Exhaled Breath Condensate Analysis in Chronic Obstructive Pulmonary Disease

Sunil K. Chhabra and Mansi Gupta

Department of Cardio-respiratory Physiology, Viswanathan Chest Hospital, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India

ABSTRACT

The increasing focus on airway inflammation in the pathogenesis of chronic obstructive pulmonary disease (COPD) has led to development and evolution of tools to measure it. Direct assessment of airway inflammation requires invasive procedures, and hence, has obvious limitations. Non-invasive methods to sample airway secretions and fluids offer exciting prospects. Analysis of exhaled breath condensate (EBC) is rapidly emerging as a novel non-invasive approach for sampling airway epithelial lining fluid and offers a convenient tool to provide biomarkers of inflammation. It has definite advantages that make it an attractive and a feasible option. It is a source of mediators and molecules that are the causes or consequences of the inflammatory process. Measurement of such markers is increasingly being explored for studying airway inflammation qualitatively and quantitatively in research studies and for potential clinical applications. These biomarkers also have the potential to develop into powerful research tools in COPD for identifying various pathways of pathogenesis of COPD that may ultimately provide specific targets for therapeutic intervention. The EBC analysis is still an evolving non-invasive method for monitoring of inflammation and oxidative stress in the airways. The limited number of studies available on EBC analysis in COPD have provided useful information although definite clinical uses are yet to be defined. Evolving technologies of genomics, proteomics, and metabonomics may provide deeper and newer insights into the molecular mechanisms underlying the pathogenesis of COPD. [Indian J Chest Dis Allied Sci 2012;54:27-37]

Key words: Chronic obstructive pulmonary disease, Exhaled breath condensate, Oxidative stress, 8-isoprostane, Hydrogen peroxide.

INTRODUCTION

A major advancement in the knowledge of pathogenesis of chronic obstructive pulmonary disease (COPD) has been the recognition that airway inflammation plays a key and a central role. It is now a part of the current definition of COPD and is believed to be the major underlying process for the altered pathophysiology and clinical manifestations. Airflow limitation has long been the pathophysiological characteristic of COPD, and therefore, lung function testing has been considered as the main investigation for diagnosis, assessment of severity, monitoring of response and for following the natural course of the disease. Indeed, spirometry is considered essential to establish permanent airflow limitation and the diagnosis of COPD. However, limitations of lung function tests in the assessment of severity and monitoring of response have been well recognised. The increasing focus on airway inflammation has led to efforts to gain insights into its nature, development and evolution in research studies with an aim to ultimately translate this knowledge into tools for diagnosis, assessment and monitoring of COPD.

Conventionally, drugs in COPD have been evaluated by changes in forced expiratory volume in one second (FEV₁) and more recently, using in addition, patient-centered clinical outcome measures. While these tools may be appropriate to evaluate therapeutic interventions such as bronchodilators, these may not serve the purpose for newer drugs that are increasingly targeted at specific pathways of the inflammatory process. Tools to measure airway inflammation are required for the evaluation of such drugs.

Oxidative stress is believed to play a major role in the pathogenesis of COPD and is characterised by an increased oxidant load and a relative or absolute deficiency of antioxidants. Oxidative processes and free radical generation lead to increased bronchial hyperresponsiveness and inflammation, apoptosis and destruction of airway epithelial cells, and impair the functions of antiproteases and surfactant. The major mediators of oxidative stress and pro-inflammatory molecules include reactive oxygen...
species (ROS) such as the superoxides and hydroxyl radicals, reactive nitrogen species (RNS), certain cytokines and eicosanoids, interleukins, tumour necrosis factor-alpha, and activated transcriptional factors such as nuclear factor kappa-B and activator protein 1. These compounds are potential candidates to serve as biomarkers of oxidative stress and airway inflammation. An ideal biomarker for COPD should be stable, be present in sufficient quantities to allow analysis, should have acceptable precision in measurement, should be responsive to change in clinical status, either worsening or improvement, with therapeutic interventions, and should be specific for the disease.

**ASSESSMENT OF AIRWAY INFLAMMATION**

Direct assessment of airway inflammation requires invasive procedures, such as biopsy and bronchoalveolar lavage that are limited by this disadvantage. These tools provide samples and specimens from limited areas of the lung. Further, these are not feasible options for advanced disease and for serial monitoring. While bronchoalveolar lavage fluid (BALF) has been used in several studies to sample the lower respiratory tract for more than two decades, its invasive nature and a definite, though small, risk of adverse events has prevented it from evolving into an acceptable clinical tool for the assessment of the airway inflammation.

