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Review

Biomarkers in Breath Condensate: A promising New Non-invasive Technique in Free Radical Research

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Oxidative stress is associated with a range of inflammatory lung diseases including asthma, adult respiratory distress syndrome, idiopathic pulmonary fibrosis, pneumonia, lung transplantation, chronic obstructive pulmonary disease, cystic fibrosis, bronchiectasis and lung cancer. Increased concentrations of reactive oxygen species (ROS) in the airways of such patients are reflected by elevated concentrations of oxidative stress markers in the breath, airways, lung tissue and blood. Traditionally, the measurement of these biomarkers has involved invasive procedures to procure the samples, or examine the compartments. As a consequence, there is a need for less invasive approaches to measure oxidative stress. Analysis of breath hydrocarbons has partly fulfilled this need, however only gas phase volatile constituents can be assessed by this approach. The collection of exhaled breath condensate (EBC) is a simple, non-invasive approach, which comprehensively samples the lower respiratory tract. It is currently used as a research and diagnostic tool in the free radical field, yielding information on redox disturbance and the degree and type of inflammation in the lung. With further technical developments, such an approach may ultimately have a role in the clinic, in helping to diagnose specific lung diseases. EBC can be exploited to assess a spectrum of potential biomarkers, thus generating a "finger print" characteristic of the disease. By assessing the nature of oxidative stress in this manner, the most appropriate therapy can be selected and the response to treatment monitored.

Keywords: Exhaled breath condensate; Biomarkers; Cytokines; Reactive oxygen species; Antioxidants; Lipid peroxides

INTRODUCTION

The lung exists in an oxygen rich environment which together with its large surface area and extensive blood supply, makes the organ susceptible to injury mediated by reactive oxygen species (ROS). Increased production of ROS has been directly linked to protein, DNA and lipid oxidation, which may cause direct lung injury or induce a variety of cellular responses, through the generation of secondary metabolic reactive species.[1,2]

There is a growing interest in identifying disease biomarkers in which oxidative stress is present (Fig. 1). Various invasive and semi-invasive means of assessing oxidative biomarkers are available; these include measurements in plasma, bronchoalveolar lavage fluid (BALF), urine and tissue biopsies. In comparison, little information is available from non-invasive techniques for evaluating oxidative stress in disease. The assessment of oxidative stress biomarkers in exhaled breath condensate (EBC) is one such approach, and is emerging as a promising area for further research. This article describes various biomarkers of oxidative stress that are present in EBC, and discusses the reproducibility, sensitivity and specificity of the methodologies used in their measurement. It also explores the utility of

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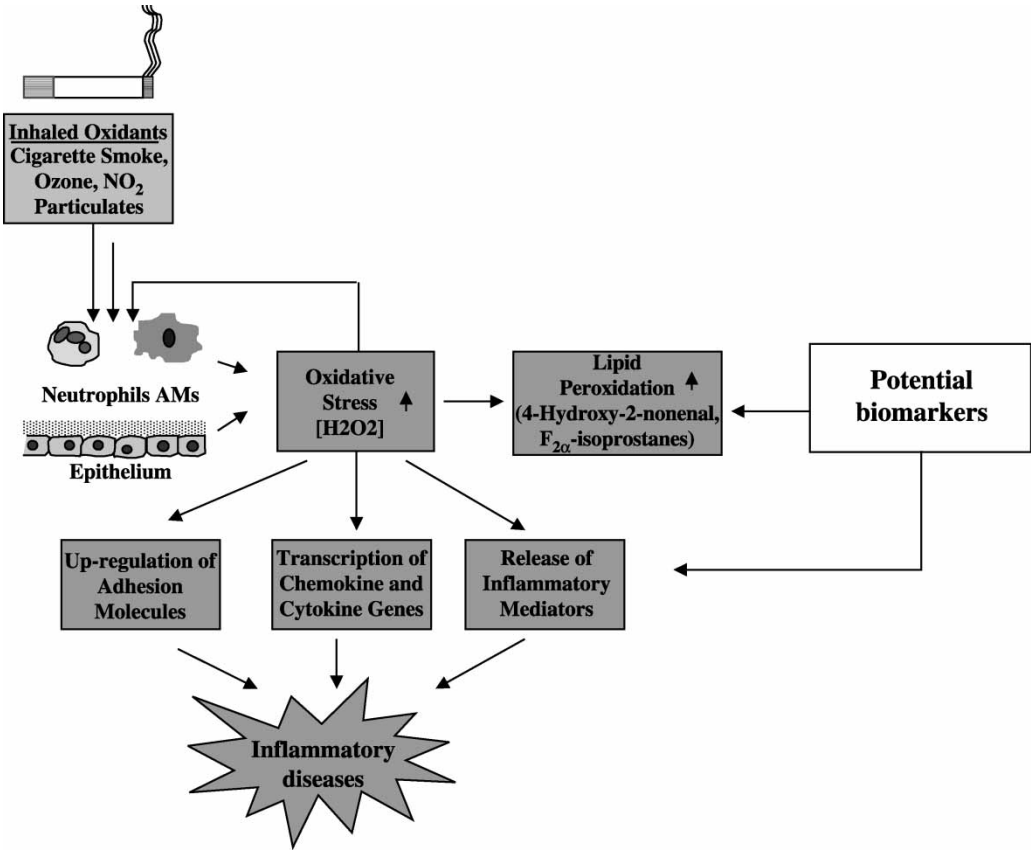

FIGURE 1 Generation of potential biomarkers in inflammatory lung diseases. Inflammatory response is mediated by oxidants either inhaled and/or released by the activated neutrophils, alveolar macrophages, eosinophils and epithelial cells leading to production of ROS and membrane lipid peroxidation. Activation of transcription of the pro-inflammatory cytokines and chemokine, up-regulation of adhesion molecules and increased release of pro-inflammatory mediators, which is detected in EBC.

exhaled oxidant biomarkers in evaluating the redox and inflammatory status of lung diseases, and in forecasting the efficacy of therapeutic interventions in clinical medicine.

