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REVIEW

Biomarkers of some pulmonary diseases in exhaled breath

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Analysis of various biomarkers in exhaled breath allows completely non-invasive monitoring of inflammation and oxidative stress in the respiratory tract in inflammatory lung diseases, including asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), bronchiectasis and interstitial lung diseases. The technique is simple to perform, may be repeated frequently, and can be applied to children, including neonates, and patients with severe disease in whom more invasive procedures are not possible. Several volatile chemicals can be measured in the breath (nitric oxide, carbon monoxide, ammonia), and many non-volatile molecules (mediators, oxidation and nitration products, proteins) may be measured in exhaled breath condensate. Exhaled breath analysis may be used to quantify inflammation and oxidative stress in the respiratory tract, in differential diagnosis of airway disease and in the monitoring of therapy. Most progress has been made with exhaled nitric oxide (NO), which is increased in atopic asthma, is correlated with other inflammatory indices and is reduced by treatment with corticosteroids and antileukotrienes, but not (β_2-agonists. In contrast, exhaled NO is normal in COPD, reduced in CF and diagnostically low in primary ciliary dyskinesia. Exhaled carbon monoxide (CO) is increased in asthma, COPD and CF. Increased concentrations of 8-isoprostane, hydrogen peroxide, nitrite and 3-nitrotyrosine are found in exhaled breath condensate in inflammatory lung diseases. Furthermore, increased levels of lipid mediators are found in these diseases, with a differential pattern depending on the nature of the disease process. In the future it is likely that smaller and more sensitive analysers will extend the discriminatory value of exhaled breath analysis and that these techniques may be available to diagnose and monitor respiratory diseases in the general practice and home setting.

Keywords: airway inflammation, oxidative stress, nitric oxide, carbon monoxide, exhaled breath condensate, non-invasive markers, asthma, chronic obstructive pulmonary disease, cystic fibrosis, bronchiectasis, interstitial lung diseases, hydrogen peroxide, eicosanoids, products of lipid peroxidation, proteins, cytokines.

Introduction

Analysis of breath constituents is a novel non-invasive way of monitoring inflammation and oxidative stress in the lungs (Kharitonov and Barnes 2001). Although most studies have focused on exhaled nitric oxide, recently several other volatile gases, including carbon monoxide, ethane and pentane, have also been used. In addition, several endogenous substances (inflammatory mediators, cytokines, oxidants) may be detected in expired breath condensates, opening up new perspectives for exhaled breath analysis.

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Many lung diseases, including asthma, chronic obstructive pulmonary disease (COPD), bronchiectasis, cystic fibrosis (CF) and interstitial lung disease, involve chronic inflammation and oxidative stress. However, these are not measured directly in routine clinical practice because of the difficulties in monitoring inflammation. In asthma fibreoptic bronchial biopsies have become the 'gold standard' for measuring inflammation in the airway wall, but this is an invasive procedure that is not suitable for routine clinical practice and cannot be repeated often. It is also unsuitable for use in children and patients with severe disease. Symptoms may not accurately reflect the extent of underlying inflammation due to differences in perception and masking by bronchodilators in airway disease. In asthma, measurement of airway hyper-responsiveness by histamine or methacholine challenge has been used as a surrogate marker of inflammation, but interpretation may be confounded by the use of bronchodilator therapy. Furthermore, it is difficult to perform this measurement in children and in patients with severe disease. This has led to the use of induced sputum to detect inflammation. This method is relatively reproducible and allows the quantification of inflammatory cells and mediators (Parameswaran *et al.* 2000). However, this technique is somewhat invasive as it involves inhalation of hypertonic saline, which may induce coughing and bronchoconstriction, and it is difficult to use in small children. Furthermore, the technique itself induces an inflammatory response, so that it is not possible to repeat measurements in less than 24 h (Nightingale *et al.* 1998).

The need to monitor inflammation in the lungs has led to the investigation of exhaled gases and condensates. Non-invasive monitoring may assist in differential diagnosis of pulmonary diseases, assessment of disease severity and response to treatment. Because these techniques are completely non-invasive, they can be used repeatedly to give information about kinetics, they can be used in patients with severe disease, which has previously been difficult to monitor, and they can be used in children, including infants. Breath analysis is currently a research procedure, but there is increasing evidence that it may have an important place in the diagnosis and management of lung diseases in the future (Kharitonov and Barnes 2000a). This will drive the development of cheaper and more convenient analysers, which can be used in a hospital and later in a general practice setting, leading eventually to the development of personal monitoring devices for use by patients.

Nitric oxide

Nitric oxide (NO) is the most extensively studied exhaled marker, and abnormalities in exhaled NO have been documented in several lung diseases (Kharitonov and Barnes 2000a), particularly asthma (Gustafsson 1998, Kharitonov 1999a, Kharitonov and Barnes 2000a). Exhaled NO measurements have been standardized in both adults and children (Kharitonov *et al.* 1997a, Anonymous 1999).

