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# Exhaled Breath Condensate in Obstructive Lung Diseases

## -A Methodological study

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*To my beloved family!*

*Det krävs ett helt nytt sätt att tänka för att lösa de problem vi skapat med det gamla sättet att tänka! ALBERT EINSTEIN*

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# ABSTRACT

Asthma and chronic obstructive pulmonary disease (COPD) are two common inflammatory airway diseases characterized by airway inflammation and mucus hypersecretion. Prediction of the outcome of these diseases may not be performed and the need for non-invasive diagnostic tools capable of identifying inflammation in asthma and COPD becomes therefore obvious. Validation, sensitivity and specificity of most non-invasive methods to detect and monitor inflammatory responses in airways are poor and there is a great need to identify and standardize less invasive, or non-invasive methods for investigation of airway inflammation. Epithelial lining fluid (ELF) covers the airway surface and contains soluble and insoluble inflammatory cell products and plasma proteins originating and passively transferred from the underlying tissue. Airborne aerosol particles containing ELF saturated with water may be recovered in exhaled air by allowing the air to pass a cold surface, creating exhaled breath condensate (EBC). EBC may then be analysed for various components of interest.

**The aims** of this thesis were (1) to explore whether a certain profile of inflammatory cell markers in EBC, saliva or serum may be identified in patients with allergic asthma or COPD, (2) to evaluate the efficacy and reproducibility of a measurable marker in EBC using either of the two condensers ECoScreen or RTube and (3) to evaluate the value of chlorine concentrations in EBC as well as reproducibility of assessments of certain compounds in EBC.

**Material and methods**: Thirty-six patients with asthma, 49 smokers or ex-smokers and 25 healthy volunteers participated in three clinical studies. In addition, efficacy, reproducibility and comparison of the two condensers were studied in an ex vivo set up using aerosols of solutions of saline, myeloperoxidase (MPO) or human neutrophil lipocalin (HNL). Aerosol boluses were transferred by means of a servo ventilator to either of the two condensers. Concentrations of chlorine (presumed to be a marker of mucous secretion) in EBC or saliva were analysed by means of a sensitive coulometric technique (AOX). The inflammatory cell markers histamine, MPO, HNL, lysozyme, cysteinyl-leukotrienes (CysLT) and eosinophil cationic protein (ECP) were analysed in EBC, saliva and/or serum by means of ELISA, RIA, EIA or immunochemical fluorescence methods, respectively. Lung function tests, including diffusion capacity were measured by standard techniques according to clinical routines.

**Results and Conclusions:** Chlorine measurements served as the main tool in our tests and intra-assay variability <10% was recorded. However, flow dependency, temperature dependency, substance dependency and concentration dependency characterized yields of EBC. Despite acceptable analytical precision, low concentration levels of inflammation markers, biological variability and occasionally contamination with saliva mean that the feasibility of the EBC method is limited. Despite biological variability, concentrations of chlorine in EBC were significantly higher during than after a mild pollen season, suggesting that chlorine concentrations in EBC are a sensitive marker of allergic airway inflammation. A vast number of confounding factors made interpretations of EBC data obtained from COPD and non-COPD patients difficult and traditional diagnostic tools, such as diffusion capacity (DLCO) and serum lysozyme appeared to best discriminate between COPD and non-COPD.

# LIST OF PAPERS

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals.

I. Davidsson A, Naidu Sjöswärd K, Lundman L, Schmekel B, Quantitative Assessment and Repeatability of Chlorine in Exhaled Breath Condensate; Comparison of Two Types of Condensators. *Respiration 2005;72:529-536*.

II. Davidsson A, Söderström M, Naidu Sjöswärd K, Schmekel B, Chlorine in Breath Condensate – A Measure of Airway Affection in Pollinosis?
*Respiration 2007;74:184-191*.

III. Davidsson A, Naidu Sjöswärd K, Schmekel B.
Efficacy of Two Breath Condensers – An in Vitro Comparative Study.
*Submitted for publication: 2008*.

IV. Davidsson A, Stratelis G, Acevedo F, Schmekel B.
Can we Predict Development of COPD?
*Submitted for publication: 2008*.

# ABBREVIATIONS

| | |
|---|---|
| AEA | Accumulated volume of exhaled air |
| AOX | Adsorbed organic halogen technique |
| ATS | American Thoracic Society |
| $AUC_{ROC}$ | Area under ROC curve |
| BAL | Bronchoalveolar lavage |
| BF | Breathing frequency |
| BMI | Body mass index |
| CFTR | Cystic fibrosis transmembrane conductance regulator |
| $CO_2$ | Carbon dioxide |
| COPD | Chronic obstructive pulmonary disease |
| CTAB | Cetyl-trimethyl ammonium bromide |
| CysLTs | Cysteinyl-leukotrienes |
| DLCO | Diffusion capacity for carbon monoxide |
| EBC | Exhaled breath condensate |
| ECP | Eosinophil cationic protein |
| ELF | Epithelial lining fluid |
| eNO | Exhaled nitric oxide |
| ERS | European Respiratory Society |
| FeNO | The fraction of eNO |
| $FEV_1$ | Forced expiratory volume during one second |
| FVC | Forced vital capacity |
| GINA | Global Initiative for Asthma |
| GOLD | Global initiative for Chronic Obstructive Lung Disease |
| HNL | Human neutrophil lipocalin |
| hs-CRP | High-sensitivity C-reactive protein |
| ICS | Inhaled corticosteroids |
| LOD | Limit of detection |
| $MEF_{50}$ | Maximal expiratory flow at 50% of forced vital capacity |
| MPO | Myeloperoxidase |
| ROC | Receiver operating characteristic |
| VAS | Visual analogue scale |
| VC | Vital capacity |
| $V_{EA}$ | Volume of exhaled air |
| Vt | Tidal volume |

# 1 INTRODUCTION

Asthma and Chronic Obstructive Pulmonary Disease (COPD) are common diseases characterised by inflammation of the airways and disturbed integrity of the epithelial cell layer of the airways[1]. Macroscopic responses to airway inflammation include airway obstruction, and mucus hypersecretion by exocytose of sub-mucosal glands and surface epithelial goblet cells[2]. The quality, as well as the quantity of mucosal secretions differs between healthy subjects and patients with respiratory diseases[3]. During periods of hypersecretion, the mucus shifts from having a protective to a potentially harmful role due to increases in viscosity which may impair mucociliary clearance with resulting accumulation of mucus in the airways[3].

## Human airway mucosa

The epithelium of the conducting airways effectively defends the body against inhaled particles and noxious agents. Proximal conducting airways are lined by ciliated epithelium and goblet cells, while distal parts are lined by thin squamous epithelial cells to facilitate gas exchange[1,4]. Lubrication of inhaled air and elimination of inhaled foreign particles are major functions of airway epithelium. Epithelial lining fluid (ELF) covers the entire airway surface[5] and is produced by serous cells, goblet cells, Clara cells, or type II alveolar cells and may also enter the mucosal surface by diffusion/transudation through the mucosa. The fluid that moistens airway surfaces is essential for preservation of the cellular milieu and ELF serves as a unique interface between inspired/expired air and the epithelium[6,7]. ELF consists of water, salts, lipids, proteins and glycoproteins[8] and secretion of liquid from submucosal glands in the tracheobronchial tree is controlled by neurogenic mechanisms[9]. Serous cells in submucosal glands secrete water by mechanisms driven by active secretion of chloride and $HCO_3^-$ involving cystic fibrosis transmembrane conductance regulator (CFTR)[10,11]. ELF may also appear on the surface of the airspace by active secretion or by passive diffusion through epithelial ion channels and pumps[12]. Molecular epithelial transport is not homogenously distributed throughout the airways; active ion transport in small airways is low compared to passive transport and the composition of ELF may differ

from that of plasma because of the low permeability rate in alveolar epithelium[12]. Fluid may also enter into the airway lumen by transudation of solutes from plasma by the action of inflammatory mediators that are suggested to elevate the local hydrostatic pressure, that in turn is supposed to push apart epithelial cells, allowing water to enter the airway lumen[13,14,4]. The integrity of the barrier function of the airway mucosa is crucial for the proper function of epithelium.

Apart from a sol phase surrounding cilia in the proximal parts of the airway, a gel phase layer is located on top of the sol phase and these two layers with disparate viscosity facilitate the upward transport of debris and mucous by mucociliary clearance. While coughing and mucociliary clearance are the most effective defence mechanisms of the proximal airways[5,1], defence against pathogens and inhaled particles in the distal parts of the airways is effected mainly by inflammatory cells acting as a cellular barrier between air and interstitium, by means of phagocytosis and other cellular defence mechanisms[15,16].

Inflammation is a complex response to noxious attacks of the airways and designed to restore tissue to its normal function[17,18]. A variety of signalling molecules, locally produced by mast cells, nerve endings, platelets and white blood cells, mediate responses to inciting agents and act as chemoattractants for perpetuating the inflammatory response[19,20]. This leads to vasodilatation and increased capillary permeability with transmigration of leukocytes and release of mediators[17,21]. Release of elastase and other compounds secreted from neutrophils, or eosinophil cationic protein (ECP) secreted from eosinophils, can set off loosening of the barrier function of epithelial cells and result in increased plasma protein leakage from the circulation to the airways[22,20]. Airway epithelium normally undergoes constant cell renewal and may be replaced with a normal structure at some time after injury[1].

# Airway remodelling in asthma and COPD

## Asthma

Asthma is a chronic inflammatory disease characterized by bronchial hyperresponsiveness and variable airflow obstruction. The prevalence of asthma has been reported to be 5-10% and more recently to be on the increase[23,24]. When uncontrolled, asthma may cause severe limitations of daily life and although rare, asthma may eventually be fatal[25]. Allergy, as well as certain environmental factors, may initiate the asthmatic inflammation[26], and the disease is defined by its clinical, physiological and pathological characteristics[27]. A clinical diagnosis of asthma is usually based on the presence of symptoms, such as episodic breathlessness, wheezing, and chest tightness[28]. Wheezing is the most typical physical symptom of airway obstruction and tends to confirm the presence of airflow limitation[27]. Airway hyper responsiveness, typical of asthma, expresses itself as episodes of wheezing, breathlessness, chest tightness and coughing, as a consequence of exposition of certain exogenous or endogenous stimuli. Although not obligatory, a reduction in forced expired airflow in spirometry readings may suggest the diagnosis. Reversibility of airways obstruction, as documented by an increase of forced expiratory volume in one second ($FEV_1$) ≥12% after inhalation of a bronchodilator compared to the pre-bronchodilator value and a history of variable airways obstruction are generally accepted as diagnostic conditions of asthma (GINA). Inhaled corticosteroids (ICS) and $\beta_2$-receptor agonists are the most common drugs used for asthma treatment. ICS has lately been considered to be the first-line therapy because of its efficacy in reducing symptom severity, such as bronchial hyperreactivity and minimising exacerbations[29]. $\beta_2$-agonists are often used on an as-needed basis because of their ability to relax airway smooth muscle and to decrease vascular permeability[30,27]. Leukotriene-1 receptor antagonist has a bronchodilating effect, reduces symptoms and airway inflammation, as well as asthma exacerbations[27]. In order to maintain good asthma control, the disease should be stable with no symptoms and airway tonus variability (GINA). If there are symptoms, this is considered as proof that the underlying inflammation has not been properly treated. Subjective symptoms and perception of chest tightness may not necessarily be paralleled by alterations in lung function[31].

