

# Exhaled Breath Condensates as a Source for Biomarkers for Characterization of Inflammatory Lung Diseases

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## ABSTRACT

Inflammatory lung diseases such as asthma and chronic obstructive pulmonary disease are common and difficult to diagnose and characterize. This is due in large part to difficulty in obtaining samples directly from the inflamed lung. The collection of lung secretions by traditional methods including bronchoalveolar lavage and induced sputum collection are limited by their invasive nature. Exhaled breath condensate (EBC) is a simple and non-invasive technique of collecting fluid samples, which are representative of airway lining fluid. Advances in collection methods and evolving molecular techniques have led to development of more sensitive assays for existing biomarkers and identification of new biomarkers, which can be potentially useful in monitoring lung inflammation. In this review, we present the current understanding of various biomarkers including small molecules (H<sub>2</sub>O<sub>2</sub>, pH and nitric oxide related biomarkers), lipid mediators (8-isprostane, leukotrienes and prostaglandins), small proteins (cytokines and chemokines) and nucleic acids (DNA and microRNAs). We also discuss the differential profile of biomarkers in recognizing different patterns of lung inflammation. As the sensitivity of methods of EBC improves, this biofluid will play an increasing role in diagnosis and monitoring of lung diseases.

**Keywords:** Exhaled Breath Condensate; Inflammation; Biomarkers; Hydrogen Peroxide; Ph; Micromnas; Nitric Oxide; Leukotrienes; Prostaglandins; Metabolomics

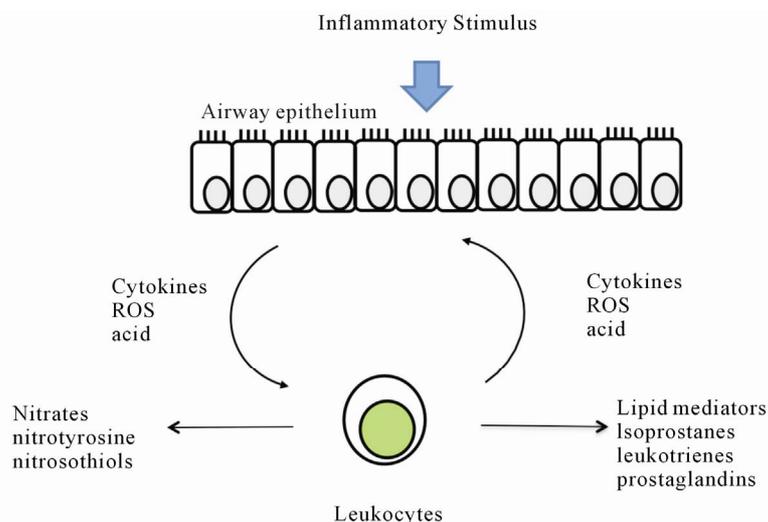
## 1. Introduction

Diseases of the lung such as asthma and chronic obstructive lung disease (COPD) are characterized by airway inflammation. These are common diseases with a US prevalence of 8.4% for asthma [1] and between 3% - 7% for COPD [2]. However, these diseases are difficult to diagnose, distinguish from each other, and characterize phenotypically. Inflammation in the airway involves an interplay between environmental stimuli, airway epithelial cells, and leukocytes (**Figure 1**). Stimuli such as infections, allergens, pollutants, oxidants, or irritants directly damage the airway cells or induce them to produce inflammatory mediators such as cytokines, reactive oxygen species (ROS), or acidic products. These mediators subsequently recruit various leukocytes to the lung, which can produce additional products, including cytokines, ROS, nitrates, and lipid mediators that contribute to the chronic inflammatory state. These mediators are found in airway lining fluid (ALF) and their measurement may serve as biomarkers to identify different inflammatory patterns in lung diseases. Their role in the

diagnoses and management of variety of lung diseases is now being recognized.

However, collection of ALF has always posed methodological challenges. The most reliable method is collection of fluid by bronchoalveolar lavage (BAL), an invasive technique that requires bronchoscopy to collect samples directly from the lower lung. Other methods, such as induced sputum, may be less invasive. However, it is difficult to reproducibly isolate high quality samples, salivary contamination is common, and it is a difficult and uncomfortable procedure for the subjects.

