

The development of exhaled breath condensate: a noninvasive method of measuring airway inflammation

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THE DEVELOPMENT OF EXHALED BREATH CONDENSATE
a noninvasive method of measuring airway inflammation

Philippe Pieter Roger Rosias

THE DEVELOPMENT OF EXHALED BREATH CONDENSATE
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PROEFSCHRIFT

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CHAPTER 1

GENERAL INTRODUCTION

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General Introduction

1.1 Respiratory disorders in children.

Children differ from adults, and have unique medical needs. It is essential to understand that the developing child is not only undergoing alveolisation, until the age of two to four years, and lung growth, but also embodies complex interactions of many processes, both endogenous, such as genetic background and immune maturation, as well as exogenous, including environmental influences and influences *in utero*.¹⁻¹⁰ Furthermore, it is equally essential to comprehend that evidence-based medicine has become the 'gold standard' for clinical practice and treatment guidelines.¹¹⁻¹² However, this concept does not apply to an individual, but to a group of individuals with clinical features sufficiently distinct from other groups to represent a useful clinical entity or distinct disease phenotype.¹³⁻¹⁴ Moreover, evidence-based guidelines do not solve their problem of inductive reasoning, as the question will always remain to which extent results are generalisable.¹⁴ Nevertheless, disease manifestations in the developing child, that sound or look similar, may be the expression of distinctly different underlying mechanisms.¹⁵ Most physicians have seen children with wheeze and/or cough, which become manifest during different time periods in childhood or adolescence, and persist and progress, or disappear, and sometimes even relapse at other time periods.

Wheezing is a common symptom of respiratory disease, and is reported in no less than 30 per cent of children during the first three years of life, with a prevalence falling to about half in older children.^{13,15-16} Especially in young children, wheeze is poorly characterised, implying a high-pitched sighing or whistling sound that is mainly heard during expiration.¹³ Common accompaniments are cough and breathlessness, and particularly during common viral respiratory tract infections, rattly sounds may mask wheeze or may be mislabelled as wheeze.^{13,17} Parental misclassification of noisy breathing in young children is widespread.^{18,19} Hence, wheezing phenotypes in children are hard to classify unequivocally.

In preschool children, exclusively viral wheeze is the most common clinical phenotype, that accounts for about two-thirds of all wheezers.¹³ It is characterised by acute episodes of wheeze, cough and breathlessness in association with viral (upper) respiratory tract infections, particularly respiratory syncytial virus, rhinovirus and parainfluenza virus, with few or no interval symptoms.^{13,20-24} Many of these children have a good prognosis since 60 per cent will have stopped wheezing by their early school years.¹⁶ This form of transient early wheeze is not related to atopy and allergic conditions.¹⁵ Risk factors associated with transient wheezing are maternal smoking during pregnancy, premature birth and low birth weight.¹⁵ Reduced lung function after birth, before any wheezing illness has occurred, may contribute to the underlying mechanism.^{5,15,25-26} Furthermore, current findings suggest that respiratory syncytial virus has a similar effect on subsequent wheeze as other confirmed viral infections, being a mainly nonallergic condition that resolves with age.^{23-24,27-30} On the other hand, a preschool persistent wheezing illness resembling classical asthma can be

distinguished from transient viral wheeze, and is characterised by chronic interval symptoms between episodes and often an atopic personal and family history.¹³

By school age, children with the atopic wheezing phenotype are most likely to develop a chronic course, with exacerbations not only induced by viral infections, but also by other factors, such as exercise, allergen exposure, irritants and stress.¹⁵ Children with atopic wheezing may develop significant impairment in lung function as well as airway hyperresponsiveness.¹⁵ However, there is also a significant number of children with the nonatopic wheezing phenotype.¹⁵ They are thought to have symptoms triggered by viral infections alone, to lack airway hyperresponsiveness, and to retain normal lung function in long-term follow-up studies.^{15,31-33}

Allergic asthma is the most persistent and usually the more severe form of recurrent wheeze, associated with immunoglobulin E-mediated immune responses to food and/or aeroallergens.¹⁵ Most children already have their first symptoms during early life.^{16,34-36} The level of allergen exposure has been fiercely discussed as a potential determinant of the development of asthma, although this is not supported by recent studies.^{15,37-39} During and after adolescence, a significant number of children lose their symptoms, which may in part be attributable to the vanishing disease expression of the nonatopic wheezing phenotype associated with viral infections.¹⁵ On the other hand, new onset of illness is also seen, particularly among female adolescents and/or associated with taking up active smoking.^{15,40} Persistent illness is associated with severe atopy and airway hyperresponsiveness.^{15,40}

The role of viral infections in exacerbating wheeze and/or inducing wheeze and asthma-like symptoms is debated extensively.^{15,24} Recent observations in infants suggest that the configuration of the immune response prior to any viral infection determines the risk of virally induced wheeze.⁴¹ However, it is also conceivable that a child's increased exposure to viral infections through day care can influence the phenotypic expression and foster a milder form of wheezing by suppressing the atopic component.^{24,42-45} Each particular virus will exert an effect depending on its own specific invasive properties, the elicited immune response, and the interacting host factors, including genetic factors.²⁴

The hygiene hypothesis, firstly proposed in 1989 by David Strachan, states that infections and unhygienic contact might actually render protection against the development of allergic illness.^{24,45} Interleukin-12 is a dominant mediator that may skew the T-helper 1 to T-helper 2 balance away from allergy-promoting T-helper 2 cells towards T-helper 1 cells.^{24,46} The so-called farm studies suggest that environmental exposure in the first years of life to nonviable microbial products, such as the evolutionarily highly conserved endotoxin from gram-negative bacteria, stimulate immune responses in a way that protects against allergies.^{24,45,47-48} However, there are conflicting data regarding the hygiene hypothesis.^{24,49-57} Alternatively, Holt *et al* have recently proposed a theoretical maturation model for the airways (and atopic asthma), based on common-shared genetic variations in kinetics of postnatal maturation of T-

helper 1 competence.⁵⁸ These collectively result in delayed maturation and 'sluggish' postnatal T-helper 1 function, and hereby increase susceptibility to both viral infection and its subsequent spread, as well as programming T-helper 2-polarized memory against aeroallergens.^{28,58-60} Nevertheless, to date a truly unifying concept has not yet emerged, but various pieces of a complex interplay between genetic background, immune response of the host, characteristics of the invading micro-organism, and the level and variety of the environmental exposure become apparent.^{5,8-9,24}

Two distinct disease entities also account for various respiratory symptoms in childhood, *in casu* 'new' bronchopulmonary dysplasia and cystic fibrosis.

Infants with bronchopulmonary dysplasia suffer prolonged (*id est* more than 28 days) oxygen dependency, troublesome respiratory symptoms, lung function abnormalities at follow-up, pulmonary and systemic hypertension, and neurodevelopmental delay.⁶¹⁻⁶³ Risk factors for the development of bronchopulmonary dysplasia include oxygen toxicity, volutrauma and infection, as well as prematurity.⁶¹ Infants described as having 'new' bronchopulmonary dysplasia, have abnormal lung growth with a markedly decreased number of alveoli, which is more prominent in 'new' bronchopulmonary dysplasia than the fibrosis and smooth muscle increase of medium-sized airways as previously seen in classical bronchopulmonary dysplasia.^{61,64-66}

Cystic fibrosis is the most common life-limiting recessive genetic multisystem disease in Caucasians, caused by a defect in the cystic fibrosis transmembrane conductance regulator gene.⁶⁷⁻⁷⁰ The phenotype of typical cystic fibrosis includes the well-known respiratory manifestations, but the hallmark is pancreatic insufficiency.^{67,69} Diagnosis is established in the majority of patients by one year of age, but in eight percent not until after ten years of age.^{69,71} Often, the problem is remembering to do the sweat test.^{67,72} Although newborn screening becomes more widely introduced, it does not preclude the need to consider cystic fibrosis in many differential diagnoses,^{67,73-74} and the difficulty will be classifying and/or diagnosing patients with atypical, difficult, subclinical or pre-cystic fibrosis.^{67,75-77}

There are less frequent or even rare but specific disorders, such as congenital airway disorders, obliterative bronchiolitis and primary ciliary dyskinesia, that may also have a wheezy component. However, they fall beyond the scope of this general introduction.

Finally, overlap or coincidental occurrence of two independent disorders in an individual creates a wide potential spectrum and at the same time it creates the sort of uncertainty that makes clinical paediatric medicine such a challenge.¹³

1.2 Chronic respiratory inflammation in children.

Having explored to some extent a wide clinical diversity of respiratory disorders from infancy to adolescence, and having designated their complex and multi-dimensional nature, comprising at least the affected phenotype, time, environment and genetic

susceptibility, it now seems appropriate to attempt to address the featuring phenotypes of chronic respiratory inflammation in children.

Asthma is characterised by a variable degree of airflow obstruction, bronchial hyperresponsiveness, and airway inflammation.⁷⁸ At autopsy, patients with fatal asthma have shown a cellular infiltrate of neutrophils and eosinophils, degranulated mast cells, reticular basement membrane thickening, loss of epithelial cell integrity, occlusion of the bronchial lumen by mucus, and hyperplasia and hypertrophy of bronchial smooth muscle and hyperplasia of goblet cells.⁷⁸ In patients with mild to moderate asthma, infiltration by lymphocytes and eosinophils can occur throughout the central and peripheral airways, often varying with disease severity.⁷⁸ In airways, resident mast cells, macrophages and epithelial cells, and recruited inflammatory cells generate chemotactic chemokines and cytokines, which activate transcription factors, such as nuclear factor- κ B, that bind to the promoter region of genes that encode inflammatory cytokines, chemokines, adhesion molecules and other proteins that induce and perpetuate inflammation.⁷⁸⁻⁷⁹

To initiate immunoglobulin E-mediated inflammation in allergic asthma, inhaled allergens must encounter dendritic cells that line the airways and then migrate to draining lymph nodes, where they present processed antigen to T and B cells.^{78,80} Interactions among these cells elicit responses that are influenced by cytokines. T-helper 2-mediated interleukin-4 and interleukin-13 promote B cells to switch to synthesise, and to release specific immunoglobulin E.^{24,78,81} This immunoglobulin binds to high-affinity immunoglobulin E receptors on the surface of mast cells in tissue or peripheral-blood basophils, and to low-affinity immunoglobulin E receptors on the surface of lymphocytes, eosinophils, platelets and macrophages.^{24,78,81} Subsequently inhaled allergen interacts with receptor-bound immunoglobulin E (cross-linking), and causes activation of the cell membrane and cytosolic pathways of mast cells and T-helper 2 cells in the airways.^{78,82-83} This causes the release of preformed mediators such as histamine, and initiates the synthesis of arachidonic acid metabolites and T-helper 2 cytokines, including interleukin-4 and interleukin-5.^{78,82-83}

However in childhood asthma, data from mucosal biopsy studies and bronchoalveolar lavage studies are inconsistent and often not in accordance with studies in adults.⁸⁴ In fact, the presence and nature of airway inflammation in common clinical expressions of childhood asthma is largely unknown, and it is quite likely that it differs considerably from the findings in adults.⁸⁴ The only really consistent finding is thickening of the reticular basement membrane, a key feature of airway wall remodelling, which was observed in almost all biopsy studies, even in young children.^{60,84-95} It is not clear whether these structural changes precede, or occur early and in parallel with, or develop as a result of airway inflammation.^{60,89,93,95-97} Nevertheless, interference with normal airway growth and lung development at crucial time periods may have particularly long-term effects and cannot be overstated.^{93,98}

Inhaled corticosteroids have become the cornerstone of maintenance therapy in childhood asthma, although it is not clear at what point such treatment should begin and indeed whether anti-inflammatory therapy can influence the natural course of the disease.^{32,78,84,99-108} A further complication is the fact that, idealised asthma guidelines do not reflect the reality of individual asthma management.^{84,109-116}

There is good evidence to show that preschool children with exclusively viral-triggered wheeze do not have chronic eosinophilic airway inflammation, and that during an acute viral-triggered attack, neutrophilic inflammation predominates.^{13,117-121} However, a minority of preschool wheezers have eosinophilic airways inflammation between viral-triggered attacks.^{13,122-126} On the other hand, it is possible that exclusively viral wheeze is atopic asthma but without the propensity to develop chronic inflammation.^{13,127-128} Furthermore, it currently remains unclear whether respiratory syncytial virus infections directly damage the infant's respiratory system and promote the development of later recurrent wheezing, or if bronchiolitis unmasks some inherent susceptibility or pre-existing lung function abnormalities, or even both.²³ The T-helper 1 to T-helper 2 paradigm is not necessarily followed,^{23,129-133} and it is plausible that small infants with a less mature immune response are unable to mount a strong antiviral T-helper 1 response, making them most vulnerable to severe disease.^{58-59,134}

In bronchopulmonary dysplasia, neutrophilic lung inflammation is a key feature.^{61,135-136} The inflammatory phase may commence antenatally, with infection being the most likely initiator.¹³⁷ Alveolar macrophages appear to mediate resolution of the acute lung injury, and are likely responsible for dysregulated lung growth, as they release growth factors.⁶¹ Alternatively, the capillary network may fail to expand, as vascular endothelial growth factor is significantly decreased in bronchopulmonary dysplasia.¹³⁸

In cystic fibrosis, there is no dispute that the two main components of airway disease are chronic infection and an exuberant host inflammatory response.⁶⁷ What is not clear is their relationship.^{67,139-145} New data are strongly suggestive that the dysregulated inflammatory response in cystic fibrosis is not related to the underlying cystic fibrosis transmembrane conductance regulator defect, but is a secondary response to prolonged and chronic infection.¹⁴⁶⁻¹⁴⁷ It would seem likely that early on in cystic fibrosis, the inflammatory response is beneficial and wards off early infection with *Pseudomonas aeruginosa*, but that in late-stage cystic fibrosis, it is clearly both ineffectual and harmful.⁶⁷ What is not known is when the transition to harm occurs, which is important, as steroids are definitely beneficial in late disease, but may not be helpful early on.^{67,148} Moreover, it is also not clear why there is persistent acute (neutrophilic), rather than chronic infection in the cystic fibrosis airway.⁶⁷

In summary, common respiratory symptoms, such as wheezing, coughing, shortness of breath, chest tightness and fatigue, in the developing child, with its specific genetic background, and undergoing alveolisation, lung growth, immune

maturation as well as environmental influences, may be attributed to different pulmonary diseases, ranging from transient viral wheeze to life-limiting cystic fibrosis, all featuring some phenotype of respiratory inflammation. Although our theoretical and clinical knowledge is increasing, and several global concepts or hypotheses have been proposed, many questions still remain unanswered and await disentanglement. The practical need for tailored individual monitoring of the natural course of disease or response to treatment, as well as the need for accurate, objective and early assessment of respiratory inflammation, in little or non-cooperative children is obvious.

1.3 Assessment of chronic respiratory disease in children.

The key question is how to identify those children with an underlying disease and separate them from the large group of children with benign and self-limiting problems.¹⁴⁹

Medical advice often relies on gathered information on symptoms and quality of life as perceived by the child or her/his parents. Each physician should balance, on one hand, his own professional findings and misunderstandings, and, on the other hand, potential parental or patient's misclassification of noisy breathing, mismatched prevalence of symptoms, poor adherence to treatment, and perception of quality of life and disease control.^{18-19,84,109-110,113,150-159} Also, disease outcomes are influenced by emotional characteristics, disease management behaviours and psychological factors.¹⁶⁰ It has become obvious that poor disease control is not only a problem of education, both for patients and providers, but also a problem of mutual communication between patients and providers.^{84,110,113}

Pulmonary function tests, mainly spirometry, are generally considered the standard criterion for objective assessment of paediatric lung disease.^{99-100,149,161} The poor cooperation of children under six years of age in performing the required manoeuvres has limited lung function evaluation.¹⁴⁹ In the 'cooperative' child (*id est* more than six years old) spirometry is the most used pulmonary function test, as, in these children, it is relatively not difficult to perform and it is repeatable.^{84,149} Paediatric guidelines have recently been published.¹⁶² In asthmatic children, spirometric parameters detect airflow limitation and help to diagnose and monitor the disease.¹⁰⁷ Forced expiratory volume in one second is the most reliable spirometric parameter and is used, along with symptom frequency and medication usage, to classify the severity of airway disease.¹⁰⁸ However, asthma is a condition with intermittent airway obstruction, and in 5 to 18 year old asthmatic children forced expiratory volume in one second is generally normal, which does not exclude disease, however.^{149,152,163-164} Whereas guidelines recommend that the monitoring of asthma should be based on symptoms and lung function tests, often insufficient attention has been paid to the underlying airway inflammation.⁸⁴ Additional markers of airway inflammation may be helpful to improve disease outcome.^{84,165-166}

The main pathological factors that underlie bronchial hyperreactivity are presumably airway inflammation and bronchial remodeling.^{149,167} Guidelines for

bronchial challenge tests have been published.¹⁶⁸⁻¹⁶⁹ However, the relationship between bronchial hyperreactivity and asthma is complex in children. While asthmatic children have bronchial hyperreactivity, bronchial hyperreactivity is not the same as asthma.^{149,170} It has also been reported in children with cystic fibrosis, allergic rhinitis and even in asymptomatic children.¹⁷¹ Indirect stimuli, such as exercise and cold air, correlate better with airway inflammation than direct stimuli, such as metacholine and histamine.^{167-168,172} Bronchial hyperreactivity may be important in difficult cases: a direct challenge will be very helpful in ruling out asthma (when negative), while it will not be able to confirm it (when positive), and conversely, an indirect challenge will be more helpful in confirming a diagnosis of asthma (when positive), than ruling it out (when negative).¹⁶⁷

Sputum induction may directly assess airway inflammation in children.¹⁷³ It implies a semi-invasive induction procedure through inhalation of an ultrasonic aerosol of hypertonic saline, and allows examination of the cellular and soluble content.¹⁷⁴⁻¹⁷⁶ Although methodological guidelines have been published, a wide variety of sputum induction and processing protocols have been used.^{175,177-180} In children, reported side-effects include not only minor symptoms and discomfort in 6 to 26 per cent, but also airway obstruction, even after beta-2-agonist-pretreatment, in 10 and 19 per cent, respectively, of children with mild and severe disease.^{173,175,181-187} In children, the reported success rate depends on disease severity and age (*id est* few children aged less than seven years), and varies from 61 to 100 per cent.^{166,173,175,181-185,188-189} All studies report high requirements for technical support and expertise to process, stain and interpret induced sputum samples, hereby limiting its use to tertiary centers.¹⁷⁶ In asthmatic children, sputum induction can provide information on eosinophilic inflammation and treatment response and is associated with several measures of asthma control.^{166,173,190-192} In children with difficult asthma, abnormal sputum cytology is only present in a minority, whereas it has been suggested recently that sputum eosinophilia in these children may predict their response to systemic corticosteroids.^{181,193} Also, in children with cystic fibrosis, limited data suggest that induced sputum may provide useful markers of inflammation and infection.^{192,194-197}

To date, endobronchial biopsy remains the 'golden standard' by which to determine airway inflammation. This invasive diagnostic procedure is safe, even in children with severe asthma, if it is performed by experienced operators with back-up from doctors skilled in airway management and the monitoring of sick children.^{93,198-202} Moreover, there is no reason not to use the opportunities afforded by clinically indicated bronchoscopy to carry out research, if sound ethical principles are followed.^{89,93,95,203-205} However, bronchoscopy has considerable limitations: (i) it is an invasive procedure for specific indications only and not suitable for repeated use;^{199,201} (ii) data are lacking on the reproducibility of several parameters in children;²⁰⁶ (iii) specimens are small and vulnerable to damage during the biopsy procedure, and can only be obtained from the larger airways; and (iv) many physiological, infectious and mechanical complications are associated with the procedure.^{174,198,201,207-208}

During bronchoscopy, bronchoalveolar lavage fluid can be obtained for microbiological and cytological analysis.^{93,117,201,209} However, there are many difficulties in the interpretation of findings: (i) bronchoalveolar lavage does not sample a static space or volume of airway surface fluid, as there is a constant exchange of fluid, electrolytes and other soluble constituents across the airway and alveolar epithelium, and as there is no practical way to obtain a pure bronchial or alveolar fraction; (ii) the concentration of any material or cell type in the bronchoalveolar lavage fluid depends on the volume of fluid used for the lavage, the efficiency of mixing and recovery (*id est* usually 40 to 60 per cent fluid return), the initial concentration in the epithelial lining fluid, and the rate of flux of the material or fluid into the bronchoalveolar lavage fluid during the procedure; and (iii) available normal values are poly-interpretable.¹⁷⁴

In summary, it may be clear that there exist many limitations of, and discrepancies between: (i) subjectively reported and objectively assessed airway symptoms; (ii) pulmonary function tests reflecting airflow limitation; and (iii) direct or indirect markers of airway inflammation. Moreover, endobronchial biopsy, bronchoalveolar lavage and sputum induction are relatively difficult direct measures of inflammation and not suitable for routine use in clinical practice, whereas it should be stressed again that these are highly valuable basic and clinical research tools.⁸⁴ Obviously, pulmonary function tests and sputum induction require more than minimal cooperation of a child, and thus, success rate substantially declines in children aged six to seven years or less. In general, procedures in favour of children should be suitable at any age, rapid and easy to perform, requiring only minimal cooperation, as well as harmless and painless, and thus, by definition, should never be invasive or semi-invasive. Furthermore, as there is such heterogeneity in chronic respiratory disease expression, it seems only logical that: (i) noninvasive specific single markers, indicating diagnosis, disease control and airway inflammation, have to be assessed at an individual level; and that (ii) if generally applied, a set of various markers is more likely to be useful in larger populations.^{84,116,210} Hence, the leitmotif being the development of novel individualised therapeutical strategies that may replace the current paradigm of strategies aimed at the whole of a certain population.⁸⁴ Therefore, it is crucial to attempt to define additional noninvasive markers that can be applied in clinical practice over a wide range, that may be specific for the evaluation and monitoring of certain disease patterns, including early disease events, and/or response to treatment, and that may give relevant information on the individual child.^{84,149,211}

1.4 Noninvasive inflammometry in children.

Having explored, in the previous chapter, the various reasons why there is a need for additional noninvasive markers of various phenomena associated with chronic respiratory diseases, especially in the growing child, we will now explore some possibilities that may address this issue. Ideally, we need a noninvasive, rapid and easy to perform method that can be used repeatedly to obtain direct samples of the airways without any risk, that is standardised and successful at any age, that requires no cooperation, and that offers direct measures that are not only sensitive and specific but also reproducible.²¹² Clearly, such a well-defined ideal does not exist in the realities

of life. Nevertheless, we may unremittingly pursue amelioration, while learning from the past. When Antoine Laurent de Lavoisier published in Paris his 'Traité Élémentaire de Chimie' in 1789, not only the French Revolution was imminent, but also modern chemistry was born by demonstrating that air is not a simple element but actually a composite.²¹³ This principle is the basis of this thesis. Indeed, exhaled air or breath potentially has many advantages that approximate the characteristics of an ideal marker of the airways. Analysis of exhaled air shows the presence of volatile substances, such as nitric oxide.^{207,212} Furthermore, exhalation against a cold surface or condenser yields exhaled breath condensate (EBC) that in turn may be collected and analysed for volatile and nonvolatile macromolecules.^{212,214}

Nitric oxide, biosynthesised from arginine and oxygen by various nitric oxide synthase enzymes, is now known as a widespread signalling molecule in various organ systems. In 1987, Salvador Moncada (married to Her Royal Highness Princess Maria-Esmeralda of Belgium) *et al* showed unambiguously that nitric oxide was made by endothelial cells and that the actions of nitric oxide (*id est* full relaxant response of blood vessels) could explain the actions of the formerly known endothelium-derived relaxing factor.²¹⁵ In 1991, Lars Gustafsson *et al* first described the presence of endogenous nitric oxide in exhaled breath of animals and humans.²¹⁶ In 1998, Robert F Furchgott, Louis J Ignarro and Ferid Murad were jointly awarded the Nobel Prize in Physiology or Medicine for their discoveries concerning nitric oxide as a signalling molecule of the cardiovascular system.²¹⁷

Since the 1990s, the exhaled nitric oxide fraction has been extensively studied and validated as a noninvasive marker in asthma that reflects eosinophilic airway inflammation in the bronchial mucosa and shows a rapid, dose-dependent response to corticosteroids.^{149,207,212,218} Exhaled nitric oxide has been standardised for use in clinical practice, and there are detailed guidelines for the online and offline measurement of exhaled nitric oxide in children aged four to five years and more.²¹⁹⁻²²⁰ Normative values have been published for children aged four years and more.²²¹ In these cooperative children, the single-breath online measurement method using a chemiluminescence analyser is the preferred technique.^{149,219-220} Results are immediately available.¹⁴⁹ Recently, compact hand-held analysers have been developed and will facilitate more widespread introduction.¹⁴⁹ Possible applications of exhaled nitric oxide inflammometry include diagnosis of asthma, prediction of steroid response, monitoring of steroid treatment and treatment compliance, steroid dose titration, prediction of exacerbation or relapse, and screening for asthma.^{149,207,212,222} Recently, several studies have shown not only the feasibility of exhaled nitric oxide measurement in paediatric clinical practice, but also suggested a significant benefit of additionally monitoring exhaled nitric oxide in childhood asthma, as treatment with inhaled corticosteroids can be adjusted individually to prevent under- or over-treatment.^{207,223-226} In children and adolescents, elevated exhaled nitric oxide has been shown to correlate well with induced sputum eosinophil counts,^{219,227-229} but not with bronchial hyperresponsiveness.²²⁹⁻²³¹ However, in adolescents in clinical remission of atopic asthma, exhaled nitric oxide and airway responsiveness were significantly correlated

with each other, as well as with eosinophil counts in bronchial biopsies.^{85,232} Also, in children with difficult asthma, after treatment with oral steroids, a correlation was seen between biopsy eosinophil score and exhaled nitric oxide.²³³

On the other hand in infants and preschool children, although suggestions for the measurement of exhaled nitric oxide have been published, more research is needed to resolve various confounding methodological issues related to spontaneous breathing with or without controlled flow rate, contamination from the upper airways, and forced exhalation.^{149,219-220,234-245}

Various other diseases have been associated with high exhaled nitric oxide values,²⁴⁶⁻²⁵⁸ variable exhaled nitric oxide values,²⁵⁹⁻²⁷⁵ and low levels of exhaled nitric oxide.²⁷⁶⁻²⁸⁸ In children with cystic fibrosis, low exhaled nitric oxide levels may be explained by deficient or reduced inducible nitric oxide synthase expression, and/or by trapping and metabolisation of nitric oxide in airways secretions and mucus.^{278-282,289} Children with primary ciliary dyskinesia, characterized by chronic airway infection and bronchiectasis due to a genetic defect leading to reduced ciliary function and impaired mucociliary clearance, have not only abnormally low exhaled nitric oxide values, but also low nasal nitric oxide that is even more discriminative and is now recommended as the screening tool of choice for primary ciliary dyskinesia.^{149,219,290}

EBC represents a bi-phasic technique, implicating at first the cooling of exhaled breath to collect the condensate, and subsequently its analysis.²¹² There is strong evidence that abnormalities in EBC composition may reflect biochemical changes of the airway lining fluid, and thus may constitute an alternative approach for noninvasive inflammometry.²¹⁴

Although the EBC procedure is experimental, the guiding and cooling of exhaled breath into a breath condenser system appears to be noninvasive, rapid and relatively easy to perform, as well as being a technique that may be used repeatedly as it does not interact with the ongoing processes in the airways, which, by consequence, may guarantee a high degree of safety to the subjected individual.

Moreover, the bi-phasic nature of the EBC technique may even offer some advantages compared with exhaled nitric oxide: (i) the collection of EBC requires only minimal cooperation of a normal (*id est* tidal) breathing subject; (ii) therefore, the potential to improve the feasibility of EBC collection, especially in the younger child; (iii) the analysis of EBC may reveal the presence of multiple biomarkers, instead of only one specific volatile substance; and (iv) hence, EBC analysis may prove to be useful in discriminating and/or monitoring more than one inflammatory lung disease.

1.5 Objectives of the thesis.

As stated previously, this thesis is based on the principle that air is a composite, and hence, analysis of breath condensate may offer new perspectives in monitoring lung inflammation.²¹³ In this thesis, we hypothesise that the noninvasive methodology of

EBC collection and analysis can be improved and adapted based on objective experimental findings, in order to evolve from a simple home made experimental research tool into a new and potentially useful means of assessing inflammatory disease status, especially in preschool children with chronic respiratory disorders. By consequence, the scope of this thesis is the further development of EBC methodology.

In **chapter 1.6** we present a state of the art on the collection and analysis of EBC in children, corresponding to our knowledge in the early years of the new millennium.

In order to facilitate future comparison and interpretation of data on this rapidly growing field of research, we proceed in **chapter 2** with an elaboration, from a paediatric point of view, of the pearls and pitfalls of each single aspect of EBC collection and preservation, and, of the markers that yet have been detected in condensate in children.

In **chapter 3** we compare new exhaled markers, comprising exhaled nitric oxide, exhaled carbon monoxide, condensate acidity, and cytokines in EBC, with conventional asthma measures, such as lung function parameters and the asthma control questionnaire of Juniper, in a cross sectional study in 32 children.

Triggered by the previous study results, we discuss in **chapter 4.1** our increasing awareness of the confounding factors due to the bi-phasic nature of EBC methodology (*id est* standardisation of collection versus reproducibility of analysis). Consequently, this leads us to proceed with research that intends to integrate more consistently the following three equivalent key issues: (i) engineering of an optimal condensate collection device, (ii) using improved or new techniques suitable to analyse the resulting condensate, and (iii) clinical application in subsets of patients with chronic respiratory disease.

In **chapter 4.2** we investigate, both *in vitro* and *in vivo*, our hypothesis that the adhesive properties of different condenser coatings interfere with the detection and measurements of eicosanoids and proteins in EBC, as this may prove to be a crucial factor to the further development of a standardised condensate collection system.

In **chapter 5** we study the reproducibility of different condensate markers, such as hydrogen peroxide, 8-isoprostane, and cytokines, using different condenser coating systems, including a newly developed glass condenser, in order to find a more reliable condenser. Furthermore, we describe the use of xMAP® technology, a liquid bead array multiplexing system to simultaneously measure multiple cytokines within a small, single condensate sample.

In **chapter 6** we investigate the use of electron paramagnetic resonance spectroscopy to directly detect the presence of free radicals, and thus directly assessing oxidative stress, in EBC in 21 children with cystic fibrosis and healthy controls. Secondly, we examine the possible source of these free radicals.

In **chapter 7** we explore, in a cross sectional design, the ability of exhaled inflammatory markers, including exhaled nitric oxide, condensate acidity, nitrite, nitrate, hydrogen peroxide, 8-isoprostane, interferon-gamma, tumor necrosis factor-alpha, interleukin-2, interleukin-4, interleukin-5 and interleukin-10 in EBC, to discriminate between cystic fibrosis and healthy controls, and to indicate disease exacerbation and severity, in a large cohort of 98 children.

In **chapter 8** we investigate the feasibility of EBC collection in 70 preschool children, with and without wheezing illness, using a newly modified glass condenser system with breath recirculation system. Furthermore, we study the profiles of interleukin-4, interleukin-5, interleukin-6, interleukin-8, interleukin-10, interleukin-12p70, interleukin-13, and tumor necrosis factor-alpha in EBC, measured by multiplexed xMAP® technology, in different preschool wheezing phenotypes.

Finally, in **chapter 9** we present the general discussion of this thesis.

1.6 New perspectives in monitoring lung inflammation: analysis of exhaled breath condensate in children.

Airway inflammation plays an important role in various respiratory disorders of childhood, including recurrent wheezing, asthma, cystic fibrosis, bronchopulmonary dysplasia, and respiratory distress syndrome.²⁹¹⁻²⁹⁴ In daily clinical practice, indirect indices of airway inflammation like symptoms and lung function measurements are routinely used for diagnosis and follow-up of inflammatory respiratory disorders. Especially in young children, these indirect indices of disease severity are less valuable than in adults; lung function studies in young children are often not possible because of lack of cooperation. Furthermore, reporting of symptoms by parents is much dependent on perception of symptoms with the potential risk for both under or overperception. Therefore in children, there is a strong need for objective and early criteria to detect and monitor airway inflammation in order to prevent or minimize the irreversible changes which are described in various chronic respiratory disorders including asthma, cystic fibrosis and bronchopulmonary dysplasia.²⁹¹⁻²⁹⁵ The analysis of airway inflammatory cells and mediators has traditionally been performed by bronchoscopy on samples of bronchial mucosal biopsies or on bronchoalveolar lavage fluid samples. In children both methods are limited in their applicability because of the invasive procedure of bronchoscopy. It is clear that in daily clinical practice, (serial) measurements of airway inflammation is not feasible using such invasive methods. A less invasive procedure to assess the presence and activity of airway inflammation would be of great benefit for early diagnosis and monitoring of inflammatory airway diseases in children.²⁹⁶

Inflammatory markers can be measured in blood or in urine.²⁹⁶ The noninvasiveness of these methods is an advantage, but their indirect nature is a limitation. Measurements of certain inflammatory markers in blood and urine represents whole body production and does not necessarily reflect production of these inflammatory markers in the respiratory tract. It is reasonable to assume that the composition of respiratory tract secretions might closer reflect airway inflammation than substances in blood or urine. Sputum is one of the respiratory secretions which can be used in the assessment of airway inflammation.²⁹⁷⁻²⁹⁸ Sputum can be obtained noninvasively, spontaneously or during chest physiotherapy. Sputum examination has been limited by difficulties in obtaining adequate samples. When sputum can not be produced spontaneously, it can be induced by inhalation of an aerosol of hypertonic saline. With this technique, sputum samples can be obtained in up to 75 to 100 per cent of asthmatic and healthy adults.²⁹⁸ In children, the succes rate in obtaining adequate samples is definitely lower.¹⁸⁸ In general sputum induction in children aged less than 6 years is not feasible. Other practical considerations of sputum induction include an inevitability of pretreatment with short acting beta-2-agonists in order to prevent an obstructive airway response provoked by inhalation of hypertonic saline, and the time-consuming procedure for inducing sputum and processing induced sputum samples. Furthermore, several studies have shown that repeated sputum induction in itself induces changes in airway inflammation within 8 to 24 hours after sputum induction, which limits the usefulness of this method for serial measurements.²⁹⁹⁻³⁰⁰

EBC is a recently rediscovered vehicle of substances from the respiratory tract, in which several potential markers of airway inflammation can be detected. In contrast with the other sampling methods mentioned above, EBC offers the advantage that it can be obtained completely noninvasively. Exhaled air carries components from the lower respiratory airways, and does not disturb the airways, in contrast to bronchial biopsies, bronchoalveolar lavage and induced sputum, and it can be obtained with minimal risk and inconvenience from both adults and children.

1.6.1 Collection of EBC in children

In children from the age of 4 years and over the EBC collection method(s) as used in adults, can be applied in almost the same way. There is a variety of condenser systems to collect EBC, although they are fundamentally similar, based on condensation of exhaled air on a cold surface. In general, EBC is collected from adults and children from the age of 4 years by tidal breathing through a mouthpiece and a two-way nonrebreathing valve, which can also serve as a saliva trap, connected to a cooled condensing system. Minimal co-operation from the child is required for this technique of EBC collection. In children, the main issue for success of EBC sampling is if they are able to use a mouthpiece properly for approximately 10 to 15 minutes. Adequate instruction of the procedure and distraction of the child, for example by using video cartoons, increases the success rate of EBC collection in children. Collecting EBC in children is not only a simple non-invasive procedure, but it is also a safe and feasible method of sampling substances from the respiratory tract. No adverse effects were seen in an EBC collection study in 93 healthy children aged 8 to 13 years, and all the children were able to complete the EBC collection procedure successfully.³⁰¹ 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.³⁰² No significant changes in forced expiratory volume in one second were found after the EBC collection. The success rate was 100 per cent starting from 4 years of age. The volume of collected condensate depends on different variables, like minute ventilation or total respired volume, condenser material and temperature, and turbulence characteristics.³⁰³ Minute ventilation seems to be the most important determinant of the condensate volume over time. So, in children it will take more time to collect the same amount of condensate as in adults. In general, a collection time of 15 to 20 minutes will yield 1 to 3 mL of EBC in children aged 4 years and over. It has been shown that the collected condensate volume significantly correlates with the age of children.³⁰²

In infants and young children, who are not able to use a mouthpiece properly, a facemask can be used. In a small number of infants, who are preferential nasal breathers, it was shown that collection of EBC through a facemask was feasible.³⁰⁴⁻³⁰⁵ In a sampling time of almost 30 minutes a mean condensate volume of 101.6 μL was recovered.³⁰⁴ In the condensate of healthy infants and cystic fibrosis infants nitrite and nitrate was detected. However, significant nasal contribution to the total nitrite to 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.³⁰⁵

Nasal collection of EBC is reported in minimal co-operative patients like healthy infants from 4 weeks of age for the analysis of hydrogen peroxide.³⁰⁶ Nasal prongs are serially connected to two polypropylene tubes, submerged in a cold trap, and in turn connected to an electric air suction pump. The condensate volume obtained, increased significantly with age, up to 18 years, and was about 45 per cent less in 1 to 6 year old children. It averaged about 20 to 30 per cent of the exhaled breath water vapour. However, contamination with nasal secretions may interfere with the actual concentration of inflammatory mediators in the lower respiratory tract.

1.6.2 Inflammatory mediators detected in EBC of children

EBC is presently an active research area. Over the last years an increasing number of EBC data are being presented. However, most data are obtained in adults. For the results of inflammatory mediators in EBC of children we will focus on data published in the international literature as peer-reviewed full articles.

A variety of inflammatory markers have been detected in EBC of children. In contrast to the condensate volume, the studied inflammatory marker levels in EBC are not age dependent.

The most commonly studied mediator is hydrogen peroxide, a marker of oxidative stress. The concentration of hydrogen peroxide in EBC of stable asthmatic children is significantly increased compared to healthy children.³⁰⁷ In stable asthmatic children the hydrogen peroxide concentrations were lower in those who used inhaled corticosteroids. In children with unstable asthma higher concentrations have been reported than in stable asthmatic children.³⁰⁸ Reference values on hydrogen peroxide have been described in a group of 93 healthy children with a mean age of 10 years.³⁰¹ Hydrogen peroxide was not age-dependent, nor was there a correlation with sex or lung function.³⁰¹ In children, it was demonstrated that atmospheric nitric oxide influenced exhaled hydrogen peroxide levels.³⁰⁹ Besides asthma, hydrogen peroxide concentration has also been detected in EBC of cystic fibrosis children with an acute infectious pulmonary exacerbation, and significantly decreased during intravenous antibiotic treatment.³¹⁰

Another marker of oxidative stress which can be measured in the EBC of children is 8-isoprostane. Children with stable asthma had higher exhaled 8-isoprostane concentrations than healthy children.³¹¹ In the asthmatic group, treatment with inhaled corticosteroids showed no difference in exhaled 8-isoprostane concentrations. The same study also showed, in contrast to the 8-isoprostane results, no significant difference in exhaled prostaglandin E2 concentrations between asthmatic and healthy children. In children with an asthma exacerbation there is a significant reduction of exhaled 8-isoprostane after a 5 day course of oral prednisone.³¹²

Leukotrienes can also be measured in EBC. In asthmatic children cysteinyl leukotrienes and leukotriene B4 were significantly increased compared with healthy children.³¹³ After a course of oral corticosteroid therapy, a reduction was seen in cysteinyl-leukotrienes in children with an asthma exacerbation.³¹²

In children with asthma and cystic fibrosis higher levels of nitrite in EBC have been found compared to children with non-asthmatic episodic cough and healthy children.³¹⁴ No significant differences were noted between the asthmatic and cystic fibrosis children, nor the episodic cough and healthy groups. In cystic fibrosis, there was no significant correlation between condensate nitrite levels and age, lung function or use of inhaled corticosteroids.³¹⁵ Children with primary ciliary dyskinesia have been shown to have very low levels of exhaled oral and nasal nitric oxide.²⁸³ However, no differences were found in the EBC levels of nitric oxide metabolites such as nitrite, nitrate, and S-nitrosothiol between children with primary ciliary dyskinesia and healthy children, despite the marked decrease in exhaled nitric oxide levels in primary ciliary dyskinesia patients.³¹⁶

Cytokines reported in EBC of children include interleukin-8, interleukin-4 and interferon-gamma. In cystic fibrosis, interleukin-8 could only be detected in 33 per cent of the condensate samples.³¹⁵ Although the interleukin-8 values were higher in cystic fibrosis children than in healthy controls, there was no statistically significant difference. Increased levels of interleukin-4 and decreased levels of interferon-gamma were detected in EBC of stable asthmatic children.³¹⁷ The use of inhaled corticosteroids was associated with a significant change in interleukin-4 concentration, in contrast to interferon-gamma concentration. Interferon-gamma was detectable in all EBC samples, whereas interleukin-4 was detectable in 92 per cent of the samples.

