

Exhaled breath condensate and airway inflammation

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Purpose of review

The collection of exhaled breath condensate (EBC) is a noninvasive method for evaluation of airway inflammation. This article reviews recent data concerning the ability of EBC markers to reflect alterations in asthma and chronic obstructive pulmonary disease or environment and occupation-induced changes.

Recent findings

The recovery of biomarkers in EBC is affected by physical characteristics of the condensing device and collecting circumstances as well as environmental conditions or exercise. The complexities of nitrogen oxide chemistry make assessment of nitric oxide metabolites in EBC and exhaled nitric oxide complementary. Analysing of EBC markers is valuable in asthma, as changes were reported irrespective of current anti-inflammatory treatment or atopic status as well as in monitoring cigarette smoking-related airway inflammation in chronic obstructive pulmonary disease patients. Hyperinflation in chronic obstructive pulmonary disease might be a potential confounder for the level of inflammation markers in EBC. In general, patterns of markers are likely to more accurately reflect the complex pathophysiological processes and therefore should be evaluated rather than a single marker.

Summary

EBC might be of particular interest in preventive medicine as inflammatory processes triggered may precede changes in lung function. Robust and easy-to-handle condensing devices and analytical methods are warranted to spread the use of EBC.

Keywords

asthma, chronic obstructive pulmonary disease, exhaled breath condensate, inflammation, occupational

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Introduction

The lung respiratory system represents the route of entry for many environmental and occupational pollutants. During the last decades, the concept of inflammation as a fundamental process of airways diseases has gained much attention. Inflammation is a hallmark in the pathophysiology cascade leading to respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD) [1]. Nevertheless, there are profound differences in the underlying airway pathophysiology between asthma and COPD, with different patterns of inflammatory cells and mediators being involved [2]. Currently induced sputum, bronchoalveolar lavage or bronchial biopsy during bronchoscopy is used for direct assessment of airway inflammation, but these methods are more or less invasive. In contrast, collection of exhaled breath condensate (EBC) is completely noninvasive and does not disturb the underlying disease process.

EBC is the liquid phase of the exhaled air sampled by cooling. This is exudated along with the gaseous phase

that contains volatile substances such as nitric oxide, carbon monoxide and hydrocarbons. Exhaled nitric oxide is the best validated gaseous constituent [3] and is used for assessing airway inflammation in clinical practice, particularly in patients with asthma [4]. EBC is mainly formed by water vapour, but volatile substances in gas phase as well as nonvolatile compounds such as proteins carried in droplets can dissolve in condensed water during the sampling. A wide variety of substances have already been detected in EBC, most of them already referred to in induced sputum or bronchoalveolar lavage (BAL) [5•].

Because EBC contains no cellular components, the evaluation and quantification of airway diseases are based on metabolic products released by cells from the lungs or substances originating from inflammatory reactions in the airway mucosa reflecting biochemical changes of airway lining fluid. Oxidative stress is functionally involved in the pathophysiology of the inflammatory process and is closely linked to nitric oxide metabolism and nitrosative stress [6]. Widely used indicators for investigation of airway diseases are listed in Table 1. Other compounds

Table 1 Important compounds in exhaled breath condensate used for diagnosis, assessment of severity and follow-up

Process	EBC compound	Detection method
Inflammation	Leukotrienes	
	LTB ₄	EIA, GC/MS, LC/MS
	CysLT (LTC ₄ , LTD ₄ , LTE ₄)	EIA, GC/MS
	Prostaglandins	
	PGE ₂	EIA, GC/MS
	PGF _{2α}	EIA
	Cytokines	
	IL-1β	EIA, multiplex bead array
	IL-4	ELISA, protein array, flow cytometry
	IL-6	EIA, multiplex bead array
	IL-8	ELISA, protein array, multiplex bead array
	TNFα	ELISA, protein array, multiplex bead array
	IFN-γ	EIA, flow cytometry
	Hydrogen ions (pH)	pH-meter, blood gas analyser
		Directly after collection
		Argon gas deaeration
		At standard EBC CO ₂ level
Oxidative stress	Hydrogen peroxide	Fluorometric assay, colorimetry, chemiluminescence
	Lipid peroxidation marker	
	8-Isoprostane	EIA, GC/MS
	Malondialdehyde	EIA, HPLC, GC/MS
Nitrosative stress	Nitrite	Fluorimetric assay, colorimetry, chemiluminescence
	Nitrate	Fluorimetric assay, colorimetry, chemiluminescence, ion chromatography
	Nitrotyrosine	EIA, HPLC, GC/MS, LC/MS
	Nitrosothiols	Fluorimetric assay, colorimetry