An emerging non-invasive technique to isolate ALF is exhaled breath condensates (EBC). Physiologically, the exhaled breath is constituted predominately by water vapor and aerosolized particles, generated by ALF. By cooling breath vapor, EBC can be collected and its biochemical composition has been found to be very similar to ALF [3]. One of the main advantages of EBC is the non-invasive collection technique, which can be conveniently performed by the patients in most age groups. The ease of collection of samples, combined with increasing

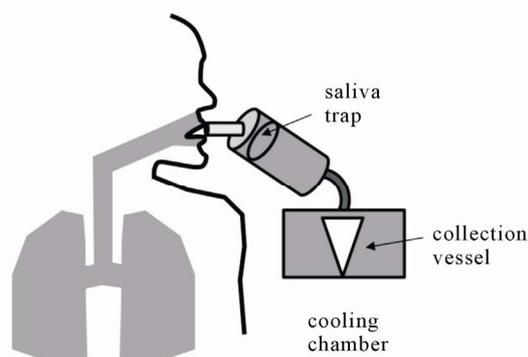


**Figure 1. Inflammatory mediators produced in airways. Stimulation of airway cells by inflammatory stimuli leads to production of inflammatory mediators such as cytokines, reactive oxygen species (ROS), or acidic products. These mediators subsequently recruit various leukocytes to the lung, which can produce additional products, including cytokines, ROS, nitrates, and lipid mediators that contribute to the chronic inflammatory state.**

sensitivity of various biomarker detection makes EBC a novel and potentially important diagnostic tool.

## 2. Collection of EBC

The principle of collecting EBC involves cooling the exhaled air below the dew point by transferring the heat to a chilled condenser surface (Figure 2). This leads to condensation of water vapor content in the breath over aerosolized ALF particles, leading to formation of enlarged EBC droplets on condenser wall [3]. The volume of EBC collected depends on multiple factors including amount of expired air, material and temperature of condenser etc. There are two commercial devices available for collection of EBC at this point-ECOScreen (VIASYS Healthcare, Hoechberg, Germany) and RTube (Respiratory Research Inc., VA) [3]. The ECOScreen uses an electric cooler, while the Rtube utilizes a metal tube that has been pre-cooled and then placed around the collection vessel. Studies have shown that level of biomarkers obtained from different collection devices varies and hence they cannot be compared directly [4]. Each has advantages, such as greater potential sensitivity of the EcoScreen versus enhanced portability and simplicity of the Rtube [5]. According to recent American Thoracic Society (ATS)/European Respiratory Society (ERS) guidelines, the subject should breath orally for around 10 minutes through the collection device, in a tidal pattern and this should allow collection of 1 - 3 ml of condensate [6]. The apparatus should have a salivary trap to minimize the salivary contamination of the condensate and contamination can further be evaluated by checking the amylase level in the collected sample [7].



**Figure 2. Schematic of EBC collection apparatus. The collection system consists of a salivary trap, a cooling mechanism, and collection vessel.**

## 3. Components of EBC and Their Role as Bio-Markers

Expired air, saturated with water vapor deposits in cooling chamber as distilled water and forms the main constituent of EBC. In addition, water-soluble volatile organic compounds (VOCs) present in gaseous phase in exhaled breath dissolve into the condensate. The third component is derived from aerosolized micro-particles which originate directly from ALF in the lower airways and contain both non-volatile constituents and dissolved VOCs [8]. Though the exact source of aerosolized particle in exhaled breath is not very clear studies have indicated a predominantly lower airway origin [9,10]. Detection of VOCs depends on multiple factors including water solubility, gas-liquid partition coefficient and temperature of condenser. The non-volatile compounds form

a broad range of molecules ranging from inorganic ions like sodium, to macromolecules such as small protein molecules like cytokines or nucleic acids.

EBC has been recognized as a source of multitude of organic and inorganic compounds including small inorganic molecules ( $H_2O_2$ , pH and nitric oxide related biomarkers), lipid mediators (8-isprostane, leukotrienes and prostaglandins), small proteins (cytokines and chemokines) and nucleic acid derivatives [11]. These biomarkers can reflect the underlying state of pulmonary inflammation and can be altered in various pulmonary diseases like asthma, COPD, bronchiectasis, infections, and lung cancer (**Table 1**).