Peripheral blood markers have been used to study airway inflammation but may not be appropriate mirrors of the airway pathology. The increase in the inflammatory cell population and markers in the airway may occur earlier than in the peripheral blood, and reflect the degree of airflow limitation better than do peripheral blood measurements. Non-invasive methods to sample airway secretions and fluids offer exciting prospects. These can provide mediators and molecules that are the causes or consequences of the inflammatory process. Measurement of such markers is increasingly being explored for studying airway inflammation qualitatively and quantitatively in research studies and for potential clinical applications. These biomarkers also have the potential to develop into powerful research tools for identifying the novel pathways of pathogenesis of COPD that may ultimately provide specific targets for therapeutic interventions.

Sputum induction is a semi-invasive technique that not only provides specimens for cytological studies of the airway lining fluid but provides a source of inflammatory mediators that can be measured in the supernatants. However, its application has largely been limited to cytological analysis though it has also been studied as a source of biomarkers reflecting oxidative stress and airway inflammation.

**EXHALED BREATH CONDENSATE ANALYSIS**

Analysis of exhaled breath is rapidly emerging as a novel and non-invasive approach for sampling airway epithelial lining fluid and offers another convenient tool to provide biomarkers. It has definite advantages that make it an attractive and a feasible option. Besides being completely non-invasive, it is suitable for serial assessments in longitudinal studies, and thus, can be used for monitoring of disease as well as serve as an outcome parameter in clinical drug trials. Other potential uses include measurement of severity of disease, and application as a diagnostic aid. It can be used in stable as well as patients with acute exacerbations and even in patients on a mechanical ventilator. It can be used in the out-patient and occupational settings, and also in field studies for epidemiological purposes. Exhaled breath analysis has been extensively examined as a mirror of the inflammatory processes in the airways in asthma and such studies are now being increasingly carried out in patients with COPD.

Exhaled breath analysis broadly focuses on two areas: measurement of exhaled nitric oxide (ENO) and the detection of biomarkers in exhaled breath condensate (EBC). The use of ENO has found limited success as a potential evaluative tool in COPD compared to its wider acceptability in the management of asthma. This scope of this review is limited to EBC analysis.

The EBC is mainly formed by water vapour but also contains several biomolecules, mainly related to neutrophil-derived products and oxidative stress (leukotrienes, prostaglandins, isoprostanes, hydrogen peroxide), endogenous airway acidification (hydrogen ions), and nitric oxide-derived products (nitrosothiols, and nitrite/nitrate). Its collection simply involves tidal breathing into a chilled collection device. Water vapour in exhaled breath is condensed and collected and various mediators can then be quantified in the condensate. The EBC provides a convenient sample of volatile substances and aerosols and reflects a summation of all areas of the lung that are ventilated.

**Exhaled Breath Condensate Analysis in COPD**

Several studies have been carried out on EBC obtained from patients with COPD. While several potential clinical applications have been explored including the ability of biomarkers to differentiate between health and disease in smokers, diagnostic specificity
for COPD, assessment of severity, phenotyping, and for evaluation of therapeutic intervention, most of the studies have been limited by small sample sizes and lack of standardised methods of collection of EBC and its analysis. The EBC analysis has the potential to offer clues to diagnosis of infectious agents and may allow risk stratification. Although most of the studies have been in stable patients, acute exacerbations of COPD (AECOPD) are characteristically associated with increased airway inflammation and oxidative stress. Therefore, EBC analysis may also be used for predicting the outcomes and monitoring the course of an exacerbation. However, such studies have been few. Clear clinical indications and utility of EBC analysis in COPD in research studies have not been defined.

EBC studies on some of the more commonly studied biomarkers in patients with COPD are reviewed below.

8-isoprostane

One of the major products of oxidative stress in the lungs is 8-isoprostane. It is a prostaglandin-F(2α) isomer that is formed in vivo by free radical-catalysed peroxidation of arachidonic acid, independent of the cyclooxygenase pathway. This has been one of the most studied biomarker in patients with COPD.

The 8-isoprostane concentrations were observed to be similar in ex-smokers and current smokers with COPD, were increased about 1.8-fold compared to healthy smokers who, in turn, had 2.2-fold higher levels than healthy non-smokers. The 8-isoprostane was similarly reported to be significantly increased in patients with COPD compared to smokers. These results were corroborated in other studies.

