

Review

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Exhaled breath condensate: a comprehensive update

Abstract: Since the late 1990s, a surge in interest in the analysis of exhaled breath condensate (EBC) resulted in the American Thoracic Society and European Respiratory Society (ATS/ERS) organising a Task Force in 2001 to develop guidelines on EBC collection and measurement of biomarkers. This Task Force published their guidelines in 2005 based on literature and expert opinions at that time, and multiple shortcomings and knowledge deficits were also identified. The clinical application of EBC collection and its biomarkers are currently still limited by several of these knowledge gaps, hence further guidelines for standardisation are required to ensure external validity. Using related articles produced since the publication of the ATS/ERS Task Force report, this paper attempts to provide a comprehensive update to the original guideline and review the methodological shortcomings identified. This review can hopefully serve as a yardstick for future studies involving this emerging clinical tool.

Keywords: biomarkers; devices; exhaled breath condensate; guideline; methodology.

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Introduction

Exhaled breath condensate (EBC) is a non-invasive method of sampling airway lining fluid (ALF). It contains

a number of molecules which may act as biomarkers that indicate disease processes occurring in the lungs or systemically.

Since the late 1990s, a surge in interest in the analysis of EBC resulted in the American Thoracic Society (ATS) and European Respiratory Society (ERS) organising a Task Force in 2001 to develop guidelines on EBC collection, measuring biomarkers and reviewing the current literature on EBC. This Task Force published their guidelines in 2005 based on the current literature and expert opinions at that time [1]. In 2012, despite the growing number of EBC-related publications, there have been no further updates in the literature comprehensively reviewing the methodological shortcomings identified by the ATS/ERS Task Force.

It is recognised that EBC collection and analysis is limited to translational research and is currently not used in clinical practice. This review aims to expand on the ATS/ERS Task Force and summarise the current literature in regards to EBC. As the technique is being utilised more frequently throughout the world, standardisation of techniques and devices is required so as to ensure that progress continues to be made and that the research is comparable between studies.

This review will outline the issues that lead to variability when examining EBC. When possible, it will aim to make recommendations based on the current peer reviewed literature to ensure that these limitations are minimised. It will also outline the biomarkers that have been studied to date in EBC, their role in various diseases and the methods used for their detection and measurement. An important focus will be placed on current assay techniques.

The reviewers of this article wish to echo the words of those from the ATS/ERS Task Force to ensure that standardisation of techniques should not stand in the way of innovation and the utility of EBC has a significant potential to grow with continued research.

Methodological issues of EBC

Origins of EBC

Air is warmed and humidified as it travels through the respiratory tree. As warm, fully saturated breath is exhaled from the lungs and comes into contact with the cold EBC collecting apparatus, it condenses into either liquid or ice depending on the cooling temperature. This condensate consists of mainly water (>99%) and a small amount of ALF [2]. This ALF is believed to have been derived from the respiratory surface lining fluids when energy from air turbulence vibrates the respiratory wall, and creates nebulised droplets. ALF droplets may also be released when closed airway or alveoli suddenly open during inspiration, providing energy to overcome surface tension [3]. The constituents of ALF are assumed to represent the respiratory tree lining fluids and their relative percentage is the essence of EBC studies.

Unlike bronchoalveolar lavage or induced sputum, EBC does not collect respiratory cells. It measures the concentration of biomarkers believed to be directly influenced by these cells, with an alteration in concentrations

indicating a corresponding change in the cellular composition and activity [4, 5].

Collection devices

There are different types of EBC collection devices used in various studies. While all devices function by cooling exhaled breath, they employ a variety of designs, cooling methods and condensation materials. These include commercial devices, such as EcoScreen® [6] (Figure 1), EcoScreen-2 [7], EcoScreen Turbo [8], RTube™ [9], and TURBO-DECCS [10], as well as custom-made devices [11] and devices for specific patient groups, including children [12], infants [13, 14] and mechanically ventilated patients [15, 16]. A comparison of biomarkers measured in EBC that were collected using different devices is shown in Table 1. The design and coating material used in the device is shown to influence the biomarker of interest. Due to distinct physical and chemical characteristics of each distinct biomarker, there is currently no universal device that is ideal for all biomarkers. The choice of an appropriate collecting device depends on the particular biomarker of interest [25].

Condensation devices

Condensation of EBC vapour can be achieved at temperatures of 4°C or lower [26]. The cooling of the exhaled breath vapour has been achieved through various means, including wet ice, wet ice with salt, dry ice, liquid nitrogen, cooling sleeve and electrical refrigerating systems [22, 26, 27]. Depending on the temperature, condensates are collected in either a liquid, solid, or a mixture of both states [26]. The condensation temperature used can influence biomarkers differently (Table 2). Each biomarker has different characteristics, thus researchers should try to optimise the collection conditions when investigating biomarkers that are near the limits of detection. The exact basis of these differences is not well understood, but will related to water solubility, degradation, either spontaneous or by enzyme activity, or to freeze-thawing and oxidation [26]. It is thus important for condensation temperatures to be reported, to facilitate comparison of data across laboratories.

Effect of respiratory patterns on EBC biomarkers

The concentration of various EBC biomarkers is found to be influenced by changes in respiratory patterns. This can



Figure 1 Picture of an EcoScreen® collection device.

Table 1 A comparison of EBC and EBC biomarkers collected using different devices.