Aldehydes and glutathione are biomarkers of oxidant induced damage and antioxidant status, respectively. Malondialdehyde and glutathione are detectable in EBC of children.³¹⁸ In children with an asthma exacerbation, malondialdehyde levels were higher and glutathione levels were lower compared to healthy children.³¹⁸ After a 5 day course of oral prednisone malondialdehyde decreased to values no longer different from those of the healthy control children, whereas glutathione levels increased. In healthy subjects as well as in asthmatic children, malondialdehyde and glutathione were negatively correlated. Malondialdehyde and glutathione concentrations were not dependent of exhaled flow rate (200, 150, 100, and 50 mL/second), and no correlation was found between spirometric values and these oxidant versus antioxidant levels in EBC.

Table 1. Inflammatory markers in EBC of children with asthma or cystic fibrosis

	Asthma	Cystic Fibrosis	References
hydrogen peroxide	↑	↑	307, 308, 310
8-isoprostane	↑	(-)	311, 312
prostaglandin E2	=	(-)	311
cysteinyl leukotrienes	↑	(-)	312, 313
leukotriene B4	↑	(-)	313
nitrite	↑	↑	314, 315
interleukin-4	↑	(-)	317
interleukin-8	(-)	=	315
interferon-gamma	↓	(-)	317
aldehyde	↑	(-)	318
glutathione	↓	(-)	318

EBC exhaled breath condensate

↑ significantly increased compared to healthy children

↓ significantly decreased compared to healthy children

= no significant difference compared to healthy children

(-) no paediatric data

Table 1 summarizes paediatric results of inflammatory markers in EBC of children with asthma or cystic fibrosis.

1.6.3 Conclusion

Assessment of inflammatory mediators in EBC is a challenging new technique for the diagnosis and management of various inflammatory lung diseases. Because of the non-invasive character of EBC and the general lack of appropriate other techniques to characterize airway inflammation in children, it is particularly interesting for paediatric respiratory medicine. EBC is not only a simple and noninvasive procedure, but it is also a safe and feasible method of sampling inflammatory mediators from the respiratory tract in children from the age of 4 years and over. A variety of inflammatory markers have been detected in EBC of children. Potentially, this noninvasive method will allow early and appropriate anti-inflammatory treatment in children, which hopefully will prevent, or at least limit, the later development of chronic respiratory diseases in adults. However, the clinical relevance of exhaled inflammatory markers first needs to be established in longitudinal studies in various respiratory disorders, with repeated measurements in individual patients.

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CHAPTER 2

EXHALED BREATH CONDENSATE IN CHILDREN: PEARLS AND PITFALLS.

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Abstract

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 other 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 emphasise on paediatric data. The apparent simplicity of the EBC method must not be over-stated, 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 standardisation 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.

Introduction

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, bronchopulmonary dysplasias and primary ciliary dyskinesia, 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.

EBC '*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 emphasise 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 formed¹⁹. 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 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

1. 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, 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 reported⁸. 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 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.

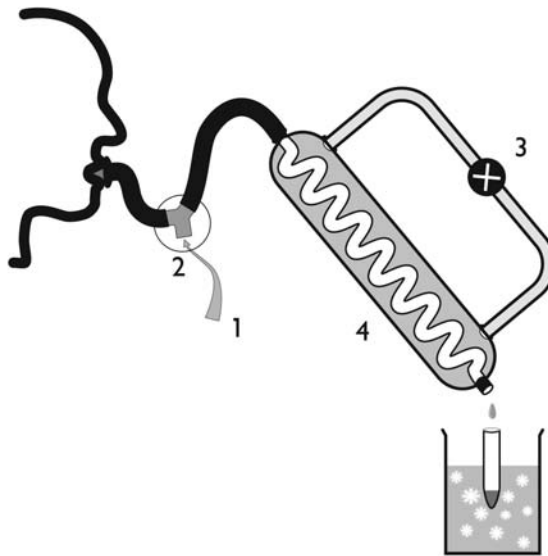


Figure 1. Schematic representation of an exhaled breath condensate collection device. The subject breathes tidally and inhales room air (1) through a two-way non-rebreathing valve (2). Exhaled air is guided by the tubing through a double-wall glass condenser (4), which is cooled by a counter-current circulating ice-water pump (3). The two-way non-rebreathing valve (2) and tubing also serve as a saliva trap.

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 standardised 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.

2. 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 if they are able to use a mouthpiece properly for 10-15 minutes. 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 years³⁵. 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 age³⁶.

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 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 time⁵.

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 facemask that separates nasal from oral exhaled air can be used. In infants, who are preferential nasal breathers, collection of EBC via a facemask 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 nitric oxide and humming, which generates an oscillating airflow and enhances paranasal sinus ventilation⁴³. Humming increases significantly (15-fold) nasal nitric oxide levels compared with quiet exhalation⁴³. 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 breathing⁴⁵.

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 mediators⁹. Leukotriene-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 of hydrogen peroxide and 8-isoprostane levels in EBC, and a delayed increase (at 18 hours after exposure) of leukotriene-B4 and thiobarbituric acid reactive substances⁵⁰.

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 minutes will yield 1-3 ml of EBC in children aged 4 years and over (own unpublished data). A rapid two-minute 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, emphasising that during EBC collection one should systematically run through a checklist of possible influencing factors or co-determinants (Table 1).

3. 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 in³.

Subsequently, each separate aliquot vial can in turn rapidly be frozen and stored at -20°C 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.

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.

Table 1. Checklist of procedure for exhaled breath condensate collection.

Condenser

- System
- Coating
- Cooling temperature

Subject

- Age
- Sexe
- Weigth
- 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 / liquid)
- Duration
- Adverse effects
- Volume of exhaled breath condensate

Preservation

- Aliquot vial volume
- Sample numbers
- Products added
- Storage temperature
- Storage time

Analysis

- Data
- Assay
- Detection limit

Analysis of EBC

EBC 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. Condensate 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 selectivity⁶³. 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 nanomol levels in aqueous matrices⁶²⁻⁶³. 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 1980⁶⁶. In the 1990s, this technique was rediscovered⁶. 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 scarce¹⁰. 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.

1. Hydrogen peroxide

Oxidative stress describes an imbalance between oxidants (reactive oxygen species) and anti-oxidants (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 pro-inflammatory mediators and protective antioxidant genes ⁷⁷. Activation of inflammatory and structural cells result in an increased production of reactive oxygen species. 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 thiobarbituric acid reactive substances. Hydrogen peroxide is less reactive than other reactive oxygen species, 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 μ mol) fluorometrical method with a stable storage time of 40 days ⁶¹.

Paediatric EBC data on hydrogen peroxide are summarised 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 nitric oxide ⁷³.

Exhaled hydrogen peroxide levels were elevated in asthmatic children compared to 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 cystic fibrosis, and significantly decreased in EBC during intravenous antibiotic treatment ⁷⁰.

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

Disease	Median value patient group (micromol)	Median value healthy controls (micromol)	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 iv	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
‡ : uM/ml
§ : nasally collected EBC
CF : cystic fibrosis
AB iv : antibiotics intravenously
ICS : inhalation corticosteroids
n.s. : not significant

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*}.

2. Eicosanoids

Eicosanoids are potent mediators of inflammation, which are derived from arachidonic acid. They include prostaglandins, thromboxane, isoprostanes, leukotrienes and lipoxins. Prostaglandins and thromboxane are formed through increased expression of inducible cyclo-oxygenase-2. Most prostaglandins and thromboxane have proinflammatory properties. Prostaglandin-E2 and Prostaglandin-I2 are anti-inflammatory.

Leukotrienes are lipid mediators derived from arachidonic acid via the 5-lipoxygenase pathway. Leukotrienes are potent constrictors and proinflammatory mediators that contribute to the pathophysiology of asthma⁹. The cysteinyl-leukotrienes (leukotriene-C₄, leukotriene-D₄ and leukotriene-E₄) are generated predominantly by mast cells and eosinophils. By contrast, leukotriene-B₄ 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-A₂⁹. F₂-isoprostanes are not only relatively stable and specific markers of lipid peroxidation, but also possess potent biological activity⁸⁹. They can be mediators of the cellular effects of oxidant stress and are a reflection of complex interactions between the reactive nitrogen species and reactive oxygen species^{8,9,89}. The most prevalent isoprostane in humans is 8-epi-PGF₂-α also known as 8-isoprostane⁹.

Lipoxin-A₄ and lipoxin-B₄ have potential anti-inflammatory action in asthma, and lipoxin-A₄ is detected in supernatant of induced sputum⁹⁰.

Eicosanoids are measured by enzyme immunoassays, using commercially available detection kits. Recently, validation of measurement of 8-isoprostanes, prostaglandin-E₂ and leukotriene-B₄ in EBC by reverse-phase high-performance liquid chromatography

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 immuno assay

ICS : inhalation corticosteroids

OCS : oral corticosteroids

RIA : radio immuno assay

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 immuno assay (Cayman Chemical, Ann Arbor, Michigan, USA)

HDM : house dust mite

ICS : inhalation corticosteroids

OCS : oral corticosteroids

ELISA : enzyme linked immuno sorbent assay (DPC Biermann, Bad-Neuheim, Germany)

CF : cystic fibrosis

PCD : primary ciliary dyskinesia

or by gas chromatography/mass spectrometry has been reported^{12,91,92*-93*}. Exhaled 8-isoprostane and prostaglandin-E2 are not flow dependent in healthy subjects^{94*}. The EBC levels of prostaglandin-E2 and thromboxane-B2 in healthy subjects with or without nose clip are comparable^{44*}.

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 immuno assay (Cayman Chemical, Ann Arbor, Michigan, USA)

IHV : isocapnic hyperventilation

ICS : inhalation corticosteroids

PCD : primary ciliary dyskinesia

Paediatric EBC data on eicosanoids are summarised in Tables 3-6.

In asthmatic children, elevated eicosanoids were found when compared with healthy children^{74,95*-101*}. An inverse correlation between exhaled cysteinyl-leukotrienes and leukotriene-B4 in children with mild persistent asthma was assessed, which may reflect the contrary effect of steroids on eosinophil and neutrophil survival⁷⁴. No significant diurnal variation in cysteinyl-leukotriene levels was found in asthmatic children^{51*}. Reduced cysteinyl-leukotrienes 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-leukotrienes and 8-isoprostanes of children with an asthma exacerbation⁷⁵. Leukotriene-B4 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*}.

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 immuno assay (Cayman Chemical, Ann Arbor, Michigan, USA)

ICS : inhalation corticosteroids

n.s. : not significant

RIA : radio immuno assay

In children with severe atopic eczema but without asthma, increased cysteinyl-leukotrienes were found in EBC ^{105*}.

In children with cystic fibrosis and primary ciliary dyskinesia, cysteinyl-leukotrienes were detected in EBC ^{106*-107*}.

Decreased concentrations of prostaglandin-E2 were reported in children with primary ciliary dyskinesia, compared with healthy controls ^{107*}. No differences were seen in the levels of leukotriene-B4 and cysteinyl-leukotrienes between these children ^{107*}.

3. Nitrogen-reactive species

Nitrite/nitrate, S-nitrosothiols and 3-nitrotyrosine are nitrogen-reactive species. The reaction between nitric oxide and superoxide anions in the airways results in the formation of peroxynitrite, a reactive oxygen species. 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 nitric oxide-derived oxidants in various disease states ^{8,9,12}. The balance between nitrite/nitrate, S-nitrosothiols, and nitrotyrosine gives insight into nitric oxide synthesis and short- and long-term changes in nitric oxide 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

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 : inhalation corticosteroids

C : colorimetric assay (Griess reaction)

CF : cystic fibrosis

n.s. : not significant

PCD : primary ciliary dyskinesia

F : fluorimetric assay (2,3 di-amino-naphtalene)

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 summarised in Tables 7 and 8.

In healthy children, nitrite values were not related to the levels of exhaled nitric oxide 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*}.

Table 8. 3-nitrotyrosine 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, moderate	13.5	5.3	<0.05	119*	EIA	-
CF	1.60	1.69	n.s.	117*	EIA	-

EBC : exhaled breath condensate

EIA : enzyme immuno assay (Cayman Chemical, Ann Arbor, Michigan, USA)

CF : cystic fibrosis

n.s. : not significant

In children with cystic fibrosis, elevated levels of nitrite were found in EBC compared to 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 cystic fibrosis ⁶⁸. In infants with cystic fibrosis, nitrite was measured using a copper baby-condensate-sampler ^{33*}. However, no statistical difference in nitrite was found between infants with cystic fibrosis and healthy infants ^{33*}. Nitrotyrosine levels did not differentiate between children with cystic fibrosis and healthy subjects, and there was no correlation with lung function ^{117*}.

In asthmatic children, significantly elevated levels of nitrite were found in EBC compared to controls ^{71,118*}. Nitrite levels were not correlated with age, family history of asthma, anti-inflammatory treatment, number of allergic sensitisations, 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 to normal children, but no correlation was observed with exhaled nitric oxide ^{119*}.

In primary ciliary dyskinesia, levels of nitrite and nitrite/nitrate were not increased ^{120*}.

4. Other markers of oxidative stress

A key feature in the pathophysiology of inflammatory lung diseases is the development of an imbalance between oxidants (reactive oxygen species) and anti-oxidants (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

Table 9. Malondialdehyde and glutathione in EBC of children

Mediator	Disease	Median value patient group (nanomol)	Median value healthy controls (nanomol)	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

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, nonanal). Aldehydes are involved in many of the pathophysiologic 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 oxidised linoleic acid and arachidonic acid, palmitoleic acid and oleic acid ⁶³. Malondialdehyde can be quantified as (volatile) thiobarbituric acid reactive substances, 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 summarised in Table 9.

In children with an asthma exacerbation, malondialdehyde levels were higher and glutathione levels were lower compared to healthy controls ⁶³. After a course of oral

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-g	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 : interleukin

ELISA : enzyme linked immuno-sorbent assay

† : R&D systems, Minneapolis, USA

EIA : enzyme immuno assay

n.s. : not significant

ICS : inhalation corticosteroids

‡ : Cayman Chemical, Ann Arbor, Michigan, USA

IFN-g : interferon gamma

§ : Pierce Endogen, Rockfort, Illinois, USA

CF : cystic fibrosis

¶ : home made

prednisone, malondialdehyde levels decreased, whereas glutathione levels increased⁶³. No differences were observed among these marker levels obtained at different flow

rates⁶³. No correlation was found between biomarker levels and spirometric values⁶³. α,β -unsaturated aldehydes were detectable only in a few EBC samples, but chemically stable saturated aldehydes were detectable in all EBC samples⁶³.

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 obstruction⁶³.

5. 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 categorised as T-cell derived lymphokines, proinflammatory cytokines, anti-inflammatory (inhibitory) cytokines and growth factors. Analysis of cytokines is performed by enzyme immunoassay, using (modified) commercially available detection kits.

Paediatric EBC data on cytokines are summarised in Table 10.

In cystic fibrosis, proinflammatory interleukin-8 was present in only 30% of the children with clinically stable disease⁶⁸. Although the values were higher than in healthy controls, there was no statistically significant difference⁶⁸.

In asthmatic children, significantly increased mean interleukin-4 levels and decreased mean interferon-gamma levels were found compared with normal children⁷². The levels of exhaled interleukin-4 were lower under steroid treatment⁷².

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

6. Immunoglobulin E

Immunoglobulin E was not demonstrated by nephelometric technique in EBC of children with allergic asthma^{32*}. However, by enzyme immunoassay, immunoglobulin-E 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 of immunoglobulin-E in EBC was found^{126*}.

7. Eosinophilic Cationic Protein

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

8. 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 anatomic-pathophysiology, 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 nitric oxide was found ^{141*}.

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

9. 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 cystic fibrosis 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 customised therapy ¹⁰.

Nevertheless, the apparent simplicity of EBC must not be over-stated, 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 micro-analysis 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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CHAPTER 3

CHILDHOOD ASTHMA: EXHALED MARKERS OF AIRWAY INFLAMMATION, ASTHMA CONTROL SCORE AND LUNG FUNCTION TESTS.

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Abstract

Exhaled markers of airway inflammation become increasingly important in the management of childhood asthma. The aims of the present study are: (i) to compare exhaled markers of inflammation (nitric oxide, carbon monoxide, acidity of breath condensate), with conventional asthma measures (lung function tests, asthma control score) in childhood asthma; and (ii) to investigate the detectability of albumin, C-reactive protein, interleukin-6, interleukin-8, tumor necrosis factor-alpha, soluble intercellular adhesion molecule-1 and soluble tumor necrosis factor-receptor-75 in exhaled breath condensate (EBC) of asthmatic children.

Thirty-two children with mild to moderate persistent asthma, and healthy controls aged 6-12 years were studied. We measured exhaled nitric oxide and carbon monoxide, and subsequently EBC was collected. Inflammatory mediators in EBC were measured using enzyme-linked immunosorbent assay. Respiratory symptoms and asthma control were assessed using the asthma control questionnaire of Juniper et al. Exhaled nitric oxide showed a significant correlation with exhaled carbon monoxide ($r = 0.59$, $p < 0.05$) and forced expiratory volume in 1 second (FEV_1) ($r = -0.59$, $p < 0.05$), but not with asthma control questionnaire score ($r = 0.48$, $p = 0.06$).

Exhaled carbon monoxide was correlated with pre-bronchodilator FEV_1 ($r = -0.45$, $p < 0.05$), but not with asthma control score ($r = 0.18$, $p = 0.35$). Acidity of EBC was significantly lower in asthmatic children than in healthy controls ($p < 0.05$), but did not correlate with any of the conventional asthma measures. We were not able to demonstrate the presence of C-reactive protein, interleukin-6, interleukin-8, tumor necrosis factor-alpha, soluble intercellular adhesion molecule-1 and soluble tumor necrosis factor-receptor-75 in EBC. Albumin was found in two EBC samples of asthmatic children.

We conclude that exhaled nitric oxide had a better correlation with lung function parameters and asthma control than exhaled carbon monoxide and acidity of EBC, in mild to moderate persistent childhood asthma. However, exhaled nitric oxide, carbon monoxide, and deaerated acidity of EBC did not differ between asthmatic children and controls, possibly because of a too homogeneous and well-controlled study population. To further evaluate the clinical utility of exhaled markers in monitoring childhood asthma, more studies are required on a wider range of asthma severity, and, preferably, with repeated measurements of markers and of asthma control.

Introduction

Asthma is a chronic lung disorder characterised by airway inflammation, variable but reversible airway obstruction, and episodic wheezing, coughing or dyspnoea^{1,2}. Non-invasive assessment of inflammatory markers in exhaled air and exhaled breath condensate (EBC) of asthmatic children becomes increasingly important^{3,4}. Airway inflammation leads to increased expression of inducible nitric oxide synthase type 2, and consequently, to an increase of exhaled nitric oxide. Indeed, exhaled nitric oxide of asthmatic patients is elevated, and shows a dose-dependent decrease during treatment with inhaled corticosteroids^{5,6}. A close correlation was found between exhaled nitric oxide and the percentage of eosinophils in bronchoalveolar lavage fluid in asthmatic children⁷. Furthermore, exhaled nitric oxide may be useful to assess clinical heterogeneity in children with moderate and difficult asthma⁸⁻¹⁰. Increased levels of exhaled carbon monoxide, by upregulation of the enzyme heme-oxygenase-1 through oxidative stress and inflammatory cytokines, were described in asthmatic patients^{11,12}. Recently, the acidity (pH) of EBC was suggested as a new marker of inflammation in adult asthmatic patients: the pH of de-aerated EBC was approximately two log orders lower in patients with acute asthma, than in healthy control subjects¹³. However, only a few studies compared these different exhaled markers of inflammation with such conventional asthma measures as lung function tests, reversibility of airway obstruction, respiratory symptoms, and asthma control. It is unknown which exhaled marker of airway inflammation is best related with conventional asthma measures.

Furthermore, data on inflammatory markers in EBC of asthmatic patients are increasing³. However, various other inflammatory parameters have not yet been evaluated in EBC. Many cytokines play a critical role in orchestrating, perpetuating and amplifying the inflammatory response in asthma¹⁴. Tumor necrosis factor-alpha and interleukin-6 are involved in many inflammatory diseases. Interleukin-6 is released in asthma, has growth-regulatory effects on many cells and is involved in T-cell activation, growth and differentiation. It is a terminal differentiation factor for B cells, and an important cofactor in interleukin-4 dependent immunoglobulin-E synthesis. Interleukin-6 may also have anti-inflammatory effects¹⁵. Interleukin-8 appears to possess chemotactic activity for primed eosinophils, however its major action is neutrophil chemoattraction and activation. Interleukin-8 may be a marker of severe asthma¹⁵. Interleukin-8 levels in bronchoalveolar lavage fluid of asthmatic children were not only strongly correlated with neutrophil counts, but also increased with more asthma symptoms¹⁶. Tumor necrosis factor-alpha may have an important amplifying effect in asthmatic inflammation. The effects of tumor necrosis factor-alpha are very similar to those of interleukin-1beta, which is an important growth factor for T-helper type 2 cells in response to antigen-primed antigen-presenting cells¹⁵. Tumor necrosis factor-alpha interacts with two cell surface receptors, tumor necrosis factor-receptor-55 and tumor necrosis factor-receptor-75. Soluble receptors may act as inhibitors of the effects of tumor necrosis factor-alpha. Tumor necrosis factor-receptor-75 is the principal receptor released by human alveolar macrophages and monocytes in the presence of interferon-

gamma¹⁵. Adhesion molecule interactions regulate the migration of inflammatory cells from the circulation into the lungs. The intercellular adhesion molecule-1 was demonstrated to mediate leukocyte rolling on inflamed pulmonary endothelium, and was shown to mediate capillary sequestration in inflamed lungs¹⁷. Soluble intercellular adhesion molecule-1 levels were significantly increased in bronchoalveolar lavage fluid from asthmatic children compared with control children, and these soluble intercellular adhesion molecule-1 levels correlated with disease activity¹⁸.

The aims of the present study in asthmatic and healthy children were: (i) to compare exhaled markers of airway inflammation (exhaled nitric oxide, exhaled carbon monoxide, and acidity of EBC) with conventional asthma measures (lung function tests and asthma control score); and (ii) to investigate the detectability of albumin, C-reactive protein, interleukin-6, interleukin-8, tumor necrosis factor-alpha, soluble intercellular adhesion molecule-1, and soluble tumor necrosis factor-receptor-75 in EBC of asthmatic children.

Subjects and Methods

Subjects

In total, 32 nonsmoking children aged 6-16 years were selected for this study (23 asthmatic children and 9 healthy controls). The asthmatic children were recruited from our outpatient clinic.

Inclusion criteria were: (i) doctor-diagnosed asthma for at least 1 year, with a history of at least two of the following: recurrent wheeze, chest tightness, shortness of breath, and/or cough; and (ii) mild or moderate persistent asthma requiring, for at least 6 months, daily treatment with inhaled corticosteroids¹⁹.

Exclusion criteria were: (i) clinical evidence of a respiratory infection in the 4 weeks prior to the study; and (ii) comorbidities such as severe mental retardation, congenital anomalies of the respiratory tract, or congenital cardiac defects. Allergy, defined as at least one positive radio-allergo-sorbent test (greater than or equal to class 2), was not an obligatory criterium for the diagnosis of asthma. The 9 healthy controls had no history of allergic rhinitis, asthma or eczema. All parents gave written informed consent. The study was approved by the Medical Ethics Committee of the University of Maastricht.

Lung function tests

Bronchodilator medication was stopped prior to lung function testing: short-acting bronchodilators at least 8 hours before the test, and long-acting bronchodilators at least 36 hours before. Dynamic spirometry was performed by means of a pneumotachograph (Masterlab Jaeger, Würzburg, Germany), with measurement of the forced expiratory volume in 1 second (FEV₁) and the forced vital capacity, according to the standards of the European Respiratory Society²⁰. The highest values of three forced expiratory manoeuvres were used for data analysis. The bronchodilating

response was assessed 15 minutes after administration of 400 µg salbutamol (Airomir®) through a spacer (Volumatic®). The bronchodilating response was expressed as the relative increase of FEV₁ compared to the predicted value of the FEV₁.

Nitric oxide in exhaled air

Exhaled nitric oxide was measured by means of the NIOX® (Aerocrine, Solna, Sweden) according to the criteria of the American Thoracic Society²¹. After inhalation of nitric oxide-free air to total lung capacity, the child exhaled slowly, keeping a constant flow rate at 50 ml/sec, guided by a balloon meter. In this way, a nitric oxide plateau was created for at least 3 seconds. Automatic control of exhaled flow rate by a dynamic flow restrictor was carried out by the apparatus, in order to achieve a constant flow rate of 50 ml/sec. The fractional exhaled nitric oxide concentration was determined by averaging the nitric oxide levels during the nitric oxide-plateau for 5-8 seconds of exhalation. The mean fractional exhaled nitric oxide concentration of three consecutive measurements was used for analysis.

Carbon monoxide in exhaled air

Exhaled carbon monoxide was measured by a modified electrochemical analyser (EC50-MICRO smokerlyzer CO monitor, Bedfont Scientific Ltd, UK), sensitive to carbon monoxide from 0-1000 parts per million by volume with a resolution of 1 part per million. Prior to the start of this study, the analyser was calibrated with a mixture of 50 part per million carbon monoxide in air. Subjects were asked to inhale maximally and hold their breath for 20 seconds before exhaling rapidly into a disposable mouthpiece²². This procedure was repeated three times, with 1 minute of normal breathing between each manoeuvre. The mean value was used for analysis²².

Exhaled breath condensate

EBC was collected on ice, while the children were wearing a noseclip and breathed quietly through a mouthpiece, connected to a two-way nonrebreathing valve and a cooled (0°C) double-jacketed 30 cm-long borosilicate glass tube²³. In order to prevent salivary contamination of EBC samples, the two-way valve and tubing were used as a saliva gravity trap. Acidification of the condensate was measured immediately after collection, using a portable pH-meter (Radiometer, type PHM201, Lyon, France)¹³. A stable pH was achieved by deaeration of the condensate with nitrogen at a flow rate of 250-500 ml/min for 10 minutes. The remaining condensate was frozen rapidly by submerging the microtubes in isopentane at -24°C, and was subsequently stored at -75°C to -80°C. Inflammatory mediators were measured in EBC using sandwich enzyme-linked immunosorbent assay, as reported previously²⁴⁻²⁹. The time interval between the collection of EBC and the measurement of mediators was not longer than 2 months.

In short, for detection of soluble tumor necrosis factor-receptor-55 and soluble tumor necrosis factor-receptor-75, monoclonal antibody MR1-1 and monoclonal antibody MR2-2 were used for coating. Specific biotin labelled polyclonal rabbit anti-human soluble tumor necrosis factor-receptor immunoglobulin-G were used as

detector reagents²⁴. Monoclonal antibody 5E1 was used as coating for the interleukin-6 enzyme-linked immunosorbent assay, and biotinylated polyclonal rabbit anti-human interleukin-6 immunoglobulin-G was used for detection²⁵. Interleukin-8 was coated by the interleukin-8 monoclonal antibody HM.5, and detected by biotinylated polyclonal rabbit anti-human interleukin-8 immunoglobulin-G²⁶. Tumor necrosis factor-alpha measurement was performed by coating with anti-human tumor necrosis factor-alpha monoclonal antibody 61E71, and detection was done with rabbit anti-human tumor necrosis factor-alpha²⁷. Enzyme-linked immunosorbent assay for soluble intercellular adhesion molecule-1 consisted of monoclonal antibody HM2 as coatings reagent, and biotinylated HM1 for detection²⁸. For detection of human albumin, plates were coated with monoclonal antibody HM17. Albumin was detected with a horseradish peroxidase-labeled polyclonal rabbit anti-human albumin immunoglobulin G (Cappel; Organon Teknika Corporation, West Chester, PA)²⁹. The C-reactive protein concentration was measured by using a polyclonal enzyme-linked immunosorbent assay, for which antibodies and standard were obtained from Dako A/S (Glostrup, Denmark). Sensitivity of the assays was 40 pg/ml for both soluble tumor necrosis factor-receptors; 20 pg/ml for interleukin-6, interleukin-8, and tumor necrosis factor-alpha; 3 ng/ml for soluble intercellular adhesion molecule-1; 8 ng/ml for albumin; and 50 pg/ml for C-reactive protein.

Immunoassay plates (NUNC Immuno Plate Maxisorp, Roskilde Denmark) were used for enzyme-linked immunosorbent assays. Biotinylated samples were detected using streptavidin-peroxidase conjugate, obtained from Zymed Laboratories Inc. (San Francisco, CA). The substrate used was 3,3',5,5'-tetramethylbenzidine (KPL, Gaithersburg, MD). Absorbance was measured spectro-photo-metrically at 450 nm using a micro-enzyme-linked immunosorbent assay autoreader.

Asthma control and symptoms

Respiratory symptoms and the degree of asthma control were measured in a standardised way by the Asthma Control Questionnaire of Juniper et al 30. The validity of the Dutch translation has been checked, and appeared to be excellent.

Power analysis and statistics

A number of 22 subjects is necessary to assess clinically relevant correlations between exhaled markers of airway inflammation and conventional measures of asthma severity with a correlation coefficient of 0.60, a power of 90%, and an alpha of 0.05. Differences between normally distributed parameters were tested with the unpaired Student's t-test, and non-normally distributed parameters with the Mann-Whitney test. Relationships between exhaled markers of airway inflammation, lung function tests and asthma control questionnaire score, were determined by Spearman correlation coefficients. $P < 0.05$ was considered statistically significant.

Results

Characteristics of the study population

No difference in age, height, weight and postbronchodilator FEV₁ were found between asthmatic children and healthy controls (Table 1). As expected, asthmatic subjects had a significantly higher asthma control questionnaire score, and a lower prebronchodilator FEV₁ than healthy controls (Table 1). The mean EBC collection time was 30 minutes in the asthmatic and healthy control groups. The mean volume of collected condensate was 2.6 ml (SD, 0.5) in the asthmatic children, 2.9 ml (SD, 0.3) in the control group, and 2.7 ml (SD, 0.5) in the total study population.

Table 1. Characteristics of study population ¹

	Healthy controls	Asthmatic children
Number	9	23
atopic / non-atopic	-	16 / 7
mild / moderate	-	13 / 10
ICS dose (µg)	-	400 (456)
Age (years)	9.4 (1.0)	10.6 (2.8)
Weight (kg)	33.6 (10.2)	35.4 (12.3)
Height (m)	1.41 (0.15)	1.42 (0.14)
Pre-FEV ₁ (% pred)	104.6 (6.9) *	96.4 (16.2)
Post-FEV ₁ (% pred)	107.6 (6.6)	103.2 (16.7)
Reversibility (% pred)	3.0 (3.8)	6.8 (8.4)
ACQ score	0 **	9.7 (8.2)

ICS beclomethasone dipropionate or equivalent

Pre-FEV₁ prebronchodilator forced expiratory volume in 1 second, in % predicted

Post-FEV₁ postbronchodilator forced expiratory volume in 1 second, in % predicted

ACQ score asthma control questionnaire score

¹median and (standard deviation)

* p=0.053, unpaired Student's t-test

** p<0.0001, unpaired Student's t-test

Exhaled nitric oxide, exhaled carbon monoxide, and acidity of EBC

Between asthmatic children and healthy controls, no significant differences in exhaled nitric oxide and exhaled carbon monoxide were found (Table 2). Only the not deaerated pH of EBC in the asthmatic group (median, 6.49) was significantly lower than in the healthy control group (median, 6.64) (unpaired Student's t-test, p<0.05).

Albumin, C-reactive protein, and cytokines in EBC

In all EBC samples, C-reactive protein was less than 50 pg/ml; interleukin-6, interleukin-8, and tumor necrosis factor-alpha were less than 20 pg/ml; soluble intercellular adhesion molecule-1 was less than 3 ng/ml, and soluble tumor necrosis factor-

Table 2. Noninvasive markers of airway inflammation (exhaled nitric oxide, exhaled carbon monoxide, and pH of exhaled breath condensate) in asthmatic children and healthy controls¹

	Healthy controls	Asthmatic children
eNO (ppb)	15.6 (8.1)	23.1 (5.0)
eCO (ppm)	2.1 (0.7)	2.3 (0.3)
pH-dea	8.11 (0.07)	8.14 (0.06)
pH-nondea	6.64 (0.05) *	6.49 (0.05)

eNO exhaled nitric oxide, in parts per billion
 eCO exhaled carbon monoxide, in parts per million
 pH-dea deaerated pH of exhaled breath condensate
 pH-nondea nondeaerated pH of exhaled breath condensate
¹median and (standard error of the mean)
 * p<0.05, unpaired Student's t-test

receptor-75 was less than 40 pg/ml. Albumine was detected in two samples of asthmatic children, with a concentration of 116 and 243 ng/ml, respectively. The highest value was found in a child with unstable disease. Albumin was not detected in EBC samples of the healthy control group.

Relationship between exhaled nitric oxide, exhaled carbon monoxide, acidity of EBC, lung function tests, and asthma control questionnaire score

In the total study population, exhaled nitric oxide showed significant correlations with exhaled carbon monoxide, and pre- and postbronchodilator FEV₁, and showed a nonsignificant correlation with asthma control score (Table 3). Figure 1 shows the

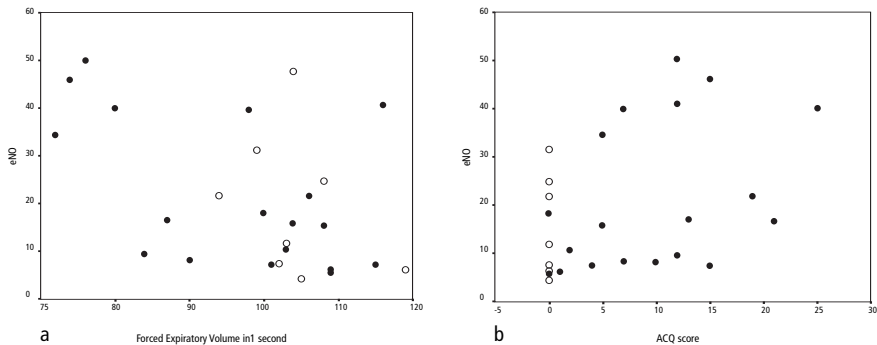


Figure 1. Scatterplots of (a) exhaled nitric oxide and prebronchodilator FEV₁ ($r = -0.59, p < 0.05$), and (b) exhaled nitric oxide and asthma control questionnaire score ($r = 0.48, p = 0.06$).

eNO exhaled nitric oxide, in parts per billion
 ACQ score asthma control questionnaire score
 (○) healthy control children
 (●) asthmatic children

Table 3. Spearman correlation coefficients between conventional asthma measures (FEV₁ and asthma control questionnaire score), and noninvasive markers of airway inflammation (exhaled nitric oxide, exhaled carbon monoxide, and pH of exhaled breath condensate) in total study population (n=32)

	eNO	eCO	pH-nondea	pH-dea	pre-FEV ₁	post-FEV ₁	ACQ
eNO	-	0.59*	-0.23	0.20	-0.59*	-0.67**	0.48†
eCO	0.59*	-	-0.41*	-0.13	-0.45*	-0.33	0.18
pH-nondea	-0.23	-0.41*	-	0.68**	0.10	0.07	-0.25
pH-dea	0.20	-0.13	0.68**	-	-0.25	-0.25	0.08
pre-FEV ₁	-0.59*	-0.45*	0.10	-0.25	-	0.86**	-0.26
post-FEV ₁	-0.67**	-0.33	0.07	-0.25	0.86**	-	-0.16
ACQ	0.48***	0.18	-0.25	0.08	-0.26	-0.16	-

eNO exhaled nitric oxide

eCO exhaled carbon monoxide

pH-nondea nondearated pH of exhaled breath condensate

pH-dea deaerated pH of exhaled breath condensate

pre-FEV₁ prebronchodilator forced expiratory volume in 1 second

post-FEV₁ postbronchodilator forced expiratory volume in 1 second

ACQ score asthma control questionnaire score

* p< 0.05

** p<0.01

*** p=0.06

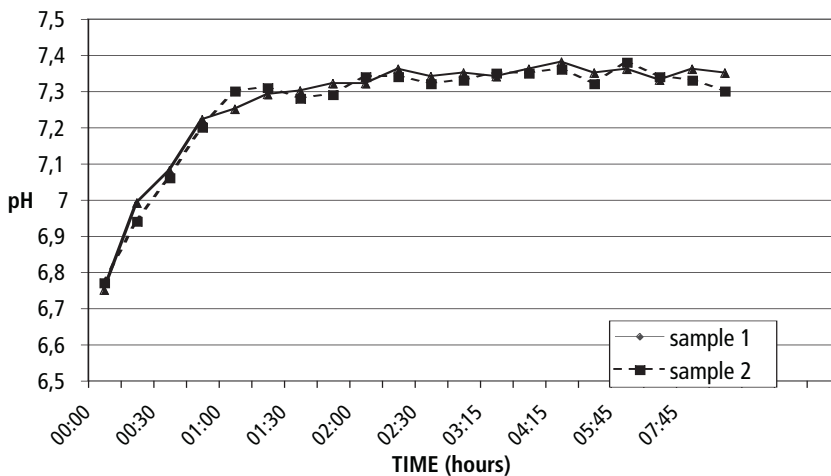


Figure 2. Nondearated pH of exhaled breath condensate with time (hours) in 2 subjects of the study population.

correlations found between exhaled nitric oxide and prebronchodilator FEV₁ ($r = -0.59$, $p < 0.05$), and exhaled nitric oxide and asthma control score ($r = 0.48$, $p = 0.06$). Exhaled carbon monoxide was significantly correlated with prebronchodilator FEV₁ and nondeaerated pH of EBC. Although the nondeaerated pH of EBC was significantly lower in asthmatics than in controls, it only correlated with exhaled carbon monoxide, and not with FEV₁, asthma control score, and exhaled nitric oxide. When correlations were analysed in asthmatics separately, comparable results were found. Deaeration of EBC did not influence the correlations of pH with the other variables. There was a substantial increase of pH levels of nondeaerated EBC samples over time (Figure 2). This effect in pH was almost 0.5 units in the first hour after EBC collection.

Discussion

Noninvasive assessment of inflammatory markers in exhaled air and EBC is a growing research field^{3,4}. This is the first study in a paediatric population comparing exhaled inflammatory markers (exhaled nitric oxide, exhaled carbon monoxide, and acidity of EBC) with lung function tests and asthma control score. Exhaled nitric oxide showed higher correlations with conventional measures of asthma, than did exhaled carbon monoxide, and pH of EBC: exhaled nitric oxide did correlate with pre- and post-bronchodilator FEV₁, and with asthma control score. Exhaled nitric oxide levels were significantly related to exhaled carbon monoxide levels, but exhaled carbon monoxide was less related to lung function parameters and symptoms than exhaled nitric oxide. Although the pH of EBC was lower in asthmatics than in healthy control children, the acidity was not related to any other parameter. It was not possible to demonstrate C-reactive protein, interleukin-6, interleukin-8, tumor necrosis factor-alpha, soluble intercellular adhesion molecule-1, and soluble tumor necrosis factor-receptor-75 in EBC.

Significant correlations between exhaled nitric oxide and lung function parameters were described in asthma previously, both cross-sectionally^{5,31} and longitudinally^{5,32}. However, several other studies found no relationship between exhaled nitric oxide and lung function tests^{33,34}. In our study, a significant correlation existed between exhaled nitric oxide and lung function tests. However, the percentage of variance in exhaled nitric oxide by changes in lung function ($= r^2$) was less than 45%, indicating that exhaled nitric oxide and lung function tests probably reflect different aspects of the airway pathophysiology^{35,36}.

With respect to exhaled carbon monoxide and lung function parameters, we found a significant but moderate correlation between exhaled carbon monoxide and prebronchodilator FEV₁. Conflicting results are reported in the adult literature^{37,38}. In childhood asthma, however, no significant correlation was found between exhaled carbon monoxide and FEV₁^{12,22,39}. The different correlations of exhaled nitric oxide and carbon monoxide with conventional asthma measures may be due to the differences in standardization and methodology. Extensive recommendations on standardization of measurements are formulated for exhaled nitric oxide, but not for

exhaled carbon monoxide ^{21,40}. Comparable to exhaled nitric oxide, it may be that exhaled carbon monoxide is flow-dependent, and that ambient air carbon monoxide levels may disturb exhaled carbon monoxide measurements. Ambient air carbon monoxide levels were not recorded in our study.

Our data suggest that exhaled nitric oxide has a better correlation with lung function parameters and asthma control in childhood asthma, than exhaled carbon monoxide. This view is supported by other recent findings ³⁹. Before oral prednisone therapy, both exhaled nitric oxide and carbon monoxide were elevated in 30 children with an asthma exacerbation, compared with healthy controls ³⁹. After steroid treatment, exhaled nitric oxide, but not exhaled carbon monoxide, significantly improved ³⁹.

We did not find a significant difference for exhaled nitric oxide or carbon monoxide, between asthmatic children and healthy controls. Several explanations are possible. (i) Maintenance treatment with inhaled corticosteroids is known to rapidly decrease exhaled nitric oxide levels, and may also decrease exhaled carbon monoxide ^{6,41,42}. All asthmatic children in our study were on long term inhalation corticosteroids. Inclusion of a group of steroid-free asthmatic children, would have facilitated interpretation of the influence of inhaled corticosteroids on exhaled markers. (ii) Most children had mild to moderate persistent asthma with fairly well-controlled disease, which may lower exhaled nitric oxide and also exhaled carbon monoxide levels ^{22,34}.

