

# Biomarkers in exhaled breath condensate: a review of collection, processing and analysis

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## Abstract

Exhaled breath condensate (EBC) is a potential rich source for countless biomarkers that can provide valuable information about respiratory as well as systemic diseases. EBC has been studied in a variety of diseases including allergic rhinitis, asthma, chronic obstructive lung disease, cystic fibrosis, lung cancer, and obstructive sleep apnea syndrome. Although numerous biomarkers have been discovered and studied in EBC, the methods of collection and biomarker detection have not been fully standardized. While leaving standardization methods up to individual labs for the present time is optimal for the continued discovery of new biomarkers in EBC, this decreases the reproducibility and generalizability of the findings. In this review we will discuss specific biomarkers studied in specific diseases as well as some of the related technical issues including collection, processing and analysis.

(Some figures in this article are in colour only in the electronic version)

## List of abbreviations

ALF	airway lining fluid	H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
AMP	adenosine monophosphate	ICS	inhaled corticosteroids
ATP	adenosine triphosphate	IFN	interferon
ATS	American Thoracic Society	IgE	immunoglobulin E
CF	cystic fibrosis	IL	interleukin
COPD	chronic obstructive pulmonary disease	IRB	institutional review board
Cr	chromium	LT	leukotriene
Cyst-LT	cysteinyl leukotriene	MDC	macrophage-derived chemokine
EBC	exhaled breath condensate	NO	nitric oxide
ECLIPSE	evaluation of COPD longitudinally to identify predictive surrogate end-points	NO <sub>x</sub>	nitric oxide metabolites
EPO	erythropoietin	OSAS	obstructive sleep apnea syndrome
FE <sub>NO</sub>	fractional exhaled nitric oxide	PG	prostaglandin
FEV <sub>1</sub>	forced expiratory volume in 1 s	TGF	tumor growth factor
GERD	gastroesophageal reflux disease	TNF	tumor necrosis factor
		TX	thromboxane

## Background

Biomarkers are substances used as indicators of a biologic state—normal or abnormal. In medicine, biomarkers are used

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to detect disease states. Detection indicates a change in the expression of a biomarker that has been found to correlate with a risk or progression of a disease or with a susceptibility of the disease to a given treatment. In respiratory disease, biomarkers are used to reflect disease processes occurring in the lungs. Biomarkers can be detected in lung tissue, bronchoalveolar lavage, sputum, peripheral blood, urine, exhaled gases and exhaled breath condensate. Physicians use these biomarkers to diagnose and monitor a variety of lung diseases.

Breath analysis, a non-invasive technique, is promising for biomarker detection. Minimally invasive procedures are ones performed with the least amount of damage to surrounding structures. The number of minimally invasive procedures performed has steadily increased in medicine, leading to greater success in the evaluation and treatment of a variety of diseases. Similarly, the field of breath research, a novel non-invasive method of examining the airways, has taken off in the medical community and is being used for diagnosing diseases and monitoring response to treatment.

In the past, invasive tests like lung biopsies were the only way to investigate the lungs and lower airways. Breath monitoring has emerged as a simple way to learn about airways. Nitric oxide (NO), found in exhaled breath, is an established biomarker for lung disease; fractional exhaled NO (FENO) is already being used to make medical decisions regarding the diagnosis and treatment of diseases, particularly asthma [1, 2]. Like spirometry and lung function tests, however, FENO may only tell part of the story of what is going on at the level of the airways. Exhaled breath condensate (EBC), another method of breath monitoring, is a technique that may provide more information about what is happening at the level of the airways.

EBC is more than a biomarker: EBC is a matrix in which countless biomarkers may be identified, similar to those found in blood, urine and the gases found in exhaled breath. EBC is obtained as breath is exhaled from the lungs into a cooled collecting device, thereby condensing the vapor and aerosolized droplets emerging with the breath (figure 1) [3]. All nonvolatile compounds found in EBC originate in the airway lining fluid (ALF) or are reaction products of volatiles that enter EBC from the gas phase. This totally non-invasive procedure has no influence on airway function or inflammation.

Guidelines were published by the American Thoracic Society (ATS) for EBC measurement in 2005 [3]. The task force reviewed the most recent studies using EBC in order to establish a consensus of guidelines for standardization of this novel method. Although numerous biomarkers have been discovered in EBC, each group has methods of EBC evaluation optimized for a specific biomarker. The task force concluded with the suggestion that each disease marker studied should be evaluated by the investigators involved. Leaving standardization methods up to individual labs for the present time is optimal for the continued discovery of new biomarkers in EBC but decreases the reproducibility of EBC as a technique.



**Figure 1.** Exhaled breath condensate schematic. As the individual inhales, air flows into the device, bypassing the cooling sleeve, as indicated by the white arrow. During exhalation air moves out through the cooling chamber as indicated by the black arrows.

### Factors effecting EBC collection

Many different methods exist for obtaining exhaled breath condensate; these methods are optimized to collect the mediator of interest. Most techniques are a modification of the most common method of EBC collection. Patients are asked to breathe tidally through a mouthpiece; this mouthpiece is connected to a collecting device that is cooled to 0 °C. Patients are usually asked to breathe through the device for anywhere from 10 to 30 min to obtain between 1 and 3 ml of condensate. Exhaled breath condensate is usually stored at -70 °C, where it can later be accessed for the detection of specific mediators. Groups that slightly alter the methods may increase the collection time to obtain more condensate or cool the collecting device to lower temperatures in order to preserve markers [4].

EBC has been applied to a variety of diseases including allergic rhinitis, asthma, chronic obstructive lung disease, cystic fibrosis, lung cancer and obstructive sleep apnea syndrome. We will discuss these diseases individually with specific attention to biomarkers that were studied in each particular disease.

### EBC in allergic rhinitis, asthma and COPD

Airway inflammation is the most studied process in EBC; inflammation is an underlying process common to allergic

rhinitis, asthma and chronic obstructive pulmonary disease. Allergic rhinitis is a clinical syndrome recognized by sneezing, nasal congestion and nasal itching; airway inflammation is involved in the pathogenesis of this condition. Similarly, asthma is a pulmonary disorder characterized by the triad of inflammation, bronchial hyperresponsiveness and reversible airway obstruction. EBC has also been used to learn about the most severe form of airway inflammation: chronic obstructive pulmonary disease (COPD). COPD is characterized by airflow limitation that is not fully reversible; this limitation is usually progressive and is associated with an abnormal inflammatory response of the lungs to noxious particles or gases. The field of non-invasive EBC monitoring has provided insight to the airways of asthmatics (table 1) [5]; EBC has also provided valuable information on the spectrum of allergic rhinitis, asthma and COPD (table 2).

Several biomarkers have been studied in these diseases including pH, nitric oxide metabolites, eicosanoids, isoprostanes, hydrogen peroxide, proteins and others. We will discuss each biomarker in detail.

### *pH*

Measuring EBC pH after deaeration for the removal of CO<sub>2</sub> is a validated technique [6, 7] that is a reproducible and relevant marker of disease [8]. A recent study suggests that this method of EBC measurement is the most reproducible method for both healthy and asthmatic subjects [9, 10]. Alteration of airway pH may serve as an innate host defense mechanism but, unregulated, it can lead to the pathological processes underlying inflammatory diseases. Acidification occurs in inflammatory processes throughout the body; it is reasonable to expect the same acidification with other inflammatory diseases like asthma [11, 12]. A decrease in the pH of airways causes bronchoconstriction [11], impairs ciliary motility [13], increases the viscosity of airway mucus [12] and damages airway epithelium [12].

The mean pH of healthy subjects is 7.7, with a normal range of 7.4–8.8 [14]. The pH of EBC collected from children with atopic dermatitis and asthma is lower than that of allergic rhinitis and healthy controls [15]. Patients with stable persistent asthma have lower EBC pH values than healthy controls; patients with severe asthma have more acidic EBC than non-severe asthmatics [6] (table 3). EBC pH values can also reflect acute exacerbations of asthma, which then normalize with anti-inflammatory therapy [7]. Airway acidification has also been found in asthmatic children [166] and children with allergic rhinitis and atopic dermatitis, suggesting that EBC pH may be a useful method for evaluating the progression of atopy to asthma [16].

