by open muscle biopsy as described previously [4, 5] in both Hospital del Mar-IMIM and Cruces Hospital (11 patients and 5 controls), while the needle

biopsy technique [12] was used in both Maastricht University and Royal Brompton Hospitals (8 patients and 2 controls). Samples were 20-30 mg size in average. Muscle specimens were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for further analysis or immersed in an alcohol-formol bath for 2h to be thereafter embedded in paraffin. All subjects were prevented from doing any potentially exhausting physical exercise 10 to 14 days before coming to the hospital to undergo the surgical procedures.

### **Muscle biopsy analyses**

All the muscle biology analyses were conducted in the same laboratory, at Hospital del Mar-IMIM, in Barcelona.

*Human cytokine antibody arrays.* The expression of thirty-six cytokines (Table 1) was detected in the quadriceps muscles of both COPD patients and healthy controls using a specific human cytokine antibody array [13, 14] (Panomics, Inc. Redwood City, CA, USA) following the manufacturer's instructions. Briefly, frozen muscle samples were first homogenized in a buffer containing 50 mM HEPES, 150 mM NaCl, 100 mM NaF, 10 mM Na Pyrophosphate, 5 mM EDTA, 10% glycerol, 0.50% Triton X-100, 2mg/ml leupeptin, 100 mM PMSF, 5mg/ml aprotinin, and 10  $\mu\text{g}/\text{ml}$  pepstatin (pH 7.5), and were then centrifuged at 1,000 g for 30 min. The pellet was discarded and the supernatant was designated as a crude homogenate. In each sample, total muscle protein level, which was in average  $\sim 8.5\text{-}9\%$  of total muscle sample weight for both patients and controls, was determined with the Bradford technique using different runs of triplicates in each case (Bio-Rad Inc., Hercules, CA). The final protein concentration in each sample was calculated from at least two Bradford measurements that were almost identical (intra-sample coefficient of variation ranged from 0.63%-1.5%). For all the array membranes, equal amounts of total protein from crude muscle homogenates were always loaded (125  $\mu\text{g}$  per sample) in identical sample volumes (1,250  $\mu\text{l}$  / array membrane) for both patients and controls. In order to save muscle specimens for

conducting further experiments, muscle samples from COPD patients were pooled from either two or three different patients. Likewise, muscles samples from the controls were also pooled from two or three different subjects. Capture antibodies specific to particular cytokines had been previously immobilized in duplicates on the array membranes, which were further incubated with blocking buffer. Crude muscle homogenates were subsequently incubated for 2h with the capture antibodies immobilized on the membranes. Following four consecutive five-minute washes, array membranes were then incubated with primary antibody (biotin-conjugated anti-cytokine mix) for 2h. Four more five-minute washes were subsequently conducted before incubation of the array membranes with streptavidin-horseradish peroxidase (HRP) conjugate for 60 min. After four five-minute washes the array membranes were finally incubated with detection solution (chemiluminescence) and exposed to radiographic films for different lengths of time. Films were further scanned with an imaging densitometer and optical densities of specific proteins were quantified with Quantity One 1-D analysis software 4.5.0 (Bio-Rad, Philadelphia, PA, USA). Optical densities (arbitrary units, a.u.) for each cytokine in each membrane were the average value of the two corresponding chemiluminescence signals (duplicates). Microarray experiments were conducted twice in order to confirm the results. Negative controls on the membranes (Table 1) had no capture antibodies on the corresponding areas, whereas positive controls for the detection system contained only streptavidin-HRP. Furthermore, negative control experiments where muscle samples were omitted and membranes were incubated only with homogenization buffer were also conducted.

**Table 1. Human cytokine antibody array: cytokines analyzed in the quadriceps muscles of both patients with COPD and control individuals**

Apol/Fas	Leptin	RANTES	ICAMP-1	IL-2	IL-7	positive control
CTLA	MIP1 $\alpha$	TGF $\beta$	VCAMP-1	IL-3	IL-8	positive control
Eotaxin	MIP1 $\beta$	INF $\gamma$	VEGF	IL-4	IL-10	negative control
GM-CSF	MIP4	TNF $\alpha$	IL-1a	IL-5	IL-12 (p40)	negative control
EGF	MIP-5	TNFR1	IL-1b	IL-6	IL-15	positive control
IP-10	MMP3	TNFR2	IL-1R $\alpha$	IL-6R	IL-17	positive control