While levels of 8-isoprostane in EBC have consistently been found to be increased in patients with COPD, these have usually been lower than those observed in induced sputum or BALF. This was true for all groups of subjects: non-smokers, healthy smokers, symptomatic smokers at risk for COPD and patients with AECOPD. Similarly, in BALF too, 8-isoprostane was found present in significantly higher concentrations than in EBC. Both correlated well with levels of leukotriene B4 (LTB4) in patients with COPD.

The relationship between EBC levels of 8-isoprostane and lung function parameters, and with other measures of severity of COPD has been investigated but found to be inconsistent and variable in different studies. In one study, levels of 8-isoprostane did not differ significantly across GOLD stages of COPD. Further, no correlations were observed between levels of 8-isoprostane and FEV₁, neutrophil count, and dyspnea scores. In another study, the increase in 8-isoprostane levels in EBC was found irrespective of the lung function impairment.

However, there was a significant correlation with emphysema scores on high resolution computed tomography (HRCT) and with Medical Research Council dyspnea scale scores. Levels of 8-isoprostane in EBC were found to be significantly lower in patients with emphysema than in patients with predominantly chronic bronchitis and correlated significantly with diffusion capacity but not with FEV₁. On the other hand, inverse correlations were observed between 8-isoprostane levels and lung function parameters in another study. Again, a recent study did not find any relationship between 8-isoprostane levels and lung function parameters but a significant positive correlation was found with the dyspnea grade. Severity-related differences in 8-isoprostane were identified according to the body mass index (BMI), obstruction, dyspnea, and exercise (BODE) index in another recent study. The last study also examined the relationship between oxidative stress and pathophysiology in COPD. In stable patients, blood oxygen levels and dynamic hyperinflation were reported related to airway 8-isoprostane levels in EBC. Concentration of 8-isoprostane were higher in patients who developed dynamic hyperinflation. End-expiratory lung volume change and partial pressure of arterial oxygen (PaO₂) independently predicted 8-isoprostane levels.

Phenotyping of COPD offers scope for an individualised approach to treatment besides identifying patients groups with different prognosis and natural history. Till now, the approach to phenotyping has been based mainly on clinical, radiological and physiological characteristics. The EBC analysis also has the potential to serve this purpose. Some studies have evaluated this potential but with opposing results. Levels of 8-isoprostane in EBC were found to be significantly lower in patients with emphysema than in patients with predominantly chronic bronchitis in one study but found to have a significant correlation with HRCT emphysema scores in another study.

The studies examining 8-isoprostane levels in EBC and their significance are summarised in table 1. In general, these studies have found patients with COPD to have higher or similar levels as current smokers and both have higher levels than non-smokers. Thus, it clearly marks oxidative stress in the airways and appears to be a useful parameter. The correlation with spirometry and severity of the disease as assessed by lung function or symptoms is modest or inconsistent. This is not surprising considering that COPD is a disease with multiple dimensions and lung function has usually been found to have modest or poor relationship with other measures of severity. Whether the oxidative stress has a consistent and predictable relationship with clinical severity is yet to be established and requires
further investigation. No study has evaluated changes in response to any therapeutic intervention. Thus, 8-isoprostane remains a promising biomarker in EBC in COPD but requires further evaluation before its utility as a clinical tool can be established.

**Hydrogen Peroxide (H$_2$O$_2$)**

In COPD patients, ROS may be produced endogenously by activated inflammatory cells including neutrophils, macrophages, and eosinophils, or exogenously, by exposures to air pollutants or cigarette smoke that act directly or by causing neutrophil influx. Even a single cigarette has been shown to cause a significant increase in EBC H$_2$O$_2$ in healthy non-smokers. Airways H$_2$O$_2$ is produced by superoxide dismutase-mediated conversion of superoxide anions. Being soluble, it appears in the exhaled breath. Therefore, H$_2$O$_2$ can serve as a direct marker of oxidative stress. The H$_2$O$_2$ measurements in EBC have been carried out in several studies in COPD.

Levels of H$_2$O$_2$ were found increased in EBC of stable patients with COPD compared to healthy controls. Patients with AECOPD had even greater levels than stable patients suggesting that oxidant production was increased further during exacerbations. While confirming increased H$_2$O$_2$ in stable COPD, it was found that patients who were current smokers did not exhale more H$_2$O$_2$ than those who were ex-smokers or those who had non-smoking COPD. Both these studies show that cigarette smoking made no difference to the EBC H$_2$O$_2$ levels and the oxidative stress was a feature intrinsic to COPD irrespective of the cause.