CELLULAR ROS AND REACTIVE NITROGEN SPECIES (RNS)

Chronic inflammatory lung diseases are characterised by the activation of epithelial cells and resident macrophages, and the recruitment and activation of neutrophils, eosinophils, monocytes and lymphocytes. The activation of macrophages, neutrophils and eosinophils generates ROS. The latter are also released by lung epithelial cells,[3] and when this occurs they stimulate inflammatory cells directly, thereby amplifying lung inflammatory and oxidant events. By virtue of their unpaired electrons, ROS are unstable compounds, capable of initiating widespread oxidative events. For example, ROS and RNS also act on certain amino acids such as methionine, tyrosine and cysteine in proteins (e.g. enzymes, kinases) profoundly altering the function of these proteins in inflammatory lung diseases.[4]

The generation of one such ROS, the superoxide anion ($O_2^{\cdot-}$), is rapidly reduced to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), whilst several transition metal salts can react non-enzymatically with H_2O_2 to form the hydroxyl radical ($^{\cdot}OH$). Most attention in the generation of $^{\cdot}OH$ *in vivo* has focused on iron, a critical element in many oxidative reactions. For example, free iron in the ferrous form catalyses the Fenton reaction and the superoxide driven Haber–Weiss reaction, generating $^{\cdot}OH$.

ROS, and in addition RNS, can also be generated intracellularly from several sources, including mitochondrial respiration, the NADPH oxidase system and xanthine/xanthine oxidase. The primary ROS-generating enzyme is NADPH oxidase, present in phagocytes and epithelial cells. Activation of this enzyme involves a complex mechanism, involving the assembly of various cytosolic and membrane-associated components, resulting in a one-electron reduction of oxygen to $O_2^{\cdot-}$, NADPH being the electron donor. The other enzymes used by phagocytes to produce ROS are heme peroxidases, myeloperoxidase (MPO) and eosinophil peroxidase (EPO). Activation of EPO produces the potent

oxidant hypochlorous acid (HOCl) and hypobromous acid (HOBr) in the presence of chloride (Cl^-) and bromide (Br^-) ions, respectively. MPO- and EPO-derived ROS can also interact with nitrite (NO_2^-) and H_2O_2 leading to the formation of RNS.

INHALED ENVIRONMENTAL OXIDANTS

Cigarette smoking and inhalation of airborne pollutants, either oxidant gases such as ozone (O_3), nitrogen dioxide (NO_2) and sulphur dioxide (SO_2), or particulate matter, directly damage the lung and activate inflammatory responses. Cigarette smoke is a complex mixture of over 4700 chemical compounds, which generate high concentrations of oxidants (10^{14}/puff).[5,6] The short-lived oxidants such as $O_2^{\cdot-}$ and nitric oxide (NO) are predominantly found in the gas-phase of cigarette smoke, and immediately react to form the highly reactive peroxynitrite ($ONOO^-$) molecule. The tar phase contains high concentrations of more stable radicals including quinone, hydroquinone and semiquinone. These can reduce O_2 to produce $O_2^{\cdot-}$, $^{\cdot}OH$ and H_2O_2.[7] The tar phase also contains an effective metal chelator binding iron to produce tar-semiquinone and tar-Fe^{2+}, which can generate H_2O_2 continuously.[7,8] The aqueous phase of cigarette smoke condensate may also undergo oxidation—owing to the presence of free iron—for a considerable period of time in the epithelial lining fluid (ELF) of smokers.[5,8]

MEMBRANE LIPID PEROXIDATION: GENERATION OF $F_{2\alpha}$/8-ISOPROSTANES, 4-HYDROXY-2-NONENAL AND ACROLEIN

The ROS ($O_2^{\cdot-}$ and $^{\cdot}OH$) generated and released by activated immune and inflammatory cells are differentially reactive, but when generated close to cell membranes, both oxidise membrane phospholipids (lipid peroxidation). This can initiate a chain reaction, which in turn can lead to the generation and accumulation of lipid peroxidation products such as malondialdehyde, F_2-isoprostanes, 4-hydroxy-2-nonenal (4-HNE) and acrolein (Fig. 2).

The peroxidative breakdown of polyunsaturated fatty acids within the membrane impairs function, inactivates membrane-bound receptors and enzymes and increases tissue permeability and cell fragility, all of which are implicated in the pathogenesis of many forms of lung injury. Compared to free radicals, lipid peroxidation aldehydes are generally stable, can diffuse within, or even escape from the cell and attack targets far from the site of the original free radical event. In addition to their cytotoxic properties, lipid peroxides are increasingly recognised as being important in signal transduction pathways, especially those activated during an inflammatory response.[9,10]

Isoprostanes are a novel class of prostanoids. Those such as 8-epi-prostaglandin $F_{2\alpha}$ and 8-iso-prostaglandin $F_{2\alpha}$/8-isoprostane (a member of the $F_{2\alpha}$-isoprostanes) are stable end products of the lipid peroxidation of arachidonic acid, circulate in plasma and are excreted in the urine.[11–13] Their formation is largely independent of cyclo-oxygenase-1 (Cox-1) and Cox-2 activity. F_2-isoprostane is a potent smooth muscle cell constrictor and a mitogen. It modulates platelet as well as other cell functions *in vitro* via prostaglandin membrane receptors (thromboxane A_2).[11,14] Isoprostanes have been used as markers of oxidative stress[14] and their measurement in biological fluids provide a quantitative index of lipid peroxidation *in vivo*.

4-HNE is a highly reactive and diffusible end product of lipid peroxidation, known to induce various cellular events, in particular proliferation, apoptosis and the activation of signalling pathways.[9,10] 4-HNE can be produced from arachidonic acid, linoleic acid or their hydroperoxides in concentrations of 10 μM–5 mM, in response to oxidative insults, and is believed to be responsible for many cytopathological effects observed during oxidative stress *in vivo*.[10,15] 4-HNE has a high affinity towards cysteine, histidine and lysine residues, altering protein function and forming protein-adducts. Many of the effects of ROS in airways may be mediated by the secondary release of inflammatory lipid mediators such as 4-HNE.