#### 4. Small Inorganic Molecules in EBC

Hydrogen peroxide ( $H_2O_2$ ) is a reactive oxygen species, generated in inflammatory cells from metabolism of superoxide anion ( $O_2^-$ ) by superoxide dismutase. Increased oxidative stress during inflammation induces respiratory bursts resulting in marked production of  $O_2^-$  which leads to increased production of  $H_2O_2$  [12]. Its levels in EBC have been measured by spectrofluorimetry [13], spectrophotometry [14], and chemiluminescence [15]. As compared to healthy nonsmokers,  $H_2O_2$  have been reported to be elevated in smokers [13], asthmatics [16] [17], COPD patients [14], bronchiectasis [18] and Acute respiratory distress syndrome (ARDS) [19]. Levels of  $H_2O_2$  have been shown significantly elevated in acute worsening of asthma [11], COPD exacerbations [20], pulmonary infection related exacerbations of cystic fibrosis [21] and may have potential role in guiding therapy in these situations.

Despite recognition of  $H_2O_2$ , as an indicator of oxidative stress, it has not been established as a reliable EBC biomarker due to several limitations. The mean levels of  $H_2O_2$  have shown a wide variability in EBC of healthy nonsmoking adults (0.01 - 0.45 micromoles) [13] and healthy children between 8 - 13 years (median  $H_2O_2$  levels was reported as 0.13  $\mu$ M, with reference range of <0.01 - 0.48  $\mu$ M) [22]. Additionally, variability of levels were found on different days of measurement on same subjects and at different times of the day [13]. The concentrations of  $H_2O_2$  in EBC was also found to be related to expiratory flow rate, suggesting a predominantly bronchial origin of  $H_2O_2$ , as compared to alveolar origin, further limiting its usefulness as a pulmonary inflammatory marker [23].

EBC pH reflects the acid/base balance of the airways. The major source of acidification of EBC includes intrinsic pulmonary inflammatory burden but there could be additional contribution from gastroesophageal reflux disease [24]. EBC pH from samples collected from oral breathing were found to be similar to endotracheal sam-

ples but different from salivary pH, supporting a predominantly lower airway origin of EBC pH [25]. Standard pH analysis is performed after de-aeration of carbon dioxide ( $CO_2$ ) with an inert gas like argon to minimize artifacts [24]. One of the main advantage of EBC pH is the fact that it has been found to be an extremely reproducible and robust parameter and does not show deviation with type of ventilation, gender, time of day collected, volume of EBC collected, duration of EBC collection, degree of hyperventilation [24] or temperature or duration of sample storage [24,25]. Normal values of gas-standardized ( $CO_2$ -free) EBC pH have been reported between 7.5 and 8.1 [24,25]. Decrease in EBC pH has been reported in both stable and unstable asthma patients as compared to healthy controls, with a greater decrease seen in unstable asthmatics treated with inhaled corticosteroids [26]. Similarly, EBC pH levels have been reported to be lower in patients with COPD compared to age and gender matched controls and correlate with disease severity, as expressed by GOLD stages, especially in ex-smokers [27]. EBC pH may have potential role in diagnosis of upper respiratory symptoms like chronic cough due to underlying acid reflux [28]. A system for continuous monitoring of EBC pH in intubated patients on mechanical ventilation has also been developed to monitor lung health in critically ill patients [24,29].

#### 5. Nitric Oxide Related Biomarkers

Nitric Oxide (NO) is an important bioactive mediator that plays important roles in multiple cellular functions. The levels of NO increase during inflammatory conditions due to activation of inducible NO synthase in response to proinflammatory cytokines [30]. NO can be measured in exhaled breath in form of Fractional excretion of NO (FeNO) and has been shown to correlate with active lower airway inflammation in asthmatics [31]. Nitrates and nitrites are products of NO metabolism that can be detected in EBC and their levels have been reported to be elevated in smokers with COPD as compared to healthy smokers [32]. NO can combine with reactive oxygen molecules to form highly reactive nitrogen species (RNS) which further covalently interact with various biomolecules to form nitroso- and nitro-molecules [33]. S-Nitrosothiols are formed by interaction of RNS with low molecular thiols and have reported to be elevated in severe asthma, COPD and cystic fibrosis [34]. 3-Nitrosotyrosine is another covalent adduct molecule generated by interaction of RNS with tyrosine with in the proteins [33] and has been shown to be elevated in patients with asthma [35] and cystic fibrosis [36]. NO and related molecules are non-specific markers of oxidative stress and may be useful in monitoring various pro-inflammatory pathologies of lower airways.

