Analysis of exhaled breath condensate in respiratory medicine: methodological aspects and potential clinical applications

Paolo Montuschi

Abstract: Analysis of exhaled breath condensate (EBC) is a noninvasive method for studying the composition of airway lining fluid and has the potential for assessing lung inflammation. EBC is mainly formed by water vapor, but also contains aerosol particles in which several biomolecules including leukotrienes, 8-isoprostanoids, prostaglandins, hydrogen peroxide, nitric oxide-derived products, and hydrogen ions, have been detected in healthy subjects. Inflammatory mediators in EBC are detected in healthy subjects and some of them are elevated in patients with different lung diseases. Analysis of EBC is completely noninvasive, is particularly suitable for longitudinal studies, and is potentially useful for assessing the response to pharmacological therapy. Identification of selective profiles of biomarkers of lung diseases might also have a diagnostic value. However, EBC analysis currently has important limitations. The lack of standardized procedures for EBC analysis and validation of some analytical techniques makes it difficult comparison of results from different laboratories. Analysis of EBC is currently more useful for relative measures than for quantitative assessment of inflammatory mediators. Reference analytical techniques are required to provide definitive evidence for the presence of some inflammatory mediators in EBC and for their accurate quantitative assessment in this biological fluid. Several methodological issues need to be addressed before EBC analysis can be considered for clinical applications. However, further research in this area is warranted due to the relative lack of noninvasive methods for assessing lung inflammation.

Keywords: exhaled breath condensate; airway inflammation; non-invasive assessment; therapeutic monitoring; asthma; chronic obstructive pulmonary disease; eicosanoids

Introduction

Inflammation has an important pathophysiological role in respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD) [Sabroe et al. 2007; Wenzel et al. 2006; Fixman et al. 2007; Barnes 2000; O’Donnell et al. 2006]. The assessment of lung inflammation is relevant for the management of chronic respiratory diseases as it may indicate that pharmacological therapy is required before onset of symptoms and decrease in lung function. Moreover, monitoring of airway inflammation might be useful in the follow-up of patients with respiratory diseases, and for guiding pharmacological therapy. Quantification of inflammation in the lungs is currently based on invasive methods including the analysis of bronchoalveolar lavage (BAL) fluid, bronchoscopy, and bronchial biopsies [Adelroth, 1998], semi-invasive methods such as sputum induction [Berlyne et al. 2000; Hargreave, 2007], and the measurement of inflammatory biomarkers in plasma and urine which are likely to reflect systemic rather than lung inflammation. Exhaled breath consists of a gaseous phase that contains volatile compounds (e.g. nitric oxide, carbon monoxide, and hydrocarbons) and a liquid phase, termed exhaled breath condensate (EBC), that contains aerosol particles in which several nonvolatile compounds have been identified [Montuschi, 2005a; Mutlu et al. 2001; Kharitonov and Barnes, 2001; Hunt, 2002; Kharitonov and Barnes, 2006; Effros et al. 2005; Horvarth et al. 2005; Cepelak et al. 2007; Montuschi, 2002; Montuschi and Barnes, 2002]. Measurement of exhaled nitric oxide (NO) is a...
well accepted, standardized, validated and widely used method for assessing airway inflammation in patients with asthma who are not being treated with inhaled glucocorticoids, can be used to monitor the response to these drugs, and to assess compliance and can predict asthma exacerbation [Anonymous, 1999; Anonymous, 2005]. Moreover, with the use of exhaled NO measurements, maintenance doses of inhaled glucocorticoids may be significantly reduced without compromising asthma control [Smith et al. 2005]. However, the clinical utility of exhaled NO measurement is currently limited to patients with asthma, as its role in the management of other respiratory diseases is not yet known.

Recently, attention has focused on EBC as a noninvasive method for studying the composition of airway lining fluid [Montuschi, 2002; Montuschi and Barnes, 2002a; Effros et al. 2005b]. Using urea, which is a freely diffusible molecule, as a marker, it has been demonstrated that a measurable fraction (1 in 24 parts) of the EBC in healthy subjects is derived from aerosolized airway lining fluid [Dwyer, 2001]. EBC analysis of inflammatory biomarkers is a noninvasive method which has the potential to be useful for monitoring airway inflammation in patients with respiratory diseases, including children [Montuschi, 2002; Montuschi and Barnes, 2002a]. As it is completely noninvasive, EBC also is suitable for longitudinal studies and for monitor the response to pharmacological therapy. Furthermore, different biomarkers might reflect the different aspects of lung inflammation or oxidative stress, which is an important component of inflammation. Identification of selective profiles of biomolecules in different inflammatory airway diseases might be relevant for differential diagnosis in respiratory medicine. Collection of EBC samples is simple, inexpensive, and safe. However, unlike exhaled NO measurement, EBC technique does not provide real-time results as EBC samples need to be assayed for different biomolecules. Several methodological issues, including standardization of EBC technique and validation of analytical methods, need to be addressed before this approach can be considered for applications in the clinical setting.

This review describes the methodology of the EBC technique, summarizes the current knowledge on the biomarkers which have been identified in EBC, discusses the advantages, the limitations, and the potential clinical applications of EBC analysis, and provides suggestions for further research in this area.

**Experimental setup**

The collection of EBC sample is simple and easy to perform. Home-made and commercially manufactured condensers are available. Home-made equipment generally consist of a mouthpiece with a one-way valve connected to a collecting system which is placed in either ice or liquid nitrogen to cool the breath [Montuschi, 2005b]. The collecting system consists of a double wall of glass, the inner wall of which is cooled by ice (Figure 1A) [Montuschi et al. 1999]. Alternatively, jacketed cooling pipes or tubes in buckets have been used [Mutlu et al. 2001]. Generally, subjects are asked to breath tidally, with a noseclip on, for 15 min through a mouthpiece connected to the condenser. Exhaled air enters and leaves the condensing chamber through one-way valves at the inlet and outlet while the chamber is kept closed. If the collecting system consists of two glass containers, EBC is collected between the two glass surfaces at the bottom of the outer glass container in a liquid form [Montuschi et al. 2005b]. Generally, 1.0–2.5 mL of EBC is collected in 15 min based on respiratory parameters (minute ventilation, respiratory rate, tidal volume), material of condenser surfaces, temperature, and turbulence of airflow.

