

Review Article

Exhaled breath condensate in children: Pearls and pitfalls

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Exhaled breath condensate (EBC) is a rapidly growing field of research in respiratory medicine. Airway inflammation is a central feature of chronic lung diseases, like asthma, cystic fibrosis, bronchopulmonary dysplasia and primary ciliary dyskinesia. EBC may be a useful technique for non-invasive assessment of markers of airway inflammation. The non-invasive character of EBC 'inflammometry' and the general lack of appropriate techniques makes it particularly interesting for paediatrics.

We provide a detailed update on the methods currently used for EBC collection and measurement of mediators. We emphasize on paediatric data. The apparent simplicity of the EBC method must not be overstated, as numerous methodological pitfalls have yet to overcome. Comparison and interpretation of data on this rapidly growing field of research is mainly hampered by the lack of standardization and the lack of specific high-sensitivity immunochemical or colorimetric assays. The initiative of the European Respiratory Society to institute a task force on this topic is a first step towards a uniform technique of EBC. Meanwhile, when using this technique or when interpreting research data, one should be fully aware of the possible methodological pitfalls.

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Coughing, wheezing and dyspnoea are common presenting respiratory symptoms in children. They constitute a major diagnostic challenge, not only due to the coexistence of different types of inflammatory lung diseases in infancy and childhood, like asthma, cystic fibrosis (CF), bronchopulmonary dysplasia and primary ciliary dyskinesia (PCD), but also due to the lack of appropriate diagnostic techniques in young children (1–4).

Markers of airway inflammation may help to: distinguish between different inflammatory phenotypes, assess the severity of airway inflammation, monitor customized treatment, and develop new therapeutic strategies, resulting in more accurate use of anti-inflammatory medication. Furthermore, it may improve compliance and disease control, and may even lead to new pathophysiological insights (5). Especially in young children, there is a strong need for a

reliable non-invasive assessment of airway inflammation or 'inflammometry'.

The collection of exhaled breath condensate (EBC) has been proposed as a simple, quick, safe, comfortable and non-invasive technique suitable for assessment of airway inflammation by obtaining samples from the lower respiratory tract of humans with inflammatory lung disease at any age, without disturbing the ongoing inflammation (6–13).

Exhaled breath condensate 'inflammometry' is particularly interesting for paediatrics. The purpose of this review is to facilitate comparison and interpretation of data on this rapidly growing field of research, by assessing its pearls and pitfalls. Therefore, we provide a detailed update on the methods currently used for EBC collection and measurement of mediators. If available, we emphasize on paediatric data,

from full papers rather than abstracts (which are known not to have undergone peer review and, therefore, are marked with an asterisk).

Principles of EBC

Exhaled breath is saturated with water vapour, which will condensate by breathing through a cooling or freezing system. Although the condensate consists mostly of water vapour, it also contains aerosol particles or respiratory fluid droplets. These droplets are released from the lung epithelial lining fluid, and they contain traces of non-volatile solutes, which can be recovered in EBC samples (6, 13–16, 17*, 18*).

The mechanism of formation of aerosol particles has not yet been fully elucidated. Aerosolization of extracellular lining fluid can occur as a result of turbulence in the airways, or during the popping-open of closed respiratory bronchioles and alveoli in the absence of bulk flow (3). The number of aerosol particles formed in the respiratory tract depends on the current velocity of air and the surface tension of extracellular lining fluid: a higher velocity and a lower surface tension favour the production of aerosols (3, 8). The balance between low velocity and high surface tension at alveolar level determines the generation of aerosols. In addition, turbulent flow and higher number of edges in branching points may facilitate aerosol formation (3, 8). The bronchial circulation is essential in humidification of inspired air, which in turn may be a determinant of the rate at which EBC is formed (19). In mechanical ventilation, increased pressure amplitude leads to an increase in EBC volume (per total respired volume), due to increased alveolar closure and reopening. This pressure amplitude-induced shear stress results in generation of aerosolized droplets (20*).

During tidal breathing, concentrations of aerosol particles range between <0.1 and 4 particles/cm³, depending upon the subject and its activity. Subjects at rest exhale lower concentrations of particles, whereas exercise causes an increase in the number of exhaled particles. Their mean diameter is <0.3 μm , and few particles ($<2\%$) have diameters >1.0 μm (6, 21, 22).

Various inflammatory diseases and each of their different stages may influence aerosol formation in a particular way. In asthma, laser spectroscopy of EBC showed different particle distribution at different stages of disease: preponderant small particles (diameter ranging from 51 to 200 nm) in severe forms of asthma, and more large particles (diameter >600 nm) in mild and moderate asthma (23*).

The exact location where aerosol particles originate in the respiratory tract and their proportional contribution to EBC composition is not fully understood (24*–26*). To our knowledge, no paediatric data on this subject have been published.