Eosinophil and mast cell infiltration of the airway mucosa is a hallmark of asthmatic inflammation. Human lung mast cells are presumed to play a major role in bronchoconstriction elicited by inhalation of adenosine[32], as well as in naturally occurring asthma[33] and the activity state of eosinophils often correlates with disease severity[34]. Biopsy and sputum specimens obtained from asthmatic patients have been shown to contain higher levels of eosinophil cationic protein (*ECP*) than those obtained from healthy control subjects[35,36]. ECP is a protein released from specific granules of eosinophils[37], it has cytotoxic properties and a capacity to kill parasites, bacteria and virus. ECP may also injure respiratory epithelial cells and stimulate airway mucus secretion, and cause the release of histamine from mast cells and basophils[38].

A wide variety of exogenous and endogenous stimuli may activate mast cells that have the capacity to release histamine, leukotrienes and prostaglandins[39]. These mediators are all potent agonists for airway smooth muscle contraction[40]. Mast cells are also responsible for synthesis and secretion of a number of chemokines that regulate eosinophil inflammation[41,42]. Mast cells may be found in epithelium, submucosa and deep in the airway walls[43]. *Histamine* is a biogenic amine synthesized by mast cells, basophils and platelets and is released as a response to various stimuli. Histamine contributes to a number of biological reactions, such as smooth muscle cell contraction, vasodilatation, increased vascular permeability and mucus secretion[44,45]. *Cysteinyl-LTs* are inflammatory lipid mediators derived from the 5-lipoxygenase pathway that is involved in the pathophysiology of asthma[46-48]. These lipids are released by circulating inflammatory cells, as well as tissue bound cells, mainly by mast cells and eosinophils. CysLTs play an important role in the acute and chronic manifestations of asthma by increasing blood flow in airway walls and vascular permeability[49,50]. They are among the most potent bronchoconstrictors, even more potent than histamine[51]. CysLTs are also a specific chemoattractant for eosinophils[52]. Lymphocytes and macrophages are together with eosinophils and mast cells also considered to play important roles in orchestrating the asthmatic inflammation. A typical inflammation seen in asthma may result in airway remodelling with microvascular leakage, epithelial disruption, mucus hypersecretion and smooth muscle hypertrophy[53]. The epithelium is not merely a passive barrier; it can also generate a range of mediators that may play a role in the inflammatory process in asthma[54].

***Due to the persistent but variable mucosal inflammation in asthma, which does not always present itself by perceivable symptoms[55] or measurable decreases of forced expiration, there is an obvious need for diagnostic tools capable of identifying ongoing inflammation in asthma.***

# Chronic obstructive pulmonary disease

The world wide prevalence of COPD has been stated to be up to 22% in males and up to 16% in females[56] and morbidity, as well as mortality, increase with age[57]. Cigarette smoking is the most important risk factor and the highest prevalence of COPD tends to occur in countries where cigarette smoking is common[58]. COPD is a progressive, inflammatory disease commonly driven by tobacco smoking and the clinical picture includes airflow limitation that is not fully reversible by bronchodilators. Airway inflammation is normally located in large, as well as in small, airways and major structural changes may develop, resulting in fibrosis and emphysema, also contributing to the decline in lung function in patients with COPD[59-61]. Typical symptoms in COPD are dyspnoea, coughing and increased mucus and sputum production stimulated by cigarette smoke, bacterial components, oxidative stress and chronic cough[62,63].

Airflow limitation in COPD is commonly measured by forced expirations by means of spirometry, and the gold standard for the diagnosis is a post-bronchodilator value of $FEV_1/FVC$ <70% in conjunction with $FEV_1$ <80% predicted normal value[58]. Bronchodilators commonly used in COPD patients include $\beta_2$-receptor agonists and anti-cholinergic drugs[64-66]. There is a dispute about the efficacy of ICS treatment; while some studies have shown that patients with severe COPD have only a modest beneficial effect of ICS treatment, others have shown that ICS treatment in patients with severe COPD results in fewer exacerbations[67]. Smoking cessation is the only known intervention that can slow down the progress of the disease[58,68,69].

Approximately $10^{17}$ oxidant molecules are inhaled in every puff by a normal smoker[70] and oxidants are supposed to trigger effects on molecules or cells primarily in airway mucosa, resulting in increased release of inflammatory mediators by activated inflammatory cells in response to cigarette smoke[70]. Submucosal glands and ciliated epithelial cells are normally found in larger bronchi[2] and they may be replaced by goblet cells in chronic bronchitis leading

to mucus hypersecretion[71]. Furthermore, pronounced goblet cell metaplasia is more commonly found in the airways of smokers with COPD than in smokers with no signs of COPD[72].

Secretion of submucosal glands is regulated by vagal muscarinic nerves and can be stimulated in several ways to increase secretion[73]. High concentrations of chloride have been found close to airway mucosal glands[74-78], and chloride secretion from submucosal glands takes place mostly in the larger airways[12] and is essential for maintaining the volume and composition of ELF[79]. A recent publication suggests that increased expression of human calcium-activated chloride channel 1 (CaCC 1) in airways contributes to mucus hypersecretion in asthma and COPD[80], suggesting a role of chlorine in mucous production.

**Lysozyme** is a widely distributed enzyme occurring in many mucosal secretions, known for its ability to break down bacterial cell membranes, thus having antibacterial properties. Lysozyme is one of the principal polypeptides of respiratory tract secretion and it occurs abundantly in bronchial mucous and accounts for >15% of the total protein content of bronchial mucous[81,82]. The protein is found in elevated levels in the lower respiratory tract in patients with chronic bronchitis[83]. Lysozyme is also secreted from activated macrophages and neutrophils, and increased concentrations of lysozyme have been shown in serum taken from smokers as compared to that of healthy never-smokers[84].

Neutrophils are commonly found in epithelium and mucosal glands in the airway mucosa in COPD patients and in smooth muscle tissue of the airways[72]. It has been hypothesized that activated neutrophils play a significant role in the pathogenesis of COPD, because of their ability to release oxygen radicals, elastase and various other cytokines. Infiltration of activated neutrophils may also be present in the bronchial mucosa in patients with COPD who have never smoked[85,86] and a marked infiltration of neutrophils and macrophages in bronchial glands has been observed in smokers with normal lung function[87].
Neutrophils and macrophages are both predominant cells in sputum and bronchoalveolar lavage (BAL) obtained from patients with COPD and while neutrophils tend to be more abundant in conducting airways, macrophages are located mainly in smaller airways[88]. Human neutrophil lipocalin (*HNL*) is a protein released from secondary granules of activated neutrophils[89]. This

protein is unique to the neutrophil[90] and hence an optimal marker for neutrophil presence and activity state[91-93].

Myeloperoxidase (MPO) is an enzyme released from primary granules of activated neutrophils[94,95] and may also be released from monocytes. Levels of MPO in BAL fluid obtained from patients with chronic bronchitis were higher than in BAL from healthy control persons[96] and serum levels of MPO were higher in patients with exacerbated COPD than in healthy volunteers[97]. Furthermore, increases of serum MPO have been associated with progression of COPD[84] and tend to decrease in conjunction with glucocorticoid treatment[98].

An increased number of neutrophils has been shown in peripheral blood obtained from patients with COPD and is significantly associated with a decline in lung function[99]. These data are compatible with the notion that the inflammation in COPD also involves systemic defence mechanisms. Inflammation in COPD may start many years prior to the onset of clinical symptoms and perpetuation of the inflammation has been suggested to be caused by increases in various cells or inflammation markers in sputum, even after a 1-year smoking cessation period. Although smoking cessation improves respiratory symptoms and forced expiration[100], there are signs of persistent airway inflammation even after smoking cessation[101,102,99] as well as increases of mucosal macrophages and sputum neutrophils[103]. Increases in subepithelial $CD4^+$ and plasma cells have also been documented after smoking cessation in patients with COPD[104].

***One of the main clinical problems concerning COPD is the difficulty in verifying the effects on the airways at an early stage, i.e. before structural changes have occurred. It is therefore important to enable assessment of airway inflammation before symptoms, such as cough and mucus formation, have arisen***.

# Tool to measure airway inflammation and remodelling

## Direct measurement

Direct measurement of remodelling and inflammation includes analyses of autopsy and surgical tissue specimens. These tissue specimens provide a global view of the pathological features and taking surgical lung biopsies are the most invasive way to get access to lung tissue. General anaesthesia is required and the procedure is associated with risks for the patient. Endobronchial or transbronchial biopsies performed by means of fiberoptic bronchoscopy are widely used in research and are today standard procedures in studies of airway inflammation and remodelling[105].

## Indirect measurements

Indirect measurements of airway remodelling include analyses of specimens, such as BAL, induced sputum, exhaled nitric oxide, blood, urine, exhaled breath condensate (EBC) or saliva. BAL is performed under local anaesthesia by means of flexible bronchoscopy and allows assessment of cellular composition or concentrations of various solutes. By inhalation of an aerosol of hypertonic saline, sputum production will be induced and expectorated and the technique is widely used in research. Induction of sputum can be performed repeatedly but patients need to be pre-treated with bronchodilators and an inadequate cellular yield is common, especially in healthy subjects. As for BAL, this method lacks the ability to sample specimens from a specific area of the airway tree and a reliable dilution factor is missing. Both these methods may by themselves induce local inflammation, presumably due to osmotic changes close in epithelial cells[106,107]. Exhaled nitric oxide (eNO) has been extensively investigated and shown to correlate with eosinophilic airway inflammation in asthmatic patients[108]. The fraction of eNO (FeNO) has been widely accepted as a method to monitor airway inflammation[109] but still the method may not be adequately validated[110,111].

***Due to the fact that validation or sensitivity and specificity of most non-invasive methods to detect and monitor inflammatory responses in airways are poor, there is a great need to identify and standardize less invasive or non-invasive methods for the investigation of airway inflammation.***

## Exhaled breath condensate

Collections of condensates of exhaled breath or saliva are the most non-invasive methods to obtain body fluids from airways. The first manuscript concerning EBC, condensing exhaled air on an ice-chilled glass surface, was published in 1980[112] and the scientific community has lately paid increasing attention to the method and more than 400 publications have been presented during the last 15 years. EBC has been suggested to be a useful tool for monitoring inflammatory processes in airways diseases[113] and is considered to be suitable for longitudinal studies and applicable in patents of all age groups and for assessing efficacy of pharmacological interventions[114]. The origin of EBC from within the airway tree has not been verified and in contrast to BAL, EBC may sample material from almost the entire respiratory tract from alveoli to mouth and reflect events within the same area[115]. Although BAL may be considered to be a more reliable method to retrieve cells and lining fluid from the respiratory tract[116], EBC has several advantages over BAL. EBC per se does not induce inflammatory changes, advanced instrumentation or premedication is not required and the procedure is easily repeated, even in patients with severe diseases[115]. Despite this, there are a number of limitations that must be resolved before EBC can be used as a validated research tool or in clinical practice.

ELF contains various volatile and non-volatile substances and these may be transported as aerosols saturated with water vapour[113]. Aerosol particles created from ELF are supposed to be formed by turbulent airflow in certain parts of the airway[117]. Particle concentration in exhaled air, as recorded by a laser light scattering particle spectrometer, ranged from less than 0.1 particles/$cm^3$ air during tidal breathing to 4 particles/$cm^3$ air during exercise[118]. Exhaled air consists mainly of water vapour, supposed to exceed 99% of the total EBC volume and 0.01-2% of the total EBC volume was suggested to be derived from ELF[119]. Not only ELF but also the mucus layer of the airways has been suggested to be recovered by EBC[120]. Evaporation loss has been estimated

to be around 30-35 mg/L air under normal circumstances and all exhaled air will not be condensed during a sampling procedure[121].

There are a number of custom-made devices designed for EBC collection, including tubing systems of different materials, length and diameter. These condensers consists of jacketed cooling pipes, tubes in buckets of ice, or glass chambers in ice. Variable efficacy of retrieving EBC was evident by the use of various *coating* materials of collectors, such as glass, silicone, teflon, "optimised glass" or Tween 20[122-124].

