Biomarkers Obtained by Non-Invasive Methods in Patients with COPD: Where do we Stand, what do we Expect?

Georgios Hillas¹, Stelios Loukides*,¹ Konstantinos Kostikas² and Petros Bakakos¹

¹Respiratory Medicine Department, University of Athens Medical School, Greece
²Respiratory Medicine Department, University of Thessaly Medical School, Greece

Abstract: Recently, there has been widespread interest in the use of non-invasive methods for the assessment of airway inflammation in a variety of lung diseases including chronic obstructive pulmonary disease (COPD).

Sputum induction is a semi-invasive technique the value of which is not restricted to sputum cell counts, as inflammatory mediators can also be measured in the supernatants. However, none of the measurable biomarkers in induced sputum is considered applicable in clinical practice. Despite the predominating sputum neutrophilia, there is increasing evidence that the presence of sputum eosinophilia predicts an objective response to steroid treatment in patients with COPD.

The commonly used Exhaled Breath Condensate (EBC) methodologies in COPD patients have considerable variability due to technical issues concerning both sample collection and analysis. Despite the above limitations, biomarkers mainly related to neutrophil derived products and oxidative stress, have been assessed for disease monitoring and response to pharmacological treatment. Endogenous airway acidification, as assessed by EBC pH, represents a measurable marker associated with oxidative stress and sputum neutrophilia.

The fraction of exhaled nitric oxide (FeNO) is the most extensively studied exhaled biomarker and increased levels of FeNO have been widely documented in patients with asthma. FeNO measurement in COPD is of limited value due to smoking effect. However, increased values of FeNO have been found in COPD patients with sputum eosinophilia. Moreover, measuring FeNO in different exhalation rates may reestablish its value in COPD.

Despite the limited use of non-invasive methods, the future direction is a challenge towards new biomarkers or a combination of them that will assist us to move from the research laboratory to daily clinical practice.

Keywords: COPD, exhaled breath condensate, sputum induction, fraction of exhaled nitric oxide, exhaled breath, biomarkers.

INTRODUCTION

A biomarker has been defined as a molecule or material (cell, tissue) that is objectively measured and evaluated as an indicator of a normal biological process, a pathogenic process or a pharmacologic response to a therapeutic intervention [1]. Although it might refer to different parameters, such as measurements of lung function [2] traditionally it is considered to refer to biological fluids or exhaled breath. An ideal biomarker is considered to show reproducibility, to be derived from a standardized procedure, to demonstrate disease specificity and last but not least to have the ability to detect changes attributed either to therapeutic interventions or changes in health status (such as exacerbations).

The need for non-invasive assessment of airway inflammation is imperative, since inflammatory airway diseases, such as chronic obstructive pulmonary disease (COPD), are characterized by variation in their clinical presentation throughout their course. The current management of COPD patients is mainly based upon clinical assessment and pulmonary function tests [3]. Moreover, there is an increasing trend in assessing the inflammatory pattern of COPD through mediators measured by non invasive techniques. Markers in biological fluids and exhaled air have been the object of intense evaluation over the past few years, with some of them reaching their introduction in clinical practice while others still remaining research tools [4].

Sputum induction is a semi-invasive technique that assisted clinical researchers to elucidate the inflammatory process of many airway diseases including COPD. Sputum sampling reflects the biofluid in the central rather than the peripheral airways [5]. Its main advantages are related to an adequate and established methodology of collection, processing and analysis. The value of sputum induction is not restricted to sputum inflammatory cells since inflammatory mediators can also be measured in the supernatants.

Exhaled volatile mediators, such as Fraction of exhaled Nitric oxide (FeNO), carbon monoxide (CO) and hydrocarbons, have also been studied in COPD [6]. FeNO is the most extensively studied marker, with limited valuable data, since it is strongly affected by smoking [7]. However, the measurement of FeNO at different flows and particularly its peripheral part mainly derived from alveoli and small airways, might offer useful information for the disease [8].

Exhaled breath condensate (EBC) is collected by cooling or freezing exhaled air. The large number of measurable molecules, the diversity of the used methodologies, as far as sample collection and processing are concerned, in addition to the lack of studies focusing on normal populations are some of the points that hamper its wide clinical use [9,10]. Despite the above limitations, it is a totally non-invasive technique that reflects biochemical changes of the airway lining fluid.

This review focuses mainly on the presentation and evaluation of non-invasive techniques in COPD. Detailed information regarding mediators, methods for collection and analysis and potential value in clinical practice will be provided.
METHODOLOGY

A Medline search was conducted using – but not limited to – the following keywords: "sputum induction", "exhaled breath condensate", "FeNO", "markers in exhaled air", "exhaled biomarkers", "assessment of airway inflammation". Manual searches were also performed and all articles retrieved from references of other articles were additionally examined. The methodologies for collection, processing and analysis were retrieved from all the selected studies.

INDUCED SPUTUM

Sputum induction is a well-tolerated, safe procedure even in those with severe disease and during exacerbations. Details of the safety precautions and methods are outlined in the European Respiratory Society guidelines [11, 12].

However, some differences in methodology still exist between various research groups. An important question, therefore, is whether these differences in methodology influence the validity and reliability of induced sputum in the assessment of airway inflammation.

The widespread application of induced sputum in a variety of airway diseases, such as COPD, and across the spectrum of disease severity has given an insight into the relationship between airway function and airway inflammation, proposed new possible disease phenotypes and defined which of these phenotypes respond to current therapy, and perhaps most importantly provided a tool to guide the clinical management of patients with airway disease [5].

Methodology

Since the first description of a standardised method in 1992 by Pin et al. [13], there has been an impressive increase in the number of papers in which researchers have used induced sputum to study various aspects of airways inflammation. Thus, in response to the interest in sputum analysis, a European Respiratory Society task force has published sputum methodology [11, 12, 14, 15].

Sputum Collection

Induced sputum must be collected early in the morning. Subjects should be pretreated with inhaled short-acting $\beta_2$-agonists. Induction is performed using an ultrasonic nebuliser. Two different approaches for induction have been used:

a) inhalation of the same (3–4.5%) or increasing (3, 4 and 5%) concentrations of aerosolized hypertonic saline over fixed time periods [13, 16].

b) inhalation of the same concentration of hypertonic saline (4.5%) over increasing time periods [17].