**Table 1. Biomarkers in EBC.**

<i>Type</i>	<i>Biomarker</i>	<i>Utility</i>	<i>Method</i>	<i>Disease associations</i>
Small inorganic molecules	H <sub>2</sub> O <sub>2</sub>	Non-specific marker of Oxidative stress	Spectrophotometry [13], SpectroFluorimetry [14], Chemiluminescence [15]	Asthma [16,17] COPD [14] Smokers [13] Bronchiectasis [18] ARDS [19]
	pH	Non-specific marker of Oxidative stress	pH meter after gas standardization [24]	Asthma [26], COPD [27], cough related to GERD [28] Acute lung injury (ALI) and ARDS [29]
Nitric oxide related biomarkers	Nitrates	Non-specific marker of Oxidative stress	Fluorimetry [32]	Elevated in smokers with COPD [32]
	S-Nitroso-thiols	Non-specific marker of Oxidative stress	Spectrophotometry [34]	Asthma Cystic Fibrosis Asthma, COPD and cystic fibrosis [34]
	3-Nitroso-tyrosine		liquid chromatography–tandem mass spectrometry [35], EIA[36]	Asthma [35], cystic fibrosis [36]
Lipid mediators	8- IP	Non-specific marker of Oxidative stress	EIA [41], Gas Chromatography/mass spectrometry [81]0	Asthma [39], Smoking and COPD [40], ALI and ARDS [42] Cystic Fibrosis [41] Exercise induced Bronchospasm (EIB) [43]
	LTB4	Neutrophil predominant inflammation	EIA [45]	COPD [45], cystic fibrosis [46], asthma [47], NSCLC [48]
	CysLTs; LTC4, LTD4, LTE4	Eosinophils and mast cell predominant inflammation	EIA [49]	Allergic asthma [49], EIB [50]
	PGD2	Mast cell predominant inflammation	EIA [49]	Allergic asthma [49]
	PGE2	Unclear role- inhibitory vs. proinflammatory	EIA [45]	COPD [45] and CF [55]
	TxA2	Broncho-constriction	RIA [56]	Asthma [56]
Cytokines and chemokines	IL-2	Acute Inflammatory cytokine	multiplex immunoassay [68]	Asthma [68]
	IL-4	Th2 Inflammatory pathway	multiplex immunoassay [68]	Asthma [68]
	IL-5	Th2 Inflammatory pathway	multiplex immunoassay [68]	Asthma [68]
	IL-6	Th17 Inflammatory pathway	multiplex immunoassay [68]	Asthma [68], COPD exacerbation [69]
	IFN-γ	Th1 Inflammatory pathway	multiplex immunoassay [68]	Asthma [68]
	IL-18	Th1 Inflammatory pathway	multiplex immunoassay [68]	Asthma [68]
	IL1β	Acute Inflammatory cytokines	multiplex immunoassay [69]	COPD exacerbation [69]
	IL-8	Neutrophilic Inflammation	multiplex immunoassay [68]	Asthma [68], COPD exacerbation [69]
	IL-10	Treg Inflammatory pathway	multiplex immunoassay [68]	Asthma [68] , COPD exacerbation [69]8]
	IL-12	Th1 Inflammatory pathway	multiplex immunoassay [69]	COPD exacerbation [69]
	RANTES	Allergic inflammation	EIA [70]	Asthma [70]

**Continued**

	MIP1 $\alpha$	Migration of T cells, NK cells, monocytes	multiplex immunoassay [68]	Asthma [68]
	eotaxin	Eosinophilic inflammation	EIA [71]	Asthma [71]
Nucleic acids	DNA	Infection	PCR [73,75]	COPD exacerbation [73], Detection of HPV in Lung Cancer [75]
	miRNAs	Allergic vs. non allergic inflammation	qPCR	Asthma and COPD

**6. Lipid Mediators**

Lipid mediators are produced in leukocytes from the enzymatic metabolism of phospholipids (**Figure 3**) and have numerous physiologic and pathologic functions. Levels may be altered in a variety of lung diseases underscoring their potential as biomarkers. Isoprostanes are prostaglandin like substances produced *in vivo* by free radical-induced peroxidation of arachidonic acid, independent of cyclooxygenase (COX) enzymes [37]. They are chemically stable molecules and their levels in body fluids have been reported as a reliable indicator of oxidative stress [38]. Elevated levels of 8-Isoprostane (8-IP) have been reported in asthma [39], healthy smokers and smokers with COPD [40], cystic fibrosis [41], acute lung injury and ARDS [42], and exercise induced bronchospasm [43].

