ATS/ERS TASK FORCE

Exhaled breath condensate: methodological recommendations and unresolved questions

I. Horváth*, J. Hunt# and P.J. Barnes*
On behalf of the ATS/ERS Task Force on Exhaled Breath Condensate


ABSTRACT: Collection of exhaled breath condensate (EBC) is a noninvasive method for obtaining samples from the lungs. EBC contains large number of mediators including adenosine, ammonia, hydrogen peroxide, isoprostanes, leukotrienes, nitrogen oxides, peptides and cytokines. Concentrations of these mediators are influenced by lung diseases and modulated by therapeutic interventions. Similarly EBC pH also changes in respiratory diseases.

The aim of the American Thoracic Society/European Respiratory Society Task Force on EBC was to identify the important methodological issues surrounding EBC collection and assay, to provide recommendations for the measurements and to highlight areas where further research is required.

Based on the currently available evidence and the consensus of the expert panel for EBC collection, the following general recommendations were put together for oral sample collection: collect during tidal breathing using a noseclip and a saliva trap; define cooling temperature and collection time (10 min is generally sufficient to obtain 1–2 mL of sample and well tolerated by patients); use inert material for condenser; do not use resistor and do not use filter between the subject and the condenser. These are only general recommendations and certain circumstances may dictate variation from them.

Important areas for future research involve: ascertaining mechanisms and site of exhaled breath condensate particle formation; determination of dilution markers; improving reproducibility; employment of EBC in longitudinal studies; and determining the utility of exhaled breath condensate measures for the management of individual patients. These studies are required before recommending this technique for use in clinical practice.

KEYWORDS: Airway inflammation, biomarkers, exhaled breath condensate, lung diseases, noninvasive monitoring, oxidative stress

CONTENTS

Executive summary .......................................................... 524
Introduction ................................................................. 525
General aspects of exhaled breath condensate collection .......................................................... 525
  Standardisation of terminology ............................................... 525
  Content of EBC .............................................................. 525
  Source of EBC ............................................................... 528

For editorial comments see page 371.
Factors which affect collecting EBC
- Condensing equipments
- Efficiency, duration and temperature of condensation
- Ambient air
- Breathing pattern
- Airway calibre and lung function
- Age and sex
- Food and drink
- Circadian rhythm
- Tobacco smoking
- Systemic diseases
- Medication
- Ventilated patients
- Safety
- Reproducibility, dilution factor, concentration of samples
- Salivary contamination
- Summary of current recommendations, requirements for EBC collection

EXECUTIVE SUMMARY
Recently there has been increasing interest in the investigation of the lungs by noninvasive means including sputum induction and measurement of biomarkers in exhaled breath including nitric oxide (NO) and those found in the cooled and condensed exhalate, termed exhaled breath condensate (EBC). Compounds identified in EBC include adenosine, ammonia, hydrogen peroxide, isoprostanes, leukotrienes, nitrogen oxides (NOx), peptides, cytokines, protons and various ions. Analysis of EBC has potential to address unmet medical needs by expanding the portfolio of noninvasive assays for the multiple coexisting pathological mechanisms underlying respiratory disorders. However this approach to studying the airway chemical and pathological environment is still in its infancy, with questions as yet remaining unanswered.

The main objective of the American Thoracic Society (ATS)/European Respiratory Society (ERS) Task Force on EBC was to develop guidelines for EBC collection and measurement of exhaled biomarkers, to make recommendations on the possible use and limits of exhaled biomarkers and to highlight those areas where further research is required. An additional objective was to provide a comprehensive review of previous studies of exhaled biomarkers in EBC, to recommend how to optimise the method and to achieve better standardisation of procedures. Based on the currently available evidence and the consensus of an expert panel, the following general recommendations for oral EBC collection are submitted: collections should occur during tidal breathing using a noseclip and a saliva trap, with a defined cooling temperature and collection time; surfaces contacting the EBC should be inert to the compounds of interest in a given study; inclusion of expiratory flow resistance or filters is not required. As biomarkers in EBC have diverse stabilities and characteristics, these are only general recommendations.

Collection of EBC is a completely noninvasive method of sampling the respiratory tract that can be repeated several times with short intervals between sampling. Collection devices can be portable and can be used in a wide range of settings including intensive care units (mechanically ventilated patients), outpatient clinics, workplaces and at home. These attributes of EBC make it a useful tool for epidemiological investigation, and to help gain understanding of the time courses of important pathological processes (oxidative stress, inflammation) in carefully designed studies of respiratory disease.

Determination of the role of EBC in diagnosis and management of individual patients, an issue distinct from large studies of disease mechanisms, awaits further investigation. EBC is a dilute, complex solution of diverse biomarkers with various chemical stabilities. Conditions and duration of storage may decrease or increase assayed concentrations of biomarkers. For many biomarkers, assays are commonly employed at or near their detection limits, leading to higher variability. Immunoassays (for cytokines, 8-isoprostane, leukotrienes and others) require individual validation in the dilute, low protein, chemically diverse EBC, which is a very different matrix than that employed in many commercially available standards. Sample concentration (lyophilisation and resuspension) and the development of more sensitive techniques are assisting in improving reproducibility. Flow-dependency and the potential for oral/upper airway/salivary contamination is likely different for each biomarker, which therefore, require individual investigation. As yet no fully validated method for calculating dilution of respiratory droplets is available and the anatomic origin of biomarkers is not precisely known. Most of these uncertainties regarding EBC assays are shared by other techniques (such as sputum induction, bronchoalveolar lavage, and exhaled NO). Details are provided regarding many of the commonly studied biomarkers, including the advantages, limitations, and potential pitfalls of the detection methods. The effect of different disease states on these EBC biomarker levels is individually summarised.

At the conclusion of the meetings of this Task Force, none of the biomarkers in EBC had been validated sufficiently for...
clinical use. It is the very diversity of EBC itself that has prevented it from achieving clinical applicability yet. With only a relatively small number of investigators studying each biomarker, the process of advancing standardised methodologies for each individual biomarker will be much slower than that for exhaled NO, for which hundreds of investigators focused on one molecule. The Task Force fully recognises this issue. However, after such validations are indeed accomplished, EBC measurements may improve clinical practice by providing useful information on those critical aspects of disease that are left completely inaccessible by current means.

The number of original publications and reviews on EBC analysis has increased sharply in recent years, as has the interest at international conferences. Clinicians are awaiting the sufficient maturation of EBC techniques to help them with their work. The Task Force, therefore, consider it important to summarise current understanding of the technique and also the limitations of knowledge in the hope that this will generate more coordinated research to find the proper place of this sampling method both in research and clinical practice.

The document is organised as follows: a general section discussing issues common to all EBC markers, followed by individual sections addressing specific biomarkers. Wherever possible, the recommendations and presented opinions are based on peer-reviewed published manuscripts (only scientific publications written in English were reviewed for this document) and not on abstracts; in the absence of clear data, we have relied on the experience of Task Force participants. When aspects of EBC collection and biomarker measurements are undetermined, this has been stated in the text. In each section unresolved issues are highlighted. The document is divided into a general section followed by sections addressing individual biomarkers and assay methodology. Wherever possible, the recommendations are based on peer-reviewed published material. In the absence of clear data, the experience of Task Force participants was relied upon. When aspects of EBC collection and mediator measurements are undetermined, this has been stated in the text. In each section unresolved issues are highlighted. Although the number of publications has doubled in each year since 1999, the total number of English-written publications dealing with breath condensate collection is still under 300, and only a few of them address methodological issues. This fact limited evidence-based recommendations. Therefore, everybody interested in the field is encouraged to bring forward data linked to methodological questions to help to better understand this sampling method and to evaluate the usefulness of any data obtained.

While the use of recommendations given (table 1 and section entitled Summary of current recommendations, requirements for EBC collection) will allow direct comparison of data obtained in different centres in future studies, this document does not intend to invalidate studies that have employed other techniques. Importantly, the Task Force does not want standardisation to inhibit innovation. There is substantial room for innovation in collection methods and assays.

INTRODUCTION

Recently there has been increasing interest in the investigation of the lungs by noninvasive means including sputum induction [1] and measurement of exhaled NO [2, 3]. Exhaled breath contains dozens of compounds, which can be measured from the cooled and condensed exhalate. After several issues regarding the problems and unsolved questions about EBC formation and collection were highlighted during an ERS Research Seminar [4], a joint Task Force of the ERS and the ATS was created.

The main objective of this Task Force was to develop guidelines for breath condensate collection and measurement of exhaled biomarkers, to make recommendations on the possible use and limits of exhaled biomarkers and to highlight those areas where further research is required. We also aimed to provide a comprehensive review of previous studies of exhaled biomarkers in EBC, to recommend how to optimise the method and to achieve better standardisation of procedures between different laboratories.

The work of this Task Force over 3 yrs was based on the exchange of knowledge between experts, and the recommendations of this document were formulated by international investigators in the field of measurements in EBC at four consecutive workshops held in Berlin in 2001, Atlanta in 2002, Stockholm in 2002 and Seattle in 2003. The initial draft was prepared by I. Horváth based on the notes taken during the workshops and submitted materials by participants and circulated among all Task Force members for review. The report was then presented to the ERS Executive Committee and ATS Board of Directors.

The document is divided into a general section followed by sections addressing individual biomarkers and assay methodology. Wherever possible, the recommendations are based on peer-reviewed published material. In the absence of clear data, the experience of Task Force participants was relied upon. When aspects of EBC collection and mediator measurements are undetermined, this has been stated in the text. In each section unresolved issues are highlighted. Although the number of publications has doubled in each year since 1999, the total number of English-written publications dealing with breath condensate collection is still under 300, and only a few of them address methodological issues. This fact limited evidence-based recommendations. Therefore, everybody interested in the field is encouraged to bring forward data linked to methodological questions to help to better understand this sampling method and to evaluate the usefulness of any data obtained.

While the use of recommendations given (table 1 and section entitled Summary of current recommendations, requirements for EBC collection) will allow direct comparison of data obtained in different centres in future studies, this document does not intend to invalidate studies that have employed other techniques. Importantly, the Task Force does not want standardisation to inhibit innovation. There is substantial room for innovation in collection methods and assays.