The choice of technique does not seem to influence the differential cell count in sputum. The duration of sputum induction varies between 15-20 min.

Sputum Processing and Analysis

Once a sputum sample has been obtained, it should be processed as soon as possible or within 2 hours in order to ensure optimum cell counting and staining [16, 17, 18]. The sample is put into preweighed polystyrene tube and weigh. Complete homogenisation can be achieved by the addition of equal volume or 4 x selected volume of diithiothreitol (DTT). Dithiothreitol breaks the disulphide bonds in mucin molecules, allowing cells to be released [19]. This is important because cells that are incompletely released from mucus tend to stain darkly, making correct identification difficult. DTT (0.1%), commonly known as 10% sputalysin solution, has been shown to be more effective at dispersing cells than phosphate-buffered saline (PBS).

The sample is aspirated and dispensed several times with disposable pipette and then agitated on vortex mixer for about 30 seconds. The duration of homogenisation varies between 10–30 minutes (usually 15 minutes). Homogenisation is feasible by using either a shaking water bath at 37 °C or a tube rocker at 22 °C [17, 20]. Filtration through a 48-mm nylon mesh is commonly used to remove mucus and debris. A single filtration step results in a slight reduction in the total cell count (TCC). However, slide quality is improved and the differential cell count (DCC) remains unchanged [21].

The TCC is performed manually using a haemocytometer. The cell viability is determined by the trypan blue exclusion method [16, 18]. Some investigators perform the TCC before centrifugation and others after centrifugation [22,23]. It is recommended that the TCC be performed before centrifugation in order to facilitate standardisation of this measurement and to allow meaningful comparisons of counts between centres and studies.

In order to separate sputum cells from the fluid phase, the sample must be centrifuged at 300–1,500×g. The duration of centrifugation is about 5–10 min [16,18,24]. The supernatant can then be stored at -70°C.

The next step is the cytospin preparation. The cell concentration is adjusted to 1.0×10³ cells mL⁻¹. Then, 40–65 µL of the sample (or 450–650×10³ cells) is added to each cyto-spin. Cytocentrifugation speeds range 10–51×g with the most common conditions being 22 ×g for 6 min [18]. There is always a risk of losing lymphocytes at lower speeds [25, 26].

Cytospin staining for DCCs can be achieved using Giemsa stain. The DCC is determined by counting a minimum of 400 nonsquamous cells and is reported as the relative numbers of macrophages, neutrophils, lymphocytes, eosinophils, and bronchial epithelial cells, expressed as a percentage of total nonsquamous cells. The percentage of squamous cells should always be reported separately.

Safety

The induction procedure is simple, safe and relatively non-invasive [11]. Sputum induction has been used safely in patients with severe COPD, but there have been no systematic studies addressing safety issues in this patient category. The ERS Task Force conclusions regarding the safety of sputum induction could serve as guidelines, particularly for those who are inexperienced in performing sputum induction procedures [11].
Clinical Applications

Sputum induction is considered to be a technique whose value is not restricted to sputum cell counts, as inflammatory mediators can also be measured in the supernatants. Additionally despite the predominating sputum neutrophilia, there is increasing evidence that the presence of sputum eosinophilia predicts an objective response to steroid treatment in patients with COPD. However none of the measurable biomarkers in sputum induction is considered applicable in clinical practice and none has been related to disease severity or progression.

Inflammatory Mediators in Supernatant

COPD patients usually produce suitable sputum spontaneously, but spontaneous sputum may contain a high proportion of dead cells [27, 28], which can potentially give misleading cell mediator measurements [29, 30]. For this reason, induced sputum has usually been the procedure of choice. It should be recognized that induced sputum may have a different composition than mucus, and may be more similar to a washing of proximal airways. Many mediators have been reported to be increased in the supernatant of patients with COPD. Most of them show a greater increase in COPD than in normal smokers, with a further increase during exacerbations.

Sputum concentrations of inflammatory mediators are generally unaffected by corticosteroids, but reduced by theophylline [31-33].

Sputum IL-8 and TNF-α have been studied more extensively. IL-8, a potent neutrophil chemoattractant, is increased in induced sputum of COPD patients compared with smokers, is related to disease severity, and further increased with exacerbations [34-36]. Increased concentrations of TNF-α and soluble TNF receptors are found in sputum of patients with COPD compared with normal smokers [37].

TNF-α, IL-8, and IL-6 concentrations have been reported higher in patients with more severe COPD compared with those with less severe COPD [38]. Broekhuizen et al. [39] reported that leptin, a hormone produced by adipocytes, is detectable in induced sputum of COPD patients and is correlated with other inflammatory markers, including TNF-α and C-reactive protein.

MMP-8 and MMP-9 [40–42], MMP-12 [43], neutrophil elastase [44] and many other proteases have been found in increased concentrations in sputum of COPD patients.

Sputum markers of structural changes in the airways have been difficult to identify. Dentener et al. [45] has recently reported that hyaluronan, a component of extracellular matrix, has been found in higher concentrations in sputum of COPD patients compared to normal smokers and non-smokers, especially in those patients with the most severe disease. This might indicate increased breakdown of extracellular matrix in COPD.

Boschetto et al. [46] found no differences in the concentrations of the tachykinin peptides substance P and neurokinin A between patients with COPD, normal smokers, and non-smokers, although there was a reduction in tachykinins during exacerbations of COPD.

The reducing and denaturant effects of DTT diminish the detectable levels of mediators such as TNF-α, LTB4 and myeloperoxidase (MPO). IL-1β, IL-6, IL-8, secretory leukocyte protease inhibitor (SLPI) and neutrophil elastase are unaffected [47]. There is also a problem with proteases in causing decreased detectable levels of IL-5, the main cytokine for eosinophil recruitment. It has been shown that IL-5 levels can be significantly increased by adding protease inhibitors [48,49]. In a recent study, Erin et al. [50] showed that after protease inhibition and optimized dialysis of sputum supernatants containing DTT, an increase in the levels of some chemokines and cytokines was detected. Accordingly, they suggested that the technique of optimized dialysis and protease inhibition should be used to partially overcome the denaturant effects of DTT and the proteolytic effect of proteases so as to identify particular cytokines and chemokines. The detection of elevated levels of particular sputum chemokines and cytokines in individual patients may provide a rationale for specific therapies.