Collection of EBC

Condenser systems

Currently, different research groups use different condenser systems, all based on the same principle of cooling exhaled air (27–31, 32*, 33*). Most widely used designs include immersion of a Teflon-lined tubing in an ice-filled bucket, and a specially designed double-wall borosilicate glass condenser system (Fig. 1). Commercially available condensers are ECOScreen (Erich Jaeger GmbH, Hoechberg, Germany) and Rtube breath condensate collector (Respiratory Research Inc., Charlottesville, VA, USA). Although cooling of exhaled air, conducted through a Teflon-lined tubing, can be readily achieved using wet ice as well as dry ice or liquid nitrogen, complete obstruction of the collection tube has been reported (8). The ECOScreen system conducts exhaled air through a lamellar condenser with a sample collection vial inserted into a cooling cuff (electric refrigerator) (12). Alternative methods are the use of countercurrent circulating ice water

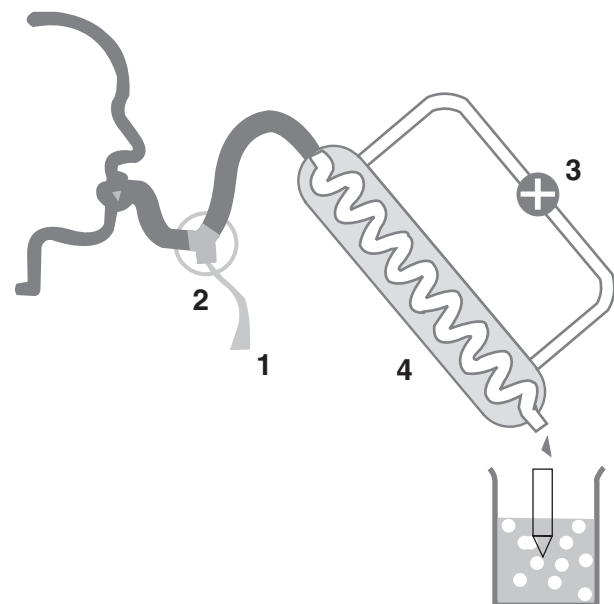


Fig. 1. Schematic representation of an exhaled breath condensate collection device: (1) inhaled room air, (2) two-way non-rebreathing valve, (3) counter-current circulating ice-water pump and (4) double-wall glass condenser.

for cooling of exhaled air which is conducted through a double-wall borosilicate glass condenser system, or the portable Rtube breath condensate collector which consists of a re-usable and pre-cooled aluminium sleeve, placed over the outside of a disposable vertical condensation or collection (polypropylene) tube, and connected to two one-way valves and a mouthpiece. The latter systems are not associated with the problem of complete obstruction.

Minimal or no adaptation is necessary for use of these condenser systems either in children, or in mechanically ventilated subjects, which are connected through the expiratory limb of the ventilatory tubing (27).

The main obstacle for developing a standardized sampling device, may be the variety of physical characteristics associated with each condenser system design. The coating or adhesive properties of the inner surface of the condenser system and even of the collection container and/or the aliquot vials have to be compared, because this can influence the concentration of the inflammatory mediator in the collected condensate. Ideal coating properties for one specific type of mediator(s) may not hold for another. When analysis of bioactive lipids is planned, polypropylene should be considered for collection to avoid the problem of adsorption that can occur with borosilicate glass or polystyrene containers (8).

Nasal collection of EBC is reported in minimal co-operative patients like children aged 4 weeks and older for the analysis of hydrogen peroxide (34). Nasal prongs are serially connected to two polypropylene tubes, submerged in a cold trap, and in turn connected to an electric air suction pump. This procedure allows the collecting of only a fraction of about 20–30% of the exhaled breath water vapour (34). However, contamination with nasal secretions may interfere with the actual concentration of the mediator in the lower respiratory tract.

EBC collecting procedure

Minimal co-operation from the subjects is required for the technique of EBC collection. In children, the main issue for inclusion for EBC sampling is whether they are able to use a mouthpiece properly for 10–15 min. Adequate instruction of the procedure and distraction of the child, for example by using video cartoons, is important. Collecting EBC is not only a simple procedure, but also a safe one. No adverse effects were seen in an EBC collection study in 93 healthy children aged 8–13 years (35). In a study of 91 children with asthma of varying severity, the EBC

procedure proved to be safe, even in children with an asthma exacerbation. The success rate was 100% starting from 4 years of age (36).

Currently, the most widely used procedure is to breath tidally and directly into the tubing system. Often, a mouthpiece is used, connected to a two-way non-rebreathing valve, which also prevents potential re-breathing of exhaled breath samples. Minute ventilation (or total respired volume) and breathing pattern are the major determinants of EBC volume over time (37–40, 41*). It is suggested to describe these respiratory parameters per EBC measurement, because their influence on mediator concentration is not yet known. Exhalation flow may influence the concentration of biomarkers in EBC as it does in exhaled nitric oxide (NO) measurements. Therefore, assessing the flow dependence of different biomarkers is crucial for the standardization and reproducibility of measurements. On the basis of the flow dependence, EBC sampling will be carried out either at a constant flow rate for a fixed time, or at a constant total exhaled volume for a variable time (5).

The use of nose clips in the prevention of contamination of the EBC with nasal air remains controversial (9, 12). Application of nose clips could open the nasopharyngeal velum, thereby contaminating the exhaled breath with nasal air. This could be relevant when measuring mediators, such as eicosanoids, which are also formed in the nose and paranasal sinuses (8, 12). Alternatively, a face mask that separates nasal from oral exhaled air can be used. In infants, who are preferential nasal breathers, collection of EBC via a face mask is feasible (42*). However, significant nasal contribution to total nitrite/nitrate was found, suggesting to limit EBC analysis in infants to compounds not affected by nasal contribution, or to limit EBC collection to sedated infants with induced oral breathing (42*).

The influence of paranasal sinuses is shown in a study on NO and humming, which generates an oscillating airflow and enhances paranasal sinus ventilation (43). Humming increases significantly (15-fold) nasal NO levels compared with quiet exhalation (43). Prostanoid levels in healthy subjects are the same with or without using a nose clip (44*). The volume of EBC is larger when subjects inhale through the nose, but mediator levels of adenosine, hypoxanthine, inosine, ammonia and thromboxane B2 are the same as with oral breathing (45).

