REVIEW

Biomarkers of lung damage associated with tobacco smoke in induced sputum

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Received 16 December 2008; accepted 1 June 2009
Available online 15 July 2009

KEYWORDS
Tobacco smoke; Induced sputum; Lung damage; Biomarkers

Summary
Cigarette smoking has been causally linked several diseases, primarily lung cancer and chronic obstructive lung disease (COPD). The diagnosis of COPD currently involves an assessment of smoking and/or occupational exposures, a history of cough, sputum and dyspnea and spirometric measures of airflow obstruction and since spirometric measures take long follow up times to detect significant changes, surrogate measures of outcome capable of predicting long-term health changes have been sought for. These include biomarkers of oxidative stress, inflammation and tissue damage in sputum, bronchoalveolar lavage, exhaled breath and serum. Published biomarker studies have not always accurately compared patients with COPD with age-matched cigarette smokers and non-smoking normal subjects without significant airflow limitation, also comparable for other exposures. Consequently, the interpretation of biomarker association studies is somewhat difficult. The purpose of this narrative review is to summarize publications reporting cellular, soluble or volatile marker of obstructive lung disease in populations of healthy non-smokers and healthy smokers, in order to determine whether the biomarkers examined could be specifically associated with exposure to tobacco smoke rather than with inflammation and airway hyper-reactivity. As induced sputum has been the most widely used investigative tool, this review has been aimed at assessing induced sputum biomarkers, referring to lung biopsy, bronchoalveolar lavage and exhaled breath markers as supporting evidence for biomarkers associations identified with induced sputum studies.

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doi:10.1016/j.rmed.2009.06.002
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Introduction

The use of tobacco, especially cigarette smoking, has been causally linked to several diseases, primarily lung cancer, chronic obstructive lung disease (COPD), and coronary artery disease. The 1964 and the 1989 United States Surgeon General’s reports pointed to a hundred-fold increase in lung cancer during the past century and to the alarming increase in chronic bronchitis and emphysema deaths.1-3 In the 40 year follow up analysis of the British doctors study of Doll and Colleagues about 15% of current cigarette smokers died of lung cancer, with a 17.4-fold increased risk of mortality risk, and 10% died of chronic bronchitis and emphysema, with a 9.9-fold increase in risk, compared to non-smokers.4

In fact, over the past forty years COPD has become a leading cause of morbidity and mortality worldwide. Currently, COPD is the fourth-leading cause of death in the USA and Europe,5 with mortality more than doubled in females over the past 20 yrs.6 However, COPD prevalence and morbidity is thought to be greatly underestimated as the disease is usually not diagnosed until clinically apparent and moderately advanced. Out of the 6.8% of the general USA population which was found to have low pulmonary function in the National Health and Nutrition Examination Survey (NHANES) study, a striking 63% of those with mildly low pulmonary function (forced expiratory volume in one second (FEV1) <80% predicted) and 44% of those with moderately to severely impaired pulmonary function (FEV1 <50% predicted) did not have yet a diagnosis of obstructive lung disease.7

Current diagnosis of COPD involves an assessment of smoking and/or occupational exposures, a history of cough, sputum and dyspnea and a measure of airflow obstruction. As spirometry is the only widely available tool to identify and monitor airflow obstruction, the diagnosis and the staging of COPD are largely based on FEV1 measures.5 The usefulness of FEV1 measures for diagnosis of COPD and for the evaluation of treatment effects has been proven in major studies conducted over the past 30 years.8,9 However, as spirometric measures do require long follow up periods to determine whether interventions tested in clinical trials determine clinically relevant changes in patient status, surrogate outcome measures capable of predicting long-term responses are been intensely sought for. A recent systematic review by Franciosi and Colleagues on surrogate outcome measures, including a number of biomarkers, has shown that endpoints commonly used to define patient clinical status and to quantify treatment effects had relatively poor sensitivity.10 Published studies examined by Franciosi and Colleagues in this review reported sufficient data for the systematic analysis of interleukin (IL)-6 and IL-8, tumor necrosis factor-alpha (TNF-α), fibrinogen, C-reactive protein (CRP), nitric oxide (NO) and carbon monoxide (CO) studies of marker levels in serum, sputum, bronchoalveolar lavage (BAL) and exhaled breath. With regard to serum measures, no sufficient data were available for the systematic review of IL-6 and IL-8, while the TNF-α data demonstrated a trend toward an association of TNF-α levels and COPD severity, although no statistically significant differences could be demonstrated in the comparison between healthy subjects and COPD patients. The same Authors did not find sufficient data for the meta-analysis of induced sputum data on TNF-α, IL-6, fibrinogen and CRP, although they found a significant difference in the levels of IL-8 of mild COPD patients compared to moderate COPD.10 The data available in the literature on the levels of IL-6, IL-8, TNF-α, fibrinogen and CRP in BAL were also scarce and only BAL IL-8 levels were found to be different in healthy subjects in comparison to patients in mild COPD stages. Finally, these meta-analyses revealed no obvious relationship between COPD severity and either NO and CO levels in exhaled breath. Collectively, the results of this systematic review may suggest that longitudinal studies and disease modelling should be the primary methods for assessing whether potential markers of disease progression for use in COPD diagnosis and clinical trials.10

More recently, the potential role of respiratory biomarkers as surrogate outcome measures in COPD pharmacological trials was the object of a section of a recent statement of an American Thoracic Society (ATS)/European Respiratory Society (ERS) Task Force on outcomes of COPD,11 where Barnes and Page described a number of markers identified in tissue biopsies, BAL, induced sputum and exhaled breath. Infiltrating, activated CD8 + T lymphocytes expressing interferon-gamma (IFN-γ), inducible protein-10 and IL-9, as well as chemokine receptors associated to a type-1 response, such as CXC chemokine receptor 3 (CXCR3) have been identified in tissue biopsies and appear to characterize the local immune response of COPD patients. In addition, airway eosinophilia and neutrophilia associated with up regulation of RANTES (regulated upon activation, normal T cell expressed and secreted) and the CX chemokine ligand 5 (CXCL5) appear to character acute COPD exacerbation.12-14 In addition, the numbers of eosinophils and the levels of leukocyte mediators such as eosinophil cationic protein (ECP), myeloperoxidase (MPO) and IL-8, have been found to be elevated in COPD patients compared to healthy non-smokers, although the levels of cellular and soluble markers were not statistically different from those found in healthy smokers. Interestingly, tryptase and histamine levels have been indicated as COPD markers, although no healthy smoker studies have been performed which could help with discriminating tobacco smoke effects form to COPD-associated inflammation.15,16 Overall, the increase in the numbers of inflammatory cells, most prominently neutrophils and, in some patients, eosinophils, together with the increase in the levels of some soluble mediators including, chiefly IL-8, have been
identified as the COPD biomarkers in induced sputum.\(^\text{17–19}\) Similarly, hydrogen peroxide (H\(_2\)O\(_2\)) and 8-Isoprostane have been identified as the biomarkers of inflammation in COPD in exhaled breath condensate (EBC).\(^\text{20,21}\)

Interestingly, in response to the question addressed by this ATS/ERS statement of whether COPD biomarkers can predict the response to therapeutic interventions, only mast cell numbers, but not the numbers of macrophages, lymphocytes and neutrophils in tissue biopsies, appear to respond to corticosteroid treatment, while the number of neutrophils and lymphocytes in the BAL have been shown to respond to inhaled corticosteroid treatment, although no long-term studies in large populations have been performed to corroborate these data.\(^\text{11}\)

Since in published COPD biomarkers studies affected subjects have not always been compared with age-matched cigarette smoking and non-smoking controls, thus making the interpretation of biomarker associations somewhat difficult, this narrative review has been designed to explore determine whether the biomarkers examined in COPD studies could be associated with exposure to tobacco smoke rather than with inflammation and airway hyperreactivity. To this end, this review summarizes lung biomarker studies investigating cellular, soluble or volatile biomarkers of obstructive lung disease in populations of healthy non-smoking and smoking subjects.

COPD biomarker studies were searched on Medline using biomarker, tobacco and smoke in addition to lung, respiratory, induced sputum, BAL and exhaled breath as key words. Papers including disease groups, such as asthma, COPD or cancer and control population characterized for smoking history were examined. Biomarker studies included induced sputum studies as well as BAL, tissue biopsy, EBC studies. As induced sputum has been validated as a sampling procedure for the evaluation of the airway both for research and clinical purposes, this review was focused upon induced sputum studies, using biopsy, BAL and EBC studies as comparators for marker validation. Animal studies were described wherever they might have been of importance to support the role of a gene/molecule in the pathogenesis of tobacco smoke-associated disease.

**Sampling procedures**

Over the past thirty years, a variety of sampling procedures have been developed to gauge lung damage and inflammation in disease states, including fiberoptic bronchoscopy (FOB) with BAL, exhaled breath analysis, EBC and sputum induction, in addition to the collection of blood and urine samples.