Measurement

Expiratory flow, soft palate closure and dead space air may all influence exhaled NO levels. Therefore exhaled NO is usually determined during single-breath exhalations against a resistance (Gustafsson *et al.* 1991, Kharitonov *et al.* 1994b,

1997a, Massaro *et al*. 1995) to prevent contamination with nasal NO (Kharitonov and Barnes 1997, Silkoff *et al*. 1997), or using reservoir collection with discarding of the dead space (Paredi *et al*. 1998). However, this method has proven difficult for some children, who may have trouble maintaining a constant flow, and recently a simple flow-driven method for online NO measurements has been developed that does not require active patient co-operation (Baraldi *et al*. 2000). Recently, single breath analysis of exhaled NO has been successfully performed in the newborn; exhaled air was sampled from the tip of a thin nasal catheter placed in the hypopharynx (Artlich *et al*. 2001). The most commonly used method to measure nasal NO is to sample nasal air directly from one nostril using the intrinsic flow of the chemiluminescence analyser (Lundberg and Weitzberg 1999). A novel method of measuring exhaled NO at several exhalation flow rates has recently been described that can be used to approximate alveolar and airway NO production (Lehtimaki *et al*. 2000). NO is continuously formed in the airways. Mixing during exhalation between the NO produced by the alveoli and the conducting airways explains its flow dependency (Silkoff *et al*. 1997) and accumulation during breath-holding (Kharitonov *et al*. 1996b). A relatively simple and robust two-compartment model of NO has been developed that is capable of simulating many important features of NO exchange in the lungs (Tsoukias and George 1998). The model assumes that the lung consists of two well-defined, separate regions: a rigid airway compartment and a well-mixed, expansile alveolar compartment. Both compartments seem to contribute to exhaled NO, and the relative contributions of each seem to be a function of minute ventilation (Tsoukias and George 1998). Finally, the model suggests that the relationship between exhaled NO at end-exhalation may be a simple, effective and reproducible technique for determining the relative contribution of the airways and alveoli to exhaled NO.

It is therefore important to register the flow rate if NO is expressed as a concentration. The flow rate recommended in 1997 by a Task Force of the European Respiratory Society is 10–15 l min^{-1} or 167–250 ml s^{-1} (Kharitonov *et al*. 1997a). Most authors have used about 100 ml s^{-1}, but a more recent recommendation from the American Thoracic Society suggests a flow rate of 50 ml s^{-1} (Anonymous 1999).

Factors affecting exhaled NO measurements

Exhaled and nasal NO in healthy subjects is independent of age, gender and lung function (Baraldi *et al*. 1999a, Ekroos *et al*. 2000). There is no evidence for significant diurnal variation (ten Hasken *et al*. 1998), and exhaled NO measurements are highly reproducible in normal subjects (Bartley *et al*. 1999, Purokivi *et al*. 2000). Different phases of the menstrual cycle may influence exhaled NO (Kharitonov *et al*. 1994a), as oestrogen activates nitric oxide synthase-3 (NOS3) in airway epithelial cells (Kirsch *et al*. 1999).

There are several major factors that can affect NO levels in normal subjects. Intravenous, inhaled or digested L-arginine, the substrate for NOS, increases exhaled NO levels in normal subjects (Kharitonov *et al*. 1995a, McKnight *et al*. 1997, Sapienza *et al*. 1998). Conversely, nebulized L-NMMA and L-NAME, non-specific inhibitors of NOS, reduce exhaled NO (Kharitonov *et al*. 1994b, Yates *et al*. 1995) and nasal NO (Holden *et al*. 1999, Sippel *et al*. 1999). Some routinely used tests can transiently reduce exhaled NO, for example, repeated spirometry

(Silkoff *et al.* 1999, Deykin *et al.* 2000), physical exercise (Phillips *et al.* 1996) and sputum induction (Piacentini *et al.* 2000b). Environmental factors such as NO, ozone and chlorine dioxide are know to increase exhaled NO levels (Nightingale *et al.* 1999, Olin *et al.* 1999, van Amsterdam *et al.* 1999b). Habitual factors such as smoking (Kharitonov *et al.* 1995c, Robbins *et al.* 1996) and alcohol ingestion (Persson and Gustafsson 1992, Yates *et al.* 1996a) reduce exhaled NO. Upper respiratory infection significantly increases exhaled NO (Kharitonov *et al.* 1995e, Murphy *et al.* 1998) and nasal NO (Ferguson and Eccles 1997).

Asthma

Increased levels of exhaled NO have been widely documented in patients with asthma (figure 1) (Kharitonov *et al.* 1994b). The increased levels of exhaled NO in asthma are predominantly of lower airway origin (Kharitonov *et al.* 1996b) and are most likely due to activation of NOS2 in airway epithelial and inflammatory cells (Hamid *et al.* 1993, Saleh *et al.* 1998) (figure 2). However, there may be a small contribution from NOS1, as polymorphisms of the NOS1 gene correlate with exhaled NO (Wechsler *et al.* 2000).