CysLT, cysteinyl leukotrienes; EBC, exhaled breath condensate; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; LTC₄, leukotriene C₄; LTD₄, leukotriene D₄; LTE₄, leukotriene E₄; PGE₂, prostaglandin E₂.

determined in EBC include proteins, DNA, keratin, electrolytes, trace elements and toxic metals. Identification of compounds depends on the analytical technology employed and is often at the limit of detection. Recommendations for EBC collection and the potential pitfalls of the technique are summarized in the American Thoracic Society/European Respiratory Society Task Force report [7].

EBC can be sampled from individuals on multiple occasions, allowing monitoring the time course of an inflammatory response as well as the response to pharmacological therapy and follow-up in longitudinal studies. This review focuses on recent data obtained from articles published since 2007 concerning applications of EBC in clinical research and practice. As the methodology for EBC sampling is important to maximize the collection of the relevant component, data concerning technical aspects are also referred to.

Impact of exhaled breath condensate collecting procedure on mediators

The composition of the condensate depends, amongst others, on the sampling equipment and cooling temperatures used [8]. Nowadays, a variety of custom-made or commercially available condenser designs are used [9]. Recent studies highlighted the point that physical charac-

teristics of the condensing device affect the recovery of widely studied and cited biomarker in EBC. These markers include hydrogen peroxide, nitric oxide-related products [10], prostanoids, leukotrienes [11], isoprostanes [12] and pH [13]. Reproducibility of biomarkers in EBC from healthy adult volunteers, however, was not influenced by the type of condensing device [14]. Normal values of these markers with methodical issues applied are listed in an outstanding summary [15^{••}]. It is obvious that one collection technique would not be optimal for all compounds of interest in studies using EBC as matrix. Therefore, the methodical setting often is based on a compromise and should be appropriately evaluated [16]. This is also true for the analytical assays applied concerning sensitivity and specificity versus availability, cost and technician time required [17].

Utility of exhaled breath condensate inflammation markers for detection of effects resulting from environmental exposition

Because of differences in perception and functional reserves of the cardiopulmonary system, the presence and extent of disease activity may not be accurately reflected by clinical symptoms or lung function tests. Measurements of biomarkers of effect in EBC are suggested as a way of exploring adverse effects in the context of air pollution exposure. There is a special focus

on markers reflecting oxidative stress as air pollutants have the ability to drive free radical reactions.

In a longitudinal assessment of lung function and symptoms, peak levels of wood-smoke particulate air pollution were associated with clinical effects on asthmatic patients but not healthy students. However, no demonstrable effect on airway inflammation and oxidative stress reflected by EBC pH and H_2O_2 could be verified even though a significant exposure could be demonstrated by higher 1-hydroxypyrene levels in urine on high pollution days used for EBC sampling [18]. During peak smog season, the EBC pH of athletes after vigorous outdoor exercise was not changed. However, the pH values at baseline were already rather low as compared with control samples or published data indicating that repetitive vigorous exercise may be a reason for airway acidification [19]. Moderate exercise on a walking treadmill placed in an indoor chamber can result in a persistent increase in EBC pH in healthy young volunteers [20]. In the context of work-related investigations, this result might gain relevance, as work is usually associated with more or less exercise. Therefore, the biomarker of interest should be evaluated according to the potential confounding of exercise when studying work-related inflammatory processes.

