

# Sputum Cytokine Profiling in COPD: Comparison Between Stable Disease and Exacerbation

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**Purpose:** Cytokines are extracellular signaling proteins that have been widely implicated in the pathogenesis of chronic obstructive pulmonary disease (COPD). Here, we investigated cytokine expression both at the mRNA and protein level in the sputum of healthy individuals, stable COPD patients, and those experiencing a severe acute exacerbation (AECOPD) requiring hospitalization.

**Patients and Methods:** Sputum was collected in 19 healthy controls, 25 clinically stable COPD patients, and 31 patients with AECOPD. In AECOPD patients sample collection was performed both at the time of hospital admission and at discharge following treatment. Sputum supernatant was analyzed by an antibody microarray detecting 120 cytokines simultaneously, while the mRNA expression of 14 selected cytokines in sputum cells was investigated by real-time PCR (qPCR).

**Results:** Proteomic analysis identified interleukin (IL)-6 and growth-regulated oncogene (GRO) $\alpha$  as the only sputum cytokines that were differentially expressed between stable COPD patients and healthy controls. At the onset of AECOPD, several cytokines exhibited altered sputum expression compared to stable COPD. Recovery from AECOPD induced significant changes in the sputum cytokine protein profile; however, the length of hospitalization was insufficient for most cytokines to return to stable levels. With regard to gene expression analysis by qPCR, we found that bone morphogenetic protein (BMP)-4 was up-regulated, while IL-1 $\alpha$ , monokine-induced by interferon- $\gamma$  (MIG), and BMP-6 were down-regulated at the mRNA level in patients with AECOPD compared to stable disease.

**Conclusion:** The sputum cytokine signature of AECOPD differs from that of stable COPD. Protein level changes are asynchronous with changes in gene expression at the mRNA level in AECOPD. The observation that the levels of most cytokines do not stabilize with acute treatment of AECOPD suggests a prolonged effect of exacerbation on the status of COPD patients.

**Keywords:** airway, biomarker, gene, inflammation, microarray, PCR

## Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory lung disease characterized by increased numbers of macrophages, neutrophils and T cells, and enhanced release of various inflammatory mediators.<sup>1</sup> Nonetheless, the airway inflammation in COPD is heterogeneous: the most common inflammatory phenotype is the neutrophil-associated COPD with inflammasome, Th1 and Th17 activation, while in a minority of patients increased eosinophilic airway inflammation with increased Th2-transcriptome signature can be observed.<sup>2</sup>

It is well established that cytokines and chemokines play a key role in the control of these inflammatory processes.<sup>3,4</sup> Cytokines secreted from T cells primarily regulate the pattern of inflammation, whereas pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$  or IL-6 amplify inflammation, in part through the activation of the transcription factor (NF)- $\kappa$ B, thereby resulting in increased expression of several inflammatory genes. Chemokines, in turn, recruit inflammatory cells from the circulation to the lungs, while growth factors maintain this inflammation and are

mainly involved in the development of the characteristic structural changes of the airways, in particular small airway disease and emphysema.<sup>5</sup>

In recent years tremendous efforts have been put forward to profile these inflammatory processes in order to better understand the molecular mechanisms underlying the disease and provide a rationale for biological agents targeting specific inflammatory pathways.<sup>6,7</sup> However, it has proved difficult to identify distinct inflammatory endotypes among COPD patients, and associations between inflammatory mechanisms and clinical manifestations of the disease remained uncertain in most cases.<sup>8</sup>

Protein microarray is an established technology to assess the proteome of biological samples.<sup>9</sup> The major advantage of using this method is that it allows the detection of a large number of factors simultaneously in a single experiment.

Sputum is a valuable source of cytokines, chemokines, and inflammatory proteins,<sup>10</sup> and there is evidence that proteomic screening of sputum yields potential biomarkers in various pulmonary diseases including asthma, COPD, cystic fibrosis (CF) and bronchiectasis.<sup>11</sup> Moreover, it appears that the sputum cytokine profiles of different pulmonary diseases are variable and can be related to the clinical status of the patients, as demonstrated recently by Eickmeier et al in the comparison between CF and COPD patients.<sup>12</sup> Nonetheless, only a few studies have applied protein microarray to sputum to compare cytokine patterns between stable and exacerbated COPD patients.

Another approach to studying airway inflammation is gene expression profiling of sputum cells which has enabled the discovery of different transcriptional phenotypes.<sup>13</sup> Using the method in patients from the ECLIPSE cohort, a large and well-characterized cohort of COPD patients, Singh et al have recently found altered gene expression profiles in frequent exacerbators<sup>14</sup> and those with more severe airflow limitation or emphysema.<sup>15</sup> Nonetheless, in these experiments, sputum proteomic analyses were not performed simultaneously. Similarly, although there are several studies investigating cytokine mRNA profiles in sputum cells,<sup>16,17</sup> none of them have attempted to compare that with sputum proteomic data, particularly in patients with acute exacerbation of COPD (AECOPD) and convalescence.