In another study, the increased levels of EBC H$_2$O$_2$ showed a positive correlation with the levels of sputum neutrophils, indicating a neutrophil-dependent mechanism for its production. The levels of H$_2$O$_2$ in severe and moderate COPD were significantly higher than in mild disease. H$_2$O$_2$ had significant correlations with FEV$_1$ and with dyspnoea scores in patients with moderate and severe disease.

### Table 1. Summary of studies on 8-isoprostane in EBC in patients with COPD

<table>
<thead>
<tr>
<th>Author and Year</th>
<th>Study Population</th>
<th>Results</th>
</tr>
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<tbody>
<tr>
<td>Montuschi et al$^{19}$ 2000</td>
<td>COPD ex-smokers (n=25) COPD current smokers (n=15) Control non-smokers (n=10) Smokers (n=12)</td>
<td>COPD ex-smokers and current smokers did not differ; Levels in patient with COPD increased 1.8-fold over healthy smokers; smokers had 2.2-fold higher levels than non-smokers; Smoking caused an acute increase in exhaled 8-isoprostane by about 50%; No correlation with severity of airways obstruction or dyspnoea</td>
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<tr>
<td>Biernacki et al$^{24}$ 2003</td>
<td>COPD acute exacerbation (n=21) before and after treatment</td>
<td>Levels were increased during exacerbation, decreased on treatment at 2 weeks and further at 2 months</td>
</tr>
<tr>
<td>Kostikas et al$^{9}$ 2003</td>
<td>COPD (n=30) Smokers, Stage 0 (n=10)</td>
<td>Levels were significantly elevated in patients with COPD; No significant difference across severity groups; No correlation with lung function and dyspnoea grades</td>
</tr>
<tr>
<td>Izquierdo et al$^{10}$ 2006</td>
<td>COPD (n=39) Control smokers (n=15)</td>
<td>Levels were significantly lower in patients with emphysema than in patients with chronic bronchitis or in the controls; correlated significantly with DLCO/VA but not with FEV$_1$</td>
</tr>
<tr>
<td>Ko et al$^{20}$ 2006</td>
<td>COPD ex-smokers (n=32) Control non-smokers (n=17)</td>
<td>COPD patients had higher levels compared to controls; Levels increased across the groups with worsening FEV$_1$</td>
</tr>
<tr>
<td>Makris et al$^{14}$ 2008</td>
<td>COPD patients (n=18) Control ex-smokers (n=5) Non-smokers (n=7)</td>
<td>Levels were significantly elevated in COPD; Levels correlated with emphysema score in HRCT and with dyspnoea scores</td>
</tr>
<tr>
<td>Mazur et al$^{12}$ 2009</td>
<td>COPD acute exacerbation (n=10) Control non-smokers (n=14) Healthy smokers (n=17) Symptomatic smokers (n=9)</td>
<td>In induced sputum, levels were at least 10-fold higher compared to EBC levels; Healthy non-smokers had the lowest levels and patients with AECOPD, the highest levels in EBC; Inverse correlations with lung function parameters</td>
</tr>
<tr>
<td>Inonu et al$^{11}$ 2011</td>
<td>COPD (n=25) Control smokers (n=26) Non-smokers (n=29)</td>
<td>COPD and smokers did not differ; Both had significantly higher levels than non-smokers; No correlation with lung function parameters but significant positive correlation with dyspnoea grade</td>
</tr>
<tr>
<td>García-Rio et al$^{15}$ 2011</td>
<td>COPD (n=76)</td>
<td>Significant severity-related differences in levels according to the BODE index; Levels were higher in those who developed dynamic hyperinflation; Hyperinflation and PaO$_2$ predicted levels on multivariate regression</td>
</tr>
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</table>

EBC=Exhaled breath condensate; COPD=Chronic obstructive pulmonary disease; FEV$_1$=Forced expiratory volume in one second; HRCT=High resolution computed tomography; AECOPD=Acute exacerbations of COPD; BODE Index=BMI, obstruction, dyspnoea, and exercise index; PaO$_2$=Partial pressure of arterial oxygen
were observed to have similar levels of $H_2O_2$ in EBC. There was no correlation between $H_2O_2$ levels and lung function parameters.\textsuperscript{11}