Sputum Cell Counts

There is a different pattern of inflammatory cells in COPD patients compared to healthy subjects. An increase in the number of total inflammatory cells, primarily in the percentage of neutrophils and sometimes of eosinophils is observed [51-53]. CD8+ T lymphocyte subpopulations are also increased in induced sputum of COPD patients [54].

Neutrophils have been studied most extensively, and are increased in number compared to matched smokers with normal lung function [34].

The raised sputum neutrophil count is related to reduced FEV1, and the increased rate of decline in FEV1, suggesting that neutrophilic airway inflammation is functionally important [40].

Several studies have reported the effects of drugs on sputum neutrophils. Most studies have shown no change in inflammatory cells with inhaled or oral corticosteroids [31, 32]. However, a reduction with oral theophylline has been reported [33].

Up to 40% of COPD patients have a sputum eosinophil count of at least 3% [52, 53]. There is increasing evidence that the presence of sputum eosinophilia predicts an objective response to oral [52, 55] and inhaled corticosteroid treatment in COPD [53]. Brightling et al. [52] reported that the response in terms of lung function, health status and exercise tolerance to a 2-week course of oral prednisolone increased as the baseline sputum eosinophil count increased, and was associated with a marked treatment-induced fall in the sputum eosinophil count, but no change in sputum markers of neutrophilic inflammation. This finding suggests that eosinophilic airway inflammation is functionally important in a subgroup of COPD patients. Thus, the beneficial effects of corticosteroids are partly due to modification of this aspect of the complex airway inflammation. Siva et al. [56] showed that a management approach over a 12-month period with the additional aim of reducing the sputum eosinophil count < 3% using corticosteroids was associated with a 62% reduction in severe exacerbations of COPD requiring hospitalization when compared to traditional symptom-based management. Based on these reports, a measurement of sput-
tum eosinophil counts can be used to identify COPD patients with corticosteroid responsive disease and to guide treatment. It should be acknowledged that patients with sputum eosinophilia and steroid responsiveness may be suffering not only from COPD but also from asthma.

EXHALED NO

Nitric oxide (NO) is the most extensively studied exhaled marker of airway inflammation. The presence of endogenous NO in exhaled breath of animals and humans was first described in 1991 [57]. The field of exhaled NO measurement has developed remarkably over the last 15 years, with more than 1,000 publications in the field. Despite the numerous publications, the field of exhaled NO measurement has been characterized from its onset by a marked variation in published fractional exhaled NO (FeNO) levels in health and disease.

NO is a free radical gas that is formed in both the upper and lower respiratory tract [58-60]. It is not stored locally and diffuses into the lumen by gaseous diffusion down a concentration gradient, thus conditioning exhaled gas with NO [61,62]. There may be significant contribution from the oropharynx [60,63].

In the lower respiratory tract, NO is produced by resident and inflammatory cells. It is generated via oxidation of L-arginine that is catalysed by the enzyme NO synthase (NOS) and yields NO and L-citrulline [64,65]. NOS exists in three distinct isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). NO derived from the constitutive isoforms of NOS (nNOS and eNOS also known as NOS1 and NOS3) and other NO-adduct molecules (nitrosothiols) are able to modulate bronchomotor tone. More specifically, in the lung NOS1 is expressed in the NANC fibers and generates NO which acts as a smooth muscle relaxer [66,67]. NOS3 has been found in pulmonary endothelium and bronchial and alveolar epithelium and is believed to regulate ciliary beat frequency [68,69]. Both NOS1 and NOS3 are corticosteroid resistant [70]. NO derived from the inductive isoform of NO synthase (NOS2), is up-regulated oropharynx via NF-kappaB-dependent pathway, such as tumor necrosis factor α, interferon γ, interleukin 1β and seems to be a pro-inflammatory mediator with immunomodulatory effects. In the respiratory tract NOS2 is expressed in epithelial cells, endothelium, airway and vascular smooth muscle, fibroblasts, mast cells and neutrophils [64].

Under physiologic conditions NO is unstable, reacting avidly with other molecules such as oxygen to form oxides of nitrogen, nitrite (NO2) and nitrate (NO3), and with superoxide anion (O2-) to form peroxynitrite (ONOO). ONOO can nitrosate proteins and lead to lipid peroxidation products, such as lipoxins [71].

Alveolar NO is probably very low because of avid uptake by hemoglobin in pulmonary capillary blood [72].

Measurement

Online measurement refers to FeNO testing with a real-time display of NO breath profiles and it is expressed in parts per billion, which is equivalent to nanoliters per liter. Offline testing refers to collection of exhalate into suitable receptacles for delayed analysis [73]. The use of FeNO measurement as a clinical tool requires the adoption of a standardized measurement technique followed by collection of reference data in all age groups. Two factors are critical in ensuring reproducible and standardized measurements of lower respiratory tract exhaled NO: (1) exclusion of nasal NO and (2) standardization of exhalation flow rate.

Expiratory Flow Rate Dependence

There is significant expiratory flow rate dependence affecting exhaled NO concentrations from the lower respiratory tract [74]. This flow dependence is characteristic of a diffusion-based process for NO transfer from airway wall to lumen and can be simply understood by faster flows minimizing the transit time of alveolar gas in the airway, and thereby reducing the amount of NO transferred. In view of this flow dependency, the use of constant expiratory flow rates is emphasized in standardized techniques.

Inhalation Phase

The patient should be seated comfortably. A nose clip should not be used, because this may allow nasal NO to accumulate and promote leakage of this NO via the posterior nasopharynx. The patient inserts a mouthpiece and inhales over 2 to 3 seconds through the mouth to total lung capacity (TLC), or near TLC if TLC is difficult, and then exhalates immediately, because breath holding may affect FeNO.

Exhalation Phase

It is common practice to display pressure or expiratory flow rate to the subject, who is requested to maintain these within a certain range [74].

During online measurement of exhaled NO, the recommended exhalation flow rate for adults and children older than 12 years as well as children younger than 12 years is 0.05 L/second and is measured at 37°C, 760 mm Hg, saturated (BTPS) in keeping with other measurements of lung function.

