

Clinical use of exhaled biomarkers in COPD

Philip O'Reilly
William Bailey

Division of Pulmonary and Critical
Care Medicine, University of Alabama
at Birmingham, Birmingham, AL, USA

Abstract: Exhaled breath analysis holds great promise as a diagnostic and investigative tool in COPD and is a new and rapidly expanding field of research in pulmonary disease. Generally speaking, exhaled breath analysis focuses on two areas: measurement of exhaled nitric oxide (ENO) and the detection of biomarkers in exhaled breath condensate (EBC). ENO measurement may not be as useful in COPD as in other pulmonary diseases, such as asthma, due to the lower levels of ENO found in COPD, although this is an area of ongoing research. Analysis of EBC for proinflammatory biomarkers is an area of great promise but its true value will not be realized until methods of collecting and analyzing EBC have been standardized. Once this is done, biomarkers detected in EBC may assist in the diagnosis of COPD, identification of preclinical disease, phenotyping of COPD patients, evaluation of response to therapies and defining the prognosis of individual patients. Identification of novel inflammatory mediators in EBC may cast new light on the pathogenesis of COPD and identify new therapeutic targets, which are badly needed in this disease.

Keywords: exhaled breath condensate, EBC, nitric oxide, ENO, COPD

Introduction

There is considerable interest in the use of biomarkers in chronic obstructive pulmonary disease (COPD) (Barnes et al 2006). Potential uses of biomarkers in COPD are many. Biomarkers could assist in the diagnosis of COPD, in dividing COPD patients into different phenotypic groups, in evaluating response to treatment and in defining the prognosis of individual patients. Biomarkers could also be a powerful research tool in COPD, identifying novel pathways of COPD pathogenesis. However, the measurement of biomarkers in COPD is difficult for several reasons. Biomarkers detected in serum or urine may not represent events in the lung but the lung itself is difficult to sample directly. Traditional methods of sampling the lung include bronchoalveolar lavage (BAL), transbronchial biopsy and sputum analysis, which are costly and involve risks to subjects. Sputum analysis requires numerous processing steps to remove mucus and other debris which may affect the detection of volatile intermediates. In contrast, exhaled breath analysis is safe, non-invasive and inexpensive and requires little or no processing. It is simpler than induced sputum collection, involving no more than tidal breathing into a collection device.

COPD is a difficult disease to study clinically. Commonly used endpoints in clinical trials, such as exacerbations or decline in forced expiratory volume in one second (FEV₁) are infrequent or change little over time. Clinical trials of interventions in COPD must enroll hundreds of patients and continue for years in order to demonstrate differences between control and treatment groups. For this reason, identification of biomarkers, that could be used as surrogate endpoints in clinical trials, has been identified as a priority by the National Institutes of Health (Croxtan et al 2002). Although the diagnosis of COPD is usually clear, distinguishing COPD from other obstructive diseases, particularly asthma, can be difficult. The presence of irreversible airflow obstruction is central to the definition of COPD but many COPD

Correspondence: Philip O'Reilly
Division of Pulmonary and Critical Care
Medicine, 215 THT, 1900 University
Boulevard, University of Alabama at
Birmingham, AL 35294-0006, USA
Tel +1 205 934 5400
Fax +1 205 934 1446

patients demonstrate significant reversibility of their airflow obstruction with inhaled bronchodilators and the degree of reversibility may change considerably between evaluations (Calverley et al 2003; NHLBI 2005). Patients with asthma can also develop irreversible airflow limitation (NAEPP 2006). For these reasons, bronchodilator reversibility testing cannot reliably distinguish asthma from COPD and there is no diagnostic test that can distinguish between asthma and COPD in all cases. New diagnostic tests, involving biomarkers of inflammation, might aid in distinguishing between these two diseases by detecting differences in airway inflammation between patients with asthma and COPD (Fabbri et al 2003). Biomarkers of airway inflammation in exhaled breath might aid in the early diagnosis of COPD before symptoms or changes in spirometry are present and may help to identify those smokers at risk of developing the disease. This would be a significant advance as most people with COPD are diagnosed late in their disease, when available therapeutic and preventive measures are limited. Biomarkers could help identify different phenotypes of COPD patients who might respond differently to therapeutic interventions such as inhaled corticosteroids and long-term oxygen therapy (Croxtton and Bailey 2006). Identification of novel biomarkers in exhaled breath could cast light on the inflammatory pathways important in COPD pathogenesis and identify potential targets for new therapies.

Identification of biomarkers in exhaled breath in lung disease has generally involved two different approaches: measurement of exhaled nitric oxide (ENO) and identification of water-soluble biomarkers in exhaled breath condensate (EBC). Of these two approaches, ENO analysis is the more studied and better standardized and is approaching clinical use in the diagnosis and management of chronic lung diseases.