A volume of 1–3 mL of liquid or solid phase of exhaled air will be trapped while breathing normally into a condenser and the concentration of some compounds tends to increase with lower temperature[125]. Optimal collecting temperature may differ between various inflammation markers and it has been assumed that heat labile mediators may be better preserved with lower temperature[126]. Samples of EBC should be frozen immediately and stored at -70°C until analysis and too many frosting-defrosting cycles should be avoided. Apart from a suggested temperature dependency, efficacy of condensate yields has also been suggested to be flow dependent ($H_2O_2$) or not dependent on airflow ($NO_x$)[127,128].

An obvious limitation of the EBC method is the fact that inflammation markers appear in low *concentrations* in EBC, often close to or below limits of detection (LOD). Another limitation of the method is the unknown *dilution* by water vapour of EBC. Several dilution markers have been suggested and concentration ratios of marker to total protein or urea have been proposed[120,119,129] and rejected[130].

Several compounds have been identified in EBC including ammonia, leukotrienes, isoprostanes, $H_2O_2$, adenosine, peptides, cytokines, nitrogen oxides ($NO_x$)[115]. Measurements of *pH* in EBC correlate with signs of airway inflammation, such as sputum eosinophilia and neutrophilia[131]. EBC pH has been suggested to mirror acute exacerbations in asthma, and treatments with anti-inflammatory drugs will normalize pH levels[132]. Independent research groups have confirmed that pH measurement in EBC are reproducible and day to day intra-subject coefficient of variation was reported to be 4.5%[115].

Measurements of hydrogen peroxide *($H_2O_2$)* in EBC have been mentioned as a useful method of assessing biological oxidative stress[131]. Various studies have

reported elevated levels of $H_2O_2$ in steroid naive asthmatic patients. Levels of $H_2O_2$ in EBC are commonly measured by spectrophotometry or spectrofluorimetry[133-135]. Data on reproducibility of $H_2O_2$ are not available.

Another marker for oxidative stress, *8-isoprostane*, shows higher levels in EBC obtained from patients with moderate to severe asthma than from those with mild asthma[49]. Levels of 8-isoprostan have also been found to vary widely in EBC obtained from healthy subjects[115]. Commercial EIA kits have been used and results validated by gas chromatography[136]. Data on reproducibility are not available[115].

*Nitric oxide (NO)* is an important regulator of the smooth muscle tone of blood vessels and bronchi. Release of NO in the lungs can be measured indirectly by quantifying nitrite, nitrate or nitrotyrosine in EBC. Concentrations of nitrite and/or nitrate have been found to be significantly higher in EBC from patients with asthma than in healthy controls[137]. Furthermore, levels of NO metabolites have been shown to decrease after treatment with ICS[138]. Data on reproducibility are not available.

A few independent research groups have reported higher levels of leukotriene or LT- metabolites in EBC from asthma patients: $LTB_4$ was higher in EBC in steroid naive atopic asthmatic children but not in atopic non-asthmatic children[136] and *CysLT* concentrations were higher in EBC obtained from patients with asthma than from healthy subjects[139,140]. They were also higher after a bronchial challenge by means of adenosine-5'-monophosphate (AMP) than after a metacholine challenge[141]. Furthermore, a bronchial challenge with allergen in allergic asthma patients also induced higher CysLT levels in EBC relative to baseline[142] and nasal corticosteroids were reported to reduce CysLT levels in EBC significantly[143]. In contrast, there are also a number of reports that CysLTs could not be detected in EBC[144], and one research group found detectable levels of $LTB_4$ in a minority of EBC samples obtained after bronchial challenge with lipopolysaccharide or a three hour stay in a pig confinement building, known to induce increased releases of $LTB_4$. All samples that displayed measurable levels of $LTB_4$ also contained significant amounts of $\alpha$-amylase, a constituent of saliva. As a consequence of these findings, saliva was suggested to be the main source of $LTB_4$ in EBC[145]. Data on reproducibility are not available.

One single report on *histamine* in EBC has been published and bronchial challenges with AMP as well as with methacholine (MCh) in both asthmatic patients and healthy subjects did not alter histamine in EBC[141].

## Saliva

Saliva is secreted from three pairs of major salivary glands and a vast number of minor salivary glands dispersed in the oral mucosa. Saliva secretion is mainly controlled by the autonomic nervous system, i.e. parasympatic and sympatic nerves[146]. Saliva secretion is increased by a number of stimuli, such as taste and olfactory stimuli, chewing, pain, aggression, while stress, anti-adrenergic or anti-cholinergic drugs will reduce production[147]. Saliva is made up largely of water, ions and proteins secreted from "acinar secretory units" of the salivary glands. Saliva is a complex biological fluid, composed of different elements, such as glycoproteins, enzymes, hormones and minerals. The electrolyte content of saliva is regulated by removal of sodium chloride during passage from gland to oral cavity and thus transformed saliva changes from an isotonic to a hypotonic solution[146].

Elements of saliva are considered to reflect the composition of circulating blood and may be used for the diagnosis of systemic disease[148,149]. Saliva does not usually contain all serum components, but serum elements may pass through by passive diffusion or by ultra filtration through the intercellular tight junctions[150]. The enzyme $\alpha$-amylase occurs abundantly in saliva, also appearing in much lower concentrations in nasal or bronchial mucus and plasma[151,81]. Concentrations of $\alpha$-amylase in saliva thus far exceed those of other body fluids and may also increase in response to physical and mental stress[152], as well as by carbohydrate intake[151]. Medication may also affect the $\alpha$-amylase content, so that beta blockers may inhibit, while beta agonists may stimulate the release[151]. The enzyme lysozyme is a normal constituent of saliva (less than 1% of total protein content) and of circulating leucocytes. In contrast, more than 15% of the total protein content in bronchial mucus is lysozyme[81].

Saliva can easily be collected non-invasively from adults or children, and has been suggested to be a good alternative to serum for analysis of various inflammatory markers[150]. The commonly used and also the simplest method of saliva sampling, is by spitting into a disposable tube. Alternatively chewing a dental cotton wad for one to two minutes (Salivette system, Sarstedt Co. Ltd,

Nümbrecht, Germany), or soaking a strip of filter paper and eluting molecules of interest[153,154] may be performed but involves a risk of unintentional stimulation of certain salivary glands (i.e. parotis) and/or adhesion of certain molecules to the collection material. Assays in saliva may be technically more reliable than the corresponding ones employed in plasma, because of the fact that saliva contains lower levels of lipids and some other interfering molecules[153]. On the other hand, a definite disadvantage of analyses in saliva is varying viscosity, as well as interference between certain components in mucous and inflammation markers of interest.

## Physiological assessment of airway remodelling

Ventilatory capacity and volumes of the lungs in healthy subject reach their maximum value at the age of 20-25 years, thereafter these factors decline linearly through middle age, with an accelerating decline in the elderly[155]. Forced expiratory volume in one second ($FEV_1$) is a reproducible surrogate marker of airway tonus (coefficient of variation <3%)[156], and also an important indicator of outcome i.e. survival and quality of life[155]. A disadvantage of this marker is that it is insensitive to ongoing airway inflammation and it is not necessarily associated with subjective perception of chest discomfort[31,157]. Annual decline of spirometry data in healthy subjects and smokers has been published and $FEV_1$ declined on an average 15 mL/year or 37 ml/year in ex-smokers[158,159] and declines of up to 80 mL/year have been published in heavy smokers[159]. Smoking cessation normally results in beneficial effects and improvement of $FEV_1$[160] and the outcome in terms of spirometry data may depend on a number of factors such as smoking history, individual sensitivity and duration of smoke cessation. Subjects with grossly impaired lung function when ceasing to smoke showed greater improvements of $FEV_1$ during the first years after cessation than those with normal, or near normal, lung function[161].

Diffusion capacity (DLCO) was first introduced by Marie and August Krogh nearly 100 years ago[162] with later modifications[163,164]. It is a single breath test used in clinical practice to estimate diffusion rate over the alveolar-capillary membrane of an inhaled bolus of a known concentration of carbon monoxide. Several factors control gas exchange, including effective membrane surface area and function, ventilation/perfusion ratio and association rate to haemoglobin. Measurement of diffusion capacity is useful in a variety of clinical settings, such as distinguishing emphysema from chronic bronchitis

and in monitoring of asthma. It is also used to predict exercise induced oxygen desaturation in COPD patients[165].

Breathlessness or dyspnoea are common symptoms in patients with chest diseases and a number of underlying causes may elicit breathlessness[166]. Perception of breathlessness, chest tightness or discomfort may not be easily quantified though they are an important issue in the management of asthma and COPD. Patients' subjective scoring of discomfort may be approximated by the individual rating on a visual analogue scale (VAS). The method has been used mainly to assess individual response to exercise or other provocations and the reliability and validity of VAS as a measure of dyspnoea has been documented in exercise and various provocation models[167,168]. VAS tends not to be applicable in long-term longitudinal studies on changes of perception of dyspnoea[169], but has been suggested to be valuable in assessing the severity of asthma[170]. VAS is constructed as a 10 cm scale with a descriptive text in the left end "none" and at the right "the worst I can imagine".

# 2 THE GENERAL AIM

To explore whether a certain profile of inflammatory cell markers in exhaled breath condensate (EBC), saliva or serum may be identified in patients with allergic asthma or chronic obstructive lung disease (COPD).

To evaluate the efficacy and reproducibility of a measurable marker in EBC using either ECoScreen or RTube

To evaluate the value of chlorine concentrations in EBC and to investigate the reproducibility of assessments of certain compounds in EBC.

# 3 MATERIALS AND METHODS

## Study design

***Paper I:*** Repeated sampling of EBC from healthy volunteers was performed in a random order, using two different condensers, ECoScreen and RTube. Subjects either managed to breathe in similar ways in separate tests (to enable evaluation of reproducibility) or altered their minute ventilation, to enable test of the influence of change in airflow rates on recovery of EBC. In a separate part of the study, EBC was collected (by means of an ECoScreen) from patients with a clinical diagnosis of asthma. To test the effect of recruitment of additional airway surfaces a resistance of 5 cm $H_2O$ was added to the outflow tract of ECoScreen. Collections of EBC from the patients with asthma were performed with, as well as without, added resistance in the exhalation circuit and these tests were performed in a random order. Endpoints were concentrations of chlorine in EBC, which were used as a tool to evaluate reproducibility or recovery.

***Paper II:*** The effect of pollen exposure on chlorine levels in EBC taken from allergic asthma patients in a longitudinal study on serum and EBC, as collected by means of an ECoScreen was evaluated. Measurements were done once during a mild pollen season and repeated a second time off season, five months later. Twenty three subjects had mild allergic asthma and the pollen exposition was coincidentally low. Endpoints were chlorine concentrations in EBC, serum concentration of ECP, forced expirations (i.e. $FEV_1$) and subjective scoring of symptoms.

***Paper III:*** Ex-vivo efficacy and reproducibility of two separate condensers (ECoScreen or RTube) were tested by condensing aerosolized solutions of HNL, MPO or chlorine. A jet nebulizer produced aerosols that were intermittently transferred by a modified servo ventilator to either of the two condensers. Bovine serum albumin (BSA 0.25 mg/mL) was added to stabilize the solutions and adherence of test substances to condensing or conducting system surfaces was tested by spraying three mL of 0.5% cetyl-trimethyl

ammonium bromide (CTAB) into the devices after each test session and analysing concentrations of test substances in lavage fluids.