FOB with BAL was introduced in mid 1970s\(^\text{22}\) to explore the lower respiratory tract (LRT) in a variety of lung disorders,\(^\text{23}\) including cigarette-induced disorders like emphysema.\(^\text{24}\) BAL is a relatively benign procedure and local adverse reactions are minor, with pulmonary infiltration and transient decrease in pulmonary function rarely observed.\(^\text{25}\) It has been widely used as a diagnostic and research tool to study interstitial lung disorders such as sarcoidosis,\(^\text{26}\) pulmonary fibrosis,\(^\text{27}\) occupational lung disorders such as silicosis and coal worker pneumoconiosis, asbestosis,\(^\text{28}\) berylliosis and hypersensitivity pneumonitis, smoke-induced lung disorders such COPD and hereditary emphysema,\(^\text{24}\) hystiocytosis-X\(^\text{28}\) and lung cancer\(^\text{29}\) as well as to investigate basic lung mucosal immunology.\(^\text{30}\) The ERS and the ATS have issued guidelines to standardize the procedure.\(^\text{25,31}\)

Exhaled breath analysis allows the measure of volatile mediators, such as NO,\(^\text{32}\) CO,\(^\text{33–35}\) ethane and pentane,\(^\text{36–38}\) in addition to nonvolatile substances in the liquid phase of exhalate such as H\(_2\)O\(_2\).\(^\text{20,39–41}\) The ATS/ERS guidelines recommend the measurement of exhaled mediators as ideally suited for the serial monitoring of patients with airways disorders.\(^\text{42}\)

EBC collection is a non-invasive sampling procedure of lower respiratory examination that has allowed the analysis of a variety of factors including ammonia, H\(_2\)O\(_2\), NO, isoprostanes, leukotrienes, adenosine, peptides and cytokines.\(^\text{43}\) This technique has been widely used in airways research and the ATS and the ERS jointly sponsored guidelines for EBC analysis.\(^\text{43}\)

Blood and urine samples have been also used to identify COPD-associated alterations, suggesting that cigarette smoke may induce systemic inflammatory responses and indicating that such samples may be suitable to screen for markers of smoke-related abnormalities, including oxidative stress and genotoxic damage.\(^\text{44}\)

**Sputum induction**

Sputum induction is a non-invasive, safe and repeatable procedure which has been developed for examining healthy and diseased subjects.\(^\text{45}\)

Sputum induction consists of inhaling an aerosol of saline solution over a determined time-period and to produce timed, forced expectoration efforts.\(^\text{46}\) It requires a high degree of cooperation from the patient and it is advisable that the procedure is performed in a quiet environment, separate from other routine activities. Sputum induction should be performed by adequately trained technologists, or nurses with sufficient experience in the procedure, under the supervision of an experienced physician. Resuscitation equipment should be available, as recommended for other specific and non-specific bronchial challenge procedures,\(^\text{47}\) and the supervising physician should be in the vicinity at all times during sputum induction in order to intervene immediately in the event of any adverse reaction. The patient must be clearly informed prior to the procedure and informed consent obtained.\(^\text{46}\)

The ERS Guidelines recommend that, as hypertonic saline may cause bronchial constriction in asthmatic subjects, short-acting \(\beta_2\)-agonist bronchodilator pre-treatment should be given to prevent excessive bronchospasm,\(^\text{47}\) and the pre-treatment of choice has generally been salbutamol, at the dosage of 200 mcg, i.e. 2 puffs, to 400 mcg i.e., 4 puffs, from a standard metered-dose inhaler.\(^\text{47}\)

Monitoring pulmonary function during sputum induction is necessary for safety reasons, in order to assess excessive bronchoconstriction. FEV\(_1\) should be measured pre-bronchodilator, post-bronchodilator and it should be monitored at intervals of 5 min during aerosol inhalation. Induction should be terminated if a fall in FEV\(_1\) of >20% compared with the post-bronchodilator value or if symptoms occur.\(^\text{47}\) At the end of the induction procedure, patients should be given an inhaled short-acting \(\beta_2\)-agonist, particularly if
there has been a fall in FEV\textsubscript{1} of >10% below the baseline value. Patients should remain in the laboratory and be monitored until their FEV\textsubscript{1} or peak expiratory flow has returned to within 5% of the baseline value.\textsuperscript{46}

The concentration of saline used for sputum induction has ranged from 0.9 to 7% in different studies and some investigators change concentration during the procedure, starting with 3% and subsequently increasing to 4 and 5%. Little is known about the effect of different saline concentrations on the levels of soluble mediators in induced sputum; however, a study has shown no difference in ECP in the supernatant of sputum induced using either isotonic or hypertonic solution.\textsuperscript{47} The consensus is therefore that a 4.5% sodium chloride solution, which is commercially available, effective and generally well tolerated, should be used as standard.

The type and output of the nebulizer are also important and ultrasonic nebulizers are recommended since other nebulizers do not usually exhibit sufficient output of saline aerosol, and there is consensus that an ultrasonic nebulizer should be used with an output of 1 ml min\textsuperscript{-1}. However, the influence of different nebulizer set-ups (length of tubing, valve, etc.) has not been systematically evaluated.\textsuperscript{47}

The duration of inhalation is an important variable, as some studies have reported that the cellular and biochemical constituents of induced sputum change during the course of sputum induction, showing that neutrophils and eosinophils dominate earlier samples whereas lymphocyte and macrophage counts are increased in the later samples, suggesting that different compartments of the respiratory tract are sampled at different procedure times.\textsuperscript{48} Induction procedures have been proposed to last 15–30 min, and 20 min is the time length recommended by the ERS panel.\textsuperscript{47} The subject may be asked to stop inhalation in order to cough up sputum after 5, 10, 15 and 20 min of induction or whenever he/she feels the urge to do so.\textsuperscript{47}

In patients with asthma or COPD sputum is spontaneously produced; in these subjects, spontaneously produced sputum contains percentages of inflammatory cells and mediators similar to those in induced sputum. It is recom-
mended to process sputum as soon as possible, within 2 h from induction, in order to ensure optimum cell counting and staining.\textsuperscript{49,50} Complete homogenization is also important and can be achieved by the use of dithiothreitol (DTT) and dithioerythritol (DTE) to break the disulphide bonds in mucin molecules, allowing cells to be released.\textsuperscript{50} After incubation in a shaking water bath at 37 °C or a tube rocker at 22 °C for 15 min,\textsuperscript{50} the sample is filtered by most investigators through a 48-mcm nylon mesh to remove mucus and debris\textsuperscript{50} and total cell count is performed manually by using a haemocytometer while determining cell viability by the trypan blue exclusion method.\textsuperscript{50,51}

Cell analysis is usually carried out using either smears or cytospin preparations. Preparation of cytospins with an optimum number of cells is felt to provide a more accurate estimate of cell distribution than smears.\textsuperscript{30,52} Cytotoxic chemical and immunocytochemical methods can be used to identify and quantify inflammatory cell populations and sub-populations in the sputum cellular phase. Flow cytometry has been applied to sputum analysis.\textsuperscript{53}

With the caveat that different sputum induction protocols might generate inter-laboratory inconsistent results, fluid-phase mediators can be measured with high reproducibility in induced sputum with a variety of immunoassays performed to measure solutes, as cytokines,\textsuperscript{54–56} and normal reference ranges derived from relatively large adult populations have been published.\textsuperscript{57}

**Soluble markers of tobacco smoke-induced lung damage**

**Chemokines and chemokine receptors**

Chemokines are low-molecular weight polypeptides involved in the migration of inflammatory cells and their receptors are thought to direct T-lymphocyte homing to the lung respiratory tract. Chemokines are classified into CXCL, CC and C subfamilies according to amino acid motifs present in their sequences. They include the growth-related oncogene-alpha (GRO-\alpha), monocyte chemotactic protein-1 (MCP-1) and IL-8, which are produced by a variety of immune and non-immune cells and whose secretion is induced by pro-inflammatory cytokines, viruses, bacterial products, the complement fragment (C)5a and leukotriene B4 (LTB4).\textsuperscript{18,58,59} Chemokine receptors identify Th1/Th2 T-cell phenotypes: Th-1 type cells preferentially express CXCR3 and CC chemokine receptor 5 (CCR5) while Th-2 type cells express CC chemokine receptor 3 (CCR3), CC chemokine receptor 4 (CCR4) and CC chemokine receptor 8 (CCR8).

Several studies on lung tissue biopsies and BAL have shown that chemokine genes are activated in COPD and in tobacco smokers. Tomaki and Coworkers have shown elevated gene expression of MCP-1, GRO-\alpha and IL-8 in COPD.\textsuperscript{60} De Boer et al. have found that MCP-1 was expressed by macrophages, T cells, endothelial and epithelial cells in lung biopsies of control individuals and smokers with COPD. In particular, MCP-1 was expressed at higher levels in the bronchioles of smokers with COPD whereby it correlated with the numbers of intraepithelial macrophages, suggesting that MCP-1 may be involved in the recruitment of macrophages into the airway epithelium in COPD.\textsuperscript{61} Interestingly, mRNA expression of MCP-1 in bronchial epithelial cells of patients with COPD has been found to be higher compared to never smokers or smokers without either airflow limitation or emphysema, suggesting a role of the bronchiolar epithelium as the source of pro-inflammatory chemokines in COPD.\textsuperscript{62} Similarly, Schulz et al (2004) showed that IL-8 and GRO-\alpha genes were expressed at higher levels by cultured bronchiolar epithelial cells from COPD-affected subjects compared to healthy smokers.\textsuperscript{63}

BAL studies have, on the other hand, indicated that chemokine genes are activated in healthy smokers. In the BAL study by Capelli and Colleagues, MCP-1 levels were higher in patients with COPD and healthy smokers than in non-smokers,\textsuperscript{64} suggesting that increased MCP-1 may be related to smoking and may be causative of the smoker’s lung inflammatory reaction. Similarly, Kuschner and Coworkers found elevated IL-8 levels in smokers, compared with non-smokers; interestingly, IL-8 elevation was cigarette exposure-dependent.\textsuperscript{65} Schulz (2003) also reported that constitutive and stimulated IL-8 gene expression in cultured bronchiolar epithelial cells from COPD subjects was significantly higher than in healthy smokers and control subjects, whereas no differences were seen between smokers and controls.\textsuperscript{66,67}
**Induced sputum studies**

Studies on induced sputum have shown that both GRO-α and MCP-1 levels are significantly increased in smokers with COPD (31 ± 11 and 0.8 ± 0.4 ng/ml, respectively) in comparison with non-smokers (2 ± 2 and 0.2 ± 0.1 ng/ml, respectively) and healthy smokers (3.0 ± 0.8 and 0.1 ± 0.04 ng/ml, respectively). A positive correlation was found between chemokine levels and the numbers of neutrophils for both GRO-α and MCP-1 ($r = 0.6, p < 0.001$; $r = 0.4, p < 0.01$, respectively).68

With regard to IL-8, Gelder and Colleagues have found that induced sputum cells expressed the IL-8 gene in 50% of normal non-smoking subjects.69 In the study of Rovina and Colleagues, IL-8 concentrations in induced sputum were increased in COPD patients (5.6 ng/ml [2.3–10]) in comparison with asymptomatic smokers (1.27 ng/ml [0.72–3.2], $p = 0.021$), which were not different from non-smokers (0.73 ng/ml [0.6–1.4], $p = 0.000$, compared to COPD).70 Yamamoto et al. also found that IL-8 expression was increased in induced sputum of patients with COPD in comparison to healthy control subjects (21.0 ± 1.8 vs. 3.3 ± 0.7, $p < 0.0001$)16 (Table 1). In this study, IL-8 sputum levels were correlated with the degree of airflow obstruction ($r = −0.78$ with FEV1/FVC, $p < 0.0001$). Collectively, and in contrast with biopsy and BAL data indicating an inciting role of tobacco smoke exposure upon heightened chemokine expression in the lung of tobacco smokers [63–69], the GRO-α, MCP-1 and IL-8 induced sputum data suggest that increased chemokine levels in sputum are related to lung inflammation, possibly with the inflammation of COPD, and not just with cigarette smoke exposure.