There is also evidence that environmental conditions (e.g. temperature and relative humidity) themselves may have an impact on EBC pH as well [21]. After arrival of a cold and humid meteorological front, EBC pH decreased significantly, whereas, in contrast, a warm and dry front results in an opposite effect. It could be speculated that these findings are related to the fact that respiratory tract infections are more likely in cold weather.

Especially, ozone is a highly reactive gas contributing to oxidative stress. Malondialdehyde is produced by reaction of oxygen radicals with the polyunsaturated fatty acid residues of phospholipids and was positively associated with indices of exposure to air pollution (PM 2.5; ozone and NO_2) in a panel study of 107 children with asthma living in Mexico City [22[•]]. Levels of malondialdehyde in EBC were significantly related to two other biological effects, namely lung function and increase in IL-8 measured in nasal lavage. The use of EBC as a matrix to assess effects of traffic-related pollutants was confirmed by EBC pH, which showed a significant inverse correlation to the level of malondialdehyde. Breathing ultrafine and fine particulate matter during outdoor exercise decreased EBC nitrite and alveolar nitric oxide in relation to observed lung function impairments [23]. The fact that a large increase in malondialdehyde was detected in parallel supports a peroxynitrite triggered lipid peroxidation. In summary, malondialdehyde could be demonstrated as a suitable marker of

oxidative/nitrosative stress in clinical settings even if malondialdehyde represents only approximately 1% of lipid peroxides.

In a more complex way, the time course relationship of the appearance of specific biomarkers along the proposed pathways leading to pulmonary function decrements and airway neutrophilia after ozone (O_3) inhalation was evaluated [24[•]]. According to the reaction of lung function after inhalation of 0.35 ppm O_3 , volunteers were placed in two groups (sensitive and nonsensitive). Both groups showed a similar peak in total nitric oxide metabolites 4 h after controlled O_3 inhalation, suggesting that O_3 exposure was effective. After inhalation of O_3 , only in the sensitive group, an exaggerated arachidonic acid metabolism, represented by leukotriene B_4 (LTB $_4$) and 8-isoprostane, was visible. The higher concentration of LTB $_4$ throughout the sampling period indicates that the lipoxygenase pathway is preferred over the free radical, nonenzymatic, or cyclooxygenase-1 pathway after exposure. Even if the inflammation marker, IL-8, was not detectable in that study, it proved useful in a large nonsmoking population of 207 employees complaining of respiratory symptoms that they perceived to be related to a water-damaged office building [25]. Only IL-8 was related with the number of symptoms and physician-diagnosed asthma, whereas nitric oxide and EBC nitrite were no strong indicators in the assessment of population health effects.

Exhaled breath condensate markers as a tool for exposure assessment/environmental confounding

Measurements of pH and hydrogen peroxide in EBC demonstrated the presence of airway inflammation in asymptomatic welders, which was modulated by the different metal fumes and gases generated according to the material and method used for the welding process [26]. In addition, the particle burden and inflammatory cells analysed in induced sputum depended on the type and duration of welding. A good correlation between chromium levels and biomarkers of oxidative stress (H_2O_2 , malondialdehyde) could also be observed in EBC of otherwise asymptomatic chrome-plating workers [27].

In COPD, patients with higher levels of toxic elements (lead, cadmium and aluminium) and lower levels of transition elements (iron and copper) are detectable as compared with nonsmoking healthy controls [28]. Assessing EBC metal level could help in evaluating the underlying mechanism of inflammatory airway diseases, as transition elements are known to be causatively involved in the generation and detoxification of reactive oxygen species as well as in scavenging of free radicals.

Metal analysis in EBC may also be suitable to assess metal dose at target level, providing a measure of exposure. With respect to respiratory physiology, it can be assumed that, in general, inhaled substances could be detected in EBC. Therefore, it seems consequent to clarify to what extent markers assessed in EBC might be originated from ambient air. Analysing cytokeratin patterns suggest that the major weight fraction of protein in EBC derives from debris shed of the stratum corneum and inhaled with inspired air [29^{••}]. In an investigation dedicated to examine physiological and methodological influences, a significant increase in the concentration of H₂O₂ occurred in the course of the day in the room where EBC samples were collected from calves [30[•]]. Ambient concentration of H₂O₂ may be attributed at least partially to the calves expiring in the room. This ambient background H₂O₂ may contribute to exhaled H₂O₂ and should be monitored to allow a proper interpretation of H₂O₂ values assessed in EBC. In contrast, the pH in ambient air condensate was nearly constant, implying that changes in the EBC pH of calves are most likely not confounded by the ambient air pH.