Exposure to proinflammatory stimuli. Elevated NO levels in atopic subjects (Adisesh *et al.* 1998, Frank *et al.* 1998) are further increased as a result of controlled allergen exposure (Kharitonov *et al.* 1995b), during the grass pollen season (Baraldi *et al.* 1999b), or during exposure to indoor allergens (Simpson *et al.* 1999, Piacentini *et al.* 2000a). Exhaled NO may represent a useful biomarker of individual exposure to air pollutants, as even healthy subjects may have elevated exhaled NO levels on days with high outdoor air pollution (Steerenberg *et al.* 1999, van Amsterdam *et al.* 1999b). This may reflect an airway inflammatory response to ozone and nitrogen dioxide (Jenkins *et al.* 1999).

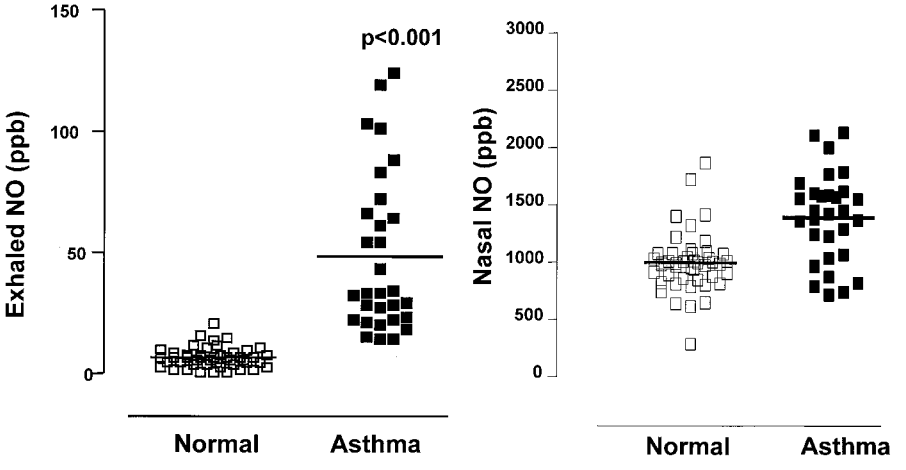

Figure 1. Exhaled NO (A) and nasal NO (B) in normal subjects and patients with asthma (Kharitonov *et al.* 1996b).

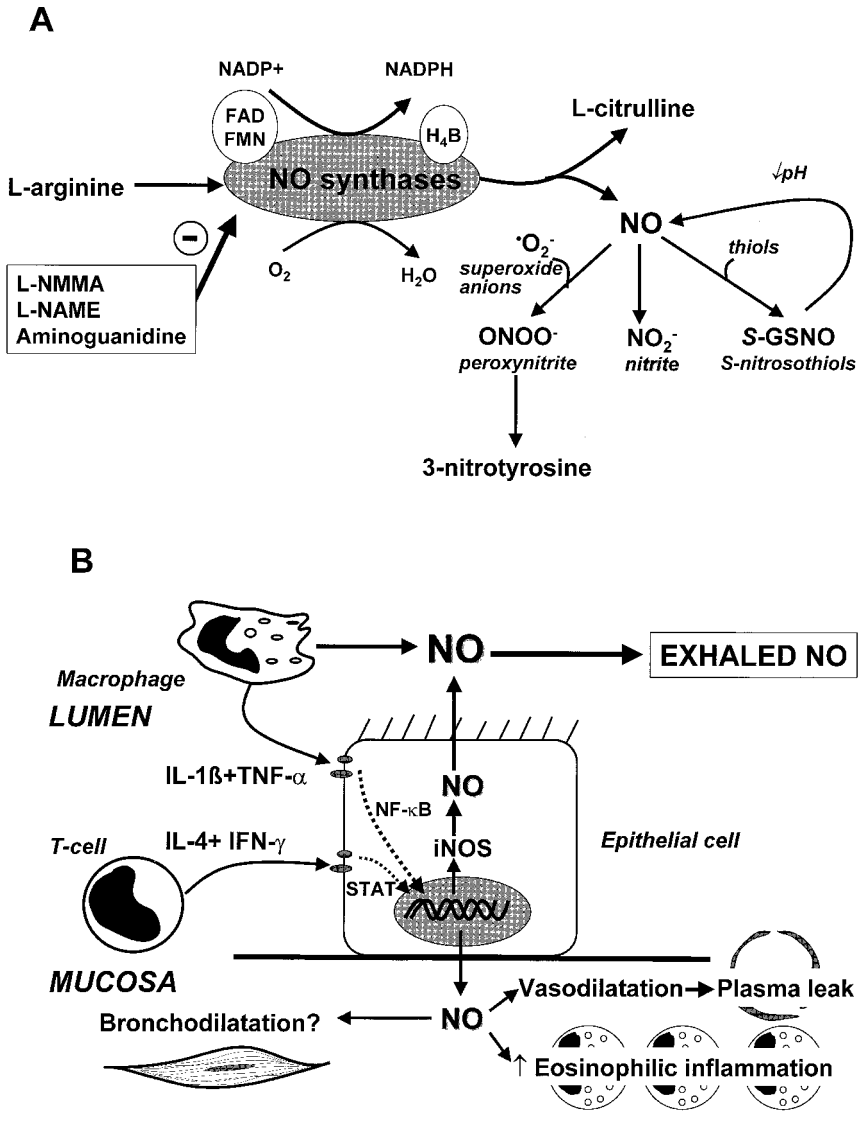

Figure 2. (A) Synthesis of NO and NO-related products. (B) Sources of NO in exhaled air.