Thus, in this study, we first analyzed the expression of 120 cytokines in the sputum supernatant of healthy individuals, stable COPD patients, and those experiencing a severe AECOPD using protein microarray. We also investigated whether treatment of AECOPD modifies the cytokine profile during the period of hospital care. Second, a subset of cytokines was selected for mRNA expression analysis by quantitative polymerase chain reaction (qPCR) and the results were compared with corresponding proteomic data.

## Materials and Methods

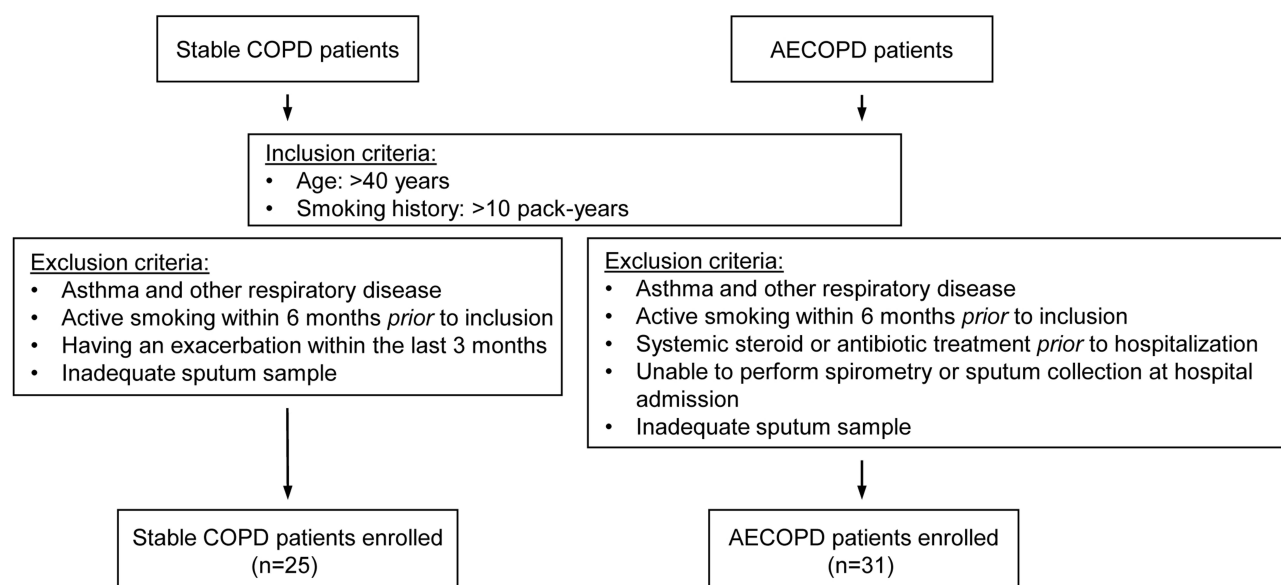
### Study Subjects

A total of 25 clinically stable, ex-smoker COPD patients and 31 ex-smoker AECOPD patients hospitalized for severe acute exacerbation were recruited for the study. Inclusion and exclusion criteria are summarized in [Figure 1](#). Diagnosis of COPD was established by chest physicians, and all patients had documented airway obstruction (post-bronchodilator forced expiratory volume in one second [FEV<sub>1</sub>]/forced vital capacity [FVC] <0.7). AECOPD was defined as increased dyspnea, cough, or sputum expectoration (quality or quantity) that led the subject to seek medical attention, as specified in international guidelines.<sup>18</sup>

Additionally, 19 never smoker or ex-smoker healthy controls were enrolled in the study. Control subjects had normal lung function values and no history of acute or chronic respiratory diseases in the previous 4 weeks. [Table 1](#) shows the characteristics of the study subjects. The research protocol was approved by the Ethics Committee of the National Koranyi Institute of Pulmonology (No: 6/2017), and all subjects gave written informed consent to participate in the study. All procedures performed in the study involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments.

### Study Design

In patients with AECOPD, spontaneously expectorated sputum was collected and fractional exhaled nitric oxide (FENO), blood gases, and lung function parameters were measured at hospital admission and on the day of hospital discharge. In stable COPD patients and healthy controls, induced sputum was collected during routine clinical visits. The smoking status of all participants



**Figure 1** Flow chart showing the study profile. COPD, chronic obstructive pulmonary disease; AECOPD, acute exacerbation of chronic obstructive pulmonary disease.

was confirmed by measuring exhaled carbon monoxide (eCO) levels using a portable CO monitor (Smokerlyzer Micro, Bedfont Scientific, Kent, UK). All subjects had eCO levels <4 ppm indicating a lack of recent smoke exposure. Sputum induction, FENO measurement, lung function, and all other laboratory measurements were performed, as previously described.<sup>19</sup>

## Sputum Processing

Sputum samples were processed as previously described.<sup>20</sup> Briefly, sputum samples were homogenized in phosphate-buffered saline (PBS) containing 0.1% dithiothreitol (DTT, Sigma-Aldrich, St. Louis, MO, USA), filtered through a 40 µm mesh, and centrifuged to separate the supernatant from cells. Cytospins were then prepared and stained with May-Grunwald-Giemsa for differential cell counting. At least 400 inflammatory cells were counted for each cytospin slide. The number of inflammatory cells was recorded as a percentage of total non-squamous cells. Finally, cells were re-suspended in 1 mL PBS and aliquoted to 10<sup>6</sup> cells per cryotube. Aliquots were centrifuged; the pellet was re-suspended in 150 µL RNAlater solution (Sigma-Aldrich) and stored at −80°C. Sputum supernatants were also stored at −80°C.