In adults, there is no consistent relationship between exhaled NO level and age [75,76]. The effect of age is limited to children and FeNO increases with age until adulthood [77]. Additionally, FeNO levels seem to be higher in men than women [78,79] although some reports conflict. It is recommended that NO analysis be performed before spirometry [80,81]. It has also been demonstrated that FeNO levels may vary with the degree of airway obstruction or after bronchodilatation [81-83], perhaps because of a mechanical effect on NO output. Patients should refrain from eating drinking and smoking for 1 hour before NO analysis. FeNO measurements should be deferred after an upper or a lower respiratory infection until recovery if possible, since viral infections may lead to increased levels of exhaled NO [84,85].

Most NO analyzers in research and clinical use employ the principle of ozone-/NO2-based chemiluminescence to measure NO. Ozone chemiluminescence analyzers are sensitive to ambient conditions, including temperature, humidity, exposure to sunlight, and so forth [86].
Staff training can be minimal, because FeNO measurement with the NO analyzer NIOX (Aerocrine, Solna, Sweden) is fully automated, and incorrect exhalation maneuvers by a patient (shorter than 10 seconds or above the certain limits of the exhaled flow) will not be accepted. Another advantage of FeNO measurement is that it does not require any extra encouragement, as may be the case with peak expiratory flow measurement. There is no "learning effect" or systematic error with serial FeNO measurements, probably because of its simplicity and high reproducibility.

Modeling NO Excretion

Several recent reports have described NO exchange dynamics using a two-compartment model of the lungs, thus an airway and an alveolar compartment [87,88]. The potential advantage of this approach is twofold. First, a greater level of specificity can be achieved for inflammatory diseases that affect primarily the airways or the alveolar regions of the lungs. Second, the parameters are independent of exhalation flow rate. At fast flow rates (>50mL/s) the alveolar contribution to the total NO value predominates, but at slower flow rates (<50mL/s) airway diffusion predominates [87]. The flow-independent parameters can be derived by measuring exhaled NO concentration at multiple exhalation flow rates. This novel approach can also be used to monitor not only the disease but also the effect of treatment with NO modulators, which may have different sites of action. The use of flow-independent nitric oxide exchange parameters may also aid understanding of the location of tobacco-induced changes in airway NO metabolism and exchange.

NO and Smoking

In a study by Malinovschi et al. reduced nitric oxide levels were found in current smokers in both the airways and alveoli. Ex-smokers showed significantly lower FeNO levels than neversmokers [89]. Passive smoking has been found to reduce levels of exhaled NO in healthy subjects and asthmatic children [90,91].

The possible mechanisms by which exhaled NO levels are reduced in smoking subjects are a potential negative feedback mechanism of the NO from the cigarette smoke, that could lead to downregulation of NO synthase (NOS) in the lungs [92], an inadequate supply of cofactors necessary for NO production, such as tetrahydrobiopterin [93], and an increase in the breakdown of NO [94].

Smoking is associated with reduced levels of tetrahydrobiopterin [93], which might reduce enzymatic NO production by uncoupling NOS, with resultant production of superoxide instead of NO [95]. Superoxide can, in turn, react with NO to form peroxynitrite. The fact that NO consumption might be increased in smokers’ airways is suggested by the increase in NO metabolites in exhaled breath condensate [96]. Therriault et al. [97] reported that the N-nitrosamine 4- (N-methylamino)-1-(3-pyridyl)-1-butanone, a component of cigarette smoke, inhibited alveolar macrophages from producing NO, a finding that might explain the lower alveolar NO in smokers. Another possible mechanism could be an increase in the permeability of the respiratory membrane for NO in chronic smokers [98].

NO and COPD

FeNO has been extensively investigated in asthma, and has been shown to correlate with predominantly eosinophilic airway inflammation and to be reduced by corticosteroid therapy [99-102]. In COPD abnormalities of FeNO are less clear cut than in asthma. Some studies suggest that FeNO is increased in patients with stable COPD, others only in patients with COPD exacerbations [103], while some report no increase. This increase during COPD exacerbations is likely due to the increase in oxidative stress, resulting in formation of peroxynitrite and then nitrate, so that NO is removed from the gaseous phase. During acute COPD exacerbations the level of eosinophilic inflammation increases which may also explain the increase seen in FeNO [104]. Besides, COPD exacerbations are often characterized by acidosis, which may have a vasodilatory influence on the vasculature and could lead to myeloperoxidase activity in the lung and subsequent increased FeNO levels [105]. Therefore the mild elevation in FeNO seen with COPD exacerbations may actually be caused by altered pulmonary function and gas exchange rather than inflammation.

Increased levels of exhaled NO have been detected in stable COPD patients in few studies [106,107], however it is not clear whether this is due to iNOS up-regulation. Di Stefano et al. [108] demonstrated an increased expression of p65, the major subunit of nuclear factor-xB (NF-xB), in the bronchial epithelium of smokers both with COPD and normal lung function. Augusti et al. [109] found that NF-xB activation and iNOS induction occur in skeletal muscle of COPD patients. These findings suggest the involvement of iNOS in the smoking-induced airway inflammation. Although iNOS has been the principal NOS isoform considered as a therapeutic target, the role of the "constitutive" NOS isoforms in airways disease is becoming increasingly important. Indeed, interpretation of studies of NOS inhibition is limited by the lack of selectivity of the agent used. However, a number of selective inhibitors have been recently developed and used as pharmacological tools [110]. The most widely used have been NG-nitro-L-arginine (L-NMMA) [111], NG-omega-nitro-L-arginine methyl ester (L-NNAME) [112] and antagonuandine (AG) [113].

Alveolar NO is elevated in conditions associated with distal lung inflammation. Alveolar NO is elevated in COPD [106] thus, the source of NO in COPD is likely to be the peripheral lung rather than the main bronchi, in contrast to asthma where airway NO is predominantly elevated [114]. Alveolar macrophages are increased in COPD patients; this could account for the elevated alveolar NO levels as these cells are able to express iNOS in response to proinflammatory cytokines [115]. nNOS expression is significantly increased in peripheral lungs from COPD patients compared with non-smokers [116], suggesting that nNOS may also contribute to the increase in alveolar NO in COPD.