Epidemiology. The diagnostic value of exhaled NO measurements to differentiate between healthy subjects with or without respiratory symptoms and patients with confirmed asthma has been recently analysed by Dupont *et al.* (1999), who demonstrated a 90% specificity and 95% positive predictive value when exhaled NO > 15 p.p.b. was used as the cut-off value for asthma.

An increased level of NO may be useful in differentiating asthma from other causes of chronic cough (Chatkin *et al.* 1999), and exhaled and nasal NO may be used to identify subjects with atopy, since non-atopic asthmatics have normal

exhaled NO (Ludviksdottir *et al.* 1999). Elevated nasal NO is also related to the size of skin test reactivity in asymptomatic asthmatic subjects (Moody *et al.* 2000). This may denote 'subclinical' airway inflammation.

Another potential use of exhaled NO levels in patient management is the prediction of future asthma. An elevated exhaled NO level may be found in patients with 'subclinical' forms of asthma (normal lung function, negative bronchodilator tests, elevated sputum eosinophilic cationic protein concentrations) (Sovijärvi *et al.* 1998, Withers *et al.* 1998). This has been investigated in epidemiological studies, in which the reservoir collection of exhaled NO was shown to be useful (Stirling *et al.* 1998, van Amsterdam *et al.* 1999a). Airway responsiveness measurements (PC_{20}) in this 'high risk' group make the combination of exhaled NO and PC_{20} a more specific test for allergic asthma. This has recently been demonstrated in a study of over 8000 adolescents in Norway (Henriksen *et al.* 2000). Because of the non-invasive nature and practicality of exhaled and nasal NO measurements, they may be used cost-effectively for screening large populations.

Disease monitoring. It is difficult to monitor the response to different classes of anti-inflammatory drugs in asthma, as there is no single test that can be used to quantify airway inflammation. Peripheral blood markers are unlikely to be adequate as the most important mediator and cellular responses occur locally within the airways. It is clear that different markers of airway inflammation should be considered together to monitor asthma (Kharitonov and Barnes 2000a).

Exhaled NO has been used to monitor the effect of anti-inflammatory treatment in asthma (Kharitonov *et al.* 1996c) and asthma exacerbations, both spontaneous and induced by steroid reduction (Kharitonov *et al.* 1996d, Jatakanon *et al.* 2000). A considerable advantage of exhaled NO is that NO levels may increase before any significant changes in other parameters, such as lung

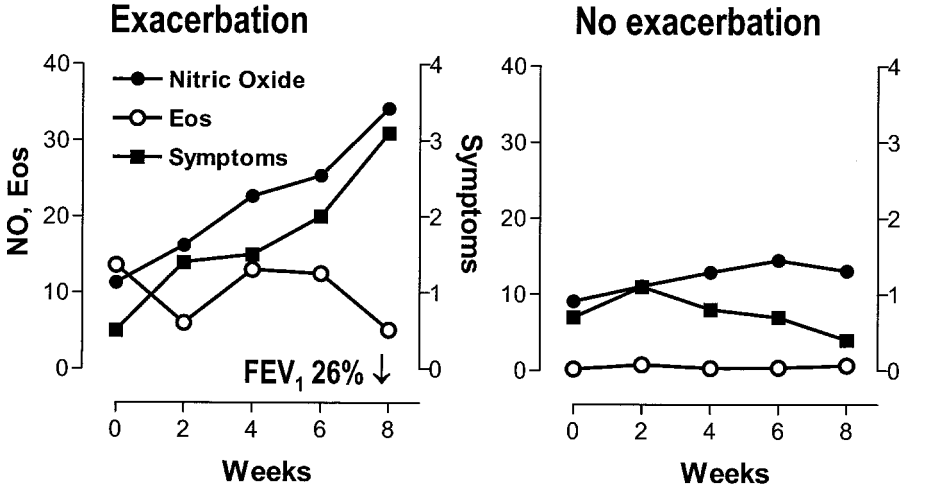

Figure 3. Exhaled NO, sputum eosinophils (Eos) and symptoms in patients with and without subsequent exacerbation following a reduction in inhaled steroids (from Jatakanon *et al.* 2000).

function or sputum eosinophils, and may therefore serve as an early warning of loss of control (Kharitonov 1999a, Jatakanon *et al.* 2000) (figure 3).

It is most likely that exhaled NO is related to asthma control rather than asthma severity (Kharitonov and Barnes 2000a), and that serial NO measurements in individual patients over time may be useful in identifying patients requiring changes in therapy. In a recent study, Sippel and co-workers have shown that exhaled NO is significantly correlated with markers of asthma control, such as asthma symptoms within the past 2 weeks, dyspnoea score, daily use of rescue medication and reversibility of airflow obstruction (Tamaoki *et al.* 2000). However, exhaled NO levels are not correlated with the following markers of asthma severity: history of respiratory failure, health care use or fixed airflow obstruction.