Salivary contamination influences the level of several markers that are detectable in EBC. Therefore, prevention of salivary contamination is important. This can be achieved by using a

gravity-saliva trap, rinsing of the mouth/mouth-washing with sodium bicarbonate 4.5% prior to collection, periodic swallowing of the accumulated saliva to maintain a dry mouth during collection, and prevention of coughing or forced expiration (8, 46*). Amylase and phosphorus measurements can be used to exclude salivary contamination of the condensate (6, 15, 47*).

Fluctuations of environmental and breath temperature during the sampling period may influence the preservation of some labile lipid mediators (9). Leukotriene (LT)-B₄ and 8-isoprostane values, but not ammonia and nitrite, are independent of collection temperature in healthy subjects (48*). Furthermore, environmental ozone concentration at the time of collecting samples is significantly related to the hydrogen peroxide concentration in EBC (49*). A study on the short-term exposure to ozone showed induction of airway inflammatory mediators: an immediate increase in hydrogen peroxide and 8-isoprostane levels in EBC, and a delayed increase (at 18 h after exposure) of LT-B₄ and thiobarbituric acid reactive substances (TBARs) (50).

It is unclear whether a circadian rhythm in mediator concentrations exists (51*–53*, 54). Afternoon collection of EBC may increase or maximize the volume of EBC (55*).

The duration of EBC collection is variable. In general, a collection time of 15–20 min will yield 1–3 ml of EBC in children aged 4 years and over (unpublished data). A rapid 2-min method of EBC collection for ammonia and pH measurements yields 0.25–0.35 ml (3, 56*).

In summary, an optimal procedure for one specific mediator may not hold for another, emphasizing that during EBC collection one should systematically run through a checklist of possible influencing factors or co-determinants (Table 1).

Preservation of EBC

In general, condensate is collected in small containers 'on ice'. This method allows subsequent pipetting of separate aliquot vials. This is not possible when the condensate is collected in frozen condition, as with the ECOscreen. The temperature of the cooling sleeve of the Rtube breath condensate collector can be chosen by the investigator, and depends on the temperature of the refrigerator where the sleeve was stored in (3).

Subsequently, each separate aliquot vial can in turn rapidly be frozen and stored at –20 to –70°C until analysis is performed. In general, an EBC sample volume of 0.05–0.5 ml is sufficient to perform one of the currently used assays.

Table 1. Checklist of procedure for exhaled breath condensate collection

Condenser
System
Coating
Cooling temperature
Subject
Age
Sex
Weight
Length
Disease (smoking/upper airway infection)
Medication
Breathing pattern (tidal breathing/forced exhalation)
Respiratory parameters (frequency/minute ventilation/total exhaled volume)
Flow
Breath temperature
Lung function parameters
Noseclip/face mask
Prevention of salivary contamination (gravity trap/rinsing mouth/swallowing)
Environment
Temperature
Ozone
Humidity
Collection
Date/hour
Phase of sample (solid/fluid)
Duration
Adverse effects
Volume of exhaled breath condensate
Preservation
Aliquot vial volume
Sample numbers
Products added
Storage temperature
Storage time
Analysis
Date
Assay
Detection limit

Sequences of changing phases (gas–fluid–frozen) may influence the concentration of some unstable EBC constituents, and should be kept limited. The value of high velocity freezing processes (for example submerging the aliquot vials in a solution of isopentane) needs to be investigated, as well as the effect of techniques to prevent rapid degradation of instable proteins or cytokines (by adding bovine serum albumin, phosphate buffer solution or proteinase inhibitors). However, bovine serum albumin may disturb the determination of some mediators, as nitrite and hydrogen peroxide. Furthermore, storage time and temperature itself may also be biomarker-dependent. Acceptable storage conditions have to be evaluated.

Analysis of EBC

Exhaled breath condensate is a human body fluid that contains not only aerosol particles

or respiratory fluid droplets with traces of non-volatile solutes, but also large amounts of pure water vapour. Therefore, changes in the concentration of inflammatory mediators in EBC may vary by a factor 100 or more, depending on variations in the concentration gradient between respiratory fluid droplets and water vapour.

The concentration of mediators or non-volatile substances in the respiratory tract lining fluid may be calculated by a method reported by Effros and co-workers, which is based upon urea or electrolyte concentrations in the condensate (14, 18*, 57, 58*, 59*). These measurements of condensate reference indicators may permit a semi-quantitative analysis of non-volatile substances in respiratory lining fluid, but need further modification (60*).

Currently, samples of EBC are mostly analysed by commercially available and sensitive assays, developed for use in other media with higher mediator concentrations than condensate. Their lower limits of detection are usually situated near the reported concentrations of markers in EBC. EBC measurements can be made more reliable by the development of new, high-sensitivity assays, adapted to the unique nature of EBC (3, 14, 61, 62). However, current immunochemical or colorimetric assays lack reference methods and are affected by poor sensitivity, specificity and selectivity (63). These assays need to be validated with more specific analytical methods, such as gas chromatography/mass spectrometry or high-performance liquid chromatography (5, 63). These techniques are considered the reference techniques for the determination of complex mixtures of organic compounds present at nmol levels in aqueous matrices (62, 63). Recently, modified analytical methods have also been described to quantitate low concentrations of the nitrite/nitrate balance as well as free 3-nitrotyrosine in EBC (64*, 65*).

Freeze-drying may be of additional value to facilitate subsequent biochemical analysis of certain non-lipid mediators.