Commercially manufactured condensers are also available [Montuschi et al. 2000; Montuschi and Barnes, 2002b]. The EcoScreen™ (Jaeger Tonnies, Hoechberg, Germany) is an electric refrigerated system which connects to a mouthpiece with a one-way valve and a collecting system connected to a power supply by an extendable arm (Figure 1B). Subjects breath through the mouthpiece that is connected to a valve block in which inspiratory and expiratory air are separated. The valve block is connected to the collecting system, consisting of a lamellar condenser, and a sample collection vial. The collecting system is inserted into a cooling cuff maintained at a cold temperature by a refrigerator. The actual temperature inside the cooling cuff is cooled down by the refrigerating system. The air flowing through the lamellar condenser condenses on the inner surface of the lamellar condenser and drops into the collecting vial. Despite it's
wide use, there is currently no evidence of advantages of the EcoScreen condenser over homemade devices, except for the possible immediate freezing of the samples. This can be important for chemically unstable compounds including leukotrienes (LTs), particularly LTE₄ and cysteinyl-LTs in general. However, as the temperature inside the cooling cuff is probably higher than −10°C throughout the test, and/or other possible technical problems that may arise, EBC samples are usually collected in a liquid or in a mixed liquid/frozen form. Inconsistencies in collection of samples (liquid, frozen, partially frozen) may affect the concentrations of chemically unstable compounds in EBC and explain part of the variability in their concentrations reported by different studies. Anyway, even if samples are collected frozen, when measuring more than one compound, samples must be thawed at the time of collection to make the required aliquots. This could be avoided by replacing the single collecting vial with some smaller, separate collecting vials. When considering a large-scale application of EBC technique, the high cost of this commercial condenser should also be considered.
The EcoScreen® II (Jaeger Tonnies, Hoechberg, Germany) has recently been manufactured. This condenser allows to measure respiratory parameters during the collection of EBC. Alternatively, the EcoScreen can be connected to a pneumotachograph and a computer for online recording of respiratory parameters [Montuschi et al. 2003a]. Another advantage of the EcoScreen II is that EBC derived from the airways or the alveoli can be collected at the same time into two separate collecting systems consisting of plastic bags inserted into a refrigerating system. This device might be useful for studying the origin of biomarkers in the lung compartments (airways vs alveoli). Compared with the material of the collecting system surface (plastics vs Teflon-coated metal) might also turned out to be more appropriate for some compounds (e.g. lipids). However, no published studies are available with this condenser.

The RTube® (Respiratory Research, Inc., Charlottesville, VA) is another commercially available condenser which has the advantage of being portable [Hunt, 2002]. This device consists of a disposable polypropylene collecting system with an exhalation valve that also serves as syringe-style plunger to pull fluid off the condenser wall (Figure 1C) [Hunt, 2002]. The refrigerating system consists of an aluminium cooling sleeve which is placed over the disposable polypropylene tube (Figure 1C). The temperature of the cooling sleeve can be chosen. The device prevents salivary contamination, and no detectable amylase concentrations in EBC have been reported [Hunt, 2002]. The RTube has been originally manufactured for measuring pH in EBC samples, but it can be used for measuring other compounds [Hunt, 2002]. As measurement of pH in EBC requires deaeration for removal of carbon dioxide, a separate device, the pHTube® (Respiratory Research, Inc., Charlottesville, VA) can be used for this purpose [Hunt, 2002]. The RTube is portable, which makes it possible to collect EBC samples at home, which is particularly suitable for longitudinal studies or when collection of several samples a day is required; the EBC sample in a polypropylene tube can be stored in a freezer at home; a sufficient volume of EBC for pH measurement can be collected in as little as 1 min [Hunt, 2002]. However, the RTube has some limitations as EBC samples need to be brought to the laboratory for biochemical assays, and storage conditions in freezer at home (−20°C) are different from those required by some chemically unstable mediators which need to be stored immediately at −80°C. One study reported that compared with RTube, collection of exhaled breath by EcoScreen allows larger volumes to be collected and detects protein and lipid mediators with greater sensitivity, which might be due to the differences in the collection temperature [Soyer et al. 2006], but further studies to address this issue are required.

Careful sterilization of the EBC equipment is required to avoid cross-contamination, although standardized procedures for sterilization of condensers are not available. It is reasonable to leave the EBC equipment in an antibacterial solution such as 1% aqueous solution of sodium hypochlorite for at least 1 h. After sterilization, the EBC collecting system needs to be washed thoroughly with water to remove the antiseptic solution and dried. Some collecting systems such as the lamellar condenser in the EcoScreen are coated with Teflon® (E.I. du Pont de Nemours & Company, Inc., Washington, DE) to avoid adhesions of biomolecules to the collecting system surfaces and the consequent artifactual decrease in their concentrations in EBC. At present, the effect of different collecting system materials on the detection of different biomolecules in EBC is largely unknown. The best collecting system material is likely to be different depending on the physicochemical properties of the biomolecule to be detected. A condenser with borosilicate glass coating has been shown to be superior to silicone, aluminium, polypropylene, and Teflon for detection of albumin in EBC [Rosias et al. 2006]. The effect of antiseptic solutions on the EBC collecting system materials are not currently known. Whether the antiseptic solutions interact and damage the material of the collecting systems needs to be clarified. Technical improvements in the design of new condensers would allow to collect simultaneously several frozen aliquots making it possible to measure different markers without thawing the whole sample. The possibility of using selective sensors to make on-line measurements of hydrogen peroxide [Gajdocsi et al. 2003] and possibly other specific inflammatory mediators in the breath is currently under investigation.