GENERAL ASPECTS OF EXHALED BREATH CONDENSATE COLLECTION

Standardisation of terminology

Based on the consensus of the Task Force participants the expression "EBC" is the preferred term to describe the method. This term should be given as a keyword in manuscripts reporting data obtained by using this technique. Of note, in previous publications several other descriptions could be found including: condensate, airway droplets, solutes, etc. As molecules from the airways may be captured by other techniques, not only by cooling exhaled breath, EBC strictly relates to exhaled samples collected by cooling the exhaled breath.

Recommendation

The terminology "EBC" should replace other descriptive terms used for this technique.

Area for further research

None.

Content of EBC

EBC contains several components. The principal component is condensed water vapour [5]. This fluid represents nearly all of the volume (>99%) of fluid collected in EBC [6]. Only a small fraction of the condensate is derived from respiratory droplets containing nonvolatile molecules (which can be both hydrophobic and water-soluble molecules) [7]. Water-soluble volatile compounds are absorbed by the condensing water during
<table>
<thead>
<tr>
<th>Standardisation issue</th>
<th>Recommendation</th>
<th>Strength of recommendation, rationale and comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General</strong></td>
<td>Standardise sampling, storage, and assay within an individual study.</td>
<td>3. Comprehensive uniform standardisation of EBC is not recommended. However, within a single study, it is recommended to identify potential confounding issues and control for them. If using different devices within one study, rigorous comparisons should be made to assure that differences in temperature, collector surface, cleaning agents, salivary trapping ability, duration of collection and other characteristics do not cause differences in marker levels.</td>
</tr>
<tr>
<td><strong>Sampling issues</strong></td>
<td>Methodology needs to be well-detailed.</td>
<td>5. Basic requirement of scientific manuscripts. Sufficient detail should be provided to assure that the technique can be reproduced.</td>
</tr>
<tr>
<td>Device</td>
<td>Manuscripts should clearly delineate the device used. If a commercial device, note the name and manufacturer and precisely specify any modifications. If a custom device, clearly detail the device, provide sufficient diagrams to allow a reasonable understanding of the equipment employed.</td>
<td>4. At the current level of knowledge, this detail is necessary to help determine what factors may indeed be relevant for the various markers. Most information for the commercial devices is readily available and does not need to be reiterated in manuscripts.</td>
</tr>
<tr>
<td>Materials</td>
<td>If a custom device, detail the materials that are employed, particularly for the surface in contact with the EBC.</td>
<td>4. This information will enhance knowledge of optimum materials for different markers. Different markers will have differing tolerance for materials that line the condensers. The materials themselves, or the compounds used to clean the equipment, may contaminate the sample. This needs to be investigated for each marker.</td>
</tr>
<tr>
<td>Temperature</td>
<td>Specify temperature or range</td>
<td>4. It remains unclear for many markers what the optimum temperature of collection is. There is an expectation that colder sampling is better for unstable mediators, however this has not been proven, and is not necessarily correct. The temperature of collection should be noted. For the commercial devices, the following recommendations are forwarded. ECoScreen: The temperature of the collector should be measured at the beginning of collection. RTube: The temperature of the cooling sleeve or the freezer in which it is kept should be measured or recorded.</td>
</tr>
<tr>
<td>Saliva trap</td>
<td>Recommended</td>
<td>5. Gross salivary contamination certainly occurs occasionally in EBC sampling. Some subjects/patients profusely drool. A clear system for preventing these occasional contaminations should be in place, or samples should be assessed for salivary contamination.</td>
</tr>
<tr>
<td>Duration of collection</td>
<td>Duration of collection should be recorded.</td>
<td>2. Sample volume, minute volume, and duration of collection are three potential, interrelated values that might be chosen for standardisation within a single study. Sample volume is difficult to determine during sample collection in most systems. Minute volume can be determined readily. Duration is simplest. Concentrations of many markers in EBC may be completely independent of any of these values. It needs to be determined for each study/marker whether these values need to be controlled.</td>
</tr>
<tr>
<td>Noseclips</td>
<td>Probably should be worn during oral EBC sampling.</td>
<td>2. There are two reasons for wearing noseclips for the collection of EBC: first is to minimise nasal airway lining fluid entry into the airstream during inhalation; second is to keep all exhaled air exiting through the mouth and not the nose. Note that the nasal contamination issues for EBC collection are entirely different than for exhaled NO, the latter of which is performed during exhalation manoeuvres only). Reasons not to wear noseclips include discomfort. There are no data to support this recommendation.</td>
</tr>
<tr>
<td>Contamination</td>
<td>Test all materials that contact EBC and assure adequate controls are in place.</td>
<td>4. NOx are notorious laboratory surface contaminants, and rigorous methods for assuring no contamination of collection equipment, pipettes or sample containers is necessary. Some sample containers may leach out NOx, which suggests that assays should be performed as soon as possible after collection. The pH of EBC may be affected by contaminants, and the system of collection and storage should be assessed for this.</td>
</tr>
</tbody>
</table>
### Subject issues

<table>
<thead>
<tr>
<th>Standardisation issue</th>
<th>Recommendation</th>
<th>Strength of recommendation, rationale and comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Storage</strong></td>
<td>Unless proven unnecessary, store samples in the coldest temperature available.</td>
<td>3. This seems a reasonable precaution, as it is likely that freezing will not significantly alter levels on markers in EBC.</td>
</tr>
<tr>
<td><strong>Stability of marker in storage</strong></td>
<td>Data should be presented regarding marker stability in EBC, or previous publications addressing the stability of the specific marker referenced.</td>
<td>4. Loss of marker over time in storage, or even with just a brief delay before analysis may lead to Type II errors and may be one cause of wide ranges of values seen in a given marker among different publications.</td>
</tr>
<tr>
<td><strong>Stabilisation of marker</strong></td>
<td>When possible, this should be performed.</td>
<td>2. Addition of a marker-free protein to EBC sample may increase or lessen the loss of an unstable marker. Early derivatisation of a reactive compound to a stable compound may allow later assay. Each marker needs to be independently analysed to determine optimum systems.</td>
</tr>
</tbody>
</table>

**Assay**

- In all cases, use assays proven to be sufficiently sensitive and specific for the marker of interest in EBC.

**Timing**

- Assays should be performed as soon as possible if any loss of marker or contamination with exogenous marker is likely to occur.

**Validation in EBC**

- Assay systems should be tested for utility in EBC.

**Imunoassays**

- Assure that nonspecific binding is identified and minimised, and that appropriate controls are performed in all cases.

**Nitrogen oxides**

- Report precisely what was measured. Do not use the term nitrogen oxides (NOx) without providing a definition for that term in the manuscript. Clearly note the NOx that are included in the assay used.

**pH**

- Report if de-aerated (or gas-standardised) and by what means. If not de-aerated, note the timing of the measurement after collection.

---

4. Loss of marker over time in storage, or even with just a brief delay before analysis may lead to Type II errors and may be one cause of wide ranges of values seen in a given marker among different publications.

2. Addition of a marker-free protein to EBC sample may increase or lessen the loss of an unstable marker. Early derivatisation of a reactive compound to a stable compound may allow later assay. Each marker needs to be independently analysed to determine optimum systems.

4. This is a reasonable precaution, as many markers are not stable. This precaution needs to be balanced with the need to maintain feasible study designs. In all cases, the effect of storage time (delay to assay) on the marker of interest in EBC should be determined.

5. Issues of assay sensitivity and specificity are of paramount importance when working with EBC. The Task Force considers it likely that the majority of variability seen within and between studies is based on difficulties with the assays.

4. Immunoassays in which the protein-poor EBC may lead to falsely elevated test results have been identified. This may result from unblocking of the immunoassay by the dilute, and potentially acidic EBC. Spike-recovery experiments using EBC as the matrix can be helpful to assuring assay functionality. Use of standard curves in an EBC or water matrix, as opposed to a proteinaceous matrix, should be considered. Also to be considered is the addition to EBC of protein so that the matrix of the standard solutions (generally containing substantial protein) and the EBC will be more similar and lead to less false positive results. Concentration of samples by lyophilisation and resuspension in 1/10th of the initial volume can bring many markers into the reliable range of immunoassays.

5. Most assay systems employed were not designed for use in EBC. EBC is a very dilute fluid that is protein and buffer poor.

4. Many NOx assays reported to date are not specific for one compound. Assays may be for nitrate, nitrite, nitrosothiol, nitrotyrosine, NO and other higher oxides of nitrogen. Chemiluminescence analysis after reduction by various methods, when carefully performed, provides sufficient sensitivity for most NOx in unconcentrated EBC. Spectrophotometric tests are less sensitive and NOx are commonly near the limits of detection, especially for nitrite and nitrosothiol, thus, these assays should be used cautiously.

Be aware of laboratory surface and supply contamination. Pay attention to potential contamination of the assay equipment (including injection supplies), storage containers, pipettes, as well as the sample collection equipment.

---

5. Both de-aerated and nonde-aerated assays have been reported. Measurement of pH after deaeration is probably the most technically validated EBC measurement in the published literature. De-aeration is a misnomer, a better term may be “gas-standardised.” Completeness of removal of CO₂ by gas standardisation is not clear. However the pH does stabilise during this process (described in text). The gas-standardised EBC pH is not a direct measurement of airway lining fluid, but appears to reflect capture of acids volatilised from the airway lining fluid. CO₂ is not appreciably more volatile from a low pH fluid, and, therefore, is not of interest in EBC when attempting to identify airway acidification. If not de-aerating, the pH will be substantially affected by CO₂ moving in and out of the EBC. Many investigators consider the CO₂ to be unwelcome noise in the system. Others consider it relevant and believe that gas standardisation is unnecessary.
Levels of some mediators have been compared between EBC tracheostomies/endotracheal tubes and EBC samples collected through the mouth. Studies are required on how and from which site of the respiratory system different substances are transported/accumulated in the exhaled breath. Accumulation of exhaled breath constituents depending on the condenser temperature. During tidal breathing, the liberated aerosol particles range between 0.1–4 particles cm⁻³ with a mean diameter of 0.3 μm [8–10], although these data do not identify the smallest particles, of which there may be substantial numbers. The mechanisms which cause airway/alveolar fluid substances or those from the mucus layer to be added to exhaled breath are not clear and further study is required.

**Recommendation**

None.

**Areas for further research**

Studies are required on how and from which site of the respiratory system different substances are transported/liberated into the exhaled air and on factors and disease states that influence this process.