There are uncertainties about the origin of $H_2O_2$ released in the lung. Fractionated samples of EBC from the airways and from the lung periphery revealed 2.6 times higher $H_2O_2$ in the former that was two-fold higher in smokers and five-fold higher in patients with COPD compared to non-smokers, suggesting that airways may be the dominant location of $H_2O_2$ production.\textsuperscript{20}

Some studies have looked at the effect of therapeutic interventions on $H_2O_2$ levels in EBC. A two-week treatment in stable patients with COPD with inhaled beclomethasone was not found to change EBC $H_2O_2$ significantly although exhaled NO was reduced.\textsuperscript{21} N-acetylcysteine, a precursor of reduced glutathione that together with glutathione peroxidase may remove $H_2O_2$, given for 12 months in stable COPD patients had no effect in the first six months but later reduced EBC $H_2O_2$ by about 2.5 fold.\textsuperscript{22} Patients with lower respiratory tract infection showed no significant changes in $H_2O_2$ concentrations in EBC on recovery and there were no significant correlations between with spirometry and serum inflammatory parameters. In contrast, several serum inflammatory markers did decrease during hospitalisation.\textsuperscript{23} On the other hand, patients with moderate to severe COPD during an exacerbation showed significant decrease in $H_2O_2$ concentrations in EBC suggesting that the oxidative stress markers like $H_2O_2$ are suitable in monitoring exacerbated COPD.\textsuperscript{7}

The studies examining $H_2O_2$ levels in EBC and their significance are summarised in table 2. From these studies, it may be concluded that $H_2O_2$ is a reliable marker of oxidative stress in patients with COPD and tends to reflect the severity of airway inflammation and clinical manifestations. Therefore, it has the potential to serve as a tool for assessment and monitoring of disease. Responsiveness to therapeutic intervention is not established.

### Cytokines and Eicosanoids

Considering the central role of airway inflammation in the pathobiology of COPD, cytokines that play a role in recruiting inflammatory cells, especially neutrophils and in activation of inflammatory cells and perpetuation of airway inflammation may be useful biomarkers in monitoring its severity.

| Table 2. Summary of studies on hydrogen peroxide ($H_2O_2$) in EBC in patients with COPD |
|---------------------------------|--|--|
| **Author** and **Year** | **Study Population** | **Results** |
| Dekhuijzen *et al*\textsuperscript{17} 1996 | COPD (n=12), COPD acute exacerbation (n=19) Controls (n=10) | Levels were highest in acute exacerbation followed by stable COPD, followed by controls |
| Nowak *et al*\textsuperscript{18} 1998 | COPD ex-smokers/smokers/non-smokers (n=32) Control non-smokers (n=17) | Levels were higher in COPD than controls; No difference between smoker and non-smoker COPD patients |
| Nowak *et al*\textsuperscript{19} 1999 | COPD ex-smokers/smokers/non-smokers (n=44) Control non-smokers (n=17) | Levels were higher in COPD than controls; No difference between smoker and non-smoker COPD patients |
| Ferreira *et al*\textsuperscript{21} 2001 | COPD non-smokers (n=20), before and after 2-week treatment with inhaled beclomethasone 500 μg twice daily | 2-week treatment with inhaled beclomethasone did not change $H_2O_2$ significantly |
| Kasielski and Nowak\textsuperscript{22} 2001 | COPD (n=22), before and after N-acetylcysteine, 600 mg once a day for 12 months | No effect of N-acetylcysteine in the first six months but later reduced $H_2O_2$ by about 2.5 fold |
| van Beurden *et al*\textsuperscript{23} 2003 | COPD (n=25) with lower respiratory tract infection | No significant changes in $H_2O_2$ concentration after treatment of infection |
| Kostikas *et al*\textsuperscript{24} 2003 | COPD (n=30), Smokers, Stage 0 (n=10) | Levels were significantly elevated in COPD patients; severe and moderate COPD had significantly higher levels than those with mild disease; significant correlation with lung function, induced sputum neutrophil counts and dyspnoea scores in moderate and severe COPD |
| Inoue *et al*\textsuperscript{25} 2011 | COPD (n=25), Healthy smokers (n=26) Non-smokers (n=29) | COPD and smokers did not differ; Both had significantly higher levels than non-smokers; no correlation with lung function parameters or dyspnoea |
Infective exacerbations of COPD may be associated by infections which are also responsible for an increased concentration of other inflammatory mediators, for example, IL-8, IL-6, endothelin-1 and tumour necrosis factor-alpha (TNF-α) levels in EBC during these exacerbations.29 Chemokines related to neutrophil and monocyte inflammation (growth-related oncogene alpha [GROalpha] and monocyte chemoattractant protein-1 [MCP-1]) have been studied in EBC of COPD patients but found to have no utility in diagnosis or assessment of the severity.30