The first study identifying surface-active properties of EBC was published in Russia in 1980 (66). In the 1990s, this technique was rediscovered (6). Since then, several inflammatory mediators have been identified in EBC. However, results from studies using a wide variety of inflammatory markers have only shown group differences between specific patient populations and healthy control subjects, but evidence for the diagnostic use of these markers in individual patients is scarce (10). Furthermore, correlations between mediators in EBC and other biological measurements of lung inflammation, as well as

the level of disease control have not yet been demonstrated sufficiently. Recently, in an animal model, a similar pattern of nitrite and total protein levels was found in EBC and bronchoalveolar lavage fluid (67*).

Although it is presently an area of active research and an increasing number of abstracts (*) are being presented, few studies have been published on children (28, 35, 36, 63, 68–75). We reviewed the available data on this age group until June 2003.

Hydrogen peroxide

Oxidative stress describes an imbalance between oxidants (reactive oxygen species, ROS) and antioxidants (superoxide dismutase, catalase, glutathione, uric acid, lactoferrin, vitamin C and vitamin E) in favour of oxidants, which contributes to the pathogenesis of several inflammatory lung diseases (76). The development of such an imbalance in lung inflammation may activate redox-sensitive transcription factors such as nuclear factor-kappa B and activator protein-1, which regulate the genes for proinflammatory mediators and protective antioxidant genes (77). Activation of inflammatory and structural cells result in an increased production of ROS. Superoxide anions are generated by nicotinamide adenine dinucleotide phosphate oxidase and then converted to hydrogen peroxide by superoxide dismutases. Hydrogen peroxide is then degraded to water by catalases. Superoxide anions and hydrogen peroxide may generate the highly reactive hydroxyl radicals, leading to peroxidative damage of airway structures and formation of volatile TBARs. Hydrogen peroxide is less reactive than other ROS, and because it is soluble, increased hydrogen peroxide in the airway equilibrates with air (9, 12, 78).

Exhaled hydrogen peroxide has potential as a marker of oxidative stress in the lungs. Exhaled hydrogen peroxide is less stable than other markers, and its concentration appears to be expiratory flow rate dependent (79). Furthermore, symptomatic upper respiratory tract infection may act as a confounder in studies on hydrogen peroxide and chronic lower airway inflammation (80). No significant circadian rhythm in hydrogen peroxide levels was reported in healthy subjects (52*). However, concentrations of exhaled hydrogen peroxide showed diurnal variation in both stable chronic obstructive pulmonary disease patients and healthy controls (54, 81). Hydrogen peroxide is measured in EBC by the spectrophotometric method or the fluorometric method with horseradish peroxidase

(82). Recently, some new techniques have been described: a fast chemiluminescent method for hydrogen peroxide measurement in EBC (83), on-line measurement of hydrogen peroxide based on a biosensor with electrochemical detection and a detection limit of 12.5 nmol (84*–86*) and a sensitive (0.02 µmol) fluorometrical method with a stable storage time of 40 days (61).

Paediatric EBC data on hydrogen peroxide are summarized in Table 2.

Reference data for exhaled hydrogen peroxide have been described in a group of 93 healthy children (35). There was no correlation between the levels of exhaled hydrogen peroxide and age, sex, or lung function in healthy children (35). Hydrogen peroxide levels in a study on 102 healthy children was influenced by atmospheric NO (73).

Exhaled hydrogen peroxide levels were elevated in asthmatic children compared with healthy controls (28, 69). Higher levels of hydrogen peroxide correlated with severity of asthma (87*). Besides diagnostic purposes, hydrogen peroxide may be used to monitor therapy. Little data are available on this topic. However, hydrogen peroxide levels were increased in 16 children with an acute infectious pulmonary exacerbation of CF, and significantly decreased in EBC during intravenous antibiotic treatment (70).

In mechanically ventilated children with various non-specified underlying conditions, hydrogen peroxide was detected in all EBC specimens, but did not correlate with the severity of illness (88*).

Eicosanoids

Eicosanoids are potent mediators of inflammation, which are derived from arachidonic acid.

They include prostaglandins (PGs), thromboxane (Tx), isoprostanes, LTs and lipoxins (LXs).

Prostaglandin and Tx are formed through increased expression of inducible cyclo-oxygenase-2. Most PGs and Tx have proinflammatory properties. PGE2 and PGI2 are anti-inflammatory.

Leukotrienes are lipid mediators derived from arachidonic acid via the 5-lipoxygenase pathway. LTs are potent constrictors and proinflammatory mediators that contribute to the pathophysiology of asthma (9). The cysteinyl-LTs (LTC4, LTD4 and LTE4) are generated predominantly by mast cells and eosinophils. By contrast, LTB4 has potent chemotactic activity towards neutrophils (9, 12).

Isoprostanes are formed by free radical-catalysed lipid peroxidation of arachidonic acid during oxidative stress (9, 12, 78). They are initially esterified in membrane phospholipids, from which they are cleaved by a phospholipase A2 (9). F2-isoprostanes are not only relatively stable and specific markers of lipid peroxidation, but also possess potent biological activity (89). They can be mediators of the cellular effects of oxidant stress and are a reflection of complex interactions between the reactive nitrogen species and ROS (8, 9, 89). The most prevalent isoprostane in humans is 8-epi-PGF2-α, also known as 8-isoprostane (9).

LXA4 and LXB4 have potential anti-inflammatory action in asthma, and LXA4 is detected in supernatant of induced sputum (90).