In addition, in the study of Costa et al., sputum levels of CX chemokine ligand 9 (CXCL9), CX chemokine ligand 10 (CXCL10), CX chemokine ligand 11 (CXCL11), and C chemokine ligand 5 (CCL5) were significantly increased in the sputum of patients with COPD when compared with non-smokers: CXCL9 − 14.3 vs. 1.4 pg/mL, $p < 0.001$; CXCL10 − 16.9 vs. 3.7 pg/mL, $p < 0.05$; CXCL11 − 58.1 vs. 33.5 pg/mL, $p < 0.05$; CCL5 − 59.9 vs. 33.5 pg/mL, $p < 0.001$. Furthermore, chemokine sputum concentrations in healthy smokers, which were not significantly different from those of COPD-affected subjects were significantly increased in comparison with non-smoking controls (CXCL9 8.5 pg/mL; CXCL10 11.3 pg/mL; CXCL11 49.8 pg/mL; CCL5 63.0 pg/mL, $p < 0.001$).71 In addition, chemokine concentrations were positively correlated with neutrophil numbers.

With regard to chemokine receptors, induced sputum studies have shown that cells from normal non-smoking subjects express chemokine receptor genes.71 In COPD, Leaky et al. showed that sputum lymphocytes from COPD patients and healthy non-smokers expressed activated phenotypes [CD103 (adhesion molecule αEβ7 integrin, the ligand for epithelial cell E-cadherin) and CD69 (IL-2 receptor (IL-2R) β-chain)]. However, lung lymphocytes in COPD expressed the chemokine receptor CXCR3 at a lower level than healthy controls.72 Contrary to the induced sputum study, Saetta and Colleagues showed, in a small study of tissue biopsies, that T cells infiltrating the peripheral airways of smokers with COPD showed increased expression of the Th1 chemokine receptor CXCR3, paralleled by the expression of its ligand CXCL10 at the epithelial level. CXCR3 expression was lower in healthy smokers, and significantly lower in non-smoking controls.73

Thus, although, sputum chemokine measures are used as a marker of airway inflammation and obstruction in smokers and COPD patients, data on chemokine receptors in relation to tobacco smoke exposure still need to be confirmed in larger studies.

**Cytokines**

**Interleukin-1**

IL-1 is one of the first cytokines ever described. The IL-1 superfamily is comprised of IL-1α, IL-1β, and the IL-1 receptor antagonist (IL-1RA), an anti-inflammatory molecule competing for the receptor binding with IL-1α and IL-1β. These factors are capable of increasing the expression of adhesion molecules on endothelial cells and enabling transmigration of leukocytes to sites of infection, hence playing an important role in the inflammatory response. IL-1 is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis.

In the experimental setting, exposure to cigarette smoke is capable of inducing lung macrophage activation and chemotaxis in mice and the administration of anti-IL-1 antibodies is capable to down-regulate macrophage activation and the ensuing lung inflammation.74

### Table 1  Chemokine levels in induced sputum of tobacco smokers with COPD, healthy tobacco smokers and healthy non-smokers.

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Healthy non-smokers</th>
<th>Healthy smokers</th>
<th>COPD smokers</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRO-α</td>
<td>2 ± 2 ng/ml</td>
<td>3.0 ± 0.5 ng/ml</td>
<td>31 ± 11 ng/ml</td>
<td>68</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.2 ± 0.1 ng/ml</td>
<td>0.1 ± 0.04 ng/ml</td>
<td>0.8 ± 0.4 ng/ml</td>
<td>70</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.73 ng/ml</td>
<td>1.27 ng/ml</td>
<td>5.6 ng/ml, $p = 0.021$</td>
<td>71</td>
</tr>
<tr>
<td>CXCL9</td>
<td>1.4 pg/ml</td>
<td>8.5 pg/ml, $a$</td>
<td>14.3 pg/ml, $a$</td>
<td>71</td>
</tr>
<tr>
<td>CXCL10</td>
<td>3.7 pg/ml</td>
<td>11.3 pg/ml, $a$</td>
<td>16.9 pg/ml, $a$</td>
<td>71</td>
</tr>
<tr>
<td>CXCL11</td>
<td>33.5 pg/ml</td>
<td>49.8 pg/ml, $a$</td>
<td>58.1 pg/ml, $a$</td>
<td>71</td>
</tr>
<tr>
<td>CCL5</td>
<td>33.5 pg/ml</td>
<td>63.0 pg/ml, $a$</td>
<td>59.9 pg/ml, $a$</td>
<td>71</td>
</tr>
</tbody>
</table>

$^a$ $p = 0.021$ compared to healthy smokers.

$^b$ $p < 0.05$ compare to healthy smokers and non-smokers.

$^c$ $p < 0.05$ compared to healthy non-smokers.

$^d$ $p < 0.05$ compare to healthy non-smokers.
In humans, tobacco smoke has been shown to cause heightened systemic IL-1 production in blood mononuclear cells and to stimulate local IL-1 responses by bronchial epithelial cells as well. Interestingly, in an explanted human bronchial epithelial cell assay, IL-1 release has been found to be higher in smokers with COPD and non-smokers compared to smokers with normal pulmonary function, suggesting that smokers who develop COPD may be particularly susceptible to tobacco smoke-induced inflammatory responses, including IL-1 release; this observation is in agreement with the results of an association study between the IL-1 gene polymorphisms C-31T and IL-1 production between sputum cells from COPD patients and non-smokers (Table 2).

Using mRNA amplification by a reverse transcriptase polymerase chain reaction (RT-PCR) technique on lung biopsies of COPD patients, higher IL-1β gene expression at mRNA level was found in COPD compared to non-diseased subjects, whereas no difference was found between smoking and non-smoking controls.

BAL studies have provided contrasting data. In the study by Kuschner and Colleagues, supernatants and cells from smokers exhibited increased levels of IL-1β in comparison to non-smokers and this increase was dependent upon the level of cigarette exposure. Similar data were obtained in the study of Ekberg-Jansson and Colleagues, who showed higher concentrations of IL-1 in conjuction with the elevation of other inflammatory markers such as HNL (Human Neutrophil Lipocalin), MPO and IL-8. In contrast, Keatings and Coworkers found increased concentrations of TNF-α in the induced sputum from COPD patients compared with the smoking and non-smoking control subjects. Similarly, in the study of Rovina and Colleagues increased levels of TNF-α were found in the induced sputum of COPD patients (total fraction 59 pg/ml, bioactive TNF-α <15 pg/ml) and healthy smokers (total fraction 37 pg/ml, bioactive TNF-α <15 pg/ml). In contrast, Dentener and Coworkers, evaluating the effect of smoke upon TNF-α production have shown that cultured sputum cells from COPD patients produced significantly less TNF-α than healthy non-smoking subjects (0.3 ng/ml in the steroid-free group and 1.0 ng/ml in the steroid-treated group vs. 2.9 ng/ml; p = 0.017 and p = 0.001, respectively), while no significant differences were found in TNF-α production between sputum cells from COPD patients and healthy smokers (2.0 ng/ml in the latter group, p = 0.08).

These results have been confirmed by Vernooy and Coworkers, who found similar concentrations of TNF-α in the induced sputum of COPD patients (total fraction 59 pg/ml, bioactive TNF-α <15 pg/ml) and healthy smokers (total fraction 37 pg/ml, bioactive TNF-α <15 pg/ml). In contrast, Keatings and Coworkers found increased concentrations of TNF-α in the induced sputum from COPD patients compared with the smoking and non-smoking control subjects.

In summary, although an effect of smoke on the release of TNF-α by immune cells has been shown, this effect has not consistently been observed in induced sputum cells in smokers without COPD (Table 2).

Granulocyte-macrophage colony-stimulating factor
Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine produced by macrophages, T cells, mast cells, endothelial cells, and fibroblasts. GM-CSF induces the production of type 1 pro-inflammatory cytokines in human peripheral blood mononuclear cells, T lymphocytes, and antigen presenting cells while down-regulating type 2 cytokines. GM-CSF prolongs neutrophil survival in a dose-dependent fashion.

Induced sputum studies. GM-CSF expression is activated by exposure to tobacco smoke. A study of induced sputum cells by Profita and Colleagues showed that sputum cells isolated from patients with COPD released higher levels of GM-CSF (599.5 [454–657] pg/ml) than sputum cells isolated from healthy smokers (294 [253–325] pg/ml, p = 0.0002), which in turn released more GM-CSF than control subjects (125 [100–150] pg/ml; p < 0.004 compared to healthy smokers and p < 0.0001 compared to COPD, respectively). These data suggest that GM-CSF may play a role in maintaining neutrophilic inflammation in the smoker’s lung (Table 2).

Vascular endothelial growth factor
Vascular endothelial growth factor (VEGF) is a potent morphogenic cytokine that modulates embryogenic angiogenesis and vasculogenesis and is expressed in a variety of cell types including epithelial cells and macrophages. VEGF
is a survival factor for epithelial and endothelial cells and constitutive VEGF expression is thought to be important in maintaining normal homeostasis of the lung structure. Elevated oxidative stress levels may induce a reduction of lung VEGF levels, hence promoting the development of emphysema, as tissue biopsy studies have shown that decreased VEGF expression is associated with increased apoptosis in the emphysematous lung.

Consistent with the experimental data indicating that cigarette smoke exposure disrupts the VEGF (165)–VEGFR-2 complex, VEGF tissue studies have shown a significant reduction of VEGF levels in smokers and COPD patients compared to non-smokers.

Consistent with tissue studies, a BAL study by Nagai et al. demonstrated that both alveolar macrophage VEGF mRNA and VEGF protein in BAL fluid were significantly decreased in current smokers compared to non-smokers. VEGF expression in alveolar macrophages was also found to be positively correlated with diffusing lung capacity for CO, indicating a possible relationship between low VEGF expression and parenchymal destruction.