Inflammatory markers in exhaled breath condensate from patients with chronic obstructive pulmonary disease

COPD is a disease with predominant neutrophilic inflammation and is common in smokers. In general, COPD disease is diagnosed when lung function parameters have become significantly reduced, reflecting a major damage of the lung. Several markers of oxidative and nitrosative damage have been detected, but because smoking is the major cause of COPD, it is to question whether observed changes are related to the impact of smoking alone or can be used to assess cigarette smoke-related lung disease. COPD patients were shown to have higher levels of nitric oxide than controls; however, within COPD patients, nitric oxide levels are not significantly different from the nonsmoking, exsmoking and smoking subgroups. In contrast, oxides of nitrogen (NO_x) levels in EBC are significantly different among different smoking status subgroups [31], and therefore, NO_x may be a more sensitive marker than nitric oxide in monitoring cigarette smoking-related airway inflammation in COPD patients. There is a greater neutrophil and lower eosinophil chemotactic activity of EBC collected from stable COPD patients as compared with controls, and current smoking favours neutrophil chemotactic activity, which could at least partially be attributed to IL-8 [32].

A hallmark of neutrophil activation is its respiratory burst in which myeloperoxidase catalyses the conversion of hydrogen peroxide (H₂O₂) and chloride ions into hypochlorous acid. Involvement of oxidative stress in the underlying pathophysiology of COPD was confirmed

by the detection of lipid peroxidation products. Among these, 8-isoprostane is widely used as a marker, but its clinical value remains largely unknown. A relationship between 8-isoprostane and lung function impairment or neutrophilic inflammation assessed in induced sputum could not be demonstrated in patients with stable COPD of different severity [33^{••}]. However, a significant correlation between 8-isoprostane and emphysema in high-resolution computed tomography was found, and 8-isoprostane levels were significantly associated with dyspnoea. Recently, for COPD patients classified at least gold class 2, a logarithmic correlation of EBC nitrite and lung function could be observed as being the strongest for the pulmonary function tests indicating hyperinflation. In contrast, EBC nitrite level was not correlated with airway obstruction or proinflammatory cytokines [34].

On the basis of these reports, we suggest estimating the extent of hyperinflation instead of the mere detection of an airflow limitation in studies concerning COPD to interpret results of EBC marker levels properly. Identifying subtypes of COPD with leading chronic bronchitis or a predominant emphysematous component may have implications for composing study subgroups and for the monitoring and management of patients with COPD.

In the airways of COPD patients, colonization and infection with bacterial or viral pathogens are common, especially with advancing progression of the disease or when bronchiectasis is present. Influence of bacteria especially on nitric oxide metabolism is best studied in cystic fibrosis, in which bacterial colonization and infection are a major topic in the airway inflammation and the clinical course. There is evidence that anaerobic bacteria can change their energy source from O₂ to nitric oxide sources, reducing nitrate to nitrite and further down to ammonium or nitric oxide. Consequently, nitrite represents an average level resulting from the complex interaction of inflammation and infection and is therefore not helpful as an additional tool to predict pulmonary exacerbation or changes in pulmonary function or scores [35].

Usually, nitrate and nitrite are considered as stable end-products of nitric oxide metabolism. However, increasing evidence suggests that nitrate and nitrite are physiologically recycled. In addition to the bacterial metabolism noted, there are different pathways in the body for the reduction of nitrite to nitric oxide, which are enhanced during acidosis (airway acidification) or hypoxia [36].