Leukotrienes are derivatives of arachidonic acid through the action of 5-Lipoxygenase (5-LO) [44]. Leukotriene (LT) B<sub>4</sub>, formed from hydrolysis of LTA<sub>4</sub> has been shown to be a major neutrophil chemoattractant. Elevated LTB<sub>4</sub> levels in EBC have been reported in neutrophilic predominant inflammatory lung pathologies including COPD [45] and cystic fibrosis [46]. Its levels have been reported to be significantly elevated in moderate to severely uncontrolled asthma patients whereas in mild episodic asthmatics, its role is not clear [47]. A recent study has also reported elevated LTB<sub>4</sub> levels in patients with Non-Small Cell Lung Cancer (NSCLC), indicating presence of neutrophilic inflammation in airways of these patients [48].

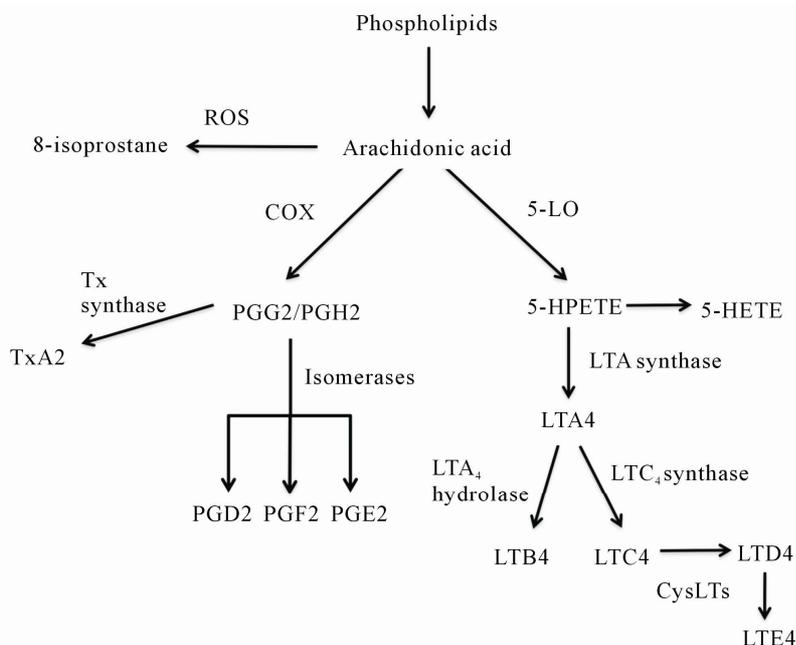
Cysteinyl-leukotrienes (CysLTs; LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub>), are produced in mast cells and eosinophils and their levels have been shown to be elevated in EBC from patients with asthma induced by allergic inflammation [49]. Another study reported elevation of CysLTs in asthma patients with exercise induced bronchospasm (EIB), with levels correlating with decrease in FEV<sub>1</sub> [50], whereas no difference was seen in levels of LTB<sub>4</sub> in EIB patients. In a recent study, multiple eicosanoids were measured in EBC samples of aspirin sensitive and aspirin tolerant asthma patients using gas chromatography/mass spectrometry and high-performance liquid chromatography [51]. The authors did not find significant difference

in concentration of leukotrienes, prostaglandins, and thromboxanes at baseline or after aspirin challenge in aspirin sensitive asthma patients as compared to aspirin tolerant asthma patients. The eicosanoids that were found to be elevated baseline in aspirin sensitive patients were 5 and 15-hydroxyeicosatetraenoic acid (HETE). Another study reported significant increase in the level of prostaglandin D<sub>2</sub> and E<sub>2</sub> metabolites and 5-, 15-HETE in aspirin intolerant asthma patients by using complementary high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC/MS) [52]. The authors proposed that highly sensitive eicosanoid profiling in EBC can be used to identify aspirin intolerant asthma phenotypes.