**Markers of inflammation in EBC**

Several biomolecules have been detected in EBC of healthy subjects and of patients with different inflammatory lung diseases (Table 1 and Figure 2). In most studies, markers in EBC were
<table>
<thead>
<tr>
<th>Biomolecule</th>
<th>Analytical method</th>
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<tr>
<td><strong>Isoprostanes</strong></td>
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<tr>
<td>8-Isoprostane (15-F2t-IsoP)</td>
<td>GC/MS [Carpenter et al. 1998], RIA [Montuschi et al. 2003a; Montuschi et al. 2003b; Mondino et al. 2004, Baraldi et al. 2003a; Montuschi et al. 2006; Montuschi et al. 2005b], EIA [Montuschi et al. 1999; Montuschi et al. 2000, Montuschi and Barnes, 2002b; Antczak et al. 2001; Shahid et al. 2005; Montuschi et al. 2002; Baraldi et al. 2003b; Kostikas et al. 2003; Biernacki et al. 2003; Carpagnano et al. 2003a; Zanconato et al. 2004]</td>
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<tr>
<td><strong>Leukotrienes</strong></td>
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<tr>
<td>LTB4</td>
<td>LC/MS/MS [Montuschi et al. 2004a; Montuschi et al. 2005a], GC/MS [Cap et al. 2004], EIA [Montuschi and Barnes, 2002b; Montuschi et al. 2003c; Antczak et al. 2001; Mondino et al. 2004; Biernacki et al. 2003; Montuschi et al. 2005b; Hanazawa et al. 2000; Csoma et al. 2002]</td>
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<tr>
<td>LTD4</td>
<td>GC/MS [Cap et al. 2004], EIA [Mondino et al. 2004; Montuschi et al. 2006; Shibata et al. 2006]</td>
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<tr>
<td>LTE4</td>
<td>GC/MS [Cap et al. 2004], EIA [Mondino et al. 2004; Shibata et al. 2006]</td>
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<td><strong>Prostanoids</strong></td>
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<tr>
<td>PGE2</td>
<td>GC/MS [Carpenter et al. 1998], RIA [Montuschi et al. 2003b; Montuschi et al. 2005b], EIA [Montuschi and Barnes, 2002b]</td>
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<td>PGF2α</td>
<td>EIA [Montuschi and Barnes, 2002a]</td>
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<tr>
<td>PGD2</td>
<td>EIA [Montuschi and Barnes, 2002b]</td>
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<tr>
<td><strong>Hydrogen ions</strong></td>
<td>pH meter or pH microelectrode [Hunt et al. 2000; Vaughan et al. 2003; Kostikas et al. 2002; Larstad et al. 2003; Shimizu et al. 2007; Nicolau et al. 2006; Walsh et al. 2006; Dupont et al. 2006; Prieto et al. 2007; Paget-Brown et al. 2005; Kullmann et al. 2004]</td>
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<tr>
<td><strong>Hydrogen peroxide</strong></td>
<td>Spectrophotometry [Horvath et al. 1998; Dekhuijzen et al. 1996], fluorometric assay [Schleiss et al. 2000; Jobsis et al. 1997], chemiluminescence [Zappacosta et al. 2001]</td>
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<tr>
<td><strong>Nitrogen reactive species</strong></td>
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<tr>
<td>Nitrite</td>
<td>Spectrophotometry [Cunningham et al. 2000; Corradi et al. 2001], fluorometric assay [Balint et al. 2001]</td>
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<tr>
<td>Nitrate</td>
<td>Fluorometric assay [Balint et al. 2001]</td>
</tr>
<tr>
<td>S-Nitrosocysteine</td>
<td>Spectrophotometry [Corradi et al. 2001], fluorometric assay [Kharitonov et al. 2002]</td>
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<tr>
<td>3-Nitrotyrosine</td>
<td>GC/MS [Larstad et al. 2005; Celio et al. 2006], LC/MS [Goen et al. 2005; Baraldi et al. 2006], HPLC [Celio et al. 2006], EIA [Hanazawa et al. 2000]</td>
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<tr>
<td>Adenosine</td>
<td>HPLC [Vass et al. 2003]</td>
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<td>Glutathione</td>
<td>HPLC [Corradi et al. 2003b]</td>
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<tr>
<td>Aldehydes</td>
<td>LC/MS [Corradi et al. 2003a; Corradi et al. 2003b], HPLC [Larstad et al. 2002]</td>
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<td>TBARS</td>
<td>Spectrofluorimetry [Nowak et al. 1999]</td>
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<td>DNA</td>
<td>PCR [Gessner et al. 2004; Carpane et al. 2005]</td>
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<tr>
<td>Electrolytes (sodium, potassium, calcium, magnesium, chloride)</td>
<td>Ion-selective electrodes [Effros et al. 2002], ion chromatography [Effros et al. 2003]</td>
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<td>Keratin</td>
<td>Proteomics [Gianazza et al. 2003], ELISA [Jackson et al. 2007]</td>
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<tr>
<td>Cytokines</td>
<td>EIA [Scheideler et al. 1993], multiplex bead array [Sack et al. 2006], flow cytometry [Robroeks et al. 2006]</td>
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<tr>
<td>IL-1β</td>
<td>Protein array [Matsunaga et al. 2006], flow cytometry [Robroeks et al. 2006], ELISA [Shahid et al. 2002]</td>
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<td>IL-2</td>
<td>EIA [Bucchioni et al. 2003; Carpane et al. 2003b], ELISA [Rozy et al. 2006]</td>
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<tr>
<td>IL-6</td>
<td>Protein array [Matsunaga et al. 2006], multiplex bead array [Sack et al. 2006], ELISA [Cunningham et al. 2000]</td>
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<td>IL-8</td>
<td>Flow cytometry [Robroeks et al. 2006], multiplex bead array [Sack et al. 2006]</td>
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Table 1. Biomolecules in EBC.
Table 1. Continued.

<table>
<thead>
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<th>Biomolecule</th>
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<td>Cytokines (continued)</td>
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<tr>
<td>IL-12p70</td>
<td>Multiplex bead array [Sack et al. 2006]</td>
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<tr>
<td>IL-17</td>
<td>Protein array [Matsunaga et al. 2006]</td>
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<tr>
<td>Interferon-γ</td>
<td>EIA [Shahid et al. 2002], flow cytometry [Robroeks et al. 2006]</td>
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<tr>
<td>IGFl</td>
<td>ELISA [Rozy et al. 2006]</td>
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<tr>
<td>Interferon-γ-inducible protein 10</td>
<td>Protein array [Matsunaga et al. 2006]</td>
</tr>
<tr>
<td>MIP-1α, MIP-1β</td>
<td>Protein array [Matsunaga et al. 2006]</td>
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<tr>
<td>PAI-1</td>
<td>ELISA [Rozy et al. 2006]</td>
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<tr>
<td>RANTES</td>
<td>Protein array [Matsunaga et al. 2006]</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Protein array [Matsunaga et al. 2006]</td>
</tr>
<tr>
<td>TNF-α</td>
<td>RIA [Scheideler et al. 1993], protein array [Matsunaga et al. 2006], ELISA [Rozy et al. 2006], multiplex bead array [Sack et al. 2006]</td>
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</tbody>
</table>

Abbreviations: GC/MS, gas chromatography/mass spectrometry; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; IGF-1, insulin-like growth factor-1; IL, interleukin; LC/MS, liquid chromatography/mass spectrometry; LT, leukotriene; MIP-1, macrophage inflammatory protein-1; PAI-1, plasminogen activator inhibitor-1; PGI2, prostaglandin; RIA, radioimmunoassay; TBARS, thiobarbituric acid reactive substances; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; Tx, thromboxane.

Figure 2. Biomarkers of inflammation and/or oxidative stress which have been detected in EBC in healthy subjects and/or in patients with airway inflammatory diseases.