Treatment monitoring. With regard to corticosteroid treatment, a large dose ($1\,mg\,kg^{-1}\,day^{-1}$ for 5 days) of oral prednisolone normalizes exhaled NO in infants and young children with wheezing exacerbations (Baraldi *et al.* 1999c), whereas the same dose in more severe asthmatic children only shifts their exhaled NO down to the levels of mild-to-moderate asthma, in spite of an improvement in lung function (Baraldi *et al.* 1997). A cumulative dose of methylprednisolone (180–500 mg) causes a 36% reduction within 50 h in the majority of severe adult patients with acute asthma (Massaro *et al.* 1995), and a combination of oral prednisolone and inhaled steroids reduces exhaled NO by 65% in children with acute asthma (Lanz *et al.* 1999).

Recently, it has been shown that NO levels correlate with the percentage improvement in the forced expiratory volume in 1 s (FEV_1) from baseline to the post-steroid (30 mg prednisolone daily for 14 days) post-bronchodilator value. A NO level of >10 p.p.b. at baseline has a positive predictive value of 83% for an improvement in FEV_1 of $\geq 15\%$, and therefore may be useful in predicting the response to a trial of oral steroid in asthma (Little *et al.* 2000).

Exhaled NO as an inflammatory marker sensitive to corticosteroids may be the ideal tool to demonstrate a dose–response effect and to adjust the dose in clinical practice. It may also be useful in patients using fixed combination inhalers (corticosteroids and long-acting β_2-agonists) to ensure that inflammation is controlled, as this may be difficult to assess on the basis of symptoms when a long-acting bronchodilator is also being taken.

Exhaled NO behaves as a 'rapid response' marker, as it is extremely sensitive to steroid treatment. It may be significantly reduced even 6 h after a single treatment with a nebulized corticosteroid (Kharitonov *et al.* 1996a) or within 2–3 days after inhaled corticosteroids (Kharitonov *et al.* 1996c), showing a maximal effect after 2–4 weeks of treatment (Kharitonov *et al.* 1996c, d, 2000a, Silkoff *et al.* 1998, Jatakanon *et al.* 1999, Lim *et al.* 1999, van Rensen *et al.* 1999).

We have demonstrated a dose-dependent reduction in exhaled NO and improvement in asthma symptoms in mild asthmatics following treatment with low doses of inhaled corticosteroids (Kharitonov *et al.* 2000a), whereas a reduction in sputum eosinophils and a similar improvement in symptoms was only observed after higher doses (Jatakanon *et al.* 1999). This suggests that exhaled NO levels may be too sensitive to determine whether inflammation is adequately controlled (Kharitonov and Barnes 2000a).

It is still uncertain whether exhaled NO is useful in directing changes in asthma therapy. Recently it has been shown that exhaled NO values above 13 p.p.b. have a sensitivity of 0.67 and a specificity of 0.65 to predict a step-up in therapy (Griese *et al.* 2000), but clearly more studies are needed using exhaled NO to direct therapy.

Corticosteroids may reduce exhaled NO by directly inhibiting the induction of NOS2 (Guo *et al.* 2000) or by suppressing the proinflammatory cytokines that induce NOS2. There is inhibition of NOS2 immunoreactivity with inhaled corticosteroid treatment in asthmatic patients, and a parallel reduction in immunoreactivity for nitrotyrosine, which may reflect local production of peroxynitrite from the interaction of NO and superoxide anions (Saleh *et al.* 1998).

Neither short-acting (Kharitonov *et al.* 1996c, Yates *et al.* 1997, Lipworth *et al.* 2000) nor long-acting (Yates *et al.* 1997, Aziz *et al.* 2000) β_2-agonists reduce exhaled NO.

The leukotriene receptor antagonist pranlukast blocks the increase in exhaled NO when inhaled corticosteroids are withdrawn (Kobayashi *et al.* 1999), and montelukast rapidly reduces exhaled NO by 15–30% in children with asthma (Bisgaard *et al.* 1999). Antileukotrienes have a moderate effect in patients with asthma and seasonal allergic rhinitis (Bratton *et al.* 1999, Wilson *et al.* 2000). Both formoterol and zafirlukast are equally effective in maintaining asthma control, and zafirlukast causes a significant reduction in exhaled NO (Lipworth *et al.* 2000).

Nebulized L-NMMA and L-NAME, which are non-selective inhibitors of NOS, both reduce exhaled NO in asthmatic patients, although this is not accompanied by any changes in lung function (Yates *et al.* 1995, Gomez *et al.* 1998). Aminoguanidine, a more selective inhibitor of NOS2, reduces exhaled NO in asthmatic patients, but has little effect in normal subjects, indicating that NOS2 is an important source of the increased exhaled NO in asthma (Yates *et al.* 1996b).

Prostaglandin (PG) E_2 downregulates NOS2 expression (D'Acquisto *et al.* 1998), and inhaled PGE_2 and $PGF_{2\alpha}$ decrease exhaled NO in normal and asthmatic subjects (Kharitonov *et al.* 1998b).