Use of ENO in COPD

Nitric oxide (NO) is a gaseous free radical which is important in many biological processes in lung physiology and is produced by many different cell types, including epithelial cells, endothelial cells, neurons and inflammatory cells in the lung (Gaston et al 1994). NO is generated in cells by nitric oxide synthases of which there are three isoforms (Moncada et al 1991). Chronic, low-level production of NO in the lung by epithelial cells and endothelial cells causes bronchodilation and vasodilation by directly causing smooth muscle relaxation. NO acts as a neurotransmitter in the non-cholinergic, non-adrenergic nervous system in the lung which mediates bronchodilation. It is also produced by inflammatory cells in

the airways, particularly macrophages, where it is important in the generation of reactive species important in pathogen-killing (Hickman-Davis et al 2002) and tissue injury in inflammation (Haddad et al 1994). The nitric oxide synthase found in inflammatory cells is inducible by pro-inflammatory cytokines. This means that large amounts of NO may be produced in the airways in inflammatory lung diseases and may be measured in exhaled breath. NO can be easily measured in exhaled air in patients with asthma where levels in excess of 100 ppb (parts per billion) can be measured compared to <20 ppb in healthy controls. ENO correlates with asthma activity and severity and has been used as part of a chronic asthma management program in children (Smith et al 2005). ENO is quickly and reproducibly measured by tidal breathing into a meter where ENO levels are instantaneously measured by chemiluminescence. Methods for the measurement of ENO have been standardized and published by the American Thoracic Society (ATS 1999). This has enabled ENO analysis to become a widely adopted clinical and research tool in respiratory disease.

The situation in COPD is less clear-cut. Several studies have demonstrated higher levels of ENO in COPD patients compared to controls. In these studies, ENO has been demonstrated to correlate with disease severity in COPD, to increase further during exacerbations and to decline after treatment with inhaled corticosteroids (Maziak et al 1998; Agusti et al 1999; Corradi et al 1999; Montuschi et al 2001). Other studies of ENO in COPD have not demonstrated elevated levels compared to controls (Clini et al 1998; Rutgers et al 1999). ENO has also been demonstrated to correlate both positively (Maziak et al 1998; Corradi et al 1999) and negatively (Clini et al 1998) with FEV₁ in COPD, perhaps reflecting its dual role in the lung as a pro-inflammatory molecule and bronchodilator. These conflicting results may be due to a number of factors. Elevation of ENO in COPD is modest and not at all of the magnitude seen in asthma. Studies of ENO in COPD have involved small numbers of subjects and have employed different methods of measuring ENO. Some studies included current smokers who have much lower ENO levels than ex-smokers, perhaps due to reaction of NO in the airways with molecules present in cigarette smoke or to inhibition of NO synthases (Kharitonov et al 1995). Nonetheless, there are potential uses for ENO analysis in the management of COPD. ENO might aid in the differential diagnosis of asthma and COPD as much higher levels of ENO are found in asthmatics. Existing data suggest that COPD patients with eosinophilic airway inflammation or with greater bronchodilator reversibility have higher levels

of ENO (Chanez et al 1997; Papi et al 2000). Such patients might be more likely to respond to treatment with inhaled corticosteroids. ENO levels might both identify these patients and detect a response to treatment. COPD patients with pulmonary hypertension and cor pulmonale may have lower ENO levels, perhaps due to impaired endothelial release of this vasodilator mediator (Clini et al 2000), and this might help identify those COPD patients who would benefit from long-term oxygen treatment (Croxtton and Bailey 2006). Answering these questions will require studies involving larger numbers of ex-smoking COPD patients.

Use of EBC in COPD

EBC collection is a promising non-invasive method for sampling the lower airways. EBC collection simply involves tidal breathing into a chilled collection device. Water vapor in exhaled breath is condensed and collected and various mediators can be then be quantified in the condensate. Mediators of interest are thought to be derived from droplets of airway epithelial lining fluid (ELF) which are mobilized from the lower airways by convection during expiration (Effros et al 2002). Numerous inflammatory mediators have been detected in EBC in several lung diseases, including asthma, COPD and the acute respiratory distress syndrome. Mediators ranging in size from simple pro-oxidant molecules to proteins have been detected. Although EBC analysis is a promising technique, there are many methodological questions associated with it that have precluded its adoption as a widespread clinical test. Different investigators have used many different types of apparatus for collecting EBC including Teflon tubing, ice baths and convection cylinders. Before EBC analysis can become main-stream, a standardized method of collecting EBC will have to be adopted. Commercially produced EBC collection devices are becoming available that may assist this process, such as the R tube[®] (Respiratory Research) and Ecoscreen[®] device (Viasys) (Montuschi 2005). The question of dilution is also a significant problem. Respiratory droplets mobilized into exhaled air are diluted in the large volume of water already present in exhaled breath, which is water-saturated. The dilution factor is extremely large and been estimated to be as high as 10,000 fold (Effros et al 2002). This causes several problems, including the fact that mediators found in EBC are usually present at very low concentrations and detectable only at the very limits of available tests. This is particularly true for proteins which due to their size are less likely to become aerosolized in respiratory droplets. The degree of dilution may vary between subjects and between tests making standardization of this technique