	EcoScreen®	RTube™	Custom-made device (material)/Other devices	Subject characteristic(s), sample size, p-value
Volume	↑ ↑	↓ = ↑	= (Siliconised glass) ↓ (PET)	Healthy controls, n=30, p<0.001 [9] Healthy controls, n=6, p<0.05 [17] – Healthy controls, n=10 [18] – Healthy controls, n=10, p<0.001 [19]
pH	= = ↑ ^a	= = ↓	= = (PET)	– Healthy controls [n=10], asthmatic patients [n=10], COPD patients [n=10] and subjects with acute cold [n=10], n=40, p=0.754 [20] – Healthy adults, n=30, p=0.419 [9] – Healthy controls, n=10 [18] – Healthy controls, n=9, p=0.059 [21] Healthy controls, n=6, p=0.34 [17] Healthy controls, n=10, p=0.46 [19] Healthy controls [n=6], asthmatic patients [n=10] and subjects with allergic rhinitis [n=7], n=23, p<0.0001 ^b and p=0.04 ^c [10]
Total protein (amount)	↑ ^a ↑	↓ = = = ↓	↓ (Glass) = (Silicone) = (Glass) = (PET) = (PET)	Healthy controls, n=12, p<0.05 ^d and p<0.001 ^e [22] Healthy controls, n=6, p=0.017 [17] Healthy controls, n=10, p=0.18 [19] Healthy controls, n=10, p=0.51 [19]
Total protein (concentration)	↑	=	= (Glass)	Healthy controls, n=12, p=0.004 [22]
Mucin	=	=	= (Siliconised glass)	Healthy controls, n=6, p=0.52 [17]
Albumin	=	=	↑ (Silicone) ↑ (Glass) = (Aluminium) = (Polypropylene) = (Teflon®) ↑ (Glass) = (Silicone) = (Aluminium) = (Polypropylene) = (Teflon®)	In vitro experiment, p=0.03 [23] Healthy controls, n=23, p<0.008 [23]
Cysteinyl leukotrienes	↑	↓		Healthy controls, n=30, p<0.001 [9]
Eotaxin	↑	↓		Healthy controls, n=30, p=0.01 [9]
8-isoprostanes	=	=	↑ (Silicone) ↑ (Glass) = (Aluminium) = (Polypropylene) = (Teflon®)	– In vitro experiment, p=0.09 [23] – Healthy controls, n=23, p<0.001 ^f , p<0.02 ^g and p=0.763 ^h [23]
Conductivity of non-lyophilised EBC		=	= (PET)	Healthy controls, n=10, p=0.087 [19]
Oxides of nitrogen	↑	=	= (Siliconised glass)	Healthy controls, n=6, p<0.001 [17]
Malondialdehyde	=		= (Tygon®)	COPD patients, n=10, coefficient correlation: r=0.9, p<0.01 [24]
Hexanal	=		=	COPD patients, n=10, r=0.9, p<0.01 [24]
Heptanal	=		=	COPD patients, n=10, r=0.9, p<0.01 [24]
Nonanal	=		=	COPD patients, n=10, r=0.8, p<0.05 [24]

↑, Significantly higher in value (p<0.05); ↓, significantly lower in value (p<0.05); =, no statistical significance (p>0.05); PET, portable EcoScreen turbo. ^amore alkaline; ^bwithout argon de-aeration prior to pH measurement; ^cwith argon de-aeration prior to pH measurement; ^dEcoScreen vs. RTube; ^eEcoScreen vs. glass device; ^fsilicone coating vs. glass, aluminium, polypropylene, Teflon coatings and EcoScreen; ^gglass coating vs. polypropylene and Teflon coatings and EcoScreen; ^hcomparison between aluminium, polypropylene, Teflon coatings and EcoScreen).

Table 2 Influence of condensation temperatures on various biomarkers.

	Change in level of biomarkers as temperature decreases	Condensation temperatures compared, °C	EBC device	Subject characteristics, sample size, p-value
Volume	↑	-10 vs. -5 -10 vs. 0 -10 vs. 5 -5 vs. 5	TURBO-DECCS	Healthy subjects, n=24, p<0.01 [27]
	↑	0 vs. 5	TURBO-DECCS	Healthy subjects, n=24, p<0.05 [27]
	↑	-50 vs. -20 -50 vs. 0	RTube	In vitro experiment [28]
	↑	-20 vs. 4	Glass CMD	Healthy subjects, n=40, p<0.0001 [26]
pH	↓ ^a	-70 vs. -20	RTube	Healthy subjects, n=12, p<0.05 [22]
Total protein (amount)	↑	-20 vs. 4	Glass CMD	Healthy subjects, n=20, p<0.0014 [26]
Total protein (concentration)	=	-70 vs. -20	RTube	Healthy subjects, n=12, p>0.05 [22]
	=	-20 vs. 4	Glass CMD	Healthy subjects, n=20, p=0.052 [26]
Hydrogen peroxide (amount)	↓	-10 vs. 5	TURBO-DECCS	Healthy subjects, n=24, p<0.05 [27]
Hydrogen peroxide (concentration)	↑	-10 vs. 0	TURBO-DECCS	Healthy subjects, n=24, p<0.01 [27]
		0 vs. 5		
	↓	-20 vs. 4	Glass CMD	Healthy subjects, n=20, p=0.009 [26]
Malondialdehyde (amount)	↓	-10 vs. 5	TURBO-DECCS	Healthy subjects, n=24, p<0.01 [27]
	↓	-10 vs. 0 -5 vs. 5	TURBO-DECCS	Healthy subjects, n=24, p<0.05 [27]
Malondialdehyde (concentration)	↑	-10 vs. 5	TURBO-DECCS	Healthy subjects, n=24, p<0.01 [26]
	↑	-10 vs. 0	TURBO-DECCS	Healthy subjects, n=24, p<0.05 [27]
Acetone	↑	-50 vs. -20 -50 vs. 0	RTube	In vitro experiment [28]
Conductivity of lyophilised EBC	↓	-10 vs. 5	TURBO-DECCS	Healthy subjects, n=24, p<0.01 [27]
	↓	-10 vs. 0 -5 vs. 5	TURBO-DECCS	Healthy subjects, n=24, p<0.05 [27]
Conductivity of vacuum-evaporated EBC	↓	-20 vs. 4	Glass CMD	Healthy subjects, n=20, p=0.042 [26]
Nitrate/nitrite (concentration)	=	-20 vs. 4	Glass CMD	Healthy subjects, n=20, p=0.63 [26]

TURBO-DECCS, transportable unit for research on biomarkers obtained from disposable exhaled condensate collection systems; CMD, custom-made device; ^amore acidic.

have significant implications in diseases such as chronic obstructive pulmonary disease (COPD) where patients' expiratory flow rate and tidal volume are reduced.