Abbreviations: COX, cyclo-oxygenase; GSH, glutathione; -GTP, -glutamyl-transpeptidase; 5-HETE, 5-hydroxyeicosatetraenoic acid; H2O2, hydrogen peroxide; 5-HPETE, 5-hydroperoxycicosatetraenoic acid; 8-IP, 8-isoprostane; 5-LO, 5-lipoxygenase; LT, leukotriene; MPD, myeloperoxidase; NADPH oxidase, reduced nicotinamide adenine dinucleotide phosphate oxidase; NO, nitric oxide; NOS, nitric oxide synthase; NO2−, nitrite; NO3−, nitrate; 3-NT, 3-nitrotyrosine; O2−, superoxide anion; ONOO−, peroxynitrite; PG, prostaglandin; ROS, reactive oxygen species; RS-NO, RS-nitrosothiols; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; Tx, thromboxane.
measured with immunoassays, which need to be validated with reference analytical methods such as mass spectrometry or high performance liquid chromatography (HPLC). These techniques also will accurately quantify the concentrations of the different markers in EBC. The presence of LTβ1 [Montuschi et al. 2004a; Montuschi et al. 2005a], 8-isoprostan e [Carpenter et al. 1998] PGE₂ [Carpenter et al. 1998], aldehydes [Corradi et al. 2003a] and free 3-nitrotyrosine in EBC has been confirmed by mass spectrometry [Goen et al. 2005; Larstad et al. 2005]. LTβ₁ [Montuschi et al. 2004a, 2005b] and aldehydes [Corradi et al. 2003] in EBC have been measured by liquid chromatography/mass spectrometry (LC/MS), whereas 8-isoprostan e and PGE₂ in EBC have been measured by gas chromatography/mass spectrometry (GC/MS) [Carpenter et al. 1998]. Free 3-nitrotyrosine has been measured by both LC/MS [Goen et al. 2005] and GC/MS [Larstad et al. 2005]. Adenosine [Vass et al. 2003], reduced glutathione [Corradi et al. 2003], and malondialdehyde (MDA) [Larstad et al. 2002] in EBC have been detected by HPLC. In patients with asthma exacerbations, pH values in EBC are more than two log orders lower than normal and normalize with glucocorticoids [Hunt et al. 2000]. Measurement of pH in EBC is very reproducible, relatively easy to perform, and provides almost real-time results [Vaughan et al. 2003]. This technique could prove to be useful for diagnosis and for monitoring therapeutic response [Vaughan et al. 2003]. pH values in EBC from patients with other lung diseases have been reported [Kostikas et al. 2002], but the biological significance of these data needs to be clarified.

There is a high interindividual variability in the total amount of proteins of concentrations in EBC in healthy subjects (from undetectable to 1.4 mg) [Scheideler et al. 1993]. At present, the reasons for this variability are unknown. Establishing the protein concentrations in EBC under standardized conditions is a priority in this research area. One study has shown that protein concentrations in EBC in 20 healthy subjects averaged 2.3 mg/dl [Effros et al. 2002].

Using immunoassays, cytokines have been detected in EBC in healthy subjects and in patients with different inflammatory airway diseases [Scheideler et al. 1993; Cunningham et al. 2000; Shahid et al. 2002]. However, immunoassays for cytokines in EBC still need to be validated by reference analytical techniques. Moreover, concentrations of cytokines reported in most studies are close to the detection limit of the assay. At these concentrations, analytical data are less reliable. However, due to its biological importance, the issue of the presence of cytokines in EBC and their accurate quantitative measurement in this biological fluid deserves further investigation.

Comparisons between absolute concentrations of EBC markers that have been reported by different studies are currently difficult due to differences in the EBC collection procedures, condensers, and sample storage and handling; differences in the analytical techniques used; incomplete identification of factors that affect EBC analysis; differences in clinical characteristics of study groups (diagnostic criteria, disease severity, pharmacological therapy); interindividual biological variability. The use of reference indicators in EBC (e.g. electrolytes or conductivity) has been proposed to take into account changes in respiratory soluble concentrations which can result from variations in the dilution of the respiratory droplets [Effros et al. 2002, 2003].

**Methodological issues**

Standardization of the EBC method and validation of the analytical techniques for measuring each inflammatory biomarker are required for comparing data from different laboratories and assessing the clinical utility of EBC analysis. Methodological issues that need to be addressed include flow dependence, time dependence, and influence of respiratory patterns; origin(s) of markers in EBC; organ specificity of EBC; possible nasal, saliva, and sputum contamination; identification of reference indicators in EBC to ascertain to what extent EBC reflects the composition of airway lining fluid and to adjust for possible changes in the dilution of respiratory droplets; influence of temperature, humidity, and collecting-system materials; reproducibility studies (between days variability, intrasubject diurnal variability); comparisons of collection devices; storage issues; possible need for sample pretreatment; for most biomolecules in EBC, the demonstration of their presence in EBC and their accurate quantitative measurement by reference analytical techniques (e.g. MS, HPLC); the validation of sensitive, specific, and reproducible immunoassays to be used routinely.
Flow dependence and influence of respiratory patterns

One study in 15 healthy adults has shown that hydrogen peroxide concentrations in EBC depend on expiratory flow rate indicating that exhaled hydrogen peroxide levels are flow dependent [Schleiss et al. 2000]. In six healthy adults, MDA concentrations in EBC collected at three different flow rates were similar [Corradi et al. 2003] indicating that MDA concentrations are not flow dependent. In four healthy children, MDA and glutathione concentrations in EBC samples collected at different flow rates were similar [Corradi et al. 2003b]. To study the influence of ventilation patterns on 8-isoprostane, a marker of lipid peroxidation, and PGE$_2$ concentrations in EBC, we asked 15 healthy adults to breath at expiratory minute ventilation of 10, 20, and 30 L/min for 10, 10, and 5 min, respectively [Montuschi et al. 2003a]. There was no difference in mean 8-isoprostane and PGE$_2$ concentrations in EBC collected at different expiratory minute ventilations indicating that the concentrations of these eicosanoids in EBC are not influenced by this respiratory parameter [Montuschi et al. 2003a]. The lack of correlation between 8-isoprostane concentrations in EBC and minute ventilation in mechanically ventilated patients supports our data [Carpenter et al. 1998]. Likewise, LTB$_4$ and LTE$_4$ concentrations in EBC were similar in five healthy subjects who breathed at 14 and 28 breaths/min for 15 min, maintaining the same tidal volume [Montuschi and Barnes, 2003b]. Other authors have demonstrated that nitrite and protein concentrations in EBC are independent of respiratory pattern [McCafferty et al. 2002a] and that ethanol concentrations in EBC samples collected after tidal breathing and deep inspiration and expiration are similar [Rickmann et al. 2001]. These findings indicate that different markers in EBC behave differently regarding flow dependence and ventilation patterns. For this reason, each marker should be studied individually.

For several markers, there is no information on the influence of airflow and/or ventilation patterns on their concentrations in EBC and additional studies are required. Moreover, the influence of ventilation patterns on markers in EBC has been mainly studied in healthy subjects and these findings cannot be directly extrapolated to patients with inflammatory lung diseases. Assessing the flow dependence of different biomarkers in EBC is important for the standardization of this technique as EBC sampling will be performed either at a constant flow rate for a fixed time, or at a constant exhaled volume for a variable time.