**EBC Acidification**

Studies in asthma have pointed to the role of airway acidification in the airway pathophysiology. It may cause bronchoconstriction, impaired ciliary motility, increased mucus production and viscosity, and airway epithelial damage.31 The EBC pH is easy to measure and therefore is a potential biomarker in airway inflammatory diseases. Exhaled carbon dioxide may be a potential confounder especially in patients with acute or chronic hypercapnoeic respiratory failure in AECOPD and stable COPD, respectively.

A comparison of asthmatics and mild COPD patients with healthy controls did not reveal significant differences.32 However, another study33 found lower pH in more severe COPD patients. Other workers reported that EBC pH was lower in COPD patients compared to asymptomatic normal smokers. It was related to disease severity, and to parameters reflecting airflow limitation, hyperinflation and air trapping.34 It was also observed that EBC pH was lower in ex-smokers than current smokers suggesting a probability of the presence of more severe disease in the former.

In a more recent study,35 EBC pH was found to be lower in patients with COPD and in smoking controls compared with non-smoking controls but was not different between COPD and smoking controls. It was not related to severity of airways obstruction or to airway inflammation assessed by sputum leukocyte counts, and was not responsive to corticosteroids. Its utility as a biomarker was questioned.35

**Other Biomarkers**

Besides the biomarkers discussed above, other workers have measured other compounds in EBC with a view to understand the nature of the inflammatory process and identify clinically useful substances.

Malondialdehyde (MDA) is a product of lipid peroxidation and has long been considered as a marker of oxidative stress. It is measured as thiobarbituric acid-reactive substances (TBARs), which are the end-products of lipid peroxidation.
Paitients with stable COPD exhibited increased lipid peroxidation, measured as TBARs, that were not found to correlate with cigarette smoking status. Two recent studies have differed in their observations. Levels of MDA were not found to be different in patients with COPD and non-smokers in one study. However, in the other, MDA was found to be significantly higher in patients with COPD compared to asthmatics and healthy controls. Further, COPD patients showed an inverse correlation between MDA concentrations and FEV₁,%. Among other biomarkers under investigation in COPD are purines that are present on airway surfaces in physiologically significant concentrations and are proposed to play a role in airway inflammation. These were found to be increased in EBC from patients with COPD, correlating with GOLD severity and FEV₁,% predicted.

**Products of Nitric Oxide**

Nitric oxide (NO) is perhaps the most extensively studied marker of airway inflammation in exhaled air. Its utility is fairly well established in asthma. However, in COPD, its status is still evolving. While larger airways are likely the primary source of exhaled NO in asthma, that in COPD appears to originate in the peripheral airways. NO lead to formation of nitrite, nitrate, and S-nitrosothiol in the epithelial lining fluid. The compounds have been measured in EBC in a few studies.

Increased levels of S-nitrosothiols have been demonstrated in EBC of patients with COPD. Liu et al measured nitrite/nitrates (NOx) levels in EBC in COPD patients and reported no significant differences in comparison with healthy non-smoking controls. Further, corticosteroid treatment was not found to have any effect on the levels.

**TECHNICAL ISSUES IN EXHALED BREATH CONDENSATE ANALYSIS**

The EBC is usually collected by asking the subject to breathe tidally using a mouthpiece and a non-rebreathing valve to separate inspiratory and expiratory air streams. The expired air is passed through a condenser, which is cooled to 0 °C or –20 °C in a refrigerated circuit. The resultant condensate is collected into a cooled vessel. It usually takes between 10 and 15 minutes to obtain 1 to 3 mL of condensate. The EBC is then stored at –20 °C or –80 °C till further analysis of the biomolecules.

Methodological issues that may influence results are important and were recently reviewed. These include techniques and procedures of collection and storage, analytical methods, inherent variation of EBC biomarkers over time, and other factors affecting EBC composition, such as smoking and demographics. These contribute to the differences and variations among different studies reviewed above. The lack of standardisation of the EBC analysis in the earlier studies makes comparison of studies difficult. Analytical methods are being improved to increase their sensitivity. To address the need for standardised methodologies of EBC analysis, American Thoracic Society/European Respiratory Society (ATS/ERS) Task Force has published methodological recommendations regarding the use of EBC.