***Paper IV***: EBC, serum and saliva collected from smokers or ex-smokers, with either clinical signs of COPD or a normal, or near normal spirometry and healthy volunteers, were evaluated by means of a cross-sectional study design. Endpoints in this study were concentrations of chlorine, lysozyme and $\alpha$-amylase in EBC, serum concentrations of lysozyme, ECP, MPO and hs-CRP, spirometry and single breath test of diffusion capacity (DLCOc).

## Patients and healthy volunteers (papers I, II, IV)

None of the study subjects participated in more than one study.

***Paper I:*** Ten healthy non-smoking volunteers and 13 non-smoking patients with a clinical diagnosis of asthma were included. Exclusion criteria were smoking, respiratory tract infection during the three weeks preceding the study, or other concurrent diseases.

***Paper II:*** Twenty-three patients with asthma were recruited from a pool of allergic asthma patients or consecutively recruited from an open asthma ward at the hospital. Inclusion criteria were a diagnosis of asthma, according to the criteria for the Global Initiative for Asthma (GINA) and allergy to common aeroallergens. Exclusion criteria were elevated levels of hs-CRP and/or any sign of infection or exacerbation of asthma. Nineteen of the 23 subjects were allergic to common aeroallergens (birch and/or grass pollen), as documented prior to the study by RAST or positive skin prick test. The remaining four patients all had a typical history of allergy to birch pollen. Eleven subjects (group A) had no regular medication with inhaled corticosteroids (ICS), and 12 (group B) had daily doses of corticosteroids (≤800 µg/day Budesonide, AstraZeneca, Södertälje, Sweden). Two patients regularly used antihistamines and three used beta agonist. Alterations in subjective rating of chest tightness made eight of the treated patients either to increase or to decrease their daily doses of ICS between the two visits at the laboratory. Rescue medication was not allowed during the 24 h preceding any test. One patient was excluded from the study due to an initial abnormally high serum value of hs-CRP assumed to depend on an acute infection.

***Paper IV:*** Twenty-seven smokers, 22 ex-smokers and 15 healthy non-smoking controls participated in this study. Twenty-nine smokers were previously enrolled in a screening study of COPD[171] and 16 smokers and ex-smokers with a clinical diagnosis of COPD, randomly selected from a general practitioners' office, participated in this study. Inclusion criteria were regular tobacco smoking for at least 20 years and exclusion criteria were significant heart or lung diseases, or any other severe concurrent disease. Subjects who had stopped smoking for at least one year prior to the study were classified as ex-smokers according to recommendations by the Society for Research on Nicotine and Tobacco[172]. Thirty subjects had normal or minor deviations from normal spirometry data and perceived no symptoms from the chest. Nineteen of the participants were categorised as having COPD according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD); eight of them were current smokers and 11were regarded as ex-smokers. Twenty age-, sex- and height matched healthy non-smoking volunteers were recruited and inclusion criteria were subjectively perceived well being and lung function tests within the normal reference range. One healthy subject was excluded from the study because of a $FEV_1$ value below the reference range, and four of the volunteers were re-categorised as ex-smokers and one of these four subjects also had COPD, thus leaving 15 healthy volunteers as reference subjects.

**Table 1.** Demographic data of subjects participating in studies I, II and IV. Data are present as median (min-max), and Mann Whitney U-tests were used in statistical evaluations. Statistically significant differences are indicated by the number of symbols; one symbol signifies $p<0.05$, two symbols $p<0.01$, three symbols $p<0.001$. ¶ refers to comparisons of COPD vs. non-COPD, Ω refers to comparisons of COPD vs. healthy volunteers, Φ refers to comparisons of non-COPD vs. healthy volunteers.

| | **Sex** (F/M) | **Age** (years) | **BMI** ($kg/cm^2$) | **Height** (cm) | **FEV$_1$** (%pred) |
|---|---|---|---|---|---|
| ***Paper I*** | | | | | |
| Healthy (n=10) | 5/5 | 25 (22-60) | 25.5(19-29) | 175 (161-184) | 102 (75-120) |
| Asthma p (n=13) | 4/9 | 44 (33-58) | 26 (21-31) | 178 (158-186) | 93 (68-122) |
| ***Paper II*** | | | | | |
| Group A (n=11) | 8/3 | 29 (19-53) | 25 (20-34) | 169 (153-189) | 93 (62-109) |
| Group B (n=12) | 8/4 | 38 (20-57) | 25 (20-39) | 170 (157-184) | 88 (19-112) |
| ***Paper IV*** | | | | | |
| COPD (n=19) | 7/12 | 68 (54-83) ¶¶¶,Ω | 24 (21-32)¶ | 171 (155-190) | 46 (29-73) ¶¶¶,ΩΩΩ |
| non-COPD (n=30) | 11/18 | 57 (47-69) ΦΦ | 27 (21-32) Φ | 175 (149-195) | 93 (75-125) |
| Healthy (n=15) | 8/7 | 60 (52-74) | 24 (22-33) | 174 (154-190) | 97 (81-121) |

# Methods

## Exhaled breath condensate

***ECoScreen***® (Eric Jaeger, Würzburg, Germany) is an electrically cooled collection system, equipped with a one-way inspiratory valve to assure that the patient does not inhale cold air during collection of EBC. Contamination by saliva was prevented/diminished by a saliva trap. The condensing area

consists of a double lumen lamellar teflon-coated aluminium tube with a condensing area of approximately 175 $cm^2$ (erroneously given as 63 $cm^2$ in paper I) and a disposable collecting cup made of polypropylene. ECoScreen maintains a stable cooling temperature of approximately -10°C during the entire condensing period.
***RTube™*** (Respiratory Research, Charlottenville, VA, USA) consists of a disposable tube made of polypropylene, and a condensing area approximately 135 $cm^2$ (erroneously given as 188 $cm^2$ in paper I). This device has two one-way valves of silicone rubber with an o-ring made of teflon. One valve ensures inhalation of room air and the second one is designed to provide maximal particle impact, serving as a saliva trap and also acts as a plunger for EBC sample collection. Cooling is accomplished by placing a pre-cooled aluminium sleeve (stored in a -70°C freezer) around the polypropylene tube.

EBC was collected according to recommendations from ATS/ERS Task Force[115]. Before EBC sampling, all subjects rinsed their mouths with de-ionized water, and then breathed normally (i.e. tidal breathing) for ten minutes through a mouthpiece connected to the condenser. Each subject wore a nose clip during EBC sampling to minimise entry of nasal airway lining fluid and to collect all exhaled air. Although both condensers are equipped with saliva traps, subjects were instructed to swallow saliva or to take a brake in order to prevent saliva contamination.
A spirometer (EcoVent, Jaeger, Wurzburg, Germany) was connected to the outflow tract of one of the condensers (ECoScreen®), to enable measurement of all air passing the condenser during sampling of EBC (papers I and IV). The volume of accumulated exhaled air ($AEA_R$) passing through the RTube during sampling was estimated by the formula:

$AEA_R$=[time (minutes) x breathing frequency/minute x Vt]

where Vt is relaxed tidal volume as measured by means of MasterScreen Capno (Eric Jaeger, (Würzburg, Germany) immediately prior to collection of EBC (paper I). $AEA_R$ has been termed $V_{EA}$ in paper II.
Increased airway pressure was accomplished in a subgroup of subjects (paper II) by adding 5 cm $H_2O$ resistance to the outflow tract of ECoScreen (Caradyne Whisoperflow CPAP, 5 cm $H_2O$, Caradyne, Galway, Ireland).

EBC was collected during 10 minutes (unless otherwise stated), condensates were then immediately removed from the condensers, volumes were

measured by a pipette, and aliquots were distributed to plastic tubes (Sarstedt AG & Co, Nümbrecht, Germany*)* and stored (-70°C) until analysis. Cleansing of the condenser was done after each use by repeated washing with hot water followed by rinsing with de-ionized water.

## Serum

Venous blood was collected according to clinical routines and it was kept at room temperature for 60±15 minutes before centrifugation at 3000 rpm for ten minutes. Supernatants were separated and stored in plastic tubes at -70°C until analysis.

## Saliva

All subjects rinsed their mouths with de-ionized water before sampling of saliva (paper IV). Spontaneously secreted saliva was collected in a plastic cup under relaxed conditions during a 2-3 minute interval, to achieve a volume of approximately 0.5-1.0 mL. Saliva was immediately transferred to a plastic tube and diluted 1:1 with 1 M acetic acid in order to decrease viscosity. Saliva was then immediately centrifuged at 3000 rpm for 10 minutes at 0°C; and supernatants were transferred to plastic tubes and kept frozen at -70°C until analysis.

## Lung function tests

Flow-volume curves were recorded by means of a Jaeger MasterScreen spirometry system (Erich Jaeger GmbH, Hoechberg, Germany). A nose clip was applied during all lung function tests. The best of three repeated measurements was documented. Forced expiratory volume over one second ($FEV_1$) and vital capacity (VC) or forced vital capacity (FVC) were measured and $FEV_1$/VC or $FEV_1$/FVC, maximal or forced expiratory flow at 50% of forced vital capacity ($MEF_{50}$ or $FEF_{50}$) were used as estimates of airway obstruction (Papers I, II and IV). Reference values for all lung function tests were used according to the clinical routine[173,174].

Tidal volume (Vt) was measured by a multiple breath test (capnography) by a $CO_2$–sensor connected to a pneumotachograph (MasterScreen Capno, Erich Jaeger AG, Würzburg, Germany). The best value of Vt (with the highest levels of CO2) was documented and used as an estimate of accumulated exhaled volume of air during EBC sampling.

Single breath tests of DLCO were carried out using a Jaeger PFT MasterScreen Labmanager, MS-PFT analyzer unit (Erich Jaeger GmbH, Würzburg, Germany), according to the clinical routine. Each patient started by breathing tidal volumes, followed by an exhalation to residual volume, and then rapidly inhaled a mixture of gas with known concentrations of CO (0.25%), He (9.5%) and $O_2$ (21%). The patient held his/her breath for 10 s and then rapidly exhaled. The expired gas was collected for analysis after discarding the initial 750 mL (i.e. dead space volume). Carbon monoxide and the tracer gas concentrations were measured, and DLCO was calculated and adjusted for haemoglobin concentration in blood and documented as percentage of predicted normal value (DLCOc% predicted). DLCO was not measured in patients who had values of $FEV_1$ <1L, (i.e. some of the COPD patients). Reference range was 75-125% of predicted normal value according to the clinical routine.

## Laboratory analysis

### *Chlorine*

***Chlorine*** was measured in saliva and EBC by means of a modified adsorbed-organic-halogen technique (AOX; DIN 34809). This method is around $10^3$ times more sensitive than the analytical methods previously published with demonstrated lack of reproducibility of chloride measurements in EBC[175]. The technique measures organic halogen compounds, e.g. chlorine, bromine and iodine, which are covalently bound to organic compounds. Of all the halogens, chlorine is the substance that is most likely to be found in exhaled condensate (due to its abundance in nature). A sample of 100 µL was combusted at 1000°C in an oxidative atmosphere (oxygen gas) and after passage through a scrubber containing concentrated sulphuric acid, the purified hydrochloric gas was carried by the oxygen stream to a coulometric titration cell (Euroglass BV,

Delft, The Netherlands) for measurement of the total chlorine content. The method was validated regularly prior to each test set by measurement of standard solutions with known concentrations of chlorine [Titrisol natriumchloride 0.1 mol/L (5.844 g/L), Merck, Darmstadt, Germany, diluted with Milli-Q® water (Millipore, Mas. USA)]. Reference concentrations used in EBC measurements were 5, 10, 20, 50 and 100 µmol/L, while concentrations of 1000, 2000, 5000 and 10,000 µmol/L were used for measurements in saliva. Milli-Q water was used as the blank. The analyses were performed within different measuring ranges. More precisely, EBC and saliva samples were analysed at 3.4-72.6 and 6400-56000 µmol/L respectively. The limit of detection (LOD) was set to 3 µmol/L (3 times the value of the blank). The recovery was 95% (duplicate analyses) and technical repeatability, i.e. coefficient of intra-assay variability (duplicate), was 0.097.