Prostaglandins are lipid mediators derived from metabolism of arachidonic acid by action of cyclooxygenase (COX) enzyme. Prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) is predominantly produced by mast cells and its levels were reported to increase in EBC after allergen-induced bronchoconstriction in asthmatic patients [49]. The levels were quantified using enzyme immunoassays (EIA) and increase in levels correlated with degree of bronchoconstriction. The role of Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is not very clear. Earlier studies reported a predominantly inhibitory role in lung inflammation [53] but more recent studies have postulated that it may also have pro-inflammatory action [54]. A significant increase of PGE<sub>2</sub> was reported in COPD patients, both steroid naive and steroid treated, as compared to healthy controls [45]. PGE<sub>2</sub> levels were also reported to be elevated in patients with stable and unstable cystic fibrosis [55]. The levels of thromboxane A<sub>2</sub> (TxA<sub>2</sub>), were reported to be increased in asthmatics as compared to healthy controls [56]. These authors also reported an improved TxA<sub>2</sub> detection rate with radioimmunoassay (RIA) as compared to standard EIA.

**7. Cytokines and Chemokines**

Cytokines and chemokines are small protein molecules involved in intercellular signaling and play an important role in inflammatory pathways in lungs and other organs. Elevated levels of cytokines have been reported in BAL specimens of patients with acute lung injury and ARDS



**Figure 3. Lipid Mediators.** Prostaglandins (PGD2, PGE2 and PGF2) and Thromboxane A2 (TxA2) are derived from arachidonic acid by COX mediated pathway. 8-Isoprostane (8-IP) is formed by free radical peroxidation of arachidonic acid, independent of cyclooxygenase (COX) enzymes. Leukotrienes (LTB4 and Cysteinyl-leukotrienes-LTC4, LTD4 and LTE4) are produced by action of 5-lipoxygenase (5-LO).

[57,58], asthma [59], COPD [60] and cystic fibrosis [61]. Elevated cytokines levels have also been reported in induced sputum samples in patients with COPD [62] and asthma [63]. EBC provides a non-invasive alternative for obtaining ALF samples and has been increasingly studied as a source for monitoring cytokines. Unlike other non-specific markers of oxidative stress, cytokines signatures can be useful in defining type of inflammation and etiology. Interaction of naïve T-cells (Th0) with other inflammatory cells and molecules leads to their differential into either T helper (Th)1 T-cells which are involved in cell mediated immunity or Th2 T-cells, which are involved with antibody mediated immunity and allergic inflammation. Increased levels of IL-4 (Th2 cytokine) and decreased interferon- $\gamma$  (Th1 cytokine) have been reported in EBC samples of asthmatic children indicating a Th2 predominant inflammation [64]. Other authors reported extremely low concentration of cytokines when collected by standard collecting apparatus and using EIA based analysis [65]. Improved detection of cytokines was reported with use of flow cytometry but this group did not find any difference in cytokine profile between asthma and COPD patients [66]. More recently, the use of glass condenser system has been reported to improve the yield of cytokines and other biomarkers in EBC samples [67]. The use of this system along with use of a more sensitive multiplex immunoassay has been reported to significantly improve the detection of cytokines in EBC [68]. This group reported increased levels of IL-2,

IL-4, IL-5, IL-6, IFN- $\gamma$ , IL-18, MIP1 $\alpha$ , RANTES, IL10, and IL-8 in EBC samples from asthmatic patients as compared to controls. Elevated levels of cytokines IL1 $\beta$ , IL-6, IL-8, IL-10, IL-12p70 have also been reported in patients with acute COPD exacerbation as compared to stable COPD, healthy smokers and healthy nonsmokers [69]. More recent studies have also reported increased levels of eosinophil specific chemokines-RANTES [70] and eotaxin [71] in EBC of asthma patients. Another potential use for cytokine monitoring can be in diagnosing airway inflammation in preschool children with wheezing [72].

## 8. Nucleic Acids

One of the main limitations of EBC is the low concentration of biomolecules, which pushes the detection limits of many assays. An emerging use of EBC is the measurement of nucleic acids, which can be amplified and detected in low numbers by PCR techniques. A few reports have demonstrated the presence of microbial DNA in EBC, and the impact on this a methodology for diagnosis of infection is emerging [73-76].

Another useful biomarker may be microRNAs (miRNAs), which are small 20 - 25 nucleotide, non-coding RNAs. MiRNAs are involved in post-transcriptional regulation of gene expression and are emerging as regulatory molecules of inflammation [77]. Differential expression of specific miRNAs in sputum of non-small

cell lung cancer patients [78], especially in adenocarcinoma [79] can potentially serve as biomarkers for early detection of lung cancers and highlights their potential role in disease diagnosis.