**Source of EBC**

EBC samples collected through the mouth versus through tracheostomies/endotracheal tubes

Levels of some mediators have been compared between EBC samples obtained directly from the lower airways through a tracheotomy tube and those collected through the mouth. Concentrations of adenosine, and thromboxane B₂ (TxB₂) and values of pH were not significantly different between these samples [11, 12]. Furthermore, although no direct comparison was made, studies using EBC collection through tracheostomy and those collecting EBC through the mouth showed similar levels of hydrogen peroxide (H₂O₂) and 8-isoprostane [5, 13–17]. These results suggest that most of these mediators are added to EBC samples in the lower airways. In contrast the concentration of ammonia is substantially lower in EBC samples obtained through tracheostomies than in those collected through the mouth suggesting that most of this compound is added to the sample from the upper airways or oropharynx [6, 11]. The potential effect of inflammatory diseases of the mouth (i.e. periodontitis, gingivitis, etc.) on EBC constituents has not yet been studied. The investigators who most intensively study EBC pH note that if the laryngopharyngeal airway is sufficiently acidic, EBC pH will be affected. Gastric acid reflux could then contribute to EBC pH decline in a subgroup of patients.

**Recommendation**

Oral/upper airway production of identified EBC constituents should be considered and appropriate control measurements are recommended to be performed to evaluate the influence of the mouth and/or upper airways.
Areas for further research
Studies with direct EBC sampling from the lower airways are needed to determine more precisely the oral/upper airway influence on other mediators. Furthermore, studies on the effect of oral diseases on the content of EBC are required.

Nasal inhalation versus oral inhalation during EBC collection, use of noseclip
In most published studies, subjects inhaled and exhaled through the mouth during collection. In some studies, subjects were asked to inhale through the nose and exhale exclusively through the mouth. There are some important differences between the two methods which may influence mediator levels found in EBC: 1) during nasal inhalation inhaled air is humidified in the upper airways; 2) mediators formed in the nose and the sinuses more likely enter the lower airways during nasal inhalation. In a study comparing the two methods (nasal inhalation-oral exhalation versus oral inhalation-oral exhalation) more EBC was collected with nasal inhalation in parallel with a larger volume of exhaled air, but no differences were found in the concentrations of adenosine, TxB2 and ammonia in healthy subjects [11]. However, in patients with inflamed upper airways higher concentrations of adenosine were found in EBC samples collected with nasal inhalation-oral exhalation than those collected with oral inhalation-oral exhalation, suggesting that adenosine produced in the inflamed upper airways is added to EBC samples collected with nasal inhalation-oral exhalation.

EBC collection with oral inhalation-oral exhalation may be performed with or without the use of a noseclip. Noseclip use prevents any accidental inhalation-exhalation through the nose during sample collection, while this cannot be safely ruled out when collecting samples without it. When tidal breathing is used for sample collection without resistance, the soft palate is not closed and air and mediators present in the nose and sinuses can be added to the sample. The use of noseclip may well minimise the aerosolisation of particles from the nasopharynx. On the other hand, volatile gases formed in the nasopharynx (such as NO) may be entrained to a greater extent in the exhaled air when noseclips are employed (as is indeed the case for NO) [2]. At the moment no data are available comparing EBC collected with and without noseclips.

Recommendation
The consensus of the expert panel was that noseclip use is advisable to ensure that no sample is lost through the nose and that inspiration bypasses the nose. The most important recommendation is that investigators should consider the potential effect of nasal airflow on EBC levels of the compound in which they are interested, and perform appropriate control experiments when feasible. In manuscripts, precise description of sampling method (route of inhalation and exhalation, use of noseclip) is required and any ongoing upper airway disease should be mentioned.

Areas for further research
Comparison of data reproducibility between EBC samples obtained with and without noseclips (oral inhalation and exhalation) is needed for mediators detectable in EBC before making explicit recommendation on this issue.

Use of resistor
For single breath exhaled NO measurement, a resistance is used to close the soft palatine to prevent NO produced in the nose and the sinuses from influencing the results [2]. Closing the velum consistently during several minutes of tidal breathing for EBC collection is a very different issue. Theoretically, it would be possible to place a resistor between the subject and the condenser during EBC collection, however, its value would be limited by the following: 1) the more effort dependent sampling technique (forced expiration instead of tidal breathing) would exclude a large number of patients who could produce EBC with the tidal breathing method, and 2) expiratory flow restriction would not be expected to prevent nasal contribution to the airstream during inspiration, during which the velum would not be kept closed regardless of expiratory resistance. It is worthy of note that all condensers have some resistance, which is negligible with the commercially available ones but should be checked in the custom-made devices. One group used a resistor when collecting EBC and found better reproducibility in EBC nitrite concentration when using it than without it [18].

Recommendation
At the moment it is not recommended to include resistance in the condensing device for EBC collection.

Areas for further research
Further studies to determine the utility of expiratory flow resistance on the concentrations of EBC mediators will be important. Design of condensers with adjustable flow restriction will be helpful in this endeavour.

Flow-dependency of EBC mediator levels
Few attempts have been made to determine the site of generation of different mediators in the EBC. In this respect, H2O2 level was shown to be flow-dependent, suggesting that it is at least partly derived from the conducting airways [19]. In the range of expiratory flows during tidal breathing no flow-dependence was found in adenosine concentration [20]. EBC pH was not affected by profound changes in flow rates [12]. Similarly the concentrations of malondialdehyde (MDA) was not flow dependent in the flow range between 50 and 200 mL s−1 measured by an ultrasonic flow sensor [21].

Recommendation
When it has not been sufficiently elucidated in the published literature, the flow dependence of mediator levels should be ascertained and incorporated into publications. This will assist in: 1) determination of the site of mediator liberation; and 2) establishing that, in the flow range found during EBC sampling, variability of mediator concentration is not caused by variation in expiratory flow.

Areas for further research
Further research is required to determine the flow-dependency and the anatomical origin of different mediators derived from the airways and/or alveoli.
FACTORS WHICH AFFECT COLLECTING EBC

Condensing equipments

Commercial versus custom-made devices

Several different devices have been designed for EBC collection including tubing of different materials (Teflon, polypropylene tubing) of differing length and diameter [5, 14, 19], or double-wall glass chambers [22] (fig. 1). These collecting vessels are cooled with wet ice [5], air [24] metal [25] or dry ice to the required temperature. Comparing a custom-made device (Teflon coated laminated metal tube cooled to -20˚C) one study showed that the use of the two devices are interchangeable when measuring MDA, hexanal, heptanal or nonanal [21]. Use of commercially available devices may help to overcome potential problems arising from the use of different devices, but no data are yet available showing that the use of these devices results in better reproducibility of data.

Commercial systems and most published custom EBC collection systems employ one-way inspiratory valves to assure that the patients do not inhale cold air that has passed through the condenser during inspiration. This is an obvious important consideration to avoid unintentional cold air challenge.

**FIGURE 1.** Schematic diagrams of exhaled breath condensate collecting devices. a) Glass chamber containing icy water in the inner glass (reproduced with permission from [22]). b) Tubing immersed in icy water (reproduced with permission from [23]).

**Recommendation**

Describe exactly what type of condensing device is used. If a home-made device is built, give details on the design (saliva trap, its resistance, material of the condensing surface, cooling method, temperature of condenser, its stability over the collecting period). When conducting a study, use the same device, or if different devices are to be used give results on the comparison of the equipments used. Current consensus is that equipment should have an inert material on the condensing surface, although different biomarkers may have different reactivities with surface materials. The collection equipment should be connected to the subject in a way that prevents gross salivary contamination, with one-way valves to prevent inhalation through the condenser and also to avoid condensation of ambient air before and/or during EBC.

**Areas for further research**

Comparison of efficacy and reproducibility of sampling (condensate volume, mediator level) between different devices should be a focus of further studies. Further studies are also required to establish if different mediators are evenly distributed in the expired air and if collection of all water vapour expired would decrease variability of EBC data.

**Efficiency, duration and temperature of condensation**

Generally, with 10 min of tidal breathing 1–3 mL of EBC can be collected from resting adult subjects (~100 µL-min⁻¹ EBC with range of 40–300 µL-min⁻¹). None of the available condensers collect all exhaled water vapour, since estimated water loss is around 30–35 mg-L⁻¹ in a wide range of ambient air temperature and humidity [26, 27]. One study estimated the water extraction by a cold trap integrated into the cooled head sampling unit and found that ~40% of water vapour was extracted with no difference in the efficiency of water vapour extraction in healthy subjects versus chronic obstructive pulmonary disease (COPD) patients [28]. Similar EBC volume was found by others using a 200 cm tubing connected to the expiratory port of a ventilator kept under saline-ice water [14].

Collection time used in most published studies is between 10–30 min with only a few studies using very short (3 min) or rather prolonged (60 min) collection time [7, 12]. In most studies 10 min has been used and this time period can be recommended for two practical reasons: 1) this collection time results in 1–2 mL of condensate from adult subjects and most children >4 yrs of age; and 2) subjects usually tolerate this period of sampling without fatigue (although loss of interest may still occur in children). At the same time, however, different study set-ups may require other collection times (i.e. shorter when time course of changes is assessed or longer when a larger amount of sample is needed). Direct comparison of different collection times has only been published regarding pH level, showing no effect of changes in collection time between 3–20 min on EBC pH in healthy subjects [12]. Although no direct comparison is available regarding other mediators, no difference can be found in the concentrations of H₂O₂, nitrite/nitrate, 8-isoprostan, adenosine and MDA between studies using 10, 15 or 20 min for EBC sampling.

**GESSNER et al.** [28] showed not only that EBC volume was linearly related to the volume expired, but also to the total protein and urea content found in EBC suggesting that these
compounds accumulated in the collecting device by a similar mechanism as expired water vapour. This even distribution, however, is not necessarily true for all substances present in EBC and needs further study. For standardisation of EBC sampling some investigators have suggested collection for a time over which a pre-defined volume of air (100 L in adults) is expired, instead of setting fixed collection time. This approach is based on the observation that EBC volume is directly proportional to the volume of exhaled air, therefore, keeping the same volume of exhalate would minimise the otherwise large inter-individual variability of EBC volume. No published data are available, however, on studies comparing these two approaches with respect to reproducibility of mediator concentrations.