Increased minute ventilation and tidal volume are independently associated with greater EBC volume [17, 19, 29] without a decrease in lyophilised conductivity [30], indicating that breathing pattern changes are capable of increasing EBC output without altering the dilution by water vapour. This is probably due to a combination of increased expired volume of breath, greater aerosolisation of ALF with increased turbulent airflow and increased recruitment of alveoli [29, 31].

EBC is thought to be produced mainly from the central rather than the peripheral lung regions, when studied using radioactive aerosol [32]. The large airways and alveoli are likely to release different biomarkers. Changes in respiratory pattern, including exercise [33], hyperventilation [19], changes in respiratory flow rate

[29] and mechanical ventilation, are found to affect different biomarkers differently (Table 3). Biomarkers that are respiratory flow-dependent generally originate from the airways [34]. Biomarkers which are non-flow-dependent originate predominately from the alveoli, where flow rate plays a minimal role in these terminal air sacs [42]. EBC should be sampled during tidal breathing, especially when measuring respiratory pattern sensitive biomarkers [1]. Additionally, monitoring and standardising expired breath volume may be necessary in future device designs to reduce variability in ventilation and hence in EBC mediator levels.

Standardisation of de-aeration and pH

EBC sample de-aeration or standardisation of gas in EBC is a common practice aimed at achieving stable EBC pH

[20, 43]. There is currently controversy over de-aeration methods.

EBC pH is a measure of airway acidification, which is unstable in its unprocessed form [1]. The variable amount of dissolved carbon dioxide is one of the main confounding factors of EBC pH. Dissolved CO₂ lowers pH and amounts are inconsistent among individuals [44, 45]. The conventional method of de-aeration is to bubble CO₂-free gas, usually with argon, in order to displace CO₂. Despite several laboratories attempting to standardise the argon de-aeration method, there is still no accepted protocol due to multiple variables involved [11, 46]. The lack of a defined argon de-aeration protocol leads to a range of de-gassing duration and flow rates among various laboratories, which may have led to the variable results obtained [20, 43, 47]. Since EBC pH of different patient groups changes differently during de-aeration [46], there is a need to standardise the argon de-aeration protocol per unit volume of EBC.

A novel approach of de-aeration is to bubble a known quantity of CO₂ into EBC samples to standardise the pCO₂ to 40 mm Hg [48]. This method has been shown to achieve more consistent EBC pH compared with non-degassed samples and samples degassed by argon at a flow-rate of 300 mL/min for 2.5–10 min [49]. More studies are needed to verify this new method of gas standardisation.

Due to the inefficiency of argon and CO₂ in removing other volatile ions and oral contaminants, including NH₄⁺/NH₃, some have suggested lyophilisation as the method of choice for removing volatile ions [50, 51]. EBC pH of lyophilised samples will be a direct measurement of non-volatile acids, such as lactic acid, that originate from epithelial fluid [50]. However, this is not widely practiced as lyophilisation is time-consuming, requires special equipment and introduces processing errors.

Duration and efficiency of EBC collection devices

Duration of EBC collection also affects EBC volumes, however, it does not affect pH [35] or concentrations of H₂O₂, nitrate/nitrite, 8-isoprostane, adenosine and malondialdehyde (MDA) [1]. Despite so, findings in animal models have identified that pCO₂ in EBC is negatively dependent on duration of collection [44]. A standardised collection time is therefore necessary in future EBC studies. A collection time of 10 min is generally sufficient to collect condensate at ~100 µL/min (range 40–300 µL/min), producing 1–2 mL of EBC per subject [52].

Table 3 Summary of different respiratory manoeuvres on EBC biomarkers.

Biomarkers	Respiratory manoeuvres	Changes in concentration	Reference
H ₂ O ₂	↑ Exhalation flow rate	↓	[34]
Volume	↑ Tidal volume/↑ Minute ventilation	↑	[17, 19, 30, 34–37]
	Resistance to outflow	↑	[17]
Protein	Changes in tidal volume/minute ventilation	NIL	[19, 37]
Total protein (amount)	Hyperventilation	↑	[19]
CysLTs	Changes in minute ventilation	NIL	[30]
	Forceful expiration		
	Airway narrowing		
	Exercise	↑ (asthmatics)	[38, 39]
		NIL (healthy controls)	[38]
Lyophilised conductivity	Changes in minute ventilation	NIL	[30]
	Forceful expiration		
	Airway narrowing		
pH	Hyperventilation/Hypoventilation	NIL	[35]
	Mechanical ventilation	↓	[40]
	Exercise	↑	[33]
Nitrite	Changes in tidal volume/minute ventilation	NIL	[37]
Nitrogen oxide	Mechanical ventilation	↑	[40]
8-Isoprostane	Mechanical ventilation	NIL	[40]
Ammonia	Exercise	↑	[33]
Propionate	Exercise	↓	[33]
Adenosine	Exercise	↑ (asthmatics)	[41]
		NIL (healthy controls)	

Reproducibility

EBC volume is arguably the only reproducible EBC parameter, provided that tidal breathing is performed during collection [1]. In general, the lack of standardisation of EBC collection methods and validation of biomarker measurement across different laboratories decrease the comparability of EBC analysis [42] despite facilitating discovery of novel biomarkers [53]. This is further complicated by the levels of many EBC biomarkers being near the lower limits of assay detection, contributing to problems of variability and validity of results [54]. Therefore, it is important to test reproducibility of each biomarker collected via various devices and to be analysed with different assays in order to find the most ideal combination for best results.

Dilution reference indicators

EBC comprises mostly condensed water vapour, and the variable dilution of the constituents has hindered its application as a representation of ALF. Without reliable measure of dilution, baseline EBC biomarker concentrations and changes in these concentrations cannot be accurately determined [55]. It is also unknown whether differences in EBC biomarker levels are due to corresponding differences in ALF or varying sizes of breath droplets arising from an identical source [3, 54, 56]. Therefore, dilution reference indicators are required to serve as comparison for calculation of true airway biomarker levels and to correct for inter-subject variability in droplet formation [42]. It must be noted that dilution is only relevant for non-volatile EBC biomarkers, and not the volatile components which are affected by different factors [57].

