**Online Data Supplement**

# CYTOKINE PROFILE IN QUADRICEPS MUSCLES OF PATIENTS WITH SEVERE CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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## 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). 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$_2$ 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 –80ºC 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 μg/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 ~ 8.5-9% 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 μg per sample) in identical sample volumes (1,250 μl / 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 | MIP1α | TGFβ | VCAMP-1 | IL-3 | IL-8 | positive control |
| Eotaxin | MIP1β | INFγ | VEGF | IL-4 | IL-10 | negative control |
| GM-CSF | MIP4 | TNFα | IL-1a | IL-5 | IL-12 (p40) | negative control |
| EGF | MIP-5 | TNFRI | IL-1b | IL-6 | IL-15 | positive control |
| IP-10 | MMP3 | TNFRII | IL-1Rα | 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 μg in 200μL 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 μL) 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).

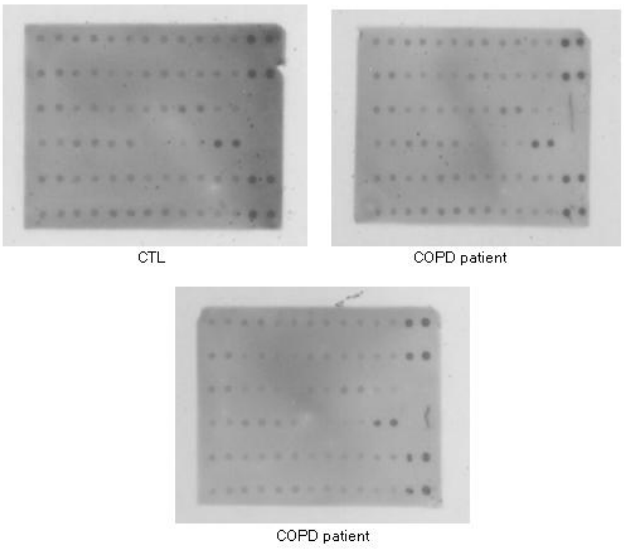
*Protein carbonyl ELISA.* Crude muscle homogenates and protein concentration were obtained from *vastus lateralis* specimens from both patients and controls as described above. Total levels of those highly reactive carbonyl groups in the protein side chains were detected by reaction (derivatization) 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 μg in 200μL 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 μL) 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 μm 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% $H_2O_2$, 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

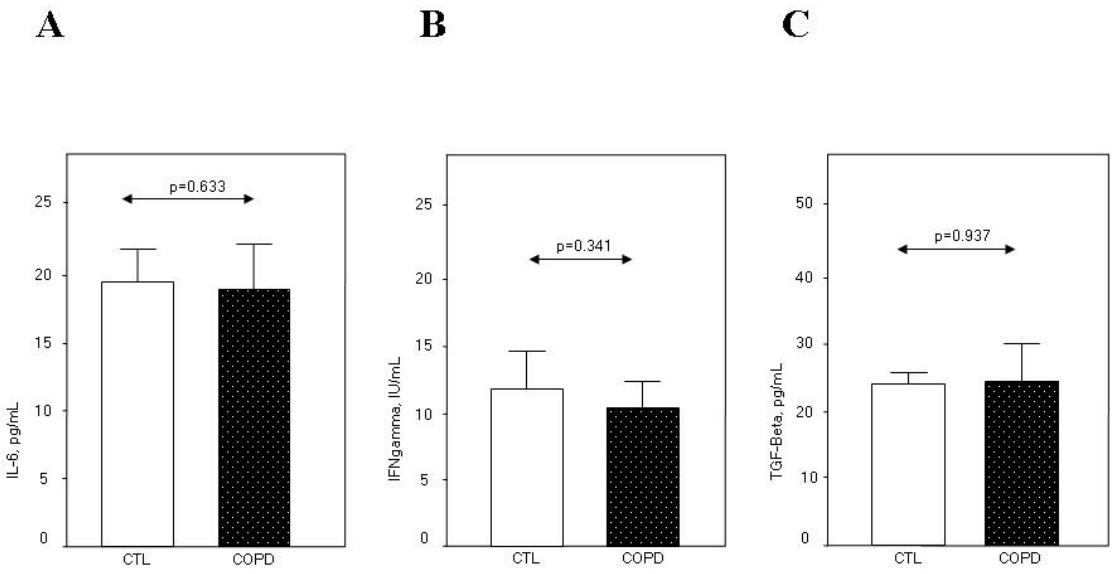
Representative images of the microarray films in pools of 2-3 muscle biopsies of two COPD patients and of one pool of three control subjects are shown in Figure 1.