Patients with COPD form more acidic EBC than patients with asthma and healthy controls (table 3) [6]. Unlike the response to treatment seen in asthma, no difference has been observed in EBC pH between patients with COPD who are treated with ICS and those that are steroid-naïve [6]. Borrill *et al* showed that EBC pH changes over time in patients with COPD, a phenomenon not seen in other inflammatory diseases of the lung [165].

Acid stress can also be used to understand the effect of environmental conditions on the airways. EBC pH is decreased in smoking asthma patients compared to non-smoking asthmatics [17]. Smoking asthma patients have features similar to the early stages of COPD [17].

Some studies, however, have found that EBC pH may not correlate with symptoms of inflammation, lung function, airway hyperresponsiveness or airway inflammation expressed by FE<sub>NO</sub> [18–20]. Although EBC pH has good repeatability in long-term assessments, EBC pH in asthmatics fluctuates more than in healthy subjects, leading to pH variability [10]. In addition, preliminary reports suggest that EBC pH may be influenced by environmental temperature and relative humidity [21], suggesting a need for larger investigational studies to standardize techniques. A study of EBC pH in COPD patients found that EBC acidification was affected by the balance of salivary acids and bases, suggesting the impact of another extrapulmonary condition on exhaled breath [22].

EBC has also been used to evaluate the influence of gastroesophageal reflux disease (GERD) on airway acidity in asthma [23]. EBC pH in asthma patients with GERD is lower than those in asthma patients without GERD (table 3) [23].

### *Nitric oxide (NO) and its metabolites*

NO plays an important role in inflammation and in the regulation of smooth muscle tone. NO levels increase in response to pro-inflammatory cytokines and oxidants [24]; this increase is detectable in the breath as fractional exhaled nitric oxide. As a free radical, NO reacts with oxygen to produce nitrogen oxides such as nitrates and nitrites. Total nitrite/nitrate (NO<sub>x</sub>) levels are elevated in patients with asthma when compared to healthy subjects; atopy is also known to increase nitrite/nitrate levels, but to a smaller degree than asthma [20]. EBC NO<sub>x</sub> levels decrease after the use of ICS in asthma, but not in COPD [25, 26]. Smoking decreases EBC NO<sub>x</sub> levels in patients with asthma, but not in COPD [26].

*Nitrite* levels in EBC are increased in COPD and may reflect the increased nitrative stress in the airways of COPD patients [27]. A strong relationship between EBC nitrite levels and hyperinflation measured by pulmonary function tests exists [28]. This suggests the possibility of using EBC nitrite levels as a biomarker for over-distension in the lungs.

*Nitrotyrosine* is formed when NO and superoxide anions create peroxynitrite, which can then react with tyrosine residues on proteins [29]. Nitrotyrosine is increased in the EBCs of steroid-naïve adults [30] and children with asthma [31]. Patients with asthma receiving oral steroids, however, have comparatively lower levels of nitrotyrosine in EBC, suggesting that systemic steroids may inhibit the inflammatory response in the airways and lead to a reduction in local oxidative stress [32, 33]. In COPD a significant negative correlation between FEV<sub>1</sub> and the amount of nitrotyrosine exists: as lung function declines measured by FEV<sub>1</sub>, the amount of oxidative stress measured by nitrotyrosine levels in EBC increases [34]. Recent work with more sensitive techniques of detecting nitrotyrosine in EBC, however, found that nitrotyrosine levels are variable and that this biomarker

**Table 1.** Major findings from studies relating to EBC in asthma. Adapted from Kostikas *et al* [5].

	pH	NO-related products	Prostanoids	Leukotrienes	8-Isoprostane	H <sub>2</sub> O <sub>2</sub>
Stable asthma	↓ [6, 20]	↑ NO <sub>x</sub> [74] ↑ nitrotyrosine [30, 31] ↑ S-nitrosothiols [27]	↔ PGE <sub>2</sub> [37–39, 41, 42] ↑ TXB2 [41]	↑ Cys-LTs [36, 38, 39, 54, 55, 57, 96, 140] ↑ LTE4 [38, 41, 42, 55, 60] ↑ LTB4 [30, 38, 41, 47, 55, 141]	↑ [6, 42, 67]	↑ [72–77]
Exacerbation	↓ [7]			↑ Cys-LTs [57, 58]	↑ [6, 67]	↑ [75]
Effect of smoking	↓ [17]	↓ NO <sub>x</sub> [25, 75]	↑ PGE2 [37]	↑ LTB4 [50]		↑ [74]
Effect of treatment	ICS ↓ [6, 7]	ICS ↓ NO <sub>x</sub> [25, 74]	ICS ↔ PGE2 [41] Montelukast ↔ PGE2 [42]	Allergen avoidance ↓ Cys-LTs [33] Nasal steroids ↓ Cys-LTs [54] Montelukast ↓ LTE4 [42, 49, 61] ICS ↓ Cys-LTs [58] ICS ↓ ↔ LTB4 [41, 47–49]	ICS ↓ or ↔ [36, 42, 58, 67, 68] Proton pump inhibitors ↓ [23]	ICS ↓ [74–77]
Atopy	↓ [16]	↑ NO <sub>2</sub> /NO <sub>3</sub> [20]		↑ Cys-LT [54] ↔ LTB4 [47]		↔ [76]

*Abbreviations:* nitrite/nitrate (NO<sub>x</sub>); prostaglandin (PG); thromboxane (TX); cysteinyl leukotrienes (Cys-LTs); leukotrienes (LT); inhaled corticosteroids (ICS).

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**Table 2.** Major findings from studies relating to EBC in COPD.

	pH	NO-related products	Prostanoids	Leukotrienes	8-Isoprostane	H <sub>2</sub> O <sub>2</sub>
Stable COPD	↓ [6]	↓ NO <sub>x</sub> [142] ↑ nitrite [27] ↑ S-nitrosothiols [27]	↑ PGE2, PGF2 $\alpha$ [43]	↑ LTB4 [44, 51]	↑ [43]	↑ [80, 81]
Exacerbation				↑ LTB4 [51]		↑ [80, 81]
Effect of smoking		↔ NO <sub>x</sub> [26]			↔ [43]	↔ [81]
Effect of treatment	ICS ↔ [6]	ICS ↔ NO <sub>x</sub> [25, 26, 109]	Non-selective COX ↓ PGE2 [45] ICS ↔ PGE2 [44]	Non-selective COX ↑ LTB4 [45] Antibiotic ↓ LTB4 [51] ICS ↔ LTB4 [44]	Antibiotic ↓ [51]	ICS ↔ ↓ [82, 83]

*Abbreviations:* cyclooxygenase inhibition (non-selective COX); nitrite/nitrate (NO<sub>x</sub>); prostaglandin (PG); thromboxane (TX); cysteinyl leukotrienes (Cys-LTs); leukotrienes (LT); inhaled corticosteroids (ICS).

**Table 3.** Comparison of EBC pH in healthy subjects, asthma, allergic rhinitis, atopic dermatitis and COPD. Data are presented as mean  $\pm$  standard deviation unless otherwise written. Adapted from [6, 16, 17, 23].

Disease condition	pH
Healthy adults [25]	7.50 $\pm$ 0.20
[165]	7.61 (CI 7.52–7.70)
In children [16]	7.78 (range 7.06–8.10)
Asthma exacerbation [25]	5.23 $\pm$ 0.21
Stable asthma [25]	7.8 $\pm$ 0.1
In children [16]	7.32 (range 6.2–8.02)
In children [166]	6.46 (IQR 5.86–6.72)
Mild asthma [25]	7.6 (CI 7.55–7.65)
Moderate asthma without GERD [25]	7.3 $\pm$ 0.60
Moderate asthma with GERD [25]	7.2 $\pm$ 0.1
Smoking asthmatics [17]	6.3 $\pm$ 0.8
Allergic rhinitis in children [16]	7.48 (range 6.90–7.98)
Atopic dermatitis in children [16]	7.40 (range 7.00–7.97)
COPD [165]	6.97 (CI 6.65–7.29)

*Abbreviations:* 95% confidence interval (CI); interquartile range (IQR); gastroesophageal reflux disease (GERD); chronic obstructive pulmonary disease (COPD).

may not be a reproducible and reliable marker for oxidative stress, particularly in children with stable asthma [35].