## Antibody Microarray

Sputum supernatant samples from all healthy control subjects and subgroups of patients with stable COPD and AECOPD (n=19 for each) were subjected to antibody microarray analysis. Both stable and exacerbated patients were randomly allocated into these subgroups. In the assay, 1 mL sputum supernatant was used to simultaneously detect 120 cytokines using Human Cytokine Antibody Array VI+VII (Raybiotech, Norcross, GA, USA), as previously described.<sup>21,22</sup> Briefly, nitrocellulose membranes coated with specific primary antibodies against human cytokines were incubated with sputum supernatant samples at room temperature (RT) for 2 hrs. The membranes were washed, and 1 mL of specific secondary, biotin-conjugated antibodies were added and incubated at RT for 2 hrs. After a wash, membranes were incubated with 2 mL of horseradish peroxidase-conjugated streptavidin at RT for 1 hr. Signal intensities were recorded using a chemiluminescence imaging system and analyzed with GeneTools software (Syngene, Cambridge, UK). The exposure time was 15 min. The raw intensity level detected at each spot was background corrected and then normalized using the average intensity of the positive control spots. Relative intensities were calculated by dividing the background-corrected and normalized intensity of each sample spot by that of the corresponding control spot.

**Table I** Demographic and Clinical Characteristics of Study Subjects

	Healthy Controls	Stable COPD	AECOPD	
<b>Subjects (n)</b>	19	25	31	
<b>Sex (male/female, n)</b>	11/8	11/14	20/11	
<b>Age (years)</b>	62.9±1.5	65.4±1.3	69.2±1.2	
<b>Smoking (pack-years)</b>	27.0±4.3	55.4±5.4	42.7±5.1	
<b>GOLD stages (n, %)</b>				
I–II.	-	15 (60)	14 (45)	
III–IV.	-	10 (40)	17 (55)	
<b>Pulmonary function</b>			<b>At admission</b>	<b>At discharge</b>
<b>FEV<sub>1</sub> (L)</b>	3.03±0.2	1.35±0.12	0.97±0.09	1.16±0.09***
<b>FEV<sub>1</sub> (% predicted)</b>	106.8±3.1	51.8±4.0	38.7±3.43	47.1±3.57***
<b>FEV<sub>1</sub>/FVC (%)</b>	77.7±1.6	53.2±1.85	46.1±2.2	50.0±1.85**
<b>Blood gases</b>				
<b>PaCO<sub>2</sub> (kPa)</b>	5.18±0.12	5.60±0.18	7.06±0.58	6.50±0.31
<b>PaO<sub>2</sub> (kPa)</b>	10.3±0.77	8.14±0.16	6.75±0.33	7.21±0.31*
<b>FENO (ppb)</b>	15.7 (13.0–23.1)	13.1 (8.2–25.5)	16.1 (10.7–28.1)	9.4 (7.0–12.9)**
<b>Laboratory data</b>				
<b>WBC (×10<sup>9</sup>/L)</b>	6.5±0.4	7.2±0.4	11.6±0.9	13.2±1.0
<b>CRP (mg/L)</b>	5.7±1.4	11.9±3.0	39.5±12.7	10.4±3.3*
<b>Sputum</b>				
<b>Total cell counts (×10<sup>6</sup>/g)</b>	0.3 (0.22–0.56)	1.3 (0.52–2.77)	2.47 (1.04–8.35)	1.92 (0.64–5.04)*
<b>Neutrophils (%)</b>	78.6 (73.4–86.1)	90.0 (83.5–93.4)	92.7 (85.1–94.7)	88.1 (77.6–93.3)
<b>Macrophages (%)</b>	16.2 (10.8–23.1)	1.22 (0.27–3.16)	3.21 (1.55–5.63)	4.64 (2.1–12.5)
<b>Lymphocytes (%)</b>	2.2 (0.82–3.58)	4.84 (2.88–8.24)	3.86 (3.2–7.05)	4.5 (3.34–8.45)
<b>Eosinophils (%)</b>	0.0 (0.0–0.32)	2.83 (0.62–4.63)	0.37 (0.0–1.04)	0.32 (0.0–0.45)

**Notes:** Data are presented as mean ± SEM or median (interquartile ranges) unless stated otherwise. \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001 vs admission.