Over the years, three dilution reference indicators (urea, total cation concentrations, and conductivity following lyophilisation) have been proposed for use in EBC. It is essential that each of these has similar plasma and ALF concentrations [58] and that they are neither synthesised nor destroyed in the lungs [55]. Urea is a near ideal indicator because besides meeting these criteria, it also diffuses passively between ALF and plasma [54, 55]. However, plasma urea concentration can be quite variable, requiring plasma measurement during each EBC analysis, and also local urea concentration may be reduced by urease-producing bacteria during infections [54]. Conductivity is the simplest method as it is sensitive, easy and quick to perform while not requiring large volumes of EBC samples [59].

There have been conflicting views on the use of dilution reference indicators. It is thought that these indicators are redundant when multiple biomarkers are measured concurrently, as their ratios may serve as a reference or when a highly sensitive and specific assay is used [3]. Currently, many laboratories have yet to incorporate this complicated calculation method in their studies [21]. This method will perhaps only receive greater acceptance when a gold standard indicator is finally defined.

Concentration of samples

As EBC is such a diluted matrix that most biomarkers are detected near the lower limits of assay sensitivity, concentration of samples is essential for successful and reliable measurement of various biomarkers. The most promising method is lyophilisation, which is the freeze-drying process that sublimates water directly from solid to gas phase, bypassing the liquid-gas transition phase, which would require heat and degradation of samples. The resultant solute can then be reconstituted [53]. This method is effective for measuring oxidative stress biomarkers (8-*iso*-prostaglandin $F_{2\alpha}$, *o*-tyrosine, 8-hydroxy-2'-deoxy-guanosine), cytokines, and proteins [60–62]. However, the lack of studies investigating its reproducibility and reliability, with estimates of sample recovery limits its utility in EBC analysis to date. Another concentration method via solid phase extraction provides better recovery for cysteinyl leukotrienes [63]. To date, methods including ultra-filtration, freeze-drying, vacuum concentration, and concentration with pyrogallol red were found to be unsuitable, but may vary for each mediator [57].

Ambient air

Ambient room air contains molecules, which can interact with EBC biomarkers through several mechanisms such as directly contributing to EBC, reacting with compounds present in EBC (thus altering or consuming biomarkers) or causing biochemical or inflammatory changes in the airways [1]. Atmospheric nitric oxide (NO) has been shown to reduce exhaled H_2O_2 concentrations [64]. Ambient H_2O_2 concentrations have also been found to correspond to approximately 30% of the H_2O_2 concentration in the EBC of healthy calves [65]. The inspired concentration of a substance may hence affect the corresponding EBC measurement.

Temperature and humidity of inhaled air [66], as well as condensation temperatures [1, 65, 67], may influence EBC constituents (due to variable heats of vaporisation and specific heat capacities compared to pure water) such as volume [26] and pH [68] but not total protein [22]. EBC samples should not be left out at room temperature after collection, due to interactions with ambient air with potential formation and degradation of unstable or volatile biomarkers such as leukotrienes, purines, H_2O_2 and volatile organic compounds (VOC) [1, 52].

Ideally, environmental humidity and temperature must be recorded along with respiratory rate, minute ventilation, total exhaled volume, exhaled breath temperature and expiratory flow rates during collection in order to assess effects on EBC volumes and/or biomarker concentrations.

Age and gender

There are conflicting data on effects of gender on EBC volume [69] and H_2O_2 levels [70]. EBC H_2O_2 [70] and exhaled NO levels [71] appear to correlate positively with age. Polar VOCs, including aldehydes, appear to be affected by gender, with increased levels in males suggesting metabolic gender differences [72]. In contrast, EBC pH does not appear to be affected by age [73] or gender [74]. EBC total protein appears to increase with age but is not affected by gender [69]. Additionally, body weight and height do not affect EBC volume and H_2O_2 concentration in adults [1].

Food and drink

Food intake and physical activity levels during the day may be responsible for changes in EBC biomarkers and may partially explain the circadian rhythm observed in biomarker concentrations [75]. Food intake prior to EBC collection has not been observed to have an effect on non-volatile mediators or pH [76]. In contrast, EBC collection 15 min after drinking 1 L of either an acidic beverage (cola) or a pH neutral beverage (water) both significantly decreased EBC pH levels [77]. Several foods and beverages contain H_2O_2 or elevate levels of oxidants in bodily fluids, thus influencing EBC oxidant concentrations [75]. In animal studies, food intake was found to double EBC H_2O_2 levels 1 h after morning feeding [65]. High dietary nitrite/nitrate concentrations can affect NO-related markers in EBC in non-inflamed airways

[78]. When measuring mediators known to be affected by certain drinks or foods, it is advisable that subjects avoid these a few hours prior to measurement (e.g., caffeinated drinks prior to measuring adenosine) [1] and should avoid drinking large volumes prior to EBC collection.

Circadian rhythm

A recent investigation indicated a circadian change in EBC H_2O_2 concentrations in healthy volunteers, with highest values in the afternoon and a nadir in the morning [70, 75]. This is also the case in calves, with significantly greater EBC H_2O_2 and pH found in the evening compared to the morning [65]. Observations of daytime variability in biomarker concentrations indicate that consistent conditions and time of day may need to be applied to the time of collection to improve reliability [75].