Eicosanoids are measured by enzyme immunoassays (EIA), using commercially available detection kits. Recently, validation of measurement of 8-isoprostanes, PGE2 and LTB4 in EBC by reverse-phase high-performance liquid

Table 2. Hydrogen peroxide in EBC of children, measured by fluorimetric assay (with horseradish peroxidase)

Disease	Median value patient group (µmol)	Median value healthy controls (µmol)	p value	Reference	Lower limit of detection (µmol)
Asthma	0.81 (†)	0.25 (†)	0.013	28	–
Asthma, stable	0.54 (†)	–	–	28	–
Asthma, acute	1.50 (†)	–	<0.001 (vs. stable asthma)	28	–
Asthma	0.60	0.15	<0.05	69	0.1
Asthma, no ICS	0.80	0.15	<0.01	69	0.1
Asthma, with ICS	0.45	0.15	n.s.	69	0.1
Asthma, severe	149.5 (‡)	10.9 (‡)	<0.001	87*	–
CF, acute infective	0.28	–	–	70	0.01
CF, after AB i.v.	0.16	–	0.002 (vs. acute infective)	70	0.01
Ventilated	0.45	–	–	88*	0.083
Healthy	–	0.13	–	35	0.01
Healthy	–	0.49 (§)	–	34	–
Healthy	–	0.51	–	73	0.05

EBC, exhaled breath condensate; †, µmol/l; ‡, µmol/ml; §, nasally collected EBC; CF, cystic fibrosis; AB i.v., antibiotics intravenously; ICS, inhaled corticosteroids; n.s., not significant.

chromatography or by gas chromatography/mass spectrometry has been reported (12, 91, 92*, 93*). Exhaled 8-isoprostane and PGE2 are not flow dependent in healthy subjects (94*). The EBC levels of PGE2 and TxB2 in healthy subjects with or without nose clip are comparable (44*).

Paediatric EBC data on eicosanoids are summarized in Tables 3–6.

In asthmatic children, elevated eicosanoids were found compared with healthy children (74, 95*–101*). An inverse correlation between exhaled cysteinyl-LTs and LTB4 in children with mild persistent asthma was assessed, which may reflect the contrary effect of steroids on eosinophil and neutrophil survival (74). No significant diurnal variation in cysteinyl-LT levels was found in asthmatic children (51*). Reduced

cysteinyl-LTs were reported after 3 months of house dust mite avoidance in allergic asthmatic children (102*). After a course of oral steroid therapy, a reduction was seen in cysteinyl-LTs and 8-isoprostanes of children with an asthma exacerbation (75). LTB4 was significantly increased after isocapnic hyperventilation with cold air in children with stable asthma, but not in the control group (103*, 104*). No correlation with spirometric measurements was found (74, 96*–99*).

In children with severe atopic eczema but without asthma, increased cysteinyl-LTs were found in EBC (105*).

In children with CF and PCD, cysteinyl-LTs were detected in EBC (106*, 107*).

Decreased concentrations of PGE2 were reported in children with PCD, compared with

Table 3. 8-isoprostanes in EBC of children

Disease	Median value patient group (pg/ml)	Median value healthy controls (pg/ml)	p value	Reference	Assay	Lower limit of detection (pg/ml)
Asthma, acute	12.00	2.60	<0.01	75	EIA	–
Asthma, after OCS	8.40	2.60	<0.001 (<0.05 vs. acute)	75	EIA	–
Asthma, no ICS	9.34	3.81	<0.01	95*	EIA	4
Asthma, with ICS	6.73	3.81	<0.01	95*	EIA	4
Asthma, no ICS	61.90	33.60	<0.01	101*	RIA	–
Asthma, with ICS	64.30	33.60	<0.01	101*	RIA	–
Asthma, with ICS	47.20	34.20	<0.008	99*	RIA	–

EBC, exhaled breath condensate; EIA, enzyme immunoassay; OCS, oral corticosteroids; ICS, inhaled corticosteroids; RIA, radio immunoassay.

Table 4. Cysteinyl-leukotrienes in EBC of children

Disease	Median value patient group (pg/ml)	Median value healthy controls (pg/ml)	p value	Reference	Assay	Lower limit of detection (pg/ml)
Asthma, mild intermittent	19.90	18.50	n.s.	74	EIA†	15
Asthma, mild persistent	27.90	18.50	<0.01	74	EIA†	15
Asthma, moderate to severe	31.50	18.50	<0.05	74	EIA†	15
Asthma, mild	19.40	17.20	–	96*	EIA†	17
Asthma, moderate	22.90	17.20	<0.001	96*	EIA†	17
Asthma, severe	27.70	17.20	–	96*	EIA†	17
Asthma, allergic	14.26	–	–	102*	EIA	–
Asthma, after 3 months HDM-free	4.60	–	<0.01 (vs. allergic asthma)	102*	EIA	–
Asthma, no ICS	12.30	4.90	0.01	97*	EIA†	17
Asthma, with ICS	10.50	4.90	0.01	97*	EIA†	17
Asthma, no ICS	30.50	15.90	<0.05	100*	EIA	–
Asthma, with ICS	31.80	15.90	<0.05	100*	EIA	–
Asthma, acute	12.70	4.30	<0.01	75	EIA†	17
Asthma, after OCS	5.20	–	<0.01 (vs. acute asthma)	75	EIA†	17
Asthma	14.70	–	–	106*	ELISA	4.5
CF	9.30	–	–	106*	ELISA	4.5
PCD	21.70	17.20	–	107*	EIA†	17

EBC, exhaled breath condensate; n.s., not significant; EIA†, enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA); HDM, house dust mite; ICS, inhaled corticosteroids; OCS, oral corticosteroids; ELISA, enzyme-linked immunosorbent assay (DPC Biemann, Bad-Neuheim, Germany); CF, cystic fibrosis; PCD, primary ciliary dyskinesia.