8-Isoprostane concentrations in EBC do not depend on the duration of EBC collection [Carpenter et al. 1998]. Conflicting results have been reported for MDA as in one study MDA concentrations in samples collected at 10 and 20 min were similar [Corradi et al. 2003a], whereas other authors showed that MDA levels decreased with prolonged sampling time [Larstad et al. 2003] in analogy to hydrogen peroxide concentrations in EBC [Svensson et al. 2003]. The lack of correlation between 8-isoprostane and PGE$_2$ concentrations in EBC collected at different expiratory minute ventilations indicating that the concentrations of these eicosanoids in EBC are not influenced by this respiratory parameter [Montuschi et al. 2003a]. The mean volume of EBC collected in a 15-min test were almost two-fold higher at 28 breaths/min compared with those at 14 breaths/min [Montuschi et al. 2002b]. In healthy subjects, EBC volume is correlated with expiratory minute ventilation, although the condenser efficiency decreases at the higher expiratory minute ventilation values [McCafferty et al. 2004]. The mean volume of EBC collected during 10 min of tidal breathing was about 58% of that collected during 20 min of tidal breathing [Corradi et al. 2003]. In healthy subjects, the volume of EBC collected is increased when subjects inhale through their noses and exhale through their mouths without wearing a noseclip probably due to an increase in minute ventilation when sample collection is performed without a noseclip [Vass et al. 2003].

Origin(s) of markers in EBC

Three aspects related to the origin of markers in EBC need to be considered: their cellular source; the compartment of the respiratory system in which they are produced (airways vs alveolar region); the extent to which EBC reflects the composition of airway lining fluid and lung inflammation.

EBC technique does not provide direct information on the inflammatory cells in the...
respiratory system. Identifying the cellular source(s) of markers in EBC requires invasive techniques such as bronchoscopy. A correlation between the concentrations of a biomolecule in EBC and a cell type can provide indirect information on the cellular source of that biomolecule. However, this does not necessarily imply that the biomolecule is released by that cell type as increased biomolecule concentrations in EBC could also reflect cell activation without increased cell counts. Flow dependence might indicate whether a certain inflammatory mediator is mainly derived from the airways or the alveoli [Schleiss et al. 2000]. If the concentrations of a biomolecule in EBC depend on expiratory flow rate, this could imply that the biomolecule is mainly derived from the airways as flow rate affects the time available for its accumulation in the airways, and therefore its EBC concentrations [Schleiss et al. 2000]. In contrast, the lack of flow dependence would indicate that the biomolecule is mainly derived from the alveolar region where flow rate has a minor role [Schleiss et al. 2000]. As it is flow dependent, hydrogen peroxide in EBC should primarily be derived from the airways [Schleiss et al. 2000], whereas 8-isoprostane [Montuschi et al. 2003a; Carpenter et al. 1998], PGE2 [Montuschi et al. 2000], and aldehydes [Corradi et al. 2003] should primarily be derived from the alveolar region. These studies were performed in healthy subjects. In patients with inflammatory lung disease, the increased numbers and/or activation of selective inflammatory cell types in different compartments of the respiratory system and the high-current velocity which can facilitate formation of aerosol particles containing nonvolatile inflammatory mediators [Hunt, 2002] might have a different impact on the concentrations of a specific biomolecule in EBC compared with healthy subjects.

The origin of nonvolatile inflammatory markers which are probably contained in aerosol particles (e.g. proteins) in EBC can also be studied by identifying cell-specific markers such as keratin and total phospholipid, which is found primarily in surfactant [Jackson et al. 2007]. Both airway and alveolar compartments contribute to the formation of EBC [Jackson et al. 2007]. Using ethanol as a model compound, it has been shown that the ratio between the two compartments depends on ventilation, with a shift towards the alveolar region during forced ventilation [Rickmann et al. 2001]. Studies on the mechanisms of EBC formation are required to established to what extent EBC reflects the composition of airway lining fluid.

Whether the concentrations of inflammatory mediators in EBC reflects lung inflammation or systemic production of these compounds, particularly under conditions of increased permeability [Carpenter et al. 1998], is still unknown. Measurement of inflammatory mediators in patients with systemic inflammatory diseases (e.g. systemic lupus erythematosus) and comparisons between concentrations of inflammatory mediators in EBC and in biological fluids (e.g. plasma and/or urine) reflecting systemic production of these compounds might be useful.

Dilution reference indicators

The use of dilution reference indicators has been proposed to 1) estimate the concentrations of nonvolatile inflammatory mediators in respiratory fluid; 2) to normalize for interindividual variations in droplet formation. Although most EBC is derived from water vapor, the presence of nonvolatile compounds in EBC indicates that droplets of the respiratory fluids are collected [Effros et al. 2002]. Calculation of airway lining fluid solute concentrations from EBC requires estimation of the dilution factor. Using electrolyte concentrations in EBC as dilution reference indicator, it has been estimated that respiratory fluid represents between 0.01 and 2% of EBC volume [Effros et al. 2002]. Due to this high variability in the dilution of respiratory droplets by water vapor, it has been argued that increased concentrations of inflammatory mediators in EBC reported in different lung diseases could reflect increased droplet formation [Effros et al. 2003]. The selective increase of biomolecules in EBC and the lack of correlation between concentrations of structurally related compounds in EBC are not consistent with this hypothesis [Montuschi and Barnes, 2002b]. Measurements of electrolytes, urea, or conductivity have been proposed as dilution reference indicators in EBC [Effros et al. 2003], but their role in EBC analysis is debated. Alternatively, EBC data can be expressed as a change in ratio of one compound to another.

Salivary contamination

Eicosanoids and other inflammatory mediators are present in saliva [Zakrzewski et al. 1987; McKinney et al. 2000] which could affect EBC.
we have provided evidence for the specificity of leukotrienes in EBC. Concentrations of adenosine and thromboxane (Tx) B2 in EBC samples through tracheostomy in mechanically ventilated patients or through the mouth in healthy subjects were similar [Vass et al. 2003]. However, most of the ammonia in the EBC is derived from the mouth, probably as ammonium gas, as indicated by undetectable concentrations of ammonia in EBC samples from patients with tracheostomies [Effros et al. 2002]. These data indicate that the effect of salivary contamination on the concentrations of biomolecules in EBC likely depend on their salivary concentrations and their physicochemical properties (volatile vs nonvolatile compounds). For this reason, the issue of potential salivary contamination of the EBC samples should be considered separately for each marker.

Validation of analytical methods
Part of the variability of biomarker concentrations in EBC could be due to analytical methods. In most studies published so far, concentrations of biomolecules in EBC were measured with commercially available immunoassay kits which have not been generally validated with reference analytical techniques. Enzyme immunoassays generally work well in buffer, but their behavior in EBC is not known. Matrix effects due to differences in the composition of EBC and immunoassay buffer could be relevant and might suggest the need for sample pre-treatment. Validation of immunoassays with reference analytical techniques (e.g. HPLC or MS) is essential for demonstrating the presence of several biomarkers in EBC and for their accurate quantitative assessment. We have provided evidence for the specificity of 8-isoprostane and PGE2 measurements in EBC by radioimmunoassays (RIAs) developed in our laboratory [Montuschi et al. 2003b] and for a commercially available assay for LTB4 [Montuschi et al. 2003b]. In these studies, RIAs for 8-isoprostane and PGE2 and EIA for LTB4 were qualitatively validated by reverse phase-HPLC [Montuschi et al. 2003c; 2003b]. Validation of immunoassays with independent assay methods is a pre-requisite for large-scale use of immunoassays in EBC analysis. As reference analytical techniques are time-consuming, very expensive and unsuitable routinely, validation of immunoassays might contribute to the development of EBC analysis in respiratory medicine.