The peripheral NO may prove to be a useful noninvasive biomarker of COPD inflammation, but further studies on reproducibility, relationship to disease severity and the effects of treatments are now needed. Moreover, it should be noted that there are currently a number of concerns regarding the technique, which is based on a mathematical model and
on generalizations, such as absence of turbulent flow, and constant airway diameter during the respiratory cycle. Additionally, NO concentrations in the peripheral lung are very low probably because of the high affinity of NO for the blood. This is likely to cancel any increase of the NO due to peripheral airway inflammation.

**NO and Corticosteroids**

Asthmatic patients treated with glucocorticosteroids (GCS) have a significantly lower FeNO concentration than GCS-naive asthmatic patients which may be explained by the inhibition of inducible NO synthase (iNOS) or type 2 NO synthase expression by GCS [117]. The mechanism by which GCS inhibit NO production might be indirect via inhibition of cytokine synthesis [118] and reduction of iNOS mRNA transcription [119] rather than a direct effect on iNOS expression.

One report suggested that FeNO falls after inhaled steroids in stable non-smoking COPD patients [120]. Moreover, patients with COPD with a partial bronchodilator response to inhaled salbutamol show a better response to ICS treatment, in reduction of elevated levels of exhaled NO, than do those without reversible airflow limitation [121]. In another study, no significant difference in FeNO levels between COPD patients treated with and without GCS was observed [122]. Histone deacetylase (HDAC) has been demonstrated to reverse inflammatory process and repress expression of the genes for inflammatory mediators. Reduced HDAC activity without increased histone acetyltransferase activity is thought to be associated with GCS resistance in COPD [123]. HDAC inhibition has been reported to increase iNOS mRNA expression in neural models of inflammation in vitro. Elevated alveolar NO is not affected by inhaled corticosteroids [106].

**Future of NO in COPD**

NO is increased in exhaled breath from very early stages of the common cold, [84,124] which often triggers COPD exacerbations. Potentially, portable NO analyzers might be used by the patients at home to alert them, in addition to symptoms, so that early detection of the onset of exacerbation and prompt treatment improves exacerbation recovery.

The recovery of COPD patients from an exacerbation is currently monitored by symptoms, purulence of sputum, and lung function. A significant proportion of patients treated for COPD exacerbation demonstrate incomplete recovery, and it is well established that frequent exacerbations contribute to decline in lung function and poor health status. Lower airway bacterial colonization in stable patients with COPD instigates airway inflammation, which leads to a protracted self-perpetuating vicious circle of progressive lung damage and disease progression. It can be speculated that normalization of alveolar NO may indicate the degree of recovery from lower airway bacterial colonization, and may serve as predictor of poor outcome if the alveolar NO levels do not respond to antibacterial treatment.

The higher presence of iNOS-positive cells in alveolar walls in patients with more severe COPD may explain a severity-related increase of the alveolar NO but this does not apply to severe emphysema that shows a lower percentage of iNOS-positive alveolar macrophages than patients with milder disease [125].

L-arginine supplementation has been studied in a variety of clinical situations in which the increase of NO production is desired. There are several reasons behind a considerable interest in the development of new compounds to act as NO donors for patients with lung diseases. NO plays an important role in bactericidal activity in the lungs, ciliary beating and mucociliary dysfunction. NO synthase (NOS) inhibitors and substrates of NOS could have great therapeutic potential in steroid-resistant pathologic conditions such as severe asthma and COPD [124].

Lack of effect of conventional ICS on alveolar NO [106] brings an important advantage of multiple expiratory flow FeNO measurements in COPD for monitoring the inflammatory process that is clearly different from asthma. It can be speculated, however, that anti-inflammatory effect of some novel formulation, for example, combination of beclomethasone dipropionate with formentoterol delivered by small-particle size aerosols, may be assessed by the multiple expiratory flow measurements in COPD. This approach may make this technique particularly valuable for assessing the anti-inflammatory effects of new therapies in COPD in the future.

**EXHALED BREATH CONDENSATE (EBC)**

**Origin-Collection**

Exhaled breath is saturated with water vapor, which constitutes its principal component and can be condensed by cooling. It also contains a wide range of volatile and non-volatile molecules. The mechanisms by which non-volatile molecules are collected in EBC are still not clarified, but it is assumed that airway surface liquid becomes aerosolized during turbulent airflow, so that EBC reflects the contents of the epithelial lining fluid [9]. EBC samples the entire respiratory tract from the mouth to the alveoli, but the exact origin of each molecule is still uncertain [9]. The comparison of mediator levels in EBC and lower airways’ samples suggests that most of these molecules are added to EBC in the lower airways [126,127] although EBC and bronchoalveolar lavage markers do not correlate directly [128].

EBC is collected by cooling or freezing the expired air. The collection procedure is totally non-invasive and the patient simply breaths tidally via a mouthpiece. The exhaled breath is directed, through non re-breathing valves that separate inspiratory and expiratory air, to a cooling device, where it is collected in a liquid or solid phase, depending on the condenser’s temperature. Several types of condensing devices have been developed, including Teflon tubes immersed in icy water, double-wall glass condensers with an inner glass chamber containing ice, double-lumen glass tubes cooled by cold air passing through the outer lumen, and more recently commercially available portable devices using an aluminum cylinder for cooling (RTube, Respiratory Research Inc., Charlottesville, VA), or refrigerating systems (Ecoscreen, Jaeger, Germany) [129]. Generally, 10-15 min-
utes of tidal breathing are sufficient for the collection of 1-3 ml of EBC. Two recent consensus statements have highlighted the necessity for better standardization of the collection and the assessment of mediators in EBC [7,9]. Studies that compared the two recommended devices have revealed some differences mainly attributed to the biomarker studied. Influencing factors involve contamination of the sample with either condenser coating or/and with plastic and glass surfaces. Regarding any risk for infection, transmission is possible through the non-disposable collection systems while it is unlikely in the disposable ones. However, a published study using a genetic based design did not observe any contamination [130]. Protective mechanisms are not related to filters since they might trap molecules in the exhaled air but they may involve one way valve devices.

The pattern of breathing has not been adequately studied so far since it is widely accepted that tidal breathing is the most appropriate pattern [9]. However, it is critical and simultaneously important to standardize the breathing pattern, especially for COPD patients since they breathe in different flow rates.