Smoking

Smoking affects concentrations of 8-isoprostane, H_2O_2 , S-nitrosothiols and nitrate in EBC [79]. In general, EBC concentrations of NO-related compounds [80], aldehydes [81, 82] and total protein may be elevated or remain unchanged with smoking [83]. Levels of 8-isoprostane, prostaglandin E_2 , leukotriene B_4 [84, 85], H_2O_2 [70] and TNF- α [80] increased, whilst levels of IL-1 β [80], glutathione (an important antioxidant) [83] and pH [76] decreased minutes after cigarette smoking. Similarly, increased levels of oxidative markers, including MDA, 8-hydroxydeoxyguanosine, superoxide dismutase and glutathione peroxidase, were identified in the EBC of smokers compared with non-smokers [86]. Smokers should thus refrain from smoking at least 3 h and preferably overnight before EBC collection to minimise effects on mediator levels.

Systemic diseases

The potential effect of systemic diseases, including pulmonary [87, 88] and extrapulmonary systemic diseases [1, 89], needs to be considered in EBC studies. An approximately 20-fold greater H_2O_2 level has been described in the EBC of uraemic patients compared to healthy controls [90]. In one study, peritoneal dialysis and haemodialysis significantly decreased exhaled H_2O_2 [91].

Medication

One inhalation of salbutamol or ipratropium had no effect on EBC H_2O_2 and thiobarbituric acid reactive substances (TBARS) concentrations in healthy subjects [70]. EBC H_2O_2 and FeNO are decreased in asthmatics following inhaled corticosteroid treatment [92, 93], although budesonide administration did not affect EBC nitrite levels in healthy subjects even after inducing an inflammatory response with ozone exposure [94]. Inhaled deionised water or 0.9% NaCl does not affect EBC H_2O_2 concentrations. However, nebulised N-acetylcysteine almost completely abolishes EBC H_2O_2 levels 30 min after administration, although this rises almost two-fold above baseline 2.5 h later [95]. A transient increase in EBC H_2O_2 following nebulised N-acetylcysteine has been demonstrated in patients with stable COPD [96], despite a decrease with long-term treatment [97].

Sample contamination

Gross salivary contamination can occur in EBC collection. There is an increased likelihood of oropharyngeal contamination with volatile biomarkers; e.g., most EBC ammonia arises from bacterial degradation of urea in the oropharynx [98]. Additionally, oropharyngeal bacterial flora may significantly contribute to EBC nitrite levels, since levels decrease following a chlorhexidine mouthwash [99]. High concentrations of eicosanoids have been detected in saliva [79], with prostaglandins and leukotrienes formed in the nose and mixing with oral expiratory air via the nasopharynx [79]. However, this can be controlled with mouth rinsing before collection, periodically swallowing saliva and using a nose clip. EBC must be checked with α -amylase assays to assess amylase concentrations which indicate salivary contamination [5], although significant salivary contamination has been ruled out by NMR spectroscopy studies of EBC [100, 101]. One investigation identified different cytokine profiles in EBC and saliva using a cytokine protein array to compare concentrations of 40 cytokines. This indicated that salivary contamination is negligible if validated collection methods are used [102]. There are also concerns with upper airway and nasal contamination. There are similar oral and nasal EBC protein patterns, which differ from those of saliva and demonstrate a lack of salivary phosphorous contamination and low EBC amylase activity [103].

Suggestions to further reduce contamination include use of VOC filters at the inhalation port to reduce ambient VOC contamination, tidal breathing to sample alveolar

air, use of a nose clip, salivary trap and Swan-neck tubing (placing condenser inlet at a higher level than the subject's mouth) [1, 52]. A nose clip prevents nasal inhalation of air and exhalation through the nose.

Disinfection of non-disposable apparatus components

Disinfection of non-disposable components of EBC apparatus is very important for sanitary purposes and accuracy of assays. While there have been no cases of infection being transmitted between subjects, it is a risk that has to be minimised particularly when using these devices in patient groups who may be immunocompromised.

Current disinfection practice described in the literature for reusable apparatus components has been inconsistent. These have ranged from simply rinsing with distilled water and air-drying to soaking to multiple disinfection steps involving enzymatic detergents and ethanol before rinsing and drying [104, 105]. It is important to ensure that detergents used are pH-neutral and non-reactive so as to limit any confounding of assays. Recent studies examining the influence of external contaminants on EBC, including disinfectants, found that standard disinfection protocols had measurable disinfectant contaminant signal when analysed by nuclear magnetic resonance spectroscopy. However, one study showed that the disinfectant Milton showed no signals [100]. Although water soaking alone was able to effectively remove most toxic components of disinfectant, ethanol washing was required for removal of unknown contaminants which may interfere with assays [104].

NMR spectroscopy-based metabolomics

Nuclear magnetic resonance (NMR) spectroscopy can be utilised to identify the biochemical profiles of metabolites in EBC samples. This is a reproducible technique and its methodology has been validated [100]. Although NMR has a lower sensitivity compared to enzyme-linked immunosorbent assay (ELISA) and mass spectrometry, it is a non-destructive analytical method that requires minimal sample preparation and has rapid acquisition time of about 10–15 min [104]. NMR-based metabolomics of EBC may have a potential use in characterising respiratory diseases as it was able to discriminate patients with COPD [104], asthma [106], stable and unstable cystic fibrosis [107] from healthy subjects. In addition, a recent NMR spectroscopic study has also shown that the repeated use of the EcoScreen®

condenser does not cause any carry-over effect and the disinfectant Milton does not alter EBC metabolomics [100], as opposed to another study that reported artificial signals when the Anacon condenser was used and disinfected with Descogen [108]. Different analytical techniques, including NMR and mass spectrometry, may be combined to improve sensitivity and further consolidate 'breathomics' [100].

Biomarkers in EBC

Hydrogen peroxide

Hydrogen peroxide is normally present in exhaled breath, probably from the pulmonary circulation [109]. Additionally, activation of airway epithelial and endothelial cells, neutrophils, alveolar macrophages and eosinophils leads to production of superoxide radicals and hence H_2O_2 production in airway inflammation. H_2O_2 is less reactive and soluble than other reactive oxygen species, with its neutral charge and low molecular weight allowing it to cross membranes to exit into the extracellular spaces. However, it is less stable than other oxidative stress markers such as the isoprostanes. H_2O_2 is volatile and readily equilibrates with air, thus its presence can be easily detected in EBC as a marker of pulmonary inflammation and oxidative stress. Compared to healthy subjects, EBC H_2O_2 has been shown to be significantly increased in patients with asthma [92], COPD [110], bronchiectasis [111], acute respiratory distress syndrome [112], interstitial lung disease [113] and cardiothoracic surgery [114], but not cystic fibrosis [115].