**Abbreviations:** FVC, forced vital capacity; FEV<sub>1</sub>, forced expiratory volume in one second; FENO, fractional exhaled nitric oxide; ppb, parts per billion; PaCO<sub>2</sub>, arterial carbon dioxide tension; PaO<sub>2</sub>, arterial oxygen tension; GOLD, global initiative for chronic obstructive pulmonary disease; CRP, C-reactive protein; WBC, white blood cells.

## RNA Isolation

Sputum samples collected from stable COPD patients and AECOPD patients were subjected to RNA isolation and subsequent gene expression analysis. Total RNA extraction was performed from 10<sup>6</sup> sputum cells using RNeasy Plus Mini Kit (Qiagen, Düsseldorf, Germany), according to the method established previously in our laboratory.<sup>20</sup> Nonetheless, even using this optimized protocol for RNA isolation from sputum, only a limited number of cytokines could be examined at mRNA level in our study (see below). The integrity of the isolated RNA was determined using the cycle threshold (Ct) of different base-pair (bp)-length amplicons of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in qPCR assays, as previously described.<sup>23</sup>

An attempt was made to isolate RNA from induced sputum of healthy controls as well. However, apart from four subjects, the amount of extracted RNA was insufficient for gene expression profiling. Therefore, samples of healthy controls were omitted from qPCR assays.

## PCR

RNA isolates were quality checked for the presence of contaminating bacterial DNA by PCR using universal bacterial primers producing a 466 bp long amplicon, as described before.<sup>24</sup> PCR was performed with Phire DNA Polymerase kit (Thermo Fisher Scientific, Waltham, MA, USA). Amplicons were identified by electrophoresis (100 V, 60 min) on 2%

agarose gel (Serva, Heidelberg, Germany) cast in TBE buffer (Duchefa Biochemie, Haarlem, Netherlands). For visualization 7  $\mu$ L GR Safe nucleic acid stain (Lab Supply Mall, Gaithersburg, MD, USA) was used.

## RT

2.0  $\mu$ g of total RNA (in 80  $\mu$ L reaction volume) was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Life Technologies, Foster City, CA, USA) with the following profile: 10 min at 25°C, 120 min at 37°C, and 5 min at 85°C.<sup>24</sup> Random hexamers (500 nmol) were used as primers.

## Real-Time PCR

Real-Time PCR (qPCR) reactions were carried out with 2  $\mu$ L cDNA sample in a total reaction mixture of 20  $\mu$ L containing 10  $\mu$ L 2 $\times$  iTaq Universal Probes Supermix (Bio-Rad, Hercules, CA, USA) and 1  $\mu$ L Taqman probe (Life Technologies) for the following 14 cytokines/chemokines: IL-1 $\alpha$ , IL-1 receptor antagonist (IL-1Ra), IL-6, IL-8, growth-regulated oncogene (GRO) $\alpha$ , regulated upon activation, normal T cell expressed and secreted (RANTES), monokine-induced by interferon- $\gamma$  (MIG), macrophage colony-stimulating factor (M-CSF), CK beta 8–1, osteoprotegerin (OPG), bone morphogenetic protein (BMP)-4, BMP-6, glial cell line-derived neurotrophic factor (GDNF) and Acrp30 (ADP). Cytokines selected for qPCR assay were chosen based on the results of the protein microarray: ie cytokines that were either differentially expressed between stable COPD and AECOPD or showed altered expression upon treatment of exacerbation. GNB2L1 and PPIA were selected as housekeeping genes that are stably expressed in lung tissue. Amplification in qPCR assays was performed with the following protocol: initial denaturation at 95°C for 30 sec, followed by 40 cycles (95°C for 15 sec, 55°C for 30 sec, and 72°C for 60 sec) as previously described.<sup>24</sup>

## Statistical Analysis

Data are presented as mean $\pm$ SEM or median with interquartile range, as appropriate. Data distribution was analyzed by the Kolmogorov–Smirnov test. Relative cytokine expressions were compared using either the Kruskal–Wallis test followed by the Dunns-test for multiple comparisons or the Wilcoxon signed-rank test. Ct values were analyzed by the Mann–Whitney test or the Kruskal–Wallis test followed by the Dunn’s test. Fold changes in gene expression levels between the groups were calculated by the pairwise fixed reallocation randomization test.<sup>25</sup> Paired Student’s *t*-test (parametric data) and the Wilcoxon signed-rank test (non-parametric data) were used to compare variables measured at the time of hospital admission and discharge. Correlation coefficients were calculated by Spearman’s method. Calculations were performed by GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). A *p*-value <0.05 was considered significant.

## Results

### Clinical Variables During Treatment of AECOPD

Exacerbations were treated with systemic glucocorticoids, bronchodilators (short- or long-acting anticholinergics and/or  $\beta_2$ -agonists), and oxygen in all cases. Antibiotics were given to 24 patients. As expected, during the course of recovery, lung function variables and arterial oxygen tension increased, while C-reactive protein (CRP), FENO levels, and sputum total cell counts decreased (Table 1).