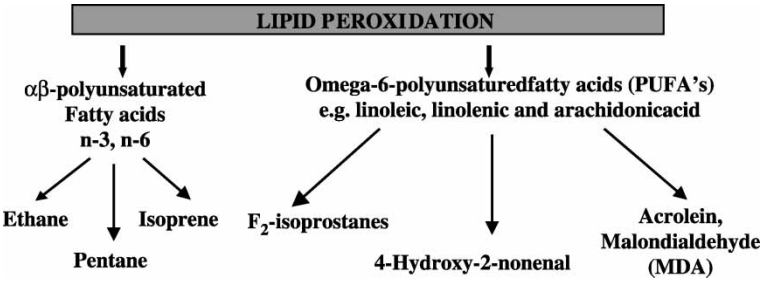

FIGURE 2 Membrane lipid peroxidation of polyunsaturated fatty acids leading to generation of various aldehydes.

Acrolein is a thiol reactive, αβ-unsaturated aldehyde present in various environmental sources, the largest source being cigarette smoke. Inhalation of acrolein induces changes in rat lung structure and function. It is known to deplete glutathione in the nasal mucosa of rats[16,17] and in alveolar epithelial cells *in vitro*.[17] Acrolein is an alkylating agent and it can react with a variety of nucleophilic sites in DNA, forming adducts with DNA bases.

EXHALED BREATH CONDENSATE

Exhaled breath consists of a gaseous phase that contains volatile substances, such as nitric oxide, carbon monoxide and hydrocarbons, and a liquid phase, termed the exhaled breath condensate (EBC), which contains water vapour and non-volatile aerosol particles.[18] It is thought that airway surface fluid becomes aerosolised during turbulent airflow, and as such, the content of the condensate originates from the bronchioles and alveoli, reflecting the composition of BALF.[19] Collection of breath condensate has several advantages over the more traditional methods of sampling the pulmonary airspaces, such as bronchoalveolar lavage (BAL). It is non-invasive and less expensive in terms of both equipment and personnel costs. The feasibility of using EBC samples to quantitate oxidant stress in patients or animal models is therefore an attractive concept. It is possible that specific biomarkers identified in EBC may be indicative of different aspects of oxidative stress and/or inflammation and as such, this non-invasive approach may be useful in categorising pulmonary diseases.[20] Furthermore, although EBC contains mainly metabolic products from the airways and the lung, it also contains products of other organs that reach the lung via the blood stream. As a consequence, the identification of products released from a particular site in the body may provide organ-specific information of oxidative stress.

COLLECTION OF EBC

The collection of EBC requires subjects to breathe tidally through a mouthpiece, which is connected to a condensing chamber for 10–15 min whilst wearing nose-clips. The collecting system can be a condensing chamber with a double wall of glass, the inner wall of which is cooled by ice (Fig. 3).[21] Exhaled air enters and leaves the chamber through one-way valves at the inlet and outlet while the chamber is kept closed. The exhaled air is then introduced, through a non-rebreathing valve, into the sampling tube where it is cooled down to −10°C. Generally, a volume of 1–2 ml of breath condensate is collected over a period of 10–15 min.[22,23] The most reliable collecting apparatus is now commercially available, namely the EcoScreen by Jaeger Inc., which is an electrically refrigerated system modified from a cold air challenge device. However, if the latter is not economically feasible, many laboratories construct and operate homemade apparatus.[23,24] Corradi and colleagues[25] have recently reported that comparable concentrations of EBC constituents can be found when breath is sampled with a homemade breath collection device versus that with a commercially available device provided the subjects are asked to breathe tidally.[25] With both approaches the collected EBC can be stored at −80°C before analysis. Agents, such as lipid peroxidation inhibitors and metal chelators may be added prior to freezing. However, standardized protocols have yet to be agreed.

A similar device to collect nasally exhaled air condensate has recently been used in children as young as four weeks of age,[26] however, nasal contamination of the condensate needs to be excluded before this method can be recommended for assessing biomarkers derived from the airways. Contamination of EBC by saliva is usually not a problem using the EcoScreen apparatus but this can be checked by measuring the salivary amylase activity,[22,23] whilst the viscosity of the samples can

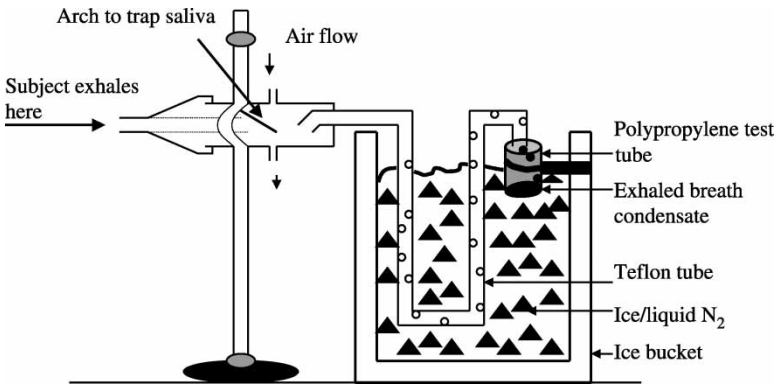

FIGURE 3 Schematic diagram showing EBC homemade collection system.

be checked with a micro-ball falling viscometer (made by Haake).

Regardless of the system employed, the collection procedure is a simple, safe and non-invasive process. Furthermore, samples can be collected from individuals on multiple occasions, allowing the time course, for example, of an inflammatory response and oxidative stress to be monitored.