Measurement of markers in EBC for assessing the response to pharmacological therapy
At present, most of the studies on the effect of anti-inflammatory drugs on markers in EBC are cross-sectional. Few single-center, generally uncontrolled, interventional studies are available. Large randomized controlled trials using a robust and standardized methodology are required to established the effect of drugs on inflammatory mediators in EBC in patients with airway inflammatory diseases.

Isoprostanes
F2-Isoprostanes, a group of 64 compounds isomeric in structure to PGF_{2\alpha}, are currently considered among the most reliable biomarkers of oxidative stress and lipid peroxidation [Montuschi et al. 2004b; Montuschi et al. 2007]. Concentrations of 8-isoprostane, the most known compound belonging to F2-isoprostanes, in EBC are increased in adults with stable asthma, reflecting the severity of the disease and the degree of inflammation [Montuschi et al. 1999]. Despite treatment with oral or high doses of inhaled glucocorticoids, patients with severe asthma have the highest concentrations of 8-isoprostane in EBC indicating that this eicosanoid is relatively resistant to inhaled glucocorticoids [Montuschi et al. 1999]. Other authors reported a similar increase in 8-isoprostane in EBC in patients with stable moderate asthma who were either steroid-naive or treated with inhaled glucocorticoids, although subgroup analysis for the effects of these drugs was not presented [Kostikas et al. 2002]. In contrast to patient with aspirin-tolerant asthma, patients with aspirin-sensitive asthma who were steroid-naive had higher 8-isoprostane concentrations in EBC compared with those who were treated with inhaled glucocorticoids [Antczak et al. 2001] indicating that the effect of glucocorticoids on 8-isoprostane might depend on asthma phenotype and/or degree of inflammation. In analogy to adults with asthma,
8-isoprostane concentrations in EBC were elevated in children with asthma who were either steroid-naive or treated with inhaled glucocorticoids compared with those in healthy children [Mondino et al. 2004; Baraldi et al. 2003a; Shahid et al. 2005]. There was no difference in 8-isoprostane levels in EBC between the two study groups [Mondino et al. 2004; Baraldi et al. 2003a; Shahid et al. 2005]. In healthy subjects, pretreatment with a high dose of inhaled budesonide (1600 µg/day for 14 days) has no effect on the increase in 8-isoprostane in EBC induced by 2 h exposure to ozone at 400 ppb [Montuschi et al. 2002]. Two open-label studies have shown that inhaled fluticasone at a dose of 100 µg twice daily for 4 weeks [Mondino et al. 2004] and oral montelukast, a LT receptor antagonist, at a dose of 5 mg once a day for 4 weeks have no effect on 8-isoprostane concentrations in EBC in atopic children with asthma [Montuschi et al. 2006].

One study reports that oral glucocorticoids reduce 8-isoprostane concentrations in EBC in patients with acute asthma [Baraldi et al. 2003b]. Compared with healthy children, 8-isoprostane concentrations in EBC were elevated in children with acute asthma [Baraldi et al. 2003b]. In these children, oral prednisolone at a dose of 1 mg/kg/day for 5 days reduced, but not normalized, exhaled 8-isoprostane concentrations [Baraldi et al. 2003b]. However, it should be noted that 8-isoprostane concentrations reported in this study were close to the detection limit of the enzyme immunoassay where data are much less reliable.

Patients with COPD have elevated 8-isoprostane concentrations in EBC [Montuschi et al. 2000; Kostikas et al. 2003]. In contrast to healthy subjects in whom smoking increases 8-isoprostane concentrations in EBC, smoking habit does not seem to affect 8-isoprostane levels in EBC in patients with COPD [Montuschi et al. 2000]. A 41% reduction of mean 8-isoprostane concentrations in EBC has been reported in patients with COPD exacerbations after antibiotic treatment [Biernacki et al. 2003]. Ibuprofen, a nonselective cyclo-oxygenase (COX) inhibitor, and rofecoxib, a COX-2 selective inhibitor, have no effect on 8-isoprostane concentrations in EBC in patients with COPD [Montuschi et al. 2005b]. The lack of effect of ibuprofen and rofecoxib on exhaled 8-isoprostane concentrations indicates that this compound is primarily formed independently of the COX pathway [Montuschi et al. 2004b; Montuschi et al. 2007; Morrow et al. 1990].

In patients with obstructive sleep apnea, a reduction in exhaled 8-isoprostane concentrations after continuous positive airway pressure therapy has been reported [Carugno et al. 2003]. However, these data need to be interpreted cautiously due to the small size effect (−1.9 pg/mL) and the reported 8-isoprostane concentrations which were close to detection limit of the immunoassay.

**Leukotrienes**

Elevated cysteinyl-LT and LTB4 concentrations in EBC have been reported in both adults and children with asthma [Montuschi and Barnes, 2002b; Montuschi et al. 2006; Antczak et al. 2001; Mondino et al. 2004; Hanazawa et al. 2000; Zanzonato et al. 2004; Carraro et al. 2005; Shibata et al. 2006; Cap et al. 2004; Csoma et al. 2002]. In one study, no difference in cysteinyl-LT concentrations in EBC was observed between steroid-naive and steroid-treated patients with aspirin-tolerant asthma, whereas steroid treatment was associated with lower concentrations of cysteinyl-LTs in patients with aspirin-sensitive asthma [Antczak et al. 2001]. These findings indicate that cysteinyl-LTs concentrations in EBC in patients with aspirin-tolerant asthma are relatively resistant to glucocorticoids, although the observational study design precludes definitive conclusions. In children with asthma, inhaled fluticasone at a dose of 100 µg twice daily for 4 weeks has a pronounced effect in reducing exhaled NO concentrations (−53%) over baseline values, but a limited effect on LTE4 concentrations (−27%) in atopic children with asthma than inhaled glucocorticoids, but a lower effect on exhaled NO concentrations (−33%) in atopic children with asthma than inhaled glucocorticoids, but a lower effect on exhaled NO concentrations (−33%) in atopic children with asthma. Although several studies reported detectable LT concentrations in EBC in patients with asthma [Montuschi and Barnes, 2002b; Montuschi et al. 2005b, 2006; Antczak et al. 2001; Mondino et al. 2004; Hanazawa et al. 2000; Zanzonato et al. 2004; Carraro et al. 2005;
Shibata et al. 2006; Cap et al. 2004; Csoma et al. 2002) and other airway inflammatory diseases, some authors were unable to detect cysteinyl-LT concentrations in EBC in adults with asthma [Sandrini et al. 2003]. Due to the lack of a standardized EBC technique, comparisons of results from different laboratories are currently difficult. In contrast to a limited effect of inhaled glucocorticoids on LT concentrations in patients with stable mild intermittent asthma, a reduction of 60% of median exhaled cysteinyl-LT concentrations in children with asthma exacerbations after oral glucocorticoids has been reported [Baraldi et al. 2003b]. This discrepancy might be partly explained by the different route of administration (inhaled vs oral) and/or the different disease severity (stable vs exacerbated asthma).