Intra-assay variability in EBC was calculated, and CV (min-max) in duplicate measurements of samples with chlorine levels below 40 µM was 8% (0-34.7%, n=108), and above this level the CV was 2% (0-6.2%, n=48).

## Biomarkers

Concentrations of ***histamine*** in EBC were determined using a commercially available, solid phase enzyme-linked immunoassay (ELISA) (IBL Immuno-Biological Laboratories, Hamburg, Germany). Analyses were carried out according to instructions from the manufacturer and adopted for acylation of urine and cell culture supernatants. Standard B was diluted (1:3) to increase sensitivity in accordance with instructions from IBL and performed according to the manufacturer, except for the modification of diminishing dilution in the acylation step (using one mL instead of two mL of Assay Buffer in the u/z standards and 0.75 mL in EBC samples). A spectrophotometer (SPECTRAmax 340, Microplate reader, Molecular Devises, Sunnyvale, CA, USA) measured optical density (OD) at 450 nm. The measured ODs of the histamine standards were used to construct a calibration curve (4-parameter logistic) against which the EBC samples were computed. Concentration of antigen in the EBC samples was inversely related to the optical density (OD). The coefficient of intra-assay variability of values in the range of interest was 30% and the detection limit of the assay was 0.3 ng/mL (2.7 nM). Values of histamine were all close to the lower LOD.

***CysLT*** was measured by means of an enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, Mich., USA). Microtitre assay plates were scanned after 60–90 min with a computer-controlled microplate reader (Victor 2 1420 multilabel counter, Wallac, Turku, Finland). Sample concentrations were calculated from a standard curve ranging from 7-1,000 pg/mL. LOD was set to 7.5 pg/L. The coefficient of intra-assay variability of values in the range of interest was 34.3%. (Measurements were done at department of Clinical and Experimental Medicine, Division of cell Biology, University of Linkoping)

Analysis of ***α-amylase*** activity was performed according to the method recommended by the Calzyme Laboratories, Inc. (http://www.calzyme.com.) and modified as follows: 50 µL *α*-amylase standard (Sigma-Aldrich Co., Stockholm, Sweden) in serial dilutions 1:2 starting from 1000 to 8 U/L in duplicates, or 50 µL of freshly-unfrozen and stirred EBC samples in triplicates were added on a 96-well plate (Nunc A/S, Roskilde, Danmark). Fifty µL of freshly prepared 1% starch solution, prepared in 20 mM Na-phosphate buffer, 6 mM NaCl pH 6.9, was added to all wells. The plate was sealed (Plate sealer, In Vitro AB, Stockholm, Sweden) and incubated for 10 min at 40$^{o}$C. The seal was then removed and 100 µL of colour reagent, [a mixture of dinitrosalicylic acid and sodium-potassium-tartrate tetra-hydrate (http://www.calzyme.com.), (Sigma-Aldrich Co., Stockholm, Sweden)] was added to all wells. The plate was again sealed and heated on a grid over the surface of a closed water bath at 95$^{o}$C for 15 minutes. Finally, the plate was allowed to stand for 15 min at room temperature (RT), centrifuged at 2000 RPM for 1 min, unsealed and read in a spectrophotometer at 540 nm and concentration of *α*-amylase was calculated. For increased sensitivity (<10U/L), the incubation time was extended up to 150 min and the starch was heated in 20 mM NaOH for 30 min at 100$^{o}$C and neutralized to pH 6.9 with HCl at RT prior the assays. The lower limit of detection (LOD) was 0.008 U/mL and CV was <10%. (Measurements were done at the Division of Physiology, Institute of Environmental Medicine, Karolinska Institute, Stockholm)

The following biomarkers were measured according to instructions given by the manufacturer, at the Dept of Medical Sciences, Section of Clinical Chemistry, University Hospital, Uppsala, Sweden
***MPO*** was measured by enzyme-linked immunoassay (ELISA) (Diagnostics Development, Uppsala, Sweden), LOD was 1.56 µg/L, and coefficient of inter-assay variability was 7% (reference range in serum of 8-250 µg/L).

***HNL*** was measured by means of HNL radioimmunoassay[176]. LOD was 1.0 μg/L and inter-assay coefficient of variation was 8% (reference range in serum 38-191 μg/L.

***ECP*** was measured by means of an immunochemical fluorescence method (Unicap ®, Pharmacia Diagnostics, Uppsala, Sweden). LOD was <2 μg/L and inter-assay variation 3% (reference range in serum of 2.3-16 μg/L).

***Lysozyme*** was measured by a radioimmunoassay (RIA). LOD was 3.7μg/L and inter-assay variability was 7% (reference range in serum of 615-1383 μg/L).

High sensitive C-reactive protein ***(hs-CRP)*** was measured according to clinical routines by a standard method (Architect, Abbott) (reference value in serum is <5 mg/L).

# Statistical analyses

Data were expressed as median value [minimum to maximum]. Mann-Whitney U-test, two-way Analysis of Variance (ANOVA) or Wilcoxon-signed rank tests for paired data, were used in statistical evaluations. Kolmogorov-Smirnov test was used for tests of normal distribution. Spearman's correlation coefficient (Rs) was used for assessments of correlations (Statistica 6.0 or 7.0, Stat Soft, Inc., Tulsa, Oklah., USA). Statistics, as well as analysis of sensitivity and specificity by means of receiver operated characteristic curves (ROC analysis), and comparisons of ROC curves as area under ROC curves ($AUC_{ROC}$) were performed by means of the commercially available computer program (MedCalc Statistical Software, Mariakerke, Belgium). Coefficient of variation (CV) was expressed by between-subject standard deviation (SD) to mean values and spread of data, i.e. standard deviation (SD)/median value were used to describe variability of gain (paper III). A two-tailed p-value <0.05 was defined as statistically significant.

# 4 RESULTS

## Reproducibility, efficacy and comparison of two condensers (papers I, III, IV)

### *In vivo study*

Chlorine was detected in all condensates and significantly higher levels were found in condensates recovered by RTube than by ECoScreen (Table 2). Ten subjects managed to breathe in a similar way during repeated collection of EBCs by means of the two separate condensers, as judged by values of ventilated volumes recorded by EcoVent or by calculated ventilation through RTube ($AEA_R$). Breathing frequencies (BF) were also comparable and, taking these data together, we judged that subjects managed to breathe in a fairly similar manner during repeated collections of EBC. Duplicate collections of condensates were performed in 10 healthy volunteers with the aim of testing variability in concentration recovery between condensers (paper I). A two-way additive effects ANOVA with machines as fixed factor and subjects as random factor was chosen. It was verified that no significant interaction exists and that the residuals do not have significantly different variance in the two machines. The analyses showed that there was a significant difference between the machines concerning concentration recovery (p=0.001). Because there is a tendency that residual standard deviation increases with increasing level, we also used the same analysis approach with log transformed data, with similar results.

In addition to those who participated in duplicate trials (ECoScreen: 3 missing values, paper I) another five healthy volunteers underwent repeated sampling of EBC with the condenser ECoScreen (paper IV), and within-subject variability was calculated in altogether 12 healthy volunteers, who managed to performed twice with similar breathing pattern by means of ECoScreen. Mean value of measurements were 12.1 μM (min to max values ranged between 4.3

and 22 μM) and differences between duplicates varied from 0.2 μM to 14.8 μM, CV 28%. There were two groups of data on coefficient of variation; while six of the 12 sets of data were below 30% the remaining sets ranged from 42 to 66%.

Between-subject variability of chlorine concentration was examined and concentrations in EBC tended to be lower when EBC was collected with RTube than ECoScreen (CV 17% vs. 31%). In contrast, between-subject variability of volume recovery was lower for ECoScreen than RTube (CV 9% vs. 15%).

Airflow dependency of volume recovery by EBC was shown in papers I, II and IV by a significant association between volume recovery and volume of exhaled air [Rs=0.63, p=0.001, n=38 (paper I) and Rs=0.55, p=0.001, n=39 (paper II) and 0.85, p<0.001, n=64, (paper IV), Figure 1], while an inverse association was shown between concentrations of chlorine in EBC and magnitude of ventilation during EBC sampling (Rs = –0.60, p<0.01, paper I, Figure 2 ).

The effect on recovery volumes and/or chlorine concentrations in EBC by increasing expiratory resistance with 5 cm $H_2O$ was evaluated in thirteen patients with mild asthma. All patients tolerated increased exhalation resistance well, although they tended to increase their voluntary tidal ventilation with approximately 4% (p=0.79). EBC volumes tended to increase but increases of volumes were restricted to patients with signs of airways obstruction as judged by lower than normal values of $FEV_1/VC$, i.e. <80% (n=9, p=0.05). Changes in EBC volumes were not accompanied by corresponding statistically significant decreases of concentrations. Changes in recovery volumes induced by increases in outflow resistance were associated with airway obstruction, as expressed by $FEV_1/VC$ percent predicted normal value (Rs=-0.65, p=0.01).

**Table 2.** Data recorded when 10 healthy volunteers sampled EBC during 10 min tidal breathing (paper I). AEA=Accumulated volume of exhaled air during ten minutes of collection of EBC. BF=breathing frequency. Data are expressed as median value (minimum-maximum). Statistically significant differences are indicated by * p=0.014

| | **ECoScreen** | **RTube** |
|---|---|---|
| EBC volume (mL) | 0.98 (0.6-1.45) | 1.00 (0.6-1.55) |
| Chlorine concentration (μM) | 9.6 (3.4-28.3) * | 30.9 (13.3-71.8) |
| AEA (L) | 72.1 (40.4-93.6) | 72 (25.9-127) |
| BF (min$^{-1}$) | 11.5 (4) | 10 (4) |

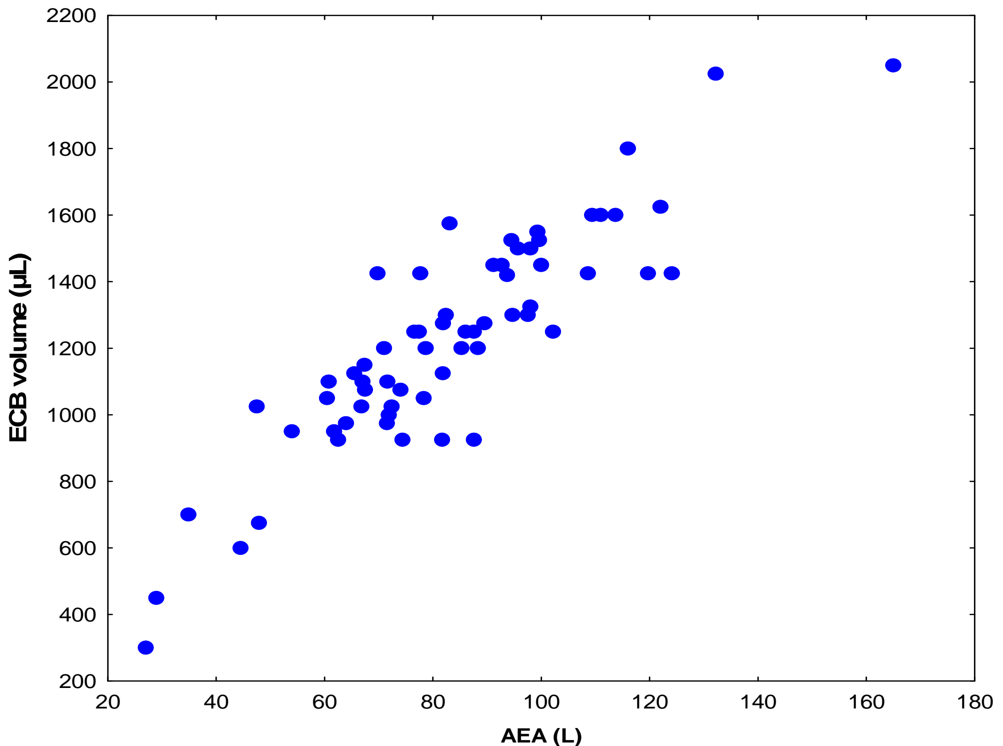


**Figure 1.** Volume of EBC versus accumulated volume of exhaled air (AEA) in 64 subjects [smokers, ex-smokers or healthy volunteers (paper IV)]. EBC was collected during 10 minutes (Spearman's rank correlation coefficient, Rs=0.85, p<0.001).