**Induced sputum studies.** In contrast with tissue and BAL studies on lung VEGF, the induced sputum study by Rivona and Coworkers showed that concentrations of VEGF were significantly higher (~1,000 pg/ml) in COPD than in asymptomatic smokers (~400 pg/ml, p = 0.024) and non-smokers (~250 pg/ml, p = 0.002), while the difference between healthy smokers and non-smokers was not significant (Table 2).

The VEGF levels in induced sputum in both groups of smokers (asymptomatic and COPD smokers) were significantly correlated with smoking history, as quantified by pack-years (r = 0.56 vs. asymptomatic smokers, p = 0.046 vs. COPD). Interestingly, VEGF levels were positively correlated with IL-8 (r = 0.636, p = 0.026, respectively) and TNF-α levels (r = 0.622, p = 0.031, respectively).

In contrast, Kanazawa et al. observed that induced sputum VEGF levels decreased with the severity of COPD (mild COPD: 1,360 ± 800 pg/ml; moderate COPD: 1,180 ± 760 pg/ml; severe COPD: 650 ± 450 pg/ml; very severe COPD: 480 ± 240 pg/ml) in comparison to healthy control subjects (1,350 ± 840 pg/ml; p = 0.007 vs. severe COPD, p = 0.002 vs. very severe COPD).

Further studies on patient populations characterized for their smoking history may be necessary to ascertain the role of VEGF as a marker of lung destruction and emphysema.

**Leptin**

Leptin is the pleiotropic molecule sharing similarities with cytokines such as IL-6, IL-11, which plays a regulatory role in innate and acquired immunity, inflammation, and hematopoiesis. Cigarette smoking is known to depress leptin levels in smokers, suggesting that cigarette smoking may increase sensitivity to leptin, as cigarette smokers have been found in a study to have lower leptin levels than non-smokers with the same BMI. Consistent with the knowledge that leptin production is acutely increased during infection and inflammation, COPD patients have been found to have higher plasmatic levels of leptin during exacerbations.

**Induced sputum studies.** Broakhuizem and Coworkers found that leptin could be detected in induced sputum of 10 out of 14 patients with moderate COPD. Statistically significant relationships were found between sputum leptin and CRP (r = 0.943, p < 0.001) and total TNF-α (r = 0.690, p < 0.01). However, no study has been carried to determine the effect of smoking upon LRT tract leptin levels, using smoking and non-smoking healthy controls.

**Other growth factors**

**Transforming growth factor-beta 1 (TGF-β1)**

TGF-β1 is a potent anti-inflammatory cytokine produced by several cell lineages, including AM. TGF-β1 also has powerful profibrotic activities since it causes smooth muscle cell hyperplasia, collagen production by fibroblasts, and differentiation of fibroblasts to myofibroblasts, cells that are important in both matrix production and extra-cellular matrix (ECM) contraction. Tobacco smoke affects the cytokine secretion as shown in a rat tracheal explant model, where cigarette smoke caused release of active TGF-β1, that was preventable by the oxidant scavenger tetramethyliourea within 1 h after exposure.

**In vitro studies** of peripheral blood mononuclear cells exposed to cigarette smoke for 1–5 min have shown that the secretion of TGF-β1 was higher in smokers than in non-smokers. However, in a BAL study of Pons and Coworkers where flow cytometry-sorted alveolar macrophages were
tested for TGF-β1 release, macrophages from patients with COPD released significantly less TGF-β1 than smokers with normal lung function and non-smokers. No TGF-β1 studies on induced sputum in healthy smokers are yet available.

**Hepatocyte growth factor (HGF)**

HGF, a heterodimeric protein obtained through the cleavage of an inactive precursor, called 90-kDa proHGF by serine protease cleavage, that has been shown to control proliferation and migration of type II pneumocytes and their differentiation into type I pneumocytes. In a rat model, decreased HGF expression is associated with emphysema.103

In contrast with experimental studies suggesting that HGF might represent a marker of susceptibility to emphysema in smokers, a lung tissue study by Bonay and Colleagues showed no differences in HGF expression at the mRNA or the protein level between emphysema patients and non-emphysema controls, irrespective of smoking history.104 No studies on HGF in induced sputum in smokers have been reported so far.

**Lymphocyte-derived cytokines**

IL-4, an 18 kDa glycoprotein, and IL-13, a 13 kDa glycoprotein produced by Th2-lymphocyte and by some epithelial cells, have similar effects, among which a potent inhibitory effect on macrophages. IL-13 overexpression in transgenic mice is capable of inducing the development of spontaneous emphysema, with mucous metaplasia and inflammation driven by the influx of macrophages and CD8 T-cells in the lung.105,106

Tobacco smoke has been shown to have systemic effects upon IL-13 production, as increased levels of IL-13 could be found in serum of smokers in a monozygotic twin study.107 Furthermore, Miotto and Coworkers showed in a lung biopsy study of smokers with COPD and asymptomatic smokers that the IL-13 and the IL-4 genes were expressed at slightly higher levels in COPD compared to healthy smokers. In contrast, the IL-5 and the IFN-γ genes were expressed at similar levels in COPD and healthy smokers.108 Similar results have been obtained by Barcelo and Coworkers in a BAL study, showing that COPD patients had higher percentages of IL-13-positive T lymphocytes compared to never smokers or smokers with normal lung function, whereby the expression of IL-13 was inversely related to the degree of airflow obstruction.109 In stark contrast, the study of Boutten and Colleagues on IL-13 and IFN-γ expression in tissue homogenates of emphysematous lungs showed that while IFN-γ expression was similar in emphysematous smokers, healthy smokers and non-smoking controls, the expression of IL-13 mRNA expression was significantly lower in emphysematous smokers compared to healthy smokers and non-smoking controls. Interestingly, no differences were found between smoking and not smoking controls.110

Consistent with the lymphocyte cytokine data, STAT4, a member of STAT family of transcription factors involved in the immune system regulation, has been implicated in COPD by Di Stefano et al., who found a significant increase in the number of phospho-(Y-693)STAT4-positive cells in the sub mucosa of COPD patients in comparison to healthy smokers and non-smokers, in a BAL and bronchial biopsy study. Similar results were obtained from bronchial epithelium and BAL cells: COPD subjects showed a significantly higher level of STAT4 expression and activation than non-smokers and healthy smoker. Interestingly, the number of phospho-(Y-693) STAT4 immunoreactive cells in the sub mucosa of smokers was inversely correlated with FEV1 (% pred) values.111

Further studies are needed to clarify the potential role of IL-13 as a marker of tobacco smoke-induced lung damage. No induced sputum studies have been reported yet assessing IL-13, nor IL-2, IL-4, and IFN-γ expression in the LRT of smokers with or without COPD.

**Oxidants and oxidative stress related factors**

**Myeloperoxidase**

MPO is the most abundant component in the azurophilic granules of leukocytes and it is found not only in neutrophils but also in monocytes and in some subtypes of tissue macrophages. MPO is secreted upon leukocyte activation, amplifies the oxidative potential of H2O2 derived from leukocyte NADPH oxidases, xanthine oxidase, uncoupled nitric oxide synthase (NOS) and various Nox isoenzymes, by forming potent oxidants capable of chlorinating and nitrating phenolic compounds. MPO-derived diffusible radical species are capable of initiating lipid peroxidation as well as promoting an array of post-translational modifications in target proteins, including halogenation, nitration and oxidative cross-linking.112

**Induced sputum studies.** Two studies have been published on MPO analysis in induced sputum. Ryttila and Coworkers reported that the numbers of MPO positive cells was greater in the induced sputum from asymptomatic smokers compared to non-smokers (p = 0.003), while no statistically significant differences were observed between smokers and ex-smokers and between healthy smokers and stage 0 COPD subjects.113

In another study from Hill et al., performed on subjects with chronic bronchitics with and without α-1 antitrypsin deficiency, MPO concentrations in induced sputum were higher in currently smoking bronchitics (0.3 units/ml) compared to who stopped smoking (0.1 units/ml), although the difference did not reach statistical significance.114

The lack of statistically significant differences between current and former smokers with COPD was confirmed by another study, which observed similar concentrations of MPO in the induced sputum of the two groups.115

Further studies could help clarifying the relationship between exposure to tobacco smoke, COPD stages and MPO expression. Furthermore, since the study of Van Schooten and Coworkers has shown that the MPO-463G → A genetic polymorphism is associated with MPO activity in the LRT as well as with the levels of smoking-related DNA adduct found in lung-derived cells, it would be reasonable to take into account the distribution of the MPO-463G → A genetic polymorphisms in assessing MPO expression in relation to tobacco smoke exposure.116

**Eosinophil peroxidase (EPO)**

EPO is a member of a family of mammalian peroxidases that includes MPO, lactoperoxidase (LPO), and thyroid peroxidase (TPO) and has been implicated in promoting
oxidative tissue damage in a variety of inflammatory conditions, including asthma.

Induced sputum studies. EPO has been implicated in lung inflammation mediated by eosinophils, and although its expression is typically higher in asthmatic patients, higher levels of the enzyme were also detected by Keatings and Colleagues in the induced sputum from COPD subjects in comparison with healthy controls. In this context it is worth noticing that the bronchial biopsy study by Amin and Coworkers has shown that EPO was increased in asymptomatic smokers compared to non-smoking subjects, thus suggesting a role of eosinophils and their products, including EPO, in the remodelling of the smoker’s airways. Currently EPO is used as a marker of eosinophils in the induced sputum and other biological samples. Further studies are needed in order to assess the role of EPO as a marker of tobacco smoke-induced inflammation.

Superoxide dismutase (SOD)
The enzyme SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. SOD is an important component of the antioxidant defense system in cells exposed to reactive oxygen species (ROS). In humans, three forms of SOD are present: SOD1 is located in the cytoplasm, SOD2 in the mitochondria and SOD3 in extracellular spaces. Extracellular SOD is expressed at particularly high concentrations in the lungs, where it has been demonstrated in bronchial lining fluid, in epithelium of Airways, and in pulmonary blood vessel walls by immunohistochemistry. This pattern of expression may reflect the importance of extracellular SOD in lung defenses against endogenous oxidative stress as well as against exogenous oxidative stress caused by inhaled air pollutants such as tobacco smoke.