*S-nitrosothiols* are produced when peroxyxynitrate reacts with thiol-containing macromolecules (i.e. cysteine and glutathione). *S-nitrosothiols* are increased in bronchial asthma, with levels directly correlating with the severity of asthma [27]. Patients with COPD also have higher *S-nitrosothiols* concentrations in EBC than healthy non-smokers [27].

#### *Eicosanoids (prostaglandins and leukotrienes)*

*Prostanoids* are synthesized by the cyclooxygenase (COX) pathway to produce prostaglandins and thromboxanes. Prostaglandin E2 (PGE2), D2 and F2a, as well as thromboxane B2 (TXB2) have been found in EBC samples [36–40]. Although the role of prostanoids is not fully understood, PGE2 is thought to be protective through its bronchodilative properties. Smoking asthmatics have higher PGE2 levels in EBC than both asthmatic non-smokers and control subjects [37]. No significant difference in PGE2 levels, however, exists between asthmatics and normal controls, despite controlling for steroid use [37]. ICS does not reduce PGE2 in EBCs from asthmatic children [41]; the same is true with leukotriene receptor antagonists (montelukast) [42]. This suggests that PGE2 may be more useful for understanding environmental effects like smoking on airways in asthma, rather than its treatment. Another eicosanoid, TXB2, is also increased in the EBC of asthmatic subjects compared with controls [40], but this finding has not been reproduced in children [41].

PGE2 and PGF2 $\alpha$  are also markedly increased in patients with COPD compared with healthy non-smokers [43]. PGE2 may have anti-inflammatory effects in the airways; raised PGE2 levels in the airways of patients with COPD could be a mechanism for counteracting the lung inflammation of this disease. Steroid-naïve and steroid-treated patients with COPD

have similar PGE2 concentrations in EBC, again suggesting that PGE2 may not be the best mediator for monitoring treatment with ICS [44]. Studies of the eicosanoid pathway and COX inhibition, however, suggest that this biomarker may be used to understand processes of airway inflammation. Non-selective COX inhibition, therefore, may have important implications for lung inflammation in COPD patients. Oral ibuprofen (non-selective COX inhibition) decreases PGE2 levels in patients with COPD; COX-2 inhibition, however has no effect on PGE2 levels in these patients [45]. This suggests that PGE2 may become useful for investigating airway inflammation and novel treatments for COPD.

*Leukotrienes* are classified into two classes: LTB4 and the cysteinyl leukotrienes (Cys-LTs: LTC4, LTD4 and LTE4). LTB4 is a chemoattractant for inflammatory cells, including neutrophils and eosinophils [46]; its exact role in allergy and asthma is unknown. LTB4 levels are elevated in the EBC of asthmatic adults [30, 38] but not in atopic children without asthma [47]. The effect of ICS use on LTB4 levels in asthma has varied in the literature [47–49]. Smoking asthmatics have higher levels of LTB4 than smokers without asthma; these values approach those of COPD patients, suggesting a shared characteristic [50].

Although LTB4 is increased in both asthma and COPD, the increase is more pronounced in COPD: LTB4 levels are increased in patients with COPD compared to healthy non-smokers [44]. ICS use has no effect on LTB4 concentrations in EBC collected from patients with COPD [44]. Antibiotic treatment, however, results in a reduction in LTB4 levels after COPD exacerbations [51]. Non-selective COX inhibition increases LTB4 in EBC; selective COX-2 inhibition has no effect on this eicosanoid [45]. The significance of these trends is unknown.

Cys-LTs are generated during the early and late phase allergic reactions, inducing smooth muscle contraction, microvascular leakage and mucous hypersecretion [52]. In fact, Cyst-LTs are 1000-fold more potent than histamine on bronchial smooth muscle tone [53]. Cys-LTs are elevated in patients with allergic rhinitis; intranasal steroid reduce these levels in EBC [54]. Cys-LT levels in EBC are increased in adults [38, 55] and children [55, 56] with asthma and are especially elevated in patients with unstable asthma [57]. These elevated levels in asthmatics approach healthy normal values after treatment with oral corticosteroids [58]. Cys-LTs in EBC have also been used to investigate other types of treatment, including leukotriene receptor antagonists like montelukast. Cys-LTs levels in EBC have not been shown to be related to the severity of exercise-induced bronchospasm or to montelukast response in children with asthma [59].

Asthmatics have elevated levels of LTE4 levels in EBC [38, 41, 42, 55, 60]. Leukotriene receptor antagonists decrease exhaled LTE4 in atopic children with asthma, but not in atopic children without asthma [42]. LTE4 may be useful for distinguishing between allergic rhinitis and asthma. A strong correlation between EBC Cyst-LTs and reticular basement membrane thickness exists, suggesting that cys-LTs may play an important role in airway remodeling [61]. Similarly, increased LTE4 levels in children with mild asthma

may be useful as a non-invasive marker for assessing airway inflammation and bronchial hyperresponsiveness in children with asthma [60].

Disorders of the upper respiratory tract, like allergic rhinitis, are commonly associated with bronchial hyperresponsiveness and asthma [62]. Leukotriene concentrations in EBC (LTB<sub>4</sub>, LTE<sub>4</sub>) are significantly increased in and after the pollen season in patients with seasonal allergic rhinitis compared to healthy controls [63]. Leukotriene levels collected in EBC decrease after pollen season compared with the seasonal baseline [63]. Patients with the highest in season leukotriene levels also had the highest post-season levels [63]. These findings suggest that leukotrienes may be an early marker of the inflammatory process in the lower airways.

### *Isoprostanes*

*Isoprostanes* are prostaglandin-like compounds formed by free-radical lipid peroxidation. Many studies have demonstrated that these compounds are accurate markers of oxidative stress in airways [64–66]. 8-Isoprostane is one of the most studied isoprostanes in EBC. Levels of 8-isoprostane are twice as high in mild asthma compared to healthy subjects; this difference is further increased in moderate to severe asthma [42, 67]. Contraindicatory results have been shown for asthma in response to ICS treatment [36, 42, 67, 68]. 8-Isoprostane levels are also elevated in patients with COPD exacerbations, but these levels are decreased after antibiotic treatment [51].

EBC levels of 8-isoprostanes are elevated in cigarette smokers without defined lung disease, but to a much greater extent in patients with COPD [43]. Despite this, 8-isoprostane levels do not differ between current smokers with COPD and ex-smokers with COPD [43]. This suggests that this isoprostane is derived from the oxidative stress specific to the inflammation underlying COPD, rather than from cigarettes alone.

8-Isoprostane levels in EBC are strongly related to small airway function in asthma, suggesting that this biomarker in EBC may be complementary to peak flow measurements currently used to examine airways in asthmatics [69]. 8-Isoprostane levels may also reflect the extension of lung damage in COPD patients [70]. 8-Isoprostane levels are inversely related to lung function in COPD: patients with poorer lung function have higher levels of 8-isoprostane detected in EBC [71].

### *Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)*

Active inflammatory cells respond to stress with a respiratory burst, resulting in the production of reactive oxygen species such as H<sub>2</sub>O<sub>2</sub>. Most studies report elevated levels of H<sub>2</sub>O<sub>2</sub> in steroid-naïve asthmatic subjects, although several studies report levels of H<sub>2</sub>O<sub>2</sub> that are below the limit of detection [72–77]. H<sub>2</sub>O<sub>2</sub> levels are influenced by disease severity [76], smoking habit [74], the presence of symptoms in unstable patients [75], but not atopy [76]. H<sub>2</sub>O<sub>2</sub> levels are thought to be related to bronchial hyperresponsiveness and bronchoconstriction [78]. Evidence that this mediator is

related to eosinophilic inflammation [76] and the products of NO metabolism [74] also exists. ICS decrease H<sub>2</sub>O<sub>2</sub> levels: stable asthmatic patients treated with ICS have lower H<sub>2</sub>O<sub>2</sub> levels compared to steroid-naïve subjects and similar to normal subjects [74–76].

Cigarette smoking causes an influx of inflammatory cells into the lower airways; smokers have a five-fold higher level of H<sub>2</sub>O<sub>2</sub> in EBC than non-smokers [79]. Patients with COPD have increased levels of H<sub>2</sub>O<sub>2</sub> compared to normal subjects; these levels are further increased during exacerbations [80, 81]. Despite the known relationship between smoking and the progression to COPD, no significant differences have been found between H<sub>2</sub>O<sub>2</sub> levels in current smokers with COPD and ex-smokers with COPD; similarly, no correlation has been found between H<sub>2</sub>O<sub>2</sub> levels in EBC and daily cigarette consumption [81]. This suggests that oxidative stress is a characteristic feature of COPD that cannot be entirely explained by the oxidants present in tobacco smoke.