extremely difficult. For example, respiratory parameters such as exhaled breath flow rate may affect the mobilization of respiratory droplets into exhaled air and greatly affect the final concentration of mediators measured in EBC. Addressing these issues will require standardization of EBC collection methodology as has been done with ENO. Recent guidelines for EBC collection issued by the American Thoracic Society and European Respiratory Society address these issues and represent an important step in this direction (Horvath et al 2005). It has been suggested that EBC mediator levels can be standardized by measuring levels of non-volatile cations in EBC using conductivity (Effros et al 2003). This is thought to reflect the degree of dilution of respiratory droplets as ELF cation levels are thought to remain relatively constant. However, in this approach, EBC samples are lyophilized to remove ammonia and this process may affect biomarker levels. These concerns notwithstanding, EBC analysis has proved to be a surprisingly robust technique for investigating lung diseases. In general, studies of EBC in COPD have shown clear differentiation between patient groups and controls, changes with therapy and good reproducibility, illustrating its potential in the investigation and management of lung diseases (Montuschi 2005).

Mediators measured in EBC in COPD have ranged from simple molecules such as hydrogen peroxide and hydrocarbons to larger molecules such as isoprostanes and cytokines. Perhaps the most studied EBC mediator in COPD is hydrogen peroxide, which is probably derived from superoxide, generated by neutrophils in the airways in COPD. Hydrogen peroxide is believed to be a biomarker for oxidative stress which is thought to be of importance in the pathogenesis of COPD (Repine et al 1997). Hydrogen peroxide levels have been demonstrated to be higher in EBC of COPD patients compared to healthy controls (Nowak et al 1999; De Benedetto et al 2000), to rise further during exacerbations (Dekhuijzen et al 1996) and to correlate with severity of COPD as measured using FEV₁ (Kostikas et al 2003). Current smoking increases hydrogen peroxide levels in EBC in healthy subjects (Nowak et al 2001) but does not appear to have this effect in stable COPD (Nowak et al 1999; Kostikas et al 2003). Hydrogen peroxide levels in EBC may, therefore, reflect underlying airway inflammation and be an appropriate biomarker for use in larger studies of COPD. Hydrogen peroxide levels in EBC were reduced by n-acetylcysteine, as anti-oxidant, in COPD patients, reflecting its use as a biomarker of oxidative stress (Kasielski and Nowak 2001; De Benedetto et al 2005). Inhaled corticosteroids were demonstrated to reduce EBC hydrogen peroxide levels in COPD

patients in one study (van Beurden et al 2003) but not in others (Ferreira et al 2001; Kostikas et al 2003). There is a need for larger studies to examine the usefulness of measuring hydrogen peroxide in EBC in COPD. Hydrogen peroxide would be an appropriate biomarker for airway inflammation in COPD as levels correlate with neutrophilic inflammation (Kostikas et al 2003), the predominant cell type in the airways in COPD. Hydrogen peroxide is easily measured in EBC using inexpensive chemical reagents.

The isoprostanes are prostaglandin-like compounds formed from the free radical-initiated peroxidation of arachidonic acid (Morrow and Roberts 1997). Isoprostanes are believed to be the most reliable biomarkers of oxidative stress in humans. They are chemically stable, are formed *in vivo* and are a reliable marker for lipid peroxidation which is a central feature of oxidative stress. Levels are present in detectable amounts in biological fluids, allowing the definition of a normal range (Roberts and Morrow 2000). One of the isoprostanes, 8-isoprostane, has been extensively investigated as a biomarker of oxidative stress in human disease including lung disease (Janssen 2001; Morrow and Roberts 2002). 8-isoprostane is a relatively abundant member of the isoprostanes and has been demonstrated to stimulate pulmonary vascular and bronchial smooth contraction *in vitro* (Kang et al 1993; Kawikova et al 1996). It may therefore be important in the pathogenesis of COPD. There have been several recent reports measuring 8-isoprostane in EBC in COPD. 8-Isoprostane levels have been demonstrated to be higher in EBC of COPD patients compared with healthy controls (Montuschi et al 2000; Kostikas et al 2003) and levels increase further during exacerbations (Biernacki et al 2003). However, there was no correlation between 8-isoprostane levels and pulmonary function measured as FEV₁ in patients with stable COPD (Montuschi et al 2000; Kostikas et al 2003). Cigarette smoking caused an acute increase in 8-isoprostane levels in EBC of healthy smokers but there was no difference in EBC 8-isoprostane levels between current and ex-smoking COPD patients (Montuschi et al 2000). 8-isoprostane levels in EBC may, therefore, reflect underlying airway inflammation and be an appropriate biomarker for use in larger studies of COPD. 8-Isoprostane may be measured in exhaled breath condensate using a well-validated commercially available enzyme immunoassay kit.