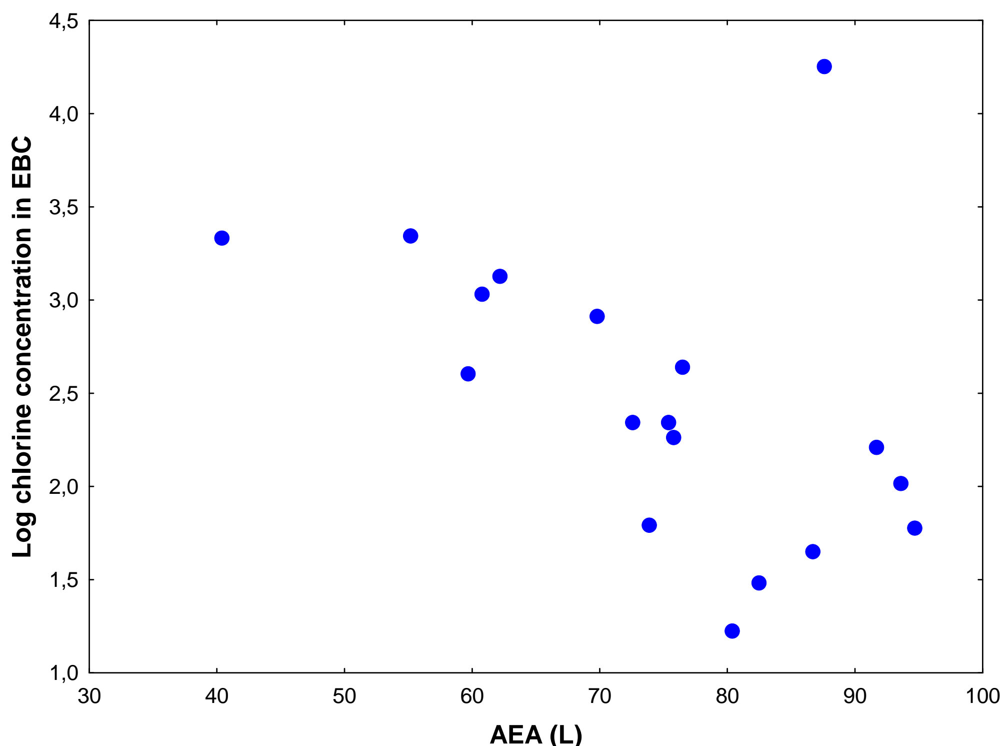


**Figure 2.** Chlorine concentrations in EBC versus accumulated volume of exhaled air (AEA) recorded in 10 healthy volunteers. EBC was collected by two different air flow rates (2 missing data). Air flow dependency was shown by an inverse correlation between concentrations of chlorine in EBC and AEA (Spearman's rank correlation coefficient, Rs=-0.60, p<0.01). [If one obvious outlier was excluded from evaluation; (Rs=0.81, p<0.001)].

## *In vitro study*

Temperature (measured by a thin thermistor, GTH 1200, Greisinger Electronic, Germany) in the condensing area of ECoScreen was approximately -2°C at start of condensation and it fell successively during ten or twenty minute's condensation and then reached -6°C and -14°C, respectively. Temperature in RTube was slightly below 0°C at the beginning of experiments and then increased successively, reaching +2°C and +7°C after 10 and 20 minutes, respectively. Volumes of condensates did not differ significantly between condensers during ten minutes of experiments (p>0.05) but in the longer experiments (20 minute condensations), volumes recovered by ECoScreen were significantly higher than those recovered by RTube (p<0.001). RTube was

significantly more efficacious than ECoScreen ($p<0.001$), when the lower concentration of saline solution was nebulized and condensed during 20 minutes (source concentration 6.4 mg/L). In contrast, using a higher concentration of the aerosol (source concentration 245 mg/L) there were no significant differences in concentrations of chlorine measured in condensates obtained by the two different devices, irrespective of nebulization-time (i.e. 10 or 20 minutes) or presence or absence of BSA.

Concentrations of MPO and HNL were higher in condensates recovered by ECoScreen than by RTube ($p<0.05$), but there was a large variability in concentrations of MPO or HNL condensates recovered by the two separate condensers. Spread of MPO retrieval ranged between 36% and 38% using ECoScreen and between 10% and 44% using RTube. Corresponding figures on spread of HNL retrieval ranged between 12% and 30% using ECoScreen and between 24% and 45% using RTube. Variability of chlorine tended to be lower and ranged between 19% and 26% using ECoScreen and between 19% and 23% using RTube

Washing the walls of the condensing and conducting systems with CTAB revealed adherence of MPO in all five lavage fluids tested [5.2 (1.7 – 10.1) μg/L], suggesting adherence of MPO to the exposed surfaces. In contrast, HNL was not measurable in any of the lavage fluids.
Degradation of 25% of HNL was noted at room temperature and degradation of MPO during similar conditions was only approximately 7%. The concentrations of MPO, as well as of HNL fell during nebulization by 43% and 13%, respectively. In contrast, the concentration of saline in the nebulizer cup increased 6-12% during 10 minutes of nebulization. Adhesion to surfaces, degradation of MPO or HNL and changes of concentrations in the nebulizer cup were used for calculation of percentage recoveries of these compounds. There were no significant differences between condensers in recovery of chlorine concentrations (range 29-43% vs. 26-40% for ECoScreen vs. RTube, respectively, $p>0.05$). Nor did we find any differences between devices in MPO concentrations (range 4-9% vs. 3-4% for ECoScreen vs. RTube, $p>0.05$). However, the recoveries of HNL tended to be higher when using ECoScreen (ranged 9-19% vs. 2-12% for ECoScreen vs. RTube, $p<0.05$).

# Asthma (paper II)

All allergic asthma patients attended at the laboratory twice: EBC, serum and lung function were recorded at visit 1, which occurred during a pollen season. All tests were repeated five months later (visit 2, off season). Levels of airborne pollen were "moderate to high" during the week around visit 1, as recorded by a pollen profile obtained from the Palyntological Laboratory, Swedish Museum of Natural History, Stockholm, Sweden.
Serum values of ECP (range 4-67 μg/L) were only moderately elevated above the reference range (2.3-16 μg/L). Lung function, as recorded by forced expirations, was also close to normal; merely seven out of 22 patients had $FEV_1$ ≤ 80% predicted normal value. In contrast, 13 patients complained of chest tightness or discomfort. Patients who had regular inhalations of ICS had lower concentrations of serum ECP than untreated patients [10 (4-36) μg/L vs. 20 (9-67) μg/L, p=0.014, median value (min-max)]. They also reported more symptoms from the chest, as documented by a higher scoring on a ten-graded visual analogue scale (VAS) [1 (1–4.5) vs. (0 (0–1), (p=0.04)]. Subjective ratings by means of a VAS, or as presence or absence of chest discomfort or recordings of $FEV_1$ were not associated to serum ECP values (p>0.05, all comparisons).

Chlorine was measurable in all EBCs obtained from the 22 asthma patients evaluated (one was withdrawn due to an abnormally high serum value of hs-CRP). There was a large spread of data on chlorine concentrations in EBC and the spread could not be explained by differences in recovery volumes or $V_{EA}$ (Rs=0.10, n.s., versus Rs=0.13, n.s.). Concentrations of chlorine in EBC obtained during visit 1, ranged from 5.1 μM to 84.2 μM and were significantly higher than those recorded in healthy subjects, as given in paper I (range 3.4-28.3 μM, p<0.01), or in paper IV (range 3.4-24 μM, p<0.001 ). There were no statistically significant associations between concentrations of chlorine in EBC and any lung function tests during the pollen season. Although close to the lower detection limit, values of CysLT, as well as histamine were recorded in all but three condensates collected at visit 1 (Table 3).

Two outliers were recorded in visit 2 and these samples contained around five or ten times higher chlorine concentrations than in the remaining EBCs. Saliva contamination was a likely cause and these samples were excluded from all evaluations. Longitudinal comparisons of recorded data were therefore performed in 20 subjects and significant post-seasonal reductions in concentrations of chlorine in EBC (p=0.004), as well as of serum ECP (p=0.004)

were recorded. Serum concentrations of ECP decreased in all but two patients: one of them increased and another diminished their daily doses of ICS due to changes in subjectively perceived chest tightness. Concentrations of chlorine in EBC decreased in all patients except for three; none of them had ICS treatment. There were no statistically significant improvements in lung function tests, or levels of CysLT or histamine in EBC collected during visit 2 relative to those recorded at visit 1 ($p>0.05$ all comparisons, Table 3). However concentrations of CysLT, as well as histamine were low; and values of CysLT were below LOD in three EBCs taken during visit 1 and in nine EBCs taken during visit 2, and histamine concentrations were below LOD in three samples taken at each one of the visits.

Although the number of participating asthma patients was low, and hence also the statistical power, the accuracy of the diagnostic tests in identifying asthma exacerbations during a pollen season was tested. This was done by means of comparisons of ROC curves and AUCs of ROC curves. Exacerbations of asthma were defined as either a decrease in forced expirations ≥20% or presence or absence of subjectively perceived symptoms from the chest. Since only minor changes were recorded in $FEV_1$, a deviation of $MEF_{50}$ or symptom score was used as a reference in comparison of ROC curves. Serum values of ECP and concentration of chlorine in EBC exhibited the largest AUCs changes of $MEF_{50}$, thus being the standard, AUC for serum ECP was 0.78 (0.56 to 0.93) and AUC for chlorine in EBC 0.78 (0.54- 0.93).

**Table 3.** Data obtained from 22 patients with allergic asthma, during a pollen season and at a follow-up visit 5 months later. During a follow-up visit 5 months post season, were two EBC samples excluded from evaluation due to presumed contamination. Levels of CysLTs were above LOD in 19 condensates sampled during pollen season and in 11 collected after the season. Histamine concentrations in EBC were above LOD in 19 samples collected during the pollen season and 17 samples collected after season. $V_{EA}$=volume of exhaled air. Median values (min-max) are given, Wilcoxon-signed rank tests for paired data was used and statistically significant differences of values recorded during after pollen season are indicated by ** p < 0.01.

| | **Pollen season** | **Post-season** |
|---|---|---|
| ECP in serum (µg/L) (n=22/22) | 13.0 (4-67) ** | 5.9 (2.5–22) |
| Chlorine in EBC, (µM) (n=22/20) | 29.1 (5.2–84.2) ** | 14.7 (6.2–52.6) |
| CysLTs in EBC, (pg/ml) (n=19/11) | 10.5 (7.4-13.5) | 10.6 (7.0-25.6) |
| Histamine in EBC, (nM) (n=19/17) | 5.5 (2.8-12.1) | 4.9 (2.9-9.9) |
| Recovered EBC volumes (mL) | 1.1 (0.5-2.0) | 1.1 (0.6–2.0) |
| $V_{EA}$ (L) | 81 (44-170) | 88 (42-205) |
| $FEV_1$ (% predicted) | 92 (19-112) | 95 (65-111) |
| $MEF_{50}$ (% predicted) | 66 (11-114) | 67 (32-150) |

# COPD (paper IV)

EBCs taken from patients with COPD contained higher concentrations of chlorine than those taken from healthy volunteers ($p<0.05$, table 4) and levels were not significantly different from values recorded in non-COPD smokers or ex-smoker. Levels of chlorine measured in reference subjects were in the same range as those previously recorded in healthy subjects (range 3.4-28.3 µM in paper I). Ventilation as assessed by EcoVent while collecting EBC did not differ in the separate groups of subjects (i.e. COPD, non-COPD and healthy volunteers). While there was a close association between ventilation and recovered EBC volumes ($R_s$=0.85, n=64 $p<0.001$), there was no association between ventilation and chlorine concentrations ($R_s$=-0.04, n.s.). Alpha-amylase was not detected in any of the EBCs, not even in the one outlier with a high value of chlorine (72.6 µM), which might suggest salivary contamination. Neither lysozyme nor MPO or ECP was detected in any of the EBCs obtained

from patients or volunteers, whether measurements were done on fresh frozen condensate or after lyophilisation.