Condensation can be achieved at temperature around 0°C using wet (salty) ice, when EBC can be collected as fluid, and it can also be collected at lower temperatures using different techniques (dry ice, liquid nitrogen, placing cooling sleeve to required temperature, cooling air to preset temperature, etc.), which results in the collection of frozen material. Regarding temperature of condensation, it should be noted that the collecting surface warms up from the exhaled air and this influences the collecting temperature. The solubility of volatile mediators in the collected samples may be influenced by temperature, and data reveal that concentration of ammonia is lower if the condensate is collected as ice rather than water. Condensing temperature and time is also important for those mediators that are unstable, such as leukotrienes and purines.

Recommendation
Investigators should report collection temperature and duration of condensation (and if time or expired volume was kept constant). Keeping the same sampling temperature and time in studies that require repeated sampling is advisable. Regarding the collection time, 10 min is recommended for most mediators, as it provides an adequate sample for assay of most mediators of interest and is well tolerated by patients.

Areas for further research
Further studies need to address the effect of collecting time and temperature on different mediator levels and to compare the reproducibility of EBC mediator measurements between sampling with set collection time versus expiratory volume. Studies on particle distribution and development of more efficient devices are also needed.

Ambient air
Ambient air contains molecules which may influence EBC composition through several possible mechanisms. Atmospheric compounds can: 1) directly contribute to EBC levels; 2) react and, therefore, change or consume molecules trapped in EBC; and 3) lead to inflammatory and biochemical changes in the airway that are subsequently reflected by changes in EBC composition. It has been demonstrated that atmospheric NO reduces exhaled H2O2 levels [29]. Ambient air that has not interacted with the respiratory system (not been inhaled) can be excluded by a device design with unidirectional flow through the condenser with the intention of minimising direct contact with ambient air. EBC samples can also interact with ambient air if samples are left exposed to room air after collection. This may result in important changes in mediator concentrations if an unstable mediator or a volatile compound is of interest or when the measured molecule or a reactive precursor molecule is present in ambient air. Finally, velocity, temperature and humidity of inhaled air may all influence the volume and content of EBC, but data are lacking on this issue.

Recommendation
Control experiments in which subjects inhale air that does not contain the compounds (or their precursors) that will be measured in EBC should be considered by each investigator. Furthermore, unless proven unequivocally to be acceptable for a given mediator, EBC samples should not be left out at room temperature after collection (the latter is important not only because of the interaction with ambient air, but also because substances may be degraded or formed in EBC more readily at this temperature).

Areas for further research
Studies with inhalation of known gas mixtures are needed to establish the effect of changes in ambient air on EBC characteristics.

Breathing pattern
Exhalation flow has an important influence on exhaled NO and it is, therefore, necessary to consider this possibility in relation to any volatile components of EBC. Exhalation flow influences the level of exhaled H2O2; at higher flows exhaled H2O2 concentration is lower, but with the low flows during tidal breathing the effect is minor [19]. A strong correlation was found between total respired volume and breath condensate volume by different authors [11, 28]. An animal study showed clear positive correlations not only between minute volume and EBC volume, but also to variables determining the breathing pattern (i.e. tidal volume per kg body weight and respiratory frequency) [30].

However, the effect of changes in minute ventilation on EBC volume and mediator concentration intra-individually in humans has not been fully published. It is known from previous studies that no consistent differences were detected in expired water content values as ventilation increased [26, 31].

No studies have been reported on the effect of breath-holding on mediator levels in EBC. No published data on the possible effect of cough (either spontaneous or induced) on EBC volume and content are available at the current time. Since this event likely influences both variables, and may even facilitate the release of “droplets”, controlled studies are required.

EBC collection appears to have no influence on lung function or exhaled NO levels [32].

Recommendation
Use tidal breathing for EBC sampling and report on the flow-dependency of the measured mediator where known.

Areas for further research
Further studies are needed to assess the effect of changing minute ventilation and coughing on the volume and content of EBC.
**Airway calibre and lung function**

EBC volume does not depend on lung function parameters including forced expiratory volume in one second and forced vital capacity (FVC) either in normal subjects or in patients with COPD [28]. At present there is no evidence to show that changes in airway calibre cause any difference in mediator release or dilution of EBC, but this question has not been studied systematically. There are data that reveal no change in EBC pH after acute airway obstruction induced by methacholine [12].

**Recommendation**

None.

**Areas for further research**

Further studies are required to assess the effect of changes in airway calibre on different mediators in EBC.

**Age and sex**

The largest database of variables affecting the concentration of an EBC biomarker is available for H$_2$O$_2$. This mediator is not age-dependent in children, but higher levels were found in older than younger adults [13, 33, 34]. Body weight or height age-dependent in children, but higher levels were found in EBC pH after acute airway obstruction induced by methacholine [12].

**Recommendation**

None.

**Areas for further research**

Further studies are required to assess the effect of changes in airway calibre on different mediators in EBC.

**Food and drink**

To the best current knowledge of the Task Force food and drink do not influence the determined nonvolatile mediators. This question, however, has not been studied systematically for all compounds of interest. When measuring mediators known to be affected by certain drinks or foods it is advisable to refrain from these for a few hours before measurement (for example to refrain from caffeinated drinks before measuring adenosine).

**Recommendation**

As study protocols are developed, evaluate the potential effect of eating, drinking, taking medication on the EBC concentration of the biomarkers of interest.

**Areas for further research**

Further studies are required to assess the effect of food and drink on different mediators in EBC.

**Circadian rhythm**

A circadian rhythm has been demonstrated for EBC H$_2$O$_2$ level both in normal subjects and patients with COPD [13, 34]. No circadian rhythm was identified for pH in a study of 152 subjects [12]. No prospective studies are available to show or rule out any diurnal variation of EBC volume or other mediators present in EBC.

**Recommendation**

In longitudinal studies consider the potential of circadian rhythm in mediator level and plan sampling to the same time of the day if diurnal variation is shown.

**Areas for further research**

Further studies are required to assess circadian rhythm of different mediators in EBC.

**Tobacco smoking**

Information is available on cigarette smoking, but not on cigar or pipe smoking. The effects on EBC biomarkers of different cigarette brands, including presence or absence of a filter and origin, have not been elucidated. Smoking (both chronic and acute smoke exposure) has considerable effect on H$_2$O$_2$, isoprostane, nitrite and nitrotyrosine levels measured in EBC [35, 39–42]. In healthy subjects cigarette smoking causes an increase in EBC H$_2$O$_2$, 8-isoprostane and nitrotyrosine concentration and in neutrophil chemotactic activity with no change in concentrations of interleukin (IL)-1β or tumour necrosis factor-α [35, 39–42]. In asthmatic patients acute smoke exposure also caused an elevation in EBC H$_2$O$_2$ level [43]. In patients with COPD no difference was observed in the mean EBC H$_2$O$_2$ and 8-isoprostane level between smokers and nonsmokers [44–46].

**Recommendation**

Smoking habit should be documented and in smokers it is advisable to refrain from smoking for at least 3 h before measurements to prevent the acute influence of smoke on mediator levels. An acute smoking group can also be considered as a control. In long-term studies changes in smoking habit should be noted (if possible avoided).

**Areas for further research**

Studies on the effect of smoking on other mediators/characteristics of EBC are needed.

**Systemic diseases**

It is important to consider the potential effect of systemic diseases including extrapulmonary systemic diseases on mediator levels in EBC. In this respect approximately 20-fold higher H$_2$O$_2$ level was described in EBC from uraemic patients compared to healthy subjects [47].

**Recommendation**

Consider systemic diseases as potential confounding factor when EBC variables are used as biomarkers of respiratory diseases.

**Area for further research**

Studies are required to determine the effect of systemic diseases on EBC variables. The potential use of EBC as a biomarker of extrapulmonary diseases should also be studied.
Medication
Several studies showed differences in EBC mediator levels in relation to medication (see details in paragraphs discussing mediator measurements). Although most studies on mediators in EBC are cross-sectional, significant differences have been demonstrated between treated and untreated groups in different airway diseases.

Recommendation
All used medication should be carefully listed and their effect on mediator level should be considered.

Areas for further research
Determination of the utility of EBC for evaluating effects of therapeutic compounds will benefit from longitudinal studies assessing the effect of medications on mediator levels.

Ventilated patients
Condensers can be connected to the outgoing limb of most respirators and samples can be collected \[5, 6, 11, 12, 14, 36, 48\], but the humidification system may contribute solutes or substantially dilute the EBC, and these factors will likely change depending on the total flow through the circuit. This approach provides an opportunity to sample the lower airways directly and to investigate patients with respiratory failure.

Recommendation
None.

Areas for further research
None highlighted.

Safety
Collection of EBC is a safe method of gaining information regarding respiratory fluids; no adverse events have been reported in over 10,000 measurements performed in different laboratories with different devices. EBC collection does not have any influence on lung function or mediator levels, and can be repeated several times with short intervals (minutes) between measurements. Since the pattern of breathing is normal, it is safer than FVC measurements, which may provoke bronchospasm in some asthmatic patients. It is noted that some people tend to hyperventilate especially at the beginning of EBC collection, but this has not led to any adverse event. Care must be taken that the risk of infection between individuals is avoided. Although no bacterial DNA fragments were detectable in EBC samples from tidally breathing cystic fibrosis (CF) patients \[49\], the possibility of other components of the collecting system transmitting microbes to subsequent users seems real. This risk of infection can be minimised by using disposable mouthpieces and tubing between the mouth and condenser and a one-way valve to avoid inhaling from the condenser. The use of disposable condensers is an alternative approach. In most pulmonary function equipment, an exhalation particle filter is employed to eliminate dilution of samples from the machine. The use of such a filter for EBC collection presents potential problems, however, the effect of an expiratory particle filter proximal to the condenser on the concentration of a compound in EBC will depend on the characteristics of the compound. Substances that are primarily derived as volatiles (by nature, uncharged) may be affected differently by a filter than charged nonvolatile macromolecules, which may be trapped more by the filter. The use of such filters inserted before the condenser is, in general, not recommended, unless appropriate studies are performed to evaluate the effects of filtering the exhaled breath on the levels of the specific mediator studied. Disinfection of reusable condensers must be carried out with special caution, since some residual disinfection agents (i.e. those work with formaldehyde) may destroy mediators collected in the disinfected collection tubes, and residual contamination from the cleaning process may affect subsequent samples.