We recently demonstrated that miRNAs are expressed in EBCs, and they can be reproducibly isolated and quantified by real time PCR (**Figure 4(a)**). In this methodology, an adapter is added to the 3' end of miRNA, and specific miRNA are amplified by using a forward primer specific to miRNAs of interest and a universal reverse primer to the adapter. After real time PCR analysis, the miRNAs were subjected to gel electrophoresis which confirmed the presence of one species at the expected size of the miRNA plus adapter sequence.

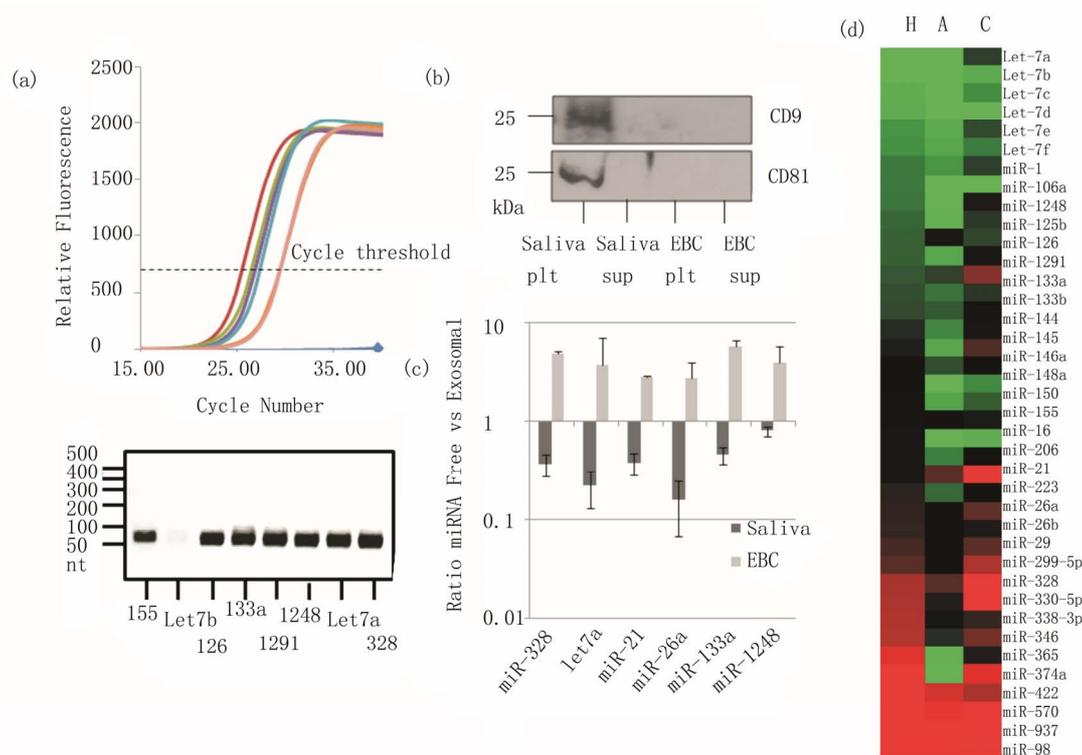
MiRNAs in can be secreted into bodily fluids from cells in exosomes, vesicles that contain miRNA, mRNA, and proteins. Exosomes can be isolated in the pellets of serum or saliva subjected to ultracentrifugation. We found that in contrast to saliva, exosomes are not present in EBC, and miRNAs exist free in solution (**Figures 4(b)** and **(c)**). We profiled miRNAs from EBC samples of asthmatics ( $n = 10$ ), COPD patients ( $n = 10$ ) and healthy controls ( $n = 14$ ), and found that miRNAs were differentially expressed between the groups (**Figure 4(d)**). For

instance, in asthmatics, miR-1248 and miR1291 expression were lower compared to COPD patients and healthy controls. These miRNAs are predicted to regulate allergic mediators, such as IL-13, IL-5, GATA3, and the high affinity IgE receptor. Thus, miRNAs may not only be used as biomarkers to differentiate between types of lung disease, but may also offer insight into the pathogenesis of disease.

We also found that small nucleolar RNAs such as SNORD44 is expressed in EBC. These RNAs have served as “housekeeping” genes to normalize expression of miRNAs between samples. Methods of normalization between subjects are essential components of EBC biomarker measurement. Additionally, since as little as 50 - 100 molecules/ml of miRNA can be reproducibly quantified using qPCR, this may be an especially promising technique.