### Antibody Microarray

Analyzing the sputum cytokine profile of stable COPD patients, only two cytokines, IL-6 and GRO $\alpha$  exhibited significantly altered expression level (2.63- and 2.24-fold increase, respectively; *p*<0.05 for each) compared with controls.

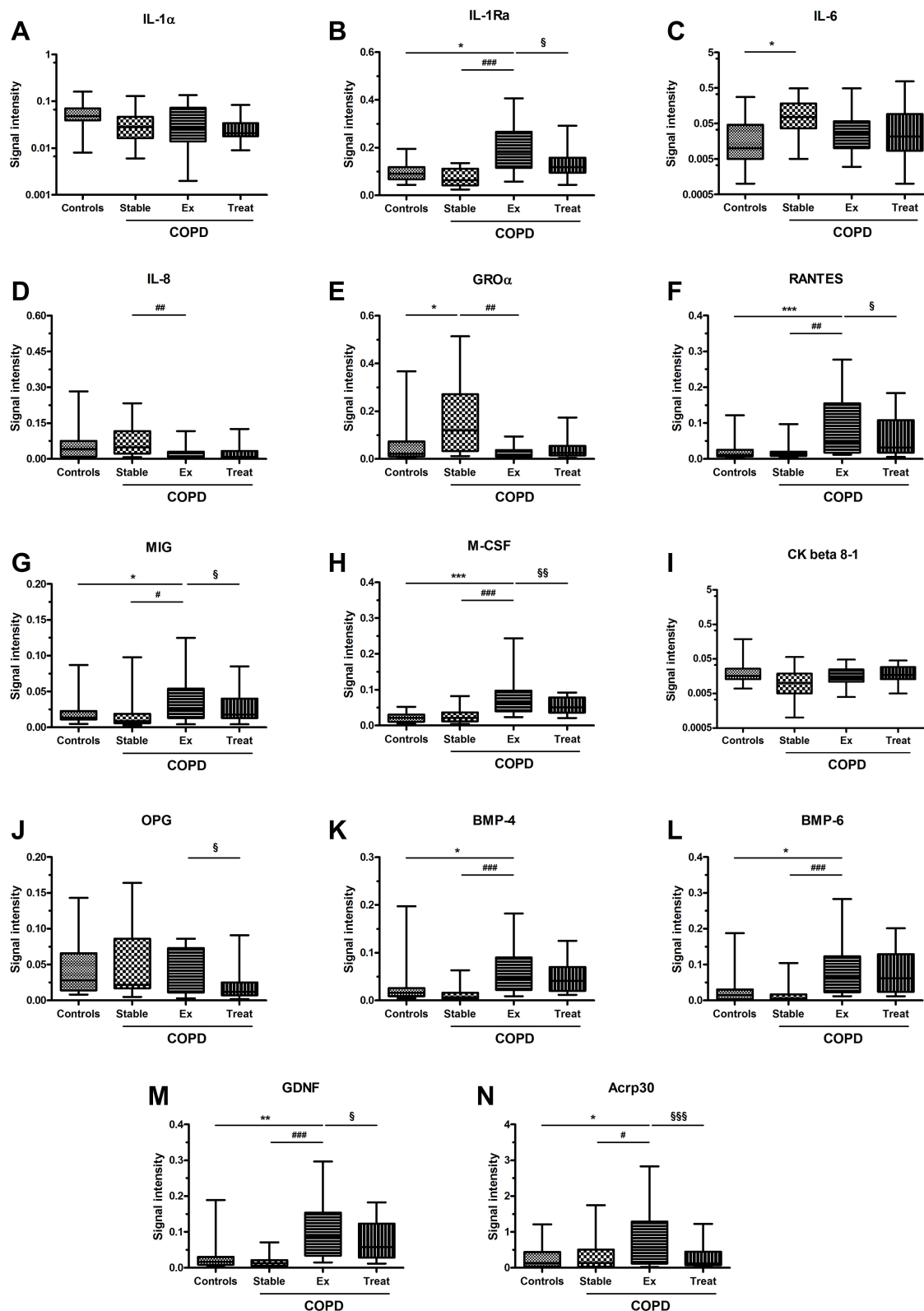
In contrast, signal intensities for several cytokines were significantly increased or decreased at the onset of AECOPD. Treatment of AECOPD resulted again in markedly different cytokine profiles in patients as evidenced by microarray analysis of sputum samples collected at the time of hospital discharge. Cytokines with significantly different expression levels are presented in Table 2, while signal intensities for the 14 cytokines selected for qPCR analysis are presented in Figure 2.