ASSAYS

To date, the majority of exhaled markers have been measured by immunoassay. Although interesting, these data now need to be validated with more specific and sophisticated analytical techniques, such as gas chromatography/mass spectrometry (GC/MS) or high-performance liquid chromatography (HPLC). Confirming the presence of these biomarkers using a more accurate analytical methodology and establishing the precise concentrations of different free radical biomarkers in EBC will increase confidence in the use of this new non-invasive technique. For example, most studies to date have used an immunoassay to measure F_2-isoprostane concentrations in EBC. This may be problematic owing to the presence of closely related substances in biological fluids that may potentially interfere with the immunoassay.[27] However, this can be resolved by using a sensitive gas chromatography/negative ion chemical ionization mass spectrometry technique to measure F_2-isoprostanes.

ELECTRON PARAMAGNETIC RESONANCE (EPR)

Spin trapping is the most direct method for the detection of highly reactive free radicals in experimental or clinical settings.[28] ROS ($O_2^{\cdot-}$,, $^{\cdot}OH$) and $NO^{\cdot}/ONOO^{-}$ can be detected in biofluids by EPR spin-trapping experiments using various spin traps, e.g. 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine (Tempone-H), nitrone spin traps phenyl-tert-butyl-nitrone (PBN), *N*-tert-butyl-α-(4-pyridyl) nitrone *N*-oxide (POBN), colloid Fe (II) diethyldithiocarbamate, and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO).[29,30] The intensity of the EPR signal corresponding to the formation of spin trap products, such as 4-oxo-tempo can be measured in terms of arbitrary units by commercially available EPR machines (X-, S- or L-bands) by various manufacturers, such as Bruker, Jeol and Magnettech GmbH. The technique of spin trapping involves the addition of a primary free radical across the double bond of a diamagnetic compound (the spin trap) to form a radical adduct more stable than the primary free radical. This technique can be applied *in vivo* in both laboratory and clinical settings where the biological fluids derived from subjects/experimental models can be directly analysed for ROS/RNS, antioxidants and radical scavengers.[30,31] Surprisingly, this technique has not been used so far to measure ROS in EBC. It is important to remember that spin-trapping is highly dependent on the spin trap used and the antioxidant status of the biofluids.

NUCLEAR MAGNETIC RESONANCE (NMR) BASED "METABOLOMICS/METABONOMICS" ANALYSIS OF BIOFLUIDS

Metabolomics (also called as metabonomics) is defined as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification".[32] High resolution H^1-NMR spectroscopic analysis of biofluids allows simultaneous detection of hundreds of low molecular weight species within a sample of body fluid, resulting in the generation of a metabolic profile or NMR "finger print" that is altered characteristically in response to physiological status.[33] Once NMR spectra are obtained, the highly complex spectra are analysed using pattern recognition and multivariant statistical methods to generate models for classification of samples.[34] This technology has been widely applied to toxicology studies with a range of biological fluids, such as urine and plasma, in both experimental animals and humans.[32,35,36] Statistical analysis of urine samples has been shown to result in inherent clustering behaviour for drugs and toxins acting on different organs, such as liver or kidney, or having different toxic mechanisms.[32] These cluster analyses are similar to those currently being developed for gene array expression analysis and proteomics, and have been demonstrated to correctly classify toxins in test samples.[35] Metobolomic analysis, therefore, seems particularly suited to the analysis of biofluids from clinical/laboratory studies, such as EBC, with the potential to simultaneously measure a range of oxidative stress products and other inflammatory markers. Analysis of such samples may lead to the identification of novel single biomarkers of interest for wider study in patient populations. Brindle and colleagues have recently showed the serum metabolome obtained from coronary heart disease healthy individuals.[37] Moreover, application of metabolomics allows the simultaneous analysis of multiple end products and it may be that these "finger prints" characteristic of disease are a more powerful and robust means by which to stratify disease severity, progression and to assess drug efficacy than the analysis of any single marker over a patient population. Thus, this technique may provide a breath profile or breathogram in EBC.

INFRARED LASER SPECTROMETER

Infrared laser spectroscopy is a promising procedure for free radical research, enabling online measurement of oxidative stress biomarkers, such as lipid peroxidation products with high sensitivity and efficiency.[38] Murtz and co-workers have developed real-time analysis of volatile ethane fractions in exhaled breath (gaseous molecular species) using laser absorption spectroscopy. The group monitored the ethane fraction exhaled by a smoker after smoking a cigarette every 30 min over a period of 4 h,[38] and observed a strong increase and subsequent decay of the ethane fraction after smoking. This method is unique, with very high sensitivity and specificity for rapid and precise breath testing. The detection limit is 300 volume parts per trillion ethane in exhaled breath with an integration time of 5 s. Another major advantage of the technique is that it allows the analysis of biomarkers without preconcentration or pre-treatment of exhaled breath. The development and introduction of this biosensor technique for immediate analysis of EBC has a potential to undertake real-time EBC monitoring of oxidative stress in animal research and clinical practice.

REPRODUCIBILITY AND STANDARDISATION

The application of any new technical procedure in investigative studies or to the clinic requires its validation in terms of sensitivity, specificity, reproducibility and correlation of the measurements with disease status. The validity of EBC as a tool for the assessment of airway oxidative stress is still questionable owing to limitations in the reproducibility of analysed oxidative biomarkers, with respect to both intra- and inter-individual variability.

It is likely that a considerable portion of cited literature differences can be explained by differences in collection procedures, sample storage and sensitivity of the analytical techniques used [enzyme immunoassay/radioimmunoassay (RIA), spectroscopy, GC/MS and HPLC]. For example, there are numerous issues affecting sample collection that need to be addressed such as device temperature, temperature of the collected fluid, saliva-trapping characteristics and contamination from non-respiratory sources.[39] Added to these, the quantity of EBC collected over a given time is itself dependent on the ventilation volume per time unit (minute volume), breathing and flow rates, exhaled air temperature and relative humidity. Changes in these parameters can cause large variations in the dilution of condensate even within individuals.