H<sub>2</sub>O<sub>2</sub> levels in EBC after the ICS treatment of COPD is controversial [82, 83]. Another treatment, however, has more promising data. Nebulized antioxidant *N*-acetylcysteine increases exhaled H<sub>2</sub>O<sub>2</sub> levels in the EBC of stable COPD patients [84]. H<sub>2</sub>O<sub>2</sub> levels have also been used as an inflammatory marker to determine the association between tissue inflammation and inflammation-associated priming of neutrophils in the peripheral blood [85]. A strong relationship exists between the levels of H<sub>2</sub>O<sub>2</sub> in EBC and neutrophil priming, suggesting that local inflammation has systemic effects on cells of the innate immune system [85].

### *Other EBC biomarkers in asthma, allergic rhinitis and COPD*

*Malondialdehyde* is generated by arachidonic and docosahexenoic acid; other classes of aldehydes are produced by lipid peroxidation. Malondialdehyde levels in EBC increase during asthma exacerbations but decrease after steroid treatment [86]. COPD have increased levels of malondialdehyde compared with healthy smokers [86]. EBC aldehydes are not strongly related to findings in induced sputum of asthmatics; this suggests that the two techniques should be evaluated independently [87].

Most *proteins*, including cytokines, are difficult to reliably measure in EBC. Cytokines help mediate inflammatory processes; several cytokines have been detected in EBC [38, 68]. Patients with COPD may have increased concentrations of pro-inflammatory mediators in EBC (IL-1 $\beta$  and IL-12) compared to healthy controls [88]. Patients with acute exacerbations of COPD have increases in IL-1 $\beta$ , IL-6, IL-8, IL-10, IL-12p70 and TNF- $\alpha$  detected in EBC compared to patients with stable COPD, smokers and healthy volunteers [88]. ICS treatment decreases levels of IL-1 $\beta$ , IL-6, IL-8, IL-10 and IL-12 with dose-dependency for IL-8, IL-1 $\beta$  and IL-12 [88]. TNF- $\alpha$  is significantly higher in the EBC of patients with COPD than non-COPD patients [89]. This group also detected erythropoietin in EBC, although no significant difference for EPO levels or correlation between EPO and TNF- $\alpha$  was found [89]. Despite these promising findings, data reproducibility has been poor for EBC studies in COPD.

Cytokines have also been investigated with asthma. Asthma is associated with eosinophilic airway inflammation and the overproduction of T-helper type 2 (Th2) lymphocyte-related cytokines. An elevated IL-4/IFN- $\gamma$  ratio is found in the EBC of children with asthma, suggesting a predominance Th2 inflammation in these airways [71]. The following cytokines, chemokines and growth factors are significantly up-regulated in asthmatic airways: IL-4, IL-8, IL-17, TNF- $\alpha$ , RANTES, IFN- $\gamma$ -inducible protein 10, TGF- $\beta$ , macrophage-derived chemokine (MDC), eotaxin and macrophage inflammatory proteins 1 $\alpha$  and 1 $\beta$  [71, 90, 91]. RANTES levels in EBC strongly correlate with airway caliber, FEV1 and respiratory resistance values in asthmatics [91]. Levels of TNF- $\alpha$  and TGF- $\beta$  measured in EBC correlate with methacholine threshold and peak expiratory flow variability [90]. EBC eotaxin and macrophage-derived chemokine levels are higher in asthmatics on ICS than the steroid-naïve asthmatics or controls [71].

Atopic dermatitis and allergic rhinitis often coexists with asthma or precede the development of asthma. EBC IL-5 levels are higher in children with atopic dermatitis, allergic rhinitis and asthma than in healthy controls [15]. Another group evaluated the relationship of chemokines (macrophage-derived chemokine, eotaxin) found in EBC with atopy-related indices [92]. They found that atopy-related indices (i.e. IgE levels and eosinophil percentage) should be considered as separate dimensions from airway inflammation in the assessment of airway inflammation. Inflammatory markers in peripheral blood and in EBC were also found to be non-overlapping factors of childhood asthma [92].

Measuring cytokine levels in EBC may be a promising approach to assess the inflammatory status of the airways in order to monitor treatment interventions and to investigate the pathophysiology of asthma in the future. The major limitation of cytokine studies is that these proteins, when actually found in EBC, are very close to the detection limit. Newer more sensitive techniques may be needed in order to make this mediator a reliable and reproducible biomarker in EBC.

Endothelins are pro-inflammatory, bronchoconstrictive and vasoconstrictive peptides that are important in airway inflammation and airway remodeling in asthma. Inflammatory cells and cytokines are thought to alter the expression and release of endothelins. Exercise-induced bronchoconstriction leads to an increase in the release of endothelin-I from the bronchial epithelium, which can be detected in EBC; this release may be important for understanding airway inflammation after post-exercise bronchoconstriction in asthmatics [93].

Adenosine, a purine nucleoside expressed in a variety of physiological cells, is present in bronchoalveolar lavage and EBC [94, 95]. Adenosine levels in EBC are significantly increased in asthmatic patients during exercise but not in healthy controls [96]. This exercise-induced change in adenosine concentration strongly correlates with a decline in FEV1. EBC adenosine is also increased in allergic rhinitis, suggesting a subclinical inflammation in lower airways of patients with allergic rhinitis [97].

Long-term exposure to tobacco smoke may increase the uptake of toxic metals in the lungs since these metals are major

contaminants in tobacco smoke. Current smokers have higher levels of lead and cadmium in EBC than healthy non-smoking subjects. Smoking COPD patients have higher levels of toxic metals than ex-smokers with COPD; ex-smokers, however, still have higher levels of these toxic metals in COPD than non-smoking controls [98]. Metallic elements in EBC may also be useful in distinguishing asthma from COPD since patients with COPD have elevated levels of toxic metals and transition elements involved in redox systems of oxidative stress [99]. These metals and elements may be useful as biomarkers of exposure and useful in distinguishing similar diseases such as allergic rhinitis, asthma and COPD.

## EBC in cystic fibrosis

Cystic fibrosis (CF) is the most common fatal inherited disease in Caucasians. An inherited mutation in a gene encoding a transmembrane protein leads to the clinical manifestations of the disease. This transmembrane protein controls the secretions of the sweat glands and the respiratory, gastrointestinal and reproductive tracts; a non-functional protein leads to altered thick secretions that lead to clinical manifestations of the disease, including chronic lung disease, exocrine pancreatic insufficiency, disease-related diabetes and liver disease. Chronic airway inflammation occurs in CF as a result of repeated bacterial lung infection and an exaggerated host response to this process [100].

Adults [101] and children [102] with cystic fibrosis have acidified airways detectable by EBC pH. This decrease in EBC pH is related to infective exacerbations of CF in children [102]. These patients also have low fractional exhaled nitric oxide (FE<sub>NO</sub>) compared to healthy subjects [103]. Ojoo *et al* investigated the relationship of airway lining fluid pH and FE<sub>NO</sub> regulation in respiratory disease [104]. This group found that patients with stable CF had lower FE<sub>NO</sub> and EBC pH than healthy controls; NO<sub>x</sub> levels, however, were significantly higher in CF than that found in healthy controls [104]. CF exacerbations lead to a further decrease in EBC pH but similar FE<sub>NO</sub> and NO<sub>x</sub> levels in EBC [104]. This group's findings suggest that EBC pH and FE<sub>NO</sub> could describe different aspects of airway inflammation. Care should be taken to differentiate the difference between what these biomarkers indicate about the pathology behind the inflammatory process.

Patients with CF have elevated levels of nitrite and nitrate in EBC compared to healthy controls [105, 106], during exacerbations and stable disease. The bacterial airway colonization and subsequent chronic airway inflammation may be detected by EBC; early recognition of airway inflammation and airway infection could lead to better clinical outcomes. Horak *et al* [107] investigated the use of EBC nitrites in monitoring CF lung disease activity in children and found no correlation between EBC nitrite levels and markers of disease severity; in fact, elevated EBC nitrite levels did not predict subsequent pulmonary exacerbations [107]. This suggests that EBC nitrite levels may not be ideal biomarkers for monitoring CF lung disease in children. Patients with CF also have elevated levels of nitrotyrosine in EBC-compared

normal subjects [108]. S-nitrosothiols are elevated in EBCs of adults with very severe CF [27].