Although serum values of hs-CRP exceeded the upper reference range in five of 19 COPD patients, there was no statistically significant difference relative to values recorded in any of the study groups, suggesting comparable activity of the systemic defence system. Lysozyme concentration in serum was significantly higher in COPD patients than in volunteers, as well as in non-COPD subjects ($p<0.01$ and $p<0.05$, table 4). Raised levels of lysozyme was not accompanied by increases in serum values of ECP or MPO, the latter ones were within their reference ranges, with the exception of two ECP and one MPO outliers. Furthermore serum concentration of lysozyme was significantly higher in subjects with abnormally low DLCOc than in those with normal diffusion capacity [1373 μg/L (1125-3117 μg/L) vs. 1083 μg/L (492-184 μg/L), $p=0.01$].

Chlorine, ECP as well as lysozyme were assessed in saliva and the spread of data of these compounds was large; more than a 1000 fold difference in highest to lowest value of salivary lysozyme values were recorded. The spread of ECP data tended to be less pronounced (140 fold) and the highest value of chlorine was nine times higher than the lowest one. The large spread in lysozyme concentrations in saliva was mainly explained by two outliers recorded in a non-COPD patient and a volunteer but, even after exclusion of these two sets of data, the spread of data was significant. While there were no major outliers in ECP data, one chlorine value described most of the large variability in chlorine values (COPD patient). There was no association between values of ECP and lysozyme in saliva, data that might suggest different sources, influx and/or degradation rates of these markers in saliva. Apart from elevated values of lysozyme in saliva from COPD patients relative to non-COPD patients, there was a trend for lower values of ECP, as well as of chlorine in saliva obtained from COPD patients than from non-COPD smokers or ex-smokers ($p<0.01$ vs. $p=0.07$).

Serum concentration of lysozyme correlated to spirometry data, i.e. $FEF_{50}$ ($R_s=-0.33$, $p<0.01$) and although not statistically significant to $FEV_1$ ($p=0.17$) and DLCOc ($R_s=-0.24$, $p=0.16$). ICS treatment and smoke cessation are obvious confounding factors. It was noted that ICS treated subjects were older ($p=0.007$), had significantly lower spirometry values expressed as percentage of normal predicted value ($p=0.01$), as well as lower values of DLCOc ($p<0.05$).

Inflammation markers did not differ from values recorded in non treated patients, suggesting suppression of inflammation markers by ICS. Except for the finding that ex-smokers were older ($p<0.01$) than those who continued to smoke, there were no significant differences in lung function (expressed as percentage of predicted normal value), or in inflammation markers measured in the group of ex-smokers and currently smoking patients. The fact that inflammation markers did not differ significantly between ICS treated and non-treated, or ex-smokers and smokers, allowed us to handle data irrespective of drug treatment or smoking cessation data.

We also evaluated data recorded in 35 smokers and ex-smokers who had attended a lung function laboratory five years prior to this study, with the aim of elucidating whether any variable could discriminate between those who had signs of progressive disease and those with a stable lung function. It was postulated that those who had a proven 5-year decrement of $FEV_1$ ≥0.35L were considered as "rapid decliners". Despite trends, there were no statistically significant differences in any of the data recorded in "rapid decliners" and "non-rapid decliners" ($p>0.05$ all comparisons).

Although DLCO was assessed merely in a limited number of patients with COPD (with consequent low statistical power), ROC analyses of possible discriminators of COPD were performed with the aim of finding a test usable in clinical practice. DLCOc ($AUC_{ROC}$ 0.85) but no other test, except serum concentration of lysozyme ($AUC_{ROC}$ 0.76) was capable of discriminating between patients with COPD and non-COPD. Although merely second-rate, the highest value of $AUC_{ROC}$ in discriminating between "rapid decliners" and "non rapid decliners" was recorded for chlorine concentrations in EBC ($AUC_{ROC}$ 0.69) and serum MPO ($AUC_{ROC}$ 0.66).

**Table 4** Results of analyses conducted in EBC, serum and saliva, from 19 patients with COPD, 30 non-COPD patients and 15 healthy volunteers. Data are presented as median value (min-max). Mann Whitney U-tests were used in statistical evaluations. Statistically significant differences are indicated by the number of symbols; one symbol signifies $p<0.05$, two symbols signifies=$p<0.01$. ¶=COPD vs. non-COPD, Ω=COPD vs. healthy volunteers, Φ=non-COPD vs. healthy volunteers

| | **COPD** | **non-COPD** | **healthy vol.** |
|---|---|---|---|
| n | 19 | 30 | 15 |
| Smokers/ex-smokers | 8/11 | 23/7 | 0/0 |
| **EBC** | | | |
| Chlorine (μM) | 13.3 (6.8-40) Ω | 10 (3.6-72.6) | 7.8 (3.4-24) |
| Amylase (U/L) | - | - | - |
| **Serum** | | | |
| Lysozyme (μg/L) | 1350 (863-3117) ¶, ΩΩ | 1119 (492-1842) | 1020 (603-1410) |
| MPO (μg/L) | 74 (33-253) | 76 (29-659) | 105 (41-162) |
| ECP (μg/L) | 5 (2-29) | 5 (2-17) | 6 (2-13) |
| hs-CRP (mg/L) | 2 (0.4-14) | 1.3 (0.3-10) | 1.2 (0.6-3.6) |
| **Saliva** | | | |
| ECP (μg/L) | 44 (2-214)¶¶ | 106 (2-282) | 64 (7-270) |
| Lysozyme (μg/L) | 3333 (15-10094) Ω | 1206 (15-14816) Φ | 6292 (79-15866) |
| Chlorine (mM) | 13.1 (6.8-56) | 16.8 (6.4-24.4) ΦΦ | 11.3 (6.8-22.0) |

# 5 DISCUSSION

Chlorine concentrations in EBC were three times higher when collected by means of RTube than by ECoScreen. The same trend was observed in the in-vitro study (paper III), when low concentrations of saline were nebulized. Differences in efficacy between the two condensers can not easily be explained, and although confounders such as contamination by saliva, in addition different condensing temperatures, different surface characteristics and different designs of devices are likely factors influencing the results. Salivary contamination of EBCs is a possible cause of differences in efficacy of the condensers. We found concentrations of chlorine in saliva obtained from healthy volunteers (11.3 mM) at similar levels as previously published (10 mM)[177]. A small droplet of saliva may contain 20 nmol of chlorine[178] and such an addition may influence the content of chlorine in EBC. Although both condensers have a saliva trap, the one in ECoScreen is small and if there is an increased salivary flow, overflow may occur and saliva may enter the condenser. For this reason, it may be more likely that saliva would enter an ECoScreen by gravitational forces than an RTube with an upright vertical position of the outflow tract. It was noted in a study by Gaber *et al*. that the addition of 0.5-1% saliva gives rise to increased $\alpha$-amylase in EBC[145]. We could not detect $\alpha$-amylase in any of the tested EBC samples, not even in those with the highest chlorine levels, although we used the same method of analysis that Gaber et al did, albeit we used an even lower limit of detection (LOD 0.008 U/mL vs. 0.078 U/mL).

Condensing temperature is higher in RTube than in ECoScreen and this may affect the outcome, a notion that is supported by observations on condensation of HNL and MPO, since we found concentrations of MPO and HNL to be higher in condensates recovered by ECoScreen than by RTube. A high temperature in the condenser may have effects on specific markers and lead to degradation and/or loss of unstable markers[126]. Progressive increases of the amount of $H_2O_2$ in EBC has been shown when cooling temperature was decreased from +5 to -10°C[125]. Apart from varying degree of degradation of both MPO and HNL, we found signs of adherence of MPO to exposed surfaces of the devices during condensation. Adherence of various molecules to collecting surfaces of condensers has previously been demonstrated and coating of all plastic surfaces with BSA or Tween 20 before EBC sampling to

reduce adherence of fatty acids derivates and proteins was suggested[124]. Despite the fact that we added BSA to the nebulised solutions of MPO or HNL, in order to stabilize these solutes, we found that variability of our biomarkers in EBC was high. However we did not coat the collection cups with BSA or Tween, proceedings that might have limited adhesion to surfaces.

Given that subjects exhaled with similar airflow rates, a wider condensing cylinder of RTube might result in lower air flow rate, within the collecting tube relative to a narrower ECoScreen, while higher airflow rate and possibly turbulent air flow might occur in the narrower condensing tube of ECoScreen. Condensation of aerosols on cold surfaces may be a time dependent process and lower airflow rates may promote condensation, a higher air flow rate, on other hand, may be counteracted by turbulence, supposed to occur in ECoScreen. Each marker has its own chemical and physical properties and RTube is the most suitable device for condensing chlorine, as shown in our studies. In contrast to the inflammatory cell markers, chlorine is stable and not degraded by a high temperature. Two-way ANOVA also confirmed that there was a statistically significant difference between the condensers concerning concentration recovery of chlorine. It was also verified that no significant interaction exists and that the residuals do not have significantly different variance in the two condensers.

We found significant associations between accumulated volume of air passing the spirometer attached to the outflow tract of the condenser and EBC volumes in all clinical studies (papers I, II and IV). Diverging results for the association between ventilated air and chlorine concentrations in EBC were noted; while there was an inverse association between the size of ventilation and chlorine levels in healthy volunteers (paper I), such an association was not obvious in papers II and IV. We therefore conclude that airflow dependent volume but not concentration recoveries occurred, supporting the notion that increased evaporation from ELF occurs with increased ventilation. These data support previously published data[129,179].