Breathing patterns must be taken into consideration with all EBC H_2O_2 measurements [67]. EBC H_2O_2 concentration varies with breathing patterns, decreasing after increased tidal volumes compared to normal breathing [75]. Other recent findings indicate that exhaled H_2O_2 concentrations can be standardised if expressed in moles per 100 L of expired air and if inhaled H_2O_2 levels are subtracted from exhaled H_2O_2 [65].

EBC de-aeration is another critical factor influencing H_2O_2 concentrations. Although it is important to remove reactive gases to stabilise EBC biomarkers and pH, research in our laboratory indicates that argon degassing lowers H_2O_2 concentrations [11]. Sample pH affects H_2O_2 quantitation, thus careful assay optimisation should be considered prior to H_2O_2 measurements [116]. EBC samples should also be rapidly stored at -80°C following collection so that H_2O_2 is not rapidly lost or oxidised [83]. Frozen samples may remain stable varying from 2–3 days to 2 months [117]. Many studies analysing EBC H_2O_2 have

used spectrophotometric colorimetric assays, with detection limits $>0.1 \mu\text{mol/L}$ [67] and differing reaction substrates, including tetramethylbenzidine, 4-hydroxyphenylacetic acid or homovanillic acid, and using either fresh or thawed EBC samples, which explain variations in H_2O_2 concentrations [118]. Other methods of EBC H_2O_2 analysis include measurement with various spectrofluorimetric techniques (fluorimetric assay) [70], as well as flow injection analysis with fluorescence detection [119] by chemiluminescent methods [120] and by immediate on-line analysis with a commercially available amperometric biosensor (Ecocheck, Jaeger, Germany) [121]. Although chemiluminescent methods have high sensitivity, detecting H_2O_2 in the nanomolar range, they are limited by poor day-to-day precision and typically require expensive equipment, which may not be suitable for routine clinical studies [67].

Arachidonic acid derivatives: eicosanoids

Eicosanoids represent a heterogeneous family of unsaturated fatty acid derivatives, arising from arachidonic acid, which is released from the cell wall by phospholipase A_2 . Cyclooxygenase or lipoxygenase enzymatic action leads to formation of prostanoids and leukotrienes, respectively, whilst actions of free-radicals lead to formation of isoprostanes [1, 83].

Prostanoids: prostaglandins, prostacyclin and thromboxanes

Prostanoids are synthesised via the cyclooxygenase pathway and include prostaglandins and thromboxanes. Prostaglandin E_2 (PGE_2) has pro-inflammatory actions and a role in inhibiting bronchoconstriction, and has been measured in EBC samples using enzyme immunoassays (EIA) and radioimmunoassay [1, 83]. Thromboxane A_2 (TxA_2) is readily converted to a chemically stable form, TxB_2 , a potent bronchoconstrictor [79]. Hence, thromboxane synthesis is usually assessed by measuring TxB_2 and EBC TxB_2 has been measured using EIA [83]. PGE_2 is present in the EBC of healthy subjects, with upper normal limits not exceeding 75 pg/mL [36], although there is variability regarding normal values [83].

Leukotrienes

Leukotrienes consist of a family of lipid mediators synthesised by leukocytes from arachidonic acid through

enzymatic catalysis of 5-lipoxygenase. They are categorised into cysteinyl leukotrienes (i.e., LTC₄, LTD₄ and LTE₄) and LTB₄. Cysteinyl leukotrienes contract airway smooth muscle, increase vascular permeability, stimulate mucus secretion and decrease mucociliary clearance [79]. LTB₄ is a potent neutrophil and eosinophil chemoattractant, contributing to oedema and local airway narrowing, and also increased mucus secretion [79, 122]. Leukotrienes have been measured in EBC using EIA, gas chromatography/mass spectroscopy (GC/MS) and liquid chromatography/mass spectroscopy (LC/MS) [83, 123, 124]. EBC LTB₄ is increased in patients with asthma [36, 125, 126], COPD [84, 127], cystic fibrosis [128] and patients undergoing cardiothoracic surgery [114]. LTB₄ concentrations in EBC are also elevated after sputum induction, indicating a pro-inflammatory effect of this procedure [129]. Cysteinyl leukotrienes have been widely assessed and are elevated in patients with asthma [36, 125, 126], including children [130–133]. This is consistent with the central role of leukotrienes in the pathophysiology of these respiratory conditions.

Isoprostanes

Isoprostanes are produced by free-radical lipid peroxidation of arachidonic acid, representing an *in vivo* marker of oxidative stress [83]. Isoprostanes have several advantages over other oxidative stress markers as they are chemically stable and are specific for lipid peroxidation. They are also useful for assessing lung oxidative stress in animal experimental models of asthma [134]. They have potent effects including smooth muscle contraction which have been implicated in the pathophysiology of various respiratory conditions [79]. 8-Isoprostane is most widely studied in EBC, usually measured using EIA although GC/MS has also been used [114]. The highest values of 8-isoprostane detected in EBC of healthy subjects were up to 50 pg/mL [83]. EBC concentrations of 8-isoprostane in the µg/mL range have also been reported and that EBC concentrations were higher than BAL concentrations [5]. One study identified the importance of the inner coating of condensing devices, with highest 8-isoprostane levels detected with glass and silicon coatings compared to the Ecoscreen® [23], which may account for variability in different studies. Levels of EBC 8-isoprostane have been shown to be increased in patients with asthma – correlating with disease severity and degree of inflammation [117, 135], COPD [83, 136], ARDS [137], cystic fibrosis [138, 139], exercise-induced asthma [140], and patients undergoing cardiothoracic surgery [114].