**Table 2** Sputum Cytokines/Chemokines with Significant Changes at the Protein Level at the Onset of AECOPD and Upon Treatment

AECOPD at Hospital Admission			AECOPD at Hospital Discharge		
Cytokine/Chemokine	Fold Change*	p-value	Cytokine/Chemokine	Fold Change <sup>#</sup>	p-value
IL-1Ra	2.66	<0.001	IL-1Ra	-1.5	<0.01
IL-2	2.66	<0.005	IL-2	-1.57	<0.05
IL-3	4.03	<0.001	RANTES	-1.42	<0.05
IL-10	13.39	<0.001	TGF- $\beta$ 1	-1.43	<0.05
IL-8	-3.03	<0.01	TGF- $\beta$ 3	-1.4	<0.05
IL-13	5.58	<0.01	MIG	-2.5	<0.05
TNF- $\alpha$	5.65	<0.001	OPG	-1.92	<0.05
GRO $\alpha$	-5.56	<0.001	PDGF-BB	-2.02	<0.05
RANTES	3.25	<0.01	Oncostatin M	-1.57	<0.05
TGF- $\beta$ 1	2.04	<0.05	M-CSF	-1.45	<0.05
INF- $\gamma$	3.09	<0.001	GDNF	-1.41	<0.05
TNF- $\beta$	1.99	<0.01	Acrp30	-2.44	<0.001
MCP-2	-1.82	<0.05			
BMP-4	4.57	<0.001			
TIMP-1	-10.0	<0.001			
TIMP-2	-3.57	<0.01			
BMP-6	4.89	<0.001			
Acrp30	2.57	<0.05			
NAP-2	2.36	<0.05			
GDNF	6.08	<0.001			
M-CSF	3.72	<0.001			
EGF	1.65	<0.05			
MIG	3.2	<0.05			
MIF	-1.75	<0.05			
Amphiregulin	-5.26	<0.001			
GM-CSF	4.13	<0.001			

**Notes:** Data are presented as fold changes in relative expression levels of cytokines. \*AECOPD onset vs stable COPD, <sup>#</sup>AECOPD treated vs AECOPD onset.

**Abbreviations:** Acrp30, Adiponectin; BMP, bone morphogenic protein; EGF, epidermal growth factor; GDNF, glial cell line-derived neurotrophic factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GRO $\alpha$ , growth-regulated oncogene  $\alpha$ ; IL, interleukin; IL-1Ra, interleukin-1 receptor antagonist; INF- $\gamma$ , interferon- $\gamma$ ; MCP-2, monocyte chemoattractant protein-2; M-CSF, macrophage colony-stimulating factor; MIF, macrophage migration inhibitory factor; MIG, monokine-induced by interferon- $\gamma$ ; OPG, osteoprotegerin; PDGF-BB, platelet-derived growth factor-BB; RANTES, regulated upon activation, normal T cell expressed and secreted; TGF- $\beta$ , transforming growth factor- $\beta$ ; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor.



**Figure 2** Signal intensities of cytokines (panel A-D) and chemokines (panel E-N) determined by antibody microarray in sputum supernatant of healthy controls (Controls), stable COPD patients (Stable) and AECOPD patients at the time of acute exacerbation (Ex) and after hospital treatment (Treat). Standard box plots with median (25th and 75th percentiles) and whiskers (at minimum and maximum values) are shown. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs controls; # $p < 0.05$ , ## $p < 0.01$  and ### $p < 0.001$  vs stable COPD; § $p < 0.05$ , §§ $p < 0.01$  and §§§ $p < 0.001$  vs after treatment.



**Table 3** Comparison of Cytokine mRNA Levels Between Different Study Groups

Cytokine/Chemokine	Stable COPD vs AECOPD at Admission		AECOPD at Admission vs AECOPD at Discharge		Stable COPD vs AECOPD at Discharge	
	Fold change	p-value	Fold change	p-value	Fold change	p-value
IL-1 $\alpha$	-2.83	<0.001	1.272	NS	-2.225	<0.05
IL-1Ra	-1.617	NS	-2.472	NS	-3.996	<0.01
IL-6	1.876	NS	-1.708	NS	1.098	NS
IL-8	-1.609	NS	-1.096	NS	-1.764	NS
GRO $\alpha$	-1.83	NS	-1.049	NS	-1.92	NS
RANTES	-1.156	NS	1.07	NS	-1.081	NS
MIG	-4.483	<0.001	1.480	NS	-3.299	<0.05
M-CSF	-1.565	NS	1.621	NS	1.036	NS
CK beta 8-I	-1.387	NS	1.328	NS	-1.044	NS
OPG	1.146	NS	-1.578	NS	-1.378	NS
BMP-4	2.538	<0.05	1.038	NS	2.634	NS
BMP-6	-3.137	<0.01	1.463	NS	-2.145	NS

**Notes:** Data are presented as fold changes in relative expression levels of cytokines. Positive values indicate up-regulation, while factors with negative values are down-regulated.

**Abbreviations:** BMP, bone morphogenetic protein; GRO $\alpha$ , growth-regulated oncogene  $\alpha$ ; IL, interleukin; IL-1Ra, interleukin-1 receptor antagonist; IL-1 $\alpha$ , interleukin-1 $\alpha$ ; M-CSF, macrophage colony-stimulating factor; MIG, monokine-induced by interferon- $\gamma$ ; OPG, osteoprotegerin; RANTES, regulated upon activation, normal T cell expressed and secreted; NS: non-significant.