Storage-Repeatability

Most of the mediators with the exception of pH and hydrogen peroxide (H$_2$O$_2$) are usually frozen for later analysis [9, 129]. This freezing procedure lacks adequate data in relation to its effect on mediators’ concentration. Limited data supports stable values within periods varying from two weeks to three months [131-133]. However, there is also data supporting different values within the same period for specific mediators [134,135]. Similar issues are arising in relation to repeatability since the already published data is poor. Again, the determinant factor is the mediator studied, since some of the mediators are less repeatable compared to others [133,136,137]. Another issue that needs clarification is the appropriate time-interval to examine repeatability, since changes due to inflammation are quite common over time in inflammatory airway diseases.

Analysis

The most common method for measuring different mediators in EBC is enzyme immunoassay (ELISA). The main advantages for this method are related to its easy performance and its good intra-assay variability. However, if one compares different studies regarding the levels of different biomarkers obtained by using the same ELISA kit, he will realize that mean values vary significantly. Another critical point is the non detectable samples [138,139]. The latter might be attributed mainly to the dilution procedure below the recommended level or to the extrapolation of the standard curve below the lowest concentration. Alternative methods for analysis are gas or liquid chromatography and mass spectrometry [9,129]. These techniques are increasing the sensitivity of measurements but they are expensive, not widely available and time consuming. Data for mediators measured with the above techniques showed conflicting results [140-142]. The above methods present less variability and adequate reproducibility although the latter needs more supporting data.

MEDIATORS

Oxidative Stress

Hydrogen Peroxide

Activated inflammatory cells such as eosinophils, macrophages and neutrophils respond with a respiratory burst, resulting in the production of reactive oxygen species, the most commonly studied to date being H$_2$O$_2$ [29]. Most commonly, H$_2$O$_2$ is measured spectrophotometrically [144,145] or spectrofluorimetrically [146–148], but various techniques have been used, including the recent development of an automated amperometric biosensor (Ecocheck, ViaSys, Germany) [149]. Usually 250 μl of 420 mM 3', 3, 5, 5' tetramethylbenzidine (dissolved in 0.42 M citrate buffer, pH 3.8) and 10 μl of 52.5 U/ml of horseradish peroxidase are reacted with 250 μl of the condensate for 20min at room temperature. Subsequently, the mixture is acidified to a pH of 1 with 10 μl of 18 N sulphuric acid. The reaction product is quantitated at the absorbency of 450 nm using a double beam spectrophotometer.

Most of the published studies have reported elevated levels of H$_2$O$_2$ with a clear discrimination between healthy subjects and COPD patients [131,145,150]. H$_2$O$_2$ is not a specific biomarker for the disease but is further elevated during exacerbations [151]. However, a significant overlap is observed mainly between healthy smokers and COPD patients. There is a positive correlation between levels of H$_2$O$_2$ and sputum neutrophils, indicating a neutrophil dependent mechanism on its release [131]. Some studies showed a decrease on H$_2$O$_2$ levels after inhaled steroid treatment but most of them lack a proper design towards the direction of control group [120,152]. In a more properly designed study a time dependent decrease of H$_2$O$_2$ levels after treatment with antioxidants was observed, indicating a potent target inflammatory group for this class of drugs [153].

8-Isoprostane

Isoprostanes are prostaglandin-like compounds formed by the free-radical lipid peroxidation of arachidonic acid and represent in vivo markers of oxidative stress. A number of studies have shown these compounds to be markers of oxidative stress and have illuminated the role of oxidant injury in association with the production of nitrogen species [154,155]. The most studied isoprostane is 8-epi-PGF2α, also known as 8-isoprostane. Many studies have reported increased 8-isoprostane levels in patients with COPD but with conflicting results regarding discrimination between different severity stages [131,138,156, 157]. Although it is considered stable, being a final product of oxidative stress, recent evidence supports that 8-isoprostane is either undetectable in the exhaled breath or demonstrates a high variability in its levels, mainly attributed to the sensitivity of ELISA measurements [129, 136, 158].

Other Markers of Oxidative Stress

Oxidation of cell membrane phospholipids produces a chain reaction, the targets of which are the polyunsaturated fatty acids, and results in the formation of unstable lipid hydroperoxides and secondary carbonyl compounds, such as aldehydic products [159]. Among these, malondialdehyde
Nitrogen Species

NO is a free radical due to its unpaired electron and it may react with oxygen to yield nitrogen oxides (NOx) or with a superoxide anion to yield peroxynitrite, a highly reactive substance that may lead to the development of NO-derived products [164,165].

Nitrate is measured as nitrite after enzymatic conversion by nitrate reductase [166]. The total NO$_2$/NO$_3$ converted nitrate plus nitrite) is measured using the Griess reaction. Enzymatic conversion is carried out as following: 60 μl of breath condensate is mixed with 10 μl NADPH 0.5 mM and 10 μl mixture of the nitrate reductase (2000 μl$^{-1}$) and FAD (50 mol l$^{-1}$). Samples are incubated for 30min at 37°C and then mixed with 10 μl LDH (100mg l$^{-1}$) and 10 μl sodium pyruvate (100 mmol l$^{-1}$). Samples are further incubated for 10min at 37°C to oxidize the excessive amounts of NADPH. After the enzymatic conversion of nitrate to nitrite the sample is assayed with 100 μl Griess reagent (Sulfanilamide 1g N-H$^+$N-1 naphthylethylenediamine 0.1 g l$^{-1}$, H$_3$PO$_4$, 25 g l$^{-1}$). After 10min of colour development at room temperature the absorbance is measured at 550nm.

Since most of stable COPD patients have low levels of FeNO due to smoking the measurement of nitrogen oxides might provide useful information regarding NO metabolism. Higher levels of NO$_2$ were observed in COPD patients compared to normal subjects either smokers or non-smokers [167]. This may be attributed to the mucus blocked exhaled NO.

Nitrosothiols (RS-NOs) are formed by interaction of NO with glutathione and may limit the detrimental effect of NO. RS-NOs are measured using a commercially available colorimetric assay kit. The assay is based upon the classic reaction of Saville and Griess [168,169]. In essence, a cleavage reaction breaks the S-N bond of RS-NOs releasing NO, which oxides rapidly to NO$_2$. NO$_2$ is then detected colorimetrically using the Griess reaction.