The analysis of EBC can also be affected by smoking and diurnal variation, as is the case for H_2O_2, which shows diurnal variations in healthy subjects.[40] H_2O_2, is in fact a good example of one that is affected by storage conditions, sample pretreatment and extent of EBC dilution. The latter can however, be checked by measuring the composition of electrolytes in EBC samples.[41] More studies are needed to define the reproducibility of biomarkers in biofluids in general, and in particular EBC. It would, therefore, be premature to provide recommendation on biomarker ranges as these may differ from one experimental or clinical condition/setting to another. However, research is in progress in many laboratories to resolve the issue of reproducibility, e.g. recent study suggested that wearing the nose clip does not affect the concentration of mediators derived in EBC whereas the volume of EBC is affected.[42] A European Respiratory Society (ERS)/American Thoracic Society (ATS) task force, as recommended[43] may be established to address issues such as reproducibility and to define the equipment and protocols best suited for the collection of EBC.

In addition to the issues mentioned above, if an increased concentration of a biomarker is detected in EBC, investigators are still faced with the problem of interpretation.[41] For example, the change could be the result of an increase in the concentration within the airway lining fluid. Alternatively, it could simply reflect a greater number of aerosolised particles being captured within the same volume of condensate. The latter is a real possibility, since the majority of lung disorders assessed using EBC have noisy/acoustic and turbulent airflow, and hence one would presume more particle formation. It is issues such as these that highlight the need to identify dilution markers before EBC is used to express airway lining fluid concentrations of individual compounds.

Further technical work and standardisation is, therefore, necessary before EBC can provide precise measurements of the concentration of a given biomarker and hence be used reliably in the clinic. Studies into the relationship between EBC biomarkers and other methods of quantifying oxidative stress (e.g. biomarkers analysis in plasma and BAL) would be beneficial however, as yet, few direct comparisons have been undertaken. Coupled to this, since the ionic/electrolyte (Na^+, K^+ and Cl^-) composition of the human airway lining fluid in health, let alone disease, is not known, a gold standard by which to make reliable comparisons does not exist. The other possibility would be to determine the urea and ammonia levels in the EBC in such a way that urea might be a suitable index of dilution for standardization of EBC. Ammonia can be detected in EBC of normal individuals but the concentration is decreased in the EBC of asthmatics due to "acidic" pH of the airways.[44] Furthermore, there are several methodological issues regarding sampling and analytical techniques of EBC needed to

be taken into account before this can be used for standardization purpose., e.g. ammonia can be expired from the nasal airways which would contaminate the EBC and the method to detect ammonia is based on urease method which converts urea into ammonia. It is recommended that ammonia is first measured by non-urease method and the concentration is subtracted from ammonia derived by the urease method. Thus, measurement of ammonia and urea as a "gold standard" is being recommended for EBC standardization.[45] Despite the problems discussed above, EBC can currently provide useful information when biomarker concentrations differ substantially between health and disease. However, clearly much work needs to be done to bring this method into routine use.

CLINICAL APPLICATIONS OF EBC

Hydrogen Peroxide

Hydrogen peroxide in exhaled breath is a direct measure of oxidant burden in the air spaces. It is soluble and equilibrates with air.[46] Smokers and patients with chronic obstructive pulmonary disease (COPD) have higher levels of exhaled H_2O_2 than healthy non-smokers,[47–49] and concentrations are even higher during exacerbations of COPD.[49] Although the definitive source of the increased H_2O_2 is unknown, in smokers it is thought to derive in part from an increased release of $O_2^{\cdot-}$ from alveolar macrophages.[50] The concentration of exhaled H_2O_2 correlated with the degree of airflow obstruction, as measured by FEV_1.[49] Increased concentrations of H_2O_2 in EBC has also been demonstrated in patients with asthma, ARDS, CF and lung cancer.[19,20]

Although the collection of EBC has been undertaken extensively in human studies the technique has also potentially enormous use in animal studies. The concentration of H_2O_2 in EBC has been studied in healthy horses, and horses affected with recurrent airway obstruction (RAO), a condition that has been used as an animal model of asthma.[51] When suffering from marked airway inflammation (as assessed by tracheal wash inflammation score and neutrophils in BAL), RAO horses had significantly higher concentrations of H_2O_2 in EBC than control horses and RAO affected horses in remission. EBC H_2O_2 levels in this animal model, therefore, provide a useful, non-invasive diagnostic indicator of the severity of neutrophilic airway inflammation. Similarly, Wilhelm and co-workers[52] have recently shown that EBC H_2O_2 levels are increased in rats made hypoxic and this correlated with lipid peroxidation levels.

Exhaled H_2O_2 may also have use in assessing antioxidant and anti-inflammatory therapies. For example, Antczak *et al.*[53] reported that a 2-week treatment course of low dose inhaled beclomethasone diproprionate decreased EBC H_2O_2 concentration in asthmatic children. A similar effect was also observed in children with stable asthma, some of who were receiving inhaled corticosteroids in a daily regimen.[54] Similarly, H_2O_2 level was decreased in patients with allergic rhinitis following triamcinolone acetonide treatments.[55] These findings suggest that the concentration of H_2O_2 in EBC is related to the severity of airway inflammation. ARDS patients treated with corticosteroids also show a tendency towards lower levels of H_2O_2 in the EBC as compared to steroid-naïve ARDS patients.[56] In another study it was observed that long-term treatment of *N*-acetyl-L-cysteine (600 mg daily) decreased EBC H_2O_2 concentrations in subjects with COPD.[57] Both asthma and CF patients with acute exacerbations have high levels of exhaled H_2O_2, which decrease during antibiotic treatment.[58] However, long-term treatment with cyclophosphamide and/or predinosone of patients with systemic sclerosis did not significantly decrease H_2O_2 concentration in EBC suggesting that restoration of H_2O_2 levels by drug treatments may be disease-specific. Furthermore, this suggests that monitoring of H_2O_2 may not be useful to study drug efficacy in certain diseases.[59]