Taking all data on chlorine concentrations in EBC together, it is concluded that the highest values of chlorine in EBC were recorded in asthma patients in the middle of a pollen season. The fall in chlorine concentrations, subjectively scored symptoms from the chest, as well as reduced serum values of ECP is liable to confirm the clinical relevance of the data on chlorine in EBC obtained from the allergic asthmatics. All scored their symptoms by VAS, and merely

thirteen patients complained of chest tightness or discomfort during the pollen season. As anticipated, there were no significant associations between scoring of chest symptoms and lung function tests or serum values of ECP[180,181]. While forced expiration (i.e. $FEV_1$) measures airflow, subjectively perceived symptoms such as chest discomfort or dyspnoea reflect more complex experiences or events and may not necessarily relate to restriction in airflow as recorded by spirometry. Our data thus confirm previous findings of poor correlation between patients' reported symptom score and $FEV_1$[31]; while some asthmatics may overestimate their symptoms, others may deny all respiratory discomfort[31]. There are also reports showing that patients with mild asthma are less likely to report their symptoms[182]. Twelve of our asthma patients had daily doses of inhaled corticosteroids. Corticoid treatment normally reduces ECP levels in serum[183], and is also known to induce apoptosis of eosinophils and down regulate production of pro-inflammatory markers, such as ECP[184]. Treatments with ICS can also increase perception of chest discomfort in subjects with asthma[185]. We found that those of our asthma patients who inhaled daily doses of corticosteroids during the pollen season reported worse VAS values than those who did not use ICS treatment. They also had significantly lower levels of ECP in serum than patients who did not use ICS treatment. ECP is a potent cytotoxic protein released in increased amounts from activated eosinophils during subacute or chronic asthma[186]. ECP also has the capacity to stimulate mucus secretion and histamine release[187,188]. We were not able to assess ECP in EBC, despite several trials and a potential association between local eosinophil activity state and chlorine in EBC, presumably secreted from mucosal glands. Although this was not possible to evaluate, it would seem likely that local eosinophils, as well as mast cells may have been activated during the pollen season.

Trials to measure CysLT or histamine in EBC, key elements in allergic inflammation, were merely partly successful, despite the fact that both CysLTs and histamine are responsible for most of early and late physiological responses to allergen provocation[189]. Although detectable in EBC, concentrations of CysLT or histamine were close to or below LOD, and furthermore the concentrations were more often below LOD in the second visit five months after pollen exposure, suggesting that concentrations were even lower after season than during season. Measurable histamine concentrations in EBC have been reported and were non-changing, as opposed to levels of CysLT, in conjunction with a challenge using inhaled AMP[141], which is known to mimic a natural challenge in allergic asthma patients[190].

Despite the fact that histamine concentrations were assayed with another, presumably more sensitive method[141] than we used, concentrations were in the same concentration range as in our study, supporting the notion that histamine is measurable in EBC but at a level that is too low to allow firm conclusions. These data taken together thus tend to support the notion of a coincidentally mild natural pollen challenge during the study period, also confirmed by low subjective scoring, the near normal spirometry data, as well as serum ECP values and furthermore low exposition as recorded by a Palyntological laboratory (though not situated in the geographical vicinity of patients during the natural challenge).

The partial failure to measure CysLT and histamine in EBC at concentration levels well above LOD, may have occurred as a consequence of adhesion of these compounds to surfaces of the condenser (ECoScreen). We did not at the time of this study suspect adhesion of inflammatory cell markers to condenser surfaces and consequently lavage of condenser surfaces by means of CTAB or other fluids, in order to estimate potential adhesion of the markers, was not done. It has been shown in this thesis (Paper III) that MPO adhered in a substantial amount to condenser walls. Adherence of various substances, such as fatty acids derivates and proteins to condenser surfaces have been reported even though surfaces were coated with detergent[191]. In contrast, it has also been shown that coating surfaces with Tween 20 or BSA before EBC sampling reduced adherence[124].

It is proposed that eosinophil related increases of airways inflammation, as mirrored by increases in serum values of ECP - albeit of limited intensity, may be responsible for elevations of chlorine in EBC in our asthmatics, possibly due to, or associated with, increases of mucous production, a typical feature of asthma[187,188]

The second highest values of chlorine in EBC were recorded in COPD patients. While asthma patients had near normal spirometry values, patients with COPD had significantly reduced forced expirations. It would therefore seem unlikely that increased airway tonus per se would result in elevated chlorine levels. Theoretically, air flow rate would increase when passing an obstructed part of conducting airways, and thus airways obstruction per se would result in higher driving force of vapour and droplet originating from ELF. This is matched with findings of higher particle/droplet concentration in exhaled air

recorded during exercise (with higher minute ventilation) than during calm tidal breathing[118].

To test the assumption that EBC originated mainly from proximal airways and using chlorine as a potential marker of mucosal gland secretion, a resistance of 5 cm $H_2O$ was added to the exhalation circuit of the condenser (ECoScreen) in some of our asthma patients (paper I). Physiological effects of addition of an exhalation resistance in the outflow tract include increase of airway mean area by opening up of atelectases, presumed to be present in our asthma patients with more severe affection of the airways[192,193]. A pressure of 5 cm $H_2O$ is unlikely to harm the respiratory tract or affect venous return to the heart[194]. Increased volume of EBC resulted from the addition of exhalation resistance in those who had the most constricted airways, but chlorine concentrations merely tended to decrease and the spread of data was large. Low statistical power may have resulted in failure to prove by statistical means a significant change in chlorine concentration in EBC. Altogether this may suggest chlorine to be more abundant close to mucosal glands in the trachea than in peripheral airways as also shown by a higher Cl/Na ratio in trachea than in peripheral airways[74]. Although distal airways have a much larger surface area than the proximal ones, suggesting that aerosol particles are likely to be recruited by evaporation, airflow rate is much lower in distal than in proximal airways and this may restrict the contribution of aerosols originating from the distal airways. Although the statistical power of our experiments was low, we suggest that our data tend to confirm the assumption that chlorine in EBC originates from larger airways, presumably from mucosal glands.

High concentrations of chlorine in EBC from patients with COPD (whether smokers or ex-smokers) may result from an alternative type of inflammation, as previously seen by biopsy findings, indicating an inflammatory profile favouring eosinophils in asthma while neutrophils and macrophages are abundant and activated in COPD[195,88,196]. It is known that smoking per se can induce inflammation[197] and that patients with COPD generally have higher serum levels of CRP than non-COPD subjects[198], as well as other signs of systemic inflammation being present in subjects with severe COPD[199,200]. Tobacco smokers have higher concentrations of MPO, lysozyme and HNL in serum than never smokers, and this may reflect an increased systemic activity of circulating monocytes and/or neutrophils[201]. We found that levels of lysozyme in serum but no other biomarker tested were significantly higher in COPD subjects relative to non-COPD subjects. Lysozyme is also secreted from

submucosal glands to help protection of the lung against bacterial infections[7], and lower levels of lysozyme can also be released by airway epithelial cells[202]. Despite abundance of lysozyme in bronchial glands, we could not measure this protein in EBC.

We found that our non-COPD patients, irrespective of current smoking habits, had spirometry recordings mainly within normal limits and only moderately increases of chlorine in EBC. Increased mucous secretion of bronchial glands is a common feature in exacerbating asthma, as well as in COPD and increases of chlorine may well be a result of enhanced mucous secretion, although we did not confirm this notion by biopsy findings or other hard data. Chlorine is, however, one of the most typical secretory products originating from bronchial glands and eosinophils may be a strong initiator of mucous secretion[203] together with a number of other inciting stimuli.

Chlorine in EBC may also unintentionally have been added by contamination from saliva droplets during collection of EBC. Saliva holds an approximately 1000 times higher concentration of chlorine than EBC, implicating that addition of merely a minimal amount of saliva would have a large impact on EBC concentrations. In spite of the fact that condensers are constructed to avoid saliva contamination, there is an obvious risk and although repeatability of chlorine measurements were quite acceptable with a coefficient of intra-assay variability <10%, we found a wide range of intra-subject variability in healthy volunteers and differences between duplicate measurements ranged between 2% and 66%. A reasonable explanation of the wide spread of these data is uncontrolled and unintentional contamination. Such uncontrolled contamination tends to invalidate EBC as a useful tool in research or clinical practice. Provided that EBC is collected without any salivary contamination, however the method may be a very useful tool in research. Comparisons of protein profiles in saliva and condensates have revealed the presence of additional proteins in EBC, that are likely to be transported in the form of aerosols from the lower respiratory tract[204]. Several proteins normally found in a high quantity in saliva were not detected in EBC, while other proteins occurring in EBC were missing in saliva[177]. It has been shown that the EBC spectra significantly differ from saliva, as assessed by nuclear magnetic resonance spectroscopy (NMR), and that NMR showed no presence of saliva signals when the EBC was collected by a condenser associated with saliva trap[205].

We also investigated whether saliva might be a useful tool in discriminating between COPD and volunteers or non-COPD smokers and ex-smokers. We found concentration data in saliva partly contradicting with ECP levels being significantly lower in saliva obtained from COPD as compared with non-COPD patients and lysozyme levels tending to be higher, while lysozyme levels in volunteers were higher than in patients. Strong binding between acid residues of carbohydrate chains of mucins and lysozyme was found when analysing sputum taken from a patient with chronic bronchitis[206]. Such strong binding may interfere with analysis of lysozyme in mucin-rich secretion, such as sputum or saliva. We did not assess viscosity of our saliva samples and one possible interpretation of our data may include such binding in a few of our saliva samples. Lower than normal values of CysLT have previously been recorded in saliva obtained from patients[145] and we suspect interaction with mucous, microscopic lacerations in oral mucosa or other causes. We conclude that interpretation of concentration data in saliva is, at present, not feasible.

Despite low statistical power in this study, we sought for an association between the clinical situation during the studied pollen season and markers of inflammation, and a ROC analysis was chosen as a tool. We postulated that either presence or absence of symptoms (i.e. a dichotomous scale) or significant decreases of lung function reflected the clinical situation during this pollen season. While only a few values of $FEV_1$ were below the stated lower reference range (i.e. a 20% decrease from predicted normal value), 17 of the values on $MEF_{50}$ were less than 80% of normal reference values. Despite awareness of the fact that a decrease of merely 20% of $MEF_{50}$ reflects a minimal change in forced expirations that may be within measurement precision and despite the fact that the statistical power was low, we performed ROC analyses to test the capacity to identify a pollen induced asthma exacerbation, by levels of chlorine in EBC or ECP levels in serum. Of all tested surrogate markers, chlorine in EBC and serum concentrations of ECP had the highest capacity to discriminate. The association between concentrations of chlorine in EBC and response to mild pollen exposure in mild allergic asthma suggest that chorine measurements in EBC were relevant and reflected a clinical situation. This assumption is supported by the finding of an $AUC_{ROC}$ of 0.78 for serum ECP concentrations as well as of chlorine concentration in EBC to discriminate between presence and absence of an allergic bronchial response. Although our data in smokers and ex-smokers are harder to interpret, due to a number of confounding factors, we suggest that measurements in this patient group tend to favour traditional tools for

discrimination of COPD patients; an AUC $_{ROC}$ of 0.85 for DLCOc and 0.76 for serum concentration of lysozyme, tend to confirm the assumption of physiological tools as superior to measurements of chlorine in EBC in this patient group. Inflammatory processes in the airways and lung parenchyma may be best reflected by measurements of DLCOc.

# 6 GENERAL CONCLUSION

## *It is concluded*

- That measurement of chlorine is acceptable as a marker in comparison of efficacy and reproducibility of EBC. Despite low concentrations in EBC, chlorine may be analysed with acceptable reproducibility by means of a modified AOX method.

- That there were signs of flow dependency (in concentrations; paper I, in volumes; papers I, II and IV), temperature dependency (when collection times exceeded ten minutes), substance dependency (adhesion of MPO to surfaces) and concentration dependency (chlorine two different concentrations; paper III)

- That despite acceptable analytical precision, the biological variability limits the feasibility of the EBC method.

- That readiness of every separate inflammation marker to adhere to condenser surfaces should be tested prior to commencement of clinical studies.

- That chlorine levels in EBC were clearly elevated despite mild pollen exposure, suggesting chlorine concentrations to be a sensitive marker of allergic airway inflammation.

- That interpretation of EBC data obtained from smokers and ex-smokers is hampered by the abundance of confounding factors and traditional tools appear to be best in discriminating between COPD and non-COPD. A low value of DLCOc, as well as a high level of lysozyme in serum discriminated best between COPD and non-COPD subjects.

- That there are a number of possible sources of error associated with the EBC technique, such as the need for assays with higher sensitivity.

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