Table 5. Leukotriene-B₄ in EBC of children

Disease	Median value patient group (pg/ml)	Median value healthy controls (pg/ml)	p value	Reference	Assay	Lower limit of detection (pg/ml)
Asthma, mild intermittent	52.7	47.9	n.s.	74	EIA†	4.4
Asthma, mild persistent	126.0	47.9	<0.0001	74	EIA†	4.4
Asthma, moderate to severe	131.9	47.9	<0.0001	74	EIA†	4.4
Asthma, mild	69.8	62.7	—	96*	EIA†	4
Asthma, moderate	137.7	62.7	<0.001	96*	EIA†	4
Asthma, severe	137.5	62.7	<0.001	96*	EIA†	4
Asthma, before IHV	25.8	—	—	103*	—	—
Asthma, after IHV	35.5	—	<0.05 (vs. before)	103*	—	—
Asthma, no ICS	26.3	19.8	n.s.	100*	EIA	—
Asthma, with ICS	24.8	19.8	n.s.	100*	EIA	—
Asthma	58.0	37.8	n.s.	98*	EIA†	—
PCD	54.7	54.5	—	107*	EIA†	4

EBC, exhaled breath condensate; n.s., not significant; EIA† enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA); IHV, isocapnic hyperventilation; ICS, inhaled corticosteroids; PCD, primary ciliary dyskinesia.

Table 6. Prostaglandin-E₂ in EBC of children

Disease	Median value patient group (pg/ml)	Median value healthy controls (pg/ml)	p value	Reference	Assay	Lower limit of detection (pg/ml)
PCD	15.7	19.7	<0.05	107*	EIA	15
Asthma, no ICS	28.8	22.6	n.s.	101*	RIA	—
Asthma, with ICS	24.0	22.6	n.s.	101*	RIA	—
Asthma, with ICS	51.5	46.8	n.s.	99*	RIA	—

EBC, exhaled breath condensate; PCD, primary ciliary dyskinesia; EIA: enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA); ICS, inhaled corticosteroids; n.s., not significant; RIA, radio immunoassay.

healthy controls (107*). No differences were seen in the levels of LTB₄ and cysteinyl-LTs between these children (107*).

Nitrogen-reactive species

Nitrite/nitrate, S-nitrosothiols and 3-nitrotyrosine are nitrogen-reactive species. The reaction between NO and superoxide anions in the airways results in the formation of peroxynitrite, a ROS. Peroxynitrite can be trapped by thiol-containing biomolecules such as cysteine and glutathione, to form S-nitrosothiols or can be oxidized to nitrate and nitrite (9, 12, 78). Peroxynitrite reacts with tyrosine residues in proteins to form the stable end product 3-nitrotyrosine, which is used as a diagnostic tool to identify involvement of NO-derived oxidants in various disease states (8, 9, 12). The balance between nitrite/nitrate, S-nitrosothiols and nitrotyrosine gives insight into NO synthesis and short- and long-term changes in NO production (9, 12).

The nitrite/nitrate balance is determined by colorimetric assay (based on the Griess reaction), by fluorometric assay (based on the 2,3-diaminonaphthalene reaction) or by an ionic chromatograph analyser (108, 109, 110*). Recently, a method based on gas chromatography/mass spectrometry was developed that allows simultaneous quantification of nitrite and nitrate in various biological fluids (111). Furthermore, a modification of the Griess method is described for use with EBC, with a detection limit of 0.640 µmol (64*). Several methods are used for the detection of S-nitrosothiols and nitrotyrosine (112–115). To quantitate low concentrations of free 3-nitrotyrosine in EBC, a mass-selective determination was developed and validated (65*).

Paediatric EBC data on nitrogen-reactive species are summarized in Tables 7 and 8.

In healthy children, nitrite values were not related to the levels of exhaled NO (73).

Although, in 23 children with and without respiratory disease, expiratory flow patterns did not significantly alter mean total nitrite/nitrate levels in EBC, large intra-subject variation was found (116*).

In children with CF, elevated levels of nitrite were found in EBC compared with controls (68, 71). There was no significant correlation between condensate nitrite values and age, lung function indices, chest radiograph score or the use of inhaled steroids (68). Nitrite levels were not correlated with the severity of CF (68). In infants with CF, nitrite was measured using a copper baby-condensate-sampler (33*). However, no statistical difference in nitrite was found between infants with CF and healthy infants (33*). Nitrotyrosine levels did not differentiate between children with CF and healthy subjects, and there was no correlation with lung function (117*).

Table 7. Nitrite in EBC of children

Disease	Median value patient group (μmol)	Median value healthy controls (μmol)	p value	Reference	assay	Lower limit of detection (μmol)
Asthma, no ICS	0.87	0.31	0.009	118*	—	—
Asthma, mild	2.02	0.41	<0.05	71	C	—
Asthma, moderate	3.51	0.41	<0.05	71	C	—
CF, mild	2.16	0.41	<0.05	71	C	—
CF, moderate	1.87	0.41	<0.05	71	C	—
CF, severe	2.13	0.41	<0.05	71	C	—
CF	3.30	1.00	0.018	68	C	0.074
CF	4.13	3.70	n.s.	33*	C	—
PCD	2.40	1.80	n.s.	120*	F	—
Cough	0.75	0.41	n.s.	71	C	—
Healthy	—	3.30	—	73	F	0.1

EBC, exhaled breath condensate; ICS, inhaled corticosteroids; C, colorimetric assay (Griess reaction); CF, cystic fibrosis; n.s., not significant; PCD, primary ciliary dyskinesia; F, fluorimetric assay, 2,3 di-amino-naphthalene.