Inflammatory markers in exhaled breath condensate from patients with asthma

Potential clinical applications of EBC in asthma were recently discussed [37,38]. In previous studies, it was demonstrated that EBC pH in asthmatic patients was

lower than in healthy controls and could be modulated with anti-inflammatory therapy. A significant lower EBC pH and elevated 8-isoprostane concentrations could be confirmed as compared with healthy controls, but in these patients with mild asthma, both markers were not correlated with pulmonary function [39]. A missing correlation between acid stress (pH) and oxidative stress (8-isoprostane) suggests that these mechanisms may have different roles in the pathogenesis of asthma even if other results indicated that toxicity and interactions of many reactive oxygen species are pH dependent.

The value of EBC pH as a potential diagnostic tool was recently addressed in symptomatic basic military trainees suspected of having asthma [40[•]]. Trainees evaluated showed symptoms resembling asthma, and EBC pH had been used as a screening tool before the diagnosis of asthma was performed by experienced physicians. Even if a significant difference between the trainees diagnosed as asthmatic or not was demonstrated, there was a considerable overlap of the pH values between the two groups and a reliable cut-off value was not discernable. In another epidemiologic setting, EBC pH could not differ between children with and without parentally reported symptoms indicative of asthma [41].

The ability to indicate an asthma diagnosis and to assess asthma severity and control of childhood asthma were evaluated with a pattern of markers [42[•]]. In a multivariate model with all determined markers considered together, only IL-4 was a significant indicator of an asthma diagnosis. Asthma control was best assessed by nitric oxide, 8-isoprostane, IFN- γ and IL-4, whereas nitric oxide, 8-isoprostane, nitrate and nitrite in condensate were the best indicators of asthma severity. H₂O₂ was not a significant indicator. In contrast, there was an inverse correlation between EBC H₂O₂ and a positive correlation between EBC pH concentration and forced expiratory volume in 1 s (FEV₁)-predicted percentage for asthmatic patients in age range of 18–64 years, respectively [43]. For all asthmatic patients, intermittent and persistent asthmatic groups, an inverse correlation between EBC H₂O₂ concentration and pH was shown. EBC H₂O₂ concentration and pH were good noninvasive predictive markers for asthma of different severity, whether it was with a persistent or intermittent course. This is in line with recent findings suggesting that acute exacerbation of asthma is associated with increased oxidative stress [44]. Patterns of markers are likely to more accurately reflect the complex pathophysiological processes in respiratory disease and could therefore meet the fact that markers of airway inflammation are differently expressed in different diseases as well as disease severities.

There is strong evidence of the value of exhaled nitric oxide in diagnosing asthma, and patients with atopic

asthma have elevated levels of exhaled nitric oxide. However, atopy in the absence of asthma was also shown to be associated with increased nitric oxide levels [45], and this confounding factor of atopic status for the diagnostic use of nitric oxide measurements was already highlighted [46]. Moreover, nitric oxide is affected by baseline anti-inflammatory therapy and therefore fails as a diagnostic marker in children on current inhaled corticosteroid treatment. Under these circumstances, EBC might be of special value, and cysteinyl leukotrienes (cysLTs) in EBC were demonstrated to be significantly higher in asthmatic patients, irrespective of anti-inflammatory treatment or allergen sensitization, respectively [47^{••}]. In general, several independent studies have indicated the presence of augmented leukotrienes in EBC of patients with asthma and were briefly reviewed in this journal [48].

Recent reports showed that markers of oxidative stress are enhanced in patients with severe asthma. Asthmatic adult patients treated with inhaled beclomethasone dipropionate and salbutamol inhalers but resulting in poorly controlled asthma showed higher H₂O₂ concentration and lower pH in EBC than individuals with stable asthma [49]. Serum malondialdehyde concentration representing peroxidation was higher in patients with poorly controlled asthma, and total antioxidant capacity in these patients was lower than in individuals with stable asthma, suggesting persistent oxidative stress. Exhaled hydrogen peroxide concentration and pH can be useful for monitoring the response to asthma treatment.