Our data showed lower pH of EBC in asthmatic children than in healthy controls, which corresponds to the results of others in adults ^{13,43}. However, this potential marker of inflammation was not correlated with lung function tests and asthma control at all. We demonstrated a substantial increase of pH level with time for nondeaerated samples of EBC, averaging about 0.5 units in the first hour after collection. Deaeration of EBC samples did not improve correlation of pH of EBC with conventional asthma measures. The influence of deaeration on the pH of EBC needs further clarification.

Albumin was detected in two EBC samples of asthmatic children, of which one had unstable disease. Furthermore, albumin was not detected in EBC of the healthy controls. In asthmatic subjects, albumin was demonstrated in induced sputum ⁴⁴. Albumin may reach the epithelial lining fluid by microvascular leakage in the airway epithelium ⁴⁴. Therefore, the detection of albumin in EBC may be related to airway inflammation in asthma.

We were not able to demonstrate the presence of C-reactive protein, interleukin-6, interleukin-8, tumor necrosis factor-alpha, soluble intercellular adhesion molecule-1, and soluble tumor necrosis factor-receptor-75 in EBC samples of our children. Possibly the detection limit of the assays used was not low enough to measure the concentration of these substances in EBC. Alternatively, the concentrations of these inflammatory mediators in EBC may have been adequately down-regulated by the long-term

treatment with inhaled corticosteroids used by our asthmatic children. On the other hand, these mediators may not be present in EBC of asthmatic children, because these cytokines are not derived from T-helper type 2 cells, in contrast to interleukin-4 and interleukin-5, which are more specific to allergic airway inflammation. Until now, interleukin-8 has only been demonstrated in EBC of children with cystic fibrosis, but not in asthma ⁴⁵. The lower detection limit of the interleukin-8 assay used by Cunningham and co-workers was 0.5 pg/ml, which is more sensitive than the limit of 20 pg/ml used in our study ⁴⁵. So far, soluble intercellular adhesion molecule-1, soluble tumor necrosis factor-receptor-75, tumor necrosis factor-alpha, and C-reactive protein have not been found in EBC of asthmatic patients. We recommend the use of assays with the lowest possible detection limit for measurement of inflammatory markers in EBC. Methodological standardizations of EBC collection systems, procedures, preservation methods, and analysis are needed, as recently reported ⁴⁶.

A limitation of our study may be the relatively small number of subjects. However, the power analysis showed that 22 subjects were sufficient to assess a clinically relevant correlation of 0.60 between exhaled inflammatory markers and conventional measures of asthma severity, with a power of 90%, and an alpha of 0.05. Therefore, the number of subjects was sufficient to answer the aims of this study.

Asthmatic heterogeneity implicates a variable degree of chronic airway inflammation, airway hyperresponsiveness, reversible airway obstruction, and potential but irreversible structural remodeling of the airways ⁴⁷. Moreover, asthmatic airway inflammation is not necessarily correlated with airway calibre ^{8,34,36}, and many inflammatory and structural changes may begin early in life ^{47,48}. Hence, a discordant pattern of generally low correlations between measures of airway inflammation, clinical parameters of disease severity, and physiological variables such as spirometric measurement of airflow obstruction, as shown in our study, may not be surprising at an early stage ^{35,49-51}. Childhood asthma management should include noninvasive integrated monitoring of all of these aspects: disease severity, disease control, airflow obstruction, airway hyperresponsiveness, airway inflammation, and airway remodeling.

In the present study, exhaled nitric oxide showed better correlations with conventional asthma measures. However, this does not imply that exhaled nitric oxide is the 'ideal' parameter of asthmatic inflammation. Pragmatically, the question remains whether exhaled nitric oxide is sensitive and specific enough to discriminate (some or all) asthmatic children from children with other chronic lung diseases, to guide adjustment of a maintenance dose of inhaled corticosteroids, to predict asthmatic exacerbations, and to improve and individualize the care for asthmatic children. Furthermore, the analysis of EBC is still hampered by the lack of methodological standardization. Numerous methodological pitfalls await further investigation, before clinical studies will help to evaluate the diagnostic and monitoring capacity in children with various chronic inflammatory lung diseases. Basically, at present it is not possible to make a judgement of whether EBC markers have value or not.

In conclusion, exhaled nitric oxide had a better correlation with lung function parameters and asthma control than did exhaled carbon monoxide and pH of EBC in mild to moderate persistent childhood asthma. However, exhaled nitric oxide, exhaled carbon monoxide, and deaerated pH of EBC did not differ between asthmatic children and healthy controls. Albumin was detected in two condensate samples of asthmatic children, but C-reactive protein, interleukin-6, interleukin-8, tumor necrosis factor-alpha, soluble intercellular adhesion molecule-1, and soluble tumor necrosis factor-receptor-75 were not found in EBC of asthmatic and healthy children. Methodological standardisation of EBC is mandatory. To further evaluate the clinical utility of exhaled markers in monitoring childhood asthma, more studies are required on a wider range of asthma severity, and preferably, with repeated measurements of markers and of asthma control.

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CHAPTER 4.1

EXHALED BREATH CONDENSATE: A SPACE ODYSSEY, WHERE NO ONE HAS GONE BEFORE ...

Rosias P, Robroeks C, Hendriks J, Dompeling E, Jöbsis Q.
European Respiratory Journal 2004; 24: 189-190.



Exhaled breath condensate: a space odyssey, where no one has gone before ...

Letter to the Editor of the European Respiratory Journal:

We read with great interest the editorial of Rahman ¹ on the reproducibility of oxidative stress biomarkers in breath condensate. As a consequence, we would like to share some of our thoughts, as ultimately, we all may want to walk on planet Mars. However, this does not mean that we are already able to lift off, as many methodological problems first need to be properly addressed.

Similarly, exhaled breath condensate (EBC) is an interesting noninvasive technique to explore inflammatory lung diseases, where no one has gone before ... Many methodological issues are still waiting to be solved, as recently reviewed ². Indeed, the development of EBC is currently hampered by many conflicting reports on biomarker reproducibility. As clearly stated, one of the main obstacles consists of current analytical problems, due to limitations of sensitivity and specificity of the assays used to date ³.

However, the statement that “now with the use of EcoScreen, collection of EBC is being standardised in many leading laboratories” may be misleading, as it suggests that “this would no longer be a confounding factor contributing to the variations in biomarkers in EBC” ¹. We want to clearly point out that the EBC collection method still remains a possible confounding factor and an important source of biomarker variability, because standardisation involves applying more than one identical collection technique. To our knowledge, there is no scientific evidence that the EcoScreen condenser would be the most valid technique to collect EBC for the measurement of inflammatory mediators in condensate. In fact, the key issue is not the reproducibility of a certain biomarker, but the reproducibility of a certain biomarker for a certain condenser system ². We compared the influence of different inner condenser coatings on the detection of human albumin and 8-isoprostane in EBC ⁴. Our data show a much greater efficiency of condenser systems with a borosilicate glass or silicone coating, compared with the EcoScreen, or condensers with aluminium, polypropylene and teflon coating. This implicates that the EcoScreen may not be the most valid apparatus, at least not for some biomarkers.

Although the need for clear-cut methodological recommendations is incontestable, one has to recognise that we are not yet able to give such recommendations. Further research on the reproducibility of biomarkers with different condenser systems is urgently needed.

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CHAPTER 4.2

BREATH CONDENSER COATINGS AFFECT MEASUREMENT OF BIOMARKERS IN EXHALED BREATH CONDENSATE

Rosias PP, Robroeks CM, Niemarkt HJ, Kester AD, Vernooij JH, Suykerbuyk J, Teunissen J, Heynens J, Hendriks HJ, Jöbsis Q, Dompeling E.
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Abstract

Exhaled breath condensate collection is not yet standardised and biomarker measurements are often close to lower detection limits. In the current study, it was hypothesised that adhesive properties of different condenser coatings interfere with measurements of eicosanoids and proteins in breath condensate.

In vitro, condensate was derived from a collection model using two test solutions (8-isoprostane and albumin) and five condenser coatings (silicone, glass, aluminium, polypropylene, and Teflon). *In vivo*, condensate was collected using these five coatings and the EcoScreen® condenser to measure 8-isoprostane, and three coatings (silicone, glass, EcoScreen®) to measure albumin.

In vitro, silicone and glass coatings had significantly higher albumin recovery compared with the other coatings. A similar trend was observed for 8-isoprostane recovery. *In vivo*, median (interquartile range) 8-isoprostane concentrations were significantly higher using silicone (9.2 (18.8) pg/mL) or glass (3.0 (4.5) pg/mL) coating, compared with aluminium (0.5 (2.4) pg/mL), polypropylene (0.5 (0.5) pg/mL), Teflon (0.5 (0.0) pg/mL), and EcoScreen® (0.5 (2.0) pg/mL). Albumin *in vivo* was mainly detectable using glass coating.

In conclusion, a condenser with silicone or glass coating is more efficient for measurement of 8-isoprostane or albumin in exhaled breath condensate, than EcoScreen®, aluminium, polypropylene or Teflon. Guidelines for exhaled breath condensate standardisation should include the most valid condenser coating to measure a specific biomarker.

Introduction

The collection of exhaled breath condensate (EBC) has been rediscovered as a simple and noninvasive technique to measure mediators of airway inflammation. EBC consists not only of water vapor, but also contains aerosolised respiratory fluid droplets released from the respiratory epithelial lining fluid. These fluid droplets contain traces of nonvolatile solutes, which can be recovered in EBC samples¹. EBC is collected by guiding and cooling exhaled air of a tidally breathing subject in a condenser system. EBC does not affect the airways; in contrast to bronchial biopsy, bronchoalveolar lavage and induced sputum. It can be obtained with minimal risk and minimal inconvenience for both adults and children². Although the American Thoracic Society and European Respiratory Society Task Force on EBC recently published general methodological recommendations on the collection of EBC, there are still some methodological pitfalls and unresolved questions³⁻⁴. The most commonly used condensers are the Ecoscreen® (Erich Jaeger, GmbH, Hochberg, Germany), RTube® (Respiratory Research Inc., Charlottesville, VA, USA), and home-made glass or Teflon devices.

Two important groups of inflammatory biomarkers in EBC of adults and children are eicosanoids and cytokines³⁻¹⁰. Eicosanoids, such as 8-isoprostane, are formed by lipid peroxidation of arachidonic acid during oxidative stress¹¹. 8-Isoprostane (molecular weight 354 Dalton) can be measured in EBC of adults and children, although reproducibility remains controversial¹²⁻¹³. Cytokines are low molecular weight (<80.000 Dalton) proteins involved in mediating inflammation and tissue repair. Reports on the detection of cytokines in EBC are incidental, with the exception of a report on interleukin-6 by Carpagnano and co-workers¹⁴. Albumin, a protein with a similar molecular weight (66.000 Dalton), was demonstrated in induced sputum and EBC in asthmatic subjects¹⁵⁻¹⁶.

The reported mean biomarker values in EBC are usually situated in the lower range of detection^{3,6}. It may be that the biomarker concentration in the epithelial lining fluid is intrinsically low, or that only minor amounts are released from the lining fluid into exhaled air. An alternative explanation may be that adhesive properties of different inner condenser coatings interfere with the detection of various inflammatory markers in EBC^{13,17}.

The aim of this study was to investigate the influence of different inner condenser coating surfaces (silicone, borosilicate glass, aluminium, polypropylene, Teflon, and EcoScreen® with a Teflon-like coating) on the measurement of 8-isoprostane and albumin in EBC using both an *in vitro* and *in vivo* approach.

Methods

In vitro studies

In close collaboration with the department of Instrument Development Engineering & Evaluation of the University of Maastricht (Maastricht, the Netherlands), an *in vitro* EBC collection model was developed consisting of a rechargeable condenser system connected in series with an ultrasonic nebuliser (Ultra-Neb™2000, DeVilbiss) and a ventilator (Servo-900C, Siemens) (figure 1A). The rechargeable condenser system consisted of a fixed 30-cm metal outer cylinder (diameter 4cm) (Felix Philips Maatmetaal BV, Maastricht, the Netherlands) connected to a counter current circulating ice-water pump at 0°C, and an exchangeable inner cylinder (diameter 2cm), as shown in figure 1B. The exchangeable inner cylinder was available in five different coatings: silicone, borosilicate glass, aluminium, polypropylene and Teflon. The borosilicate glass tubes



1A. *In vitro* exhaled breath condensate collection model consisting of a rechargeable condenser system, connected in series with an ultrasonic nebuliser and a ventilator. The nebuliser could be filled with a test solution of a known concentration of 8-isoprostane or human albumin. The nebulised test solution is propelled by the ventilator through the condenser system.

1B. Schematic representation of the rechargeable condenser tube system, which consisted of a fixed 30cm metal outer cylinder (diameter 4cm), connected to a counter-current circulating ice-water pump, and an exchangeable inner cylinder (diameter 2cm). The exchangeable inner cylinder was available in five different coatings: silicone, borosilicate glass, aluminium, polypropylene, and Teflon. The outer surface of this inner cylinder was in direct contact with the counter-current circulating ice-water, while its inner surface was in direct contact with the exhaled breath or the volatile substances derived from the nebuliser and propelled by the ventilator.

Figure 1A-1B. The rechargeable exhaled breath condenser system

were manufactured by Louwers Glass and Ceramic Technologies (Hapert, the Netherlands). Identical glass tubes were coated internally with silicone by Imbreglon BV (Beuningen, the Netherlands). The aluminium tubes were manufactured by Felix Philips Maatmetaal BV. Identical aluminium tubes were coated internally with Tempcoat 1011F (Teflon) by Imbreglon BV. The polypropylene tubes were produced by Applikon BV

(Schiedam, the Netherlands). The outer surface of the inner cylinder was in direct contact with the counter current circulating ice-water, while its inner surface was in direct contact with the exhaled breath or the volatile substances derived from the nebuliser and propelled by the ventilator. The nebuliser was filled with a biomarker test solution (42 pg/mL 8-isoprostane or 0.1 mg/mL albumin in a 22 mL saline 0.9% solution), which was nebulised and propelled by a ventilator through the condenser system, simulating tidal breathing for 15 minutes (ventilator settings: inspiration time 33%, respiratory frequency 20/minute, tidal volume 200 mL). The construction of the collection device only allowed contact between the propelled air/exhaled breath and one specific type of inner coating that was chosen at that moment (figure 1B). The condensate was collected at the open end of the condenser device directly into vials. For each coating, 15 experiments were performed (see the power analysis in the Statistics section). After each experiment, the coated tubes were rinsed repeatedly with bi-distilled water and subsequently dried at room air for at least 24 hours. Multiple coated tubes of each type were available to allow a smooth continuation of the experiments, without any delay due to the drying of the coated tube that was used in the preceding test. The biomarker condensate recovery percentage was defined as the ratio of biomarker concentration in condensate, to biomarker concentration in the test solution.

***In vivo* 8-isoprostane study**

As 8-isoprostane has been detected in EBC of healthy subjects, the influence of condenser coatings on 8-isoprostane measurements *in vivo* was studied in 28 healthy volunteers (see the power analysis in the Statistics section). Standardised questionnaires from the International Study of Asthma and Allergies in Childhood (ISAAC) were completed to exclude the presence of respiratory infections, asthma or allergic disease¹⁸. Each subject was asked to breathe tidally, while wearing a nose-clip, into a mouthpiece connected to a two-way non-rebreathing valve that was in turn connected by tubing to the condenser. The two-way valve and tubing also served as a saliva trap. In this way, each subject exhaled during 15 minutes along each of the five coatings in random order, as well as along the EcoScreen[®], a commercial condenser system with a modified Teflon coating.

***In vivo* albumin study**

In contrast to 8-isoprostane, the *in vivo* albumin study was planned in asthmatic children, as the chance on positive albumin detections is larger in a disease population¹⁶. EBC was collected in 40 asthmatic children. Inclusion criteria were doctor-diagnosed mild or moderate persistent asthma requiring daily inhaled corticosteroids for at least 6 months¹⁹. Exclusion criteria were clinical evidence of respiratory infection 4 weeks prior to study, and co-morbidity (mental retardation, respiratory tract anomalies, or cardiac defects). In contrast to the *in vivo* 8-isoprostane study, the asthmatic children in the albumin study were only asked to exhale tidally along one type of coating (silicone or glass or EcoScreen[®]), to allow parallel group comparison. Children were randomly allocated to one out of three coating groups.

Sample processing

Immediately after collection, the condensate samples were snap-frozen at -78°C using dry ice and subsequently stored at -80°C until analysis. A reaction mixture of the immunoassay was not added to the condensate samples before storage. In order to maintain optimal condensate sample preservation, for subsequent 8-isoprostane and albumin measurement, samples were analysed within 6 to 8 weeks of storage at -80°C . Samples were only defrosted once, at the time of analysis. All isoprostane and albumin concentrations were determined in duplicate by specific enzyme immunoassay (Cayman Chemical®, Ann Arbor, MI, USA) and sandwich enzyme-linked immunosorbent assay, respectively ¹⁶. The lower limit of detection was 2.45 and 32 pg/mL, respectively.

Statistics

The condensate recovery percentage of a specific coating was the primary outcome measure of the *in vitro* experiments. Mean values and standard deviations of recovery percentages were calculated. A comparison between the five different coatings and between the two biomarkers *in vitro* was made by means of univariate analysis of variance (ANOVA). Power analysis demonstrated a required number of 15 experiments in each group, in order to detect a relevant difference between coatings of 25% with a power of 90%, an alpha of 0.05 and a standard deviation of 20% (the standard deviation in the first set of 10 experiments was 20%).

In the *in vivo* studies, the number of positive detections, median and mean values, standard deviations (based on the first 15 subjects) and interquartile ranges were calculated. Power calculation showed that 23 patients are needed to detect a 10% difference in biomarker concentration with a power of 90%, an alpha of 0.05, and a standard deviation of 14% (paired t-test). Due to a number of negative detections, the Wilcoxon paired signed-rank test was used to compare data within subjects. A sample with negative detection is not considered as a missing value, because it actually informs us that the measurement of a marker was below the detection limit. Therefore, the negative detection is given an arbitrary value of 0.5.

Ethics

All subjects gave written informed consent. The study was approved by the Medical Ethics Committee of the University Hospital of Maastricht.

Results

in vitro study of albumin and 8-isoprostane

Albumin was detected in 93% of condensate samples (figure 2). Overall, the mean albumin recovery in condensate was only 52% using this experimental model. The albumin recovery percentages varied from 22.9% using a Teflon coating, to 69.4 and 94.7% using a glass and silicone coating, respectively. The albumin recovery percentage was significantly higher using the silicone and glass coating compared with the other coatings ($p=0.03$, ANOVA). No other statistically significant differences were present.

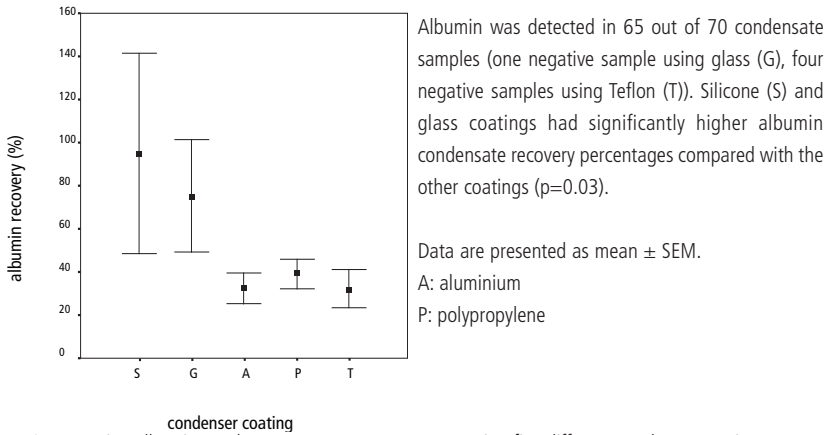


Figure 2. *In vitro* albumin condensate recovery percentages using five different condenser coatings.

Isoprostane was detected in 77 out of 78 condensate samples (figure 3). The isoprostane recovery percentages varied from 93.1% using an aluminium coating to 102.7 and 105.6% using a glass and silicone coating, respectively, which did not reach statistical significance (ANOVA, F-test, $p=0.09$). Overall, the mean 8-isoprostane recovery in condensate was close to 100%, which was significantly higher than the overall albumin recovery percentage of 52% (ANOVA, $p=0.0001$).

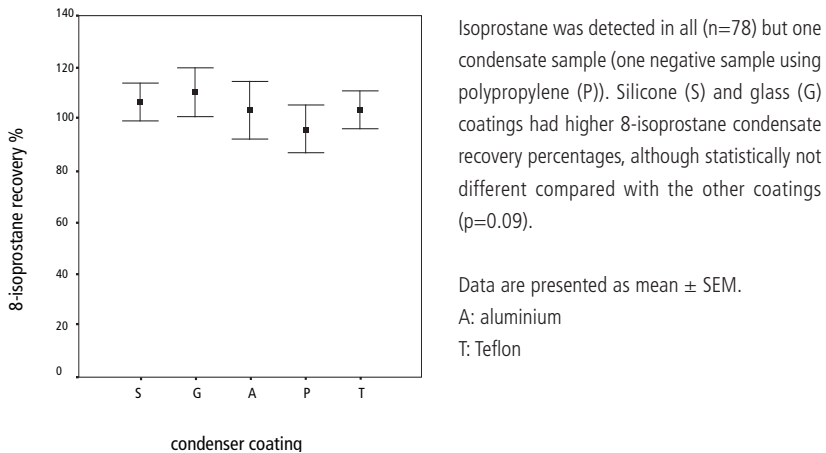


Figure 3. *In vitro* 8-isoprostane condensate recovery percentages using five different condenser coatings.

***In vivo* study of 8-isoprostane**

Subject characteristics are shown in table 1. Isoprostane was detected in 41% of condensate samples (figure 4). For each coating, the number of positive / negative isoprostane detections in EBC was 23 / 5 (silicone), 15 / 13 (glass), 11 / 17 (aluminium), 7 / 21 (polypropylene), 5 / 23 (Teflon) and 8 / 20 (EcoScreen®). The median 8-isoprostane concentrations in EBC in 28 healthy subjects ranged from 0.5 pg/mL for aluminium

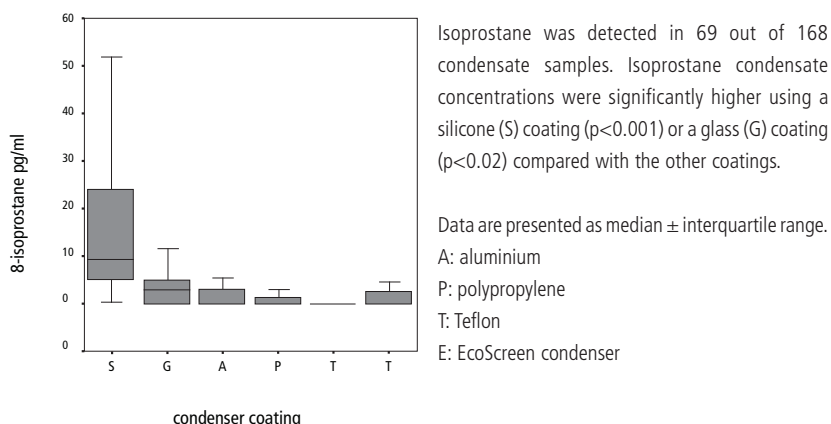


Figure 4. *In vivo* 8-isoprostane condensate concentrations in 28 healthy volunteers using six different condenser coatings.

(interquartile range 0.5-2.9), polypropylene (0.5-1.0), Teflon (0.5-0.5) and the EcoScreen[®] (0.5-2.5), to 3.0 pg/mL (0.5-5.0) pg/mL and 9.2 (5.1-23.9) pg/mL for the glass and silicone coating, respectively. Condensate 8-isoprostane concentrations were significantly higher using silicone coating compared with each of the other coatings ($p < 0.001$, Wilcoxon signed-rank test). Similarly, glass coating was better than polypropylene, Teflon and the EcoScreen[®] ($p < 0.02$, Wilcoxon test). The 8-isoprostane concentrations obtained with aluminium, polypropylene, Teflon and the EcoScreen[®] did not differ from each other ($p = 0.763$, Friedman test). As median values and interquartile ranges in smokers and nonsmokers were comparable, smoking was not a confounder in this study.

***In vivo* study of albumin**

Subject characteristics are shown in table 1. Albumin was detected in 8 out of 40 condensate samples (0.04-0.24 $\mu\text{g/mL}$). Positive detections were almost exclusively found with a glass coating: 7 out of 13 samples using a glass coating, 1 out of 14 samples using the EcoScreen[®], and none out of 13 samples using a silicone coating

Table 1. Subject characteristics.

	Healthy subjects 8-isoprostane study	Asthmatic subjects albumin study
Subjects	28	40
Male	10	27
Smoking	5	0
Atopic	0	36
Age yrs	26 (20-57)	11 (5-17)

Data are presented as n or median (range)

($p < 0.008$, Chi-square test). Disease stability, lung function and atopic status were not confounding factors, as these characteristics were equally distributed across the three coating groups (data not shown).

Discussion

Both *in vitro* and *in vivo*, the present study demonstrates a significant influence of the different inner condenser coatings on the measurement of eicosanoid and protein biomarkers in EBC. In particular, silicone and glass coatings were superior to aluminium, polypropylene and Teflon with respect to the recovery of both 8-isoprostane and albumin in EBC *in vitro* and *in vivo*.

A careful MEDLINE online database search for all available studies on 8-isoprostane in EBC, demonstrates data in line with the results of our study. When considering an identical assay (enzyme- or radio-immunoassay), higher 8-isoprostane concentrations were reported using a glass coating compared with the EcoScreen[®]. *In vivo* using the EcoScreen[®], present 8-isoprostane concentrations (median (range) 3.5 (2.5-4.8) pg/mL) were comparable to the values reported by Van Hoydonck et al (4.6 (3.9-7.7) pg/mL). Specific data on detectability of isoprostane in EBC are scarce. The current study reports 41% of detectability in volunteers, which is in agreement with the findings (36%) of Van Hoydonck et al in smokers²⁰.

In vitro, the overall mean albumin condensate recovery percentage was 52%, compared with 100% isoprostane recovery. This suggests a more difficult condensate recovery of protein biomarkers, which is supported by the present limited number of positive albumin detections *in vivo*. Also, the relatively few positive studies on cytokines or proteins in EBC *versus* several positive reports on 8-isoprostane and leukotrienes in EBC may suggest that the recovery of proteins in EBC is less easily than of eicosanoids. Recently, Tufvesson and Bjermer also showed that the detection of eicosanoids and cytokines in EBC in asthmatic subjects may be facilitated by coating of all collection surfaces with Tween 20 and bovine serum albumin, respectively, combined with vacuum-centrifugation of condensate samples²¹. Moreover, 30-fold differences in condensate levels of interleukin-4 in asthmatic children were found using different condenser systems and enzyme immunoassays²²⁻²⁴. The latter finding indicates that variability of biomarker levels in condensate may also be attributed to intrinsic analytical problems of currently used immunoassays, as illustrated recently for thromboxane-A2 metabolite measurements in EBC²⁴.

In vitro, the mean 8-isoprostane condensate recovery percentages did not differ significantly between the condenser coatings ($p=0.09$). An explanation may be the higher overall mean condensate recovery of isoprostane, in contrast to albumin, which may illustrate a different behavior of these biomarkers during the expiratory phase of the EBC collection. In turn, this may be a reflection of difficulties experienced by molecules with a higher molecular weight, in their transition from respiratory fluid

droplets and exhaled air, to condensate. Conversely, overload in the 8-isoprostane *in vitro* tests seems unlikely when considering that the absolute amount in the test tubes must have been very close to concentrations found *in vivo*. Hence, it may be that the nebuliser set-up is producing 8-isoprostane aerosols that are more effectively condensed as compared to the real EBC *in vivo*. Therefore, further studies on the optimal condenser coating should always include *in vivo* experiments.

The EcoScreen® is a commonly used and commercially available condenser system with distinctive physical characteristics. In contrast to the current authors' collection system and the RTube®, which collect EBC in the liquid phase, the EcoScreen® collects EBC mainly as ice, which may be associated with more drastic freeze-thaw cycles that can affect the molecules collected^{23,25}. The EcoScreen® is a condenser system based on a modified inner Teflon coating. The 8-isoprostane EBC concentrations were similar for the EcoScreen® and the Teflon coating, suggesting no significant influence from the specific physical characteristics of the EcoScreen® condenser device *in vivo*.

A different design of the *in vivo* 8-isoprostane and albumin study was chosen, although ideally both study parts should have had a similar design. However, considerations in support of differing designs were decisive: (i) subjects with asthma are more likely to have positive albumin detection in EBC than healthy controls¹⁶; and (ii) a 90 minutes EBC collection period, like in the *in vivo* 8-isoprostane study, was neither realistic nor feasible in the asthmatic children of the *in vivo* albumin study. Therefore, a between-subject (parallel groups), rather than an intra-subject, comparison of condenser coatings was chosen for the *in vivo* albumin study.

Standardisation of the EBC collection method implicates more than applying one identical collection technique. It implicates the development of a condenser system with the highest and least varying recovery percentages. Optimisation of condensate recovery percentages may lead to an improvement of biomarker reproducibility. Therefore, the current authors recommend the use of a condenser system with an inner glass or silicone coating for the measurement of 8-isoprostane and albumin in EBC. The reproducibility of measurements by using different condenser coatings should be evaluated *in vivo*.

An explanation for this superiority of glass and silicone coatings may be that water acts as a bipolar vehicle. The pH of exhaled breath ranges from 6.50 to 7.80, whereas the isoelectrical point of human albumin is situated at pH 4.80^{3,26}. In other words, at higher pH values (such as reported in EBC) albumin acts as a negatively charged molecule. Therefore, the negatively charged albumin in exhaled breath may be repulsed by glass that is also negatively charged. This may promote the recovery of albumin in EBC. In contrast, the same albumin molecule may be attracted by aluminum metal ions that are positively charged. This may hamper the recovery of albumin in EBC in this case. Furthermore, 8-isoprostane acts in an extremely hydrophobic manner and will stick to neutral surfaces (such as Teflon) in the presence of aqueous solutions, to lipids and to detergents.

Not only adhesive and electrical properties of the condensation equipment may affect whether a compound is collected in EBC, but also issues such as the length of the condensation tube, thermal conduction of the coating, and solute solubility, volatility, and its dissociation constant ($pK_a = -\log K_a$).

Finally, these findings in 8-isoprostane and albumin do not necessarily hold for all other biomarkers. Every biomarker has its own chemical and physical characteristics. Currently, one may speculate that most eicosanoids act in a similar way. Likewise, small proteins such as cytokines may act differently from eicosanoids, but similarly when compared one cytokine to another.

The present authors conclude that a condenser system with a silicone or glass coating is more efficient for the measurement of 8-isoprostane or albumin in EBC, compared with the EcoScreen®, or condensers with aluminium, polypropylene or Teflon coating. The increased efficiency of the borosilicate glass and silicone coatings may be due to less adhesive properties resulting in more biomarker recovery in the condensate. To establish methodological standardisation of the collection of EBC, the most valid and inert condenser coating has to be applied for the measurement of a specific inflammatory biomarker.

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CHAPTER 5

BIOMARKER REPRODUCIBILITY IN EXHALED BREATH CONDENSATE COLLECTED WITH DIFFERENT CONDENSERS

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Abstract

Optimal collection and analysis of exhaled breath condensate (EBC) are prerequisites for standardisation and reproducibility of assessments. We aimed to assess reproducibility of EBC volume, hydrogen peroxide, 8-isoprostane, and cytokine measurements using different condensers, including a newly developed glass condenser.

At four points in time, 30 healthy subjects performed sequential EBC collections randomly using four condensers: glass, silicone, EcoScreen®, and an optimised glass condenser. In small EBC samples, hydrogen peroxide was measured by spectrophotometer, 8-isoprostane by enzyme immunoassay, and cytokines by multiplexed xMAP® technology.

The optimised glass condenser yielded significantly more EBC volume (median 2025µL, interquartile range 1056), reproducibility of EBC volume was comparable with EcoScreen® (coefficient of variation: 19-20%), but was significantly better compared to silicone and glass (coefficient of variation: 29-37%). The new condenser was associated with significantly more detections of hydrogen peroxide, 8-isoprostane, interleukin-2, interleukin-4, interleukin-5, interleukin-13, and tumor necrosis factor-alpha. Isoprostane concentrations were significantly higher using the new condenser, whereas hydrogen peroxide and cytokine concentrations were not. Reproducibility of biomarkers was equally variable for all condenser types.

In conclusion, significantly more EBC volume and biomarker detections were found using the optimised glass condenser, including higher 8-isoprostane levels. However, biomarker reproducibility in EBC in healthy adults was not influenced by the type of condenser.

Introduction

The collection of exhaled breath condensate (EBC) is a non-invasive, safe technique to obtain direct samples from the lower respiratory tract, without disturbing an ongoing inflammation¹⁻³. Analysis of EBC reveals the presence of inflammatory markers such as eicosanoids, hydrogen peroxide, and cytokines¹⁻³. Although the American Thoracic Society and European Respiratory Society Task Force on EBC published general methodological recommendations on the collection and analysis of EBC, there are still some unresolved methodological pitfalls, as illustrated by the use of various nonstandardised collection systems¹⁻³.

Optimal condensate collection, and optimal biomarker detection and measurement in EBC are reciprocal prerequisites for any standardisation. However, current condensation systems are suboptimal, with relatively short-measured, open ended designs and loss of noncondensed exhaled breath, as reflected by variable EBC volumes and biomarker reproducibility¹. Logically, modification by using guided breath flows, enlarged condensation surface, and optimised condensate recovery may improve condensation. Moreover, current designs have different inner coatings, featuring different adhesive interactions with exhaled markers⁴. Recently, loss of biomarker within the sampling system was demonstrated, *in vitro* and *in vivo*, for 8-isoprostane and albumin, at the expense of nonglass condenser systems, and in favour of glass and silicone condensers⁴. To assess clinical relevance, a study on the reproducibility of these biomarker measurements *in vivo*, using different condenser coatings including glass, is needed⁴. Furthermore, conventional biomarker assays are not always suitable for use in even large sample volumes of condensate, a bio fluid highly 'diluted' by water vapour⁵, whereas new analytical techniques are rapidly emerging and may offer new perspectives⁶⁻⁷. Recently, multiplexed cytometric bead array was used in children to simultaneously measure different cytokines in only 50µL condensate samples, however, the detection level did not reach 50%⁶. Liquid bead-based multiplexing xMAP® technology (Luminex Corporation, Austin, USA), based on flow cytometry and (faster) liquid microspheres reaction kinetics, is less laborious, highly sensitive and specific, allows simultaneous measurements in small sample sizes, and improves interarray reproducibility⁷. Therefore, we hypothesised that optimised condensate collection with minimal adhesive properties, improves the reproducibility of different measurements in EBC.

The aim of the present study is to assess the effect of four different condensers (glass, silicone, EcoScreen®, and a new, optimised glass condenser) on the reproducibility of breath condensate volume, and the detection, concentration and reproducibility of inflammatory biomarkers, including hydrogen peroxide, 8-isoprostane, interleukin-2, interleukin-4, interleukin-5, interleukin-6, interleukin-8, interleukin-10, interleukin-13, and tumor necrosis factor-alpha, in EBC in healthy nonasthmatic adults.

Methods

Study subjects

We recruited 30 eligible healthy nonasthmatic adult volunteers (table 1) among medical students and staff at the University Hospital Maastricht, based on the following criteria: each volunteer was able to breath tidally into a mouthpiece for at least 15 minutes; exclusion criteria were: history of asthma, upper or lower airway infection, and use of antibiotics, corticosteroids, cromoglycate, nedocromil, theophylline or leukotrien-antagonist. Nonasthmatic healthy adults were chosen to eliminate possible confounding factors attributable to heterogeneous disease expression and/or variability of disease control.

Table 1. Subject characteristics of healthy nonasthmatic adult volunteers (n=30).

male : female ratio	19 : 11
age (in years)*	23 (14)
weight (in kg)*	75 (25)
height (in cm)*	180 (14)
non smoking	28
history of eczema and/or hay-fever	6

* median and (interquartile range)

Study design

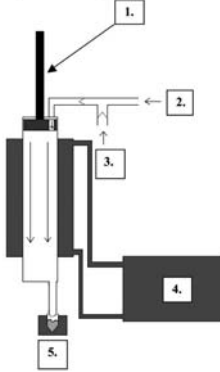
To assess within-day, between-day and between-week reproducibility, each volunteer was asked to perform four tests: on the first day in the morning (test 1) and afternoon (test 2), the day after at the same time as test 1 (test 3), and one week later at the same time (test 4). In turn, each test consisted of four sequential EBC collections, using ad random different types of condenser.

Exhaled breath condensate collection

EBC was collected using one of the following condensers: the commercial Teflon-like EcoScreen[®], a condenser with exchangeable inner cylinder of silicone or glass, as described previously ⁴, and a new, optimised glass condenser that was developed in close collaboration with the Department of Instrument Development Engineering & Evaluation of the Maastricht University (patent number EP 07102586), as extensively described in figure 1. Briefly, the inclined condensation surface is enlarged (using a length of 90cm), condensate recovery is optimised (using a downwards moveable plunger), and breath flows are turbিনately directed towards the condenser wall (by the plunger's multiple breath channels).

To perform one EBC collection, each subject was asked to exhale tidally, while using a nose clip, through a mouthpiece and two-way nonbreathing valve connected with the condenser, during a fixed period of 15 minutes.

Description of the new, optimised glass condenser system.



A

1. inclined glass condenser with a tube length of 90 cm (to enlarge the condensation surface), and downwards moveable plunger (to optimise condensate recovery from the inner condenser wall), with tangential and axial breath flow channels (to turbinate direct the tidally exhaled breath flows towards the cooled inner condenser wall)
2. swan-neck tubing (serving as gravitational saliva trap) and two-way nonbreathing valve, connected to a mouthpiece
3. entrance of inspired room air
4. cooling unit consisting of a counter-current circulating ice-water pump
5. removable, cooled glass sample vial (to collect exhaled breath condensate)



B

Photograph of the glass condenser tube with plunger *in situ*.



C

Photographic detail of the glass condenser tube with downwards moveable plunger *in situ*. The plunger has three tangential and axial breath flow channels (to turbinate direct the tidally exhaled breath flows towards the cooled inner condenser wall).

Figure 1A, 1B, and 1C.

The new optimised glass condenser system.

Condensate sample processing

Immediately after collection, condensate samples were snap-frozen at -78°C using dry ice and stored at -80°C . Analysis was performed within three months from sampling time.

Hydrogen peroxide was measured in $50\mu\text{L}$ EBC, in duplicate by spectrophotometer (Perkin Elmer® UV-VIS Lambda 10 Spectrometer, Shelton/Norwalk, Connecticut, USA) with a lower detection limit of $0.05\mu\text{M}$, as described previously⁸.

Isoprostane was measured in $100\mu\text{L}$ EBC, by specific enzyme immunoassay (Cayman Chemical®, Ann Arbor, Michigan, USA), that was modified to reach a lower limit of detection of 1.0pg/mL . The standard curve of this assay ranged from 250pg/mL to 1.95pg/mL . We were able to report 8-isoprostane values as low as 1.0pg/mL , as a logit/log transformation was used. Isoprostane recovery experiments were performed,

and coefficients of variation of the absorption signals were assessed. When spiking for the lower 8-isoprostane values of 3.9pg/mL and 7.8pg/mL, an isoprostane recovery of 92% (coefficient of variation of concentration 16%), and 95% (coefficient of variation of concentration 15%) were found, respectively. A coefficient of variation (of absorption signals) below 15% was considered highly acceptable. Hence, the corresponding coefficient of variation of concentrations may be higher. Therefore, the coefficients of variation found in the 8-isoprostane recovery experiments using these low values, were considered good. Standard curves, patient samples and quality control samples of 2.5pg/mL and 10pg/mL isoprostane were assayed in triplicate. Intra-assay variation of standard curves, patient samples and quality control samples should be less than 15%, otherwise all samples measured in that assay were excluded and reanalysed. Finally, in all accepted samples, 8-isoprostane concentrations were determined from mean absorption signal intensities.