In a single study, higher levels of RS-NOs were observed in COPD patients, indicating an increased antioxidative activity that could eliminate the harmful effects of NO [170]. Despite this, further studies are needed in order to define properly the above mediator’s variability or/and clinical value in a more detailed manner.

Cytokines

Cytokines and chemokines are involved in many aspects of the disease process in COPD, including recruitment of neutrophils, macrophages, T-cells and B-cells, airway wall remodeling, goblet cell metaplasia and epithelial cell hyperplasia and the induction of emphysema. Measuring different cytokines in COPD could lead to a better phenotyping of the disease as well as to clarification of underlying mechanisms. However, this effort is hampered by the low sensitivity of ELISA methodology in detecting cytokines’ concentration. Most studies have reported values below or near the limit of the standard curve of the ELISA method [171,172]. An alternative and promising method for cytokine detection is a method using a multiplex array kit [173]. In this method EBC is collected by inserting a special conduit from the EcoScreen breath condensate collecting device into the expiratory limb of the ventilator tubing after the Y-shaped connecting piece directly for a 20-min time period. Humidification of inspiratory gas is achieved using heat humidifiers. To fit characteristics of the multiplex array, 2 ml of EBC fluid is lyophilized with an evaporator centrifuge. For cytokine detection, the pellet is resuspended in 60 μl water. By this procedure the concentration is increased 33-fold. A multiplex fluorescent bead immunoassay is taken to detect cytokine concentrations. Eighteen microliters of a mixture of six bead populations with distinct fluorescence intensities and coated with capture antibodies specific for IL-1β, IL-6, IL-8, IL-10, TNF-α, and IL-12p70 proteins are incubated with 18 μl deionized water in duplicates. Cytokines in test samples and recombinant standards as bound to capture beads are detected by PE-conjugated detection antibodies and measured in a flow cytometer. To reduce loss of cytokines by surface absorption and to consider matrix dependency as typical for immunoassays, the effect of added protein is investigated. The complete test procedure is investigated for linearity through the measurement of nine standards from 2.5 to 500pg/ml and calculating the coefficient of correlation. Linear range and detection limit are determined for every singular cytokine to define optimal range for cytokine detection.

With this technique increased levels of all cytokines were identified in COPD exacerbations [174]. Despite these promising results the whole procedure needs further validation mainly towards the direction of reproducibility and sensitivity.

Leukotriene B$_4$ (LTB$_4$)-Prostaglandins

Cysteinyl leukotrienes have been connected with the asthmatic response, whereas the role of LTB$_4$ has been connected to neutrophilic inflammation. LTB$_4$ is produced by constitutive cells (eg, mast cells and macrophages) and infiltrating cells (eg, neutrophils and eosinophils). LTB$_4$ has no direct action on airway smooth muscle, but it may contribute to bronchoconstriction by increasing vascular permeability and mucus secretion. It is considered as one of the main mediators responsible for neutrophil recruitment [175]. Increased levels of LTB$_4$ have been reported in stable COPD.
with further increase during exacerbations [135,138,176].

Despite the above results, LTB₄ levels varied significantly within studies although the same ELISA kit was used. Possible explanations are related to the effect of oral contamination, time of storage as well as the instability of the mediator which was confirmed in one of the studies [135]. Interestingly, LTB₄ in EBC correlates significantly with the respective concentration of the mediator in induced sputum [135]. This might reflect a common source of the two biological fluids mainly referring to central airways. In a study by Kostikas et al. [135] increased levels of LTB₄ were found in those COPD patients showing similar reversibility in airway obstruction to that of asthmatic smokers. Limited data exists for prostaglandins with a selective increase in prostaglandin E₂ (PGE₂) in patients with COPD which may be relatively resistant to inhaled corticosteroid therapy [176]. PGE₂ is usually measured by radioimmunoassay using specific antisera. An interesting observation regarding therapeutic response for both mediators is published by Montuschi et al. [177]. Non-selective Cyclooxygenase (COX) inhibition decreases PGE₂ and increases LTB₄ in EBC, whereas selective COX-2 inhibition has no effect on these eicosanoids. PGE₂ in EBC is primarily derived from COX-1 activity, and COX inhibition may redirect arachidonic acid metabolism towards the 5-lipoxygenase pathway.

**pH**

Measurement of pH with pH electrodes after deaeration for the removal of CO₂ with a CO₂-free gas (e.g. argon or nitrogen) is a technically validated EBC measurement in the published literature [9,129,178]. Measurement of EBC pH is a simple, robust, reproducible and relevant marker of airways disease [179]. In healthy subjects, the mean EBC pH after deaeration is 7.7 with a range of normal 7.4–8.8 [180]. However, a recent study suggests that the standardization of EBC pH to a certain level of PCO₂ in EBC represents the most reproducible method for the measurement of EBC pH [181]. There is a debate as to whether orally collected EBC pH assays reflect acidification of the lower airways, because the high ammonia content of the mouth may conceivably interfere with the assay [182]. This concern has not been proven and extensive data does not reveal an effect of oral ammonia on EBC pH assays [182,183]. Measurement of pH of deareated EBC is not affected by hyperventilation, temperature of collection, duration of collection or storage [9,129], but is dependent on the collection devices used [134,184]. Patients with COPD had low pH levels with clear differentiation between patients and normal subjects [178,185]. The variability of EBC pH in COPD patients is mainly due to changes in airway pH over times, which are not seen in healthy nonsmoking subjects [185]. A significant association between pH levels and sputum neutrophilia was observed [178]. Its association with lung function impairment is still not clear since conflicting results have been reported [178,185].

**Other Exhaled Gases**

**CO**

Although its measurement is considered easy, its utility remains low. Exhaled CO is elevated in patients with COPD compared to normal smokers. Despite this, many methodological issues mainly attributed to factors such as environmental CO levels and passive smoking led to no further evaluation of this biomarker [6].

**Hydrocarbons**

Volatile hydrocarbons such as ethane and pentane are biomarkers of lipid peroxidation and have been detected in exhaled breath. Exhaled air is usually collected during a flow and pressure-controlled exhalation into a reservoir discarding dead space air contaminated with ambient air. Ethane content is analyzed using gas chromatography. Levels of exhaled ethane are strictly exhalation flow-dependent with good reproducibility. COPD patients showed elevated levels of exhaled ethane [186, 187]. Ethane also correlated with disease severity as assessed by forced expiratory volume in one second (FEV₁).