## Gene Expression Analysis

From the 14 cytokines selected for qPCR analysis, BMP-4 mRNA was up-regulated ( $p<0.05$ ), while IL-1 $\alpha$ , MIG and BMP-6 mRNAs were down-regulated at the onset of AECOPD compared to stable COPD ( $p<0.001$ ,  $p<0.001$  and  $p<0.01$ , respectively; Table 3). The initial phase of recovery from exacerbation did not result in significant changes in cytokine gene expressions ( $p>0.05$  for each) at the time of hospital discharge. Consequently, IL-1 $\alpha$ , and MIG were still expressed at a lower level compared to the stable state ( $p<0.05$  for each). Finally, IL-1Ra was down-regulated in patients with AECOPD, reaching statistical significance by the time of discharge ( $p<0.01$ ). GDNF and ADP genes were not detectable at the mRNA level.

## Correlations Between Gene Expression Levels and Clinical Variables

In stable COPD patients, BMP-4 gene expression showed significant correlations with sputum total cell counts ( $r=0.528$ ,  $p<0.05$ ) and CRP level ( $r=0.452$ ,  $p<0.05$ ), while CK beta 8-I was associated with sputum neutrophil cell ratio ( $r=0.546$ ,  $p<0.01$ ). M-CSF gene expression also correlated with CRP level ( $r=0.437$ ,  $p<0.05$ ).

At the onset of AECOPD, a positive correlation was found between sputum lymphocyte cell ratio and IL-8, GRO $\alpha$  and BMP-6 gene expression levels ( $r=0.445$ ,  $p<0.05$ ,  $r=0.369$ ,  $p<0.05$ ,  $r=0.491$ ,  $p<0.05$ , respectively). In contrast, CRP level was negatively associated with RANTES, MIG and GRO $\alpha$  expression ( $r=-0.444$ ,  $p<0.05$ ;  $r=-0.444$ ,  $p<0.05$  and  $r=-0.397$ ,  $p<0.05$ , respectively) at this time point. Treatment of AECOPD resulted in the loss of all these correlations (data not shown). No other clinically important correlations were detected.

## Discussion

The most striking finding of our study is that the cytokine profile of clinically stable COPD patients and those experiencing a severe acute exacerbation share little in common. In addition, our study demonstrates that changes in cytokine gene expression at the protein level are often asynchronous with mRNA level changes in AECOPD.

Previous COPD microarray studies used blood as the sample of choice.<sup>26–28</sup> However, a respiratory sample, such as sputum is preferable as it is lung-specific, while blood contains a mixture of signature markers of several pathophysiological processes unfolding simultaneously in a patient.<sup>29</sup> There were attempts to use exhaled breath condensate (EBC) to



profile cytokines locally in the airways.<sup>30</sup> Nonetheless, measurement of biomarkers in EBC has often poor reproducibility rendering this approach less suitable for assessing inflammatory processes in the airways.

Our findings that IL-6 and GRO $\alpha$  expression levels were elevated in stable COPD patients are consistent with the important roles they play in the pathogenesis of COPD. GRO $\alpha$  is a CXC chemokine with strong activating and chemotactic abilities for neutrophils, while IL-6 is an established pro-inflammatory cytokine that is involved in many of the systemic features of the disease.<sup>31</sup>

In AECOPD several cytokines displayed altered signal intensities. For example, the expression level of IL-1Ra, RANTES, MIG, M-CSF, BMP-4, BMP-6, GDNF, and Acrp30 was increased at the onset of AECOPD, while recovery from exacerbation lead to a decrease in signal intensities for Acrp30, IL-1Ra, MIG, RANTES, and M-CSF. Among these factors, RANTES is a potent chemoattractant that has been implicated in recruiting neutrophils and/or eosinophils into the airways during exacerbations.<sup>32</sup> It may also be an important mediator of rhinovirus-induced inflammatory responses.<sup>33,34</sup> Similarly, M-CSF has recently been implicated in COPD, particularly in exacerbations, as an important regulator of the differentiation and survival of inflammatory cells.<sup>31</sup>

Additionally, the expression of TNF- $\alpha$ , a key upstream inflammatory cytokine with a wide range of effects, was elevated in AECOPD.<sup>35</sup> TNF- $\alpha$  has an important role in Th1-mediated immune responses, augmenting both IP-10 and MIG signaling downstream.<sup>36</sup> These chemokines are induced by interferon (INF)- $\gamma$ , likely, as part of a Th1-mediated immune response. Moreover, there is evidence from animal studies that respiratory syncytial virus infections, as frequent causes of exacerbations, enhance inflammation, apoptosis, and lung tissue destruction through the up-regulation of cytokines including INF- $\gamma$ , RANTES, IL-13, MIG, and GM-CSF.<sup>37</sup> Of importance, all these factors showed increased expression in AECOPD in our study. These cytokines are not only involved in local tissue injury but may promote anti-inflammatory responses that eradicate infectious agents.<sup>38,39</sup> Overall, we presume that cytokines identified in AECOPD are primarily involved in the inflammatory responses initiated by bacterial or viral pathogens, as major causes of exacerbations, explaining the markedly different cytokine profile between exacerbated and stable patients.