Recommendation
Do not use filters between the subject and the condenser. Take special care to disinfect reusable collecting devices, and assure that residual cleaning agents do not adversely effect the assays for, or levels of, the biomarkers of interest.

Areas for further research
Further studies are needed to assess if infective agents including viruses can be demonstrated in EBC.

Reproducibility, dilution factor, concentration of samples
With tidal breathing methods the volume of EBC is a reproducible characteristic of EBC, which is not surprising since exhaled air is nearly saturated with water at body temperature \[5, 11\]. Mediator levels in EBC are more variable than EBC volume even if reference techniques such as HPLC are used for determination, but with sensitive assays good reproducibility has been found for adenosine, aldehydes, glutathione and pH in EBC \[11, 12, 20, 50\]. The cause of variability is not completely understood, but two components are likely to be involved: changing dilution (for nonvolatiles) and large same sample variability of some of the assay techniques used. The latter in most cases is due to the fact that most biomarkers in EBC are at the low end of assay sensitivity, making variability more likely.

Dilution factor
Some attempts have been made to assess the dilution of alveolar lining fluid (ALF) in EBC samples and to standardise EBC by using exhaled volume \[5\], exhaled ions \[6, 51, 52\], urea \[6, 53\], protein concentration \[28\] or conductance of lyophilised EBC by using exhaled volume \[5\], exhaled ions \[6, 51, 52\], urea \[6, 53\], protein concentration \[28\] or conductance of lyophilised samples \[51\] as “internal standards”, or by using external dilution markers. The importance of a dilution factor is based on the assumption that the ratio of liberated solutes to exhaled water vapour is unpredictable and can change, but a so called “dilution factor” can be determined from each EBC sample by determining the concentration of a substance in EBC, which has a well known concentration in sera and diffuses through the cell membranes, but is not produced in the alveoli or airways. Such a dilution marker would make it possible to calculate the “real airway level” of determined mediators in EBC. It has been suggested that concentrations of electrolytes or urea or the measurement of conductance can be used for estimating dilution of EBC \[6, 51\]. The simplest of these is the measurement of conductivity of lyophilised samples \[51\]. One study found only a small inter-day intra-subject variability in the concentration of sodium and chloride concentrations in EBC samples from healthy subjects and patients with CF, suggesting that variable dilution is not likely a major cause of
variable biomarker levels [52]. In contrast, when measuring urea as a dilution factor for nonvolatile compounds in EBC, considerable within-subject variability was found with a dilution factor ranging between 8,300 and 48,400 [53].

EBC collection offers an advantage over collecting bronchoalveolar lavage (BAL) because it is completely noninvasive, no medication is required to employ the technique and no external fluid is added to the airways [54]. ALF is diluted to a great extent in EBC [6] and EBC compounds are likely to represent not only ALF, but also the mucus layer of the airways [53]. It has not yet been convincingly demonstrated that better reproducibility can be achieved by normalising EBC data with a dilution factor. Although dilution may be a factor influencing EBC data, it is unlikely that changes in mediator levels observed in different airway diseases can be completely explained by changes in droplet release or formation. Furthermore, it is important to note that this approach cannot be used for volatile compounds in EBC, for which other aspects need to be considered [53].

Detection limit of the assay techniques, sample concentration

Most of the mediators found in EBC are in the lower range of detection of the assay techniques currently available, where the intra- and inter-assay variability of methods is large (see Measurements of mediators in EBC). Exceptions, in which levels are fully in the range of available assays, include total protein, nitrate, pH and ammonia. Reporting inter-assay and intra-assay variation given by the manufacturer may be misleading when values are around the lower detection limit and, therefore, they should be determined for the concentration range found in EBC samples to enable any power calculation for studies using the measurement of given mediator in EBC. In this respect more sensitive methods for detection and very careful handling of samples are required. Another source of concern is that standard curves for immunoassays should be generated using standards that are similar to the EBC matrix, or at least that the assay be validated to assure that nonspecific results/false positives are avoided.

A potential option to overcome this problem is the concentration of samples. Lyophilisation and vacuum-evaporation has been used by some investigators. These approaches were used in some studies (i.e. 3-, 4-fold concentration for measurement of nitrotyrosine and leukotriene B4 (LTB4); see below, or lyophilisation for conductivity measurement; [51]), however these methodologies were not systemically evaluated (i.e. data on mediator recovery have not been published). Volatile, semi-volatile, and unstable substances (i.e. ammonia, H2O2, leukotrienes) will or may be lost during freeze-drying.

**Recommendation**

Give details of intra-assay and inter-assay reproducibility measurements carried out using EBC samples by using appropriate means of evaluation (i.e. Bland-Altman test when appropriate, or coefficient of variation) [55]. Specify the lower limit of detection and the definition used for it (this may vary even between commercial kits). If there are values under the detection limit consider them as such and use appropriate test for statistical analysis. Whenever possible, use methods which give data well above the detection limit. In case of sample concentration give adequate data on recovery. Rigorously determine specificity of the assay.

**Areas for further research**

Adequate studies on reproducibility measurement and reference data are required. Development of more sensitive and specific assays for most mediators found in EBC and data on results of sample concentration are also needed.

**Salivary contamination**

Studies on the protein content of EBC collected through the mouth show that EBC contains molecules not present in saliva [56] and the electrolyte ratios of saliva differ from those in the orally collected condensate [6]. This information suggests that saliva is not the dominant contributor to EBC.

However, saliva contains many of the mediators that are also present in the lower airways, and indeed it is reasonable to consider that there is some exchange of compounds between the saliva and the lower airways. This is particularly relevant for the compounds found at higher concentrations, including volatile substances. Since we are interested in lower airway sampling, salivary contamination should be rigorously excluded when collecting exhaled breath. Various techniques can be used to avoid gross salivary contamination, such as the use of a saliva trap, placing the condenser at a higher level than the mouth making it more unlikely that saliva can enter the collecting device and separating the mouth-piece from the condenser by a length of tubing. Detecting salivary amylase is a frequently used method to exclude saliva contamination in EBC. Several studies have demonstrated that if care is taken to exclude saliva from EBC samples amylase can be detected only in a small portion of samples with levels approximately 10,000 times lower that those in saliva [6, 20]. However, amylase measurements are not specific for salivary amylase and amylase can also be found in the lungs, so positive results of the test do not necessarily mean salivary contamination. Furthermore, the dilution of airway lining fluid is great in EBC, and care must be taken in interpreting amylase data. A negative signal does not completely exclude minute contribution from the mouth. Furthermore, the majority of proteins recovered from EBC collected from isolated lower airway were also found in saliva, suggesting that these proteins are present in both compartments (i.e. saliva and secretions of the lower airways) [56]. Both the anatomical and biochemical sources are still an issue with exhaled NO [57], and these issues should be considered no less important for EBC. It is, therefore, reasonable to assume that there is some degree of oral contamination of EBC because of particles being formed there as well. To complicate matters, lower airway-derived compounds can be trapped in the mouth. To allow the assessment of potential sample “contamination” by the mouth, it will be useful to seek changes in salivary concentration of the compounds that are being assayed in EBC. Whenever possible, studies should also be conducted in patients with endotracheal tubes or tracheotomies.

**Recommendation**

According to current experience there is no need to determine and report salivary amylase level in EBC samples, but efforts should be made to prevent salivary contamination.
Area for future research
More sensitive assays to exclude oropharyngeal contamination are of interest.

Summary of current recommendations, requirements for EBC collection
Based on the consensus of the expert panel and on the published data detailed above, the following recommendations should be observed for EBC collection (table 1): oral sample collection should be performed with subjects in sitting position wearing a noseclip while tidal breathing. Collection time and temperature can vary depending on the study objective, but it should be kept the same within any one study and precisely reported. It is required to have a condensing device with inert material on the collecting surface, containing a sufficient salivary trap, having a mouth piece with separated inlet (as an inhalation port) and outlet (to direct exhaled breath toward the condensing apparatus), with low resistance without a filter between the subject and the condensing chamber.

When reporting data on EBC detailed methodology for sample collection should be provided. This includes the description of the sampling device, the collecting surface material, the volume of the dead space (if possible), the duration and temperature of collection, breathing pattern, use of noseclip and route of inhalation, method and duration of sample storage. Additionally, intra-assay and inter-assay variability of the assay technique, and intra-subject variability of the mediator should be reported. Lastly, details on participants should contain information on upper airway disease, smoking habit, and medication.

MEASUREMENTS OF MEDIATORS IN EBC
Storage of EBC samples
Because on-line measurements are not available for most mediators present in EBC with the exception of a few assays including those for pH and H₂O₂, EBC samples are usually stored before mediator measurements.

EBC samples should be immediately frozen after collection and stored at -70°C until mediator determination is performed. It is advisable to test the stability of mediators at the storage temperature if not published previously. Assays clearly should show quite good agreement of data [62, 64, 65] or a rather high coefficient of variation within repeated measurements [19, 34, 63]. The mean coefficient of variation over 21 days was 45% showing quite good agreement of data [62, 65]. The lower limit of detection with the fluorimetric method is 0.1 µmol·L⁻¹, but in one study a lower detection limit (0.005 µmol·L⁻¹) was reported by using automated flow injection to detect the fluorescence of the reaction product [62]. Most groups have reported 0.1 µm·L⁻¹ as the detection limit for the colorimetric assay [45], but one recent study used 0.11 µmol·L⁻¹ for detection limit [63]. Values presented both for healthy individuals and patients with different respiratory disorders vary widely, most of them are close to the lower limit of detection and often include zero (see more details below).

Data on reproducibility have not been given in many publications and the reported values vary between studies showing quite good agreement of data [62, 64, 65] or a rather high coefficient of variation within repeated measurements [19, 34, 63]. The mean coefficient of variation over 21 days was 45% in COPD patients and 43% in healthy subjects [34]. Due to the variability of data, concern has been raised regarding the reproducibility of the colorimetric assay in the range of values found in EBC and the usefulness of this measurement for monitoring oxidative stress in the airways [15, 36, 63, 66]. Therefore, the use of more sensitive methods is advisable for peroxide measurement and further studies are required to establish the usefulness of other methods (i.e. chemiluminescence or other) for H₂O₂ measurements in EBC [59, 67–70].