Recent data indicates that EBC can be stored up to 40 days without any loss of H_2O_2 activity.[60] However, as mentioned above, the variability of measured exhaled H_2O_2, and the presence of confounding factors (e.g. increased generation of ROS by cigarette smoke-mediated redox cycling), raises concerns over its reproducibility as a biomarker of oxidative stress in smokers and in smoking-related diseases. This is further complicated by the increased concentration of H_2O_2 in EBC during a common cold.[61]

The use of different measurement techniques and the variation in reported H_2O_2 values indicate that H_2O_2 levels might be difficult to standardize in EBC.[60,62] It is also possible that the concentrations of some of oxidative stress biomarkers are variable and below the detection limit of the available assay methods (Table I). Therefore, in order to use these EBC markers for research or clinical purposes, more sensitive and specific assays may be required. A recent attempt by Corradi and colleagues employing liquid chromatography coupled with atmospheric pressure chemical ionisation tandem mass spectrometry revealed different classes of aldehydes (malondialdehyde, hexanal and heptanal) in EBC.[25] The concentrations of these aldehydes were increased in smokers compared to non-smoking control subjects but malondialdehyde was only increased in patients with COPD compared to smokers suggesting the specificity and selectivity of

TABLE I Various biomarkers in EBC and their measurements

Biomarker	Analysis	Values in EBC	References
ROS: superoxide anion, hydroxyl radical and hydrogen peroxide	Chemiluminescence, enzymatic, spectroscopy, ESR/EPR	H_2O_2 (0.02–0.5 μM)	[19,20,58,100,103]
RNS: nitric oxide (nitrite and nitrate), peroxynitrite, nitrotyrosine, Nitrosothiols	Electrometry, spectroscopy, GC/MS, HPLC, immunoassay, ESR/EPR	3-Nitrotyrosine (0.66–6.3 ng/ml); nitrite/nitrate (0.64–20.2 μM); Nitrosothiol (0.08–0.46 μM)	[63,66–68,71,72,79,80,82–88]
Lipid peroxidation products: TBARS, malondialdehydes, 4-HNE, F_2-isoprostanes	Colorimetry, ELISA, enzyme immunoassay (EIA), GC/MS, HPLC	MDA (35.6 ± 4.0 nmol/l) by GC/MS; TBARS (~0.5 μM) F_2-isoprostanes (3.9–15.8 pg/ml) by EIA kit; and (80–120 pg/ml) by GC/MS in COPD; low detection limit in non-smoking controls)	[23,25,89,90,95,96,100–106,121]
Cytokines, chemokines and growth factors: IL-1β, TNF-α, IL-4, IL-6, IF-γ, IL-10, VEGF, soluble interleukin-2 receptor protein (sIL-2R), IF-γ	ELISA, luminex, RIA, GC/MS	IL-4 (35.7 ± 6.2 pg/ml), IL-6 (~3 pg/ml), IL-10 (~20 ng/ml); IL-8 (~10 pg/ml); IF-γ (3.7 ± 0.2 pg/ml); IL-1β (370 pg/ml); TNF-α (120 pg/ml); sIL-2R (2159 U/ml); VEGF (2.5 pg/ml)	[22,86,107–110,122,126–128]
LTs and prostaglandins (PGs)	ELISA, HPLC, RIA, GC/MS	Thromboxane B_2 (25 to 30 pg/ml); leukotriene B4 (7.7 ± 0.5 pg/ml); cys-Lts (1.4 ± 0.5 pg/ml); PGE_2 (44.3 pg/ml)	[42,111,112,116,117,119]
pH	Electrometry, colorimetry	Normal (pH 7.57); Asthma (acute pH 5.23, stable pH 7.43); COPD (pH 7.16); Cystic fibrosis (acute pH 5.32, chronic pH 5.88)	[120,121,129]
Antioxidants: total antioxidant capacity, glutathione, vitamin E, C, uric acid, albumin and other metabolites	Spectrophotometry, immunoassay, HPLC, GC/MS, NMR/metabolomics (metabonomics)	Glutathione (14.1 ± 0.8 nM)	[2,32,37,96,124]

the EBC constituents in health and disease.[25] Thus, more specific and sensitive assays can be developed for oxidative stress biomarkers.

Nitric Oxide, Peroxynitrite and 3-nitrotyrosine

Nitric oxide (NO) has been used as a marker of airway inflammation, and indirectly as a measure of oxidative stress. Its synthesis and release in the lungs can be measured indirectly by quantifying stable, NO-derived products in EBC.

NO concentrations are elevated in the exhaled air of patients with asthma,[63,64] and this has been suggested to contribute to airway oedema and inflammation.[64–66] These elevations decrease significantly when inhaled corticosteroids are administered.[67,68] Further work has shown that a combined increase of NO and H_2O_2 in EBC of asthmatics is associated with a pro-oxidant activity in airway walls, resulting in lipid peroxidation and nitration of proteins.[63,69–71]

Patients with COPD who have stopped smoking also appear to have elevated exhaled NO levels compared with non-smoking healthy subjects,[72–74] although these concentrations are only mildly elevated compared with those measured in patients with untreated asthma.[72] In addition, a prospective randomised, placebo-controlled, cross-over trial has shown that inhaled beclomethasone reduces exhaled NO levels in stable non-smoking patients with COPD, a finding compatible with an anti-inflammatory mechanism of action.[75]

Reaction of NO and $O_2^{\cdot-}$ results in the formation of the peroxynitrite anion ($ONOO^-$). This is a highly reactive oxidant species that can induce lipid peroxidation, tyrosine nitration and MAP kinase activation.[76,77] It also inhibits pulmonary surfactant production, promotes hyperresponsiveness in the airways of guinea pigs and damages pulmonary epithelial cells.[46,66,77,78] Moreover, $ONOO^-$ can add a nitro group to the 3-position adjacent to the hydroxyl group of tyrosine to produce the stable product nitrotyrosine.[79] 3-Nitrotyrosine concentrations are elevated in the exhaled breath of patients with asthma,[80] an observation supported by strong immunoreactivity for nitrotyrosine in the airway epithelium, lung parenchyma and inflammatory cells in the airways of asthmatics.[80,81] Whilst 3-nitrotyrosine concentrations are reduced in patients with severe asthma receiving steroid therapy, in moderate asthma during inhaled steroid withdrawal, they increase in association with worsening of symptoms and deterioration of lung function.[80] Observations in stable asthma patients also indicate elevated 3-nitrotyrosine concentrations in EBC,[80,82] while increased concentrations and a relationship with FEV_1 have been demonstrated in patients with COPD.[83]