## 9. Limitations and Future Directions

EBC is a promising technology that allows insight into pulmonary pathophysiology in a non-invasive way. The concentration of biomolecules in EBC is highly diluted (up to 2000 to 10,000 fold) by condensed water vapor [6],



**Figure 4. MiRNA expression in EBC:** (a) real time PCR analysis of miRNAs and analysis of the products by gel electrophoresis; (b) Saliva or EBC were subjected to ultracentrifugation to pellet exosomes. Western blot for the exosome-specific proteins CD81 and CD9 showed their presence in the ultracentrifugation pellet (plt) of the saliva but not pellet of EBC or the supernatant (sup) or EBC or saliva; (c) Real time PCR analysis of miRNA expression demonstrates that they are present in the exosomal fraction of saliva but in the supernatant of EBC; (d) Heat map showing average expression of miRNA in healthy subjects (H), asthmatics (A), or COPD patients (C).

barely at lower detection limit by standard immunoassay techniques. The role of dilution indicators including conductivity of sample [80], urea and electrolytes [10] have been proposed for standardization of EBC samples but have not yet been established. In the absence of standardization, the wide variability of biomolecules concentration has become one of the main limitations of this technology. Using ratios of related biomarkers instead of absolute values, or normalization of data to housekeeping genes in the case of miRNA, may be useful as standardization measures.

With the development of newer analytical methods like liquid chromatography/mass spectrometry (LC/MS) [81], GC/MS, HPCL, radioimmunoassay (RIA) [56] and multiplex immunoassays [68] and use of glass condenser system for EBC collection [68], there have been significant improvement in the yield and detection threshold of various biomarkers. Another important development is application of metabolomics, which is study of measuring biomolecules in metabolic pathway in body fluids. In a recent study [82], nuclear magnetic resonance technique (NMR) spectroscopy was applied to analyze metabolomics in EBC samples from healthy subjects, COPD patients and patients with laryngectomy and significant difference in their metabolic fingerprints were reported in-between study groups.

In summary, EBC is an exciting technology, allowing an insight into biomolecule milieu of airway lining fluid. It is an important source of biomarkers, both non-specific markers of oxidative stress and specific biomarkers indicating different aspects of pulmonary inflammation. As the ability to comprehensively measure many markers increase via high-throughput methods, EBC will likely play a prominent role in disease diagnosis and characterization. At present, it is primarily used in research, but is emerging as a novel tool in diagnosing and managing inflammatory lung conditions and developing personalized pharmacological strategies.

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## Abbreviations

COPD: Chronic obstructive pulmonary disease	EIB: Exercise induced bronchospasm
EBC: Exhaled breath condensate	HETE: Hydroxyeicosatetraenoic acid
H <sub>2</sub> O <sub>2</sub> : Hydrogen peroxide	PGD <sub>2</sub> : Prostaglandin D <sub>2</sub>
DNA: Deoxyribonucleic acid	EIA: Enzyme immunoassays
RNA: Ribonucleic acid	PGE <sub>2</sub> : Prostaglandin E <sub>2</sub>
MiRNAs: microRNAs	TxA <sub>2</sub> : Thromboxane A <sub>2</sub>
ROS: Reactive oxygen species	RIA: Radioimmunoassay
ALF: Airway lining fluid	Th0: Naïve T-cells
BAL: Bronchoalveolar lavage	Th: T helper
VOCs: Volatile organic compounds	IL: Interleukin
O <sub>2</sub> <sup>-</sup> : superoxide anion	IFN-γ: Interferon-gamma
ARDS: Acute respiratory distress syndrome	RANTES: Regulated and normal T cell expressed and secreted
FeNO: Fractional excretion of NO	MIP1α: Macrophage Inflammatory Proteins1α
RNS: Reactive nitrogen species	PCR: Polymerase chain reaction
NO: Nitric oxide	Treg: Regulatory T cells
CO <sub>2</sub> : Carbon dioxide	NK cells: Natural killer cells
COX: Cyclooxygenase	NMR: Nuclear magnetic resonance
8-IP- 8-Isoprostane	HPCL: High performance liquid chromatography
5-LO: 5-Lipoxygenase	GC/MS: Gas chromatography/mass spectrometry
LT: Leukotriene	LC/MS: Liquid chromatography/mass spectrometry
NSCLC: Non-small cell lung cancer	
CysLTs: Cysteinyl-leukotrienes	