BAL and blood studies have shown that SOD1 and SOD3 expression is reduced in the lung and the blood of tobacco smokers. In a study performed by Di Silvestro and Coworkers on BAL samples from normal non-smokers, healthy smokers and asthmatic subjects, SOD activity was detected in all samples. Unlike serum or some other fluids, in which the enzyme SOD3 accounts for virtually all SOD activity in the BAL fluid. Interestingly, SOD activity was lower in smokers than in non-smoking controls or asthmatics, suggesting an important role of cigarette smoke in reducing the local anti-oxidative power in the lung.

The above lung data were confirmed in the study of Kim and Colleagues, in which extracellular SOD activity was quantified on blood samples and SOD3 was found to be lower in smokers in comparison with age-matched non-smokers, suggesting a negative effect on the oxidant defense systems.

In contrast, the study of Hulea and Coworkers on blood samples from 200 subjects, smokers and non-smokers, showed that while in smokers of 18–45 years of age the changes of the plasma pro-oxidant and antioxidant parameters were not significantly different from those of the age-matched controls, in the 46–80 age group they were. In smokers, both antioxidant erythrocyte enzymes, glutathione peroxidase (GSH-Px) and SOD, exhibited increased activity in the 18–45 age group and decreased activity in the 46–80 age group. No study has been yet published on SOD expression in induced sputum in smokers versus non-smokers.

Glutathione S-transferase
The glutathione S-transferase (GST) family of enzymes comprises a long list of cytosolic, mitochondrial, and microsomal proteins catalyzing the conjugation of reduced glutathione via the sulfhydryl group to electrophilic centers on a wide variety of substrates, both endogenous and xenobiotic. An activity that’s useful in the detoxification of endogenous compounds such as peroxidized lipids as well as in the metabolism of xenobiotics, in particular in protecting against oxidative damage from tobacco smoke components. A number of polymorphisms of the GST genes have been identified which are associated with the levels of enzymatic activity and with lung cancer and emphysema/COPD risk. Although GST might represent an interesting marker of the response of the LRT to tobacco smoke exposure, no induced sputum studies have been published so far.

Cytochrome P450 1A1 and P450 1A6
Cytochrome P450 enzymes catalyze the first step of the metabolism and subsequent elimination of hydrophobic xenobiotics. However, the activity of some isoforms, including Cytochrome P450 1A1 (CYP1A1), may result in cellular insults such as oxidative stress and activation of procarcinogen compounds into reactive metabolites. CYP1A1 is involved in the biotransformation of tobacco-derived polycyclic aromatic hydrocarbons (PAH) into carcinoogenic metabolites. CYP1A1 is inducible by PAHs (Polycyclic aromatic hydrocarbons) and is presumed to be important in pulmonary carcinogenesis. The CYP1A1-dependent monoxygenase transforms selected xenobiotics (including PAHs procarcinogens) into potent carcinogetic metabolites. CYP1A1 is induced in the lung up to 100-fold by tobacco smoking. No studies have been reported so far on the analysis of lung CYP1A1 gene expression using sputum induction or other non-invasive sampling procedures.

Isoprostanes
8-Isoprostane is the best characterized compound belonging to F2-isoprostanes, a group of stable Prostaglandin F2a (PGF2a) isomers formed by the free radical peroxidation of arachidonic acid independent of the action of cyclooxygenase. 8-Isoprostane is considered an ideal marker for investigating the pathophysiology of oxidative injury. Although 8-Isoprostane may be produced by cyclooxygenase (COX)-1 and COX-2 activity in some cells and tissues, it can be considered a reliable biomarker of lipid peroxidation caused by ROS.

Induced sputum studies. Levels of 8-isoprostane have been measured in induced sputum. In a recent study, Kinnula and Coworkers found that 8-isoprostane levels in induced sputum were higher in healthy smokers (108.4 pg/ml) than in non-smokers (15.3 pg/mL, p = 0.005), who were not significantly different from ex-smokers. Interestingly, healthy smoker levels were not significantly different from stage 0 COPD subjects (66.6 pg/mL). In contrast, 8-isoprostane levels were significantly increased in COPD (202.2 pg/mL in stage I–III, p < 0.0001 and p = 0.02 compared with non-
smokers and healthy smokers, respectively). In addition, a statistically significant correlation was found between 8-isoprostanet levels, lung function parameters (FEV₁/FVC ratio: $r = -0.66$, $p < 0.0001$; FEV₁: $r = -0.48$, $p = 0.006$) and smoking history (pack-years, $r = 0.56$, $p = 0.001$). Finally, 8-isoprostane sputum levels were positively correlated with neutrophils counts in sputum (total neutrophils $r = 0.37$, $p = 0.02$). In conclusion, 8-iso-prostanet levels in healthy smokers, COPD ex-smokers and current smokers were similar. Interestingly, 8-Isoprostane levels in COPD ex-smokers with COPD, 8-Isoprostane was higher than in healthy smokers. 8-Isoprostane levels in COPD ex-smokers and current smokers were similar. Interestingly, EBC 8-isoprostane concentrations increased 15 min after smoking, compared to basal levels. 21

Similar data were obtained by Montuschi and Colleagues, in an EBC cross-sectional study evaluating the non-smoking and smoking controls in comparison with current smokers with COPD. In this study, 8-Isoprostane levels were detectable in the EBC of healthy non-smokers and were increased in healthy smokers, COPD ex-smokers and COPD current smokers. In ex-smokers with COPD and current smokers with COPD, 8-Isoprostane was higher than in healthy smokers. 8-Isoprostane levels in COPD ex-smokers and current smokers were similar. Interestingly, EBC 8-isoprostane concentrations increased 15 min after smoking, compared to basal levels. 21 In conclusion, 8-isoprostane measures in EBC appear to be a non-invasive and reliable measure of oxidative stress and a candidate marker of tobacco smoke-induced lipid peroxidation.

**Reactive nitrogen species (RNS)**

Increased production of NO during inflammatory and immune processes affecting the respiratory tract can cause respiratory tract injury thus contributing to the pathophysiology of inflammatory airway diseases, as NO can react with superoxide anion released from inflammatory cells, yielding the potent oxidant peroxynitrite. Peroxynitrite adds a nitro group to the 3-position adjacent to the hydroxyl group of tyrosine to produce the stable product nitrotyrosine. NO also reacts directly with O₂ to form nitrite, the oxidation of which, by neutrophil-derived MPO or by other related peroxidases, yields nitryl chloride and nitrogen dioxide. Although nitration of tyrosine is generally attributed to peroxynitrite, the peroxidase-dependent nitrite oxidation pathway is also involved.

**Induced sputum studies.** Nitrotyrosine is considered an indicator of the production of RNS: in COPD patients, the numbers of nitrotyrosine-immunopositive cell have been shown to be related to the number of iNOS-positive cells and to airflow reduction (FEV₁). 132 Nitrotyrosine-tagged cells have been measured in induced sputum studies showing markedly positive reactions for iNOS and nitrotyrosine in smokers as compared to non-smokers. In smokers, the total number of iNOS-positive cells was higher than in non-smokers ($p = 0.004$, respectively). In addition, there was a statistically significant difference between never smokers and ex-smokers ($p = 0.04$), while there was no significant difference between asymptomatic smokers and stage 0 COPD subjects. Similarly, smokers showed a higher number of nitrotyrosine-positive cells than non-smokers ($p = 0.003$), while there was no difference between healthy smokers and stage 0 COPD subjects or between never smokers and ex-smokers. The total number of iNOS-positive cells was negatively correlated with airflow measures [MEF-50 ($r = -0.567$, $p = 0.006$)].

The available data suggest that oxidative stress imposed by RNS may be exaggerated in the airways of current smokers and in COPD patients. Further, the counts of nitrotyrosine-positive cells were significantly correlated with the airway obstructive changes in COPD. Hyper production of RNS may be an important factor in the pathogenesis of COPD. NO produced in the airways, presumably via iNOS, is consumed by its reaction with superoxide anion and/or peroxidase-dependent mechanisms. However, no major differences could be observed between asymptomatic smokers and subjects with GOLD stage 0 COPD.

**Proteases and antiproteases**

**Alpha-1-antitrypsin**

Alpha-1-antitrypsin ($\alpha$-1-AT) is a serine protease and trypsin inhibitor protecting the lung parenchyma from the action proteases, chiefly of neutrophil elastase. $\alpha$-1-AT concentration in the blood varies from 150 to 350 mg/dL. Emphysema is associated with inherited $\alpha$-1-AT deficiency and inactivation of $\alpha$-1-AT has been postulated as a risk factor for developing emphysema among smokers with normal levels of $\alpha$-1-AT. 133

**Induced sputum studies.** In the study of Vignola and Coworkers, $\alpha$-1-AT levels were found to be elevated in chronic inflammatory disorders of the lung such as COPD and asthma as compared to normal controls, although no difference was found between the $\alpha$-1-AT levels of asymptomatic smokers and those of non-smoking controls. 134 The results of this study are at odds with those obtained in BAL and serum studies where $\alpha$-1-AT levels were found to be increased in active smokers. In this regard, Olsen et al. have shown that the concentration of $\alpha$-1-AT was increased in the BAL fluid of smokers compared to non-smokers (0.306 ± 0.057 vs. 0.184 ± 0.025 mg/100 cc, $p = 0.06$).
suggesting that increased α-1-AT levels in the LRT of cigarette smoker might be protective against proteolysis. The fundamental study of Ogushi and Colleagues on BAL fluid and plasma showed that while smoker plasma α-1-AT had similar activity as that of non-smokers, α-1-AT from the LRT of smokers had significantly lower activity than that of non-smokers. The α-1-AT from the BAL of smokers was significantly less active than that of autologous plasma, suggesting that cigarette smoking may be associated with a decrease in the LRT neutrophil elastase (NE) inhibitory capacity, thus increasing the vulnerability of the lung to elastolytic destruction and the risk of developing emphysema.

Thus, the data regarding the levels of α-1-AT in the alveolar spaces, in the serum and the bronchial mucosal surface raise questions on the effect of tobacco smoke exposure upon this key protective enzyme, although the discrepancy between induced sputum and BAL data may be related to the different compartments of the respiratory tract sampled by different procedures.

**Alpha-1-antichymotrypsin**

Alpha-1-antichymotrypsin (α-1-AC) is a serine protease inhibitor of chymotrypsin-like proteinases, including pancreatic chymotrypsin, neutrophil cathepsin G, mast cell chymase that is synthesized by hepatocytes and alveolar macrophages.