Table 8. 3-nitrotyrosine in EBC of children

Disease	Median value patient group (ng/ml)	Median value healthy controls (ng/ml)	p value	Reference	Assay	Lower limit of detection (ng/ml)
Asthma, moderate	13.5	5.3	<0.05	119*	EIA	—
CF	1.60	1.69	n.s.	117*	EIA	—

EBC, exhaled breath condensate; EIA, enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA); CF, cystic fibrosis; n.s., not significant.

In asthmatic children, significantly elevated levels of nitrite were found in EBC compared with controls (71, 118*). Nitrite levels were not correlated with age, family history of asthma, anti-inflammatory treatment, number of allergic sensitizations or the severity of asthma (71).

After treatment with inhaled corticosteroids, a significant reduction in nitrite was only observed in asthmatic patients with a clinical improvement (118*). Nitrotyrosine levels were increased in asthmatic children compared with normal children, but no correlation was observed with exhaled NO (119*).

In PCD, levels of nitrite and nitrite/nitrate were not increased (120*).

Other markers of oxidative stress

A key feature in the pathophysiology of inflammatory lung diseases is the development of an imbalance between oxidants (ROS) and antioxidants (glutathione), known as oxidative stress (76).

Glutathione is formed by the selenium-dependent enzyme glutathione peroxidase and it is a vital intra- and extra-cellular protective antioxidant against oxidative/nitrosative stresses, which play a key role in the control of proinflammatory processes in the lung (76, 77). Glutathione is important in immunomodulation,

remodelling of the extracellular matrix, apoptosis and mitochondrial respiration (77). Glutathione can be measured in EBC by modified high-performance liquid chromatography with fluorescence detection (63).

Reactive oxygen species generated close to cell membranes deplete intracellular glutathione and oxidize membrane phospholipids (membrane lipid peroxidation), which may continue in a chain reaction (77). Endogenously generated aldehydic lipid peroxidation products are malondialdehyde, α,β -unsaturated aldehydes and saturated aldehydes (hexanal, heptanal and nonanal). Aldehydes are involved in many of the pathophysiological effects associated with oxidative stress in cells and tissues (77, 121). Malondialdehyde is generated mainly by arachidonic acid and docosahexenoic acid. α,β -unsaturated aldehydes are formed by lipid peroxidation of ω -6 (arachidonic acid and linoleic acid) and ω -3 (oleic acid) polyunsaturated fatty acids. Saturated aldehydes are breakdown products of oxidized linoleic acid and arachidonic acid, palmitoleic acid and oleic acid (63). Malondialdehyde can be quantified as (volatile) TBAR, but the colorimetric assay lacks specificity (63). Recently, malondialdehyde was measured by high-performance liquid chromatography separation and fluorescence detection (with a detection limit of 4.1 nmol) or after derivatization by

liquid chromatography-tandem mass spectrometry (62, 63, 122). In subjects with asthma and chronic obstructive pulmonary disease, the concentration of aldehydes in condensate was not affected by expiratory flow rates, sampling time, total exhaled air volume and total condensate volume (63, 122).

Paediatric EBC data on glutathione and malondialdehyde are summarized in Table 9.

In children with an asthma exacerbation, malondialdehyde levels were higher and glutathione levels were lower compared with healthy controls (63). After a course of oral prednisone, malondialdehyde levels decreased, whereas glutathione levels increased (63). No differences were observed among these marker levels obtained at different flow rates (63). No correlation was found between biomarker levels and spirometric values (63). α,β -unsaturated aldehydes were detectable only in a few EBC samples, but chemically stable saturated aldehydes were detectable in all EBC samples (63).

Furthermore, the observed bi-directional changes in malondialdehyde and glutathione levels in EBC of asthmatic children show that EBC analysis does not simply represent alterations of turbulent airflow and airway obstruction (63).

Cytokines

Cytokines are intercellular low molecular weight (< 80 kDa) immune regulatory peptides involved in controlling the local and/or systemic consequences of injury, inflammation, haematopoiesis and tissue repair. Their effects start by binding to specific high-affinity cell surface receptors that cause alteration of gene expression in the target cells. An increasing number of cytokines are being described. They are categorized as T-cell derived lymphokines, proinflammatory cytokines, anti-inflammatory (inhibitory) cytokines and growth factors. Analysis of cytokines is performed by EIA, using (modified) commercially available detection kits.

Paediatric EBC data on cytokines are summarized in Table 10.

In CF, proinflammatory interleukin(IL)-8 was present in only 30% of the children with clinically stable disease (68). Although the values were higher than in healthy controls, there was no statistically significant difference (68).

In asthmatic children, significantly increased mean IL-4 levels and decreased mean interferon (IFN)- γ levels were found compared with normal children (72). The levels of exhaled IL-4 were lower under steroid treatment (72).

Table 9. Malondialdehyde and glutathione in EBC of children

Mediator	Disease	Median value patient group (nmol)	Median value healthy controls (nmol)	p value	Reference
Malondialdehyde	Asthma, acute	30.2	19.4	0.002	63
	Asthma, after OCS	18.5		0.001 (vs. acute)	63
Glutathione	Asthma, acute	5.96	14.1	<0.0001	63
	Asthma, after OCS	8.44		0.04 (vs. acute)	63

EBC, exhaled breath condensate; OCS, oral corticosteroids.