Chronic pulmonary infections often determine the course and prognosis of CF [109]. Opportunistic bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* are a major cause of the chronic airway neutrophil-dominated inflammation [110]. Neutrophils release many mediators, including eicosanoids, which can be detected in EBC. LTB<sub>4</sub> levels in EBC are increased in children with CF compared to healthy controls; this difference is enhanced during a CF exacerbation [102]. LTB<sub>4</sub>, a biomarker for neutrophilic inflammation, is elevated in the EBC of children with CF with active bacterial airway infections [48]. CF children colonized with *P. aeruginosa* have higher levels of LTB<sub>4</sub> in EBC than those colonized with *S. aureus* [48].

8-Isoprostane levels are increased three-fold in patients with CF compared to healthy controls [111]. No relationship exists between EBC pH and 8-isoprostane levels in children with CF [102].

H<sub>2</sub>O<sub>2</sub> levels, thought to be indicative of oxidative stress levels, are reduced during the antibiotic treatment of patients with infective exacerbations of CF [112].

IFN- $\gamma$  levels in EBC are significantly higher in children with CF compared to healthy controls. Robroeks *et al* used multivariate logistic regression models to evaluate biomarkers in EBC [113]. This group found that the presence of CF is best indicated by 8-isoprostane, nitrite and IFN- $\gamma$  levels in EBC [113]. An exacerbation of CF, however, is best indicated by 8-isoprostane and nitrite levels. CF severity is best indicated by EBC acidity [113].

IL-10, IL-4, TNF- $\alpha$  and IFN- $\gamma$  are in the EBCs of CF patients [113]. EBC levels of IL-8 are elevated in CF patients colonized by *P. aeruginosa* and *S. aureus*; IL-8 levels are also elevated in CF patients that are not colonized, compared to healthy children [114].

Purines are established biomarkers of airway inflammation in respiratory disease [95]. Purines mediate airway mucus clearance, airway surface liquid volume, ciliary function and mucin secretion [115, 116]. Purines are also involved in inflammatory cell responses: neutrophils release ATP when activated [117, 118]. ATP levels are increased in CF relative to controls; these levels decrease after the treatment of pulmonary exacerbation due to CF [119]. Esther *et al* developed a sensitive and specific liquid chromatography/tandem mass spectrometry method to detect adenyl purines as biomarkers from EBC; urea was detected as a dilution marker in EBC [120]. The AMP/urea ratio is elevated in EBC collected from patients with CF, compared to that from healthy controls [120]. This group's findings suggest that EBC can be used to detect a biomarker for airway inflammation and to control for variable dilution [120]. Purines are candidate biomarkers of neutrophilic airway inflammation.

EBC has also been used to evaluate the effectiveness of glucose as a biomarker for airway inflammation in cystic fibrosis [121]. The respiratory tract has low levels of glucose: in animals, glucose concentration in respiratory tract lining fluid is 3–20 times lower than plasma glucose concentration (1, 22). In humans, respiratory tract glucose concentrations

are also low, but they are elevated by inflammation and hyperglycemia [121]. Breath glucose from EBC of CF patients is higher than expected for blood glucose in CF patients and in CF-related diabetes [121]. This group's data suggest that EBC may be a useful estimate of respiratory fluid glucose concentration and that the effects of lung disease and hyperglycemia can be distinguished by this method.

## EBC in lung cancers

Lung cancer is the most common cancer in the world. One current limitation in diagnosis is that lung cancer is typically detected through chest x-ray and sputum cytology. As a result, a considerable amount of research has been directed toward the early detection of tumor markers before the cancer becomes clinically observable and unresponsive to treatment. Many different research groups have brought forward potential lung cancer biomarkers; however, none have proven to be useful in a clinical setting for the early diagnosis of lung cancer. Currently, an accurate diagnosis is usually only made when the disease has further progressed and is less responsive or unresponsive to therapeutic intervention. Unfortunately, in lung cancer there are no sensitive and specific biomarkers, such as prostate-specific antigen in prostate cancer. Several biomarkers will probably have to be used together, including P53, ras and the methylation of different genes.

Several articles reported that EBC may present as a simple non-invasive alternative lung cancer biomarkers. Chromate workers with and without lung cancer have higher pulmonary tissue Cr levels than controls [122]. By using electrothermal atomic absorption, Cr can be detected in EBC from patients and controls. Significant increase in the levels of Cr-EBC after surgical intervention in non-small cell lung cancer patients gives new information for sensitivity of Cr-EBC as a biomarker of lung exposure. The problem is that pulmonary Cr levels have never been standardized and different values have been reported in control subjects [123, 124]. Certainly, tissue Cr level is affected by age, gender and region and even by lifestyle therefore before using Cr-EBC as a biomarker for lung cancer, it is necessary to standardize the Cr levels in pulmonary tissue.

The alteration of DNA in EBC have been reported as significantly more frequently than those of blood DNA [125]. This result suggests that the detection of abnormalities in EBC could be a marker and identify patients with high risk of lung cancer. Carpagnano *et al* showed that whole blood DNA shows significantly lower microsatellite alteration frequency compared with EBC DNA [126]. This alteration in EBC could be a significant marker for tumorigenesis and could become a useful biomarker for early lung cancer diagnosis.

## EBC in sleep apnea

Obstructive sleep apnea syndrome is characterized by repetitive episodes of upper airway obstruction during sleep and occur in approximately 9% of men and 4% of women [127]. The inflammation in the airway plays a key role in the pathogenesis of OSAS but still the mechanism is unknown [128]. Recently, researchers are increasingly looking for new

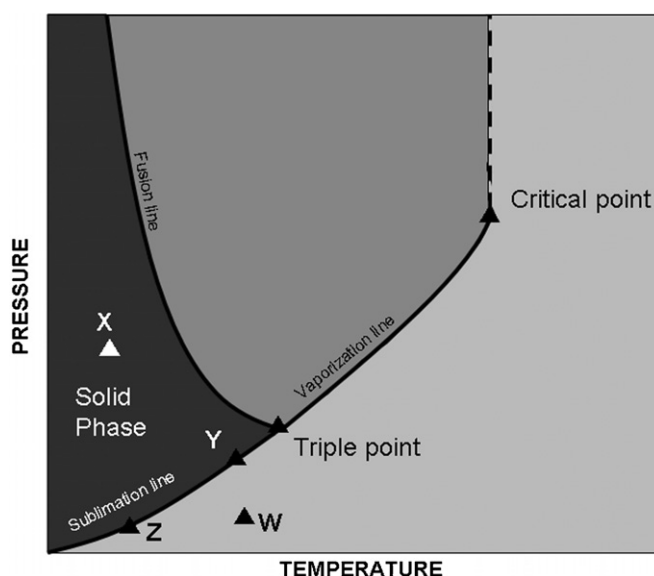
markers of airways inflammation although there are very few studies made on EBC in OSAS. Carpagnano *et al* are the first group that showed the exhaled pH in patients with OSAS [129]. They suggest that obese patients with OSAS and obese patients without OSAS present upper airway inflammation which could be monitored by the exhaled pH in EBC. There are also studies that shows the exhaled breath markers such as 8-isoprostane [130, 131], leukotrienes, nitrates, H<sub>2</sub>O<sub>2</sub> and pH correlates with the severity of OSAS [130]. There are also pro-inflammatory cytokines such as TNF- $\alpha$  and IL-6 which are the strongest predictors of apnea-hypopnea index change. Li *et al* showed that monitoring IL-6 and TNF- $\alpha$  could be useful for follow-up procedures [131]. Goldbart *et al* suggest that EBC could be a useful tool as a non-invasive biomarker for upper airway inflammation in children with sleep-disordered breathing. They showed that eicosanoids are measurable in EBC and there is a difference in these inflammatory mediators that emerge among children with and without sleep-disordered breathing which could be used as a non-invasive tool in the clinic.