Inflammatory markers in exhaled breath condensate after allergen inhalation

Bronchoprovocation test with allergen is the 'gold standard' for the diagnosis of environmental or occupational asthma. Its use is limited by the fact that repeated lung function measurement over 24 h is needed to detect a late response, patients are often on permanent antiasthmatic treatment or bronchoprovocation test cannot be performed because of an advanced or persisting airway obstruction. Analyses of LTB₄, cysLT and 8-isoprostane in EBC for monitoring of bronchoprovocation test in patients with suspected occupational airway disease revealed a high interindividual variation in levels of these markers, and no visible similarity was observed [50]. However, provocations were performed with different allergens. When a homogeneous patient group with confirmed topic asthma to *Dermatophagoides pteronyssinus* was exposed to the specific allergen, an isolated early asthmatic response (EAR) was obvious in the reactive patients after inhalation [51[•]]. In patients with EAR, both cysLTs and prostaglandin D₂ (PGD₂) in EBC were pronouncedly increased after allergen challenge. The increased levels of cysLT and PGD₂ detected correlated

with each other and could be correlated with the extent of FEV₁ impairment. Therefore, in patients with allergen-induced EAR, EBC might be a useful matrix to monitor the generation of mast cell-associated mediators.

Conclusion

EBC collection is a noninvasive and repeatable tool for assessing inflammatory processes in airway diseases. However, there are still many methodological limitations, and interpretation of findings is hampered by the fact that the most widely used devices differ significantly in the collection efficiency of markers of interest. Therefore, there is research ongoing to determine the components of the airway lining fluid environment in order to better understand the disease using sophisticated and reliable collecting and assay techniques only available in some laboratories. On the contrary, EBC should be further evaluated to facilitate the diagnosis and monitoring of disease activity. Therefore, low-priced and easy-to-handle equipment suitable for field studies is preferred (Fig. 1). EBC might be of particular interest in preventive medicine, as inflammatory processes triggered by environmental and occupational pollutants may precede changes in lung function.

EBC assays can reflect acid stress, oxidative stress and inflammation. Many of the recent studies of EBC related to hydrogen peroxide, nitrogen oxides and pH. The complexities of nitrogen oxide chemistry make EBC NO_x assays and exhaled nitric oxide complementary, not equivalent. NO_x concentrations may also reflect bacterial burdens, and in contrast to being stable end-products of nitric oxide metabolism, nitrate and nitrite are

physiologically recycled. There is general consensus regarding the utility of EBC pH as a simple integrative marker of airway inflammation. Evidence of lipid oxidation can be derived from the presence of substances such as malonaldehyde and 8-isoprostane. Compounds in EBC are often below or close to their quantification limit when applying conventional assays. However, a sample with negative detection gives the information of a marker below the lower detection limit, thereby useful in the sense of a screening parameter. The impact of atopic status for studying asthmatic patients is well known, and there is adding complexity in COPD, stressing hyperinflation as a potential confounder for the level of inflammation markers in EBC.

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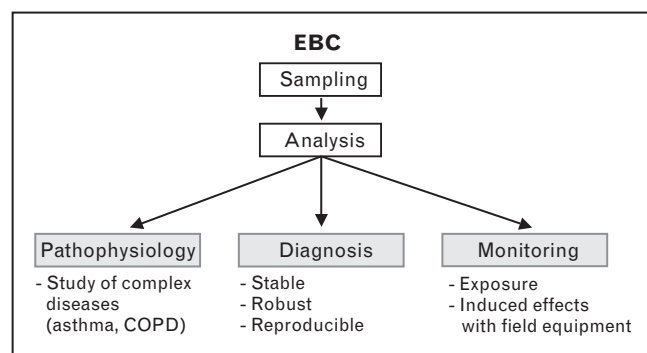
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Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 80–81).

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Figure 1 Methodological issues and different options for the use of exhaled breath condensate



Analysis of EBC can be used to study the pathophysiology of complex diseases such as asthma and COPD. For using EBC analysis in the diagnosis of airway diseases and effects of treatment, the methods have to be stable, robust and reproducible. Another option is the implementation of EBC analysis in field studies with adequate, easy-to-handle equipment to monitor exposure and the induced effects. COPD, chronic obstructive pulmonary disease; EBC, exhaled breath condensate.

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