COPD

Exhaled NO levels in stable COPD (Kharitonov *et al.* 1995c, Robbins *et al.* 1996, Rutgers *et al.* 1999) and chronic bronchitis (Von Essen *et al.* 1998) are lower than in either smoking or non-smoking asthmatics (Verleden *et al.* 1999) and are not different from normal subjects. This reduction in exhaled NO is due to the effect of tobacco smoking, which downregulates epithelial NOS (Su *et al.* 1998), and may reflect increased oxidative stress that may consume NO in the formation of peroxynitrite (Eiserich *et al.* 1998).

Patients with unstable COPD, however, have high NO levels compared with stable smokers or ex-smokers with COPD (Maziak *et al.* 1998), which may be explained by increased neutrophilic inflammation and oxidant/antioxidant imbalance. Eosinophils that are capable of expressing NOS2 and producing NO are present in exacerbations of COPD (Saetta *et al.* 1994). Pulmonary hypertension has the opposite effect, as COPD patients with cor pulmonale have low exhaled NO levels (Clini *et al.* 2000), which may reflect impaired endothelial NO release.

A small proportion of patients with COPD appear to respond to corticosteroids; these patients, who are likely to have coexistent asthma, have an increased

proportion of eosinophils in induced sputum (Fujimoto *et al.* 1999). These patients also have an increase in exhaled NO (Papi *et al.* 2000). This suggests that exhaled NO may be useful in predicting which COPD patients will respond to long-term inhaled corticosteroid treatment.

CF

Exhaled and nasal NO levels are significantly lower in CF than in normal subjects, despite the intense neutrophilic inflammation in the airways (Balfour-Lynn *et al.* 1996, Thomas *et al.* 2000) (figure 4A), leading to the release of superoxide anions, which convert NO to nitrate and may result in the formation of peroxynitrite. Although there is a trend toward both exhaled and nasal NO being higher in patients who were not homozygous for the ΔF508 CF transmembrane regulator mutation, there is no strong association between exhaled NO and disease severity in CF (Antuni *et al.* 2000) or infection with *Pseudomonas* (Thomas *et al.* 2000).

Bronchiectasis and primary ciliary dyskinesia

An increase in exhaled NO is found in bronchiectasis and the increase in NO is related to the extent of disease as measured by a computed tomography score (Kharitonov *et al.* 1995d). Primary ciliary dyskinesia (PCD), including Kartagener's syndrome, is a genetic disease characterized by defective motility

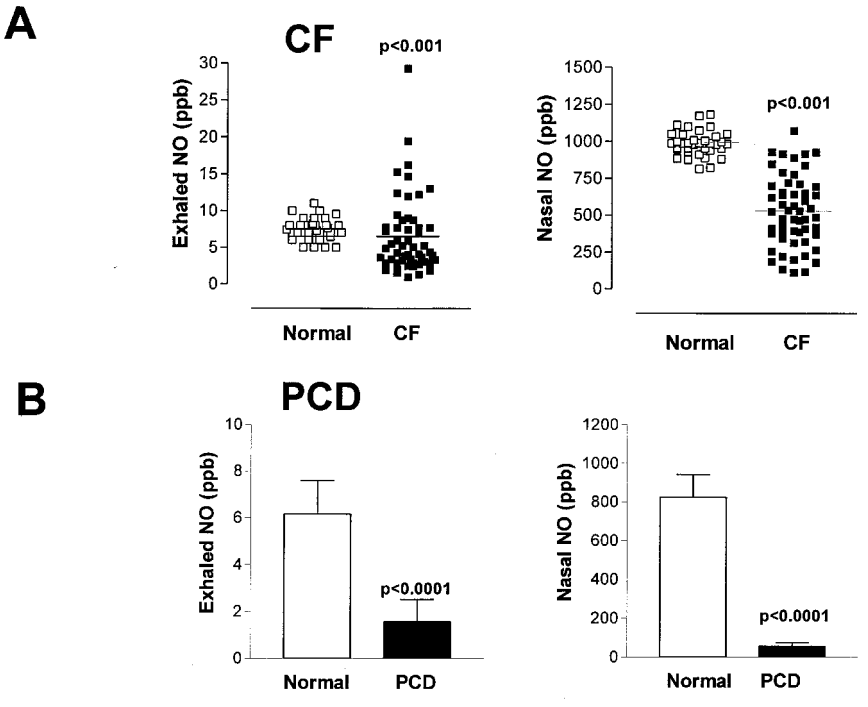

Figure 4. Exhaled and nasal NO in CF (A) (from Thomas *et al.* 2000) and in PCD (B) (from Loukides *et al.* 1998b).

of cilia, in which the levels of exhaled NO are very low compared with normal subjects (Loukides *et al.* 1998b) (Figure 4B). Such low values of exhaled and nasal NO are not seen in any other condition and are therefore of diagnostic value. Measurement of exhaled NO might be used as a screening procedure to detect PCD amongst patients with recurrent chest infections or male infertility due to immotile spermatozoa, and the diagnosis of PCD is then confirmed by the saccharine test, nasal NO, ciliary beat frequency and electron microscopy (Bush 2000). Low levels of exhaled and nasal NO in PCD patients are related to mucociliary dysfunction (Loukides *et al.* 1998b, Tamaoki *et al.* 2000), and treatment with the NO donor L-arginine increases nasal NO and also improves mucociliary transport in PCD patients (Loukides *et al.* 1998b, Kharitonov and Barnes 2000a). The mechanism for such a low NO production by nasal and airway epithelia in PCD is unknown, but it might be linked to genetic abnormalities in NOS2 gene expression, as in CF.