Table 10. Cytokines in EBC of children

Cytokine	Disease	Median value patient group (pg/ml)	Median value healthy controls (pg/ml)	p value	Reference	Assay	Lower limit of detection (pg/ml)
IL-4	Atopy	1.94	—	—	125*	ELISA†	0.13
	Asthma, atopic	7.68	1.34	—	124*	EIA	—
	Allergic rhinitis	5.36	1.34	—	124*	EIA	—
	Allergic rhinitis	1.78	1.34	n.s.	123*	EIA	—
	Asthma, atopic	1.54	1.34	n.s.	123*	EIA	—
	Asthma, acute	5.67	—	n.s. (vs. atopic asthma)	123*	EIA	—
	Asthma, no ICS	53.7	35.7	<0.05	72	ELISA‡	20
	Asthma, with ICS	37.5		<0.05 (vs. no ICS)	72	ELISA‡	20
IFN- γ	Asthma, no ICS	3.7	5.1	<0.01	72	EIA§	0.5
	Asthma, with ICS	4.1		n.s. (vs. no ICS)	72	EIA§	0.5
IL-8	CF	47	10	n.s.	68	ELISA¶	0.5

EBC, exhaled breath condensate; IL-4, interleukin 4; ELISA, enzyme-linked immunosorbent assay; †, R & D systems, Minneapolis, MN, USA; EIA, enzyme immunoassay; n.s., not significant; ICS, inhaled corticosteroids; ‡, Cayman Chemical, Ann Arbor, MI, USA; IFN- γ , interferon- γ ; §, Pierce Endogen, Rockford, IL, USA; CF, cystic fibrosis; ¶, home made.

In atopic children with stable asthma or allergic rhinitis, the concentrations of IL-4 were statistically not different from those in non-atopic controls (123*, 124*). After allergen exposure or asthma exacerbation, higher levels of IL-4 were found (123*, 125*).

Immunoglobulin E

Immunoglobulin E (IgE) was not demonstrated by nephelometric technique in EBC of children with allergic asthma (32*). However, by EIA, IgE was detected in all samples of atopic children and controls with considerable overlap, values ranging from 20 to 230 IU/ml (123*–126*). After allergen exposure, an increase in IgE in EBC was found (126*).

Eosinophilic cationic protein

In EBC of children with asthma and healthy controls, eosinophilic cationic protein was detected in similar concentration (127*).

Acidity of EBC

The degree of airway acidification or acidopnea may serve as a simple and inexpensive method to monitor respiratory inflammation. However, the measurement of the pH of EBC remains controversial because of methodological issues and the possible influence of salivary contamination (128–135, 136*–139*). Furthermore, the complex determinants of the pH of EBC need to be clarified. A direct comparison between the pH of EBC and *in situ* measurement of bronchial mucosal fluid pH in mechanically ventilated adults showed significantly higher *in situ* pH measurements (140*). These results suggest different anatomopathophysiology, or may reflect that vaporization by itself may favour the lower pH of EBC (140*).

In asthmatic children, no significant correlation between EBC acidity, lung function, exhaled carbon monoxide and exhaled NO was found (141*).

In children with CF, the pH of EBC was decreased during infective exacerbations, but there was no significant difference between stable children with CF and healthy controls (142*). The reduced pH of EBC in children with CF was associated with a lower level of EBC ammonium (143*).

Bacteria and inflammatory cells

Reports on negative detection results are scanty, but their interpretation may guide further research developments.

No bacteria or cells were detectable in EBC of 14 CF patients, aged 7–28 years (144*). Cells could not be demonstrated in freshly obtained EBC of 11 atopic children, aged 6–15 years, as assessed by fluorescence activated cell sorter (own data).

Pearls and pitfalls of EBC

Assessment of inflammatory markers in EBC is a challenging new technique for the diagnosis and management of various lung diseases. The non-invasive character, the direct information on airway inflammation, the relatively easy collection procedure and the general lack of appropriate techniques of pulmonary function testing in young children makes it particularly interesting and potentially useful.

The management of different types of chronic respiratory inflammation in childhood may improve by further developing EBC into an easy, reliable and 'daily practice' clinical tool which may provide us with characteristic profiles of different biomarkers for different stages of inflammatory lung disease. Hence, it may enlarge our understanding of the disease process and may provide the potential for more appropriate customized therapy (10).

Nevertheless, the apparent simplicity of EBC must not be overstated, as numerous methodological pitfalls are identified and several problems still have to be solved. Comparison and interpretation of data on this rapidly growing field of research is mainly hampered by the lack of standardization and the lack of specific high-sensitivity immunochemical or colorimetric assays (Tables 1–10). The application of proteomics, with high resolution two-dimensional gel electrophoresis and microanalysis of protein spots, may allow the recognition of particular protein patterns in different disease and may result in the recognition of new diagnostic proteins or therapeutic targets (4, 145*). Similarly, the use of fluorescence detection and mass spectrometry-related techniques, and of high-performance liquid chromatography, may offer advantages not only in terms of sensitivity, but also in terms of discriminative power and quantitative reliability (63). The initiative of the European Respiratory Society to institute a task force on this topic is a first step towards a uniform technique of EBC.

Future efforts should be directed towards: (i) optimizing the EBC condenser system and the EBC storage procedures; (ii) standardization of the measurement procedures of inflammatory

markers in EBC; (iii) improving the sensitivity of the assays of inflammatory markers in EBC; (iv) validation or correlation of markers against more direct measurements of airway inflammation (biopsies) in groups with various inflammatory disorders and in healthy subjects; (v) reproducibility, normal variation (intra- and inter-day) and reference ranges (age dependent) of the inflammatory marker; (vi) evaluation of the sensitivity and specificity of the various markers or profiles of markers in differentiating inflammatory airway disorders; (vii) predictive value of the various exhaled inflammatory markers for early diagnosis and exacerbations of disease; and (viii) assessing the usefulness of EBC for the guiding of anti-inflammatory treatment, resulting in better outcome of disease.

In this way, it will become clear whether EBC is the challenging new technique, able to improve diagnosis and management of various chronic respiratory disorders in childhood.

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