Numerous other inflammatory mediators have been detected in EBC in COPD (Montuschi 2005). Additional biomarkers of oxidative stress, such as aldehydes, have been detected (Rahman et al 2002; Corradi et al 2003) but are unlikely to be useful clinically due to chemical instability,

lack of specificity, contributions from dietary sources and lack of specificity and imprecision of the analytical methodology. EBC pH has been demonstrated to be lower in COPD patients compared with asthmatics and healthy controls and is thought to be a biomarker for airway inflammation (Kostikas et al 2002). Recent reports have described the detection of cytokines in EBC. Interleukin-6 was higher in EBC of COPD patients than healthy non-smokers (Bucchioni et al 2003). Growth-related oncogene alpha levels were lower in COPD patients than non-smoking controls and monocyte chemoattractant protein-1 levels were the same in both groups (Ko et al 2006). The significance of these data is uncertain and cytokines were detected in EBC in very low quantities (pg/ml). Nonetheless, it is exciting that macromolecules such as cytokines can be detected in unconcentrated EBC and illustrates the great potential of EBC analysis in COPD.

New directions – collagen degradation products

The hallmark of emphysematous COPD is alveolar destruction. Central to this process is the destruction of structural proteins in the lung by proteolytic enzymes, particularly matrix metalloproteases (MMP's), secreted by inflammatory cells which are initially attracted to the airways by cigarette smoke (Shapiro and Ingenito 2005). Mice deficient in MMP-12 (Hautamaki et al 1997) or neutrophil elastase (Shapiro et al 2003) do not develop emphysema despite long-term exposure to cigarette smoke and elastin degradation products have been detected in the urine of patients with COPD (Stone et al 1995). We have identified a novel tripeptide, with the sequence proline-glycine-proline (PGP), which is a collagen breakdown product and a neutrophil chemoattractant (Weathington et al 2006). We have detected PGP in sputum and BAL fluid (BALF) of some patients with COPD and cystic fibrosis but not in patients with asthma (Weathington et al 2006). We suspect that PGP may contribute to airway inflammation in chronic lung diseases with a large neutrophilic component, such as COPD and cystic fibrosis, and that PGP may be a biomarker for these diseases. Interestingly, only the COPD patients with emphysema detected on chest imaging were positive for PGP. Generation of PGP in the lung may therefore be a critical point marking the transition of COPD to an emphysematous phenotype. PGP may become a useful tool in distinguishing COPD from asthma and emphysematous from non-emphysematous COPD. PGP antagonists may be useful therapies for COPD directed at neutrophilic airway inflammation.

We have also detected PGP in unconcentrated EBC from some COPD patients using liquid chromatography and mass spectrometry as well as in EBC from a subset of healthy smokers (O'Reilly et al 2006). This means that PGP may enable the detection of preclinical COPD in at risk smokers many years before the development of symptoms or airflow obstruction. The combination of EBC collection and proteomic analysis by mass spectrometry holds great promise for the noninvasive analysis of PGP-containing peptides and other biomarkers in COPD. We have detected PGP in unconcentrated BALF and EBC of COPD patients in similar concentrations (200–300 pg/ml). As the dilution of ELF is much greater in EBC (as high as 10,000-fold) than in BALF (approximately 100-fold), this would suggest that PGP is preferentially concentrated in EBC compared with BALF. There may be a partitioning effect with EBC collection sampling larger airways where PGP is generated and BAL being more likely to sample smaller airways and alveoli where there is less PGP. This may help explain why EBC has been a robust tool for sampling inflammatory mediators in the airways, as is the experience of numerous investigators, despite the concerns over dilution and reproducibility.

Conclusion

The analysis of exhaled breath mediators in COPD is very much a work in progress. ENO measurement has found its greatest application in the management of asthma and may not be as useful in COPD due to the lower levels of ENO found in this disease. This reflects differences in airway inflammation between asthma and COPD which are still not fully understood. EBC analysis is an area of great promise despite the problems over dilution and reproducibility. Widespread adoption of this technique as a research or clinical tool must await the adoption of common standards and methodology. Crucially, the true potential of EBC will not be realized until we have identified novel biomarkers, detectable in EBC, that are of value in the diagnosis of COPD, identification of preclinical disease, phenotyping of COPD patients, evaluating response to therapies and defining prognosis of individual patients. Identification of novel inflammatory mediators, such as PGP-containing proteins, may cast new light on the pathogenesis of COPD and identify new therapeutic targets, which are badly needed in COPD.

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