Interstitial lung diseases

In patients with systemic sclerosis who have developed pulmonary hypertension, there is a reduction in exhaled NO compared with normal subjects and patients with interstitial lung disease without pulmonary hypertension (Kharitonov *et al.* 1997b, Rolla *et al.* 2000). There is strong expression of nitrotyrosine and NOS2 in macrophages, neutrophils and alveolar epithelium in the lungs of patients with idiopathic pulmonary fibrosis with active inflammation during the early to intermediate stage of the disease (Saleh *et al.* 1997). This is consistent with elevated levels of exhaled NO in patients with fibrosing alveolitis. Increased exhaled NO levels are associated with disease activity, as assessed by bronchoalveolar lavage (BAL) lymphocyte counts, and are reduced in patients treated with corticosteroids (Paredi *et al.* 1999b). Cytokines, including tumour necrosis factor-α (TNFα) and interferon-γ, are increased in the pulmonary inflammation of sarcoidosis, and there is an upregulation of NOS2 in the respiratory epithelium and granulomata (Moodley *et al.* 1999). The magnitude of the rise in exhaled NO in sarcoidosis may be related to the activity of the disease and is reduced by steroid therapy. This is perhaps the reason behind two conflicting observations reporting either elevated (Moodley *et al.* 1999) or normal (O'Donnell *et al.* 1997) exhaled NO in patients with active pulmonary sarcoidosis.

Occupational diseases

Laboratory animal allergy (LAA) is among the highest occupational risks for asthma. Exhaled NO is raised in subjects with LAA symptoms and correlates with symptom severity (Adisesh *et al.* 1998). The progressive increase in exhaled NO from asymptomatic to early LAA to symptomatic asthma suggests that exhaled NO measurements may be useful in monitoring occupational asthmas and the environmental health effects of air pollution in epidemiological surveys (van Amsterdam *et al.* 2000). Recently, measurement of exhaled NO and induced sputum were evaluated in occupational asthma. Aluminium potroom workers (exposure to dust and fluorides) with asthma-like symptoms had higher concentrations of exhaled NO than those with no symptoms (Lund *et al.* 2000), suggesting that exhaled NO may be an early marker of airway inflammation in

potroom workers. High levels of exhaled NO and asthma-like symptoms in subjects with occupational exposure to high levels of ozone and chlorine dioxide (Olin *et al.* 1999), or in swine confinement workers (Von Essen *et al.* 1998), may indicate the presence of chronic airway inflammation.

Infections

Exhaled, but not nasal NO, is elevated during viral infections in adults and children (Kharitonov *et al.* 1995e, Ferguson and Eccles, 1997). Exhaled NO is also increased in experimental human influenza (Murphy *et al.* 1998) and rhinovirus infection (de Gouw *et al.* 1998). The increase in NO production during viral infection is likely to be protective, as NO inhibits virus replication either by inhibiting viral RNA synthesis, or/and by S-nitrosylation of the cysteine proteases that are critical for the virulence and replication of viruses (Saura *et al.* 1999). Exhaled (Loveless *et al.* 1997) and nasal NO (Palm *et al.* 2000) in human immunodeficiency virus (HIV)-positive individuals is less than in control subjects, and NO synthesis is further depressed in terminally ill HIV patients (Evans *et al.* 1994), suggesting that low NO may indicate a mechanism of impaired host defence in HIV infection. NO plays an important role in resistance to *Mycobacterium tuberculosis* infection, and exposure of extracellular *M. tuberculosis* to <100 p.p.m. of NO for a short period (<24 h) results in microbial killing (Long *et al.* 1999). Elevated exhaled NO and NOS2 expression in alveolar macrophages is found in patients with active tuberculosis and is reduced with antituberculosis therapy (Wang *et al.* 1998). Nitrate concentrations are significantly higher in BAL in immunosuppressed children with pneumonia than in normal control subjects (Grasemann *et al.* 1997), and elevated exhaled NO levels are found in patients with lower respiratory tract inflammation and chronic bronchitis (Von Essen *et al.* 1998).

Lung cancer

The levels of nitrite in epithelial lining fluid and exhaled NO are significantly higher in patients with lung cancer compared with control subjects, and are correlated with the intensity of NOS2 expression in alveolar macrophages (Liu *et al.* 1998). The level of nitrite is also significantly higher in epithelial lining fluid from cancer patients, but the increased NO production is not specific to the tumour site and may be due to a tumour-associated non-specific immunological and inflammatory mechanism.