**Electronic Nose (e-Nose)**

Exhaled breath contains a mixture of hundreds of volatile organic compounds (VOCs). The electronic nose (e-nose) analyzes VOCs by composite nanosensor arrays (“breathomics”) with learning algorithms [188]. This is based on pattern recognition without analyzing the individual molecular components, which potentially suffices for diagnostic objectives [189]. In a recent study, it was found that VOC-patterns of exhaled breath could discriminate patients with lung cancer from COPD patients as well as healthy controls [190]. This indicates that the VOC-patterns in exhaled air differ between two separate smoking-related lung diseases, which warrants further steps towards diagnostic validation of electronic noses in lung cancer and COPD. The main intention of using electronic noses is not to assess the pathophysiological cause of a disease but to evaluate their diagnostic accuracy for a future application as a clinical test. The identification of specific VOCs provides vital information on the specific molecular pathways involved and is certainly relevant as an aid in optimizing specific sensors for future clinical purposes.

**DISCUSSION**

Most important findings are summarized on Table 1. The widespread application of induced sputum in COPD, and across the spectrum of disease severity has given an insight into the relationship between airway function and airway inflammation, has proposed new possible disease phenotypes and defined which of these phenotypes respond to current therapy, thus providing a tool to guide the clinical management of COPD patients [5].

Induced sputum samples predominantly the large airways and may not reflect the peripheral inflammation in COPD. IL-8, TNF-α and IL-6 levels are increased in induced sputum of COPD patients compared to healthy smokers and are related to the severity of the disease [34-38]. MMP-8, MMP-9, MMP-12, neutrophil elastase and many other proteases have been also found in increased concentrations in induced sputum of COPD patients [40-44]. Recent studies using dialysis to remove dithiothreitol which disrupts sulphhydryl bonds and thus may alter proteins show that such processing may allow the detection of high levels of important inflammatory and...
Table 1. Major Findings from Non-Invasive Assessment of COPD

<table>
<thead>
<tr>
<th>INDUCED SPUTUM</th>
<th>FeNO</th>
<th>EBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑ TNF-α [54, 56, 57, 58]</td>
<td>↑ in exacerbations [101,104]</td>
<td>↑ H2O2 [131,145,154,155]</td>
</tr>
<tr>
<td>↑ MMPs [40,41,42, 43]</td>
<td>↑ neutrophil elastase [44]</td>
<td>↑ aldehydes [148,150]</td>
</tr>
<tr>
<td>↑ neutrophils [34,40,51]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>↑ eosinophils [52,53,55,56]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

↑ = increased levels, ↓ = decreased levels, ~= at normal range.

Abbreviations: COPD= Chronic Obstructive Pulmonary Disease, H2O2= Hydrogen peroxide, IL-8=interleukin-8, IL-6= interleukin-6, LTB4=Leukotriene B4, MMPs=metaloproteinases, NO=Nitric oxide, TNF-α= Tumor Necrosis Factor-alpha.

The great interest for EBC grew rapidly and accumulating evidence suggests that it has the potential of becoming a validated method which may provide valuable information in the understanding of the pathways propagating airway inflammation. Although promising, EBC is currently used only as a research tool, due to the lack of appropriate standardization and the absence of reference values. The large number of measurable biomarkers, the high degree of within-subject variability, the differences in mediators’ values despite the common use of collection devices and finally the diversity of the used methodologies are some of the points that hamper its wide clinical application [9,10].

In terms of updated validated results and the adequate differentiation between health and disease, EBC pH seems to represent the most promising marker. However, it still remains unresolved whether its highly variable values within disease might indicate a specific phenotype reflecting a distinct functional or inflammatory pattern of the disease [177,184]. Another interesting biomarker measured instantly and adjusted for minute ventilation is H2O2. Most of the studies support a good differentiation between health and disease and additionally it seems to characterize properly the presence of exacerbation. However, it cannot be a disease specific biomarker since similar values were observed in other airway diseases [149]. The rest of EBC measurements need methodological optimization and validated measurements within centers.

What do we expect from EBC in COPD? Apart from the evaluation of new biomarkers, the standardization of the already existing procedures is warranted for its introduction in clinical practice.

What do we expect from biomarkers obtained by non-invasive methods in COPD? COPD is an heterogenous syndrome that encompasses a variety of obstructive diseases that differ in terms of the mechanisms and the response to therapy. Accordingly, it seems crucial to identify the different disease phenotypes within the range of this syndrome. The ideal approach is not to measure any single biomarker in order to detect elevated or decreased levels but to try to identify the particular phenotype that is related to the specific biomarker and the underlying mechanism. Most likely a single biomarker is not sufficient and the combination of more than one may approach more effectively the recognition of the phenotype. We believe that we are not close but we are...
definitely not that far from this attainment. We still have a lot to learn and improve in the field of biomarkers but we have to always remember that the understanding of the pathophysiology of a world epidemic disease is essential to improve the management of these patients as well as to evaluate the plausible effects of new treatment regimens.

REFERENCES


Assessment of COPD by Non-Invasive Methods

Current Medicinal Chemistry, 2009 Vol. 16, No. 22 2835


Li, C.G.; Rand, M.J. Evidence that part of the NANC relaxant response to guinea pig trachea to electrical field stimulation is mediated by nitric oxide. Br. J. Pharmacol., 1991, 102, 91-94.


Assessment of COPD by Non-Invasive Methods

Current Medicinal Chemistry, 2009 Vol. 16, No. 22 2837


[130] Hyslop, P.A.; Sklar, L.A. A quantitative fluorimetric assay for the determination of oxidant production by polymorphonuclear leukocytes: its use in the simultaneous fluorimetric assay of cellular acti-

[131] Ruch, W.; Hooper, P.H.; Bagnoli, M. Assay of H2O2 production by macrophages and neutrophils with homovanillic acid and horse-


Ko, F.W.; Lau, C.Y.; Leung, T.F.; Wong, G.W.; Lam, C.W.; Hui, D.S. Exhaled breath condensate levels of 8-isoprostane, growth related oncogene 

2838

Current Medicinal Chemistry, 2009 Vol. 16, No. 22

Hillas et al.


Balbi, B. COPD: is chemotaxis the key? Chest, 2003, 123, 983-86.