Rationale for measurement of mediators
EBC sampling is performed with three major aims: 1) to learn more about the pathological mechanisms of airway diseases by detecting changes in mediator levels; 2) to learn more about the composition of the airway lining fluid; and 3) to use the determined mediators as exhaled biomarkers of airway diseases.

H₂O₂
Source
H₂O₂ is produced after converting superoxide anions O₂⁻ to H₂O₂ by superoxide dismutase in several cell types [15, 61]. In the respiratory system H₂O₂ may be released both from inflammatory and structural cells including neutrophils, eosinophils, macrophages and epithelial cells. H₂O₂ is a volatile molecule, which has been demonstrated in EBC by several investigators [4].

Protocols, recommendations and potential pitfalls
H₂O₂ in EBC is unstable, therefore, EBC should be rapidly frozen after collection and kept at ≤-70°C until the determination of its peroxide concentration. Different data have been published on the stability of H₂O₂ in frozen EBC samples varying between from 2-3 days to 2 months [33, 46]. One option to circumvent the problem of instability is to add assay reagents immediately after collection of EBC and store the stable reaction product frozen until the measurement [58]. Saliva contains 10–100-fold higher H₂O₂ concentration than EBC, therefore, exclusion of saliva is of great importance. Nasal and oral EBC collection have been compared and showed that the two methods are not interchangeable when measuring this mediator [38]. The most frequently used methods of measuring H₂O₂ in EBC are the colorimetric or fluorimetric measurements, which are based on the ability of H₂O₂ to react with suitable substrates [4, 15]. The lower limit of detection with the fluorimetric method is 0.1 µmol·L⁻¹, but in one study a lower detection limit (0.005 µmol·L⁻¹) was reported by using automated flow injection to detect the fluorescence of the reaction product [62]. Most groups have reported 0.1 µmol·L⁻¹ as the detection limit for the colorimetric assay [45], but one recent study used 0.31 µmol·L⁻¹ for detection limit [63]. Values presented both for healthy individuals and patients with different respiratory disorders vary widely, most of them are close to the lower limit of detection and often include zero (see more details below).

Data on reproducibility have not been given in many publications and the reported values vary between studies showing quite good agreement of data [62, 64, 65] or a rather high coefficient of variation within repeated measurements [19, 34, 63]. The mean coefficient of variation over 21 days was 45% in COPD patients and 43% in healthy subjects [34]. Due to the variability of data, concern has been raised regarding the reproducibility of the colorimetric assay in the range of values found in EBC and the usefulness of this measurement for monitoring oxidative stress in the airways [15, 36, 63, 66]. Therefore, the use of more sensitive methods is advisable for peroxide measurement and further studies are required to establish the usefulness of other methods (i.e. chemiluminescence or other) for H₂O₂ measurements in EBC [59, 67–70].

Diseases states that influence H₂O₂
In healthy, young, nonsmoking individuals H₂O₂ levels diverge from 0 to 0.9 µmol·L⁻¹. Higher H₂O₂ concentrations...
were found in healthy elderly persons, smokers and ex-smokers [13]. A correlation between age and H$_2$O$_2$ concentration has also been reported. Furthermore, a circadian rhythm of exhaled H$_2$O$_2$ was shown in healthy subjects with highest levels at 12:00 and 24:00 h and also in patients with COPD [13]. Increase in EBC H$_2$O$_2$ level was found in asthma [22, 58, 60, 71–73], healthy smokers [35, 39], COPD [34, 44, 45, 59, 65, 74, 75], bronchiectasis [76, 77], CF [78, 79] and acute respiratory distress syndrome (ARDS) [5, 80, 81], acute hypoxaemic respiratory failure [14], reperfusion injury [82], allergic rhinitis [83], common cold [84], post-operative period after lung resection [85], systemic sclerosis [86] and in experimental models of hypoxia/reoxygenation [87, 88].

In asthma, levels were related to the eosinophil differential counts in induced sputum and also to airway responsiveness [22]. In patients with exacerbations higher peroxide levels were reported than in patients with stable disease and treatment with corticosteroids lowered its concentration, but no change was reported after treatment with montelukast [58, 72, 73].

Expired H$_2$O$_2$ level was shown to be elevated both in healthy smokers [39] and also in patients with COPD compared with values from healthy nonsmokers with no significant difference between their values [44–46]. In COPD patients with exacerbations, higher levels of expired H$_2$O$_2$ were found than in patients in stable condition [45]. Inhaled steroids had no effect on H$_2$O$_2$ level in this disease in some studies [45, 74], but caused a decrease in another investigation [89]. Long-term antioxidant treatment with N-acetylcysteine decreased exhaled H$_2$O$_2$ concentration in patients with COPD [75]. Experimental data using a horse model of COPD showed a positive correlation between H$_2$O$_2$ in EBC and the numbers of neutrophils in BAL fluid indicating the significant relationship between H$_2$O$_2$ in EBC and the degree of inflammation [90].

EBC H$_2$O$_2$ concentrations were elevated in bronchiectasis with a significant inverse correlation between lung function and exhaled H$_2$O$_2$ level [76, 77]. In CF an increase in exhaled H$_2$O$_2$ concentration has also been described, although not consistently [64, 78, 79]. Treatment with antibiotics caused a decrease in H$_2$O$_2$ level in CF [79].

Level of validation
Independent groups confirmed the presence of H$_2$O$_2$ in EBC using different assay techniques, and have reported day to day intra-subject coefficient of variation is 43% in healthy subjects [33].

**Nitrogen oxides and related products**

**Nitrite/nitrate**

**Source**
Nitrite (NO$_2$) and nitrate (NO$_3$) are nitrogen redox forms that are present in the epithelial lining fluid of the human respiratory tract. The term nitrogen oxides (NOx) incorporates these two ions, as well as other oxides of nitrogen.

**Protocols, recommendations and potential pitfalls**
NO$_2$ and NO$_3$ have been detected in EBC by using spectrophotometric assays (Griess reaction), a fluorimetric method (2,3-diaminonaphthalene (DAN) reaction), chemiluminescence assays or ion chromatography followed by conductivity measurement [91–104]. The reported detection limit of the DAN assay is 0.1 μM and that of the Griess reaction is higher [91, 92]. NO$_2$ tends to be found in condensate in the 1 μM range, which is, therefore, close to the detection limit for these assays. Data on day-to-day reproducibility has not been published. The chemiluminescence technique is sensitive in the nM range and NO$_2$ / NO$_3$ have been detected by this method in BAL [93, 94]. Caution must be taken when interpreting NO$_2$ assays, as the compound cannot be considered stable at low pH, and even at neutral pH, NO$_3$ is considered stable. NO$_3$ levels tend to be roughly 5–10 fold higher than NO$_2$ levels, although this may vary in part because of changes in airway oxidative conditions. Since most combined NO$_2$ / NO$_3$ assays rely on the formation of NO$_2$ from NO$_3$, variability of data may arise partially from differences in the efficacy of the reduction method chosen. In this respect, enzymatic reduction was found to be better for NO$_3$ determination in EBC than cadmium reduction [95].

NOx are present on every laboratory surface, including glassware and pipette tips. Therefore, investigators should take great precautions to avoid contamination of the sample. Experience suggests that it is necessary to thoroughly rinse with highly pure (distilled/de-ionised) water any material that might come in contact with EBC, including devices used for collection, processing and assaying EBC. It is best to perform this rinsing very soon before use, because readily diffusible ambient NO becomes oxidised and contaminates surfaces rapidly.

**Diseases states that influence nitrite/nitrate concentration in EBC**
Concentrations of NO$_2$ and/or NO$_2$+NO$_3$ were found to be significantly higher in asthma, CF and bronchiectasis compared with healthy controls [18, 96–99, 101, 102], but in mild asthma and CF some studies showed no increase [91, 103, 105]. In smokers both increases and normal concentrations of EBC NO$_2$ have been described [40, 42, 103]. In patients with exacerbations of asthma, higher NO$_3$ levels were observed compared with stable patients and treatment with inhaled steroids resulted in a significant decrease in its concentration [96, 98]. In children with asthma and CF higher EBC NO$_2$ levels were found than in children with nonasthmatic, episodic cough [98]. In CF, NO$_2$ level in EBC was elevated or normal in contrast to decreased level of exhaled NO [18, 91, 92]. Similarly, in primary ciliary dyskinesia (PCD), a genetic disorder associated with low levels of exhaled NO, normal NO$_2$ levels were described in EBC [106]. The dissociation between exhaled NO levels and EBC NO$_2$ concentrations observed in CF, PCD and smoking healthy subjects emphasise further the need for more research on biochemical changes in the airway walls. Conflicting reports of exhaled NO$_3$ concentrations have been published showing unchanged or increased NO$_3$ levels in different airway conditions [101, 102]. EBC NO$_3$ level was demonstrated to be elevated in patients with acute lung injury [107].

**Level of validation**
Independent groups confirmed the presence of NO$_3$ in EBC using different assay techniques. There are no reported data on day-to-day intra-subject coefficient of variation.
Nitrotyrosine

Source
The reaction of NO and superoxide anions in the airways leads to the formation of peroxynitrite, which is a highly reactive oxidant species. Peroxynitrite reacts with tyrosine residues of proteins to form the stable product nitrotyrosine, which may be detected with specific antibodies [108].

Protocols, recommendations and potential pitfalls
EBC nitrotyrosine has been measured by specific enzyme immunoassay (EIA), which has a detection limit if 3.9 ng·mL⁻¹. Nitrotyrosine has not been detected in all EBC samples (not even when samples were concentrated three-fold). The range of nitrotyrosine concentrations in EBC of healthy subjects is 0–14 ng·mL⁻¹ [91, 108]. Day to day variability was reported on seven healthy subjects finding a coefficient of variation of 6% [91]. Using mass spectrometry (MS) technique nitrotyrosine can be measured in picomolar range [109]. Although not universally recognised, the commercial ELISA assays (which are sandwich ELISAs) presumably quantify proteins nitrated at more than one site, rather than single amino acids. Thus, when nitrotyrosine levels are reported from sandwich ELISA assays, what likely is meant is that nitrated proteins are present.

Disease states influencing nitrotyrosine concentration in EBC
Nitrotyrosine concentrations were found to be increased in patients with asthma compared with healthy controls with a relation between exhaled nitrotyrosine and NO levels in these patients [109]. In CF patients, levels of EBC nitrotyrosine were also elevated, despite the normal level of NO₂ and NO₃ and a decreased level of exhaled NO in these patients as compared with those of normal subjects [91]. An inverse correlation between the levels of nitrotyrosine and the severity of lung disease was also found in this study [91].