It appears that the kinetics of the cytokines investigated in our study display a considerable difference during the course of AECOPD and the 7–14 day long hospitalization was insufficient for a number of cytokines to return to levels observed in stable COPD. This is perhaps not so surprising, as some cytokines may be involved in the recovery from exacerbation, while others may be responsible for the long-term effects of exacerbation on the patients' condition. To investigate these issues, the recovery from exacerbation should be examined on a longer time scale.

Another salient finding of this study was that changes in gene expression observed at the protein level did not mirror those at the mRNA level for most cytokines investigated in our study. For example, while IL-1 $\alpha$ , BMP-6, and MIG mRNA expressions were significantly down-regulated in AECOPD compared to stable disease, protein levels of these factors were either unchanged (IL-1 $\alpha$ ) or elevated (BMP-6, MIG). Similarly, changes in mRNA levels for IL-1Ra, M-CSF, and RANTES did not correlate with protein level changes during the course of AECOPD.

In contrast, changes in BMP-4 mRNA levels were closely mirrored at the protein level, both at the onset of AECOPD and after treatment. Similarly, GRO $\alpha$  and IL-8 protein and mRNA levels moved in the same direction, although these changes only reached significance at the protein level. With regard to OPG, a down-regulation upon treatment was observed both at the mRNA and protein levels.

Although biological processes are typically driven by proteins, mRNA signatures are often used as a proxy to describe changes in functional pathways. However, our data is another case in point that changes in mRNA levels are not necessarily paralleled by similar changes in the corresponding protein levels. Post-transcriptional and post-translational modifications can profoundly affect the kinetics of mRNAs and proteins, which may partly explain the mismatch observed in our study between some mRNAs and their corresponding protein levels, in line with previous reports.<sup>40,41</sup>

Recently, BMP-4 was found to promote airway basal progenitor cell differentiation to smoking-related phenotypes that may be important in the development of airway remodeling in COPD.<sup>42</sup> Therefore, it is of note that we found a correlation between BMP-4 gene expression and sputum cell count and systemic inflammation marker level. Moreover, we can speculate that the sustained up-regulation of BMP-4 gene expression could be one of the factors supporting tissue remodeling in AECOPD.

Several lines of evidence indicate that cigarette smoking is a confounding factor in the measurements of cytokines in the airways.<sup>1</sup> Since our patient recruitment strategy called for ex-smokers only, acute smoke exposure did not influence our results.

Again, it is generally assumed that besides their barrier function epithelial cells are capable of influencing inflammatory responses in the airways, at least in asthma.<sup>43</sup> Therefore, it is tempting to speculate that airway epithelial cells could also be involved in the production of the various cytokines/chemokines detected by the microarray in our study. On the other hand, it is also possible that the increased INF- $\gamma$  expression in patients with AECOPD exerted an antagonistic regulatory effect on the gene expression profile of epithelial cells compared to IL-4, as previously suggested in some cell culture studies.<sup>44</sup>

Note that 60% of our stable COPD patients had GOLD stage I or stage II disease. We might have been able to detect more significant differences between the cytokine profiles of healthy controls and the stable COPD group if we had included patients with more advanced disease.

Finally, we believe that our findings have implications for further research. First, our proteomic study has revealed several novel cytokines potentially involved in the development and the long-term effects of AECOPD. However, the exact pathogenic role of these factors remains unclear and should be investigated in the future. Research should also address whether these cytokines could be used as therapeutic targets in the management of patients with AECOPD. Moreover, it would be worthy evaluating the relationship between cytokine patterns and long-term patients' outcomes in terms of lung function decline, exacerbation frequency or treatment responsiveness. If such associations can be demonstrated, this would warrant applying our findings to phenotype COPD patients based on cytokine patterns, both in stable state and AECOPD. Finally, further studies are needed to compare the cytokine profile of the upper airways with the proteomic data obtained in this experiment in the sputum as a special biomatrix. Indeed, a recent study investigating the relationship of protein biomarkers in the upper versus the lower airways could identify several potential nasal proxy candidates for the lower airways in patients with asthma.<sup>45</sup>

## Conclusion

In conclusion, using the highly sensitive antibody microarray technology, we demonstrated that the airway cytokine profile of stable COPD patients and those with AECOPD is markedly different. Moreover, changes in mRNA levels are not necessarily accompanied by similar changes in the corresponding protein levels in AECOPD, and it appears that the level of most cytokines is not stabilized during the time frame of a standard hospitalization indicating a long-lasting effect of exacerbation on the status of COPD patients. Overall, our data suggest that antibody microarray analysis is a suitable tool to characterize changes in the lung proteome. It is tempting to speculate that COPD patients can be phenotyped based on sputum cytokine patterns; however, this should be investigated in further studies involving a larger cohort.

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## Disclosure

The authors report no conflicts of interest in this work.

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