S-nitrosothiols

Nitric oxide can be trapped by thiol-containing biomolecules, such as cysteine and glutathione, to form *S*-nitrosothiols—metabolites with bronchodilating effects. Increased levels of *S*-nitrosothiols have been reported in the EBC of smokers and patients with asthma and cystic fibrosis,[84] while concentrations are reduced in asthmatics after 3 weeks of treatment with (400 μg daily) inhaled budesonide.[85]

Nitrite and Nitrate

NO_2^- and nitrate (NO_3^-) are end products of NO metabolism. Whilst NO_2^- is increased in EBC of patients with lung diseases,[83,84] smoking causes an acute increase in NO_3^-/ but not NO_2^-.[82] Exhaled NO_2^- is also increased in adults[86] and children[87] with cystic fibrosis compared to age-related controls. Furthermore, total EBC NO_2^-/NO_3^- concentrations were found to be significantly higher in cystic fibrosis patients and asthmatics compared to healthy controls.[86–88]

MEASUREMENT OF LIPID PEROXIDATION END PRODUCTS

The non-invasive measurement of aldehydes in exhaled breath may provide a useful tool to assess lipid peroxidation *in vivo* and in doing so, the clinical status of patients with chronic inflammatory diseases can be monitored.

F_2-isoprostanes

Increased EBC concentrations of 8-isoprostane (8-isoPGF$_{2\alpha}$) have been found in patients with asthma,[23] in line with findings in plasma[89] and BALF.[89] Interestingly, elevated EBC concentrations of 8-isoPGF$_{2\alpha}$ in mild asthmatics were not found to be affected by inhaled corticosteroids, suggesting that this therapy may not be effective in inhibiting lipid peroxidation in this condition.[85] 8-isoPGF$_{2\alpha}$ concentrations are also elevated in the expired breath of smokers and in patients with COPD.[90]

Measurements of F_2-isoprostanes have also been made in non-pulmonary diseases. Plasma levels are elevated in patients with cardiovascular disease and it has been suggested that this may be a useful biomarker of risk.[91] In addition, elevated concentrations of 8-isoPGF$_{2\alpha}$ have been demonstrated in a variety of clinical syndromes thought to be associated with oxidant stress, including acetaminophen and paraquat poisoning, coronary reperfusion with thrombolytic agents, and alcohol intake in cirrhotic patients.[92] F_2-isoprostane concentrations are also elevated in response to oxidant stress in animal models.[93] The concentration of 8-isoprostane levels was not decreased in patients with asthma after a 5-day course of oral prednisone, suggesting that corticosteroids may not be fully effective in response to oxidative stress in children with an exacerbation of asthma.[94]

4-Hydroxy-2-nonenal

Increased concentrations of 4-HNE-modified protein is present in airway and alveolar epithelial cells, endothelial cells and neutrophils in smokers with COPD compared to subjects without airway obstruction.[95] This demonstrates not only the presence of 4-HNE, but that 4-HNE is associated with modification of proteins in lung cells to a greater extent in patients with COPD. The increased number of 4-HNE-adducts in alveolar epithelium, airway endothelium and neutrophils was inversely correlated with FEV_1, suggesting a role for 4-HNE in the pathogenesis of COPD. Again, these findings suggest that the measurement of 4-HNE-adducts in EBC may have potential in monitoring oxidative stress *in vivo*.[95]

Malondialdehyde

Recent studies have evaluated malondialdehyde and glutathione concentrations in the EBC of control children and children with asthma, both during exacerbation and after a 5-day course of therapy with oral prednisone.[96] Both malondialdehyde and glutathione were detectable, and found to be modified during asthma exacerbation (increased aldehyde and decreased glutathione concentrations). After a 5-day course with oral prednisone these changes were reversed. Similarly, Corradi and co-workers have recently shown higher concentrations of malondialdehyde in the EBC obtained from COPD patients.[25] However, the intervention study has not been conducted in COPD.

Analysis of EBC also has the potential to be used in the prenatal diagnosis of foetal hypoxia, since significantly higher levels of diene conjugates and malondialdehydes have been found in pregnant women who gave birth to babies with severe foetal and neonatal hypoxia.[97]

Hydrocarbons and Thiobarbituric Acid Reactive Substances (TBARS)

The determination of hydrocarbons and TBARS in EBC has also been proposed as a means to assess lipid peroxidation *in vivo*. Concentrations of both biomarkers are increased in patients with asthma,[98–100] in line with the analysis of plasma concentrations, which in turn are negatively correlated with FEV_1.[101] Patients with COPD have been

reported as having elevated levels of exhaled ethane, and the latter correlated with disease severity as assessed by FEV_1.[102] Furthermore, lower exhaled ethane concentrations were found in steroid treated patients. TBARS are also elevated in breath condensate, plasma and in the lungs of patients with stable COPD,[103–106] and in one study, the increase in EBC TBARS was again correlated with a decline in FEV_1.[103] These findings can be interpreted that oxidative stress and lipid peroxidation are increased in the airways of patients with asthma and COPD.