Cytokines were measured in 50µL EBC, using the liquid bead-based multiplexing xMAP® technology (Luminex Corporation, Austin, USA). Multiplex immunoassay was performed as described previously^{7,9-10}. The corresponding lower limits of cytokine and chemokine detection were: interleukin-2 (1.0pg/mL), interleukin-4 (1.2pg/mL), interleukin-5 (1.2pg/mL), interleukin-6 (0.4pg/mL), interleukin-8 (1.1pg/mL), interleukin-10 (1.2pg/mL), interleukin-13 (1.0pg/mL), and tumor necrosis factor-alpha (1.3pg/mL). Measurement and data analysis were performed using the Bioplex 100 system and Bioplex Manager software version 3.0 (Bio-Rad Laboratories, Hercules, CA). All multiplex immunoassays were performed in 96 well format 1.2µm filter bottom plates (Millipore, Amsterdam, The Netherlands) and a 12 point standard curve in duplicate was included on every plate. In order to minimise interassay variation, positive and negative control samples were included. As far as possible, EBC samples from one donor series of experiments were run on one plate. Three EBC samples were spiked with 100 or 10pg/mL cytokines (interleukin-2, interleukin-4, interleukin-5, interleukin-6, interleukin-8, interleukin-10, interleukin-13, and tumor necrosis factor-alpha), and measured them in quadruplicate. The mean coefficient of variation of these cytokine measurements was 12.2%. The recovery of 100pg/mL spiked cytokine was 103% (range from 71 to 129%). At 10pg/mL, the mean recovery was somewhat lower at 89% (range from 64% in interleukin-13, to 111% in interleukin-4). It was concluded that there was a slight matrix effect of EBC, but this did not result in an overestimation of cytokine measurements.

Statistics

Statistical calculations were performed using SPSS 11.5 (SPSS Inc, Chicago, USA). Not normally distributed data were expressed as median and interquartile range. Normally distributed data were expressed as mean and standard error. To estimate variance within a single method of measurement (coating), coefficient of variation (= (standard deviation/mean) x100%) were used. The coefficient of variation was calculated as the mean of individual coefficients of variation, calculated over the two or four relevant measurements. Within-subjects, coefficients of variation were used as data distribution was normal. When the distribution of individual coefficients of variation was not

normal, the nonparametric Friedman test was used to see if these coefficients of variation were different between coatings. To test for differences among normally distributed repeated measures, analysis of variance (ANOVA) was used. The Chi-square test was used to evaluate statistically the differences between proportions for four groups in a data set. Condensate samples with biomarker concentration below the lower detection limit, *in strictu sensu* negative detections, were not considered as missing values, because they actually informed us that marker concentrations were below the lower detection limit. Therefore, these negative detections were given an arbitrary value between zero and the lower detection limit: 0.025 μ M for hydrogen peroxide, 0.1pg/mL for 8-isoprostane, and 0.1pg/mL for cytokines. Samples were defined missing if the EBC volume, yielded after 15 minutes collection time, was zero, or insufficient to analyse.

Power calculation

Ideally, power calculations should be based on the expected changes in biomarker concentrations and on their variability. However, these changes in biomarker concentrations and their variability were unknown. In fact, the objective of this study was to answer this question. Therefore, we took the uncertainty of the estimated variability as a basis for power calculations. For each of the estimated variances (within-day, between-day and between-week) we have data from 30 volunteers. Using standard results for the variance of a chi-square random variable, we infer that, with 30 subjects, the relative confidence limits (relative to the observed value of the variance) will be 0.634 and 1.807. For the standard deviation this implies relative limits of 0.80 and 1.34¹¹.

Ethics

All parents gave written informed consent. The study was approved by the Medical Ethics Committee of the University Hospital of Maastricht.

Results

EBC volume

In 5 out of 480 manoeuvres, we were not able to collect condensate due to erroneously connected tubing. EBC collection by the optimised glass condenser yielded significantly more median condensate volume, compared to the other condensers ($p=0.001$, Friedman) (table 2). Within-day, between-day, between-week and overall reproducibility of EBC volume (expressed as coefficient of variation) were comparable in the new condenser and EcoScreen[®] ($p=0.715$, Chi-square), but was significantly better compared to silicone and glass ($p<0.028$, Chi-square).

Hydrogen peroxide measurements in EBC

Overall, 29% of hydrogen peroxide measurements were missing (table 3). Significantly more positive hydrogen peroxide detections were found using the optimised glass condenser, compared to silicone and glass ($p<0.050$, Chi-square). Median hydrogen peroxide concentrations and reproducibility (expressed as coefficient of variation) did

Table 2. Volume of exhaled breath condensate, and reproducibility (expressed as coefficients of variation) in four different condenser systems.

EBC	Silicone	Glass	New	EcoScreen	p-value
missings	4/120	0/120	1/120	0/120	-
volume *	525 (319)	712 (297)	2025 (1056)	1237 (759)	0.001 †
CVin-day *	20 (27)	21 (19)	11 (15)	9 (21)	0.002 †
CVbe-day *	28 (36)	30 (26)	11 (26)	13 (23)	0.027 †
CVbe-week *	35 (36)	26 (28)	15 (22)	15 (21)	0.003 †
CVerall *	37 (22)	29 (16)	20 (17)	19 (27)	0.001 †

EBC	exhaled breath condensate
New	optimised glass condenser
missings	number of failed EBC collections, after 15 minutes, to total number of tests
volume	volume of EBC, in μL
CV	coefficient of variation, in percentage
in-day	within day
be-day	between day
be-week	between week
*	expressed as median (interquartile range)
†	Friedman test

Table 3. Hydrogen peroxide measurements in exhaled breath condensate: detection, concentration, and reproducibility (expressed as coefficients of variation) in four different condenser systems.

H₂O₂	Silicone	Glass	New	EcoScreen	p-value
missings	59/120	49/120	10/120	23/120	-
detections	52/9	62/9	77/33	75/22	0.003 †
conc *	2.6 (2.7)	2.1 (2.3)	1.8 (1.6)	2.2 (2.5)	0.286 ‡
CVin-day *	17 (25)	12 (28)	46 (71)	13 (21)	0.271 ‡
CVbe-day *	22 (33)	30 (42)	30 (118)	16 (52)	0.821 ‡
CVbe-week *	18 (46)	26 (42)	32 (44)	12 (32)	0.256 ‡
CVerall *	23 (25)	29 (21)	24 (52)	22 (13)	0.080 ‡

H ₂ O ₂	hydrogen peroxide
New	optimised glass condenser
missings	number of missing measurements to total number of tests
detections	number of positive to negative detections
conc	concentration of H ₂ O ₂ in EBC, in μM
CV	coefficient of variation, in percentage
in-day	within day
be-day	between day
be-week	between week
*	expressed as median (interquartile range)
†	Chi-square test
‡	Friedman test

not significantly differ between the four condensers ($p=0.286$ and $p>0.080$, respectively, Friedman).

8-Isoprostane measurements in EBC

Overall, 13% of 8-isoprostane measurements were missing (table 4). Significantly more positive 8-isoprostane detections were found using the optimised glass condenser, compared to silicone and glass ($p<0.023$, Chi-square). The median concentration of 8-isoprostane was significantly higher using the new condenser compared with the other three condensers ($p=0.001$, Friedman). Statistically, 8-isoprostane reproducibility (expressed as coefficient of variation) did not significantly differ between the four condensers ($p>0.151$, Friedman).

Table 4. Isoprostane measurements in exhaled breath condensate: detection, concentration, and reproducibility (expressed as coefficients of variation) in four different condenser systems.

8-IP	Silicone	Glass	New	EcoScreen	p-value
missings	27/120	16/120	9/120	10/120	-
detections	89/4	98/6	110/1	109/1	0.001 †
conc *	2.0 (2.2)	2.9 (2.3)	3.6 (2.7)	2.5 (1.8)	0.001 ‡
CVin-day *	6 (29)	20 (55)	23 (35)	24 (29)	0.297 ‡
CVbe-day *	31 (65)	26 (57)	23 (32)	15 (32)	0.559 ‡
CVbe-week *	22 (42)	22 (57)	15 (31)	25 (28)	0.954 ‡
CVoverall *	31 (57)	48 (55)	29 (22)	26 (26)	0.151 ‡

8-IP	8-isoprostane
New	optimised glass condenser
missings	number of missing measurements to total number of tests
detections	number of positive to negative detections
conc	concentration of 8-IP in EBC, in pg/ml
CV	coefficient of variation, in percentage
in-day	within day
be-day	between day
be-week	between week
*	expressed as median (interquartile range)
†	Chi-square test
‡	Friedman test

Cytokine measurements in EBC

Overall, 20% of cytokine measurements were missing (table 5.a). Overall, the levels of cytokine detection using xMAP's technology were: interleukin-2 (68%), interleukin-4 (97%), interleukin-5 (73%), interleukin-6 (46%), interleukin-8 (46%), interleukin-10 (61%), interleukin-13 (70%) and tumor necrosis factor-alpha (64%). The optimised glass condenser had significantly more positive detections of interleukin-2, interleukin-4, interleukin-5, interleukin-13 and tumor necrosis factor-alpha, compared to silicone

Table 5.a. Number of positive to negative detections of cytokines in exhaled breath condensate, collected with four different condenser systems.

detections	Silicone	Glass	New	EcoScreen	p-value *
missings	49/120	37/120	1/120	10/120	-
IL-2	50/21	60/23	82/37	68/42	0.001
IL-4	69/2	81/2	113/6	108/2	0.001
IL-5	58/13	65/18	87/32	70/40	0.001
IL-6	41/30	45/38	48/71	43/67	0.811
IL-8	44/27	45/38	44/75	44/66	0.999
IL-10	54/17	57/26	63/56	61/49	0.654
IL-13	53/18	58/25	88/31	71/39	0.001
TNF-alpha	52/19	57/26	72/47	65/45	0.050

detections number of positive to negative detections

New optimised glass condenser

missings number of missing measurements to total number of tests

IL interleukin

TNF tumor necrosis factor

* Chi-square test

Table 5.b. Concentration of cytokine measurements in exhaled breath condensate, collected with four different condenser systems.

conc	Silicone	Glass	New	EcoScreen	p-value
missings	49/120	37/120	1/120	10/120	-
IL-2 *	1.9 (5.0)	1.6 (4.4)	1.2 (3.5)	1.6 (4.0)	0.494
IL-4 †	5.8 (0.3)	5.1 (0.3)	4.7 (0.3)	5.4 (0.3)	0.129
IL-5 *	4.4 (13.8)	3.0 (12.1)	3.2 (11.4)	3.1 (12.1)	0.544
IL-6 *	0.9 (4.3)	0.9 (4.5)	0.7 (3.8)	0.6 (4.4)	0.113
IL-8 *	2.0 (4.6)	2.1 (4.0)	2.7 (4.8)	2.1 (3.9)	0.341
IL-10 †	1.5 (0.3)	1.6 (0.9)	1.4 (0.8)	1.3 (0.7)	0.872
IL-13 †	6.6 (1.1)	6.6 (1.1)	6.3 (0.7)	5.9 (0.8)	0.946
TNF-alpha †	2.1 (0.4)	2.0 (0.4)	1.8 (0.3)	1.7 (0.3)	0.876

conc concentration in EBC, in pg/ml

New optimised glass condenser

missings number of missing measurements to total number of tests

IL interleukin

TNF tumor necrosis factor

* expressed as median (interquartile range), Friedman test

† expressed as mean (standard error), analysis of variance

and glass ($p < 0.050$, Chi-square), and more interleukin-5 and interleukin-13 detections, compared to EcoScreen® ($p < 0.021$, Chi-square). Cytokine concentrations were not significantly different between the four condenser types ($p > 0.113$, Friedman or ANOVA, respectively) (table 5.b). Reproducibility (expressed as coefficient of variation) of cytokine measurements did not significantly differ, neither overall (table 5.c), nor within-day, between-day and between-week (data not shown). Best range of overall coefficients of variation was found for the measurement of interleukin-6 (11-14%), interleukin-8 (2-15%), interleukin-10 (11-30%) and tumor necrosis factor-alpha (8-22%). When coefficients of variation were evaluated using the positive values only (without negative and/or arbitrary values), also no differences between condensers were found (data not shown).

Table 5.c. Overall reproducibility (expressed as coefficients of variation) of cytokine measurements in exhaled breath condensate, collected with four different condenser systems.

CV overall	Silicone	Glass	New	EcoScreen	p-value
missings	49/120	37/120	1/120	10/120	-
IL-2 †	80 (12)	75 (11)	84 (10)	70 (11)	0.802
IL-4 †	33 (7)	32 (6)	36 (5)	26 (4)	0.494
IL-5 †	74 (13)	71 (12)	73 (11)	81 (13)	0.941
IL-6 *	12 (77)	12 (68)	14 (78)	11 (69)	0.690
IL-8 *	15 (28)	12 (21)	2 (18)	9 (25)	0.766
IL-10 *	30 (48)	17 (48)	16 (39)	11 (53)	0.719
IL-13 †	51 (11)	68 (12)	73 (10)	63 (10)	0.574
TNF-alpha *	17 (45)	22 (64)	19 (62)	8 (68)	0.878

CV coefficient of variation, in percentage

New optimised glass condenser

missings number of missing measurements to total number of tests

IL interleukin

TNF tumor necrosis factor

* expressed as median (interquartile range), Friedman test

† expressed as mean (standard error), analysis of variance

Discussion

We demonstrated that EBC volume, and the detection of biomarkers were significantly influenced by the condenser system, in favour of the new glass condenser, whereas biomarker reproducibility was not influenced by the type of condenser. The proposed optimised glass condenser yielded significantly more condensate volume compared to the silicone, glass and EcoScreen® condensers. Reproducibility of EBC volume was comparable for the new condenser and EcoScreen®, and was significantly better compared to the other two condenser types.

In EBC collected with this new condenser, significantly more positive hydrogen peroxide, 8-isoprostane, interleukin-2, interleukin-4, interleukin-5, interleukin-13 and tumor necrosis factor-alpha detections were found, supporting improved sampling of EBC. Moreover, 8-isoprostane concentrations were significantly increased in EBC yielded by the new condenser, compared to the other three condensers, which is in accordance with former findings⁴. On the other hand, reproducibility of hydrogen peroxide, 8-isoprostane, and cytokine measurements in EBC did not significantly differ between the four condensers, suggesting no significant influence of the type of condenser coating on reproducibility. In literature and to date, no formal study addressing this issue has been published, although reproducibility using other analytical techniques has been reported¹²⁻¹⁴, and/or could not be calculated due to small number of subjects¹⁵⁻¹⁷.

Variations in biomarker measurements in EBC may be attributed to variations in the dilution and/or quality of condensate that is influenced by different collection techniques and procedures, sample processing and storage conditions, and/or sensitivity of the analytical techniques used¹⁷. The levels of the highly volatile hydrogen peroxide in EBC may be susceptible to different cooling temperatures during collection¹⁸, circadian rhythm¹⁹, flow dependency²⁰, different methods of measurement with widely varying values (even in healthy subjects) close to lower detection limits^{3,17,21-22}, and high chemical reactivity with salivary and exhaled compounds by which (some) hydrogen peroxide is consumed over time during collection and storage^{3,17,22}. Isoprostanes are relatively stable end-products of *in vivo* arachidonate peroxidation, and are measured in EBC by immunoassay, that may be influenced by cross reactivity with closely related substances¹⁷. Other confounding factors may be age, diets rich in antioxidants, and smoking habits¹⁷.

The use of coefficients of variation is not always the ideal way of expressing variability: when mean values are low, coefficients of variation can be abnormally high. Therefore, we also expressed our results as standard deviations and intraclass correlation coefficients. When using both of these alternative expression methods, results were comparable: no difference in biomarker variability between condensers was found (data not shown). Furthermore, biomarker levels were assessed in condensate originating from healthy adults, and thus, may have been more pronounced in a steroid naïve population with documented chronic respiratory inflammation, and comparable levels of disease control. Hence, one may expect increased mean concentrations, and lowered coefficients of variation in diseased subjects. On the other hand, with these very low concentrations of cytokines and 8-isoprostane, we cannot rule out an influence of analytical variability.

Cytokines were simultaneously measured by xMAP's technology in small 50µL EBC samples. The overall level of detection was 46-97%, that was much better compared to cytometric bead array in small samples in children (<50%), and in large 1000-2000µL lyophilised samples in adults (3-100%)^{6,23-26}. When compared with conventional (solid-phase) immunoassays, multiplexed immunoassays detect bioactive

and inactive molecules, have a growing analytical range, are rapid (hours instead of days), have good precision (coefficient of variation: 10-15%), are not interfered by drugs, and have simple protocols ⁷.

Currently, different nonstandardised techniques to collect EBC are in use ¹⁻³. All systems are based on the cooling of exhaled breath, whereas their design may vary from immersed plastic tubing, over glass distilling columns, to commercial systems, such as the lamellar Teflon-like EcoScreen[®] condenser (Erich Jaeger GmbH, Hoechberg, Germany), the hand-held disposable polypropylene RTube[™] (Respiratory Inc., Charlottesville, Virginia, USA), and the thermostatically-controlled polyethylene Turbo-Deccs (ItalChill, Parma, Italy) ^{1-3,27}. These designs implicate relatively short-measured and open-ended systems that tolerate the needless loss of noncondensed exhaled breath, whether initially, or after a prolonged collection time. Moreover, also biomarkers may be lost within these collection systems, as recently demonstrated for 8-isoprostane and albumin measurements, *in vitro* and *in vivo*, in nonglass condensers (including EcoScreen[®]) ⁴. This superiority of glass coatings may be mainly related to the behaviour of water as bipolar vehicle ⁴. Three other studies report the influence of sampling systems on biomarkers in EBC ²⁸⁻³⁰. Tufvesson *et al* proposed to coat EcoScreen[®] collection surfaces with bovine serum albumin and Tween-20, to measure cytokines and eicosanoids, respectively ²⁸. However, they also reported possible false-positive (eicosanoid) results, and the need of sample concentration, by vacuum-centrifugation, prior to analysis ²⁸. Soyer *et al* found significantly higher cysteinyl leukotrienes and eotaxin using EcoScreen[®], compared to RTube[™], due to susceptibility of precooled RTube[™] sleeves to (increased) ambient temperatures during collection, and due to different materials that could affect sample recovery ²⁹. Prieto *et al* compared RTube[™] and EcoScreen[®], and reported that EBC pH values are dependent on the collection device used ³⁰. Furthermore, the EcoScreen[®] has been associated with deposition of frozen condensate on its lamellar walls. For all the above mentioned reasons, we developed an optimised glass condenser system. The new glass condenser had an improved condensation process and condensate recovery, using an inclined and enlarged condensation surface, with a condensate sweeping plunger, having tangentially and axially guiding breath flow channels.

In the present study, these improvements resulted in significantly increased EBC volumes, and increased biomarker detections with the new glass condenser, compared to silicone, glass and EcoScreen[®]. This suggests both an improved condensation process, and an increased opportunity to perform a broad spectrum of analyses. Moreover, the optimised glass and EcoScreen[®] condenser were both significantly associated with less variation in the generated EBC volume, compared to the other condensers. Hereby, reducing a possible confounding influence of the variable quantity of EBC collected over a given time, and even within individuals.

Optimisation of EBC collection, using the modified new glass condenser with statistically equivalent coefficients of variation compared to the commercial EcoScreen[®], and optimisation of EBC analysis, using rapid, multiplexed measurement

of cytokines in small EBC sample sizes, may all together open a window of opportunities, even in strained collection procedures, such as in young or dyspnoeic subjects (with less sustained efforts to cope with sampling procedures), by allowing the search for, and identification of, particular profiles of different exhaled markers, involved in the regulation of chronic respiratory inflammation, for diagnostic and monitoring purposes.

In conclusion, the optimised glass condenser yielded significantly more EBC volume, with good reproducibility. Furthermore, significantly more positive detections of hydrogen peroxide, 8-isoprostane, interleukin-2, interleukin-4, interleukin-5, interleukin-13 and tumor necrosis factor-alpha were found in EBC collected with the new condenser, hereby offering an increased capacity to analyse for complex biomarker profiles. Moreover, concentrations of 8-isoprostane were significantly increased using the optimised glass condenser, compared to the other three condensers. However, reproducibility of biomarker measurements in EBC was not influenced by the type of condenser.

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CHAPTER 6

FREE RADICALS IN EXHALED BREATH CONDENSATE IN CYSTIC FIBROSIS AND HEALTHY SUBJECTS

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Abstract

Many markers of airway inflammation and oxidative stress can be measured noninvasively in exhaled breath condensate. However, no attempt has been made to directly detect free radicals using electron paramagnetic resonance spectroscopy.

Condensate was collected in 14 children with cystic fibrosis and seven healthy subjects. Free radicals were trapped by 5,5-dimethyl-1-pyrroline-N-oxide. Electron paramagnetic resonance spectra were recorded using a Bruker EMX[®] spectrometer. Secondly, to study the source of oxygen centered radical formation, catalase or hydrogen peroxide was added to the condensate.

Radicals were detected in 18 out of 21 condensate samples. Analysis of spectra indicated that both oxygen and carbon centered radicals were trapped. Within-subject reproducibility was good in all but one subject. Quantitatively, there was a trend towards higher maximal peak heights of both oxygen and carbon centered radicals in the children with cystic fibrosis. Catalase completely suppressed the signals in condensate. Addition of hydrogen peroxide resulted in increased radical signal intensity.

Detection of free radicals in exhaled breath condensate of children with cystic fibrosis and healthy subjects is feasible using EPR spectroscopy.

Introduction

The collection of exhaled breath condensate (EBC) is a simple and noninvasive technique to measure mediators of airway inflammation. EBC consists not only of water vapor, but also contains aerosolized respiratory fluid droplets released from the respiratory epithelial lining fluid. These fluid droplets contain traces of nonvolatile solutes, which can be recovered in EBC samples¹. EBC is collected by guiding and cooling exhaled air of a tidally breathing subject through a condenser system. The collection of EBC does not disturb the airways, in contrast to bronchial biopsy, bronchoalveolar lavage and induced sputum, and thus, can be obtained with minimal risk and inconvenience in both adults and children². Many inflammatory mediators, such as 8-isoprostane, have been demonstrated in EBC of adults and children with chronic inflammatory lung diseases³⁻¹².

Oxidative stress appears to play an important role in several inflammatory lung diseases, including asthma and cystic fibrosis. Oxidative stress is defined as an imbalance between oxidants (reactive oxygen species, and reactive nitrogen species) and antioxidants (superoxide dismutase, catalase, glutathione, uric acid, lactoferrin, vitamin C, vitamin E) in favor of oxidants¹³. By virtue of their unpaired electrons, many reactive oxygen species are unstable compounds, capable of initiating widespread oxidative events. Increased production of reactive oxygen species has been directly linked to protein, DNA and lipid oxidation, which may cause direct lung injury or induce a variety of cellular responses, through the generation of secondary metabolic reactive species¹⁴.

The measurement of F2-isoprostanes is currently the most reliable approach to assess oxidative stress status *in vivo*¹¹. In EBC, 8-isoprostane can be measured in both adults and children, although reproducibility remains controversial^{11-12,15-16}. On the other hand, spin trapping is the only direct method for the detection of free radicals^{14,17}. Reactive oxygen and nitrogen species can be detected by electron paramagnetic resonance spectroscopy using various spin traps, e.g. 5,5-dimethyl-1-pyrroline N-oxide (DMPO)^{14,18,19}.

However, as the increased production of radicals appears to play an important role in inflammatory lung diseases, as far as we know, no attempt has been made to detect free radicals in EBC. The aim of this study was the detection of free radicals in EBC of children with cystic fibrosis using electron paramagnetic resonance spectroscopy.

Materials and methods

Study subjects

EBC was collected in 14 children with cystic fibrosis, routinely attending the cystic fibrosis centre of the Maastricht University Hospital, and seven healthy controls (including one adult). Cystic fibrosis was defined as a positive quantitative sweat test in conjunction with chronic airway obstruction and/or recurrent infections of the airways and/or exocrine pancreatic insufficiency and maldigestion. Children with cystic

fibrosis were clinically stable at the time of the assessment. Healthy subjects were asked to complete a standardized questionnaire of the International Study of Asthma and Allergies in Childhood (ISAAC) in order to exclude the presence of asthma or allergic disease²⁰. The subject characteristics are shown in Table I. The study was approved by the medical ethical committee of the University of Maastricht.

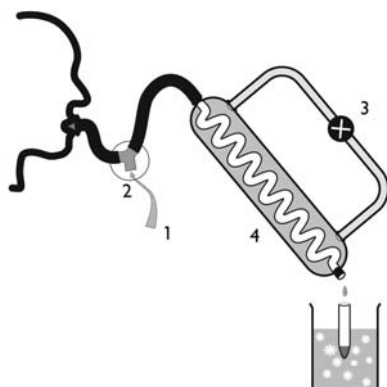
Table I. Subject characteristics.

	Cystic fibrosis	Healthy subjects
Number of subjects	14	7
Age (years)	14 (6 – 17)*	13 (9 - 25)*
Male / female ratio	9 / 5	4 / 3
Weight (kg)	38 (18 – 60)*	44 (37 - 85)*
Height (cm)	153 (114 – 176)*	164 (144 - 179)*
FEV ₁ (%predicted)	66 (31 – 108)*	-
Use of corticosteroids	4	0
Use of antibiotics	4	0

*median and (range)
FEV₁ forced expiratory volume in 1 second

Collection of exhaled breath condensate

Each subject was asked to exhale tidally, while wearing a nose clip, through a mouthpiece connected to a two-way non-rebreathing valve and a cooled (0°C) double-jacketed 50-cm borosilicate glass tube as shown in figure 1 and described previously^{3,10}. In order to prevent salivary contamination of EBC samples, the two-way valve and tubing were used as a saliva gravity trap. Condensate was collected at the open end of the glass condenser, as shown in figure 1, directly into vials containing spin trap (DMPO) (Table II). Thus, no resistance was created during exhalation. Each vial was wrapped in aluminum paper to



The subject breathes tidally and inhales room air (1) through a two-way non-rebreathing valve (2). Exhaled air is guided by the tubing through a double-wall glass condenser (4), which is cooled by a counter-current circulating ice-water pump (3). The two-way non-rebreathing valve (2) and tubing also serve as a saliva trap.

Figure 1. Schematic representation of the exhaled breath condensate collection device.

protect DMPO against daylight. The condensate collection continued until the minimal required volume of EBC was obtained necessary to perform one test (Table II). Samples were analyzed immediately. Four different test conditions were applied, as shown in Table II. Test 1-4 were performed to study DMPO-condensate samples under different volumes and concentrations of EBC and DMPO (Table II).

Table II. Specific test conditions.

	CF	Healthy	EBC	DMPO
Test 1	2	1	1 mL	1 mL (50 mM)
Test 2	4	-	0.5 mL	0.5 mL (100 mM)
Test 3	5	6	0.5 mL	0.25 mL (100 mM)
Test 4	3	-	0.3 mL	0.25 mL (100 mM)

CF number of cystic fibrosis patients

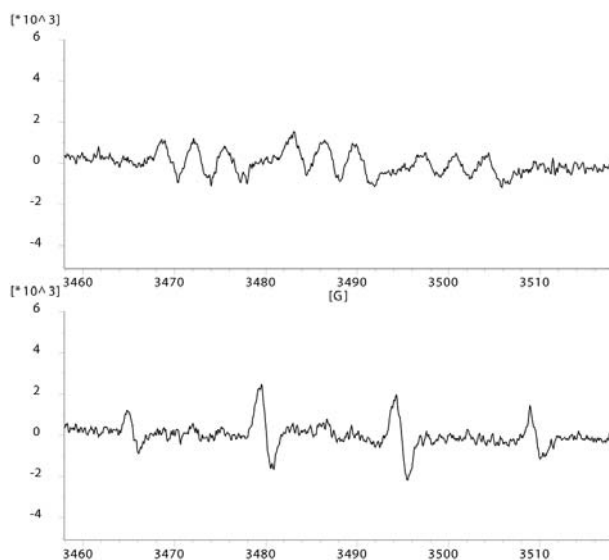
Healthy number of healthy subjects

EBC volume of collected exhaled breath condensate

DMPO volume and (concentration) of spin trap (5,5-dimethyl-1-pyrroline N-oxide)

Electron paramagnetic resonance spectroscopy

Electron paramagnetic resonance spectra of the DMPO trapped radicals were recorded at room temperature using a Bruker EMX[®] electron paramagnetic resonance spectrometer (Bruker Biospin GmbH, Karlsruhe, Germany). The experimental conditions were as follows: Klystron frequency 9.85 GHz, microwave power 30 mW, field



The upper part of figure 2 shows the signal of 5,5-dimethyl-1-pyrroline N-oxide (DMPO) in water, or the DMPO-background signal. The lower part of figure 2 shows the condensate-specific radical adduct signal after subtraction of the DMPO-background signal.

X-axis: magnetic field strength (Gauss)

Y-axis: intensity of the electron paramagnetic resonance signal

Figure 2. The DMPO-background signal and the condensate-specific radical adduct signal.

modulation amplitude 2G at 100 kHz frequency, time constant 40.96 msec and 1.0×10^6 gain, and a sweep width of 60G. Each sample was scanned 50 times. The signal of DMPO in H₂O was subtracted from the DMPO-condensate signal to obtain condensate-specific spectra (Figure 2).

Within-subject reproducibility of the electron paramagnetic resonance signals was assessed in five subjects by measuring electron paramagnetic resonance spectra in EBC collected with a one-hour interval. The results were expressed as coefficients of variation ($CV = [SD/mean] \times 100\%$).

In order to quantify the method, we assigned to each electron paramagnetic resonance signal an arbitrary unit derived from its maximal peak height, as illustrated in figure 3.

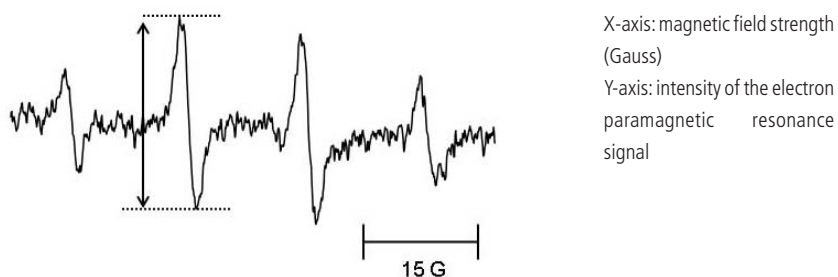


Figure 3. Quantification of electron paramagnetic resonance spectra in exhaled breath condensate by assessing maximal peak height in arbitrary units.

Additionally, test A and B were designed to study the source of oxygen centered radical formation. In test A, catalase (50 μ L of a 5000 U/mL catalase solution) was added to the DMPO vials of test three. Catalase converts hydrogen peroxide in water and oxygen. Therefore, catalase was expected to reduce free radical signals derived from hydrogen peroxide. In test B, hydrogen peroxide (2 μ L of a 9.7 M hydrogen peroxide solution) was added to the DMPO vials of test four. When hydrogen peroxide is considered to be the main source of free radical generation, hydrogen peroxide should amplify free radical signals.

Finally, saliva and sputum were investigated on the presence of free radical signals, in order to exclude free radical contamination by these biofluids.

Results

The median duration of EBC collection was 16.5 minutes (range 13-18 minutes). As shown in Table III, optimal capture of free radical adducts was obtained using 0.25-0.50 mL DMPO (100 mM) and 0.5 mL condensate. Free radical adduct signals were detected in 18 out of 21 DMPO-condensate samples of children with cystic fibrosis and healthy controls (Table III).

Table III. Detection of oxygen (O) and carbon (C) centered radical adduct signals in exhaled breath condensate of children with cystic fibrosis and healthy controls.

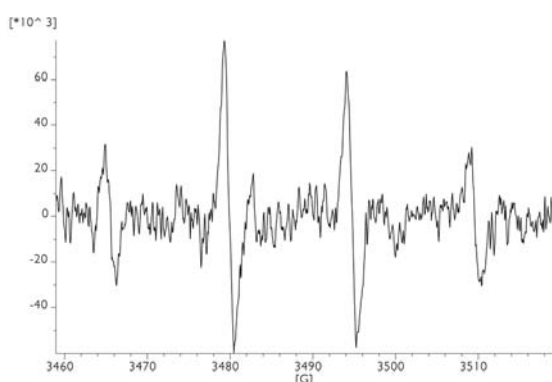
	Cystic Fibrosis			Healthy Controls		
	O	C	O or C	O	C	O or C
Test 1	1/2	0/2	1/2	0/1	0/1	0/1
Test 2	1/4	3/4	4/4	-	-	-
Test 3	5/5	3/5	5/5	6/6	4/6	6/6
Test 4	2/3	0/3	2/3	-	-	-
Total			12/14			6/7

O number of oxygen centered radical adduct signals to number of subjects

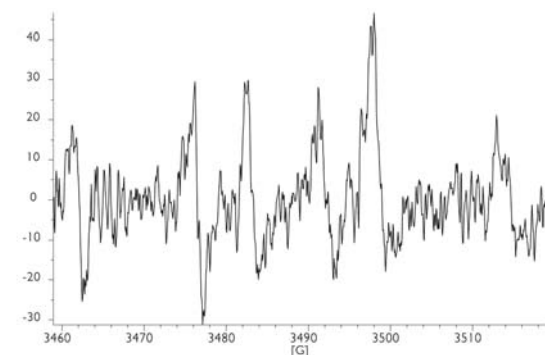
C number of carbon centered radical adduct signals to number of subjects

O or C number of free radical adduct signals to number of subjects

Total total number of free radical adduct signals to total number of subjects



DMPO: 5,5-dimethyl-1-pyrroline N-oxide
X-axis: magnetic field strength (Gauss)
Y-axis: intensity of the electron paramagnetic resonance signal

Figure 4A. Oxygen (1:2:2:1) centered radical adduct signal in the mixture of condensate and spin trap (DMPO).

DMPO: 5,5-dimethyl-1-pyrroline N-oxide
X-axis: magnetic field strength (Gauss)
Y-axis: intensity of the electron paramagnetic resonance signal

Figure 4B. Carbon (1:1:1:1:1) centered radical adduct signal in the mixture of condensate and spin trap (DMPO).

Analysis of electron paramagnetic resonance spectra indicated that both oxygen and carbon centered radicals were trapped by DMPO, as shown in Figure 4A and 4B respectively.

Within-subject reproducibility of electron paramagnetic resonance spectra in EBC was good in four subjects (coefficients of variation ranged from five to twelve percent) (figure 5). However, in one subject the coefficient of variation was 87%.

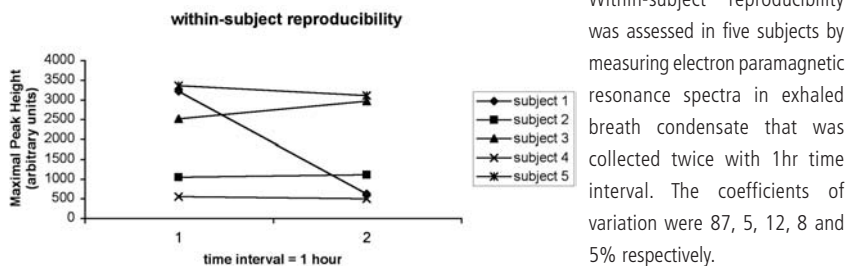


Figure 5. Within-subject reproducibility of electron paramagnetic resonance spectra in exhaled breath condensate.

Quantitative results expressed as maximal peak height (in arbitrary units) of both oxygen and carbon centered radicals in children with cystic fibrosis and healthy controls had a normal distribution and are shown in figure 6. The maximal peak heights of oxygen as well as carbon centered radical adduct signals seemed higher in the cystic fibrosis group compared with the healthy controls. However, the difference in oxygen and carbon centered radicals failed to reach statistical significance ($p=0.828$ and $p=0.253$ respectively, Student's t-test).

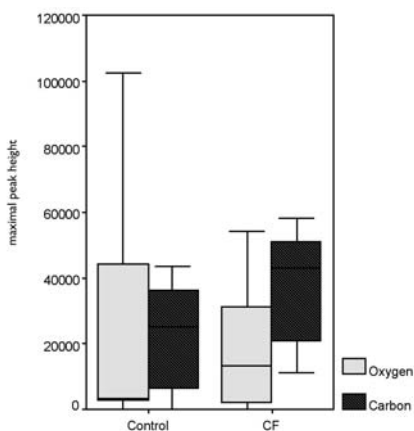


Figure 6. Quantitative results expressed as maximal peak height (in arbitrary units) of both oxygen and carbon centered radical adduct signals in exhaled breath condensate in children with cystic fibrosis and healthy controls.

Within-subject reproducibility was assessed in five subjects by measuring electron paramagnetic resonance spectra in exhaled breath condensate that was collected twice with 1hr time interval. The coefficients of variation were 87, 5, 12, 8 and 5% respectively.

The maximal peak height of oxygen centered radical adduct signals (oxygen) and carbon centered radical adduct signals (carbon) did not differ significantly between the children with cystic fibrosis (CF) and healthy control group ($p=0.828$ and $p=0.253$ respectively, t-test).

Catalase completely suppressed most oxygen centered radical adduct signals in EBC of children with cystic fibrosis (four out of five) and healthy controls (three out of six) (Figure 7A). On the other hand, addition of hydrogen peroxide resulted in a substantial increase of oxygen centered radical adduct signals in EBC of children with cystic fibrosis (two out of three) (Figure 7B).

Saliva contained little or no free radicals (three out of four samples were negative). It was not possible to detect free radicals in sputum (n=1) due to its viscosity.

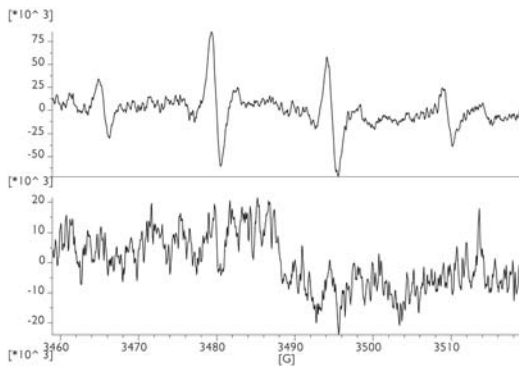


Figure 7A. Suppression of oxygen centered radical adduct signal in the mixture of condensate, spin trap (DMPO) and catalase.

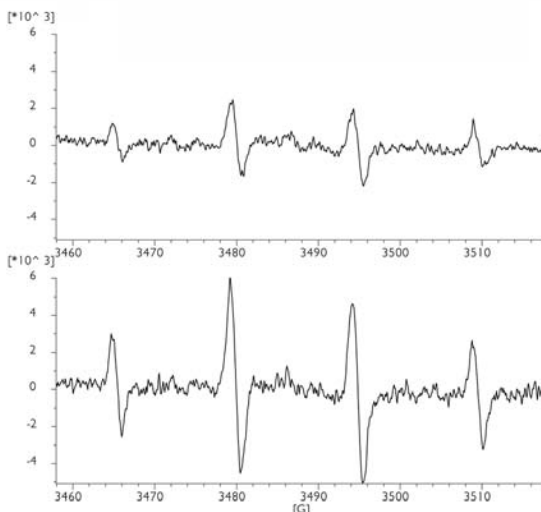


Figure 7B. Amplification of oxygen centered radical adduct signal in the mixture of condensate, spin trap (DMPO) and hydrogen peroxide.

The upper part of figure 7A shows an oxygen centered radical adduct signal in the mixture of condensate and spin trap (5,5-dimethyl-1-pyrroline N-oxide, or DMPO). The lower part of figure 7A shows that subsequent addition of catalase results in suppression of the oxygen centered radical adduct signal.

X-axis: magnetic field strength (Gauss)

Y-axis: intensity of the electron paramagnetic resonance signal

The upper part of figure 7B shows an oxygen centered radical adduct signal in the mixture of condensate and spin trap (5,5-dimethyl-1-pyrroline N-oxide, or DMPO). The lower part of figure 7B shows that subsequent addition of hydrogen peroxide results in amplification of the oxygen centered radical adduct signal.

X-axis: magnetic field strength (Gauss)

Y-axis: intensity of the electron paramagnetic resonance signal

Discussion

The presence of oxidative stress in children with cystic fibrosis was assessed by direct measurement of free radicals in EBC using electron paramagnetic resonance spectroscopy. Oxygen as well as carbon centered radicals were trapped by DMPO in EBC in children with cystic fibrosis and healthy controls. Quantification demonstrated a difference that failed to reach statistical significance. Short-term reproducibility was good, except in one subject. Furthermore, most oxygen centered radical adduct signals were completely suppressed by catalase, while signal amplification by hydrogen peroxide was observed, indicating that hydrogen peroxide is a main source for the generation of free radicals in EBC.

Although within-subject reproducibility was good in four subjects, reproducibility was questionable in one subject, maybe as a consequence of physical exertion between the two tests.

Our quantitative approach using maximal peak height of an electron paramagnetic resonance signal in EBC did not show a statistical significant difference in oxygen or carbon centered radicals, between the cystic fibrosis children and healthy controls. However, there was a trend towards higher maximal peak heights of both oxygen and carbon centered radicals in the cystic fibrosis group, that may reach statistical significance in a larger study population.

Various indirect biomarkers of oxidative stress in condensate of subjects with chronic respiratory inflammation are known, as recently reviewed ^{3,14}. Currently, comparative reports on the direct measurement of free radicals in EBC are, to our knowledge, not available.

Oxidative stress appears to play an important role in several inflammatory lung diseases. It is defined as an imbalance between oxidants (reactive oxygen species, reactive nitrogen species) and antioxidants (e.g. catalase) in favor of oxidants ¹³. Chronic inflammatory lung diseases, such as asthma and cystic fibrosis, are characterized by the activation of epithelial cells and resident macrophages, and the recruitment and activation of neutrophils, eosinophils, monocytes and lymphocytes ¹⁴. The activation of macrophages, neutrophils and eosinophils results in the generation of reactive oxygen species ¹⁴. Lung epithelial cells also release reactive oxygen species, and when this occurs these substances stimulate inflammatory cells directly, thereby amplifying lung inflammatory and oxidant events ^{14,21}.