Nitric-oxide (NO)-related products

NO has been one of the most extensively studied exhaled biomarkers of inflammation, especially in asthma and COPD [141]. NO plays an important role in inflammation, regulation of smooth muscle tone, and levels increase in response to pro-inflammatory cytokines and oxidants [53]. NO is a free radical which readily reacts with oxygen to form nitrogen oxides (NO_x) or reacts with superoxide anion to form peroxynitrate – a reactive substance that may lead to production of NO-derived products. Nitrite (NO₂⁻) and nitrate (NO₃⁻) are readily found in ALF and are produced by the reaction of NO with oxygen and the decomposition of peroxynitrate. S-Nitrosothiols are produced by interaction of peroxynitrate with thiol-containing substances, including cysteine and glutathiones, which act as antioxidants limiting the nitrosative stress of NO-related products [83]. Nitrotyrosine is produced by the reaction of peroxynitrate with protein tyrosine residues. Nitrotyrosine can alternatively be formed by nitration of proteins using myeloperoxidase, a heme enzyme in neutrophils [142]. NO synthesis and release in the respiratory system have been indirectly assessed in EBC by quantifying levels of nitrate/nitrite, nitrotyrosine and S-nitrosothiols [83, 143]. Nitrate and nitrite have been measured in EBC of patients with asthma [53, 93], COPD [53, 144], pulmonary fibrosis [145], bronchiectasis, cystic fibrosis [146], acute lung injury [147], primary ciliary dyskinesia [148] and community acquired pneumonia [149]. However, conflicting results exist between increased or unchanged nitrate/nitrite levels in some respiratory conditions [149]. Nitrotyrosine has been found to be elevated in patients with asthma [125], COPD [53] and cystic fibrosis [150] although one study did not find it a useful marker of nitrosative stress when comparing stable patients with healthy subjects [151].

EBC nitrite and nitrate are measured by colorimetric (Griess reaction), fluorimetric [using the 2,3-diaminonaphthalene (DAN) reaction] and chemiluminescence assays as well as liquid, gas and ion chromatography [152]. The reported detection limit of the DAN assay is 0.1 µm whilst the Griess reaction is higher. EBC nitrite is often detected in the 0.1 µm range, which is close to the detection limits of the former assays [1]. Nitrate is often measured indirectly following incubation with nitrate reductase [117, 152] and tends to be 5–10-folds higher than nitrite levels although this may reflect changes in inflammatory airway conditions [1]. Nitrotyrosine is best measured with mass spectrometry with the greatest sensitivity (measured in the picomolar range) [125], but can also be measured using enzyme immunoassays or high performance liquid chromatography (HPLC) [1, 83]. S-nitrosothiols are usually

measured using colorimetric assays [83, 152], but can be sensitively measured using chemiluminescence analysis after reduction to NO in copper-cysteine or using ultraviolet light [153].

There is considerable variability in the normal ranges for nitrite/nitrate [83], with high day-to-day variability of EBC NO_x measurements [105]. This may relate to different assays used for measurement and sample contamination. Laboratory contamination is a major problem as nitrogen oxides are present on laboratory surfaces, glassware and pipette tips [154]. Ambient nitric oxide is readily diffusible and can become oxidised, thus rapidly contaminating surfaces. Hence, precautions should be taken by rinsing collecting equipment and glassware with purified (distilled/de-ionised) water and appropriate control samples without EBC [1].

Cytokines, chemokines and growth factors

Most proteins, including cytokines, are difficult to measure reliably in EBC. Cytokines mediate inflammatory processes and have been measured in EBC by enzyme and radioimmunoassay, either individually or on multiplex platforms. Limiting factors in measurement have included low limits of detection, matrix effects and intra- and inter-assay variability [117, 155]. Recent studies have measured many different EBC cytokines, chemokines and growth factors in various pulmonary diseases using multiplex analysis and protein arrays, allowing sensitive detection of multiple factors using small volumes of EBC [61, 87, 102, 156]. The major limitation that remains is that EBC cytokine levels are usually close to assay detection limits. This may be overcome by lyophilisation, which enables removal of water and other volatile substances, but may degrade biomarkers [53]. EBC lyophilisation can concentrate samples up to a factor of 30 times, thus improving sensitivity [61]. There was no relationship between EBC cytokine concentrations and storage times up to 1 year in samples stored at -80°C [157]. With newer technologies, EBC cytokine measurement may become useful in assessing airway inflammatory status for treatment monitoring and investigating disease pathophysiology [156].

Aldehydes and thiobarbituric acid-reactive substances (TBARs)

Aldehydes (MDA, hexanals, heptanals and nonenals) are lipid peroxides, measured in EBC as markers of

oxidant-induced damage [81]. MDA is generated by arachidonic acid and docosahexanoic acid, and is regarded as a TBAR. EBC MDA levels are usually increased during asthma exacerbations and in COPD patients [158] compared with healthy smokers [81], and higher in smokers than non-smokers [82]. Conversely, reduced EBC glutathione indicates reduced antioxidant capacity [1, 159]. Increased MDA and decreased glutathione have been demonstrated in asthmatic children and patients with allergic rhinitis compared with healthy controls [24, 160]. These substances may be formed during sample preparation. Although the measurement is simple, current colorimetric assays and LC/MS lack specificity, with day-to-day intra-subject coefficient of variations for different aldehydes being 12%–20% [24]. Hence, these substances are not frequently used as markers of lipid peroxidation [1]. Novel measurement techniques utilise an alternate isotope-coded derivatisation assay (AIDA) in the LC/MS measurement of aldehydes, with results in good agreement with external calibration methods [161].

Adenosine

Adenosine triphosphate and its metabolites, including adenosine – a purine nucleoside, have been detected in EBC [117]. EBC adenosine has been shown to be significantly greater in patients with asthma, cystic fibrosis [162], allergic rhinitis during exercise [41] and in allergic rhinitis [163]. EBC is a useful method of measuring purinergic molecules compared with other sampling techniques, due to absence of confounding factors such as low EBC cellular and protein content [164]. EBC adenosine has been measured using LC/MS and recently using HPLC [165]. Luciferin-luciferase assays have been used for measurement of ATP as luciferin is a substrate oxidised by ATP [164]. In order to overcome varying dilution of airway droplets, the simultaneous measurement of purines and a dilution marker have been recently validated [162, 164].