### EBC measurement and analysis techniques

The statement released by the ATS/ERS and several other groups have reviewed the technical aspects of EBC [3, 5, 132]. We focused our review on papers published after ATS/ERS guidelines. Of these 76 recent papers on EBC, 26 papers studied asthma, 15 papers investigated COPD, 7 papers evaluated CF and the remainder evaluated other populations (healthy reference, obstructive sleep apnea, bronchiectasis, asbestosis, allergic rhinitis, lung cancer) (table 4). The most popular EBC collection device was the Ecoscreen EBC collecting device (27 studies), followed by the R-tube (12 studies) and glass condensing systems (11 studies) (table 5).

Standard techniques for detecting markers in EBC include enzyme-linked immunoassays, pH measurement and fluorometric assays. We found that several new high-sensitivity techniques are being successfully utilized for biomarker detection in EBC, including liquid chromatography/electrospray ionization tandem mass spectrometry (table 5). These techniques may be more sensitive than previous methods and increase the ability of detecting important markers.

Novel techniques of EBC analysis are promising for learning more about the pathobiology of asthma. A recent group used metabolomics in EBC and discovered novel compounds. This suggests the possibility of creating biochemical fingerprints for each disease in the near future [133]. EBC has also been applied to other types of biomarkers. Corhay *et al* [134] used EBC to compare the eosinophil and neutrophil chemotactic activity of patients with COPD and healthy subjects. This group found that current smoking favored neutrophil chemotactic activity and that COPD patients had more neutrophil chemotactic activity than eosinophilic activity in EBC, compared to healthy controls.



**Figure 2.** Phase diagram illustrating the lyophilization process in detail. Figure adapted from Labconco protocol on freeze-drying [135]. Most samples are frozen below the point at which the entire suspension is completely frozen (eutectic point) (X) and raised to just below the critical temperature (Y). The pressure is lowered (Z) to encourage the free flow of water molecules from the product. The collecting system of the lyophilizer acts as a cold trap with a low temperature (W) to collect moisture, leaving the frozen sample.

### Lyophilization

Perhaps the most promising methods to increase the utility of EBC is lyophilization. In this method, freeze-drying of EBC is used to concentrate the sample. One of the largest obstacles to the field of EBC research is biomarker detection. Since the majority of the sample consists of condensed water vapor, most biomarkers exist in EBC at very low concentrations. When detected in EBC, these biomarkers are very close to the detection limit for that marker. This proximity decreases the reproducibility and reliability of the use of EBC in biomarkers. The dilution problem has partly been addressed by lyophilization, a freeze-drying process that allows for concentrating a sample. Normally, a solution can be concentrated by adding heat and evaporating free water; this process of heating, however, can significantly damage a sample. Lyophilization is a method of dehydrating a delicate sample that would otherwise be damaged by the drying process. By regulating the temperature and pressure of the sample, freeze-drying brings the sample system around the triple point of a typical phase diagram, thereby avoiding the liquid-gas transition seen in ordinary drying (figure 2). Water is removed from the sample after it is frozen and placed under a vacuum, allowing the ice to change directly from solid to vapor without passing through a liquid phase; this allows for the removal of free water without excessive heating of the product (figure 2).

The process of lyophilization consists of three steps: pre-freezing, primary drying and secondary drying [135]. Samples are pre-frozen before starting lyophilization at the eutectic temperature, the temperature at which all areas of the concentrated solute are frozen. Eutectics are mixtures of

**Table 4.** Demographic information of EBC papers reviewed. Papers were grouped by those that studied healthy reference populations (A) asthma, allergic rhinitis, atopic dermatitis and COPD (B); obstructive sleep apnea (C); cystic fibrosis (D); other lung diseases (E).

First author	Year	Population studied	Sample size
(A)			
Czebe [143]	2008	Healthy reference, adult	12
Do [144]	2008	Healthy reference, adult, smokers and non-smokers	12
Hoffmann [145]	2008	Healthy reference, adult	6
Hu [146]	2008	Healthy reference, adults; rheumatoid arthritis	22
Kullmann [21]	2008	Healthy reference, adults	12
Balbi [147]	2007	Healthy reference, adult	Review
Bloemen [148]	2007	Healthy reference, adult	21
Conventz [149]	2007	Healthy, adults	27
Gaber [150]	2006	Healthy, adults	34
Paget-Brown [14]	2006	Healthy reference, adult and children	404
Schumann [89]	2006	Healthy reference, adult	22
(B)			
Accordino [10]	2008	Asthmatic, adults	42
Akpinar-Elci [151]	2008	Environmental exposure, water damage and asthma	371
Bayley [152]	2008	Bronchiectasis, COPD	61
Makris [70]	2008	COPD, adults	30
Raissy [59]	2008	Asthma, children	11
Romieu [153]	2008	Asthma, pollution exposure, children	107
Zhao [19]	2008	Asthma, adults	64
Carraro [133]	2007	Asthmatic, children	36
Corhay [134]	2007	COPD, adults	110
Fireman [154]	2007	Obstructive lung disease, interstitial lung disease, persistent cough, adults	75
Gessner [28]	2007	COPD, adults	112
Liu [26]	2007	COPD, adults, smoking status	176
Noble [155]	2007	Asthma, adults	41
Prieto [156]	2007	Asthma, allergic rhinitis, healthy adults	23
Robroeks [157]	2007	Asthma, children	114
Rysz [84]	2007	COPD, adults	19
Shimizu [23]	2007	Asthma, GERD adults	Review
Vogelberg [158]	2007	Bronchitis, children	94
Zietkowski [93]	2007	Asthma, adults	26
Baraldi [31]	2006	Asthma, children	48
Bodini [33]	2006	Asthma, children	10
Boulet [17]	2006	Asthma, smoking status, adults	49
Brunetti [16]	2006	Asthma, allergic rhinitis, atopic dermatitis, children	186
Effros [22]	2006	COPD, adults	20
Failla [54]	2006	Allergic rhinitis, adults	69
Ko [71]	2006	Asthma, adults	64
Ko [71]	2006	COPD, adults	32
Leung [166]	2006	Asthma, children	58
Lex [61]	2006	Asthma, children	29
Matsunaga [90]	2006	Asthma, adults	36
Montuschi [42]	2006	Asthma, children	33
Mutti [99]	2006	COPD and asthma, adults; smokers	160
Mutti and Corradi [98]	2006	COPD, smoking, adults	Review
Nicolaou [18]	2006	Asthma, children	630
Oudijk [85]	2006	COPD, adults	10
Profita [15]	2006	Asthma, atopic dermatitis, allergic rhinitis, children	91
Ratnawati [20]	2006	Asthma, children	92
Shibata [60]	2006	Asthma, children	43

**Table 4.** (Continued.)

First author	Year	Population studied	Sample size
Vass [97]	2006	Allergic rhinitis, adults	42
Borrill [165]	2005	COPD, adults	48
Cap [63]	2005	Allergic rhinitis, adults	79
Gessner [88]	2005	COPD, smoking, adults	120
Leung [92]	2005	Asthma, children	92
Montuschi [47]	2005	COPD, adults	30
Ojoo [104]	2005	Asthma, CF, adults	45
(C)			
Carpagnano [129]	2008	OSA, obese	60
Li [159]	2008	OSA, smokers	90
(D)			
Esther [119]	2008	CF, children and adults	58
Esther [120]	2008	CF, adults	14
Robroeks [113]	2008	CF, children	98
Baker [121]	2007	CF and DM, adults	56
Horak [107]	2007	Cystic fibrosis, children	32
Celio [35]	2006	Cystic fibrosis, asthma, children	56
Bodini [114]	2005	CF, children	40
(E)			
Gessner [160]	2008	Respiratory failure, ARDS, adults	30
Gogate [161]	2008	Review, children	Review
Hunt [132]	2007	Review	Review
Lehtonen [162]	2007	Asbestosis	30
Hunt [132]	2007	Review	Review
Kharitonov [139]	2006	EBC review	Review
ATS [3]	2005	Review	Review

*Abbreviations:* chronic obstructive pulmonary disease (COPD); obstructive sleep apnea (OSA); cystic fibrosis (CF); acute respiratory distress syndrome (ARDS); diabetes mellitus (DM); gastroesophageal reflux disease (GERD).

substances that freeze at lower temperatures than water. When an aqueous suspension is cooled, water is separated from the lower freezing solute as it changes to ice. This creates pockets of concentrated solute with a lower freezing temperature than water. The product may appear to be frozen at this point but is not until all of the solute in the suspension is frozen. The eutectic temperature is the temperature at which all of the eutectic mixture is frozen in the suspension. Next, in primary drying free water in the form of ice is removed via sublimation: ice evaporates off as vapor. This is accomplished by decreasing the pressure surrounding the samples and by adding small amounts of energy in the form of heat (figure 2). It is important to note, however, that very little energy is added to prevent the sample from crossing the damaging triple point of the phase diagram; crossing this point would lead to the damage seen in traditional drying. Secondary drying is a final isothermal desorption process that removes residual moisture [135]. After lyophilization is complete, all free water has been removed and solute remains. This solute can then be reconstituted in a solution of choice.