In a genetic study among the Swedish population, α-1-AC deficiency was found to be transmitted with an autosomal dominant inheritance pattern. Interestingly, gene mutations have been associated with increased prevalence of COPD.

No induced sputum studies have been yet published on α-1-AC levels in tobacco smokers.

**Secretory leukocyte proteinase inhibitor**

Secretory leukoprotease inhibitor (SLPI) is a serine protease inhibitor produced in the lung by tracheal, bronchial, bronchiolar epithelial cells, type II pneumocytes cells as well as by monocytes, alveolar macrophages and neutrophils. In addition to its antiprotease action, SLPI has antibacterial properties against Gram-positive and Gram-negative bacteria and is up-regulated by “alarm signals” such as bacterial lipopolysaccharides and cytokines.

Susceptibility of SLPI to cigarette smoke was demonstrated by Cavarra and Colleagues who have shown in a mouse model that cigarette smoke exposure reduces by 50% SLPI inhibitory activity. In a BAL study on normal non-smoking volunteers, Vogelmeier and Coworkers showed that SLPI is present in the lung epithelial fluid, at levels similar to those of α1-AT, although more than 50% of the antiprotease recovered by BAL is inactive.

**Induced sputum studies.** Culpitt and Coworkers evaluated the concentration of SLPI in the induced sputum of a group of COPD patients. These authors showed that this enzyme could be measured in induced sputum.

No data are yet available on induced sputum SLPI levels in healthy smokers or non-smoking controls.

**Alpha-2-macroglobulin**

Alpha-2-Macroglobulin (α-2-M) is a large plasma protein produced by the liver that is comprised of four identical subunits bound together by 5–5 bonds, capable of inactivating a variety of proteinases, including serine-, cysteine-, aspartic and metalloproteinases. The α-2-M structure includes a 35 amino acid “bait” region which is cleaved by proteinases making them susceptible of being captured by α-2-M so that the proteinase-α-2-M complex is recognized by macrophage receptors and cleared.

Modulation of α-2-M expression in response to tobacco smoke exposure was suggested by Tappia and Coworkers who showed that plasma concentrations of α-2-M are increased in smokers, compared to non-smokers.

**Induced sputum studies.** Measurable levels of α-2-M have been detected in I.S. samples of healthy non-smoking subjects (2.72 ± 1.01 μg/ml) by Halldorsdottir and Coworkers, and the quantification of the levels of α-2-M in I.S. has been proposed as a marker of bronchial exudation in asthma research.

No data on the levels of α-2-M in induced sputum in smokers are yet available.

**Matrix metalloproteinases and tissue inhibitor of metalloproteinases**

Metalloproteinases (MMPs) are a family of structurally related enzymes capable of degrading all components of the ECM; more than twenty members of the MMP family have been identified so far. The enzymes of the MMP family share 32–49% amino acid similarity and have similar structural domains. MMP-9, that is predominantly produced by macrophages and is also found in neutrophil granules, is the major elastolytic MMP. Lung macrophages and epithelial cells produce tissue inhibitors of metalloproteinases (TIMPs) which selectively inhibit MMPs. MMPs and TIMPs, which are thought to play key roles in tissue remodelling of the airways in COPD and asthma patients, been shown to be induced by oxidants, including peroxynitrite and hydrogen peroxide, bacterial products such as LPS, by cytokines including IL-1β, PDGF and TNF-α.

**Induced sputum studies.** With regard to the MMPs capable of degrading elastase i.e., MMP-2, MMP-7, MMP-9 and MMP-12, a number of studies have looked at their expression and concentrations in cells and supernatants obtained by sputum induction (Table 4).

Aviles and Colleagues evaluated normal non-smoking controls, healthy smokers and COPD patients for MMP expression in induced sputum. Healthy smokers were found to have a higher concentration of total MMP-9 (273 ± 277 ng/ml vs. 128 ± 146 ng/ml) than non-smoking controls. Patients with COPD had the highest concentrations of total (477 ± 262 ng/ml) and active MMP-9 (178 ± 126 ng/ml), compared to both healthy smokers and non-smoking controls (p < 0.05). In the same study, healthy smokers were found to have a higher ratio of total MMP-9 to TIMP-1 (0.16 ± 0.14 vs. 0.08 ± 0.06) than non-smoking controls; patients with COPD had the lowest concentrations of TIMP-1 (1.044 ± 1.036 μg/ml). When all groups were considered together, there was an inverse relationship between the MMP-9/TIMP-1 ratio and airflow measures (FEV1); when the COPD group in isolation was considered, the correlation was stronger (r = −0.56; p = 0.002), and an even stronger correlation was observed when the extent of smoking history was taken into account (r = −0.81; p < 0.0001).
Similarly, in a recent study by Ilumet and Coworkers, MMP-9 levels were significantly elevated both in asymptomatic smokers compared to non-smokers ($p = 0.01$) and in Stage 0 COPD as well ($p < 0.001$); there was no difference however between healthy smokers and smokers with COPD. A study by Vernooy and Coworkers confirmed the expression of MMP-9 activity in the induced sputum of healthy smokers and mild-to-moderate COPD patients: they found that mean total MMP-9 activity was significantly higher in the COPD group when compared to that of the healthy smokers group ($p < 0.05$) and that active MMP-9 was detectable in $76\%$ of COPD patients compared to $41\%$ of healthy smokers ($p < 0.05$), whereas MMP-2 activity was below the detection threshold.

Elevated MMP-9 activity was found in healthy smokers with subclinical emphysema. Per mg albumin of BAL fluid.

### Table 4: Proteinases in induced sputum of tobacco smokers with COPD, healthy tobacco smokers and healthy non-smokers.

<table>
<thead>
<tr>
<th>Proteinase</th>
<th>Healthy non-smokers</th>
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<th>COPD non-smokers</th>
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<td>MMP-9</td>
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<td>41%(^a)</td>
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<td>MMP-9</td>
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</table>

\(n/a\): not available.
\(^a\): % of subjects with MMP activity.
\(^b\): Former smokers.
\(^c\): Per mg albumin of BAL fluid.
\(^d\): Smokers with subclinical emphysema.
\(^e\): \(p < 0.05\) in comparison with other groups.

The elevation of the expression of MMP-9 in COPD was confirmed in a study by Vignola and Coworkers, where the analysis of the levels of MMP-9 and TIMP-1 in the induced sputum of patients with chronic bronchitis and healthy non-smokers showed that MMP-9 and TIMP-1 levels were significantly increased in COPD (80.5 vs. 23 ng/ml, \(p < 0.008\), and 475 vs. 60 ng/ml, \(p < 0.0003\)). However, this study did not evaluate healthy smokers.

Demedts and Colleagues evaluated MMP-12 in induced sputum from non-smoking controls, healthy smokers and COPD patients. Non-smokers (4.2 ng/ml), healthy smokers (6.7 ng/ml) and former smokers (6.1 ng/ml) had significantly lower levels of MMP-12 than patients with COPD (17.5 ng/ml), although there were no significant differences in MMP-12 levels among the three control groups. Interestingly, MMP-12 levels in induced sputum from COPD patients who were actively smoking (16.1 ng/ml) did not differ significantly from those in COPD patients who had quit smoking (18.2 ng/ml, \(p = 0.87\)). Furthermore, MMP-12 specific activity (detected by cleavage of a fluorescence quenched substrate) was significantly higher in COPD patients than in controls (4.11 vs. 0.14 µg/µl, \(p = 0.0002\)). As in other studies, an inverse correlation was found between MMP-12 protein levels in induced sputum and airflow measures.

MMP-12-expressing cells in induced sputum were quantified by immunocytochemical staining by Babusyte and Colleagues, who showed that healthy smokers had higher percentages of MMP-12 positive cells than healthy never smokers. Further, COPD smokers had a higher number of MMP-12-expressing cells than COPD ex-smokers, although the difference was not statistically significant. The numbers of MMP-12 positive macrophages correlated with the numbers of pack-years in COPD smokers ($r = 0.54$, \(p < 0.03\)), COPD ex-smokers ($r = 0.64$, \(p < 0.05\)) and healthy smokers ($r = 0.78$, \(p < 0.05\)).

Culpitt and Colleagues were able to confirm that the levels of MMP-9 and TIMP-1 were significantly higher in normal smokers compared to non-smoking controls. They also found that the levels of MMP-1, MMP-8, in addition to MMP-9 and TIMP-1, were significantly higher in the induced sputum of COPD patients than in healthy smokers or non-smoking controls.

TIMP-1 was also measured in the studies of Boschetto and Coworkers, who showed no significant differences in the concentration of TIMP-1 in induced sputum of COPD patients with and without emphysema (2.3 vs. 7.5 ng/ml) in comparison to non-smoking controls, and of Vignola and Colleagues who detected TIMP-1 in induced sputum COPD patients.

With regard to the MMPs capable of degrading triple helix native collagen i.e. MMP-1, MMP-8 and MMP-13, the study by Vernooy and Coworkers showed that active MMP-1 could be found in a small proportion of COPD patients and healthy smokers while MMP-8 was the dominant MMP collagenase in both COPD patients and healthy smokers. The mean total MMP-8 activity was significantly higher in the COPD group ($p < 0.05$), and active MMP-8 was found in 35% of the COPD patients compared to 6% of the healthy smoker controls ($p < 0.05$), whereas MMP-2 and MMP-13 activities were below the detection threshold. When subjects were classified according to their current smoking behavior, and MMP activity was compared, no differences in the activity were observed between individuals who were current smokers and those who were ex-smokers in both groups (COPD patients and healthy subjects). A significant correlation between both active and total MMP-8 and MMP-9 activity with the absolute number of neutrophils was observed in the sputum of COPD patients ($r = 0.668$, \(p = 0.003\) and $r = 0.655$, \(p = 0.004\), respectively).

In Ilumet’s study, only MMP-8 induced sputum concentrations differentiated GOLD Stage 0 COPD patients from asymptomatic smokers ($p = 0.02$). Interestingly, MMP-8 levels correlated significantly with alterations in small...
airway flow parameters (MEF-50, MEF-25) (p = 0.005 and p = 0.0004), which are considered a good measure of bronchial obstruction in COPD, and with markers of neutrophil activation such as MPO and lactoferrin.149

Thus, MMP-9, MMP-12 and MMP-8 appear to be expressed at higher levels in COPD patients compared to healthy smokers. While MMP-9 is over-expressed in healthy smokers, MMP-8 may differentiate GOLD Stage 0 COPD, i.e., subclinical chronic obstructive bronchitis from healthy smokers.