Cytokines

The measurement of lung cytokines using EBC analysis is a novel but increasingly popular approach. This approach has been further advanced by the introduction of luminex technology based on cytometrix bead analysis. Using this technique, multiple cytokines, chemokines and growth factors can be measured simultaneously in a very small quantity of biological sample. Interleukin-1β, TNF-α, interleukin-6 and interleukin-8 have all been reported in EBC from human subjects,[20,21] whilst interleukin-10 has been detected in EBC from pigs, and reportedly increases during ischaemia–reperfusion injury after experimental transplantation.[107] Carpagnano and co-workers have recently shown that IL-6 levels were increased in current smokers compared to non-smokers and the concentration was correlated with the number of cigarettes smoked per day and lung function.[108] Furthermore, the concentration of IL-6 is increased in EBC obtained from patients with cystic fibrosis.[109]

Asthma is characterized by an imbalance of T helper (Th)-2 cells, which secrete interleukin (IL)-4 and Th1 cells, which secrete interferon (IFN)-γ. These cytokines have been studied in EBC of normal children and those with asthma either untreated or treated with inhaled corticosteroids.[110] Concentrations of IFN-γ were lower and IL-4 elevated in EBC of children with asthma, while IL-4 concentrations were lower in those children who were on high dose inhaled corticosteroids compared with those on low doses of steroids. As a consequence the ratio of IL-4/IFN-γ was significantly higher in untreated children with asthma compared to that in healthy control children or those children on inhaled steroid therapy. This finding is consistent with the predominance of Th2 cells in the airways of children with asthma. EBC analysis may, therefore, become useful in studying allergic inflammation, and optimising corticosteroid therapy in children with asthma. Furthermore, these data confirm that ratios of EBC biomarkers can provide valuable information on redox status (e.g. glutathione, H_2O_2 and nitrite to nitrate) and inflammation (e.g. IL-4 to IFN-γ) status.

Leukotrienes (LTs)

LTs are a family of lipid mediators derived from arachidonic acid via the 5-lipoxygenease pathways. They are potent constrictor and pro-inflammatory mediators that contribute to the pathophysiology of asthma. The cysteinyl leukotrienes (cys-LTs) LTC4, LTD4 and LTE4 are primarily generated by mast cells and eosinophils,[111] whilst LTB4 is a potent chemoattractant of neutrophils.[112] cys-LTs and LTB4 in EBC, measured using ELISA, are elevated in patients, including children, with mild and moderate to severe asthma treated or untreated with inhaled steroids.[113–115] Furthermore, steroid withdrawal in patients with moderate asthma, which leads to a worsening of the condition, has been found to further increase LTB4, LTC4, LTD4 and LTE4 in EBC. Elevated concentrations of LTB4 have also been reported in smokers, patients with COPD and cystic fibrosis[108,109,116,117] and in healthy calves during experimental chest infection.[118] The levels of prostaglandin E2 (PGE_2) was also increased in patients with COPD.[119] These studies support the use of exhaled cys-LTs, LTB_4 and PGE_2 as non-invasive markers of airway inflammation.

pH

The pH of EBC collected from patients with acute asthma is more than two log orders lower than normal, but normalises with corticosteroid therapy.[120] Similar findings have been reported in COPD and bronchiectasis.[121] This may be due to airway acidification leading to lowered ammonia levels in these diseases. The assay used to determine EBC pH is easy and furthermore, measurements are highly reproducible. As such, EBC pH is another parameter that may become useful clinically for disease diagnosis and monitoring of therapy.

Proteins/Proteomic

The distinct nature of proteins present in EBC can be measured qualitatively and quantitatively by either direct protein assay or by more sophisticated proteomic technique. Earlier work of Scheideler and co-workers have revealed that the amount of proteins in the EBC varies from 4 μg to 1.4 mg.[122] Griese and co-workers have recently used this technique to measure the components of proteins present in EBC obtained from healthy subjects by oral and nasal means of exhaling breath.[123] They found three spots (72 kDa/isoelectric point (pI) 6.6–7.0, 66 kDa/pI 5.9–6.7 and 45–48 kDa/pI 8.0–8.6 present in these subjects. This suggests that the quantification of specific proteins by this technique may provide a pattern of distinct proteins in health and disease.

Antioxidants

As evidence mounts for the involvement of oxidative stress in a range of pulmonary diseases, there is increasing interest in the antioxidant status of the lungs in health and disease.[104,124,125] The use of EBC to measure lung antioxidant status is, therefore, an attractive prospect. Indeed, this simple non-invasive method could be used to monitor oxidative stress in disease and the response to antioxidant or anti-inflammatory treatment. Given that the extracellular antioxidant concentrations in the lungs are generally determined in diluted lung lining fluid, i.e. BAL very sensitive assays are required for the provision of reproducible data. Clearly, such approaches are feasible in breath condensate as demonstrated by the recent report of glutathione in EBC by Corradi *et al.*[25,96] Preliminary work in the author's (F.J. Kelly) laboratory has demonstrated the presence of low concentrations of other antioxidants such as ascorbate and urate in EBC. However, given the highly oxidizing environment of EBC (high concentrations of H_2O_2) most antioxidants appear to be present only in their oxidized form and it is as yet unclear if, and how, these measurements provide meaningful information about the *in vivo* antioxidant status of the airways. The recent introduction of the new technique of metabolomics, which is based on ^{1}H-NMR spectroscopy, may be useful in this respect, as a "metabolome" (total small molecules including antioxidants of a cell), can be determined in EBC.[32,37]

CONCLUSIONS

EBC is a simple, safe and non-invasive procedure that can be successfully applied to both human and animal studies. The utility and relevance of EBC assays in assessing biomarkers of oxidative stress and studying mechanisms of lung disease is exemplified by the growing list of respiratory conditions investigated by the procedure (asthma, adult respiratory distress syndrome, idiopathic pulmonary fibrosis, pneumonia, lung transplantation, COPD, cystic fibrosis, bronchiectasis and lung cancer), and the wide array of biologically relevant compounds that can be measured. Although EBC is currently a research tool, it has the potential to be of great benefit in clinical practice in helping to diagnose disease, in identifying patients who could possibly benefit from a given antioxidant or anti-inflammatory therapy and in monitoring the disease and response to treatment. In addition, within the clinical trial setting, EBC assays can satisfy the increasing demand to measure biomarkers as a means to assess the efficacy of new drugs in an easily accessible and cost-effective manner.

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