Although lyophilization may be an effective tool for concentrating dilute samples that would be damaged by traditional drying techniques, the process of lyophilization is the most complex, timely and expensive form of drying.

Lyophilization has been used by many groups evaluating EBC biomarkers [28, 59]. Despite the growing trend of using this technique to concentrate EBC for biomarker detection, very little is known about the reproducibility and reliability of this method.

## Discussion

Exhaled breath condensate is a promising non-invasive technique for learning about the airways. EBC provides a real-time assessment of pulmonary pathobiology. Patients whose condition limits testing ability by other methods (i.e. children) are able to successfully participate in this technique. The simplicity and low cost of the collection device will allow for the use of this technique in large longitudinal studies, such as what the ECLIPSE study did for biomarkers in EBC in evaluating COPD [136].

Airway acidification has also been found in allergic rhinitis and atopic dermatitis, suggesting that EBC pH may be a useful method for evaluating the progression of atopy to asthma [16]. Studies in COPD suggest the need for EBC pH in screening large populations for airway inflammation to better understand how pH changes relate to COPD, particularly with the phenomenon of changing pH

**Table 5.** EBC techniques of papers reviewed. Papers were grouped by EBC collection device: R-tube (A), Ecoscreen (B) or other (C). Papers that utilized lyophilization are listed in (D).

First author	Year	EBC collection device	EBC component measured	EBC analysis	Lyophilization
<b>(A)</b>					
Bayley [152]	2008	R-tube	Leukotriene B4, IL-8, secretory protease inhibitor, $\alpha$ 1-antitrypsin, myeloperoxidase	ELISA; myeloperoxidase by chromogenic substrate assay	No
Czebe [143]	2008	Ecoscreen, R-tube, Anacon	pH, protein, leukotriene	pH; protein via Bradford method; leukotrienes by ELISA	No
Do [144]	2008	R-tube	pH, NH <sub>4</sub> <sup>+</sup>	pH meter; HPLC (NH <sub>4</sub> <sup>+</sup> )	No
Esther [119]	2008	R-tube	Purines (ATP, ADP, AMP, adenosine)	Luciferin–luciferase luminescence assay	Yes
Esther [120]	2008	R-tube	Adenyl purines, urea	LC-MS	Yes
Kullmann [21]	2008	R-tube	pH	pH meter	No
Raissy [59]	2008	R-tube	Cysteinyl leukotrienes	ELISA	Yes
Romieu [153]	2008	R-tube	Malondialdehyde	Fluorescence	Unknown
Baker [121]	2007	R-tube	Glucose	Anion-exchange chromatography with pulsed amperometric detection	Yes
Bloemen [148]	2007	R-tube	Variability of pH, volume, protein content	Protein-fluorescence spectrophotometer; pH, biotrode	No
Prieto [156]	2007	R-tube, Ecoscreen	pH	pH meter	No
Leung [166]	2006	Ecoscreen, R-tube	pH, 8-isoprostane, cysteinyl leukotrienes, leukotriene B4	pH meter, ELISA	No
Nicolaou [18]	2006	R-tube	pH	pH meter	No
Paget-Brown [14]	2006	R-tube	pH	pH meter	No
Leung [92]	2005	R-tube	MDC, eotaxin, leukotriene B4	ELISA (MDC, eotaxin), LTB4 (acetylcholinesterase competitive enzyme immunoassay)	No
<b>(B)</b>					
Carpagnano [129]	2008	Ecoscreen	pH	pH meter	No
Czebe [143]	2008	Ecoscreen, R-tube, Anacon	pH, protein, leukotriene	pH; protein via Bradford method; ELISA	No
Gessner [28]	2008	Ecoscreen	Protein (cytokeratins 2, 9, 10); IL-6, IL-8	ELISA (IL-6, IL-8); Western blot (proteins)	Yes
Hoffmann [145]	2008	Ecoscreen	Cytokeratins	Mass spectrometry	Yes
Li [159]	2008	Ecoscreen	IL-6, IL-10, TNF- $\alpha$ , 8-isoprostane	ELISA	No
Zhao [19]	2008	Ecoscreen	pH, 8-isoprostane	ELISA (8-isoprostane), pH meter	No
Corhay [134]	2007	Ecoscreen	Eosinophil and neutrophil chemotactic activity	Microchambers and chemotactic index	No
Gessner [28]	2007	Ecoscreen	IL-8, IL-1 $\beta$ , IL-6, IL-10, IL-12, TNF- $\alpha$ , nitrite	GREISS reaction (nitrite), multiplex immunoassay (cytokines)	No
Lehtonen [162]	2007	Ecoscreen	Leukotriene B4, 8-isoprostane	ELISA	No
Noble [155]	2007	Ecoscreen	pH	pH meter	No
Piotrowski [163]	2007	Ecoscreen	8-Isoprostane, cysteinyl leukotrienes, leukotriene B4	ELISA	Unknown
Prieto [156]	2007	R-tube, Ecoscreen	pH	pH meter	No
Vogelberg [158]	2007	Ecoscreen	pH	pH meter	No
Zietkowski [93]	2007	Ecoscreen	Endothelin-1	ELISA	No
Celio [35]	2006	EcoScreen	3-nitrotyrosine,	GC-NICI-MS; HPLC	Yes
Gaber [150]	2006	Ecoscreen	Leukotriene B4	ELISA	No
Ko [71]	2006	Ecoscreen	Eotaxin, MDC	ELISA	No
Ko [164]	2006	Ecoscreen	8-Isoprostane, growth related oncogene- $\alpha$ , monocyte chemoattractant protein-1	Sandwich enzyme gimmunoassays	No

Table 5. (Continued.)