Besides self-fabricated collection systems, the most commonly used commercially available devices for EBC collection are the portable R Tube (Charlottesville, Virginia, USA) and non-portable EcoScreen (Jaeger, Wuerzburg Germany). pH measurements in EBC collected by these two systems are repeatable and reproducible.

Use of different methods of collection has been found to give different results that do not follow a uniform pattern. From the comparative studies so far, it appears that the possible differences between devices appear to vary with the biomarker measured and with the presence or absence of the disease. The physical and chemical properties of the surface of the collecting device may affect the yield by different capacities for adsorption.

The effect of differing expiratory flow rates has not been examined in patients with COPD. The differences between normals and patients may arise because patients have a different breathing pattern. Whether expiratory flow rate and tidal volume need to be controlled has not been addressed in studies in COPD. Theoretically, hyperventilation, forced exhalation, greater turbulence in more severe airways obstruction or larger tidal volumes should yield higher concentrations of the EBC markers by favouring greater aerosolisation of airway lining fluid. In one study, in patients with asthma, EBC H₂O₂ was shown to vary inversely with the expiratory flow rate during collection. However, the pattern of breathing had no significant effect on concentrations of LTB4, LTE4, prostaglandin (PG) E₂, pH, NO₂⁻ or total protein. The EBC collection is invariably done using oral breathing. The nasal and oral contamination while collection may change the pH, NO metabolites and leukotrienes like LTB₄. One concern has been oral contamination with ammonia that can affect EBC pH measurements. However, studies have shown that this is unlikely to have any significant effect.

Oral contamination may also affect EBC NO metabolites. Nitrate levels in EBC are influenced by dietary intake. Nitrate is reduced to nitrite by bacterial activity that takes place primarily in the oropharyngeal tract of healthy subjects.
Oropharyngeal nitrite possibly contributes to exhaled NO in non-inflamed airways. The LTB4 levels have been reported to be raised in EBC samples that contained salivary amylase. Therefore, the contribution of oral contamination should be investigated with respect to each specific EBC mediator.

The concentrations of mediators measured in the EBC are likely to be affected by the water vapour content and variable degrees of aerosolisation. These may not reflect the true levels in the airway lining fluid. These factors may explain the differing levels reported for different mediators even in normal non-smoking controls as well as the fairly wide within-session variations. Possible solutions to such sources of error include corrections by dilutional factors estimated from another consistent constituent of EBC or report ratios of mediators rather than absolute levels. These approaches need validation. As most specimens are stored and kept frozen at −20 °C or −80 °C till analysis, the levels of markers may be affected. However, this has not been found to be true for most markers for storage periods up to 2 or 3 months.

The extent of nasal contamination as well as the effect of using a nose-clip to occlude the nose is largely unknown in patients with COPD. Whether a nose clip should be worn is not certain. However, the reproducibility of EBC pH (see below) was similar, with and without a nose clip.

The acute effect of smoking is an increase in the oxidative stress. Smoking was shown to cause an acute increase in exhaled 8-isoprostane in COPD patients by about 50%. In normal subjects, acute smoking was found to increase levels of H2O2. On the other hand, the acute effect of smoking a single cigarette on other EBC compounds such as NO3, nitrosothiols, nitrotyrosine and certain cytokines is variable or inconsistent.

The ATS/ERS Task Force methodological recommendations on EBC include the following general suggestions to ensure uniformity in methodology among studies: collect during tidal breathing using a nose clip and a saliva trap; define cooling temperature and collection time (10 min is generally sufficient to obtain 1-2 mL of sample and is well tolerated by patients); use inert material for condenser; do not use resistor and do not use a filter between the subject and the condenser. In addition, specific recommendations on methodology of collection, storage and analysis have been made for individual biomarkers.

Several other potential confounders including age, sex, diet, drugs and race as well as other biological factors such as circadian fluctuations may influence results. The available information on the effect of these factors on EBC composition is scanty. The EBC pH was found not to be influenced by age.

collected EBC seven times every 4 hour during 24 hours and three times every 7 days during two consecutive weeks and observed diurnal variation in H2O2 levels with two-peak values at 12:00 and 24:00 hour. The mean H2O2 concentration estimated over the whole two-week period was higher in patients above 40 years regardless of smoking habit and it positively correlated with age in never-smoked subjects. Neither moderate exercise nor one puff of salbutamol nor ipratropium influenced significantly the concentration of H2O2 and TBARs in EBC. The mean H2O2 concentration was found to be increased significantly during the day in both the COPD patients and controls. The effect of diet on nitrate levels was discussed above.