By virtue of their unpaired electrons, oxygen species are reactive compounds, that cause widespread oxidative events ¹⁴. One such species, the superoxide anion, is rapidly reduced to hydrogen peroxide by superoxide dismutase ¹⁴. The combination of hydrogen peroxide with transition metal ions yields the hydroxyl radical ¹⁴. Reactive oxygen and nitrogen species can also be generated intracellular from several sources, including mitochondrial respiration, the NADPH oxidase system and xanthine/xanthine

oxidase, leading to cell necrosis or apoptosis¹⁴. When generated close to cell membranes, the reactive oxygen species oxidize membrane phospholipids (lipid peroxidation)¹⁴. This initiates a chain reaction, which in turn leads to the generation and accumulation of lipid peroxidation products such as malondialdehyde, F2-isoprostanes, 4-hydroxy-2-nonenal and acrolein¹⁴. The peroxidative breakdown of polyunsaturated fatty acids within the membrane impairs function, inactivates membrane-bound receptors and enzymes and increases tissue permeability and cell fragility¹⁴. In addition to their cytotoxic properties, lipid peroxides are increasingly recognized as being important in redox-sensitive signal transduction pathways, especially those activated during an inflammatory response^{14,22,23}.

To study the source of the formation of oxygen centered radicals, two additional tests (test A and B) were performed. Test A shows the absence of a radical adduct signal in the mixture condensate, catalase and spin trap (in seven out of eleven samples), which suggests that hydrogen peroxide may play an important role in the generation of oxygen centered radicals measured in EBC after spin trapping. Increased concentrations of hydrogen peroxide in EBC have been demonstrated in patients with asthma and cystic fibrosis³. In sixteen children with cystic fibrosis, Jöbbsis et al reported increased hydrogen peroxide levels in EBC, and a significant decrease of these levels during intravenous antibiotic treatment²⁴. Test B demonstrates an increased formation of most oxygen centered radicals (in two out of three samples), after addition of hydrogen peroxide. These amplified electron paramagnetic resonance signals suggests that EBC may contain compounds that enable the formation of radicals from hydrogen peroxide, such as transition metal ions and/or compounds that reduce these ions to their Fenton active states. Griese and co-workers found higher concentrations of zinc in EBC in cystic fibrosis patients, compared to healthy controls²⁵. Zinc is known as a redox-active metal that may generate both hydrogen peroxide and hydroxyl radicals by Fenton reaction²⁶.

Carbon centered radicals may originate from various sources, such as fatty acids, alkane, ethanol or carbohydrates. However, we are not able to exclude with certainty whether free radicals are formed *in situ* in the collection vial. A possible explanation for the presence of carbon centered radicals, in the absence of oxygen centered radicals, may be that carbon-containing molecules react with hydroxyl radicals. By consequence, carbon containing molecules become carbon centered radicals and the former hydroxyl radical is no longer an oxygen centered radical. Similarly, hydroxyl radicals may be formed *in situ* from hydrogen peroxide in the presence of certain compounds such as transition metals. In order to reduce the risk of *in situ* free radical formation, one may develop a collecting device that allows direct contact between exhaled air and a DMPO containing filter. Spin trapping remains highly dependent on the spin trap used and the antioxidant status of the condensate²⁷. Furthermore, the outcome of electron paramagnetic resonance results is not only influenced by the amount of free radicals trapped by constituent compounds of the EBC and the presence of some electrolyte ions, such as sodium and potassium, but also influenced by the spin trap-free radical interaction (each free radical has a specific affinity for a given spin trap)²⁷.

Finally, as most saliva samples were negative, free radicals in EBC do not likely result from contamination with this biofluid. This suggests that free radicals in EBC may originate from the lower respiratory tract, providing further support that EBC reflects the epithelial lining fluid.

Free radicals were also present in condensate of healthy subjects. This may not be surprising, considering that the lung normally exists in an oxygen rich environment which makes it susceptible to oxidative stress, and considering that electron paramagnetic resonance spectroscopy allows direct but semi-quantitative measurement of free radicals^{14,28}. We did not find free radicals in three out of 21 condensate samples, including one healthy control, one child with cystic fibrosis on longterm prednisone treatment, and one child with cystic fibrosis on antibiotics. Corticosteroids may have an antioxidant effect by decreasing the numbers as well as the oxidative and chemotactic responses of neutrophils, whereas antibiotics may have a certain role in reducing oxidative stress by reducing infection and thereby lung inflammation²⁹.

We aimed at assessing the potential use of electron paramagnetic resonance spectroscopy in the detection of free radicals in EBC in children with cystic fibrosis and healthy subjects. Future studies should include a larger number of subjects and a wider range of disease severity, with repeated measurements of conventional markers and free radicals in EBC.

Other disturbing factors may include counteracting effects of exercise, dietary components, diurnal rhythm, duration of EBC collection and analysis, partial exposition of DMPO to daylight, environmental temperature and inhaled air particles of diverse origin^{27,30,31}.

As we report on a more direct and noninvasive assessment of oxidative stress, one may speculate on clinical implications. However, the future challenge is to define the source of free radical formation, and to extend our understanding of the discriminative power of electron paramagnetic resonance spectroscopy in the detection of (exacerbations of) chronic lung disease, monitoring of disease severity, and disease progression.

We conclude that detection of free radicals in EBC of children with cystic fibrosis and healthy subjects is feasible using electron paramagnetic resonance spectroscopy. Furthermore, it is likely that hydrogen peroxide is crucial for the generation of oxygen centered radicals detected in EBC. Moreover, EBC may also contain compounds that enable radical formation.

Acknowledgements

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CHAPTER 7

BIOMARKERS IN EXHALED BREATH CONDENSATE INDICATE PRESENCE AND SEVERITY OF CYSTIC FIBROSIS IN CHILDREN

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Abstract

Chronic airway inflammation is present in cystic fibrosis. Noninvasive inflammometry may be useful in disease management. The aim of the present cross-sectional study in children with cystic fibrosis was to investigate: (i) the ability of fractional exhaled nitric oxide and other exhaled inflammatory markers (exhaled breath condensate acidity, nitrite, nitrate, hydrogen peroxide, 8-isoprostane, and Th1/Th2 cytokines) to indicate (exacerbations of) cystic fibrosis; and (ii) the ability of these noninvasive inflammatory markers to indicate disease severity in cystic fibrosis.

In 98 children (48 cystic fibrosis / 50 controls) exhaled nitric oxide was measured using the NIOX®. Subsequently, exhaled breath condensate was collected and analysed, and lung function tests were performed by means of dynamic spirometry.

In cystic fibrosis, condensate interferon-gamma and nitrite concentrations were significantly higher, and exhaled nitric oxide levels were significantly lower, compared to controls (3.3 (\pm 0.3) pg/mL, 2.2 (\pm 0.2) μ M, 10.0 (\pm 1.2) ppb, *versus* 2.6 (\pm 0.2) pg/mL, 1.4 (\pm 0.1) μ M, 15.4 (\pm 1.4) ppb, respectively). Using multivariate logistic regression models, the *presence* of cystic fibrosis was best indicated by combined condensate 8-isoprostane, nitrite and interferon-gamma (sensitivity 78%, specificity 83%; area under receiver operating characteristic curve 0.906, p <0.001). An *exacerbation* of cystic fibrosis was best indicated by condensate 8-isoprostane and nitrite (sensitivity 40%, specificity 97%, area under receiver operating characteristic curve 0.838, p =0.009). Most indicative biomarkers of cystic fibrosis *severity* were exhaled nitric oxide, and condensate acidity (sensitivity 96%, specificity 67%; area under receiver operating characteristic curve 0.751, p =0.008).

In this cross-sectional study in children, the combination of different exhaled inflammatory markers could indicate the presence of cystic fibrosis disease, exacerbation, and severity. Longitudinal data are necessary to further confirm the role of these exhaled markers in the management of cystic fibrosis in children.

Introduction

Cystic fibrosis is an autosomal recessive disorder, caused by genetic mutations in the cystic fibrosis transmembrane conductance regulator protein. This results in impaired or absent transport of chloride through cell-membranes, with impaired mucociliary clearance, and viscous mucus in the airways. Moreover, defective mucociliary action results in inability of the airways to clear bacteria, e.g. *Pseudomonas aeruginosa*¹. Chronic airway infection, inflammation and oxidative stress are the characteristics of cystic fibrosis disease, which are mainly due to neutrophilic immune responses². Pulmonary disease is the leading cause of morbidity and mortality in patients with cystic fibrosis³.

Currently, monitoring of pulmonary disease in cystic fibrosis is based on clinical features and pulmonary function tests. However, these tests may not reflect the onset, origin or severity of airway inflammation⁴. The noninvasive assessment of inflammatory markers in exhaled breath condensate (EBC) may be a promising tool in patients with chronic lung diseases⁴⁻⁶. In cystic fibrosis, noninvasive inflammatory markers may be helpful in the early detection of cystic fibrosis lung disease in infants, the prediction and follow-up of an exacerbation, and the evaluation of treatment effects.

Several research groups detected altered inflammatory markers, such as leukotriene-B₄, interleukin-6, and free radicals, in EBC of patients with cystic fibrosis, compared to controls⁷⁻¹⁰. Jöbsis *et al* showed increased exhaled hydrogen peroxide concentrations during cystic fibrosis exacerbations⁸. Recently, the presence of interleukin-4, interleukin-10, tumor necrosis factor-alpha, and interferon-gamma was demonstrated in EBC of patients with cystic fibrosis¹⁰. However, study populations were often small, and data on noninvasive markers in cystic fibrosis limited and inconsistent. In addition, the relationships between noninvasive inflammatory markers, and the severity or exacerbations of cystic fibrosis disease, are still unknown. Therefore, we assessed different inflammatory markers in EBC in almost 100 children with cystic fibrosis and controls, comparing the ability of these markers to indicate (exacerbations of) cystic fibrosis, and/or disease severity. Because of oxidative stress and airway inflammation, hydrogen peroxide, 8-isoprostane, and nitrite/nitrate are upregulated in the airways of cystic fibrosis patients, and acidity is increased. Conversely, fractional exhaled nitric oxide levels have shown to be lower in cystic fibrosis.

The aim of the present cross-sectional study in children with cystic fibrosis was to investigate: (i) the ability of fractional exhaled nitric oxide and other exhaled inflammatory markers (condensate acidity, nitrite, nitrate, hydrogen peroxide, 8-isoprostane, and Th1/Th2 cytokines) to indicate (exacerbations of) cystic fibrosis; and (ii) the ability of these noninvasive inflammatory markers to indicate disease severity in cystic fibrosis.

Methods

Study subjects

We included 48 children with cystic fibrosis, and 50 control children without a history of lung disease or atopy. Informed consent was obtained from all subjects. The study was approved by the Medical Ethics Committee of the University Hospital of Maastricht.

Cystic fibrosis population

Children known with cystic fibrosis were recruited from the outpatient clinics. Cystic fibrosis disease was defined as a combination of typical clinical features (persistent pulmonary problems, meconium ileus, failure to thrive, steatorrhoe), and an abnormal sweat test (chloride > 60mM). An exacerbation of cystic fibrosis was diagnosed by the paediatric pulmonologist based on a significant increase in cystic fibrosis related symptoms, and/or a decrease of at least 10% in forced expiratory volume in one second, or forced vital capacity compared to previous measurements¹¹. Height and weight were expressed as Z-scores (age and gender specific standard deviation scores). Cystic fibrosis severity was assessed using the Shwachman-Kulczycki score.

Control population

Control children without lung disease were recruited from the outpatient clinic of the University Hospital Maastricht. The reasons of consultation were constipation and enuresis nocturna. All children completed the questionnaire from the International Study of Asthma and Allergies in Childhood (ISAAC) to exclude children with a (history of) airway or allergy complaints, or asthmatic disease¹².

Exclusion criteria

Exclusion criteria were: (i) diseases that may interfere with the results of the study (upper airway infection, heart disease, anatomic abnormalities of the airways, and other chronic inflammatory diseases, such as Crohns disease and rheumatoid arthritis); (ii) mental retardation; (iii) inability to perform the EBC collection procedure; (iv) active smoking; and (v) use of the following medication: papaverin, sodium nitroprusside, angiotensin-converting enzyme inhibitors, oxymetazoline, L-arginine, or nitric oxide synthase inhibitors.

Design and Measurements

The design of this study was cross-sectional. At first, fractional exhaled nitric oxide was measured. Subsequently, EBC was collected, and lung function was measured by means of dynamic spirometry. All tests were performed within one hour.

Nitric oxide in exhaled air

Fractional exhaled nitric oxide was measured using a NIOX[®] chemiluminescence analyser (Aerocrine, Solna, Sweden). Measurements were assessed according to the recommendations of the American Thoracic Society and European Respiratory Society for children⁶. The mean nitric oxide value of the plateau reached during the last 3 seconds of exhalation, was used to obtain the fractional exhaled nitric oxide value of that measurement⁶. The mean fractional exhaled nitric oxide of three measurements was used for analysis.

In 14 patients with cystic fibrosis, fractional exhaled nitric oxide was measured using the offline method, because there was not a NIOX® analyser available in these clinics. The first 4 seconds of exhaled air were discarded and during the next 4 seconds, exhaled air was collected in an inert balloon. Fractional exhaled nitric oxide levels were analysed on the NIOX® chemiluminescence analyser within 8 hours¹³. The exhalation flow rate during these measurements was 50 mL/sec.

Exhaled breath condensate

EBC was collected using a home-made 50-cm double-wall borosilicate glass condenser, continuously cooled by circulating ice water (1°C), as described previously^{5,10,14}. Subjects were asked to breath tidally for 30 minutes through a mouthpiece, while wearing a nose-clip. The mouthpiece was connected to a two-way non-rebreathing valve (Hans Rudolph Inc, series 1420, Kansas City, USA), which also served as a gravitational saliva trap. In addition, children were allowed to swallow saliva during EBC collection.

The acidity of the EBC was measured immediately after collection (Radiometer®, type PHM201, Radiometer Nederland BV, Zoetermeer, the Netherlands). Samples were not deaerated¹⁴. Subsequently, the EBC samples were frozen and stored at -80°C in 1mL Eppendorf® tubes.

In EBC, nitrite and nitrate concentrations were assessed using a fluorimetric assay (Cayman Chemical, Ann Arbor, USA)¹⁵. To measure 8-isoprostane in EBC, an enzyme immunoassay was used (Cayman Chemical, Ann Arbor, USA), which was modified in order to lower the detection limit from 5.0 pg/mL (80% B/B₀) to 0.5 pg/mL. Standard curves, patient samples and quality control samples of 2.5 pg/mL and 10 pg/mL, were assayed in triplicate. The coefficients of variation of the absorption signal were assessed. Intra-assay variation of standard curves, patient samples and quality control samples should be less than 15%, otherwise all samples measured in that assay were excluded and re-analysed. Finally, in all accepted samples, 8-isoprostane concentrations were determined from mean absorption signal intensities.

Hydrogen peroxide measurements in EBC were based on a method previously described by Dekhuijzen *et al*¹⁶. Briefly, EBC was mixed with 0.4M citrate buffer, horseradish peroxidase solution (50U/mL), and tetramethylbenzidine (20 mM), and incubated for 20 minutes at room temperature. Subsequently, the sample was mixed with sulphuric acid (2M). The tetramethylbenzidine oxidation product, as a measure of the amount of hydrogen peroxide, was determined spectrometrically at 450nm using a Perkin Elmer UV-VIS Spectrometer Lambda 10 (Norwalk, CT, USA).

Cytokines (interleukin-2, interleukin-4, interleukin-5, interleukin-10, tumor necrosis factor-alpha, and interferon-gamma) were assessed in 50 µL samples of EBC with flow cytometry (multiplexed cytometric bead array (CBA), BD Bioscience®, San Diego, USA), as described previously¹⁰.

Lung function tests

Bronchodilator medication was stopped prior to lung function testing: shortacting bronchodilators for at least 8 hours, and longacting bronchodilators at least for 36 hours. Dynamic spirometry was performed by means of the Flowscreen® (Jaeger®, Wuerzburg, Germany). The lung function parameters were assessed according to European Respiratory Society standards. The highest values of one of three correct performed manoeuvres were used for analysis. Recorded parameters were: forced expiratory volume in one second, and forced vital capacity. In addition, in the cystic fibrosis population, residual volume, total lung capacity, and intrathoracic gas volume were assessed by body plethysmography (Jaeger®, Würzburg, Germany). The reversibility on a bronchodilator was determined 15 minutes after inhalation of 400µg salbutamol. Reversibility was defined as an increase in measured forced expiratory volume in one second of at least 9% of predicted value.

Statistical analysis

Statistical calculations were performed using SPSS 11.5 (SPSS Inc., Chicago, USA). Data were expressed as mean ± standard error of the mean, or as median (interquartile range) values for normal, or not normal distributed continuous variables, respectively. Comparison between groups was performed using the chi-square for categorical variables, the Student's t-tests for normal distributed parameters, and, Mann Whitney U-test was used when the distribution of the parameters was considered not normal. P-values < 0.05 were considered statistically significant.

At first, the influence of separate inflammatory markers was tested in univariate logistic regression analyses. Thereafter, the different inflammatory markers were combined in multivariate logistic regression models with cystic fibrosis disease, cystic fibrosis exacerbation, and cystic fibrosis severity as dependent binary variables. Age, height, weight, forced expiratory volume in one second as per centage of predicted value, and the forced expiratory volume in one second to vital capacity ratio were introduced in the model as covariates, but were removed if p-values exceeded 0.10, in order to keep the degrees of freedom as high as possible. The stepwise backward method was applied to reduce the number of independent variables, and to assess which inflammatory markers were most predictive: only inflammatory markers with a p-value <0.05 were kept in the final models. Based on these models, receiver operating characteristic curves were obtained. EBC samples with negative detections were not considered as missings, because they actually inform us that the measurements of inflammatory markers were below the lower detection limit. Therefore, the negative detections were given an arbitrary value between the lower detection limit and zero.

Power analysis

The standard error of the sensitivity and the specificity of an inflammatory marker will be less than 5%, given a population of 50 children with cystic fibrosis and 50 controls, and an assumed sensitivity of 70%. This was considered sufficiently accurate for the purpose of this study.

Results

Population characteristics

Two of the 48 patients with cystic fibrosis were older than 18 (21 and 25 years, respectively). Children with cystic fibrosis (n=48) were older, smaller, weighed less and had more airway obstruction compared to controls (n=50) (Table 1; $p < 0.05$). Exacerbation was diagnosed in 13% of the cystic fibrosis patients. 24 Patients were treated with prophylactic antibiotics. All of these patients were receiving antibiotics orally (trimethoprim/sulphamethoxazol 17%, azitromycin 27%, amoxicillin/clavulanic acid 4%, and flucloxacillin 2%). In addition, 11 patients received inhaled antibiotics (8 (17% tobramycin, and 6 (13%) colistin). Therapeutic antibiotic treatment was given to the six patients with an exacerbation (orally: azitromycin 2(4%), ciprofloxacin 2(4%), amoxicillin/clavulanic acid 1(2%), and, intravenously: tobramycin 3(6%), ceftazidim 1(2%), piperacillin/tazobactam 1(2%)).

Collection of EBC and FeNO measurements

The mean EBC volume collected was 3.1 ± 0.2 mL and 3.0 ± 0.2 mL in the cystic fibrosis population and control population, respectively. No side effects were reported during or following exhaled nitric oxide measurements and EBC collection.

Detection of inflammatory markers

Detections of inflammatory markers in EBC are shown in Table 2. Nitrite, nitrate, hydrogen peroxide, and 8-isoprostane were measured in all EBC samples. The level of cytokine detection in EBC ranged up to 42%, and 20% in children with cystic fibrosis, and healthy controls, respectively. It was not possible to detect interleukin-5. Interferon-gamma and interleukin-4 were detected significantly more often in EBC samples of cystic fibrosis patients (42%, and 13% respectively), compared to samples originating from healthy controls (20%, and 2% respectively; Chi-square test, $p < 0.05$).

Concentration of inflammatory markers

Mean concentrations of interferon-gamma and nitrite were significantly higher in the cystic fibrosis group (3.3 ± 0.3 pg/mL, and 2.2 ± 0.2 μ M, respectively) compared to controls (2.6 ± 0.2 pg/mL, and 1.4 ± 0.1 μ M, respectively; Student's t-test, $p < 0.05$) (Table 2). In contrast, fractional exhaled nitric oxide was significantly lower in children with cystic fibrosis (10.0 ± 1.2 ppb), compared to control children (15.4 ± 1.4 ppb; $p < 0.05$). No influence was present of inhaled corticosteroids, use of antibiotics, or presence of atopy.

Cystic fibrosis disease

Multivariate logistic regression models showed that EBC 8-isoprostane, nitrite, and interferon-gamma, and forced expiratory volume in one second, were all significant indicators for cystic fibrosis disease (Table 3). The model including exhaled markers only, had a sensitivity of 78%, a specificity of 92%, and a area under the receiver operating characteristic curve of 0.906 (Figure 1A).

Table 1. Clinical characteristics of the study population*

	cystic fibrosis (n=48)		controls (n=50)
age (yrs)	13.0 ± 0.6	†	10.0 ± 0.4
height (cm)	148 ± 3		142 ± 2
Z-score height #	-1.0 ± 0.2	†	0.1 ± 0.2
Z-score weight	-1.0 ± 0.1	†	0.5 ± 0.2
sex (male / female)	27 / 21		27 / 23
FEV ₁ % predicted	76 ± 4	†	102 ± 2
FEV ₁ /VC (%)	77 ± 2	†	88 ± 1
TLC % predicted	104 ± 2		NA
RV % predicted	174 ± 10		NA
ITVG % predicted	123 ± 4		NA
reversibility §	23 %		NA
cystic fibrosis exacerbation	6 (13 %)		NA
atopy **	17 (36 %)		NA
positive sputum cultures:			
- <i>Pseudomonas aeruginosa</i>	23 (58 %)		NA
- <i>Staphylococcus aureus</i>	29 (60 %)		NA
- <i>Haemophilus influenzae</i>	14 (29 %)		NA
- <i>Haemophilus parainfluenzae</i>	3 (6 %)		NA
- <i>Aspergillus fumigatus</i>	17 (35 %)		NA
- <i>Candida albicans</i>	6 (13 %)		NA
- Hemolytic <i>Streptococcus</i> group A or B	8 (17 %)		NA
therapy:			
- rhDNase	25 (52 %)		NA
- antacids	24 (50 %)		NA
- corticosteroids	13 (27 %)		NA
- therapeutic antibiotics	7 (15 %)		NA
- prophylactic antibiotics	24 (50 %)		NA

FEV₁, forced expiratory volume in one second; FVC, forced vital capacity; ITVG, intrathoracic gas volume; NA, not applicable; rhDNase, recombinant human desoxyribonuclease; RV, residual volume; TLC, total lung capacity; VC, vital capacity.

* data are shown as mean ± SEM, except indicated otherwise

standard deviation of height corrected for age and sex, according to Dutch standard values

|| standard deviation of weight corrected for age and sex, according to Dutch standard values

§ defined as an increase in FEV₁ after 400 µg salbutamol of 9% of predicted or more

† significantly different from controls (p<0.05)

** defined as total IgE level exceeds 20 kU/L, and/or positive Phadiatop, and/or positive radioallergo-sorbent test

Table 2. Concentrations, and number of positive detections of inflammatory markers in exhaled breath condensate.

exhaled inflammatory marker	units	detection limit	cystic fibrosis (n=48)			controls (n=50)	
			N%	mean ± SEM		N%	mean ± SEM
FeNO	ppb		90	10.0 ± 1.2	‡	100	15.4 ± 1.4
pH			100	6.3 ± 0.1		100	6.3 ± 0.0
nitrite	µM	0.05	100	2.2 ± 0.2	‡	100	1.4 ± 0.1
nitrate	µM	0.05	100	14.4 ± 2.0		100	16.5 ± 1.4
H ₂ O ₂	µM	0.1	100	2.4 ± 0.2		100	2.2 ± 0.3
8-isoprostane	pg/mL	0.5	100	4.3 ± 0.5		100	4.7 ± 0.6
IFN-γ	pg/mL	1.5	42	3.3 ± 0.3	† ‡	20	2.6 ± 0.2
TNF-α	pg/mL	1.2	15	1.6 ± 0.1		15	1.6 ± 0.1
IL-2	pg/mL	1.2	2	1.2		0	ND
IL-4	pg/mL	1.4	13	1.8 ± 0.1	†	2	2.0
IL-5	pg/mL	1.1	0	ND		0	ND
IL-10	pg/mL	1.1	10	1.3 ± 0.1		6	1.4 ± 0.2

FeNO, fractional exhaled nitric oxide; H₂O₂, hydrogen peroxide; IFN-γ, interferon-gamma; IL, interleukin; ND, not detectable; pH, condensate acidity; SEM, standard error of the mean; TNF-α, tumor necrosis factor-alpha.

N% number of positive detections, as percentage of samples above detection limit

† p<0.05 positive detections of cystic fibrosis *versus* controls

‡ p<0.05 concentrations of inflammatory markers in cystic fibrosis *versus* controls

Table 3. Multivariate logistic regression model to indicate the *presence* of cystic fibrosis disease.

	p(-2LL)	β ± SE	OR (CI 95%)
8-isoprostane	0.02	-0.08 ± 0.76	0.92 (0.88-0.98)
nitrite	0.03	0.75 ± 0.37	2.11 (1.35-3.25)
IFN-γ	0.02	0.74 ± 0.35	2.10 (1.17-2.54)
FEV ₁ % predicted	<0.01	-0.07 ± 0.02	0.93 (0.89-0.97)

β, coefficient of the predictor variables; CI, confidence interval; FEV₁, forced expiratory volume in one second; IFN-γ, interferon-gamma; OR, odds ratio; p(-2LL), significance of change in -2 log likelihood-ratio; SE, standard error.

Cystic fibrosis exacerbation

Six (13%) of the cystic fibrosis population had an exacerbation, based on an increase in symptoms, and/or decrease in lung function parameters. In the sputum of these patients, the following micro-organisms were cultured: *Pseudomonas aeruginosa* (100%), *Staphylococcus aureus* (67%), *Haemophilus influenzae* (33%), *Aspergillus fumigatus* (83%), *Candida albicans* (17%), Hemolytic *Streptococcus* group A or B (17%). In a multivariate analysis, 8-

Figure 1A. Receiver operating characteristic (ROC) curve of the multivariate backward logistic regression model of exhaled breath condensate 8-isoprostane, nitrite and interferon-gamma, to indicate the presence of cystic fibrosis (CF) disease.

The area under the curve is 0.906. The maximum sensitivity is 78%, with a specificity 92%.

0 = Controls
1 = Cystic fibrosis

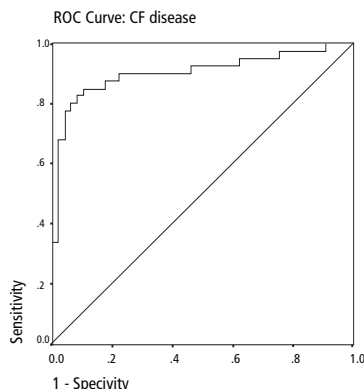


Table 4. Multivariate logistic regression model to indicate cystic fibrosis exacerbation.

	p(-2LL)	B ± SE	OR (CI 95%)
8-isoprostane	0.03	-0.42 ± 0.21	0.70 (0.44-0.99)
nitrite	0.01	-1.34 ± 0.65	0.26 (0.07-0.93)

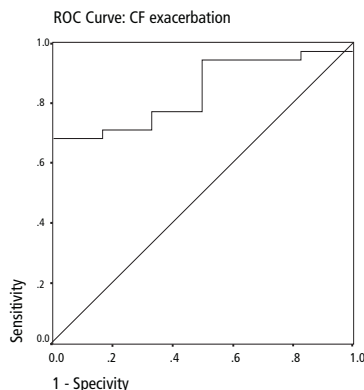
The definition of poor cystic fibrosis lung disease control was based on the presence or severity of symptoms, and decrease in lung function parameters.

B, coefficient of the predictor variables; CI, confidence interval; OR, odds ratio; p(-2LL), significance of change in -2 log likelihood-ratio; SE, standard error.

Figure 1B. Receiver operating characteristic (ROC) curve of the multivariate backward logistic regression model of exhaled breath condensate 8-isoprostane and nitrite, to indicate cystic fibrosis (CF) exacerbation.

The area under the curve is 0.838. The maximum sensitivity is 40%, with a specificity 97%.

0 = Stable CF
1 = CF exacerbation



isoprostane and nitrite indicated a cystic fibrosis exacerbation (Table 4). However, sensitivity was only 40%, with a corresponding specificity of 97% (area under the receiver operating characteristic curve of 0.838, $p=0.009$) (Figure 1B). Correction for age, gender, presence of atopy, lung function parameters, or use of antibiotics, did not influence the results.

Cystic fibrosis severity

In this part of the analysis, the cystic fibrosis severity classes were reduced to two groups, according to the Shwachman-Kulczycki score: group 1 ($n=15$) consisted of patients with no excellent severity status, whereas group 2 ($n=33$) consisted of children with an excellent status. In the multivariate logistic regression model, fractional exhaled nitric oxide, EBC acidity, and forced expiratory volume in one second to vital capacity ratio were significant markers to indicate cystic fibrosis severity (Table 5). The model including exhaled markers only, had a sensitivity of 96%, and a specificity of 67% (area under the receiver operating characteristic curve of 0.751, $p=0.008$) (Figure 1C).

Table 5. Multivariate logistic regression model to indicate cystic fibrosis lung disease severity.

	p(-2LL)	$\beta \pm SE$	OR (CI 95%)
FeNO	0.01	-0.19 ± 0.09	0.83 (0.70-0.98)
pH	0.01	-6.04 ± 2.66	0.02 (0.00-0.44)
FEV ₁ /VC %	<0.01	0.19 ± 0.08	1.21 (1.03-1.41)

Cystic fibrosis severity was classified using the Shwachman-Kulczycki score.

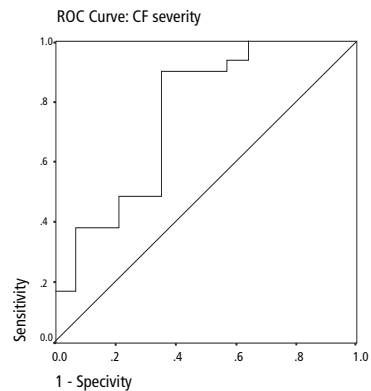
β , coefficient of the predictor variables; CI, confidence interval; FeNO, fractional exhaled nitric oxide; FEV₁, forced expiratory volume in one second; OR, odds ratio; p(-2LL), significance of change in -2 log likelihood-ratio; pH, condensate acidity; SE, standard error; VC, vital capacity.

Figure 1C. Receiver operating characteristic (ROC) curve of the multivariate backward logistic regression model of fractional exhaled nitric oxide, and exhaled breath condensate acidity, to indicate cystic fibrosis (CF) lung disease severity.

Cystic fibrosis severity was classified using the Shwachman-Kulczycki score. The area under the curve is 0.751. The maximum sensitivity is 96% with a specificity 67%.

0 = Not excellent CF status

1 = Excellent CF status



Discussion

This study is based on a relatively large study population of 98 children, of whom 48 children with cystic fibrosis, and simultaneous measurement of several exhaled inflammatory biomarkers, as well as conventional (noninvasive) disease measures, in order to allow a comparison between inflammatory markers, and to assess relationships with cystic fibrosis exacerbation and disease severity. The presented data show that different profiles of noninvasive inflammatory markers in exhaled air and breath condensate are able to reflect different aspects of cystic fibrosis disease in children with good sensitivity and specificity: (i) EBC 8-isoprostane, nitrite and interferon-gamma concentrations were able to indicate the presence of cystic fibrosis disease; (ii) EBC 8-isoprostane and nitrite indicated cystic fibrosis exacerbation; and (iii) cystic fibrosis lung disease severity was assessed by fractional exhaled nitric oxide and EBC acidity.

To our knowledge, no comparable studies on noninvasive profiles of inflammatory markers in children with cystic fibrosis have (yet) been published. However, several of these markers have been studied separately. In the cystic fibrosis population, fractional exhaled nitric oxide levels were reduced when compared with control population, which is in accordance with available data in literature^{8,17-25}. Furthermore, we found significantly higher concentrations of nitrite in EBC originating from children with cystic fibrosis, compared to controls. These results are similar to the findings of Formanek *et al*, Cunningham *et al*, as well as Ho, Ojoo and Tate *et al* in young adults with cystic fibrosis^{17-18,26-28}. Lower fractional exhaled nitric oxide levels and higher nitrite concentrations in the cystic fibrosis population may be explained by several hypotheses^{17,29}. On one hand, the viscous mucus in cystic fibrosis airways may impair diffusion of gaseous nitric oxide into the airway lumen, which may result in conversion of nitric oxide into nitrite¹⁸. On the other hand, decreased expression of inducible nitric oxide synthase II in epithelial cells may result in low fractional exhaled nitric oxide levels in children with cystic fibrosis^{25,30-33}.

Interferon-gamma and interleukin-4 were detected more often in EBC samples originating from children with cystic fibrosis, and interferon-gamma condensate concentrations were higher in this group, compared to controls. This is in accordance with the findings of Wojnarowski *et al*, which demonstrated increased transcription of interferon-gamma and interleukin-4 in bronchial biopsies from children with cystic fibrosis, whereas normal controls did not express these cytokines³⁴. Immunoregulatory effects of interferon-gamma on various cells, are extensive and diverse³⁵⁻³⁶. Interferon-gamma and interleukin-4 upregulate class II molecules on macrophages and dendritic cells. Thereby, the antigen presenting capacity of epithelial and endothelial cells, among others, is being induced³⁶. Moreover in cystic fibrosis, defensive mechanisms to antigens are impaired, and by consequence, additional mechanisms need to be addressed to resolve ongoing inflammatory processes and infections³⁷. Interleukin-5 was not detected in any EBC sample, which is in agreement with the findings of Armstrong *et al* in bronchoalveolar lavage fluid, and may be related to the presence

of proteases in cystic fibrosis airways³⁸. In general, cytokine concentrations in EBC were relatively low, and close to the detection limits of the assays. Previously, we reported that interferon-gamma, interleukin-2, interleukin-4, and interleukin-10 were detected in 8-25% of the samples originating from children with cystic fibrosis, whereas tumor necrosis factor-alpha and interleukin-5 were not detected¹⁰. In literature, no other comparative data on multiplexed cytometric bead array in small EBC sample sizes in children with cystic fibrosis are yet available.

EBC acidity, and concentrations of 8-isoprostane and hydrogen peroxide did not differ between cystic fibrosis and control populations, which is in accordance with the results of Jöbbsis *et al*, and Ho *et al*^{8,39}. On the other hand, other research groups demonstrated lower EBC pH levels and higher 8-isoprostane concentrations in children with cystic fibrosis⁴⁰⁻⁴². This discrepancy may be attributed to the mild to moderate cystic fibrosis severity in this study population, as, in the multivariate models, condensate 8-isoprostane was a significant indicator of cystic fibrosis disease and exacerbation, while EBC pH significantly contributed to the indication model of cystic fibrosis severity.

Several limitations of this study on noninvasive measurements of inflammatory biomarkers in children have to be acknowledged. At first, the cystic fibrosis population mainly consisted of patients with mild to moderate disease, whereas no subjects were actually categorised into a severe disease category. Therefore, results cannot be generalised to the latter type of patients. Secondly, not all cytokines were detected in all EBC samples. However, the lack to detect (some) cytokines does not necessarily implicate that they are not present, as the used detection method may lack sufficient sensitivity, and as there is an overall agreement that cytokines are involved in the pathophysiology of cystic fibrosis. Therefore, a sample with negative detection was not considered as a missing value, as it actually informed us that the measurement of an inflammatory marker was below the lower detection limit. Thirdly, and obviously, the clinical relevance of these findings for the individual child at long term level remains yet to be determined. Although the presented data may suggest that the combined use of exhaled inflammatory markers may be helpful in the management of cystic fibrosis disease, longitudinal study designs are needed to assess whether these markers are able to: (i) indicate the early onset of cystic fibrosis lung disease in young children, (ii) detect exacerbations in an early stage, and (iii) monitor disease exacerbations and treatment effects.

Finally, the presented findings emphasise the importance of evaluating different profiles of different exhaled inflammatory markers, for their 'synergistic' ability to reflect different aspects of chronic lung disease, as current disease management is mainly based on symptoms and deterioration of lung function parameters, which may not appropriately reflect ongoing inflammatory processes, and as a single inflammatory marker may not be able to properly reflect (an aspect of) an evolving chronic inflammatory process. In future, inflammatory markers in EBC may be a valuable additional tool to detect and monitor inflammatory activity in patients with cystic fibrosis, which ultimately may improve quality of live in children with cystic fibrosis.

In conclusion, this cross-sectional study in children demonstrated that different profiles of noninvasive inflammatory markers, such as condensate 8-isoprostane, nitrite, interferon-gamma, acidity, and fractional exhaled nitric oxide, were able to indicate the presence of cystic fibrosis disease, disease exacerbation, and/or cystic fibrosis lung disease severity, with good sensitivity and specificity. Longitudinal data are necessary to further confirm the role of these exhaled markers in the management of cystic fibrosis in children.

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CHAPTER 8

NEW METHOD TO COLLECT EXHALED BREATH CONDENSATE, AND TO ASSESS AIRWAY INFLAMMATION IN WHEEZING PRESCHOOL CHILDREN

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Submitted

Abstract

Exhaled breath condensate is a promising noninvasive method to assess respiratory inflammation in adults and children. So far, condensate collection is mainly feasible in children aged 4 years and over, and condensate analysis is hampered by insufficiently sensitive bioassays. We hypothesised that a new optimised glass condenser, combined with an ultrasensitive multiplexed assay, enables adequate collection, and cytokine measurements in exhaled breath condensate in preschool children.

To assess feasibility and efficacy of a new method of exhaled breath condensate collection in 70 nonsedated preschool children; and to assess cytokine concentrations in condensate (interleukin-4, interleukin-5, interleukin-6, interleukin-8, interleukin-10, interleukin-12p70, interleukin-13, tumor necrosis factor-alpha) in preschool children with atopic asthma, recurrent viral wheeze, and healthy controls.

The overall success rate of condensate collection was 83%. Median condensate volume was 400 μ L. The detection of cytokines was successful in 95-100% of samples. In the asthmatic group, tumor necrosis factor-alpha concentrations were significantly decreased, and the ratio's of interleukin-5 to tumor necrosis factor-alpha, and interleukin-13 to tumor necrosis factor-alpha were higher, compared to nonasthmatic children.

Noninvasive assessment of cytokines in exhaled breath condensate in preschool children is possible, by using a new optimised glass condenser system in combination with the ultrasensitive multiplexed liquid bead array analysis. This new method may help to discriminate between wheezing phenotypes in these children.

Introduction

Preschool wheezing is reported in no less than 30% of children, and may indicate a trivial cold, as well as an ongoing chronic lung inflammation, such as asthma ¹⁻². Atopic asthma is associated with a cytokine pattern compatible with predominant activation of the T-helper cell type 2 mediated immune response ³. However, in the developing preschool child, accurate assessment of different wheezing phenotypes is currently difficult ¹⁻⁸. Moreover, proper diagnosis is hampered by the current lack of a reliable routine technique to assess different types of respiratory inflammation in preschool children.

Ideally, such a technique would be noninvasive, rapid and easy to perform, without any risks, successful at any age, standardised, sensitive, specific and highly reproducible. The collection of exhaled breath condensate (EBC) is a noninvasive technique to obtain direct samples from the lower respiratory tract, without disturbing an ongoing inflammation ⁹⁻¹¹. In children, analysis of EBC reveals the presence of various types of inflammatory biomarkers, including cytokines ⁹⁻¹⁵.

General methodological recommendations on EBC collection are published ¹¹. So far, the collection of EBC was mainly feasible in children aged four years and over ^{11,16}. In preschool children, feasibility is substantially hampered by the need for procedural sedation, or, in nonsedated preschool children, by relatively too long EBC sampling times (from a toddler's point of view). Also, suboptimal condensate analysis due to the use of immunoassays with questionable reproducibility, that often require relatively too large sample volumes, contributes to this problem ^{10,17-21}.

Recently, the efficacy of different open-ended EBC collection systems was compared, and a condenser with silicone or glass coating was found to be more efficient for the measurement of 8-isoprostane or albumin in exhaled breath condensate, than EcoScreen[®], aluminum, polypropylene or Teflon[™] ¹⁷.

Taken together, this has led us to make the following methodological adjustments for the purpose of this study: (i) the collection of EBC using an optimised closed glass condenser system; and (ii) the simultaneous measurement of cytokines in the resulting EBC by means of xMAP[®] technology, an ultrasensitive multiplexed liquid bead array that requires only small sample volumes. We hypothesised that the combination of these methodological adjustments enhances the feasibility and efficacy of EBC collection and analysis in preschool children with and without wheezing.