LTB4, but not LTE4, concentrations in EBC are elevated in patients with COPD [Montuschi et al. 2003d; Kostikas et al. 2003]. Steroid-naïve and steroid-treated patients with stable COPD have similar LTB4 concentrations in EBC [Montuschi et al. 2003d]. In patients with COPD exacerbations, LTB4 concentrations in EBC were decreased 2 weeks after treatment with antibiotics and this effect was maintained after 2 months [Biernacki et al. 2003]. LTB4 concentrations in EBC are also increased in patients with cystic fibrosis exacerbations and are reduced after 2 weeks of antibiotic treatment [Carpagnano et al. 2003b].

Prostanoids

PGE2 concentrations in EBC are elevated in steroid-naïve and steroid-treated patients with stable COPD and correlate with LTB4 concentrations in EBC indicating that the greater the lung inflammation, the higher the production of PGE2 which may have anti-inflammatory effects in the airways [Montuschi et al. 2003d]. PGE2 concentrations in EBC in steroid-naïve and steroid-treated patients with COPD were similar [Montuschi et al. 2003d]. Noneselective inhibition of COX by ibuprofen reduces PGE2 concentrations in EBC in patients with COPD, whereas rofecoxib, a selective COX-2 inhibitor, has no effect [Montuschi et al. 2005b] with potential implications for the modulation of airway inflammation in COPD. There was no difference in PGE2 concentrations in EBC between patients with asthma and healthy subjects [Mondino et al. 2004; Baraldi et al. 2003] and between patients with asthma who were either steroid-naïve or treated with inhaled glucocorticoids [Mondino et al. 2004; Baraldi et al. 2003a].

TxB2, the stable hydrolysis product of TxA2, a potent bronchoconstrictor, was detected in some patients with asthma who were steroid-naïve [Montuschi and Barnes, 2002b], whereas it was not detected in either patients with COPD or healthy subjects [Montuschi et al. 2003d]. Other authors reported detectable TxB2 concentrations in EBC in most of the healthy subjects [Huszar et al. 2005].

PGD2 and PGF2α, which are bronchoconstrictors, were detected in some patients with asthma and healthy subjects [Montuschi and Barnes, 2002b]. In those subjects, in whom these eicosanoids were detectable there was no difference in their concentrations between the two groups [Montuschi and Barnes, 2004b].

pH

Measurement of pH is very reproducible and might prove clinically useful for the diagnosis and monitoring of pharmacological therapy in inflammatory airway diseases [Hunt, 2002; Vaughan et al. 2003].

pH of deaerated EBC is over two log orders lower in adults with acute asthma than in healthy subjects and normalizes with glucocorticoid therapy [Hunt et al. 2000]. pH values in EBC in children with acute asthma are lower than those in children with stable asthma and normalize with inhaled glucocorticoid therapy [Brunetti et al. 2006]. Treatment with lansoprazol, a proton pump inhibitor, for two months increases pH values in EBC in patients with moderate asthma and gastroesophageal reflux disease, but not in asthmatic patients without gastroesophageal reflux disease [Shimizu et al. 2007]. Mean pH values are lower in patients with COPD than in patients with asthma and healthy subjects [Kostikas et al. 2002]. One study reported no difference in EBC pH between children with and without parentally reported symptoms suggestive of asthma and no consistent association between EBC pH and lung function, AHR, and airway inflammation [Nicolau et al. 2006]. In patients with COPD, pH values in EBC are negatively correlated with sputum neutrophils and hydrogen peroxide concentrations in EBC [Kostikas et al. 2002]. In one study, there were no differences in pH values between patients with COPD who were steroid-naïve and
those who were treated with inhaled glucocorticoids [Kosikas et al. 2002]. EBC pH can be continuously monitored in mechanically ventilated patients and pH values normalize with recovery [Walsh et al. 2006]. pH values in EBC are lower in patients with allograft rejection after lung transplantation compared with those without rejection or healthy subjects [Dupont et al. 2006].

The effect of inhaled glucocorticoids on hydrogen peroxide concentrations in EBC in patients with COPD has not been established [Ferreira et al. 2001; van Beurden et al. 2003a]. One double-blind crossover placebo-controlled study performed in 20 stable nonsmoking patients with COPD showed that beclomethasone at a dose of 500 µg twice daily for 2 weeks had no effect on hydrogen peroxide concentrations in EBC [Ferreira et al. 2001]. In contrast, another study reported that glucocorticoids with more peripheral deposition (HFA-beclomethasone dipropionate at a dose of 400 µg twice daily for 4 weeks) and with more central deposition (fluticasone dipropionate at a dose of 375 µg twice daily for 4 weeks) reduced hydrogen peroxide concentrations in EBC in patients with stable moderate COPD [van Beurden et al. 2003a]. In 25 patients with COPD who were admitted to hospital because of exacerbations due to lower respiratory tract infections, exhaled hydrogen peroxide concentrations were not reduced during treatment with intravenous dexamethasone, inhaled salbutamol/ipratropium and antibiotics as needed [Kasielski et al. 2001]. In a double-blind, double-dummy, placebo controlled, parallel group study in 44 patients with stable COPD, treatment with oral N-acetyl-cysteine, which can have antioxidant effects, at a dose of 600 mg a day for 12 months reduced hydrogen peroxide concentrations in EBC starting from 9 months [van Beurden et al. 2003b]. Despite this evidence of reduced oxidant stress, a multicenter randomized placebo controlled trial has shown that N-acetyl-cysteine is ineffective at prevention of deterioration in lung function and prevention of exacerbations in patients with COPD [Decramer et al. 2005]. Moreover, hydrogen peroxide concentrations in EBC in patients with COPD are transiently increased 30 min after nebulization of N-acetyl-cysteine.

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Elevated levels of hydrogen peroxide in EBC are reduced, but not normalized, after two months of antibiotic treatment in patients with active tuberculosis [Kwiatkowska et al. 2007].

The clinical significance of these findings is currently unknown. More controlled studies to establish the effects of pharmacological therapy on exhaled hydrogen peroxide in patients with COPD are required. As with other biomolecules, a reliable methodology for EBC analysis is required for planning and interpreting the results of interventional studies.