First author	Year	EBC collection device	EBC component measured	EBC analysis	Lyophilization
Leung [166]	2006	Ecoscreen, R-tube	pH, 8-isoprostane, cysteinyl leukotrienes, leukotriene B4	pH meter, ELISA	No
Lex [61]	2006	Ecoscreen	Cysteinyl leukotrienes	ELISA	No
Matsunaga [90]	2006	Ecoscreen	Cytokines	Protein array	No
Montuschi [42]	2006	Ecoscreen	Leukotriene E4, 8-isoprostane, prostaglandin E2	ELISA (LTE4), HPLC (8-isoprostane, gprostaglandin E2)	No
Profita [15]	2006	Ecoscreen	pH, IL-5	ELISA (IL-5), pH meter	No
Schumann [89]	2006	Ecoscreen	Erythropoietin, TNF- $\alpha$	Cytometric bead arrays	No
Vass [97]	2006	Ecoscreen	Adenosine	HPLC	No
Borrill [165]	2005	Ecoscreen	pH	pH meter	No
Cap [63]	2005	Ecoscreen	Leukotrienes B4, C4, D4, E4	GC-MS	No
Gessner [88]	2005	Ecoscreen	Cytokines (IL-1 $\beta$ , IL-6, IL-8, IL-10, IL-12, TNF- $\alpha$ )	Cytometric bead arrays	Yes
Montuschi [47]	2005	Ecoscreen	PGE2, LTB4	ELISA (LTB4), Radioimmunoassays (PGE2)	No
(C)					
Accordino [10]	2008	Tygon tube	pH	Dearation, pH meter	No
Akpinar-Elci [151]	2008	Corr-A-Flex II	IL-8, nitrate	Immunoassay; Chemiluminescence	No
Czebe [143]	2008	Ecoscreen, R-tube, Anacon	pH, protein, leukotriene	pH I; protein via Bradford method; ELISA	No
Hu [146]	2008	Unknown	H <sub>2</sub> O <sub>2</sub>	Chemiluminescence	No
Makris [70]	2008	Glass condensing chamber	8-Isoprostane	ELISA	No
Robroeks [113]	2008	Glass condensing chamber	pH, NOx, 8-isoprostane, H <sub>2</sub> O <sub>2</sub> , IFN- $\gamma$	pH meter, fluoremetric assay (NOx), ELISA (8-isoprostane), spectrophotometry (H <sub>2</sub> O <sub>2</sub> )	No
Carraro [133]	2007	Tygon tube	Low molecular weight metabolites	Metabolomics, NMR spectra	Yes
Fireman [154]	2007	Teflon	H <sub>2</sub> O <sub>2</sub>	Colorimetry	No
Horak [107]	2007	Teflon	Nitrites	Griess assay	No
Liu [26]	2007	Glass condensing chamber	Nitrite/nitrate	Griess method	No
Robroeks [157]	2007	Unknown	NOx, H <sub>2</sub> O <sub>2</sub> , 8-isoprostane, IFN- $\gamma$ , TNF- $\alpha$ , IL-2, IL-4, IL-5, IL-10, pH	Flow cytometry (Cytokines); ELISA (8-isoprostane), fluorometry (NOx)	No
Rysz [84]	2007	Glass condensing chamber	H <sub>2</sub> O <sub>2</sub>	Spectrophotometry	No
Baraldi [31]	2006	Glass condensing chamber	3-nitrotyrosine	LC-MS	No
Bodini [33]	2006	Glass condensing chamber	Nitrotyrosine	ELISA	No
Boulet [17]	2006	Unknown	pH	pH meter	No
Brunetti [16]	2006	Teflon	pH	pH meter	No
Effros [22]	2006	Polycarbonate condenser; Corr-a-Flex	pH, acetate, NH <sub>4</sub> <sup>+</sup>	pH meter	Yes
Failla [54]	2006	Custom	Cysteinyl leukotrienes	ELISA	No
Mutti [99]	2006	TURBO DECCS	Metallic elements and serum pneumoproteins	Inductively coupled plasma-mass spectrometry and electrothermal atomic absorption spectroscopy	No
Oudijk [85]	2006	Glass condensing chamber	H <sub>2</sub> O <sub>2</sub>	Spectrophotometry	No
Ratnawati [20]	2006	Glass condensing chamber	NOx levels, pH	Fluorescence and pH meter	No
Shibata [60]	2006	Unknown	LTE4	ELISA	Yes
Bodini [114]	2005	Glass condensing chamber	pH, LTB4, IL-8	ELISA (LTB4, IL-8), pH meter	No
Ojoo [104]	2005	Glass condensing chamber	pH, NOx	pH meter, colorimetry (NOx)	No
(D)					
Esther [119]	2008	R-tube	Purines (ATP, ADP, AMP, adenosine)	Luciferin-luciferase luminescence assay	Yes
Esther [120]	2008	R-tube	Adenyl purines, urea	LC-MS	Yes

**Table 5.** (Continued.)

First author	Year	EBC collection device	EBC component measured	EBC analysis	Lyophilization
Gessner [28]	2008	Ecoscreen	Protein (cytokeratins 2, 9, 10); IL-6, IL-8	ELISA (IL6, IL8); Western blot (proteins)	Yes
Hoffmann [145]	2008	Ecoscreen	Cytokeratins	Mass spectrometry	Yes
Raissy [59]	2008	R-tube	Cysteinyl leukotrienes	ELISA	Yes
Baker [121]	2007	R-tube	Glucose	Anion-exchange chromatography with pulsed amperometric detection	Yes
Carraro [133]	2007	Tygon tube	Low molecular weight metabolites	Metabolomics, NMR spectra	Yes
Celio [35]	2006	EcoScreen	3-nitrotyrosine,	GC-NICI-MS; HPLC	Yes
Effros [22]	2006	Polycarbonate condenser; Corr-a-Flex	pH, acetate, NH <sub>4</sub> <sup>+</sup>	pH meter	Yes
Shibata [60]	2006	Unknown	LTE4	ELISA	Yes
Gessner [88]	2005	Ecoscreen	Cytokines (IL-1 $\beta$ , IL-6, IL-8, IL-10, IL-12, TNF- $\alpha$ )	Multiplex chemiluminescent immunoassay system (cytometric bead arrays)	Yes

*Abbreviations:* interleukin (IL); enzyme-linked immunoassay (ELISA); nitrate/nitrite ratio (NO<sub>x</sub>); not applicable (NA); liquid chromatography–tandem mass spectrometry (LC-MS); gas chromatography/negative ion chemical ionization/mass spectrometry (GC/NICI/MS); high-performance liquid chromatography (HPLC); gas chromatography–mass spectrometry (GC-MS); macrophage-derived chemokine (MDC).

with time [165]. The next goal in understanding pH as a biomarker in EBC is to explain the effect of treatment on EBC pH in different diseases. This may help elucidate the differences between inflammatory processes in each disease. Similarly, extrapulmonary conditions may affect the airways and, ultimately, the content of the breath exhaled. These extrapulmonary conditions need to be better understood in order to account for their effect in future studies, thereby eliminating possible confounding effects on measured EBC pH.

Although studies of nitric oxide metabolites have been promising, not enough data exists to understand its relationship with FE<sub>NO</sub> and other parameters of lung function for asthma and COPD. This finding suggests the importance of evaluating oxidative stress in the airways by evaluating several different mediators. Although nitrotyrosine has not proven to be a reliable marker in EBC for asthma, it appears as though the same is not true for COPD. Patients with COPD may produce consistently higher levels of this biomarker in EBC, enough to be useful as a marker for monitoring airway inflammation and its relationship to other markers of lung function. Larger studies should be done to investigate the role of these compounds in oxidative stress and asthma to determine whether these compounds have pathophysiological significance.

LTB<sub>4</sub> is promising for investigating the differences in airway inflammation of asthma, atopy and COPD, but more work should be done to evaluate the effect of different treatments for these airway diseases. Cyst-LTs have been well studied in asthma and allergic rhinitis; treatment monitoring with ICS is a promising future for Cyst-LTs in EBC. LTE<sub>4</sub> may prove to be especially useful in understanding the pathobiology of inflammation in the airways of patients with asthma. These findings suggest that leukotrienes may be an early marker of the inflammatory process in the lower airways.

8-Isoprostane illustrates an increased oxidative stress in the airways and increased levels have been found in patients

with more severe COPD. This biomarker must be assessed to learn about disease progression in COPD patients. Hydrogen peroxide levels in EBC have been used to learn more about the clinical syndrome of asthma and may prove to be an important tool for investigating the pathophysiology of inflammatory lung diseases like asthma. More studies must be done to establish the effects of therapy on H<sub>2</sub>O<sub>2</sub> levels in EBC for patients with COPD.

Further investigations must be done in order to determine a consensus regarding EBC technique in order to reduce the effect of confounders on data interpretation. Guidelines similar to the existing recommendations for FE<sub>NO</sub> measurement [137] and sputum eosinophils collection [138] are needed.

Several things must be improved and many questions must be answered in order to improve the routine use of EBCs. One of the largest problems with EBC is the need for a sensitive and specific assay for detecting biomarkers. Work has been done with novel techniques but high cost and complicated procedures hinder their application. EBC will also need to be standardized as a technique. Since different mediators detected in EBC arrive from different parts of the airway, it is unlikely that one technique will be the best for obtaining all biomarkers. More studies must be done in order to improve the understanding of where these markers originate before techniques can be standardized. Once these issues are resolved, reference values for each biomarker found in EBC should be created in order to use these biomarker levels to aid in diagnosing and treating conditions, similar to what exists for FE<sub>NO</sub>.

Future work for EBC is promising. Many groups [139] have suggested that a combination of biomarkers (FE<sub>NO</sub>, EBC pH, EBC H<sub>2</sub>O<sub>2</sub> levels, etc) would enhance our understanding of the airways; in fact, it is possible that ‘disease breathprints’ can be created for each disease. Although many biomarkers have been detected in EBC, little information exists on how

these biomarkers are related to clinical outcomes. The use of EBC in large samples and the future reference values of each biomarker may be used to predict disease progression, disease stability and response to current and novel therapies.

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