*Definition of abbreviations:* CTLA, cytotoxic T lymphocyte antigen; GM-CSF, granulocyte-macrophage colony-stimulating factor; EGF, epidermal growth factor; IP, interferon-gamma-inducible protein-10; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinases; RANTES, regulated upon activation normal T cell-expressed and secreted; TGF, transforming growth factor; IFN, interferon gamma; TNF, tumor necrosis factor; R, receptor; ICAM, intercellular adhesion molecule; VCAMP; vascular cell adhesion molecule; VEGF, vascular endothelial growth factor; IL, interleukin.

*Cytokine ELISA.* After a careful analysis of the microarray cytokine profile in the quadriceps of both COPD patients and controls and on the basis of former studies [2, 3, 15-21], the protein expression of the cytokines TNF-alpha, TNF-alpha receptors I and II, IL-6, interferon-gamma, TGF-beta, and VEGF was quantified in all the muscles using specific sandwich ELISA kits (Biosource Europe, Nivelles, Belgium) for each cytokine. Frozen samples from quadriceps muscles from both COPD and controls were homogenized and protein concentration calculated as described above. For all the samples, from both COPD patients and controls, equal amounts of total protein from muscle homogenates were always loaded in triplicates (20  $\mu$ g in 200 $\mu$ L total volume each singlet for all the triplicates of all the study samples) onto the ELISA plates. All samples were incubated with the specific primary antibodies and were always run together in each assay. Before commencing the assay,

samples and reagents were equilibrated to room temperature. A standard curve was always run with each assay run. Standards (200  $\mu$ L) were performed as indicated by the manufacturer's instructions. Protocol was also followed according to the corresponding manufacturer's instructions for each cytokine. Absorbances were read at 450 nm using as a reference filter that of 655 nm. Intra-assay coefficients of variation for the different cytokines ranged from 1.1% to 1.4%. Inter-assay coefficients of variation for the same cytokines ranged from 4% to 9%. The minimum detectable concentration of each of the cytokines in muscles was set to be 3 pg/mL (Biosource Europe, Nivelles, Belgium).

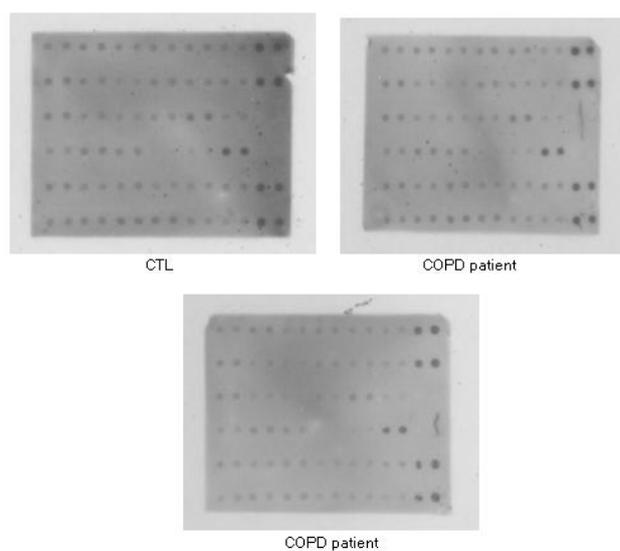
*Protein carbonyl ELISA.* Crude muscle homogenates and protein concentration were obtained from *vastus lateralis* specimens from both patients and controls as described above. Total levels of those highly reactive carbonyl groups in the protein side chains were detected by reaction (derivatization) with 2,4-dinitrophenylhydrazine (DNPH), resulting in the formation of 2,4-dinitrophenylhydrazone (DNP) [22] using the protein carbonyl enzyme immuno-assay kit (Zenith Technologies Corp. Ltd., Dunedin, New Zealand). The DNP-derivatized proteins were subsequently incubated with primary anti-DNP antibody along with the rest of the reagents following the manufacturer's instructions. For all the samples, from both COPD patients and controls, equal amounts of total protein from muscle homogenates were always loaded in triplicates (20  $\mu$ g in 200 $\mu$ L total volume each singlet for all the triplicates of all the study samples) onto the ELISA plates. All samples were all run together in each assay. Before commencing the assay, samples and reagents were equilibrated to room temperature. A standard curve was always run with each assay run. Standards (200  $\mu$ L) were performed as indicated by the manufacturer's instructions. The entire protocol was followed according to the corresponding manufacturer's instructions. Absorbances were read at 450 nm using as a reference filter that of 655 nm.