Induced sputum data were confirmed by a BAL study by Russell and Coworkers who examined the production and activity of MMP-9 from in vitro stimulated macrophages in smokers with COPD, healthy smokers, and non-smokers, found that alveolar macrophages from COPD patients had a significantly greater level of MMP-9 activity than healthy smokers and non-smokers, with macrophages from healthy smokers releasing higher levels of MMP-9 than macrophages from non-smokers.157 Similar data were also obtained for MMP-12 by Babusyte and Colleagues who confirmed the results in the two different tissue compartment: sputum induced and BAL.153

Neutrophil elastase (NE)

Human NE is a 29 kDa serine protease stored in azurophilic granules of mature neutrophils that’s capable of intracellular degradation of proteins during phagocytosis and extracellular degradation of connective tissue during an inflammatory process. Being able of destroying cross-linked elastin and the major forms of collagen, NE is required both for normal tissue turnover and for host defense, but it is also potentially harmful for its ability to destroy normal tissues. As such, it plays a central role in the pathogenesis of pulmonary emphysema by destroying the alveolar once anti-proteases such as α1-AT are inactivated.

Tobacco smoke has both acute and chronic effects on NE expression. Weitz and Colleagues evaluated the levels of the NE-derived fibrinogen peptide A-α1-21 in healthy volunteers to find fivefold higher levels in smokers compared to non-smokers (2.0 nmol/L vs. 0.4 nmol/L; p < 0.0001). Smoking three cigarettes after 12 h of abstinence induced a marked raise in fibrinopeptide levels (1.8 nmol/L vs. 0.4 nmol/L; p < 0.0001).158 Similarly, Hind and Coworkers evaluated NE concentrations in current smokers, ex-smokers and never smokers and showed that smokers had significantly higher NE concentrations in plasma than ex-smokers or never smokers. Interestingly, higher NE levels were associated with lower FEV1 and a higher annual decline in FEV1 in the preceding 10 years.159

Induced sputum studies. The NE activity in induced sputum was measured by Van Overveld and Coworkers in a population including current and former smokers with COPD. Although asymptomatic smokers and non-smokers were not evaluated in this study, this report indicated that NE was markedly elevated in current smokers versus former smokers with COPD (36.4 ± 12.0 vs. 346.2 ± 72.1 nmol/l).155 (Table 4).

In addition, Vignola and Coworkers found that in healthy smokers the level of total and active elastase were significantly higher than in controls subjects (p < 0.03, p < 0.002, respectively), but significantly lower than in chronic bronchitis patients (p < 0.006, p < 0.002, respectively).134 The data seem to confirm those reported in a study performed on BAL fluids by Yoshioka et al., who found that NE-α1-protease inhibitor complex concentrations were significantly increased in smokers with subclinical emphysema (0.52 ± 0.10 µg/mg albumin) compared to ex-smokers (0.21 ± 0.03 µg/mg albumin, p < 0.01).160

Leukocyte pro-inflammatory and antibacterial products

Leukotriene B4

LTB4, a dihydroxy fatty acid derived from arachidonic acid through the 5-LO pathway, is released by neutrophils and macrophages and plays a regulatory role in the immune response to antigenic stimulation; it is a potent chemotactic and chemokinetic agent for human polymorphonuclear leukocytes and a margination factor for monocytes and macrophages. In addition, LTB4 stimulates cytotoxic T cell, natural killer cell and suppressor T cell activity.

With regard to the source of airflow LTB4, Laviolette and Coworkers evaluated lung alveolar macrophages obtained from smokers and showed that they released 80 to 90% less LTB4 in vitro, as compared to non-smoker macrophages. In contrast, blood polymorphonuclear leukocytes from smokers and non-smokers showed no difference in the synthesis of 5-lipoxygenase products,161 a finding confirmed by Tardiff et al.162

Induced sputum studies. The levels of LTB4 in the airways were evaluated, by Kostikas and Colleagues, in a population of COPD patients and healthy smokers. This study showed that COPD patients had significantly higher levels of LTB4 (2.64 ± 0.6 pg/mL) in comparison with healthy smokers (1.45 ± 0.4 pg/mL, p < 0.0001).163 LTB4 values were higher in COPD patients with reversible airway obstruction (3.07 ± 0.3 pg/mL) compared to patients without airflow reversibility (2.24 ± 0.5 pg/mL, p = 0.002). Values of LTB4 in the sputum supernatant of patients with reversible and non-reversible COPD presented a significant positive correlation with sputum neutrophil levels (r = 0.71, p = 0.001). The study might suggest that 5-lipoxygenase products are released at higher levels in smokers. However, non-smoking controls were not evaluated in the study.

In this regard, the EBM study of Carpanagno and Colleagues, evaluating the levels of LTB4 in healthy smokers and non-smokers, showed increased LTB4 levels (9.4 ± 0.4 pg/mL) in smokers compared to non-smokers (6.1 ± 0.3 pg/mL, p < 0.001).164 Further studies may confirm the role of tobacco smoke in augmenting leukotriene levels in the airways of smokers and COPD patients.

Human neutrophil lipocalin

HNL, also called neutrophil gelatinase-associated lipocalin (NGAL), is a specific constituent of neutrophil granulocyte granules.165 HNL is secreted more readily from the neutrophils than any other neutrophil specific marker and increased concentrations of HNL have been seen in sera of patients with acute bacterial infections.166,167 HNL can be used as a neutrophil marker in biological samples.119

Induced sputum studies. A study from Keatings and Colleagues showed that HNL was markedly elevated in the induced sputum from patients with COPD compared to non-
smokers.\textsuperscript{117} However, this study did not include an asymptomatic smoker control group.

In this regard, a BAL study from Ekberg-Jansson and Coworkers showed that concentrations of HNL were higher in healthy smokers (with normal FEV₁) than in non-smokers (85 vs. 54 \(\mu g/l\), \(p = 0.05\)). HNL levels in BAL were positively correlated with BAL neutrophils (\(r = 0.62, p < 0.0001\)) and with emphysematous lesions seen on HRCT scan (\(r = 0.71, p = 0.01\))\textsuperscript{77} (Table 5). Together, the data may indicate HNL as a candidate marker of neutrophil-mediated lung damage in cigarette smokers.

### Human cationic antimicrobial protein-18 (hCAP-18)

The hCAP-18 is an antimicrobial member of the cathelicidin family produced by respiratory epithelia and by polymorphonuclear leukocytes.\textsuperscript{168}

**Induced sputum studies.** Xiao and Coworkers showed that COPD patients had significantly higher levels (75.3 \(\pm 38.9\) ng/ml) of sputum hCAP-18 compared to control non-smoking subjects (39.9 \(\pm 24.2\) ng/ml, \(p < 0.009\)). hCAP-18 heavily stained the cytoplasmic granules of sputum neutrophils, suggesting that a major source of sputum hCAP-18 was represented by infiltrating neutrophils. Importantly, hCAP-18 expression showed a strong correlation to the decrease of airflow, as measured by FEV₁ (\% pred).\textsuperscript{166} Although these data may suggest a role for hCAP as a smoke-related inflammation marker, the lack of an appropriate asymptomatic smoker control group in the only study published so far, calls for further confirmatory studies.

### Eosinophil cationic protein

The ECP is usually associated with the eosinophil granulocyte. ECP is a member of the RNase A superfamily of proteins involved in inflammatory processes mediated by eosinophils. ECP is bactericidal, helminthotoxic and cytotoxic to tracheal epithelium cells as well as to several mammalian cell lines.\textsuperscript{169}

**Induced sputum studies.** Rytila and Coworkers showed that ECP levels in induced sputum of smokers are higher, although not in a statistically significant fashion, than those in non-smokers (109 \(\pm 245\) vs. 26 \(\pm 20\) \(\mu g/ml\), \(p = 0.266\)).\textsuperscript{113} This study was largely confirmed by Balzano and Coworkers, who found higher ECP levels in induced sputum from patients with COPD (711.5 \(\pm 1,179.9\) \(\mu g/l\)) than asymptomatic smokers (138.8 \(\pm 176.7\) \(\mu g/l\)) and non-smoking controls (81.7 \(\pm 72.3\) \(\mu g/l\)) (\(p < 0.01\)). In this study, the difference between healthy smokers and non-smokers was not statistically significant however, although statistical significance (\(p < 0.05\)) was reached for the comparison between COPD patients and smoking controls. Significant correlations were found between FEV₁/FVC ratio and sputum ECP levels (\(r = -0.56, p = 0.0126\)) and between neutrophil sputum count and ECP concentration (\(r = 0.54, p = 0.0152\)).\textsuperscript{170} The COPD data were confirmed by a study, evaluating ECP concentrations in BAL samples in smokers with COPD, which showed that ECP levels were higher in smokers with COPD than in non-smoking controls.\textsuperscript{16} In support to the susceptibility of ECP expression to tobacco smoke exposure, a study of ECP serum levels by Jensen and Coworkers showed that ECP concentrations in blood were more elevated in smokers compared to non-smoker controls. Strikingly, ECP was linearly associated with daily cigarette consumption and FEV₁. After smoking cessation, ECP levels decreased within 6 months.\textsuperscript{171} Together, the data indicate ECP as a marker of inflammation associated with cigarette smoke, although induced sputum data fail to differentiate healthy smokers from non-smokers and from smokers with obstructive disease (Table 5).

### Toll-like receptor 2 (TLR2)

Pathogen recognition receptors (PRR) are important receptors expressed on the macrophage’s cell surface mediating the interaction between pathogen-associated molecular patterns (PAMPs) and the cell. Toll-like receptor 4 (TLR4), together with CD14 and the MD2 adapter molecule, serves as receptor for components from Gram-negative bacteria such as LPS. TLR2 predominantly recognizes components from Gram-positive bacteria such as lipoteichoic acid (LTA) and peptidoglycan (PGN).\textsuperscript{172}

A BAL study by Droemann and Coworkers on macrophage mRNA and protein expression of TLR2 of COPD patients, healthy smokers and non-smoking controls showed that the expression of TLR2 in COPD patients and smokers was reduced in comparison with non-smokers. Furthermore, exposure to LPS led to an increased protein and mRNA expression in non-smokers but not in smoker and COPD patients macrophages.\textsuperscript{172} No studies on the TLR2 expression in induced sputum from smokers are available.