Therefore, the aims of the present study were: (i) to assess the feasibility and efficacy of EBC collection in nonsedated preschool children, using a modified, closed, glass condenser, and (ii) to assess cytokine profiles (interleukin-4, interleukin-5, interleukin-6, interleukin-8, interleukin-10, interleukin-12p70, interleukin-13, and tumor necrosis factor-alpha) in EBC using multiplexed xMAP[®] technology in preschool children with different wheezing phenotypes.

Methods

Study subjects

Eligible preschool children were recruited at the paediatric outpatient department of the Maasland Hospital of Sittard and the University Hospital of Maastricht. According to parent-completed questionnaires, medical documents, and information of the responsible paediatric pulmonologist, children were allocated to one of the following study groups:

1. The *doctor's diagnosed atopic asthma* group characterised by: (i) recurrent wheezing, (ii) not 'exclusively' triggered by viral infections, (iii) with a documented history of a favourable corticosteroid effect, (iv) positive family history of atopy in the first degree (parent or sibling with asthma, hay fever, food allergy, or eczema), and (v) atopy (positive Phadiatop or radioallergo-sorbent test class \geq 2, and/or elevated blood level of total immunoglobulin-E_s). Current corticosteroid maintenance therapy was not an obligatory criterion.
2. The *viral-triggered recurrent wheeze* group characterised by: (i) recurrent wheezing, (ii) 'exclusively' triggered by viral infections, (iii) without a documented history of steroid therapy, or with documented history of no favourable corticosteroid effect, and (iv) without corticosteroid maintenance therapy in the preceding three months. Lack of atopy was not an obligatory criterion.
3. The *healthy control* group characterised by: (i) the absence of recurrent wheezing, and (ii) without a history of corticosteroid maintenance therapy. Lack of atopy was not an obligatory criterion.
4. The *unclassifiable recurrent wheeze* group consisted of infants and toddlers with recurrent wheezing, but not eligible for the asthmatic or viral wheeze group. They participated only in the feasibility study.

Overall exclusion criteria were cardiac disease, anatomic or congenital defects of the airways or lungs, syndromes, systemic disease, and/or mental retardation. Presumed inability to use a face mask did not preclude participation into this study. Subject characteristics are shown in Table 1.

Exhaled breath condensate collection

This cross sectional study design comprised one attempt, between wheezing episodes, to collect EBC in a preschool child. Precedingly, parents received thorough instructions on the collection procedure by one of the coaching childhood asthma nurses, and were asked to bring along the child's favourite cartoon, cd-song, booklet, and/or puppet.

EBC was collected using a modified closed glass condenser system that was developed in close collaboration with the Department of Instrument Development Engineering & Evaluation of the Maastricht University (patent number EP 07102586). The EBC collection system consisted of an inclined and closed glass condenser with a tube length of 90cm (to enlarge the condensation surface to 1046cm²), a downwards moveable plunger (to optimise condensate recovery from the inner wall of the condenser), with tangential and axial breath flow channels (to turbulently direct the tidal breath flows towards the cooled

Table 1. Characteristics of 70 preschool children, allocated to one of the four study groups, based on parent-completed questionnaires and available medical documents.

Study group	Asthma	Viral	Control	Unclassifiable
Number	23	22	20	5
Age in months ^A	31 (19) ^C	28 (16) ^C	13 (18) ^C	25 (15)
range	11-47	5-47	2-41	14-47
Male / female ratio	13 / 10	17 / 5	12 / 8	2 / 3
Weight in kg ^A	14 (3) ^D	13 (2) ^D	11 (5) ^D	12 (3)
Z-score weight ^A	0 (1.4)	0 (2.5)	0 (1.1)	0 (1.0)
Height in cm ^A	95 (14)	94 (17)	85 (23)	92 (15)
Z-score height ^A	0.0 (1.5)	-0.3 (2.1)	0.5 (2.1)	-1.0 (2.0)
Elevated IgE / Pos RAST ^B	23 (100)	4 (18)	0 (0)	2 (40)
Current use of steroids ^B	15 (65)	-	-	5 (100)
Inhaled steroid dose ^B	400 (100)	-	-	400 (200)

Asthma, doctor's diagnosed atopic asthma group; **Control**, healthy control group;

Elevated IgE, elevated blood level of total immunoglobulin-E; **Pos RAST**, positive radioallergo-sorbent test \geq class 2; **Unclassifiable**, unclassifiable recurrent wheezing group; **Viral**, viral-triggered recurrent wheezing group.

^Amedian (interquartile range); ^Babsolute number (percentage); ^C $p = 0.012$ (multiple comparisons, Hochberg correction); ^D $p = 0.046$ (multiple comparisons, Hochberg correction).

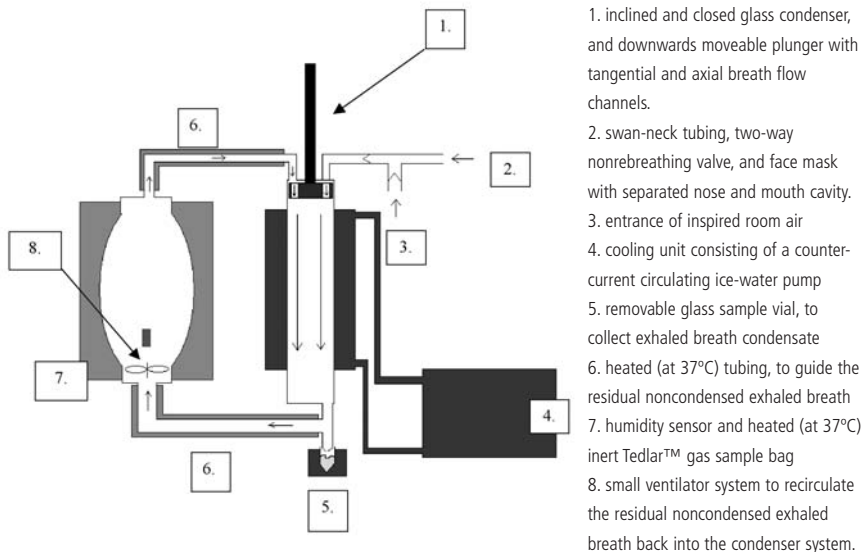


Figure 1. Schematic representation of the new closed glass condenser system, that enabled condensate collection in preschool children before and after breath recirculation.

inner condenser wall), and a unique breath recirculation system, heated at 37°C (Figure 1). The breath recirculation system consisted of heated tubing (to guide the residual noncondensed exhaled breath, and to avoid condensation outside the condenser tube), a humidity sensor, a heated inert Tedlar™ gas sample bag (to collect all residual noncondensed exhaled breath), and a small ventilator system to recirculate the residual noncondensed exhaled breath back into the cooled condenser system (to allow secondary condensation of exhaled breath) (Figure 1).

The child exhaled tidally, as long as possible, through a face mask which separated exhaled air from nose and mouth cavity, and with two-way nonrebreathing valve (8900 pediatric sizes oro-nasal masks, Hans Rudolph Inc., Kansas City, Missouri, USA), connected to the glass condenser system by means of a swan-neck tubing (serving as gravitational saliva trap). During this condensation process, the formerly lost noncondensed exhaled breath was separately being collected in the gradually inflating inert Tedlar™ gas sample bag. When the child ceased the procedure, EBC was collected by pushing the plunger downwards. Subsequently, the plunger was pulled up again, and the separately collected exhaled breath was recirculated from the Tedlar™ gas sample bag back into the condenser system, to perform a secondary breath condensation. The recirculation was stopped when the degree of humidity was $\leq 22\%$.

To improve the toddler's coping with distress from the unfamiliar procedure, they were distracted while breathing tidally through a facemask. We primarily combined strict parental cooperation, with an age appropriate cartoon, that was already started prior to entrance. In case of imminent failure to cope, a more active strategy was used on parent's advice, including children's stories, puppetry, and/or children's songs.

EBC collection was defined successful when at least 60µL condensate was produced, comprising the minimal required volume of 50µL to perform one multiplexed cytokine analysis, and an additional 10µL to compensate for loss due to sample handling.

After each procedure, the nondisposable parts of the condenser were disconnected, rinsed repeatedly with bi-distilled water, and subsequently dried at room air for at least 24 hours.

Condensate sample processing

Immediately after collection, condensate samples were snap-frozen at -78°C using dry ice and stored at -80°C. Analysis was performed within three months from sampling time. Cytokines in EBC were measured in duplicate using a liquid bead array multiplexing system or xMAP® technology (Luminex Corporation, Austin, USA). Multiplex immunoassay was performed as described previously, to measure the following cytokines (with corresponding lower limits of detection): interleukin-4 (1.2 pg/mL), interleukin-5 (1.2 pg/mL), interleukin-6 (2.4 pg/mL), chemokine interleukin-8 (2.9 pg/mL), interleukin-10 (2.3 pg/mL), interleukin-12p70 (1.2 pg/mL), interleukin-13 (1.0 pg/mL), and tumor necrosis factor-alpha (1.3 pg/mL)²²⁻²⁴. Measurement and data analysis were performed

using the Bioplex 100 system and Bioplex Manager software version 3.0 (Bio-Rad Laboratories, Hercules, CA). All multiplex immunoassays were performed in 96 well format 1.2µm filter bottom plates (Millipore, Amsterdam, The Netherlands) and a 12 point standard curve in duplicate was included on every plate. In order to minimise interassay variation, positive and negative control samples were included.

In earlier experiments, the short-term intrasubject variability of cytokine measurements in EBC was determined in 59 older children (18 asthmatics, 21 children with cystic fibrosis, and 20 healthy controls), aged 6 to 16 years. The coefficient of variation was 17.9% for interleukin-4, 11.9% for interleukin-5, 13.0% for interleukin-6, 6.1% for interleukin-8, 14.9% for interleukin-10, 26.9% for interleukin-12p70, 22.6% for interleukin-13, and 15.8% for tumor necrosis factor-alpha²⁵.

Statistics

Statistical calculations were performed using SPSS 11.5 (SPSS Inc, Chicago, USA). Normally distributed data were expressed as mean and standard error. The Student's t-test was used to test for differences among dependent, paired or repeated samples. Pearson's correlation coefficients were used to assess the degree of association between two normally distributed variables. Analysis of variance (ANOVA) was used to test for differences among normally distributed variables for more than two study groups.

Not normally distributed data were expressed as median with interquartile ranges (IQR). The Wilcoxon signed rank test was used to test for differences among not normally distributed paired data, whereas the Mann-Whitney U test was used for unpaired data. Spearman's rank correlation coefficients were used to assess the degree of association between two not normally distributed variables. The Kruskal-Wallis test was used to test for differences among non-normal variables for more than two study groups. The Hochberg correction for multiple comparisons was applied. The Chi-square test was used to evaluate the distribution of categorical variables (number of occurrences) in four study groups.

Power Calculation

Assuming a success rate of 80%, the standard error of feasibility is 5.1% using n=60 subjects. This was considered sufficiently accurate for the purpose of this study.

Ethics

The study was approved by the Medical Ethics Committee of the University Hospital of Maastricht and the Maasland Hospital Sittard. All parents gave written informed consent.

Results

Feasibility of EBC collection

A total number of 70 preschool children participated. Feasibility of the new EBC collection method in preschool children is shown in Table 2.

Table 2. Feasibility of exhaled breath condensate collection, before and after breath recirculation (BR), in 70 preschool children.

Study group	Asthma	Viral	Control	Unclass.	p value
Number	23	22	20	5	-
Age ^A	31 (19) ^C	28 (16) ^C	13 (18) ^C	25 (15)	-
Before BR					
Duration ^A	11 (9) ^D	12 (6) ^D	6 (6) ^D	14 (1)	0.004 ^F
Volume ^A	200 (250) ^E	300 (280) ^E	0 (245) ^E	300 (100)	0.031 ^F
Success rate ^B	15 (65)	20 (91)	6 (30)	4 (80)	-
After BR					
Volume ^A	367 (620)	340 (362)	130 (417)	575 (287)	0.385 ^F
Total					
Volume ^A	300 (500)	410 (400)	295 (357)	800 (150)	0.351 ^F
Success rate ^B	17 (74)	21 (95)	15 (75)	5 (100)	<0.001 ^G

After BR, after breath recirculation; **Age**, in months; **Asthma**, doctor's diagnosed atopic asthma group; **Before BR**, before breath recirculation; **Control**, healthy control group; **Duration**, duration of condensate collection in minutes; **Success**, exhaled breath condensate collection was defined successful when at least 60 μL of condensate was yielded; **Total**, before and after breath recirculation; **Unclass.**, unclassifiable recurrent wheezing group, excluded for statistical analysis for reasons of disproportionality; **Viral**, viral-triggered recurrent wheezing group; **Volume**, condensate volume in μL . ^Amedian (interquartile range); ^Babsolute number (percentage); ^C $p = 0.012$ (multiple comparisons, Hochberg correction); ^D $p \leq 0.012$ (multiple comparisons, Hochberg correction); ^E $p = 0.039$ (multiple comparisons, Hochberg correction); ^Fanalysis of variance (ANOVA); ^Gchi square test.

Before breath recirculation, the median duration of EBC collection was 10 minutes (IQR 9), the median EBC volume was 200 μL (IQR 270), and the success rate was 64% in these preschool children. After breath recirculation, the median EBC volume was 225 μL (IQR 495).

In total, the median EBC volume was 400 μL (IQR 450). The success rate of EBC collection was 83% in these preschool children, but differed significantly between the four study groups ($p < 0.001$, Chi square).

In the healthy group, the median duration of EBC collection was 6 minutes, and median EBC volume before breath recirculation was zero μL , whereas both were significantly decreased compared to the asthmatic and viral wheeze group ($p < 0.031$, ANOVA).

A younger age was significantly correlated with less duration of EBC collection time ($R = 0.481$, $p < 0.001$, Spearman correlation coefficient). A duration of EBC collection time of 10 minutes or more, was associated with successful EBC collection before breath recirculation.

Table 3. Distractive measures for preschool children during the exhaled breath condensate collection procedure.

Study group	Asthma	Viral	Control	Unclass.	total (%)
Number	23	22	20	5	70 (100)
Success	17	21	15	5	58 (83)
Not started	1	1	2	0	4 (6)
Distraction by:					
Parent	19	17	17	4	57 (86)
Cartoon	20	14	11	5	50 (76)
Book	10	5	5	4	24 (36)
Puppet	5	3	2	0	10 (15)
Song	0	6	2	0	8 (12)

Asthma, doctor's diagnosed atopic asthma group; **Control**, healthy control group; **Success**, exhaled breath condensate collection was defined successful when at least 60 μL of condensate was yielded; **Unclass.**, unclassifiable recurrent wheezing group; **Viral**, viral-triggered recurrent wheezing group.

Table 3 shows the distractive measures used during EBC collection. Four children refused to start due to sudden anxiety, associated with insufficient parental stimulation and/or recent ear-nose-throat surgery (adenoidectomy, and/or tonsillectomy, and/or myringotomy with insertion of tympanostomy tube). Distraction was mainly achieved by combined parental cooperation and cartoon watching. The Hans Rudolph face mask was applied in 60 children, whereas five preferred the babyhaler[®] and one the nebuchamber[®] face mask. Two collection procedures were not successful due to leakage of exhaled breath from defective Tedlar[™] gas sample bags. No adverse effects on respiration, nor other side-effects, were seen during the collection procedures.

Cytokine measurements before and after breath recirculation

Table 4 shows the detection and concentration of interleukin-4, interleukin-5, interleukin-6, interleukin-8, interleukin-10, interleukin-12p70, interleukin-13, and tumor necrosis factor-alpha in EBC collected before, as well as after breath recirculation, in 45 preschool children. The detection of cytokines in EBC was successful in 95 to 100% of the collected 50 μL condensate samples. There was no statistical difference between cytokine concentrations before and after breath recirculation ($p > 0.117$, Wilcoxon).

Cytokines in subgroups with and without wheeze

For further cytokine analysis between the different study groups, the separately collected condensate samples before as well as after breath recirculation in each subject, were considered as one donor series. Between the asthmatic ($n=17$), viral wheeze ($n=21$) and healthy ($n=15$) group, the median cytokine concentrations of interleukin-4, interleukin-5, interleukin-6, interleukin-8, interleukin-10, interleukin-12p70, and interleukin-13 did not differ (data not shown), whereas the median concentration of tumor necrosis factor-alpha was significantly decreased in the asthmatic group (median 38.8pg/mL in

Table 4. Detection (%) and concentration (pg/mL) of cytokines in exhaled breath condensate collected before and after breath recirculation (BR) in 45 preschool children.

EBC	before BR		after BR		p value ^C
	% ^A	pg/mL ^B	% ^A	pg/mL ^B	
IL-4	100	2.1 (0.7)	100	2.2 (0.9)	0.346
IL-5	98	16.7 (1.6)	97	16.7 (1.6)	0.278
IL-6	96	3.3 (0.9)	95	2.9 (0.4)	0.611
IL-8	100	3.9 (1.0)	100	4.4 (1.1)	0.117
IL-10	98	4.7 (0.7)	100	4.7 (0.5)	0.455
IL-12p70	100	3.4 (0.4)	100	3.4 (0.3)	0.301
IL-13	100	8.5 (1.8)	100	8.5 (1.4)	0.603
TNF-alpha	96	40.9 (5.8)	95	40.9 (6.1)	0.746

after BR, after breath recirculation; **before BR**, before breath recirculation; **EBC**, exhaled breath condensate.

^Adetection in percentage; ^Bmedian concentration (interquartile range) in pg/mL; ^CWilcoxon signed rank test.

asthma, 42.9pg/mL in viral wheeze, and 40.9pg/mL in healthy controls, respectively) ($p=0.030$, Kruskal Wallis). A weak correlation existed between age and cytokine concentrations ($-0.120 < R < 0.392$, Spearman).

Table 5. Cytokine profile in exhaled breath condensate in 53 doctor's diagnosed atopic asthmatic (AA) and nonasthmatic (nonA) preschool children.

EBC	AA	nonA	p-value ^B
Number	17	36	-
TNF-alpha ^A	38.8 (4.3)	41.7 (3.4)	0.019
IL-5 / TNF-alpha ^A	0.45 (0.05)	0.40 (0.06)	0.032
IL-13 / TNF-alpha ^A	0.24 (0.06)	0.20 (0.03)	0.049
IL-4 ^A	2.1 (0.6)	2.0 (0.7)	NS
IL-5 ^A	17.1 (2.0)	16.9 (1.6)	NS
IL-6 ^A	3.2 (0.7)	3.1 (0.7)	NS
IL-8 ^A	4.1 (1.0)	4.0 (0.5)	NS
IL-10 ^A	4.7 (1.0)	4.9 (0.5)	NS
IL-12p70 ^A	3.5 (0.7)	3.4 (0.2)	NS
IL-13 ^A	8.8 (1.5)	8.5 (1.8)	NS

AA, doctor's diagnosed atopic asthma group; **EBC**, exhaled breath condensate; **nonA**, nonasthmatic preschool children consisting of the viral-triggered recurrent wheezing group and healthy group; **NS**, not significant.

^Amedian (interquartile range); ^BMann Whitney U test.

In atopic asthmatic children, inhaled corticosteroids did in general not influence cytokine levels in EBC, with one exception: tumor necrosis factor-alpha concentrations were significantly lowered in children with inhaled corticosteroid therapy, compared to steroid naïve children (median 37.1 pg/mL *versus* 41.7 pg/mL, respectively) ($p=0.013$, Mann Whitney U).

When asthmatic and nonasthmatic (viral wheeze and healthy) children were compared, tumor necrosis factor-alpha concentrations in the asthmatic group (median 38.8 pg/mL, IQR 4.3) were significantly decreased *versus* nonasthmatic children

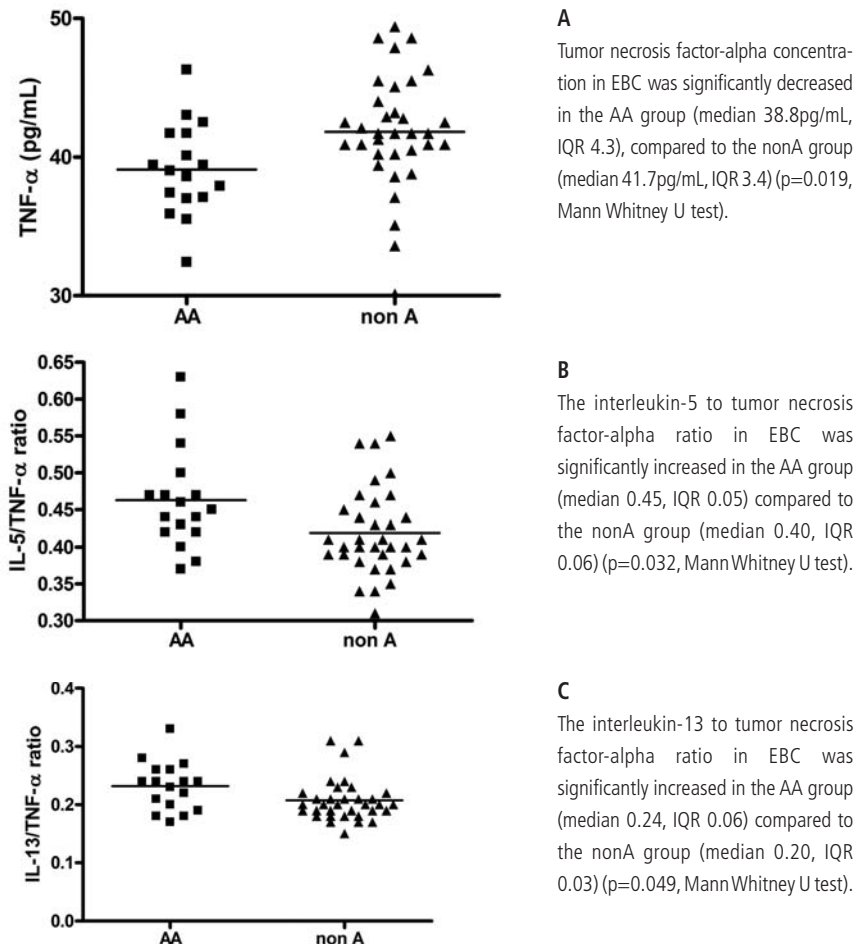


Figure 2A- 2B-2C. Th2/Th1 cytokine profiles in exhaled breath condensate (EBC) in doctor's diagnosed atopic asthmatic (AA) and nonasthmatic (nonA) preschool children.

AA, doctor's diagnosed atopic asthma group; **EBC**, exhaled breath condensate; **IL**, interleukin; **IQR**, interquartile range; **nonA**, nonasthmatic preschool children consisting of the viral-triggered recurrent wheezing group and healthy group; **Th**, T-helper cell; **TNF- α** , tumor necrosis factor-alpha.

(median 41.7pg/mL, IQR 3.4) ($p=0.019$, Mann Whitney U) (Table 5, Figure 2a). The ratios of interleukin-5 to tumor necrosis factor-alpha, and interleukin-13 to tumor necrosis factor-alpha in the asthmatic group (median 0.45, IQR 0.05, and median 0.24, IQR 0.06, respectively) were significantly higher compared to nonasthmatic children (median 0.40, IQR 0.06, and median 0.20, IQR 0.03, respectively) ($p=0.032$, and $p=0.049$, respectively, Mann Whitney U) (Table 5, Figures 2b-2c).

Discussion

We demonstrated the feasibility and efficacy of EBC collection and analysis in preschool children with and without wheezing, using a novel closed glass condenser with breath recirculation system, and using multiplexed liquid bead array: (i) the overall success rate of the new EBC collection method was 83%, with a median EBC volume of 400 μ L; (ii) the detection of cytokine profiles in 50 μ L EBC was successful in 95 to 100% of the collected samples; (iii) similar cytokine concentrations were found in EBC collected before as well as after breath recirculation; and (iv) in the atopic asthmatic group tumor necrosis factor-alpha concentrations in EBC were significantly decreased, whereas interleukin-5 to tumor necrosis factor-alpha, and interleukin-13 to tumor necrosis factor-alpha ratio's were significantly increased, compared to preschool children with viral wheeze and healthy controls.

We simultaneously introduced novel techniques to optimise the condensation process (Figure 1), and to improve small condensate sample analysis, in nonsedated preschool children with and without recurrent wheezing. Cumulatively, this new collection method with breath recirculation resulted in an overall success rate of 83%, compared to 64% when using the 'usual' (before breath recirculation) procedure. Overall, the median total volume of EBC was 400 μ L in this study. In the literature, Moeller et al proposed a method to collect, both orally and nasally, a median EBC volume of 282 μ L, using two disposable Plastipack syringes, in tidal breathing infants, however, during sedated sleep and without assessment of markers in the collected condensate¹⁹. Griese et al presented a nasal polypropylene collection system with air suction pump, to measure hydrogen peroxide and 8-isoprostane, and reported 85 μ L min^{-1} of nasally collected EBC in healthy children aged 4 weeks to 18 years, however, with contamination by nasal secretions²⁶⁻²⁷. The correlation between age and duration of EBC collection is in accordance with the significantly increased volume of collected EBC with age, reported by both Griese et al, and Moeller et al^{19,25}. Irrespective of the duration of EBC collection, breath recirculation allowed condensation of the totally exhaled breath volume within the closed glass system, in contrast to maximally 30% using the open-ended nasal suction system of Griese et al²⁶⁻²⁷. Hence, our concept of breath recirculation enabled not only the collection of an increased volume of EBC, but also, in the younger toddlers, with limited cooperation in time (often less than 10 minutes), breath recirculation was often crucial to yield a minimal but sufficient volume of condensate.

Furthermore, cytokine concentrations in EBC collected before and after breath recirculation did not differ significantly, demonstrating that concentrations were not affected by the breath recirculation process, and illustrating an efficiency to generate additional and equivalent condensate.

In the literature, vastly different, nonstandardised EBC collection techniques are available, including the immersion of plastic tubing in an ice-filled bucket, a double-wall glass distilling column with counter-current ice-water pump, an ice-filled and double-chambered glass container, the commercial EcoScreen® (Erich Jaeger GmbH, Hoechberg, Germany), RTube™ (Respiratory Inc., Charlottesville, Virginia, USA), and Turbo-Deccs (ItalChill, Parma, Italy)^{9-11,28-29}. All systems are based on the principle of constantly cooling exhaled air, whereas the handheld plastic RTube™, placed in a pre-cooled sleeve, is highly susceptible to (higher) ambient temperatures during collection^{9-11,30}. Moreover, these systems have an open ended and relatively short measured design, resulting in the variable loss of noncondensed exhaled breath, as condensation does not immediately occur, and/or does not continue once the condensation surface is completely saturated.

Additionally, exhaled biomarkers may be lost in different EBC sampling systems, due to adhesive interactions between condenser coatings and exhaled biomarkers^{17,31}. Therefore, Tufvesson et al proposed to coat the EcoScreen® collection surfaces with bovine serum albumin and Tween-20, to measure cytokines and eicosanoids, respectively³¹. However, they reported possible false-positive eicosanoid results, and the need of vacuum-centrifugation prior to analysis³¹. Recently, we compared six condenser systems, and found a glass or silicone condenser more efficient, both *in vitro* and *in vivo*, for the recovery of 8-isoprostane and albumin in EBC, compared to aluminium, polypropylene, Teflon and the EcoScreen® condenser¹⁷.

In ventilated neonates, Cheah et al described a polyvinylchloride tubing, immersed in wet ice, to collect EBC, however, with insufficient sensitivity to detect hydrogen peroxide¹⁸. Other techniques for EBC collection in ventilated infants, children and young adults, have recently been proposed³²⁻³³.

Multiplexed liquid bead array was used to measure cytokines more accurately in only 50µL sample volumes of EBC. The detection level of cytokines was high, ranging from 95 to 100%. Multiplexed liquid bead array is based on flow cytometry and microspheres (with faster and more reproducible liquid reaction kinetics, than solid planar assays), and allows multiplexing of up to 100 assays within one sample²². Reasons to introduce this technique were: (a) lack of sensitivity of conventional enzyme-linked immunosorbent assays to detect cytokines in 200 to 500µL EBC³⁴; and (b) variable level of simultaneous cytokine detection by cytometric bead array, ranging from 0 to 25%, in 50µL EBC in children¹³, and from 3 to 100%, in 1000 to 2000µL lyophilised EBC in adults³⁵⁻³⁷. When compared with conventional assays, multiplexed immunoassays are highly sensitive and specific, detect bioactive and inactive molecules, are rapid (hours instead of day(s)), have good (but not yet excellent) precision with

coefficients of variation ranging from 10 to 15%, are not interfered by drugs, have a simple protocol, require small sample volumes, and allow simultaneous detection ²².

In preschool children with different wheezing phenotypes, the central issue consists of finding a noninvasive measure to identify underlying (persisting) airway disease, such as atopic asthma. In contrast to preschool children with viral wheeze and healthy children, atopic asthma is characterised by a predominantly T-helper cell type 2 mediated chronic eosinophilic airway inflammation ^{1,3,6}. Statistical analysis revealed a significantly decreased tumor necrosis factor-alpha in EBC in the atopic asthmatic group, compared to viral wheeze and healthy controls. Steroid-treated atopic asthmatic children had lowered levels of tumor necrosis factor-alpha in EBC. This is in accordance with studies on bronchial biopsies in atopic asthmatic inflammation showing significantly decreased levels of tumor necrosis factor-alpha after eight weeks of budesonide treatment ³⁸.

As supported by the findings of Shahid et al, who reported increased levels of interleukin-4 and decreased interferon-gamma in EBC in asthmatic children, the ratio's of interleukin-5 to tumor necrosis factor-alpha, and interleukin-13 to tumor necrosis factor-alpha in EBC were significantly increased in the asthmatic group, compared to nonasthmatics, although the difference between these fractions of interleukin-5 and interleukin-13 to tumor necrosis factor-alpha were mainly due to the lowered levels of tumor necrosis factor-alpha in EBC in asthmatic preschool children ¹⁵.

The phenotypic classification in this study was performed on basis of parentally reported symptoms, a documented reaction on (inhaled) corticosteroids, medical records, and information of the responsible paediatric pulmonologist. Although a certain asthma diagnosis cannot be proven in this age group, a reasonable accuracy can be obtained using simple, clinically based parameters ³⁹. It is likely that children of the asthmatic group had allergic asthma on basis of recurrent wheezing that was not 'exclusively' viral triggered, a documented positive steroid response, the presence of allergic sensitisation in these children, and a positive family history of atopy in the first degree. On the other hand, we acknowledge that the presence of atopy in four children in the viral wheeze group may have influenced cytokine concentrations. Similarly, upper airway contamination through the use of facemasks without separated nose and mouth cavity, may have been another possible confounding factor in six children. Age was not a confounder in this study, as cytokine concentrations were not significantly related to age. This is particularly important as a difference of age existed between the healthy children and the children with wheeze (Table 1). Further evaluation of repeated cytokine profile measurements in distinct wheezing phenotypes is warranted.

Finally, we speculate that the proposed method of EBC collection may open a new window of opportunities in pediatric research, as it may allow noninvasive inflammatory profiling in nonsedated preschool children.

In conclusion, we demonstrated the feasibility and efficacy of a novel closed glass condenser with breath recirculation system to collect EBC in nonsedated preschool children with and without wheezing illness, irrespective of the duration of EBC collection. Multiplexed liquid bead array enabled more accurate cytokine measurement in only small EBC sample sizes. In atopic asthmatic preschool children, tumor necrosis factor-alpha concentrations in EBC were significantly decreased, whereas the ratio's of interleukin-5 to tumor necrosis factor-alpha, and interleukin-13 to tumor necrosis factor-alpha were significantly increased, compared to nonasthmatic children.

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CHAPTER 9

GENERAL DISCUSSION

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General Discussion

The front cover of this thesis reminds us that in the eighth century before Christ, Homeros has written the *Odyssey*, an epic poem telling of the wanderings of Odysseus, a greek hero of the Trojan war and king of Ithaca.¹ It took Odysseus ten long years to return to his homeland and to finally regain the heart of his beloved queen Penelope.¹ The methodological development of exhaled breath condensate (EBC) collection and analysis, the subject of this thesis, is also to some extent a telling of the long voyage we have made, instead of a clearcut report of stepwise proceedings, before our destination came into sight. In this way, the heart of Penelope consists of accurate diagnosis and customised care based on noninvasive techniques for all children with chronic respiratory inflammation.

9.1. What is exhaled breath condensate ?

In 1980, the pilot article on EBC entitled 'Surface-active properties of the exhaled air condensate, a new method of studying lung function', was published in Russian by Sidorenko *et alia*.² The *perestroika* reforms and the policy of *glasnost* hastened the end of the former Soviet Union in 1991.³ Soon after, EBC was rediscovered as a bi-phasic technique implicating, at first, the cooling of exhaled breath to collect the condensate, and, secondly, the analysis of the collected condensate.⁴ EBC contains respiratory droplets with volatile and (mainly) nonvolatile macromolecules that are released from the epithelial lining fluid of the airways, and that, subsequently, are 'drowned' in an excess of water vapour.⁴⁻⁵ There is evidence that abnormalities in EBC composition may reflect biochemical changes of the airway lining fluid.⁴ Moreover, the guiding and cooling of exhaled breath into a breath condenser system appears to constitute a noninvasive, rapid and relatively easy to perform method, as well as a technique that may be used repeatedly as it does not interact with the ongoing (patho)physiological processes in the airways, which, by consequence, may guarantee a high degree of safety to the subjected individual.⁶

9.2. Why should we study EBC ?

In this section we substantiate the further methodological development of EBC collection and analysis, as argued in chapter 1.

Evidence-based medicine is the 'gold standard' for clinical practice and treatment guidelines, and is largely dependent upon distinct disease phenotypes.⁷⁻⁸ However, daily clinical reality confronts us with a natural diversity of complaints, which often reflects various dimensions of a broad spectrum of diseases.⁹⁻¹⁰ Heterogeneity is the hallmark of chronic respiratory inflammation, especially in growing children.¹⁰⁻¹⁷ However, current management guidelines are mainly based on symptoms and pulmonary function tests, and do not succeed in achieving optimal disease control.¹⁸⁻¹⁹ This is, at least in part, due to the lack of a tailored individual approach, especially in the younger child.¹⁸⁻¹⁹

Moreover, current conventional disease measures have some important limitations. There are many discrepancies between: (i) subjectively reported and objectively assessed airway symptoms; (ii) pulmonary function tests reflecting airflow limitation; and (iii) direct or indirect markers of airway inflammation, such as bronchoalveolar lavage fluid or bronchial challenge tests, respectively. Endobronchial biopsy, bronchoalveolar lavage and sputum induction are relatively difficult direct measures of airway inflammation and not suitable for routine use in clinical pediatric practice. Pulmonary function tests and sputum induction require more than minimal cooperation of a child, and thus, success rate substantially declines in children aged six to seven years or less. In general, procedures in favour of children should be suitable at any age, rapid and easy to perform, requiring only minimal cooperation, as well as harmless and painless, and thus, by definition, should never be invasive or semi-invasive. As chronic respiratory disease expression is heterogeneous, it seems only logical that noninvasive specific single markers (indicating diagnosis, disease control and/or airway inflammation), have to be assessed at an individual level. Moreover, if generally applied, a set of various markers is more likely to be useful in larger populations.

Therefore, it is crucial to define additional noninvasive markers of inflammation, in particular in children with chronic respiratory disorders, that can be applied in clinical practice over a wide range, that may be specific for the evaluation and monitoring of certain disease patterns, including early disease events, and/or response to treatment, and that may give relevant information on the individual child. One such noninvasive marker, nitric oxide, was discovered in human exhaled breath by Gustafsson *et alia* in 1991.²⁰ Exhaled nitric oxide fraction is a marker of eosinophilic asthmatic inflammation.²¹ Only recently, in 2005, the first randomised controlled trial in asthmatic children on titrating inhaled corticosteroids using exhaled nitric oxide was published.²²

Compared to current disease measures, and in particular exhaled nitric oxide, the noninvasive collection and analysis of EBC offers some theoretical advantages comprising: (i) an EBC collection procedure that may require only uncontrolled tidal breathing, instead of constant expiratory flow rates (with or without the use of dynamic flow restrictors);²¹ (ii) the potential of EBC to reveal the presence of multiple nonvolatile biomarkers, that play a role in the pathophysiology of various respiratory disorders, in contrast to only one specific volatile substance such as nitric oxide; and, subsequently, (iii) the potential to be useful in discriminating and/or monitoring of more than one inflammatory lung disease in subjects of all ages. Therefore, the development of EBC methodology may ultimately result in a promising new tool to measure various markers of chronic respiratory disease, especially in preschool children.

9.3. Pearls and pitfalls of EBC

In this section we separately discuss our findings, as presented in chapter 1.6, chapter 2 and chapter 4.1, because these insights, in their very essence, were formative to our further research efforts.

In chapter 1.6, we presented the methodology of EBC, corresponding to our knowledge at that time, and we found it to offer challenging new perspectives for the diagnosis and management of various inflammatory lung diseases, of particular interest for paediatric respiratory medicine. Moreover, EBC procedures are well tolerated, and even safe in children, aged four years and above, with and without asthma exacerbation.⁶

Subsequently, we proceeded with a systematic elaboration from a pediatric point of view, presented in chapter 2, of the pearls and pitfalls of each single aspect of EBC methodology, comprising the collection, preservation and analysis of EBC. We identified many confounding factors that were, in general, attributable to the lack of a standardised collection technique and procedure, as well as to the lack of specific and highly sensitive analytical techniques.

By consequence, we did not wish to overstate the apparent simplicity of EBC. Moreover, we were triggered off by, on one hand, the constant flux of reports on highly variable marker concentrations in condensate in clinical studies using more or less inappropriate assays, and, on the other hand, by the various methods of EBC collection, as systematically reviewed in chapter 2.

Hence, we put forward that these two key issues (*id est* variable marker concentrations in EBC, and various methods of EBC collection) might very well be mutually linked, at least in part, and until proven otherwise, as argued in chapter 4.1. All the above has led us to direct our research efforts primarily towards optimising the condensate collection system for the measurement of a given type of biomarker, prior to any standardisation at all, rather than focussing on pure clinical 'hit-and-run' EBC trials, that usually provide only limited cross sectional data in relatively small populations, with only median group differences, using very different techniques, including assays with inappropriate lower detection limits and poor reproducibility. On the other hand, we could not neglect the fundamental reasons why we were and still are involved in research on EBC. In fact, we ultimately want to develop a new and noninvasive tool to assess chronic respiratory inflammation, especially in preschool children. Thus, we simultaneously performed two clinical trials, although one may argue their early or preliminary status.

We now invite the interested reader to join us in systematically evaluating four topics on EBC that are addressed by this thesis, although there inevitably exists some overlap: (i) cross sectional studies in children with asthma and cystic fibrosis (chapter 9.4), (ii) development of a new condenser system (chapter 9.5), (iii) new sensitive techniques for the detection and measurement of biomarkers in EBC (chapter 9.6), and (iv) application of new techniques in preschool children (chapter 9.7).

9.4. Cross sectional studies in children

In this section we will mainly discuss our findings in two – early – cross sectional, clinical studies, presented in chapter 3 and chapter 7, in children with asthma and

cystic fibrosis, respectively. In this – early – perspective, we have put on trial our knowledge on EBC at that time, in order to further direct and adjust our research planning.

9.4.1. A cross sectional study in children with asthma

In chapter 3, we presented a cross sectional clinical trial in a relatively homogenous and well-controlled population of 23 asthmatic children and 9 healthy controls. New exhaled markers, comprising exhaled nitric oxide, exhaled carbon monoxide, condensate acidity (pH) and cytokines (*in casu* interleukin-6, interleukin-8, tumor necrosis factor-alpha, soluble tumor necrosis factor-alpha receptor 75, soluble intercellular adhesion molecule-1), as well as albumin and C-reactive protein in EBC, collected using a cooled borosilicate glass distilling column, were compared with conventional disease measures, such as lung function parameters and the asthma control questionnaire of Juniper.

At first, it was shown that, conventional specific solid-phase enzyme-linked immunosorbent assays, in particular designed for use in blood- and/or bronchoalveolar lavage fluid-samples (with lower limits of detection ranging from 20 to 50 pg/mL), were not sensitive enough to detect interleukin-6, interleukin-8, tumor necrosis factor-alpha, soluble tumor necrosis factor-alpha receptor 75, and C-reactive protein in 200 to 500µL of EBC. On the other hand, ultrasensitive enzyme immunoassays were only able to detect albumin in two out of 32 EBC samples (with a lower limit of 8 ng/mL), and were not able to detect soluble intercellular adhesion molecule-1 in EBC (with a lower limit of 3 ng/mL). Thus, conventional and even ultrasensitive solid-phase enzyme immunoassays were not able to smoothly detect small proteins in condensate samples, mainly due to lower detection limits that were not suitable for use in this 'new' water vapour-dominated medium. Huszar *et alia* compared two types of (similar) immunoassays to measure thromboxane A2 concentrations in EBC collected with the commercially available EcoScreen® condenser.²³ They reported vastly different absolute values, due to intrinsic analytical problems associated with these assays.²³ Similarly, 30-fold differences in condensate levels of interleukin-4 in asthmatic children were found using different enzyme immunoassays, as well as different condenser systems.²⁴⁻²⁵ On the other hand, Leung *et alia* also used ultrasensitive sandwich enzyme immunoassays, and reported detectable and reproducible measurements of growth factors (*in casu* macrophage-derived chemokine, thymus and activation regulated chemokine, epidermal growth factor, AA isoform of platelet-derived growth factor, and vascular endothelial growth factor) and cytokines (*in casu* tumor necrosis factor-alpha and interleukin-4) in EBC collected in asthmatic children with the RTube™ condenser.^{24,26-27} Detectable levels were ranging from 27 to 100 per cent.²⁴ In children with cystic fibrosis, Bodini *et alia* found interleukin-8 detectable using a similarly ultrasensitive immunoassay in 90 per cent of EBC samples collected with a glass condenser.²⁸ However, these authors did not report the volume of EBC that was required to perform each measurement.^{24,26-28} It may be clear that these conventional immunoassays are not yet optimal for routine use in EBC.