Level of validation
Only one research group demonstrated the presence of nitrosothiols in EBC using the same assay technique in two separate studies, there are no reported data on day-to-day intra-subject coefficient of variation.

Adenosine

Source
Adenosine is formed during the degradation of adenosine triphosphate (ATP) and has a wide range of effects in the respiratory system through its specific receptors [112].

Protocols, recommendations and potential pitfalls
EBC adenosine can be determined by HPLC. The method used for plasma adenosine measurement has been validated for EBC [20, 113]. The detection limit of the method is 2 nmol·L⁻¹ (signal to noise ratio at least 3:1) The inter-assay reproducibility of EBC adenosine measurement was found to be <10% [11, 20]. The range of healthy adenosine values was found to be between 0–20 nmol·L⁻¹; EBC levels were several-fold lower than those in plasma or BAL, however, taking dilution factor of EBC into account EBC and BAL adenosine values resulted in similar airway concentrations [114, 115].

Disease states that increase EBC adenosine concentrations
EBC adenosine level was found to be elevated in patients with allergic rhinitis [11] and in asthmatic patients with substantial overlap with healthy values in both conditions [20]. In patients with worsening of asthma symptoms, EBC adenosine concentration was higher than in stable disease and adenosine level in EBC showed a positive correlation with exhaled NO concentration in asthma [20].

Level of validation
Only one research group demonstrated the presence of adenosine in EBC using the same assay technique in different studies. Mean intra-day coefficient of variation is 10% in healthy subjects [20]. There are no reported data on day-to-day intra-subject coefficient of variation.

Arachidonic acid metabolites
Prostaglandines and thromboxanes
Source
Arachidonic acid, released from the cell wall by phospholipase A₂, is converted to prostaglandin endoperoxides by cyclo-oxygenase. Endoperoxides are then converted to prostaglandins, prostacyclin and thromboxane A₂ (TXA₂) [116]. TXA₂ is rapidly converted to TXB₂, a chemically stable but biologically inactive metabolite (one of the further metabolic processes TXB₂ undergoes is the common β-oxidation resulting in the formation of 2,3-dinor TXB₂). Thus, thromboxane synthesis in biological tissues has been monitored by measuring TXB₂.

Protocols, recommendations and potential pitfalls
Prostaglandin E₂ (PGE₂) and TXB₂ can be measured by EIA and also by radioimmunoassay (RIA) [11, 117–120]. The specificity of PGE₂ measurement by RIA has been validated by reverse phase HPLC [118]. The detection limit of the EIA for TXB₂ is 13 pg·mL⁻¹ and that of the RIA is 13.6 pg·mL⁻¹. While TXB₂ was detected only in a small part of EBC samples by EIA [119],
higher TxB2 values were found by RIA (normal range between 0–200 pg·mL\(^{-1}\)) [11]. The observed difference is likely attributable to the 100% cross-reactivity of the RIA assay, but not the EIA method, with 2,3-dinor-TxB2. The difference in specificity between the two kits, therefore, needs to be taken into account when comparing data. Reproducibility data have not been published on TxB2 measurements. The detection limit for PGE2 (EIA) is 8 pg·mL\(^{-1}\), the repeatability data showed an intra-class correlation coefficient of 0.79 [120].

Diseases states that influence eicosanoid concentration

No significant differences were found in exhaled PGE2, PGD2 and TxB2 levels between healthy subjects and patients with asthma, whereas PGE2 concentrations were found to be increased in EBC in patients with COPD and in asthmatic smokers [120–122].

Level of validation

Independent research groups demonstrated the presence of PGE2, PGD2 and TxB2 in EBC using different assay techniques, there are no reported data on day-to-day intra-subject coefficient of variation.

Leukotrienes

Source

The Cys-LTs (leukotriene (LT)C\(_4\), LTD\(_4\), LT\(_D4\) are released from inflammatory cells of the airways, particularly mast cells and eosinophils, and play a role in asthmatic airway inflammation [123]. LTB\(_4\) is formed from arachidonic acid as a result of enzymatic hydrolysis of LTA\(_4\), a potent activator of neutrophils and a proinflammatory mediator [124].

Protocols, recommendations and potential pitfalls

Cys-LTs (LTC\(_4\)/D\(_4\)/E\(_4\)) and LTB\(_4\) can be measured by EIA with detection limit of 15 pg·mL\(^{-1}\) and 4.4 pg·mL\(^{-1}\), respectively [30, 125–132]. The range of cys-LT concentrations in EBC of healthy subjects varies between 0–25 pg·mL\(^{-1}\) and that of LTB\(_4\) is between 0–220 pg·mL\(^{-1}\) [73, 109, 125–132]. In one study authors could not detect cys-LTs at all in EBC [73]. The specificity of the immunoreactivity of the LTB\(_4\) EIA assay was confirmed by using reverse-phase high performance liquid chromatography [133]. The correlation coefficient for two repeated LTB\(_4\) measurements was 0.76 [133].

Diseases states which influence LT concentrations

An elevation in exhaled LTB\(_4\) level was demonstrated in calves after experimental respiratory tract infection [30]. Cys-LTs and LTB\(_4\) were found to be elevated in asthmatic patients as compared with normal subjects [109, 117, 125–128]. Furthermore, elevated concentration of cys-LT found during exacerbations in asthmatic children was decreased significantly after prednisone treatment [126]. In COPD, LTB\(_4\) was increased in the stable state and further increased during exacerbations and decreased following antibiotic treatment [129]. An increased concentration of LTB\(_4\) was also found in CF patients [130] and in patients undergoing lobectomy, but not after cardiopulmonary bypass [48].

Level of validation

Independent research groups demonstrated the presence of Cys-LTs and LTB\(_4\) in EBC using different assay techniques. Reported day to day intra-subject coefficient of variation for LTB\(_4\) is 2% in a small group of healthy subjects [125].

8-isoprostane

Source

8-isoprostane, a stable prostaglandin-like product, is formed from arachidonic acid by the nonenzymatic action of reactive oxygen species, and, therefore, it is suggested to be a marker of oxidative activities and oxidative stress [134].

Protocols, recommendations and potential pitfalls

Most studies used commercial EIA kits to measure 8-isoprostane with a detection limit of 3.9 pg·mL\(^{-1}\) to detect 8-isoprostane in EBC [17, 128, 135, 136]. The assay was validated directly by gas chromatography/MS showing high correlation between added known amounts of 8-isoprostane and the concentration measured with the EIA [118]. 8-isoprostane levels in EBC samples from healthy subjects varied between zero and 40 pg·mL\(^{-1}\) and the average concentration given in different studies show pronounced differences. Reproducibility of this assay has been determined by different groups with contradictory results [63, 65]. A more sensitive and specific method to assay EBC isoprostane is gas chromatography/MS. Using this method CARPENTER et al. [16] found 8-isoprostane only in some healthy subjects, but MOLONEY et al. [48] presented data demonstrating detectable level of this molecule in EBC from all subjects tested.

Disease states influencing 8-isoprostane concentration in EBC

8-isoprostane concentration was found to be elevated in asthma [17, 117, 121, 126, 128], COPD [41, 129], interstitial lung disease [135], CF [136], ARDS [9], pulmonary sarcoidosis [137], obstructive sleep apnoea [138], and also in healthy subjects after ozone-inhalation [139]. In asthma, the increase observed in EBC 8-isoprostane level was related to the severity of the disease and a relative resistance of 8-isoprostane to steroids has been reported in children with asthma exacerbation [17, 121, 126, 128]. 8-isoprostane was also found to be increased during COPD exacerbation and decreased after treatment [40, 129].

Level of validation

Independent research groups demonstrated the presence of 8-isoprostane in EBC using different assay techniques. There are no reported data on day to day intra-subject coefficient of variation.

Other markers of oxidative stress

Source

Aldehydes (malondialdehyde, 4-hydroxyhexanoyl, 4-hydroxy-nonenal, hexanal, heptanal and nonanal) are lipid peroxides which reflect oxidant-induced damage [21]. Conversely, reduced glutathione reflects the antioxidant capacity [140].

Protocols, recommendations and potential pitfalls

Measurement of thiobarbituric acid-reactive substances is a simple, but nonspecific method for the assessment of lipid peroxidation, therefore, it is not recommended as a marker of lipid peroxidation [44].

Aldehydes were detected in EBC using liquid chromatography-tandem MS which had a detection limit for different aldehydes
between 0.31–1.07 nM [21, 141]. Glutathione was determined by high performance liquid chromatography with fluorescence detection with a detection limit of 2.0 nM [142]. The range of aldehydes in healthy subjects was between 15–55 nM and that of reduced glutathione was 11–17 nM. The mean coefficient of variation (%) of aldehyde measurements in EBC was between 12–20% for the different aldehydes. Oxidised glutathione has been reported to be below detection limits in EBC.

Disease states that influence aldehyde and glutathione concentrations

In asthma elevated aldehyde levels and decreased glutathione levels were detected in patients with exacerbations and these values returned towards the levels found in normal subjects after appropriate treatment [142]. Furthermore, levels of aldehydes were also increased in COPD [21].

Level of validation

Only one research group demonstrated the presence of glutathione in EBC in one study. The presence of aldehydes in EBC was also demonstrated only by one group in two studies using the same assay techniques in both studies. Reported day to day intra-subject coefficient of variations for different aldehydes are between 12–20% [142]. There are no reported data on day-to-day intra-subject coefficient of variation on EBC glutathione.

pH

Source

Airway pH homeostasis is maintained by a balance of different buffer systems and the production and release of acids and bases in the airways.