*Immunohistochemistry.* Muscle morphometry in both patients and controls was assessed as previously described. [4, 6] Muscle samples were immersed in subsequent baths of different degrees of alcohol, formol, and xylol, before being finally embedded in paraffin. Slides were formerly fixed in amino propyl-triethoxilane and acetone, and dried by heat (60°C). Three  $\mu\text{m}$  muscle paraffin-embedded sections were obtained using a microtome. All sections were deparaffinated, and incubated with citric acid solution in a pressure cooker (antigen retrieval protocol). Slides were then blocked in 3%  $\text{H}_2\text{O}_2$ , incubated for 30 min at room temperature in a humid chamber with monoclonal anti-MyHC-I (clone MHC, Biogenesis Inc., England, UK, 1/20 dilution), monoclonal anti-MyHC-II (clone MY-32, Sigma, St. Louis, MO, USA, 1/100 dilution) antibodies. After several washes in phosphate buffered saline (PBS), slides were incubated for 30 min with biotinylated universal secondary antibody followed by incubation (30 min) with horseradish peroxidase (HRP)-conjugated streptavidin and diaminobenzidine (kit LSAB+HRP, Dako Cytomation Inc., Carpinteria, CA, USA) as a substrate. Negative control slides were exposed only to secondary antibodies. Slides were counterstained with hematoxylin, dehydrated and mounted for conventional microscopy. At least 100 fibers were counted in each specimen.

## RESULTS

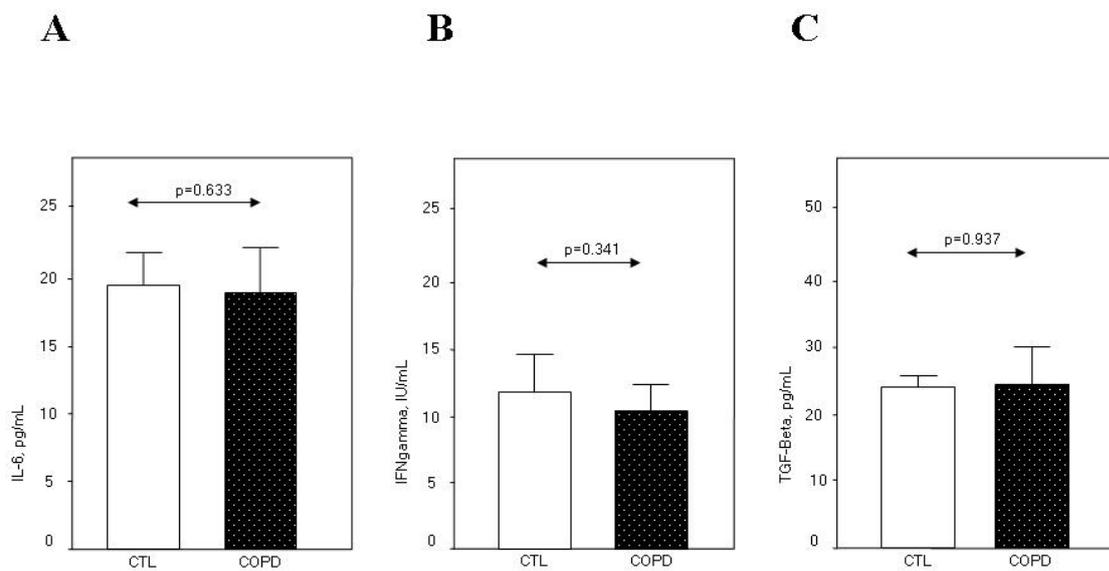
Representative images of the microarray films in pools of 2-3 muscle biopsies of two COPD patients and of one pool of three control subjects are shown in Figure 1.

E. Barreiro et al. Figure 1



*Expression of IL-6, Interferon-gamma, and TGF-beta.* Muscle protein levels (ELISA measurements) of the cytokines IL-6, interferon-gamma, and TGF-beta did not significantly differ between COPD patients and controls (Figures 2A, 2B, and 2C, respectively).

E. Barreiro et al. Figure 2



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