Transitional metals

Transitional metals are d-block metals that form ions with two or more oxidative states. Iron (10–30 μm), copper (13–22 μm) and zinc (12–20 μm) are the most abundant redox-active transitional metals in the human body. Pivotal transitional metal-catalysed oxidative stress pathways include the production of superoxides from NADPH-dependent

oxidase with subsequent production of hydroxyl radicals through the Fenton and Haber-Weiss reaction. Furthermore, iron and copper play key a role in the catalysis of many other endogenous reactions, generating reactive intermediates (Table 4).

Metal-catalysed reaction oxygen species can initiate destructive processes including lipid peroxidation and enzyme inactivation. Metal-catalysed formation of $\cdot\text{OH}$ can result in removal of a hydrogen atom from unsaturated fatty acid, leading to lipid radical formation, destruction of cell membrane integrity and cell death. Binding of endogenous enzymes to redox-active transition metals can initiate protein oxidation and inactivation.

Many studies have examined the correlation between oxidative stress and the presence of COPD, with several oxidant biomarkers identified to be elevated in this chronic disease [166, 167]. Hydrogen peroxide (H_2O_2), a by-product of many transition metal catalysed reactions, is an example of a strong oxidant elevated in EBC of COPD patients. Other oxidative stress markers including 8-isoprostane [167] and nitric oxide related products [168] have also been demonstrated to be elevated in the EBC of COPD patients. Novel biomarkers with potential utility for early diagnosis and prognosis of COPD are of significant interest.

Exhaled breath DNA

Small amounts of DNA and epithelial cells, which are believed to originate from the lower respiratory tract, are also detectable in EBC [169–171]. Oxidative stress to cells in the respiratory tract results in DNA mutations, which lead to changes in cell cycling, growth promotion, DNA repair, apoptosis, and can induce angiogenesis and cellular invasion. These mutations can potentially be detected in the EBC of patients with lung cancer using PCR technology [172].

Table 4 Redox-active metal-catalysed formation of reactive oxygen intermediates.

$\cdot\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{OH}\cdot$ (metal-catalysed Haber-Weiss reaction)
LOOH (lipid peroxide) $\rightarrow \text{LO}\cdot, \text{LO}_2\cdot$ (alkoxyl and peroxy radical)
Ascorbic acid + $\text{O}_2 \rightarrow$ ascorbyl radical, $\cdot\text{OH}$, H_2O_2
$\text{NAD(P)H} + \text{O}_2 \rightarrow \text{NAD(P)}\cdot, \cdot\text{O}_2^-, \cdot\text{OH}, \text{H}_2\text{O}_2$
Catecholamines + $\text{O}_2 \rightarrow \cdot\text{O}_2^-, \cdot\text{OH}, \text{H}_2\text{O}_2$
$\text{RSH}(\text{thiols}) + \text{O}_2 \rightarrow \text{O}_2^-, \cdot\text{OH}\cdot\text{H}_2\text{O}_2, \text{RS}\cdot$ (thiyl radical)

It has been reported that a significantly greater proportion of patients with non-small cell lung cancer have 3p microsatellite alterations or loss of heterozygosity in EBC compared to controls [173], with overlapping profiles of microsatellite alterations in DNA from EBC and tumour tissues [174]. It has also been demonstrated that p53 mutations are present in EBC of patients with lung cancer, while no mutations were found in controls [175]. The oncogene KRAS has also been found in EBC of patients with lung cancer and interestingly levels were shown to decrease significantly following surgical tumour resection [176]. Detection of the epidermal growth factor receptor (*EGFR*) gene mutation has also been demonstrated in the EBC of a patient with squamous cell carcinoma of the lung [177]. Gene promoter methylation can also be detected in the EBC of lung cancer patients and is significantly associated with lung cancer status [178]. Genetic alterations consistent with neoplasia could be a significant marker of tumorigenesis, which may be useful for early lung cancer diagnosis.

Human papilloma virus (HPV) DNA has also been identified at significantly greater frequencies in the EBC of patients with lung cancer compared to controls [179]. In addition, EBC has been used for rapid detection of microbial DNA and RNA to demonstrate bacterial and viral lung infections [180] and in infective exacerbations of COPD [181]. However, results have been disappointing for detection of *Mycobacterium tuberculosis* DNA in patients with tuberculosis [182, 183] as well as *Pseudomonas aeruginosa* and *Burkholderia cepacia* from patients with cystic fibrosis [184]. This may relate to the dilute nature of EBC and small quantities of DNA present in these infections.

Conclusions

The clinical application of EBC collection and its biomarkers are limited by several factors and standardisation is required to ensure external validity. This review has outlined the limitations in EBC collection techniques which may contribute to the variability in the results. Issues relating to many of the factors affecting the reliability of EBC analysis, such as gas-standardisation when examining pH and sample contamination are yet to be resolved to date. Therefore, further research is still required.

The recent use of metabolomics, which is able to detect several metabolites simultaneously, has important utility and is opening up several new opportunities to

develop breath patterns that may be able to characterise disease states. Despite this, the major limitation to the clinical use of EBC is still the inadequate sensitivity of assays even with recent developments in lyophilisation and assay techniques.

Overall, the technique of EBC has been developing since the ATS/ERS Task Force publication and will continue to grow over the coming years. With continued research, EBC analysis may one day be used in clinical practice in the near future.

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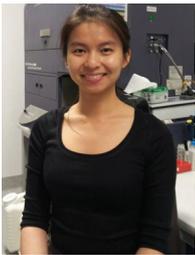
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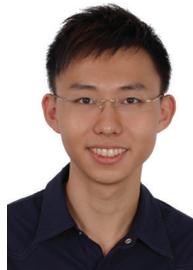
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