Secondly, our childhood asthma study had shown that not-deaerated condensate pH was significantly lowered in the asthmatic group, compared to controls, whereas this was not found using deaerated condensate pH. At that time, the methodology of condensate pH measurements was quite controversial, and even provoked a fierce polemic in literature whether to deaerate EBC samples or not.²⁹⁻³⁷ Meanwhile, this particular acid issue has been extensively addressed by several other research groups.³⁸⁻⁴⁷ Condensate pH is not stable due to the presence of ambient and exhaled carbon dioxide.⁴⁸⁻⁴⁹ Therefore, deaeration or gas standardisation involves the bubbling a carbon dioxide-free gas through the EBC sample for several minutes, to remove carbon dioxide and bicarbonate from EBC, and to rise and stabilise EBC pH.⁴⁸⁻⁴⁹ Nowadays, the American Thoracic Society / European Respiratory Society Task Force on EBC states that gas standardisation is useful when samples cannot be assayed immediately, when one is not interested in exhaled carbon dioxide levels, or when the condensate pH is expected to be particularly acidic.⁴⁸⁻⁴⁹

Thirdly, we reported a discordant pattern of generally low correlations between measures of airway inflammation, clinical parameters of disease severity, and physiological variables such as spirometric assessment of airway calibre. These few positive findings are likely related to the very absence of any methodological EBC standardisation, including the use of inappropriate analytical techniques, and/or to the relatively homogenous and well-controlled study population. However, it is also plausible that this may have been a first subtle indication that the measurement of airway inflammation does reflect some unmet aspects of chronic respiratory disease. Airway inflammation is not the same as subjectively reported airway symptoms, nor is it the same as pulmonary function tests that only reflect airflow limitation. Hence, this may suggest an additional value of exhaled measures of inflammation, for the noninvasive management of childhood asthma.

Nevertheless, a rapid and ill-considered implementation of EBC in clinical trials may be not more than walking on thin ice. Recently, this was demonstrated, again, in a prospective study in 40 children with stable asthma eligible for inhaled steroid reduction.⁵⁰ In this study, the authors report that EBC measurements of nitrite/nitrate levels and cysteinyl leukotrienes proved to be disappointing clinical tools, although they add that their results cannot be taken to suggest that EBC has no role in the monitoring of childhood asthma.⁵⁰ However, some alternative and plausible methodological explanations have not been highlighted by this study, such as the use of a specific solid-phase enzyme immunoassay to measure cysteinyl leukotrienes, with a relatively high lower-limit of detection of 15.0 pg/mL, designed for application in other mediums, but not EBC.⁵⁰ Moreover, the authors mentioned they had to make arbitrary choices of what to measure due to the small volumes of condensate obtained by the commercially available EcoScreen® condenser, indicating an inefficient condensation process, or the need of large sample volumes required for analysis.⁵⁰

9.4.2. A cross sectional study in children with cystic fibrosis

In chapter 7, we explored the ability of different exhaled inflammatory markers, including exhaled nitric oxide, condensate pH, nitrite, nitrate, hydrogen peroxide, 8-

isoprostane, and various T helper-1 and T helper-2 cytokines, such as interferon-gamma, tumor necrosis factor-alpha, interleukin-2, interleukin-4, interleukin-5 and interleukin-10 in EBC, to discriminate between a large cohort of 48 children with cystic fibrosis and 50 healthy controls in a cross sectional study design. Furthermore, we used multiplexed cytometric bead array, as previously described by our research group, to measure several cytokines, simultaneously, in EBC collected using a cooled borosilicate glass distilling column.⁵¹

The cross sectional cystic fibrosis study showed that multiplexed detection of cytokines in only 50µL of EBC was possible, using cytometric bead array. This technique consists of a series of spectrally discrete particles that can be used to capture and quantitate soluble analytes (*in casu* cytokines).⁵²⁻⁵³ The analyte is then measured by detection of a fluorescence-based emission and flow cytometric analysis.⁵²⁻⁵³ In short, cytometric bead array generates data that are comparable to enzyme linked immuno sorbent assays, but in a multiplexed or simultaneous fashion, and obtains more results from a single small sample.⁵²⁻⁵³ In the cystic fibrosis study, interleukin-5 was the only nondetectable cytokine in EBC, compared to several nondetectable cytokines in our first report on cytometric bead array (tumor necrosis factor-alpha and interleukin-5 were nondetectable in children with cystic fibrosis, whereas interleukin-2, interleukin-4 and interleukin-5 were nondetectable in healthy control children).⁵¹ The lack to detect any interleukin-5 is in agreement with the findings of Armstrong *et alia* in bronchoalveolar lavage fluid, and may be related to the presence of proteases in cystic fibrosis airways.⁵⁴⁻⁵⁵ The lack to detect (some) cytokines does not necessarily implicate that they are not present, as the detection method may lack sufficient sensitivity. In the cystic fibrosis study, the levels of detection of the other cytokines (interferon-gamma, tumor necrosis factor-alpha, interleukin-2, interleukin-4 and interleukin-10) ranged up to 42, and 20 per cent in children with cystic fibrosis, and healthy control children, respectively, compared to 25 and 14 per cent in our first report.⁵¹

Significantly more detections of interferon-gamma and interleukin-4 were found in EBC in children with cystic fibrosis compared to controls. Moreover, cytometric bead array did require only a small EBC sample size, as mentioned above, and in contrast to other reports, as noted below.⁵⁶⁻⁵⁸

In literature, no comparative data on cytometric bead array in EBC in children are (yet) available. On the other hand, in adults with acute lung injury or chronic obstructive pulmonary disease, cytometric bead array has also been described for the measurement of interleukin-1-beta, interleukin-6, interleukin-8, interleukin-10, tumor necrosis factor-alpha and interleukin-12p70 in EBC, collected with the EcoScreen[®], and only after lyophilisation (or freeze-drying) of 2000µL EBC and cryopreservation.⁵⁶⁻⁵⁷ The reported levels of cytokine detection in these subjects ranged from 37 to 100 per cent.⁵⁷ Recently, Matsunaga *et alia* also reported the use of a membrane protein array to measure 40 different cytokines and chemokines in 1000µL EBC in adult asthmatics using the EcoScreen[®], and found relative cytokine levels in 3 to 27 per cent of the samples.⁵⁸ Interleukin-4, interleukin-8, interleukin-17, tumor necrosis factor-

alpha, regulated on activation normal T-cell expressed and secreted (RANTES), interferon-gamma-inducible protein 10, transforming growth factor-beta, and macrophage inflammatory protein 1-alpha and 1-beta were significantly upregulated in asthmatics compared with healthy controls.⁵⁸ Matsunaga *et alia* refer to a confirmation of good correlations between the relative levels obtained by using this method and the actual levels obtained by means of quantitative assays.⁵⁸⁻⁵⁹ In 22 adults, Schumann *et alia* reported the detection of erythropoietin and tumor necrosis factor-alpha in EBC collected using the EcoScreen®, in 68 and 59 per cent, respectively.⁶⁰ However, they did not report further details on the required volume of EBC, nor on sample processing.⁶⁰

Furthermore, in the cross sectional cystic fibrosis study, the concentrations of interferon-gamma and nitrite in EBC were significantly increased, whereas exhaled nitric oxide levels were significantly decreased in the cystic fibrosis group, compared to controls. These results are in accordance with available data in literature on nitric oxide,⁶¹⁻⁷⁰ nitrite (assessed in young adults with cystic fibrosis),^{62-63,71-73} as well as interferon-gamma (assessed in bronchial biopsies).⁷⁴ Several hypotheses may explain lowered exhaled nitric oxide levels and higher nitrite concentrations in cystic fibrosis, including: (i) impaired diffusion of gaseous nitric oxide into the airway lumen due to viscous mucus in the cystic fibrosis airways, which in turn may result in conversion of nitric oxide into nitrite;^{63,75} (ii) decreased expression of inducible nitric oxide synthase II in epithelial cells;^{70,76-80} and (iii) increased arginase activity in cystic fibrosis airways.⁸¹ Hence, we provided evidence for the discriminating ability, between children with cystic fibrosis and healthy controls, of three separate, exhaled inflammatory markers. Obviously, the clinical relevance of these findings for the individual child at long-term level remains yet to be determined. The concentration of hydrogen peroxide in EBC did not differ between children with cystic fibrosis and controls, which is in accordance with other reports.^{61,82} Also, condensate pH and the concentration of 8-isoprostane in EBC did not significantly differ between these study groups, which may be attributed to the mild to moderate cystic fibrosis severity in the study population. On the other hand, Montuschi *et alia* found significantly increased 8-isoprostane concentrations in EBC in 19 adult patients with cystic fibrosis, compared to normal subjects,⁸³ whereas Carpagnano *et alia* found significantly lowered condensate pH in children with cystic fibrosis, than in healthy controls, and a further decrease in 10 children with cystic fibrosis exacerbation, compared to 10 stable cystic fibrosis patients.⁸⁴

Using multivariate logistic regression models, a **diagnosis** of cystic fibrosis was best indicated by 8-isoprostane, nitrite and interferon-gamma in EBC. This particular profile of combined exhaled inflammatory markers, was able to discriminate children with cystic fibrosis from healthy controls, with an acceptable ratio of sensitivity (78 per cent) to specificity (92 per cent), and an area under the receiver operating characteristic curve of 0.906. An **exacerbation** of cystic fibrosis was best indicated by 8-isoprostane and nitrite, with a sensitivity of 40 per cent, a specificity of 97 per cent, and an area under the receiver operating characteristic curve of 0.838. Most indicative biomarkers of cystic fibrosis **severity** were exhaled nitric oxide and condensate pH, with a sensitivity

of 96 per cent, a specificity of 67 per cent, and an area under the receiver operating characteristic curve of 0.751. In literature, to date, no comparable data are available. However, to clearly state, these indicative profiles do not at all intend to be an alternative assessment of cystic fibrosis diagnosis, as this would be almost redundant and even out of the question. In our opinion, such disease discriminating profiles of exhaled markers have to be identified, prior to any longitudinal clinical study on the noninvasive monitoring of chronic respiratory disease. Moreover, this study illustrates that the combination of various noninvasive markers into several specific profiles, may indicate different aspects of chronic respiratory disease. Hence, these combined noninvasive markers may even provide additional information, in contrast to each marker separately. *Exempli gratia*, in cystic fibrosis exhaled nitric oxide levels are usually low, whereas nitric oxide may indicate disease severity when combined with condensate pH. This interesting 'synergistic' effect warrants further exploration in a longitudinal setting.

In summary, it may be clear that the need for additional improvement of EBC methodology, prior to longitudinal clinical studies, was still incontestable.

9.5. Development of a new condenser system

In this section we will chronologically describe, in four phases, the development of a new breath condenser system, based on findings originating from chapter 4.2, chapter 5 and chapter 8.

9.5.1. Phase 1: the Babel confusion

In the methodological review, presented in chapter 2, we provided an overview of the vastly different techniques to collect EBC, at that time. Most widely used designs included immersion of a Teflon-lined tubing in an ice-filled bucket, and a double-wall borosilicate glass distilling column connected to a counter-current ice-water pump. Commercially available systems included the lamellar Teflon-like EcoScreen® condenser (Erich Jaeger GmbH, Hoechberg, Germany) and the hand-held disposable polypropylene RTube™ breath condensate collector (Respiratory Inc., Charlottesville, Virginia, USA). The EcoScreen® conducts exhaled air through a lamellar condenser with a sample collection vial inserted into an electrical cooling cuff. This design has been associated with deposition of frozen condensate on its inner lamellar walls. Likewise, immersed plastic tubing was even associated with complete obstruction of the collection lumen. Most of these condenser systems are connected to a two-way non-rebreathing valve and a mouth piece, whereas the RTube™, a portable hand-held condensate collector which consists of a re-usable and precooled aluminium sleeve that is placed over the outside of a disposable polypropylene vertical condensation or collection tube, is connected to one-way valves and a mouth piece. Recently, the thermostatically controlled polyethylene Turbo-Deccs (ItalChill, Parma, Italy) has joined this list.⁸⁵ All systems are based on the principle of constantly cooling exhaled air, whereas the handheld plastic RTube™, placed in a pre-cooled sleeve, is highly susceptible to (higher) ambient temperatures during EBC collection.⁸⁶ Hence, the

confusing variety of physical characteristics associated with each specific condenser design constitutes one of the main obstacles for a smooth development of a standardised EBC sampling device.

9.5.2. Phase 2: the condenser coating study

As stated in chapter 4.1, we argued that the variability of biomarker concentrations may be associated, at least in part, and until proven otherwise, with the various designs of EBC collection. This culminated in the so-called coating study, as presented in chapter 4.2, and investigated the influence of different adhesive properties of inner condenser coating surfaces (*in casu* glass, silicone, aluminium, polypropylene, Teflon and the EcoScreen® condenser) on the measurement of eicosanoids (*in casu* 8-isoprostane) and proteins (*in casu* human albumin) in condensate. We have shown that a condenser with a glass coating was more efficient for the measurement of these molecules in EBC, both *in vitro* as well as *in vivo*, when compared with other coatings, including the commercially available and Teflon-like EcoScreen® condenser.

The reported *in vivo* detectability and concentrations of 8-isoprostane in EBC in healthy subjects, were in line with available, but scarce data in literature.⁸⁷ Moreover, when reviewing literature, according to an identical assay (enzyme or radio-immunoassay), and different condenser coatings, also higher 8-isoprostane concentrations were reported using a glass condenser compared with the EcoScreen® condenser.^{83,87-100}

On the other hand, the reported isoprostane condensate recovery percentages *in vitro* were high and did not differ significantly between the condenser coatings. This may be a reflection of the higher overall mean condensate recovery of isoprostane (100 per cent) in contrast to albumin (52 per cent), which may in turn illustrate a different behaviour of these molecules during the expiratory phase of the EBC collection, as molecules with a higher molecular weight (*id est* 354 Dalton versus up to 80.000 Dalton, for 8-isoprostane and albumin, respectively) may experience more difficulties in their transition from respiratory fluid droplets and exhaled air, into the condensate. Conversely, an isoprostane overload in the *in vitro* experiments seems unlikely, when considering that the absolute amount in the condensate sample vials must have been very close to the concentrations found *in vivo*, as an isoprostane test solution of 42 pg/mL was nebulised. This may indicate that the nebuliser set-up was producing isoprostane aerosols that have been more effectively condensed as compared to the real EBC *in vivo*, and/or that the upper out of range plateau zone of the immunoassay was reached.

We also report in this study, a limited number of positive albumin detections *in vivo*, which in turn is in agreement with (i) the relatively few positive studies, at that time, on cytokines or proteins in EBC versus several positive reports on 8-isoprostane and leukotrienes in EBC; and with (ii) the methodological concerns raised by Tufvesson *et alia* on evaporation, loss of biomarkers in the condensate collection system and low biomarker concentrations in EBC (as further discussed).¹⁰¹

A possible plausible explanation for the superiority of glass coatings is mainly related to the fact that water acts as a bipolar vehicle, whereas the pH of exhaled breath ranges from 6.50 to 7.80, and the isoelectrical point of human albumin is situated at pH 4.80.¹⁰² In other words, at higher pH values (such as reported in EBC) albumin acts as a negatively charged molecule in exhaled breath, that may be repulsed by the negatively charged glass condenser wall, and thus, may promote the recovery of albumin in EBC. In contrast, the same negatively charged albumin molecule may be attracted by aluminium metal ions that are positively charged, and thus, may hamper the recovery in EBC. Also, 8-isoprostane acts in an extremely hydrophobic manner, but will stick to neutral surfaces (such as Teflon) in the presence of aqueous solutions, to lipids and to detergents.

Furthermore, we acknowledge that variability of biomarker concentrations in EBC may, in part, be due to reported intrinsic problems of different immunoassays (as already discussed).²³⁻²⁵ Thus, to actually comprehend the possible impact or relevance of our finding, the reproducibility of the measurement of 8-isoprostane (or other eicosanoids) and proteins such as cytokines, should be additionally evaluated *in vivo* by using different condenser coatings as well as a reliable ultrasensitive analytical method (this issue will be addressed in the next chapter).

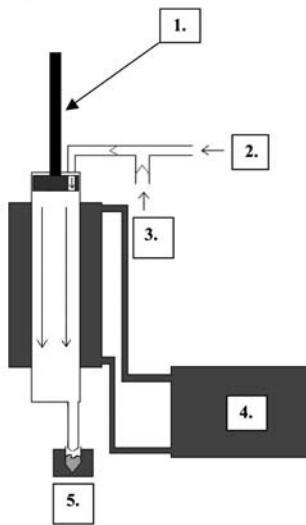
We believe that borosilicate glass, which is readily available and easy to use, should be considered as a more appropriate, instead of inferior, basic material for condenser systems than the Teflon-coated EcoScreen[®], at least for the measurement of eicosanoids and small proteins in EBC. Hereby, not only offering new and more perspectives to further develop the condensate collection system, but also illustrating that future guidelines for EBC standardisation should be prone to include the most valid condenser coating(s) to collect condensate and subsequently measure a specific disease marker.

Up to date, three other studies on the potential influence of sampling systems on biomarkers in the EBC have been published.^{86,101,103} Tufvesson *et alia* proposed a novel method to collect EBC using the EcoScreen[®] condenser of which all collection surfaces previously had been coated with bovine serum albumin (1 per cent) and Tween-20 (0.01 per cent), when measuring cytokines and eicosanoids, respectively.¹⁰¹ However, they also reported some major limitations, as (i) the preceding coating procedure lasts 30 minutes, (ii) the Tween-20 coating may lead to false-positive (eicosanoid) results, and (iii) a 5 to 10-fold concentration of the condensate sample was necessary, by vacuum-centrifugator, prior to analysis.¹⁰¹ Soyer *et alia* compared two commonly used commercially available EBC collection systems, the RTube[™] and EcoScreen[®] condensers.⁸⁶ They found that there was no difference for the measurement of acidity, but the concentrations of cysteinyl leukotrienes and eotaxin were significantly higher in samples collected with the EcoScreen[®], than with the RTube[™].⁸⁶ Soyer *et alia* attributed these differences mainly to (i) the susceptibility of pre-cooled RTube[™] sleeves to (increased) ambient temperatures over the recommended time course of a collection manoeuvre, whereas the highest

temperatures were measured at the one-way valve, near the mouth, however (ii) they also stated that differences in the materials could affect sample recovery.⁸⁶ Recently, Prieto *et alia* compared the RTube™ and EcoScreen®, and reported that condensate pH values are dependent upon the collection device used.¹⁰³

9.5.3. Phase 3: a new condenser concept emerges

The next step in our search for a more reliable condenser device, was to introduce a newly developed glass condenser system, as presented in chapter 5. Briefly, the condensation surface was enlarged using a glass condenser tube length of 90 cm, condensate recovery was optimised using an inclined condensation tube with a downwards moveable condensate sweeping plunger, and exhaled breath flows were turbulently directed towards the cooled inner condenser wall by the plunger's multiple tangential and axial breath channels (Figure 1).



1. inclined glass condenser with a tube length of 90 cm (to enlarge the condensation surface), and downwards moveable plunger (to optimise condensate recovery from the inner condenser wall), with tangential and axial breath channels (to turbulently direct the tidally exhaled breath flows towards the cooled inner condenser wall)
2. swan-neck tubing (serving as gravitational saliva trap) and two-way nonbreathing valve, connected to a mouthpiece
3. entrance of inspired room air
4. cooling unit consisting of a counter-current circulating ice-water pump
5. removable, cooled glass sample vial (to collect exhaled breath condensate)

Figure 1. Description of the new, optimised glass condenser system, as presented in chapter 5.

In this study, 30 healthy adult subjects were chosen in order to eliminate possible confounding factors attributable to heterogenous disease expression and/or variability of disease control. Each subject was asked to perform four tests at different points in time, to assess within-day, between-day and between-week reproducibility of condensate volume, and of the measurements of hydrogen peroxide, 8-isoprostane, interleukin-2, interleukin-4, interleukin-5, interleukin-6, interleukin-8, interleukin-10, interleukin-13, and tumor necrosis factor-alpha (using a new technique for the detection of cytokines, as will be further discussed in chapter 9.6). Each of these four tests consisted of four sequential EBC collections, during 15 minutes, using ad random different types of condenser systems (the new optimised glass condenser, a silicone condenser, the former glass condenser, and the EcoScreen®).

We demonstrated that the newly developed optimised glass condenser yielded significantly more condensate volume (2025 μ L), compared to the silicone, (former) glass and EcoScreen[®] condensers (525 μ L, 712 μ L, and 1237 μ L respectively), suggesting both an improved condensation process, and an increased opportunity to perform a broad spectrum of analyses using the new optimised condenser. Reproducibility of EBC volume, expressed by means of percentage of coefficient of variation, was comparable for the new condenser and EcoScreen[®] (ranging from 11 to 20 per cent, and from 9 to 19 per cent, respectively), but was significantly better compared to the silicone and (former) glass condenser (ranging from 20 to 37 per cent, and from 21 to 30 per cent, respectively). Hence, the optimised glass and EcoScreen[®] condenser were both significantly associated with less variation in the generated EBC volume, compared to the other condensers. Hereby, reducing a possible confounding influence of the variable quantity of EBC collected over a given time, and even within individuals.

In EBC collected with the new glass condenser system, significantly more positive hydrogen peroxide, 8-isoprostane, interleukin-2, interleukin-4, interleukin-5, interleukin-13, and tumor necrosis factor-alpha detections were found, supporting an improved sampling of EBC. Moreover, 8-isoprostane concentrations were significantly increased in EBC yielded by the new condenser, compared to the other three condensers, which is in accordance with the findings in the former coating study. On the other hand, reproducibility of hydrogen peroxide, 8-isoprostane and cytokine measurements in EBC, expressed by means of percentage of coefficient of variation, was equally variable for all condenser types, suggesting no significant influence of the type of condenser coating on reproducibility of biomarker measurements in EBC in healthy adults. In literature and to date, no formal study addressing this issue has been published, although biomarker reproducibility using other analytical techniques has been reported,¹⁰⁴⁻¹⁰⁶ and/or could not be calculated due to small number of subjects.^{87,107-108}

Variations in biomarker measurements in EBC may be attributed to variations in the dilution and/or quality of condensate, that is influenced by different collection techniques and procedures, sample processing and storage conditions, and/or sensitivity of the analytical techniques used.¹⁰⁷ The levels of the highly volatile hydrogen peroxide in EBC are susceptible to (yet) uncleared methodological issues that may be attributed to: (i) different cooling temperatures during collection;¹⁰⁹ (ii) a circadian rhythm of exhaled hydrogen peroxide levels;¹¹⁰ (iii) the different methods of measurement with widely varying values for both healthy individuals and patients with respiratory disorders, with most of these values being close to the lower limits of detection;^{82,87-88,104-105,111-124} (iv) evidence of hydrogen peroxide flow dependency, as nontidal breathing or lower controlled exhalation flows lead to significantly higher hydrogen peroxide levels in EBC, when compared with higher controlled flows, and as tidal breathing during EBC collection incorporates multiple different flows in each breath;¹¹⁵ and (v) the high level of chemical reactivity of hydrogen peroxide with salivary contamination, and exhaled nitrogen oxides and ammonia, forming various other compounds, including hydroxylamine, peroxyxynitrite and nitrate, by which

hydrogen peroxide levels in EBC decline over time in storage, and by which it is likely that (some) hydrogen peroxide is consumed even during the time of collection.^{48-49,119,125-127} Isoprostanes are relatively stable end-products of *in vivo* arachidonate peroxidation, and are measured in EBC by immunoassay, that may be influenced by crossreactivity with closely related substances.¹⁰⁷ We used commercially available enzyme immunoassays, that are very sensitive but lack in specificity, to measure 8-isoprostane in 100µL of EBC, instead of radioimmunoassays that now have been qualitatively validated for these measurements, by reverse phase high performance liquid chromatography, as described by Montuschi *et alia*.¹²⁸⁻¹²⁹ However, the use of both enzyme immunoassays and radioimmunoassays may be associated with cross reactivity of antibodies.¹⁰⁶ On the other hand, Cap *et alia* have used gas chromatography/mass spectrometry to quantify exhaled eicosanoids (*in casu* leukotrienes).¹³⁰ Although the specificity of mass spectrometry detection ensures an unequivocal determination of the analysed substances, gas chromatography requires, in contrast to liquid chromatography, derivatisation procedures and corresponding sample pre-treatment for nonvolatile compounds.¹³⁰ Therefore, to avoid more sources of errors, Gonzalez-Reche *et alia* recently presented a validated method for the simultaneous determination of arachidonic acid metabolites in 1000µL of EBC by liquid chromatography-electrospray ionisation tandem mass spectrometry, although this method still included an automated sample enrichment step.¹⁰⁶ Other confounding factors may be age, diets rich in antioxidants, and smoking habits.¹⁰⁷

Our findings on biomarker reproducibility were not altered by expressing the results as intraclass correlation coefficients or standard deviations, instead of coefficients of variation. Biomarker levels were assessed in condensate originating from healthy adults, and thus, may have been more pronounced in a steroid naïve population with documented chronic respiratory inflammation, and comparable levels of disease control. In diseased subjects, one may expect increased mean concentrations, and lowered coefficients of variation.

To recapitulate, the proposed new glass condenser had an improved condensation process and condensate recovery, using an inclined and enlarged condensation surface, with a condensate sweeping plunger, having tangentially and axially guiding breath flow channels. These improvements resulted in significantly increased EBC volumes and biomarker detections, including higher 8-isoprostane EBC levels, demonstrating a significant influence of the condenser system, in favour of the new optimised glass condenser. However, reproducibility of biomarker measurements in EBC in healthy adults was not influenced by the type of condenser.

9.5.4. Phase 4: the new condenser system

Finally, we introduced, in chapter 8, a new method for the collection of EBC, before and after breath recirculation, and demonstrated its feasibility in 70 preschool children, aged 4 years or less, with and without wheezing illness. Furthermore, we used the same analytical technique as previously mentioned to measure cytokines in the collected EBC (as will be further discussed in chapter 9.6 and 9.7).

Preschool children, and in particular toddlers, do not simply intend to follow any instruction (to breath tidally into a facemask with separated nose and mouth cavity) unless they are willing to cooperate. Also, if toddlers tend to cooperate, they often do not sustain their effort for a relatively long time (initially condensation of exhaled breath often takes at least 5 to 10 minutes depending on the available condensation surface), as their willingness to cooperate may rapidly disappear, and/or as they can be distracted by anything at all. The collection of EBC in preschool children requires short sampling times. However, these are not always offered by the currently used and less efficient condensers, with open ended and relatively short-measured designs. These condensers are characterised by the loss of a highly variable amount of noncondensed exhaled breath, as condensation does not immediately occur, and/or does not continue to occur once the condensation surface is completely saturated. In addition, exhaled biomarkers may be lost in different EBC sampling systems, due to adhesive interactions between condenser coatings and exhaled biomarkers, as already discussed. Furthermore, EBC collection in preschool children is also hampered by suboptimal condensate analysis, associated with techniques that require relatively large volumes of EBC, and by consequence, necessitate again a prolongation of sampling times. For all the above reasons, a modification of the glass condenser was mandatory, in such a way that the condensation process was optimised with a high efficiency-to-time ratio. Hence, we were searching a design that would allow maximal condensation of all the exhaled breath that was gathered, whether in only a few minutes or after a prolonged period of time.

In short, the glass condenser system as previously described in chapter 5 (with enlarged condensation surface, inclined and optimised condensate recovery with plunger, and turbinate directed breath flow channels), was now closed and connected to a unique heated (at 37 degrees Celsius) breath recirculator system (Figure 2). The breath recirculation system itself consists of heated tubing (to guide the residual noncondensed exhaled breath, and to avoid condensation outside the condenser tube), a humidity sensor, a heated inert Tedlar™ gas sample bag (to collect all residual noncondensed exhaled breath), and a small ventilator system (to recirculate the residual noncondensed exhaled breath back into the condenser system for secondary condensation of exhaled breath).

The child exhaled tidally, as long as possible, through a face mask, with separated nose/mouth cavity, and two-way nonrebreathing valve, that was connected to the glass condenser system. During this condensation process, the formerly lost noncondensed exhaled breath was separately being collected in a gradually inflating inert Tedlar™ gas sample bag. When the child ceased this procedure, EBC was collected by pushing downwards the plunger. Subsequently, the plunger was pulled up again, and the separately collected exhaled breath was recirculated, from the Tedlar™ gas sample bag, back into the condenser system, to perform a secondary breath condensation, until the degree of relative humidity was less than 22 per cent.

This new concept of EBC collection before and after breath recirculation, allowed condensation of the totally exhaled breath volume, whether gathered after only a few minutes of EBC collection, or after a prolonged period. Moreover, the concept enabled

the collection of significantly more EBC volume compared to the 'usual' (before breath recirculation) procedure, whereas, in toddlers with limited cooperation in time (and more often seen in the healthy group that was relatively unknown with the use of face masks), breath recirculation was often crucial to yield minimal but sufficient condensate volume. The cumulative nature of these methodological modifications resulted in an overall success rate of 83%. Furthermore, the main issue was whether the concentration of cytokines, measured in the collected EBC, was affected by the recirculation process or not. We demonstrated that all cytokine concentrations in EBC collected before and after breath recirculation, were comparable and did not differ significantly. Hence, illustrating the efficiency of the breath recirculation system in generating an additional amount of equivalent condensate.

In literature, some attempts have been described to collect EBC in infants.^{117,131} Griese *et alia* presented a nasal collection system, that consisted of two serially connected polypropylene tubes and an electrical air suction pump, for the measurement of hydrogen peroxide and 8-isoprostane.¹³¹⁻¹³² However, they reported difficulties related to contamination with nasal secretions, and to the collection of only 20 to 30 per cent of the totally exhaled breath volume.¹³¹⁻¹³² Cheah *et alia* described a bedside method for the collection of EBC, based on polyvinylchloride tubing, that was immersed in a column of wet ice, for the measurement of hydrogen peroxide in neonates who were ventilated or receiving nasal continuous positive airway pressure.¹¹⁷ However, they reported insufficient sensitivity to use their system for the detection of exhaled hydrogen peroxide.¹¹⁷ Recently, Moeller *et alia* proposed, a feasible and safe method to collect EBC, both orally and nasally, in tidally breathing infants.¹³³ However, this method required sedated sleep, and was described without the assessment of any marker in the collected condensate.¹³³ Last but not least, other techniques for the collection of EBC from ventilated patients, including infants, children and young adults, have recently been proposed.¹³⁴⁻¹³⁵

In our study, successful EBC collection was associated with an optimal distraction of the preschool children. We primarily combined strict parental cooperation (86 per cent), after preceding instructions by the coaching childhood asthma nurse, with an age appropriate cartoon (76 per cent), that was already started prior to entrance. In case of imminent failure to cope, a more active strategy was used on parent's advice, including children's stories (36 per cent), puppetry (15 per cent), and/or children's songs (12 per cent). Distraction was mainly hampered by insufficient parental stimulation and/or a history of ear-nose-throat surgery.

In literature, a variety of different distractors have been used for non-pharmacological pain or distress management in children of all ages, including watching cartoons,¹³⁶⁻¹³⁸ using party blowers,¹³⁹ looking through kaleidoscopes,¹⁴⁰ blowing bubbles,¹⁴¹⁻¹⁴² non-procedural talk,¹⁴³ listening to short stories,¹⁴⁴ humour,¹⁴⁵ listening to music,¹⁴⁶ puppetry,¹⁴⁷⁻¹⁴⁸ and virtual reality glasses.¹⁴⁹⁻¹⁵⁰ The management of a child's distress does not equal pain management. However, the latter may provide some hints to improve the child's coping with distress due to a noninvasive and

painless but unfamiliar procedure, such as the collection of EBC, in particular in preschool children. In literature, distraction performed by mothers has been reported to be an effective analgesic factor.¹⁴³⁻¹⁴⁴ Moreover, children state that just having their parents present provides the most comfort when in pain, and they will recall that they were not left alone on a stressful occasion.^{136,151} Cohen *et alia* found that 4 to 6 year old children watching a popular cartoon series felt less pain, whereas Cassidy *et alia* found that watching television during an immunisation injection was not effective in reducing pain in a group of 5 year old children.¹³⁷⁻¹³⁸ Mason *et alia* suggested that a passive strategy, such as watching television, may be more effective than an active one, such as distraction with an interactive toy, for decreasing pain, because children's distress interfered with their ability to interact with the distractor.¹⁴⁴

Hence, we introduced a novel, highly efficient, closed concept that enabled the collection of EBC, both before and after breath recirculation, and yielded sufficient as well as equivalent condensate in the 'diagnostically-nearly-unaddressed' population of (nonsedated) preschool children with or without wheezing illness.

9.6. New sensitive techniques for the detection and measurement of biomarkers in EBC

In this section we will discuss our findings, mainly related to the analysis of EBC to measure cytokines, and free radicals (as markers of oxidative stress), using new sensitive techniques as applied in chapters 5 and 8, and chapter 6, respectively.

9.6.1. Cytokines in EBC

9.6.1.1. Why and how we tried to detect cytokines in EBC

Cytokines are intercellular immune regulatory peptides involved in the orchestration of many different aspects of local and/or systemic inflammatory processes, including tissue repair, and thus, cytokines are associated with various chronic respiratory disorders, as indicated in chapter 1. This also implicates that these mediators constitute interesting research targets as the changing level of different cytokines, or in other words specific profiles of cytokines, may reflect parallel changes at certain stages in specific types of chronic respiratory inflammation. Furthermore, cytokines are low molecular weight proteins (ranging from 4.000 to 50.000 Dalton), that may be able to shift or migrate from the intrapulmonary compartment into the airway lumen, and, thus, may be exhaled, as suggested by two incidental reports, at that time, on their detection in EBC in children.^{25,71} Cunningham *et alia* reported the detection of the pro-inflammatory interleukin-8 in EBC in 30 per cent of children with clinically stable cystic fibrosis.⁷¹ Shahid *et alia* reported significantly increased mean levels of interleukin-4 and decreased mean levels of interferon-gamma in asthmatic children, compared with healthy controls, whereas lower levels of interleukin-4 were found under corticosteroid treatment.²⁵ Hence, we were primarily interested in the detection of these small proteins in EBC in children, but at the same time, we were also very aware of the difficulties that we were facing, as our review (presented in chapter 2) had also made clear that EBC methodology was significantly hampered by the lack of specific and highly sensitive analytical techniques. In the early childhood asthma study, presented in chapter 3,

it was shown that conventional and even ultrasensitive specific solid-phase enzyme immunoassays were not able to sufficiently detect cytokines in EBC (chapter 9.4 above). Subsequently, a new technique, multiplexed cytometric bead array, was used in the early cystic fibrosis study, presented in chapter 7, and allowed the simultaneous measurement of several cytokines in only small condensate samples (chapter 9.4 above). However, the level of cytokine detection still did not reach 50 per cent, as already discussed.

Therefore, we were unremittingly pursuing amelioration. Moreover, all the above may only illustrate the very importance of rapidly transferring novel developments of 'nano-molecular' analytical technology, into the field of condensate-related research.

9.6.1.2. Multiplexed liquid bead array and the measurement of cytokines in EBC

In chapter 5, we not only investigated the *in vivo* reproducibility of different biomarkers in EBC using four different condenser types (including the new open glass condenser), but we also introduced a novel technique to, both more rapidly and precisely, detect and measure several cytokines simultaneously. Luminex's xMAP technology is a liquid bead array multiplexing system, built on proven, existing technology, including traditional chemistry, flow cytometry, microspheres (characterised by liquid reaction kinetics that are faster and more reproducible, than with solid planar assays), laser technology and digital signal processing, that are combined in a unique way and allow multiplexing of up to 100 unique assays within a single sample.¹⁵²⁻¹⁵³

When compared with conventional bioassays and solid-phase enzyme immunoassays, multiplexed immunoassays are highly sensitive (a few pg/mL), allow quantitative detection (in contrast to semiquantitative bioassays), have high specificity, detect bioactive as well as inactive molecules, have a growing analytical range (cytokines, phosphoproteins, antibodies), are rapid (hours instead of day(s)), have good (but not yet excellent) precision (coefficients of variation range from 10 to 15 per cent), are not interfered by drugs, have a simple protocol, require small sample volumes, allow simultaneous detection, and can be applied for multiple species (not only human).¹⁵³ However, the initial set-up time is consuming and expensive.¹⁵³

Cytokines were simultaneously measured by means of xMAP's technology in small EBC sample volumes of 50 μ L. All cytokines (*id est* interleukin-2, interleukin-4, interleukin-5, interleukin-6, interleukin-8, interleukin-10, interleukin-13, and tumor necrosis factor-alpha) were now detectable in EBC, compared to our early studies based on cytometric bead array with 1 to 4 not detectable cytokines in EBC.⁵¹ The overall level of cytokine detection using multiplexed liquid bead array was 46 to 97 per cent, whereas the detection level using cytometric bead array was less than 50 per cent in similarly small EBC samples in children,⁵¹ and 3 to 100 per cent in large 1000 to 2000 μ L lyophilised EBC samples in adults.^{56-58,60}

In EBC collected with the new (open) glass condenser, significantly more detections of interleukin-2, interleukin-4, interleukin-5, and interleukin-13, and tumor necrosis

factor-alpha were found, compared to the other condenser types. However, cytokine concentrations in EBC in healthy adults, and reproducibility of these cytokine measurements expressed as coefficients of variation (ranging from 5 to 78 per cent), did not significantly differ and were equally variable for all types of condenser (as previously discussed).

In chapter 8, the same multiplexed liquid bead array technique was applied for the measurement of cytokines in 50 μ L of EBC collected with the new closed glass condenser, before and after breath recirculation, in preschool children with and without wheezing illness. Also in this study, all cytokines (*id est* interleukin-4, interleukin-5, interleukin-6, interleukin-8, interleukin-10, interleukin 12p70, interleukin-13, and tumor necrosis factor-alpha) were detectable in EBC, and the level of cytokine detection in EBC ranged from 95 to 100 per cent.

Hence, we believe that optimisation of EBC collection, using the new glass condenser(s), and optimisation of EBC analysis, using rapid multiplexed xMAP measurement of cytokines in small EBC sample sizes, may all together open a window of opportunities, even in strained collection procedures, such as in young or dyspnoeic subjects (with less sustained efforts to cope with sampling procedures), by allowing the search for, and identification of, particular profiles of different exhaled markers, involved in the regulation of chronic respiratory inflammation, for diagnostic and monitoring purposes.

On one hand, we acknowledge the fact that the introduction of novel analytical technique may be considered as a major confounding factor, as this technique still awaits further investigation of the reproducibility, sensibility, specificity and validity of its generated data on cytokines in EBC. For this purpose, the Standards for Reporting of Diagnostic Accuracy (STARD) initiative provides recommendations, including a checklist and flow diagram, towards complete and accurate reporting on diagnostic accuracy.¹⁵⁴⁻¹⁵⁶ On the other hand, we ultimately do not see an alternative approach other than the one proposed, to gradually introduce such novelties, because the first step, in our opinion, will always consist of establishing an accurate and rapid analytical technique, prior to any application in longitudinal clinical trials.

9.6.2. Free radicals in EBC

Oxidative stress, defined as an imbalance between oxidants (such as reactive oxygen species) and antioxidants (such as vitamin C) in favour of oxidants, also appears to play an important role in several inflammatory lung diseases.^{107,157} Hence, in our search for the application of new analytical techniques in EBC, we were also interested in the assessment of free radicals, that are considered the most direct markers of oxidative stress.^{107,157}

In the study, as presented in chapter 6, we used the most direct method to assess oxidative stress status *in vivo* in children with cystic fibrosis and healthy control children. This study did demonstrate the feasibility of electron paramagnetic resonance spectroscopy to directly and semi-quantitatively detect free radicals, *in casu* both

oxygen- and carbon-centered radicals, in 500 μ L of EBC. We found a trend towards higher maximal peak heights of oxygen- as well as carbon-centered radicals in EBC in children with cystic fibrosis, compared to healthy controls. However, this difference did not reach statistical significance, and may be attributed to the small study population, and/or to the fact that also a normal lung exists in an oxygen-rich environment that makes this lung also susceptible to oxidative stress.