The large number of measurable biomarkers and the diversity of the methodologies hamper the reproduction of wide clinical application and development of any consensus. Koutsokera et al have summarised the ranges of concentrations of different biomarkers include H2O2, NO-related products, arachidonic acid metabolites and pH in the studies that have been carried out so far. The only one marker with established reference values in healthy subjects is EBC pH, whereas the others need further refinement and standardisation of the methodologies. Until the technical aspects of measurements are sorted out, EBC analysis is likely to remain confined to research studies. Even though it appears to be a very promising tool, its adoption as a clinical tool in near future is unlikely.

Reproducibility of Exhaled Breath Condensate

Variations on repeated measurements are an important consideration in deciding the utility of an assay. These variations may be natural or biological or may simply reflect the effect of technical factors reviewed above. Variations in any assay must be known before significance can be assigned to changes observed in response to changed clinical status or therapeutic intervention. A change with any intervention should be shown to be greater than spontaneous variability to attribute it to the beneficial effect of treatment. Variations may be within-assay (several measurements performed during the single assay) or short term (within a day) or over a longer term (over a few days) in a stable condition. As airway inflammation is likely to be variable over time due to variations in environmental exposures, infections and disease-related factors, normal subjects can be expected to show much greater stability compared to patients and any variability in the former is likely to reflect the imprecision in the assay that may be an inherent limitation in the method or may be influenced by other technical factors.

Van Hoydonck et al concluded that levels of 8-isoprostane and H2O2 cannot be reproducibly
assessed in EBC from healthy smokers because of their low concentration and/or the lack of sensitivity of the available assays. Within-assay coefficient of variation of EBC 8-isoprostane was 29.2% that was lower than the corresponding values for within-day (65.3%) and between-day (79.1%) variations. Using the Bland Altman method, there were wide limits of agreement for within-day and between-day reproducibility. While within-assay variability and group mean changes were small, considerable within-day and between-day variability raises questions about its suitability for monitoring and assessment of response to therapeutic interventions. The results were similar with LTB4. In contrast, pH was reported to have a much better stability—within-assay, within-day, and between-day. The EBC pH was observed to be robust and reproducible and not affected by several patient-related and technical factors in health. The H$_2$O$_2$ has been shown to have had a better stability and repeatability compared to 8-isoprostane in patients with COPD. However, it needs to be noted that all the studies looking at precision of measurements over time had small sample sizes. An important consideration in looking at repeatability and reproducibility of measurements is the statistical method used. Inappropriate methods may underestimate variation. Correlations and comparisons of group means can hide vital differences and agreement analysis is the preferred method to look at repeatability. The issue of short-term and long-term stability needs to be addressed for several other mediators, in health and disease, and the minimal clinically important change remains to be defined.

**Safety Issues**

The studies in patients with COPD have used different EBC collection devices and in none of these any adverse effects have been reported. A major concern is the possibility of transmission of infection between individuals. Filters cannot be used in the inlets of the collecting devices as these may absorb some of exhaled substances. Use of disposable systems is certainly an effective way of preventing any such transmission of infection between patients but this adds to the total costs. Use of non-disposable EBC collection systems, however, was not shown to facilitate transmission of infection in EBC studies in patients with cystic fibrosis.

**CONCLUSIONS**

Exhaled breath condensate analysis is an evolving non-invasive tool for monitoring of inflammation and oxidative stress in COPD. It may provide an insight into the complex pathways of the origin and perpetuation of airway inflammation in COPD. Potential clinical applications include diagnosis, monitoring of severity and evaluation of therapeutic interventions, phenotyping and prognostication. While studies so far have focused on measurements of levels of markers such as 8-isoprostone, hydrogen peroxide, products of NO, lipid mediators, eicosanoids and airway fluid acidification, this method of studying airway inflammation is still in its infancy. Evolving technologies of genomics, proteomics, and metabonomics may provide deeper and newer insights into the molecular mechanisms underlying the pathogenesis of COPD and identify targets for therapeutic interventions permitting specific interventions unlike the present treatment strategies that are largely symptomatic and supportive.
REFERENCES