Protocols, recommendations and potential pitfalls

The acidity (pH) of EBC can be readily measured with pH electrodes and indicator dyes [12, 143]. In healthy subjects, the pH of EBC immediately tested tends to be unstable. To enhance the stability of the readings, de-aeration (gas standardisation) with a CO2 free gas (such as argon, nitrogen oxygen or another CO2-free gas) can be performed. During de-aeration, the pH gradually rises to a point when stable reading can be obtained (at this point, no further CO2 can be removed by de-aeration). In healthy subjects, EBC pH after de-aeration has a mean pH of 7.7, with a range of normal considered by the investigators to be 7.4–8.8. These values are obtained from orally collected EBC samples. From intubated subjects without lung disease, the mean pH of de-areated samples is likewise 7.7 with no difference from matched oral collections [12]. In another study, the pH of EBC samples from intubated subjects undergoing cardiothoracic surgery was reported to be between 5–7 [48]. There is a debate as to whether orally collected EBC pH assays reflects acidification of the lower airways, because of high ammonia content of the mouth [144] conceivably interferes with the assay. However, this concern has not been proven. Extensive data do not reveal an effect of oral ammonia on EBC pH assays [145]. Intra-day and intra-week coefficients of variation of EBC pH measurements in healthy subjects are reported to be 3.5% and 4.5% respectively [12]. The pH of de-areated EBC is not affected by hyperventilation, duration of collection (3–7 min), duration or manner of storage (up to 2 yrs), oral versus endotracheal collection, exclusion of oral ammonia (a base), or acute airway obstruction with methacholine [12].

Disease states that influence EBC pH

Up to three log order decreases in EBC pH have been described in acute asthma [102, 143], CF [146], COPD, bronchiectasis [102] and acute lung injury [48, 147]. In asthma, pH rose back to normal after successful treatment with steroids [143]. Furthermore, pH levels were related to eosinophilic or neutrophilic inflammation of the airways [102]. At the moment no published data are available on direct comparison of EBC pH values and pH measured directly in the airways.

Level of validation

Independent research groups confirmed that pH is easily assayed in EBC and data are reproducible. Reported day to day intra-subject coefficient of variation of EBC pH is 4.5% [12].

Ammonia (NH₃/NH₄⁺)

Source

Ammonia produced during the urease hydrolysis and/or glutaminase activity in the airways may have important functions, including pH regulation [148].

Protocols, recommendations and potential pitfalls

Ammonia is a volatile compound, and collection temperature and duration influences its level in EBC. NH₄⁺ can be readily measured spectrophotometrically in EBC [148]. Most of EBC ammonia arises from the mouth [6, 11, 53].

Disease states that influence ammonia concentration in EBC

The concentration of ammonia in EBC was found to be between 14–1220 μM in healthy subjects and is lower in asthma [147]. It has been suggested that the decreased level of exhaled ammonia in asthma reflects reduced pulmonary production [147], but this has been disputed [149]. Previous observations showed that the pH of the mouth has a profound effect on the release of NH₃ into the exhaled air [150].

Level of validation

Independent research groups demonstrated the presence of ammonia in EBC by different assay techniques. Mean intra-day coefficient of variation is 60% with a broad range from 10–180%. Intra-week coefficient of variation of 64%, with a similarly broad range of variabilities [145].

Cytokines

Source

Inflammatory cells and structural cells of the pulmonary system are all able to form different cytokines [151].

Protocols, recommendations and potential pitfalls

Cytokine concentrations in EBC samples are usually quantified by EIA/ELISA kits. The manufacturer’s description provides a detection limit and also an intra- and inter-assay variability. Caution must be taken when using these methods, because the concentration of several cytokines are at around the lower limit of detection in uncentrulated EBC samples, where variability is higher than the values given in the manuals (values may fall out of the linear range of the standard curve). To circumvent...
this problem samples can be concentrated before measurement. Assurance of the validity of these assays using EBC samples is not ascertained. Several different cytokines have been described to be present in EBC [7, 42, 64, 138, 152–156], although some of them, such as IL-8 could only be detected in small proportion of subjects [64] and values often include zero.

Disease states that influence cytokine concentrations
Increased levels of IL-4 and a decreased level of interferon-γ were described in EBC of asthmatic children [152]. Steroid treatment was associated with a significant reduction in IL-4 concentration of some tested markers including pH, H₂O₂, adenosine, 8-isoprostane [11, 12, 19, 20, 28, 32, 34], enabling the method to be used both for demonstrating short-term effect of interventions (i.e. acute effect of smoking, medications) and also in longitudinal studies. Collection devices can be portable and can be used in a wide range of settings including intensive care units (mechanically ventilated patients), outpatient clinics and home.

Currently available assay techniques are not sensitive enough for many biomarkers, contributing to reported variability. Many assays are laborious and time-consuming. As yet no fully validated method for calculating dilution of respiratory droplets is available and the anatomic origin of biomarkers is not precisely known. These and the other unsolved issues regarding this technique limited the present Task Force recommendations to several aspects of the technique. Therefore, its primary aims could not be fully met. There is agreement on the recommendations that could be made based on the currently available evidence (or in some cases based on the consensus of the expert panel), clear consensus on areas of uncertainties, and delineation of needed further studies. Not surprisingly, the Task Force group was faced with many of the same problems and limitations associated with other methods of sampling the airways (sputum induction, BAL, and exhaled NO) [2, 57, 162]. The number of original publications and reviews on EBC analysis [23, 24, 163–173], and presentations at international conferences has increased sharply in recent years with editorials and correspondences in respiratory journals debating the value of this sampling method [144, 149, 174–181]. The rapid expansion of research on EBC is reflected by the fact that during the ATS/ERS evaluation and approval of this Task Force report >40 publications appeared using this method in peer-reviewed journals [182–228]. The Task Force considered it important to put together this report summarising their current understanding of the technique, and also the limitations of their knowledge, in the hopes of catalysing research that improves the methodologies and to find the proper place of this sampling method both in research and clinical practice.

**FUTURE DEVELOPMENTS**

It is clear that EBC contains many potential biomarkers. It is now important to optimise their measurement and study the clinical value of monitoring biomarkers in the breath in a variety of lung diseases and to establish the reproducibility of these measurements. This is a complex task as each biomarker needs to be considered individually because of differing solubility, stability, volatility and amount.

One of the current limitations of EBC measurements is the low concentration of many biomarkers so that their measurement is limited by the sensitivity of assays. It is likely that ever more sensitive assays will be available as more potent antibodies are
developed and new molecular detection techniques are introduced. Metabonomics is a recent technique that may be particularly applicable to EBC analysis. Metabonomics involves the detection of hundred of thousands of metabolites in a biological fluid usually using high resolution nuclear magnetic resonance spectrometry or liquid chromatography/MS. Powerful pattern recognition computer programs recognise patterns of metabolites that are sensitive to disease, effects of treatment and disease severity. Metabonomics of EBC (a “breathogram”) may, therefore, prove to be useful in screening lung diseases, following disease progression, predicting responses to treatment and in monitoring of response to therapy [229, 230].

One relative disadvantage of EBC measurements is that they require a subsequent analysis and it is likely that there will be important advances in on-line detection of particular biomarkers using sensitive biosensors. For example, it is possible to detect H$_2$O$_2$ on-line (real-time) using a silver electrode or by coating a platinum electrode or polymer with horseradish peroxidase [69, 70, 231]. Similar enzyme detector systems may also be developed for real-time monitoring of various lipid mediators, including 8-isoprostane. It is relatively easy to monitor pH of EBC and this is readily amenable to real-time detection. Several molecular biosensors are now in development and have the potential to detect very low concentrations of various relevant biomarkers. Ultimately it may be desirable to collect EBC to monitor patients in clinical practice using disposable detector sticks.

Proteomics, which applies high resolution gel electrophoresis or MS to detect multiple proteins in biological samples, may also be a useful approach to analysis of the proteins in EBC. This may reveal disease-specific patterns and may lead to the identification of novel proteins for detection of disease and identification of new therapeutic targets. However, there are several technical problems that need to be overcome before this becomes a useful approach.

It is clear that exhaled breath condensate is an exciting new approach to monitoring lung diseases that may have great potential in the future. These guidelines provide the first step in standardising measurements and encouraging research in this new field.

ACKNOWLEDGEMENTS

Task Force participants (who attended one or more meetings). Those participants who helping in the writing up of the Report are listed as co-authors: K. Alving (Stockholm, Sweden); A. Antczak (Lodz, Poland); B. Balint (Deszk, Hungary); E. Baraldi (Padova, Italy); G. Becher (Berlin, Germany); W.J.C. van Beurden (Nijmegen, Netherlands); A. Blomberg (Umea, Sweden); M. Corradi (Parma, Italy); R. Dekhuijzen (Nijmegen, Netherlands); R.A. Dweik (Cleveland, OH, USA); T. Dwyer (Jackson, MS, USA); R. Effros (Milwaukee, WI, USA); S. Erzurum (Cleveland, OH, USA); J. Freels (Tucson, AZ, USA); B. Gaston (Charlottesville, VA, USA); C. Gessner (Leipzig, Germany); M. Goldman (Los Angeles, CA, USA); A. Greening (Edinburgh, UK); L.P. Ho (Oxford, UK); J.M. Hohlfeld (Hannover, Germany); J. Hunt (Charlottesville, VA, USA); Q. Jóbis (Maastricht, Netherlands); S.A. Kharitonov (London, UK); F. Kelly (London, UK); D. Laskowski (Cleveland, OH, USA); C. Lehmann (Hannover, Germany); A. Lindstrom (Research Triangle Park, NC, USA); S. Loukides (Athens, Greece); D. Marlin (Kentford, UK); P. Montuschi (Rome, Italy); A-C. Olin (Gothenburg, Sweden); A.E. Redington (Hull, UK); P. Reinhold (Jena, Germany); E.L.J. van Rensen (Leiden, Netherlands); R. Robbins (Tucson, AZ, USA); M. Rothe (Freiburg, Germany); I. Rubinstein (Chicago, IL, USA); P. Silkoff (Denver, CO, USA); H-J. Smith (Gauting, Germany); W.R. Steinhaeusser (Marburg, Germany); W.G. Teague (Atlanta, GA, USA); K. Toren (Gothenburg, Sweden); G. Vass (Budapest, Hungary); J. Vaughan (Charlottesville, VA, USA); C. Vogelberg (Dresden, Germany); and H. Wirtz (Leipzig, Germany).

REFERENCES


Bucchioni E, Kharitonov SA, Allegra L, Barnes PL. High levels of interleukin-6 in the exhaled breath condensate in patients with COPD. Respir Med 2003; 97: 1299–1302.


Hyde RW. “I don’t know what you guys are measuring but you sure are measuring it!” A fair criticism of measurements of exhaled condensates? Am J Respir Crit Care Med 2002; 165: 561–564.