On the other hand, we found that this direct and nearly online technique required quite sophisticated equipment and personnel, which makes it not readily available for practical use in a clinical setting.

We also examined the possible source of these free radicals. The findings of this study suggested that hydrogen peroxide is likely crucial for the generation of oxygen centered radicals, and that EBC may also contain compounds that enable *in situ* radical formation. Furthermore, free radicals do not likely result from salivary contamination, and thus, may originate from the lower respiratory tract. By consequence, this may provide further support that the content of EBC reflects the ongoing processes in the epithelial lining fluid.

Recently, Carraro *et alia* reported the application of metabolomics in the analysis of EBC, in asthmatic children.¹⁵⁸ Metabolomic analysis, by means of high resolution ¹H nuclear magnetic resonance spectroscopy and multivariate statistical methods, provides biochemical profiles of low molecular weight proton-containing endogenous metabolites in biological fluids, without requiring any preselection of measurable analytes, and allows both the detection of known metabolites as well as the prediction of unknown metabolites and novel biomarkers.¹⁵⁸ Discrimination between asthmatic children and healthy controls, using the combination of exhaled nitric oxide and forced expiratory volume in one second had a success rate of 81 per cent, whereas selected nuclear magnetic resonance signals, corresponding to acetylated and to oxidized compounds, had a success rate of 86 per cent.¹⁵⁸

9.6.3. The measurement of other inflammatory markers in EBC

Although we were primarily focused on the measurement of cytokines and free radicals in EBC, from a condensate analytical point of view, we concomitantly measured other inflammatory markers in EBC, including hydrogen peroxide, nitrite, nitrate, condensate acidity, and 8-isoprostane. However, due to the increasing complexity of our own research efforts, and due to the fact that several other research groups have addressed or still investigate the methodology of these specific markers in EBC, we chose to incorporate the recommendations and workshop proceedings published by the American Thoracic Society & European Respiratory Society Task Force on EBC,^{48-49,159} or to take into account, if possible, new and relevant methodological findings, as listed below.

9.6.3.1. The measurement of hydrogen peroxide in EBC

Superoxide anions ($O_2^{\cdot-}$) are rapidly reduced to hydrogen peroxide (H_2O_2) by superoxide dismutase in several cell types.^{48,107} In the respiratory system, hydrogen peroxide may be

released both from inflammatory and structural cells, including neutrophils, eosinophils, macrophages and epithelial cells.⁴⁸ The most frequently used methods of measuring hydrogen peroxide in EBC are the colorimetric and fluorimetric assays, that are based on the ability of hydrogen peroxide to react with suitable substrates.⁴⁸ Hydrogen peroxide is assayed by colorimetry using horseradish peroxidase-catalysed oxidation of tetramethylbenzidine, as described by Gallati and Pracht.^{107,160} Using the colorimetric method, most groups have reported 0.1 μM as detection limit.⁴⁸ The fluorimetric technique, described by Ruch *et alia*, uses the reaction substrates 3,3',5,5'-tetramethylbenzidine, p-hydroxyphenylacetic acid, scopoletin, phenolsulphonphthalein, luminol 5-amino-1,2,3,4-tetrahydrophthalazine-1,4-dione or homovanillic acid.^{107,161} Using the fluorimetric method, the lower limit of detection is 0.1 μM , although in one study this was lowered to 0.0005 μM using automated flow injection to detect the fluorescence of the reaction product.^{48,113} Recently, an optimisation of this technique by increasing the buffering capacity of the reaction mix, has been published.¹⁰⁵ Hydrogen peroxide, being highly volatile, evaporates rapidly and therefore, could be trapped by adding the substrates to the EBC immediately after sample collection and kept frozen until analysis.¹⁰⁷ New techniques, such as online or real-time measurements using sensitive biosensors have been developed, although the imprecision in the low range is still high (28 per cent), but declines with higher concentrations of hydrogen peroxide.^{104,107}

Data on hydrogen peroxide vary considerably, and most of them are even close to the lower limit of detection.⁴⁸ Therefore, concern has been raised regarding the reproducibility of the colorimetric assay, and the use of more sensitive methods is advised.^{48,119-123} Furthermore, as already discussed in chapter 9.5, the levels of the highly volatile hydrogen peroxide in EBC are susceptible to (yet) uncleared methodological issues that may be attributed to a possible circadian rhythm,¹¹⁰ flow dependency,¹¹⁵ rapid chemical reactivity,^{119,125-127} and susceptibility to different cooling temperatures.¹⁰⁹ Day-to-day intra-subject coefficient of variation was 43 per cent in healthy subjects,⁴⁸ whereas we reported, in chapter 5, overall coefficients of variation ranging from 22 to 29 per cent.

9.6.3.2. The measurement of nitrogen oxides and related products in EBC

Nitrite (NO_2^-) and nitrate (NO_3^-) are nitrogen redox forms that are present in the epithelial lining fluid of the human respiratory tract.^{48,107} The term nitrogen oxides (NOx) incorporates these two ions, as well as other oxides of nitrogen.^{48,107}

Nitrite and nitrate have been detected in EBC by using spectrophotometric assays (Griess reaction), a fluorimetric method (2,3-diaminonaphthalene (DAN) reaction), chemiluminescence assays, or ion chromatography followed by conductivity measurement.^{48,71-72,162-174} The reported detection limit of the DAN assay is 0.1 μM , and that of the Griess reaction is higher.⁴⁸ The chemiluminescence technique is sensitive in the nanoM range.⁴⁸ Caution must be taken when interpreting nitrite assays, as the compound cannot be considered stable at low pH, and even at neutral pH.⁴⁸ Nitrate is considered stable, whereas its levels tend to be roughly 5 to 10 fold

higher than nitrite levels, although this may vary in part because of changes in airway oxidative conditions.⁴⁸ Most combined nitrite/nitrate assays rely on the formation of nitrite from nitrate.⁴⁸ In this respect, enzymatic reduction was found to be better for nitrate determination in EBC, than cadmium reduction.^{48,166}

Nitrogen oxides are present on every laboratory surface, including glassware and pipette tips.⁴⁸ Readily diffusible ambient nitric oxide becomes oxidised and contaminates surfaces rapidly.⁴⁸ Therefore, great precautions should be taken to avoid sample contamination.⁴⁸ It is necessary to thoroughly rinse with highly pure water, any material that might come in contact with EBC, including devices used for collection, processing and assaying EBC.⁴⁸

The reaction of nitric oxide and superoxide anions in the airways leads to the formation of peroxynitrite (ONOO⁻), which is a highly reactive oxidant species.^{48,107} Peroxynitrite reacts with tyrosine residues of proteins to form the stable product nitrotyrosine, that may be detected with specific antibodies, using a specific enzyme immunoassay with a detection limit of 3.9 ng/mL.^{48,175} However, commercial enzyme linked immunosorbent assays presumably quantify proteins nitrated at more than one site, rather than single amino acids.⁴⁸ Likely this means that nitrotyrosine levels mainly consist of nitrated proteins.⁴⁸

Nitrosothiols (RS-NOs) are formed by interaction of nitric oxide or nitrite with glutathione or other thiol containing molecules.^{48,107,176-177} Nitrosothiols can be measured by colorimetric assay, based on the cleavage reaction of nitrosothiols releasing nitric oxide, which is then oxidised to nitrite and detected by the assay.⁴⁸ The detection limit is 0.025 μ M.⁴⁸ A more sensitive method is chemiluminescence analysis after reduction to nitric oxide in copper-cysteine or by ultraviolet light.⁴⁸

There are no reported data on day-to-day intra-subject coefficient of variation of nitrogen oxides.⁴⁸

9.6.3.3. The measurement of condensate acidity (pH)

Airway pH homeostasis is maintained by a balance of different buffer systems and the production and release of acids and bases in the airways.⁴⁸ The acidity of EBC can be readily measured with pH electrodes and indicator dyes.^{31,39,48,178} Gas standardisation (with a carbondioxide-free gas, such as argon and nitrogen) is recommended to stabilise pH of EBC, as already discussed in chapter 9.4.^{42,44,48} There is a debate as to whether orally collected EBC pH reflects acidification of the lower airways, because of high ammonia content of the mouth.^{35-37,40,47-48,179-180} The pH of deaerated EBC is not affected by hyperventilation, duration of collection, duration or manner of storage (up to two years), and oral *versus* endotracheal collection.^{38,48,178} Recently, normative data for deaerated EBC pH in all ages have been published,⁴³ as well as in a prospective population-based birth cohort of 630 children.⁴⁵ Reported day-to-day intra-subject coefficient of variation of deaerated EBC pH is 4.5 per cent, and 5 per cent using non deaerated EBC pH.^{41,48,178}

9.6.3.4. The measurement of 8-isoprostane in EBC

8-Isoprostane, a stable prostaglandin-like product, is formed from arachidonic acid by the nonenzymatic action of reactive oxygen species, and, therefore, it is suggested to be a proportional marker of oxidative stress.^{48,107} Commercial enzyme immunoassays are used to measure 8-isoprostane in EBC, with a detection limit of 3.9 pg/mL.^{48,107} The assay was validated directly by gas chromatography / mass spectroscopy showing high correlation between added known amounts of 8-isoprostane and the concentration measured with the assay.^{48,128} However, reproducibility remains contradictory.^{48,107} Enzyme immunoassays may give false values owing to the presence of closely related substances in biological fluids that may potentially interfere with the assay.¹⁰⁷ The potential disadvantage of measuring isoprostanes is that they are unstable at room temperature or on ice, and rapidly degrade *ex vivo*.¹⁰⁷ Agents such as lipid peroxidation inhibitors and metal chelators may be added prior to freezing to stabilise (F₂-)isoprostanes.¹⁰⁷ A more sensitive and specific method to assay isoprostane in EBC is gas chromatography / mass spectroscopy.⁴⁸ Recently, Gonzalez-Reche *et alia* presented a validated method for the simultaneous determination of arachidonic acid metabolites in 1000µL of EBC by liquid chromatography-electrospray ionisation tandem mass spectrometry, as already discussed in chapter 9.5.¹⁰⁶ There are no reported data on day-to-day intra-subject coefficient of variation whereas we reported, in chapter 5, overall coefficients of variation ranging from 26 to 48 per cent in healthy adults.⁴⁸

9.6.3.5. The measurement of prostaglandines and thromboxanes in EBC

Arachidonic acid, released from the cell wall by phospholipase A₂, is converted to prostaglandin endoperoxides by cyclo-oxygenase.⁴⁸ Endoperoxides are then converted to prostaglandins, prostacyclin and thromboxane A₂.^{48,181} Thromboxane A₂ is rapidly converted to thromboxane B₂, a chemically stable but biologically inactive metabolite, that can be measured.⁴⁸

Prostaglandin E₂ and thromboxane B₂ can be measured by enzyme immunoassay and by radio immunoassay.^{48,128,182-185} The specificity of prostaglandin E₂ measurement by radio immunoassay has been validated by reverse phase high performance liquid chromatography.^{48,128} The detection limit for thromboxane B₂ is 13 pg/mL for the enzyme immunoassay, and 13.6 pg/mL for the radio immunoassay.⁴⁸ Higher thromboxane B₂ values were found by radio immunoassay, which is likely attributable to the 100 per cent crossreactivity of this assay (but not the enzyme immunoassay) with 2,3-dinor-thromboxane B₂.⁴⁸ The detection limit for prostaglandin E₂ is 8 pg/mL using the enzyme immunoassay.⁴⁸ There are no reported data on day-to-day intra-subject coefficient of variation.⁴⁸

9.6.3.6. The measurement of leukotrienes in EBC

The cysteinyl-leukotrienes (leukotriene C₄, leukotriene D₄, and leukotriene E₄) are released from inflammatory cells of the airways, in particular mast cells and eosinophils, and play a role in asthmatic airway inflammation.^{48,186} Leukotriene B₄ is formed from arachidonic acid as a result of enzymatic hydrolysis of leukotriene A₄, a potent activator of neutrophils and a proinflammatory mediator.^{48,187} Leukotrienes can be measured by enzyme immunoassay with detection limit of 15 pg/mL and 4.4 pg/mL,

respectively.⁴⁸ The specificity of the immunoreactivity of the leukotriene B4 enzyme immunoassay was confirmed by using reverse phase high performance liquid chromatography.^{48,188} The correlation coefficient for two repeated leukotriene B4 measurements was 0.76.^{48,188} Reported day-to-day intra-subject coefficient of variation for leukotriene B4 is 2 per cent in a small group of healthy subjects.^{48,189}

9.6.3.7. The measurement of other markers of oxidative stress in EBC

Aldehydes (malondialdehyde, 4-hydroxyhexanal, 4-hydroxynonenal, hexanal, heptanal and nonanal) are lipid peroxides which reflect oxidant-induced damage.^{48,107,190} Conversely, reduced glutathione reflects the antioxidant capacity.^{48,191} Aldehydes were detected in EBC using liquid chromatography-tandem mass spectroscopy which had a detection limit for different aldehydes between 0.31 and 1.07 nM.^{48,190,192} Glutathione was determined by high performance liquid chromatography with fluorescence detection, with a detection limit of 2.0 nM.^{48,193} Reported day-to-day intra-subject coefficients of variation for different aldehydes are between 12 and 20 per cent.^{48,193} There are no reported data on day-to-day intra-subject coefficients of variation on EBC glutathione. When interpreting the results obtained for markers of oxidative stress, it should be borne in mind that malondialdehyde is not a specific product of lipid peroxidation.¹⁰⁷ Although the levels of malondialdehyde and isoprostanes correlate well *in vitro*, there is 25,000 times more malondialdehyde as compared to isoprostanes, and the *in vivo* levels of isoprostanes are 28-fold higher than those of malondialdehyde.¹⁰⁷ The level of oxidative biomarkers in EBC can be affected by diet, smoking, diurnal variation and age.¹⁰⁷

9.6.3.8. The measurement of adenosine in EBC

Adenosine is formed during the degradation of adenosine triphosphate (ATP) and has a wide range of effects in the respiratory system through its specific receptors.^{48,194} Adenosine can be determined by high performance liquid chromatography, and has been validated for EBC.^{48,195,196} The detection limit is 2 nmol/L.⁴⁸ There are no reported data on day-to-day intra-subject coefficient of variation, whereas mean intra-day coefficient of variation is 10 per cent in healthy subjects.^{48,196}

9.6.3.9. The measurement of ammonia in EBC

Ammonia (NH₄⁺) produced during the urease hydrolysis and/or glutaminase activity in the airways may have important functions, including pH regulation.^{29,48} Ammonia is a volatile compound, and collection temperature and duration influences its level in EBC.⁴⁸ Ammonia can be readily measured spectrophotometrically in EBC.^{29,48} Most of EBC ammonia arises from the mouth.^{5,48,182,197} Mean intra-day coefficient of variation is 60 per cent, with a broad range from 10 to 180 per cent.⁴⁸

9.6.3.10. The measurement of other molecules in EBC

EBC contains other molecules that can provide important information, such as p53 mutation,¹⁹⁸⁻¹⁹⁹ deoxyribonucleic acid,^{4,200} hepatocyte growth factor,²⁰¹ erythropoietin,⁶⁰ vitronectin, and endothelin-1.²⁰² EBC collection may also be useful for rapid detection of infectious processes in the lung using polymerase chain reaction technology, such as tuberculosis.^{48,200,203}

Table 1. State of the art on biomarkers in EBC, including condensate pH, in children with asthma or cystic fibrosis.

Biomarker in EBC	Asthma	CF	Reference
Hydrogen peroxide	↑	↑	61,124,209
8-isoprostane	↑	(-)	92,93,95,99,129
Prostaglandin E2	=	(-)	93
Cysteinyl-leukotrienes	= / ↑	(-)	50§,92,95,99,129,189,210*-212
Leukotriene B4	= / ↑	↑	26,28,99,189,212,213
Nitrite	= / ↑	↑	46,50§,71,72
Nitrotyrosine	↓ / = / ↑	=	214‡,215,216
Interleukin-4	↑	(-)	24,25
Interleukin-8	(-)	↑	28,71
Interleukin-10	=	=	51
Interferon-gamma	↓ / =	=	25,51
TNF-alpha	=	(-)	24
PDGF, VEGF, EGF	=	(-)	24
MDC	↑	(-)	26,27
Eotaxin	↑	(-)	26
Aldehyde	↑	(-)	193
Glutathione	↓	(-)	193
Metabolomics †	↑	(-)	158
Ammonia	↓	(-)	212,219
Condensate pH	↓ / =	↓	28,45-46,84,217-219

EBC exhaled breath condensate

CF cystic fibrosis

TNF-alpha tumor necrosis factor-alpha

PDGF platelet-derived growth factor

VEGF vascular endothelial growth factor

EGF epidermal growth factor

MDC macrophage-derived chemokine

↑ significantly increased compared to healthy children

↓ significantly decreased compared to healthy children

= no significant difference compared to healthy children

(-) no paediatric data

* levels in asthma decreased if treated with montelukast

§ levels in asthma unchanged compared with asthma exacerbation

† oxidised and acetylated compounds

‡ levels in asthma decreased after treatment with inhaled corticosteroids

9.6.3.11. Dilution factor of EBC

The importance of a dilution factor is still being debated, and based on the assumption that the ratio of liberated solutes to exhaled water vapour is variable.⁴⁸ A so called

dilution factor can be determined from each EBC sample by determining the concentration of a substance in EBC, which has a well known concentration in sera and diffuses through the cell membranes, but is not produced in the alveoli or airways.⁴⁸ Such a dilution marker would make it possible to calculate the real airway level of determined mediators in EBC.⁴⁸ Many attempts have been made.^{5,197,204-207} The most simple of these is the measurement of conductivity of lyophilised EBC samples.²⁰⁶ Recently, this method was used by Baker *et alia* to develop the noninvasive measurement of breath glucose concentrations.²⁰⁸ These were obtained by multiplying condensate glucose concentrations by calculated dilution factors (estimated by dividing serum total cation concentration by condensate total cation concentration).²⁰⁸ They reported an elevated breath-to-blood glucose ratio in adult patients with cystic fibrosis, compared to healthy patients and diabetes mellitus patients.²⁰⁸

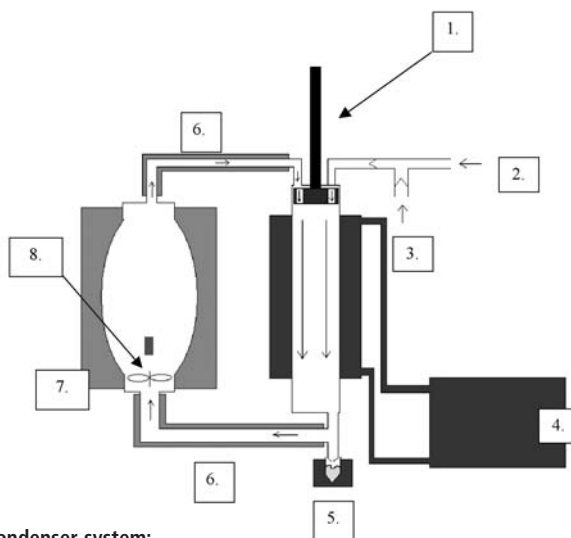
In Table 1, we provide a state of the art on the current measurements of biomarkers in EBC, including condensate pH, in children with asthma and cystic fibrosis.

Finally, we want to emphasize again that EBC constitutes a very different matrix than those employed in 'conventional' research and current clinical practice, and that various potential biomarkers (yet) have been detected in this exciting matrix. Furthermore, it may be clear that each of these biomarkers has its own physical, pathophysiological, biochemical, electrical, and/or even quantum-physical characteristics. Thus, this very diversity itself is of vital importance to its potential value in noninvasive inflammometry, but simultaneously, it is the main complicating factor in a smooth and stepwise advancement of research efforts.

9.7. Application of new techniques in preschool children

Finally, in chapter 8, we not only demonstrated the feasibility of a novel method to collect EBC in 70 nonsedated preschool children with and without wheezing illness (Figure 2). Also, we assessed EBC cytokine profiles, by multiplexed liquid bead array, in probably atopic asthmatic and nonasthmatic preschool children, and found significantly discriminating T-helper 2 to T-helper 1 cytokine ratios in EBC.

In children, wheezing is reported in no less than 30 per cent during the first three years of life.⁹ In preschool children, wheeze is poorly characterised, and may indicate a trivial viral cold, as well as an ongoing chronic lung inflammation.^{9,220} On one hand, exclusively viral-triggered wheeze accounts for about two-third of wheezy children, and is characterised by acute episodes associated with viral (upper) respiratory tract infections.^{9,220} Between episodes, viral wheeze is not associated with eosinophilic airway inflammation, which has important therapeutic implications.⁹ These children have a good prognosis since 60 per cent will stop wheezing by the early school years.²²¹ Transient, viral-triggered wheeze is not related to atopy and allergic conditions, whereas maternal smoking during pregnancy, premature birth and low birth weight are known risk factors.¹¹ On the other hand, atopic asthma is associated with a cytokine pattern compatible with predominant activation of the T-helper 2



Description of the new condenser system:

1. inclined and closed glass condenser with a tube length of 90 cm (to enlarge the condensation surface), and downwards moveable plunger (to optimise condensate recovery from the inner wall of the condenser), with tangential and axial breath channels (to turbulently direct the tidal breath flows towards the cooled inner condenser wall)
2. swan-neck tubing (serving as gravitational saliva trap) and two-way nonbreathing valve, connected to a face mask with separated nose and mouth cavity (8900 pediatric sizes oro-nasal masks, Hans Rudolph Inc., Kansas City, Missouri, USA)
3. entrance of inspired room air
4. cooling unit consisting of a counter-current circulating ice-water pump
5. removable glass sample vial (to collect exhaled breath condensate)
6. heated (at 37°C) tubing (to guide the residual noncondensed exhaled breath, and to avoid condensation outside the condenser tube)
7. humidity sensor and heated (at 37°C) inert Tedlar™ gas sample bag (to collect all residual noncondensed exhaled breath)
8. small ventilator system to recirculate the residual noncondensed exhaled breath back into the condenser system (to allow secondary condensation of exhaled breath)

Description of the new EBC collection procedure:

The child exhaled tidally, as long as possible, through a face mask, with separated nose/mouth cavity, and two-way nonbreathing valve (2), that was connected to the glass condenser system (1). During this condensation process, the formerly lost noncondensed exhaled breath was separately being collected in a gradually inflating inert Tedlar™ gas sample bag (6-7). When the child ceased this procedure, EBC was collected by pushing downwards the plunger (1). Subsequently, the plunger was pulled up again (1), and the separately collected exhaled breath was recirculated (8), from the Tedlar™ gas sample bag (7), back into the condenser system (1), to perform a secondary breath condensation, until the degree of humidity was $\leq 22\%$.

Figure 2. Description of the new glass condenser system and exhaled breath condensate collection procedure. The new system enabled condensate collection in preschool children before and after breath recirculation.

mediated immune response,²²²⁻²²³ and is characterised by chronic interval symptoms, with often an atopic personal or family history.⁹ Moreover, asthma is a heterogeneous disease, and diagnosis is hampered by features of the developing child undergoing (lung)growth, immune maturation and environmental influences.^{12-13,15}

Furthermore, accurate diagnosis is hampered by the current lack of a reliable routine technique to assess different types of respiratory inflammation in preschool children.

Hence, these confounding issues make it hard to classify a wheezing phenotype in preschool children unequivocally.

By consequence, the central issue, in preschool children with different wheezing phenotypes, consists of finding a noninvasive measure to identify and discriminate underlying (persisting) airway disease, such as atopic asthmatic airway inflammation,²²²⁻²²³ from nonasthmatic states, such as recurrent viral wheeze and healthy children.^{9,15} Accordingly, statistical analysis revealed several significant differences between probably atopic asthmatic and nonasthmatic preschool children: tumor necrosis factor-alpha concentrations in EBC were significantly decreased, whereas the ratios of interleukin-5 to tumor necrosis factor-alpha and interleukin-13 to tumor necrosis factor-alpha in EBC were significantly increased in the probably atopic asthmatic group, compared to nonasthmatics. This is in accordance with the results of Shahid *et alia*, who reported an increased ratio of interleukin-4 to interferon-gamma in EBC in asthmatic children, aged 4.5 to 17 years.²⁵ These findings may provide evidence for early skewing of the T-helper 2 to T-helper 1 balance in favour of an allergy-promoting T-helper 2 response.²²²⁻²²³

Although an accurate and firm asthma diagnosis cannot be proven in this young paediatric age group, a reasonable accuracy can be obtained using simple, clinically based parameters, as demonstrated by Castro-Rodriguez *et alia*.²²⁴ They developed two clinical indices for the prediction of asthma, based on the Tucson Children's Respiratory Study.²²⁴ A stringent index included frequent wheezing during the first three years of life, and either one major risk factor (parental history of asthma or eczema), or two out of three minor risk factors (eosinophilia more than 4 per cent, wheezing without colds, and allergic rhinitis).²²⁴ A loose index required any wheezing during the first 3 years of life, plus the same combination of risk factors described previously.²²⁴ Children with a positive loose index were 2.6 to 5.5 times more likely to have active asthma between ages 6 and 13, than children with a negative loose index. Risk of having subsequent asthma increased to 4.3 to 9.8 times, when a stringent index was used.²²⁴ Furthermore, they reported that 59 per cent of children with a positive loose index, and 76 per cent of those with a positive stringent index had active asthma in at least one survey during the school years.²²⁴ Over 95 per cent of children with a negative stringent index never had active asthma between ages 6 and 13.²²⁴

Therefore, the phenotypic classification of probably atopic asthmatic preschool children, in our study, was carefully assessed on basis of parentally reported symptoms

including recurrent wheezing that was not 'exclusively' viral triggered, a documented positive response on (inhaled) corticosteroids, and medical records and information of the responsible paediatric pulmonologist on the presence of allergic sensitisation in these children, and a positive family history of atopy in the first degree. Hence, it is likely that these children had 'true' allergic asthma. Furthermore, support for an accurate asthma diagnosis comes from the significantly elevated T-helper 2 to T-helper 1 ratios in the probably atopic asthmatic group, compared to the nonasthmatic children in this study.

In conclusion, we demonstrated the feasibility of a novel method to collect EBC in preschool children, and subsequently assessed significantly elevated ratios of T-helper 2 to T-helper 1 cytokines in EBC in probably atopic asthmatic preschool children, compared to nonasthmatic preschool children.

9.8. What do we add ?

In this thesis, we described the development of EBC into a feasible and highly efficient, noninvasive method to assess, in children of all ages, various biomarkers of inflammation and oxidative stress. EBC is a bi-phasic technique implicating the cooling of exhaled breath to collect the condensate, and subsequently, the analysis of the collected condensate.

The first part of this thesis focused on EBC collection. Initially, condensate was collected using a wide diversity of condenser designs (as reviewed), including a borosilicate glass distilling column, as used in two - early - cross sectional studies in children, aged 5 years or more, with asthma and cystic fibrosis, respectively. Subsequently, we demonstrated that a borosilicate glass condenser was more efficient to measure 8-isoprostane and albumin in EBC, compared to five other condenser types. We mainly attributed this significant difference to distinct adhesive interactions between the inner condenser coatings and the exhaled biomarkers. Accordingly, glass was used as basic material to further optimise the condensation process. Hence, we presented an open glass condenser system with enlarged condenser surface, improved condensate recovery using an inclined condenser tube setting and a condensate sweeping plunger, with several tangential and axial breath flow channels to guide exhaled breath towards the cooled inner condenser wall (as shown in Figure 1). We demonstrated, in healthy adults, that the open glass condenser not only yielded significantly more EBC volume, but was also associated with more detections of hydrogen peroxide, 8-isoprostane, and several cytokines in the collected EBC, than other condenser types. Succeedingly, the condenser system was modified for use in preschool children. For this purpose, the glass condenser was closed with a unique, heated (at 37 degrees Celsius), breath recirculation system, in such a way that the formerly lost noncondensed exhaled breath was separately being collected in a gradually inflating inert Tedlar™ gas sample bag (as shown in Figure 2). When the child ceased the 'usual' EBC collection procedure, the separately collected exhaled breath was recirculated, from the gas sample bag, back into the condenser system, to

perform a secondary breath condensation. This new concept of EBC collection before and after breath recirculation, allowed condensation of the totally exhaled breath volume, whether gathered after only a few minutes of EBC collection, or after a prolonged period. Moreover, this concept enabled the collection of significantly more EBC volume compared to the 'usual' (before breath recirculation) procedure, whereas in toddlers with limited cooperation in time, breath recirculation was often crucial to yield minimal but sufficient condensate volume (to analyse for biomarkers). Cumulatively, the success rate was 83 per cent in preschool children with and without wheezing illness. Furthermore, we demonstrated that all cytokine concentrations in EBC, collected before and after breath recirculation, were comparable, which illustrated the efficiency of the new method of EBC collection in preschool children.

The second part of this thesis focused on EBC analysis. Initially, an increasing number of biomarkers were detected (or not) in EBC, using a wide diversity of conventional assays (as reviewed). Incidental reports on detected biomarkers included hydrogen peroxide, nitrogen oxides, eicosanoids, (three) cytokines, and aldehyde/gluthatione (as reviewed). EBC consists of respiratory droplets with volatile and (mainly) nonvolatile macromolecules that are released from the epithelial lining fluid of the airways, and than 'drowned' in an excess of water vapour. Moreover, many different condenser designs were in use, and most conventional assays were not appropriate for use in this new medium as they were lacking sufficient sensitivity. Not surprisingly, biomarker reproducibility was often poor, and sometimes even highly questionable. Several research groups addressed these issues for specific biomarker measurements mainly by introducing analytical modifications, and/or by exploring in various study populations. Meanwhile, we demonstrated a significant interaction between the method of EBC collection and its subsequent analysis, and found glass condensers more efficient (as stated above). Also, we focused on the measurement of cytokines, as potentially highly interesting markers of airway inflammation in EBC, and on free radicals, as 'almost' direct markers of oxidative stress. On one hand, we tried to detect cytokines in large EBC samples by means of conventional specific assays, however without successful cytokine detection. Subsequently, we used multiplexed cytometric bead array to measure simultaneously different cytokines in small EBC samples, and reached a level of detection of nearly 50 per cent, in five out of six assayed cytokines. Succeedingly, we were able to increase the cytokine detection level at 95 to 100 per cent, in all (eight) assayed cytokines, by introducing multiplexed liquid bead array. On the other hand, we were also interested in markers of oxidative stress in EBC. We demonstrated the presence of free radicals in EBC by means of electron paramagnetic resonance spectroscopy, although this procedure is not likely suitable for future routine use. However, the presence of these free radicals in EBC may provide some further evidence for its reflection of the airway lining fluid.

The final part of this thesis focused at the clinical application of EBC. The cross sectional childhood asthma study showed low correlations between single exhaled inflammatory markers and conventional asthma measures. In children with cystic fibrosis, other exhaled inflammatory markers, including exhaled nitric oxide, were

differently combined into several specific profiles, and were able to indicate diagnosis, exacerbation, and (to a lesser extent) disease severity, though confirmation in a longitudinal context is needed. In preschool children with and without different wheezing phenotypes, the new closed glass condenser with breath recirculation system, and multiplexed liquid bead array were used to assess T-helper 1 and T-helper 2 cytokine profiles in EBC. Hence, we demonstrated – *noninvasively* – significantly elevated T-helper 2 to T-helper 1 cytokine ratios in probably atopic asthmatic preschool children, compared to nonasthmatic children.

In summary, the noninvasive methodology of EBC collection and analysis has developed into a feasible and highly efficient method, that enabled *noninvasive inflammometric profiling* in nonsedated preschool children with different wheezing phenotypes. We believe that *noninvasive inflammometric profiling* will contribute to an improvement of diagnosis and customised care in children of all ages with chronic respiratory disease, whereas this inevitably will generate new insights that may ultimately lead to alter some of the previously agreed disease management strategies.

9.9. What may or should be ?

Obviously, the need for a standardised EBC methodology is incontestable.^{48-49,159} Although substantial progress has been made by improving and adapting the condenser system, as well as by introducing new ultrasensitive analytical techniques, some issues still remain unresolved and await proper investigation, as listed below. More than ever, the joint American Thoracic Society & European Respiratory Society Task Force on EBC should diversify research efforts according to these issues, and coordinate multicentre research groups respectively.

- (i) the origin and physiology of EBC
- (ii) biomarker-tailored EBC collection method(s) and procedure(s)
- (iii) subject-tailored EBC collection method(s) and procedure(s)
- (iv) international consensus for methodological standardisation
- (v) identification of biomarker profiles in different respiratory disorders
- (vi) application in longitudinal noninvasive inflammometric clinical trials
- (vii) validation of biomarker profiles through correlation with conventional direct measurements of inflammation, pulmonary function tests, and symptoms

(i) The anatomical origin and (patho)physiology of EBC remains to date largely unknown. Increasing insights in the mechanisms regulating the formation of EBC, including the exact anatomical sites of EBC particle formation, the influence of temperature, exhalation flow, salivary contamination,²²⁵ and respiratory physiology itself,²²⁶⁻²²⁸ as well as the so-called 'dilutional' effects of water vapour on the content of EBC, including a possible marker of solubility,^{5,197,208,229-230} may contribute to an improvement of the EBC collection procedure resulting in adequate sample acquisition, and may clarify whether measurements should be standardised according to dilution, and/or expired breath volume, or not. Hence, these understandings may contribute to improved reproducibility.

(ii) A biomarker-tailored EBC collection method and procedure implicates, at least, that for each type of marker the most valid condenser system is used, as well as the implementation of 'nano-molecular' analytical techniques that offer the best sensitivity and reproducibility. We advise the use of glass condensers to measure cytokines and 8-isoprostane in EBC. Furthermore, we advise the use of multiplexed liquid bead array to measure cytokines in EBC. However, it is not yet possible to give such recommendations for the measurement of other markers, as the possible influence of condenser coating and analytical technique are yet unknown.

(iii) A subject-tailored EBC collection method and procedure should be further modified for use in all ages, including newborns, as well as for use in ventilated subjects, including prematurely born infants prone to bronchopulmonary dysplasia.

(iv) International consensus for methodological standardisation of a particular EBC methodology and procedure for a given type of biomarker may be achieved through integration of both biomarker-tailored and subject-tailored EBC methodology.

(v) Identification of potentially specific and useful profiles of noninvasive biomarkers, measured in EBC and/or exhaled air, in different respiratory diseases. The combination of several noninvasive markers may provide additional information, as shown in the cross sectional cystic fibrosis study. *Exempli gratia*, Battaglia *et alia* reported the association of FeNO and 8-isoprostane in EBC, with small airways function assessed by the single breath nitrogen test, in steroid-naïve mild adult asthma, suggesting that these combined markers reflect small airway inflammation, and favouring for them a complementary role to spirometry in the monitoring of asthma.²³¹

Also, this disease profile identification step may include a more general approach using factor analysis, which can help to reduce a large number of disease features, *in casu* biomarkers in EBC and other conventional or new disease measures, into a relatively small number of independent factors, and thus, grouping associated or correlated features.²⁶ Factor analysis is essentially free of a predetermined hypothesis on any interrelated features (or biomarkers), and thus, factor analysis can be viewed as a hypothesis generating tool.²⁶

Subsequently, cluster analysis may be used to detect clusters among a sample of individuals.^{9,232} Cluster analysis encompasses a number of different algorithms and methods for grouping objects of similar kind into respective categories.²³² A general question facing many researchers, is how to organise observed data into meaningful structures. Cluster analysis is an exploratory data analysis tool which aims at sorting different objects into groups in a way that the degree of association between two objects is maximal if they belong to the same group and minimal otherwise.²³² Given the above, cluster analysis can be used to discover structures in data without providing an explanation or interpretation.²³²

We deal with clustering in almost every aspect of daily life.²³² *Exempli gratia*, the way food and beverage are displayed in supermarkets. Also, according to the modern taxonomical system employed in biology, man belongs to the primates, the mammals, the amniotes, the vertebrates, and the animals.^{10,232} Note how in this classification, the higher level of aggregation the less similar are the members in the respective class.²³² Cluster analysis is not as much a typical statistical test as it is a collection of different algorithms that put objects into clusters according to well defined similarity rules.²³² Cluster analysis methods are mostly used when we do not have any *a priori* hypotheses, but are still in the exploratory phase of our research.²³² In a sense, cluster analysis finds the most significant solution possible.²³² In general, whenever one needs to classify a mountain of information into manageable meaningful piles, cluster analysis is of great utility.²³²

Hence, cluster analysis may detect clusters of correlated disease features, comprising biomarkers in EBC and other conventional or new disease measures, among a sample of individuals.^{9,26,232-235} Succeedingly, the utility of these clusters can be evaluated in clinical practice, which may accordingly lead to refining of the disease model or profile.^{9,26,232-235}

(vi) Application of exhaled biomarker profiles in longitudinal noninvasive inflammometric clinical trials. This requires the establishment of normal variations and age-dependent reference ranges in healthy control children, and an evaluation of the best sensitivity and specificity for each profile of biomarkers in healthy children and children with respiratory disease.

As correlation analysis in cross sectional observations cannot prove either the presence or the direction of causality, longitudinal clinical studies need to evaluate these different profiles of markers in the monitoring of various respiratory disorders. This includes an evaluation of their potentially predictive value for response to treatment, and exacerbation or relapse of disease, as well as their correlations with exhaled nitric oxide, pulmonary function tests and other conventional measures of disease control, such as symptom score, usage of rescue medication and quality of life questionnaires.

Ultimately, this may lead to the inclusion of *noninvasive inflammometric profiling* into specific management guidelines, and thus, enable future individualised therapeutic strategies.

(vii) Validation of biomarker profiles through correlation with direct measurements of inflammation, including induced sputum, bronchoalveolar lavage and endobronchial biopsies. Recently, Fireman *et alia* demonstrated a differential pattern of inflammation assessed by induced sputum eosinophil count and colorimetric measurement of hydrogen peroxide in EBC in adult patients with obstructive compared to restrictive lung diseases and persistent cough.²³⁶ Moreover, Fireman *et alia* reported no detectable levels of hydrogen peroxide in EBC in subjects with interstitial lung disease, in contrast to adults with obstructive lung disease, hence suggesting evidence for the specificity of hydrogen peroxide measured in EBC, which is mainly derived from the airways or

bronchial compartment, and only to a lesser extent from the alveolar compartment.²³⁶ Jackson *et alia* reported no correlation between markers (*in casu* 8-isoprostane, hydrogen peroxide, total nitrogen oxides, acidity, total protein, total phospholipid and keratin) in EBC and bronchoalveolar lavage, in 49 adult patients undergoing bronchoscopy for diverse reasons, including lung transplantation.²³⁷ Gessner *et alia* reported no significant correlations between cytokine levels (*in casu* interleukin-1-beta, interleukin-6, interleukin-8, interleukin-10, interleukin-12p70 and tumor necrosis factor-alpha) in bronchoalveolar lavage fluid and EBC in a limited population of 14 adult subjects with an acute exacerbation of chronic obstructive pulmonary disease, requiring bronchoalveolar lavage for microbiological reasons.⁵⁷ On the other hand, Lex *et alia* reported, in a subgroup of asthmatic children who were not treated with montelukast (but with high doses of inhaled and/or systemic steroids), a significant relationship between cysteinyl leukotrienes in EBC and reticular basement membrane thickening in endobronchial biopsies.²¹⁰ Obviously, these findings are highly intriguing, whether positive or negative, however, we strongly insist upon cautious interpretation in view of the many currently not (yet) standardised aspects of EBC methodology. To state once more, the need for standardised EBC methodology is incontestable, and should primarily and urgently be addressed using a coordinated multicenter approach.

Finally, we also want to point out that trained household dogs were able to detect biopsy-confirmed lung and breast cancer with 0.99 and 0.88 specificity, respectively.²³⁸ Currently more than 500 trace compounds in exhaled breath are known as volatile organic compounds (VOCs).²³⁹ Volatile organic compounds can be produced anywhere in the body, and are transported via the bloodstream, and exhaled through the lung.²⁴⁰ Therefore, looking at a set of volatile organic compounds may enable recognition and monitoring of different complex processes in the body.²³⁹ However, to date, huge variations exist between results of different studies, due to lack of standardisation, and associated technical problems of sampling and (automated and real time) analysis.²³⁹⁻²⁴²

Thus, *noninvasive inflammometric profiling* may ultimately encompass the assessment of various biomarkers in EBC, with concomitant volatile organic compounds, and may lead to the routine use of individual breathograms that may guide customised management strategies in chronic respiratory disorders and beyond ...

9.10. Epilogue

To recapitulate, Penelope's heart may not yet have been conquered entirely, but this thesis offers at least an improved EBC methodology, and thus, a spark of new perspectives for the management of both the younger as well as the older children with chronic respiratory inflammation. Methodological standardisation of EBC collection and analysis remains a prerequisite, and future investigations will demonstrate whether accurate diagnosis and customised care for all children with chronic respiratory inflammation can be achieved by additional routine *noninvasive inflammometric*

profiling. In that way, EBC may prove to be the mythological *lanus bifrons*,¹ the old roman god with two foreheads or faces, as shown on the back cover of this thesis, and often depicted with two keys, one closing the gateway of the past, the other one opening the gateway of the future ...

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