**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**



**REFERENCES**

1 Fabbri LM, Hurd SS. Global strategy for the diagnosis, management and prevention of COPD: 2003 update. *Eur Respir J* 2003;**22**:1-2.

2 Montes de Oca M, Torres SH, De Sanctis J, *et al*. Skeletal muscle inflammation and nitric oxide in patients with COPD. *Eur Respir J* 2005;**26**:390-97.

3 Koechlin C, Maltais F, Saey D, *et al*. Hypoxaemia enhances peripheral muscle oxidative stress in chronic obstructive pulmonary disease. *Thorax* 2005;**60**:834-41.

4 Barreiro E, Gea J, Corominas JM, *et al*. Nitric oxide synthases and protein oxidation in the quadriceps femoris of COPD patients. *Am J Respir Cell Mol Biol* 2003;**29**:771-78.

5 Barreiro E, Gea J, Matar G, *et al*. Expression and carbonylation of creatine kinase in the quadriceps femoris muscles of patients with chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* 2005;**33**:636-42.

6 Barreiro E, de la Puente B, Minguella, *et al*. Oxidative stress and respiratory muscle dysfunction in severe chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2005;**171**: 1116-24.

7 Steiner MC, Barton RL, Singh SJ, Morgan MD. Bedside methods versus dual energy X-ray absorptiometry for body composition measurement in COPD. *Eur Respir J* 2002;**19**: 626-31.

8 Quanjer PH, Tammeling GJ, Cotes JE, *et al*. Lung volumes and forced ventilatory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J Suppl* 1993;**16**:5-40.

9 Jones NL, Makrides L, Hitchcock J, *et al*. Normal standards for an incremental progressive cycle ergometer test. *Am Rev Respir Dis* 1985;**131**:700-8.

10 Coronell C, Orozco-Levi M, Mendez R, *et al*. Relevance of assessing quadriceps endurance in patients with COPD. *Eur Respir J* 2004;**24**:129-36.

11 Hopkinson NS, Nickol AH, Payne J, *et al*. Angiotensin converting enzyme genotype and strength in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2004;**170**: 395-99.

12 Bergstrom L. Muscle electrolytes in man. Determination by neutron activation analysis on needle biopsy specimens. A study on normal subjects, kidney patients, and patients with chronic diarrhea. *Scan J Clin Lab Invest* 1962;**68**:1-110.

13 Huang RP. Detection of multiple proteins in an antibody-based protein microarray system. *J Immunol Methods* 2001;**255**:1-13.

14 Lin Y, Huang R, Cao X, *et al*. Detection of multiple cytokines by protein arrays from cell lysate and tissue lysate. *Clin Chem Lab Med* 2003;**41**:139-45.

15 Di Francia M, Barbier D, Mege JL, *et al*. Tumor necrosis factor-alpha levels and weight loss in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1994;**150**: 1453-55.

16 Schols AM, Buurman WA, Staal van den Brekel AJ, *et al*. Evidence for a relation between metabolic derangements and increased levels of inflammatory mediators in a subgroup of patients with chronic obstructive pulmonary disease. *Thorax* 1996;**51**:819-24.

17 Takabatake N, Nakamura H, Abe S, *et al.* The relationship between chronic hypoxemia and activation of the tumor necrosis factor-alpha system in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2000;**161**: 1179-84.

18 Takabatake N, Nakamura H, Abe S, *et al*. Circulating leptin in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 1999;**159**:1215-19.

19 Van Helvoort HA, Heijdra YF, Thijs HM, *et al*. Exercise-induced systemic effects in muscle-wasted patients with COPD. *Med Sci Sports Exerc* 2006;**38**:1543-52.

20 Rabinovich RA, Figueras M, Ardite E, *et al*. Increased tumour necrosis factor-alpha plasma levels during moderate-intensity exercise in COPD patients. *Eur Respir J* 2003;**21**: 789-94.

21 Vassilakopoulos T, Divangahi M, Rallis G, *et al*. Differential cytokine gene expression in the diaphragm in response to strenuous resistive breathing. *Am J Respir Crit Care Med* 2004;**170**:154-61.

22 Levine RL, Williams JA, Stadtman ER, *et al*. Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol* 1994;**233**:346-57.