Nitrogen reactive species
High levels of nitrite/nitrate have been found in EBC of adults [Ganas et al. 2001] and children with asthma [Ratnawati et al. 2006]. In one study, nitrite/nitrate concentrations in EBC were lower in adults with asthma who were treated with inhaled glucocorticoids compared with those in steroid-naïve adults with asthma [Ganas et al. 2001]. Increase in nitrite concentrations in EBC is more pronounced during asthma exacerbations [Hunt et al. 1995] and severe asthma [Corradi et al. 2001]. In a double blind, parallel group, placebo controlled, randomised trial, inhaled budesonide at a dose of 100 µg or 400 µg once daily for 3 weeks caused a rapid decrease in nitrite/nitrate concentrations in EBC which was not dose-dependent [Kharitonov et al. 2002]. Nitrite/nitrate concentrations returned to baseline values one week after treatment withdrawal [Kharitonov et al. 2002].

A significant reduction in exhaled S-nitrosothiols, which are derived from interaction of NO or reactive nitrogen species with thiol groups, compared with pretreatment levels was only seen at the end of weeks 1 and 3 of treatment with high dose budesonide [Kharitonov et al. 2002]. However, whereas reduction in nitrite/nitrate concentrations in EBC is likely to contribute to the anti-inflammatory effects of glucocorticoids in the airways, the reduction in S-nitrosothiols might have a different biological significance as S-nitrosothiols have bronchodilator effects and reduced concentrations of these compounds in the airways has been causally linked to acute respiratory failure in children [Gaston et al. 1998].

Some studies reported elevated levels of 3-nitrotyrosine in EBC in both adults [Hanaazawa et al. 2000] and children with asthma [Baraldi et al. 2006] and in patients with cystic fibrosis [Balint et al. 2001]. In a single-blind placebo-controlled study, inhaled flunisolide at a dose of 800 µg a day for 8 weeks reduced 3-nitrotyrosine concentrations in EBC in children with mild-to-moderate asthma [Bodini et al. 2006]. However, a study in which 3-nitrotyrosine was measured with both GC/MS and HPLC showed that free 3-nitrotyrosine in EBC fails as a marker for oxidative stress in children with stable cystic fibrosis and asthma [Celio et al. 2006] although larger study groups are required to draw definitive conclusions.

Other markers
Using immunoassay kits, cytokines including interleukin (IL)-1β, [Scheideler et al. 1993; Sack et al. 2006] tumor necrosis factor-α (TNF-α) [Scheideler et al. 1993; Sack et al. 2006], IL-4 [Shahid et al. 2002; Matsunaga et al. 2006; Robroeks et al. 2006], IL-5 [Proflat et al. 2006], IL-6 [Sandrini et al. 2003; Sack et al. 2006; Rozy et al. 2006], IL-8 [Cunningham et al. 2000; Sack et al. 2006; Matsunaga et al. 2006], IL-10 [Sack et al. 2006; Robroeks et al. 2006], IL-12p70 [Sacks et al. 2006] insulin-like growth factor-1 [Rozy et al. 2006], interferon-γ, [Shahid et al. 2002; Robroeks et al. 2006] plasminogen activator inhibitor-1 [Rozy et al. 2006] were detected in EBC in patients with different lung diseases. However, concentrations of cytokines are generally close to the detection limit of the immunoassay [Buccioni et al. 2003; Cunningham et al. 2000; Shahid et al. 2002; Carpagnano et al. 2003b; Robroeks et al. 2006]. At these concentrations the analytical variability is high and the reliability of data is questionable. As with other immunoassays matrix effects due to differences in composition between assay buffer and EBC may play a role.

Using a chemiluminescence-based membrane protein array, expression of IL-4, IL-8, IL-17, TNF-α, RANTES, interferon-γ-inducible protein 10, transforming growth factor-β, and macrophage inflammatory protein 1α and 1β in EBC were found elevated in steroid-naïve
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patients with asthma [Matsunage et al. 2006]. However, using flow cytometry, IL-2, IL-4, interferon-\(\gamma\), and IL-10 were detected only in 16, 16, 11, and 9%, respectively, of all samples in patients with asthma and cystic fibrosis at EBC concentrations which were close to the detection limit of the assay [Robroeks et al. 2006]. Using a multiplex fluorescent bead immunoassay (cytometric bead array), IL-1\(\beta\), IL-6, IL-8, IL-10, TNF-\(\alpha\), and IL-12p70 have been found elevated in EBC patients with acute lung injury compared with healthy subjects [Sack et al. 2006]. However, cytokines concentrations in EBC were in the order of few pg/ml. Protein array systems are convenient for analyzing several biomolecules at the same time, but the development of more sensitive assays and the identification of cytokines in EBC with reference analytical techniques still stand.

Keratin concentrations have been detected in EBC [Jackson et al. 2007; Gianazza et al. 2003] and found elevated in healthy smokers [Gianazza et al. 2003].

Some authors reported that DNA can be detected in EBC [Gessner et al. 2004; Carpagnano et al. 2005] whereas other authors failed to detect Pseudomonas aeruginosa and B. Cepacia by PCR in the EBC of patients with cystic fibrosis [Vogelberg et al. 2003]. Whether gene expression analysis in EBC will have diagnostic relevance is not known.

Conclusions
Measurement of biomolecules in EBC might provide insights into the pathophysiology of lung diseases. Selective profiles of inflammatory mediators in EBC might have a differential diagnostic value in respiratory medicine. As it is completely noninvasive, analysis of EBC is potentially useful for assessing lung inflammation, monitoring pharmacological therapy, testing new drugs for lung diseases, and clarifying the mechanism(s) of action of the existing drugs, providing a more rational pharmacological basis for their administration. However, EBC analysis is currently limited to research purposes, due to the lack of standardization and methodological limitations that make it difficult to compare data from different laboratories. Future research in this area should include: the identification of reference values for the different inflammatory markers; large longitudinal studies to ascertain if sequential EBC analysis in the individual patient reflects the degree of inflammation and/or disease severity; studies of the relationships between EBC markers and symptoms, pulmonary function, and other indices of airway inflammation (e.g. exhaled NO and eosinophil counts in sputum); the accurate quantitative assessment of biomolecules and the identification of profiles of biomolecules in EBC in different lung diseases; large prospective controlled interventional studies to establish the usefulness of EBC analysis for monitoring pharmacological therapy in lung diseases, as the available studies are mainly cross-sectional; studies to establish the usefulness of EBC analysis for predicting therapeutic response and assessment of new drugs; identification of other biomolecules in EBC; studies to establish the feasibility and significance of gene expression analysis in EBC; studies on drug disposition (e.g. antibiotics); studies on formation of EBC, its origin in the respiratory system and its relationships with airway lining fluid.

Whether and when EBC analysis will be applicable in the clinical setting cannot be anticipated. Due to the important pathophysiological role of inflammation in lung diseases such as asthma and COPD, the relative lack of noninvasive techniques for monitoring airway inflammation and therapy, and the importance of its potential applications, further research on EBC analysis is warranted.

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Conflict of interest statement
The authors declare that there is no conflict of interest.

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