### Protease activated receptor 2 (PAR2)

Protease activated receptors (PARs) are members of the seven transmembrane G-protein-coupled receptor superfamily. There are 4 known PARs numbered from one to four, and they are expressed on platelets, endothelial cells, myocytes and neurons. PARs play an important role in most components of injury responses, including cell proliferation, migration, matrix remodelling, and inflammation. PAR2 is preferentially activated by trypsin and tryptase.\textsuperscript{173}

<table>
<thead>
<tr>
<th>Table 5</th>
<th>Leukocyte products in induced sputum of tobacco smokers with COPD, healthy tobacco smokers and healthy non-smokers.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy non-smokers</td>
<td>Healthy smokers</td>
</tr>
<tr>
<td>HNL</td>
<td>54 (\mu g/l)</td>
</tr>
<tr>
<td>ECP</td>
<td>26 (\pm 20) (\mu g/ml)</td>
</tr>
<tr>
<td>ECP</td>
<td>81.7 (\pm 72.3) (\mu g/l)</td>
</tr>
</tbody>
</table>

\(^{a}\) \(\mu g/mL\) of BAL fluid.

\(^{b}\) \(p = 0.05\).
Studies on bronchial biopsies and BAL cells seem to suggest that the expression of PAR genes might be variably modulated by exposure to tobacco smoke as well as by inflammation. A study by Miotti and Mapp on tissue biopsies of the central airways of smokers and non-smokers showed that PAR2 was expressed in bronchial smooth muscle, epithelium, glands, and in the endothelium and smooth muscle of bronchial vessels. PAR2 expression was similar in the central airways of smokers and non-smokers, but, among smokers expression was increased in smokers with symptoms of chronic bronchitis with normal lung function compared to smokers with both symptoms and airway obstruction.174

In a BAL study by Roche and Colleagues on cultured alveolar macrophages, PAR1 mRNA expression was lower in smokers compared to non-smokers while PAR2 mRNA expression was higher in smokers. In contrast, no difference was observed when cultured macrophages were evaluated for protein expression.175 No studies on PAR1 or PAR2 expression in the LRT of smokers using induced sputum have been published so far.

Discussion and prospectives

Induced sputum is a non-invasive, standardized sampling procedure, amenable to diagnosis and monitoring of patients with inflammatory airways diseases by allowing access to specific cell types and cell products from the airways and has been widely used in asthma and COPD biomarker research.179 Biomarkers can be defined as molecules which indicate, or are associated with, an alteration in physiology and may be of clinical utility in assessing disease activity:179: in this context, the paramount importance of determining the role to active tobacco smoke in modulating the expression of molecules putatively amenable to monitoring disease activity and progression in COPD patients, a disorders strongly associated with tobacco smoke is apparent. This review summarizes the data obtained from 16 studies assessing the expression of 19 induced sputum biomarkers in populations of healthy and COPD affected tobacco smokers and in healthy non-smokers and the results of 16 studies on the expression of the same biomarkers in tissue biopsies, bronchoalveolar lavage and exhaled breath condensate as comparators, as well as the data and on 20 additional potential biomarkers relevant to COPD inflammation and tobacco smoke exposure.

For the purpose of this analysis, the respiratory biomarkers examined could be classified into four groups, in relation to their association with exposure to tobacco smoke and/or with COPD (Table 6):

<table>
<thead>
<tr>
<th>Group</th>
<th>Suggested biomarkers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>GM-CSF, MPP9, LTB4, 8-isoprostane</td>
</tr>
<tr>
<td>Group 2</td>
<td>GROα, MCP-1, IL-8, NHL, hCAP18, ECP, α1-AT</td>
</tr>
<tr>
<td>Group 3</td>
<td>CXCL9, CXCL10, CXCL11, CCL5, NE</td>
</tr>
<tr>
<td>Group 4</td>
<td>SOD, leptin, TGF-βd</td>
</tr>
<tr>
<td>Group 5</td>
<td>TNF-α, VEGF, MPO, EPO</td>
</tr>
</tbody>
</table>

Table 6 Summary classification of lung damage biomarkers associated with tobacco smoke in induced sputum.

- Group 1: biomarkers of response to tobacco smoke exposure, associated with COPD activity, which are expressed at higher levels in healthy smokers than in non-smokers and at higher levels in COPD than in healthy smokers
- Group 2: biomarkers of COPD inflammation whose expression level is not associated with tobacco smoke exposure, as it is higher in COPD patients than in controls, with no significant differences between healthy smokers and non-smokers
- Group 3: biomarkers of response to tobacco smoke exposure, not associated with COPD activity
- Group 4: biomarkers negatively associated with COPD and/or tobacco smoke exposure
- Group 5: molecules variably associated with COPD and/or tobacco smoke

To group 1 can be assigned the cytokine GM-CSF, the elastolytic protease MPP-9 and the collagenolytic protease MMP-8. The leukocyte chemo-attractant LTB4 was also found to overexpressed in COPD compared to smokers, with BAL studies showing that smokers express higher LTB4 levels than non-smokers. Similarly, the oxidative stress marker 8-isoprostane was overexpressed in smokers with moderate-severe COPD compared to healthy smokers, which in turn showed higher expression than non-smokers. Similarly, nitro-peroxidative activity that has been shown to be higher in COPD than in normal, as nitrotyrosine immunoreactive cells have been shown in higher percentages in COPD induced sputum than in normal non-smokers and also in smokers compared to non-smokers, although no study directly compared COPD and non-COPD affected smokers.

To group 2 can be assigned the chemokines GRO-α, MCP-1 and IL-8 and the leukocyte markers NHL, hCAP-18 and ECP and the antiprotease, the anti-protease α1-AT, in one induced sputum study. However, contrary to induced sputum studies, BAL studies showed that IL-8 levels were smoke exposure-related. Similarly, a BAL study showed that α1-AT levels are higher in smokers compared to non-smokers, albeit in the presence of reduced activity.

To group 3 can be assigned the chemokines CXCL9, CXCL10, CXCL11, CCL5 and the protease NE. However, no NE study is available comparing COPD to normal smokers.
To group 4 could be assigned the antioxidant factor SOD, for it decline in the BAL of smokers, leptin and TGF-β, although no induced sputum studies are available, to our knowledge, for these markers.

Finally, there were molecules variably associated with COPD and/or tobacco smoke (group 5). Among growth factors, TNF-α and VEGF have been evaluated with contrasting results, as both up-regulation and down-regulation of their expression were shown in induced sputum and/or BAL studies. Among oxidative stress related molecules MPO was associated with smoking history, but no data were available to analyze the association with COPD and EPO was associated with COPD, but no sufficient data were available to assess an association with smoking.

A number of potential COPD biomarkers have not been examined in induced sputum studies yet. The cytokine IL-1, although modulated by tobacco smoke exposure in in vitro studies has not been shown to be consistently associated either with smoke or with COPD. The cytokines IL-4 and IL-13 have been associated with tobacco smoking in BAL and blood studies, the macrophage markers PAR2, TLR2, the antiprotease factors α-1 AC, SLPI, α-2-M, associated with susceptibility to emphysema in genetic studies, the oxidative detoxification and the xenobiotic detoxification related enzymes of the GST and the Cytochrome P450, the cytokine-like molecule leptin, the HGF, and TGF-β, the cytokine the have not been tested, to our knowledge, in induced sputum studies.

Not unexpectedly, given the differences in the compartment of the airways and the lung parenchyma explored by sampling procedures such as induced sputum and BAL or tissue biopsies, a number of inconsistencies have emerged which make the characterization of respiratory biomarkers difficult. In addition, most studies evaluated small populations thus being unable to reveal potential dose response effects of tobacco smoke exposure upon marker expression. In this regard, Vestbo and coworkers have recently published the design of a large COPD biomarker study including about 3000 subjects including COPD patients and both smoking and non-smoking controls.180

The data summarized in this review suggest that, with the above caveats, the studies here reviewed suggest that group 1 and group 2 respiratory biomarkers might be used as indicators of COPD disease in populations of persons at risk. Interestingly, group 4 biomarkers might instead be used to predict disease susceptibility. Studies are needed to confirm the ability of the biomarkers categorized above to predict disease severity and response to treatment in COPD patients, it is though foreseeable that characterization of COPD markers for their association with tobacco smoke exposure might be of help in better determine disease phenotypes.

Finally, it is worth mentioning that the studies described here have all been carried out following the traditional approach of laboratory or animal model biomarker studies i.e., they all focussed on a single gene or protein or on a few genes/proteins in combination, identified based on prior knowledge or suspected mechanisms of emphysema/chronic bronchitis/COPD pathogenesis. As elegantly pointed out in a recent review of gene association studies with emphysema risk, newer genetics techniques (e.g., genome-wide association studies) allow for the comprehensive evaluation of the entire genome without prior assumptions regarding disease biology.176 As genome-wide studies may lead to the identification of novel disease candidate genes, to be henceforth subjected to further in vitro and/or in vivo experimentation, new “omic” techniques such as transcriptomics and proteomics can afford the investigator with powerful research tools with which to explore gene expression alterations in the lung exposed to tobacco smoke.

In this regard, recent gene and protein expression studies have been carried out using transcriptomics and proteomics on BAL macrophages and “brushed” bronchial epithelial cells, although none on induced sputum samples.177,178 By using of gene chips and proteomic techniques, these studies have led to the identification of several hundreds of genes/proteins which were modulated by exposure of tobacco smoke. Interesting, the larger group of genes and proteins was represented by oxidative stress-related genes and proteins, a number were related to apoptosis or cell proliferation and to inflammation/repair. It is likely that the genome/proteome/metabolome-wide approaches, coupled with rigorous selection of populations of homogeneously exposed subjects will greatly improve upon our ability to identify expression markers indicative of tobacco smoke-associated respiratory disease risk.

Conflicts of interest

None of the authors have a conflict of interest to declare in relation to this work.

Acknowledgments

C. Saltini is the recipient of a research grant from Research and Drug Development srl. Rome, Italy. A. Comandini is a post-graduate fellow of the Doctorate in Thoracic Sciences, University of Bologna, Italy.

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