Adult respiratory distress syndrome

Adult respiratory distress syndrome (ARDS) is associated with a neutrophilic alveolar inflammation. In animal models of ARDS induced by endotoxin there is increased production of NO (Stewart *et al.* 1995). Exhaled NO values are low, presumably because of the concomitant oxidative stress and consumption of NO by superoxide anions to form peroxynitrite (Brett and Evans, 1998). Association of reduced exhaled NO levels with the increases in the pulmonary artery pressure and the alveolar–arterial oxygen difference and the decrease in lung compliance (Ishibe

et al. 2000) suggest that exhaled NO may an indicator of lung injury in adult patients after cardiopulmonary bypass.

Carbon monoxide

Carbon monoxide (CO) is a gas that can be formed endogenously and is detectable in exhaled air. There are three major sources of CO in exhaled air: enzymatic degradation of haem, non-haem-related release (lipid peroxidation, xenobiotics, bacteria) and exogenous CO. The predominant endogenous source of CO (~85%) in the body is from the degradation of haemoglobin by the enzyme haem oxygenase (HO), and approximately 15% arises from degradation of myoglobin, catalase, NOSs, guanylyl cyclase and cytochromes (Berk *et al.* 1974).

The alveoli are the predominant site of exhaled CO in normal subjects (Kharitonov *et al.* 2000b). The fact that breathing through the nose increases the CO levels obtained in the exhaled air (Andersson *et al.* 2000) suggests that the nose and paranasal sinuses may also contribute to the CO production of the human airways.

The use of exhaled CO as a marker to assess different diseases (cardiovascular, diabetes and nephritis) was first described in Russia in 1972 (Nikberg *et al.* 1972). Over the last 20 years exhaled CO has been measured to identify current and passive smokers, to monitor bilirubin production, including hyperbilirubinaemia in newborns, and in the assessment of lung diffusion capacity. CO can be quantified by a number of different techniques. In humans most of the measurements have been made using electrochemical CO sensors. The sensor is selective, gives reproducible results (Kharitonov *et al.* 1998a) and is inexpensive. End-tidal exhaled CO measurements can be made during a single exhalation and is a routine procedure in cooperative adults. It can also be easily performed in children over 5 years of age (Uasuf *et al.* 1999). A method for measuring CO in nasally sampled exhaled air in non-cooperative neonates has been developed that involves the relatively non-invasive placement of a small catheter into the posterior of the nasopharynx and collection of breath samples either manually or automatically (Vreman *et al.* 1996).

Asthma

Elevated levels of exhaled CO have been reported in stable asthma (Zayasu *et al.* 1997), with normal levels in patients treated with inhaled corticosteroids. The difference in exhaled CO between normal and asthmatic subjects, however, is much less than for exhaled NO (Kharitonov 1999b), and the effect of inhaled steroids on exhaled CO in mild asthma patients, as has been reported recently, is negligible (Lim *et al.* 2000). Significantly elevated CO levels are found in patients with severe asthma (Stirling *et al.* 2000), including patients treated with 30 mg of prednisolone for 2 weeks (Biernacki *et al.* 1999). In view of the simplicity of CO measurements and the portability of CO analysers, exhaled CO may be useful for the non-invasive monitoring of paediatric asthma. For example, children with persistent asthma despite treatment with steroids, which reduces their NO levels, have significantly higher exhaled CO compared with those with infrequent episodic asthma (Uasuf *et al.* 1999).

COPD

A major limitation of exhaled CO in COPD is the marked effects of cigarette smoking, which masks any increase that may occur due to the disease process. There is no difference in exhaled CO in patients with chronic bronchitis (without airflow obstruction) when compared with normal subjects (Delen *et al.* 2000). However, exhaled CO levels are elevated in ex-smoking COPD patients (Culpitt *et al.* 1998), suggesting ongoing oxidative stress or inflammation. HO is induced in fibroblasts exposed to cigarette smoke (Muller and Gebel 1998). There is an increase in exhaled CO during acute exacerbations of COPD, with a decline after recovery (Biernacki *et al.* 1998).

Bronchiectasis, CF and interstitial lung disease

Exhaled CO levels are elevated in patients with bronchiectasis, irrespective of whether or not they are treated with inhaled corticosteroids (Horvath *et al.* 1998b). In contrast to NO, exhaled CO levels are markedly elevated in stable CF patients (Paredi *et al.* 1999c, 2000), increase further during exacerbations and are reduced with antibacterial treatment (Antuni *et al.* 2000) (figure 5). We have shown that patients homozygous for the CF transmembrane regulator ΔF508 mutation have higher exhaled CO levels than heterozygous patients (Paredi *et al.* 1999c).

Elevation of exhaled CO is related to lung function deterioration (Antuni *et al.* 1999a) and impaired gas transfer in patients with cryptogenic fibrosing alveolitis and scleroderma (Antuni *et al.* 1999b). Elevated levels of exhaled CO in patients with fibrosing alveolitis are also associated with disease activity as assessed by BAL cell counts (Paredi *et al.* 1999b). This suggests that exhaled CO may be used to monitor disease progression and response to therapy in interstitial lung diseases.

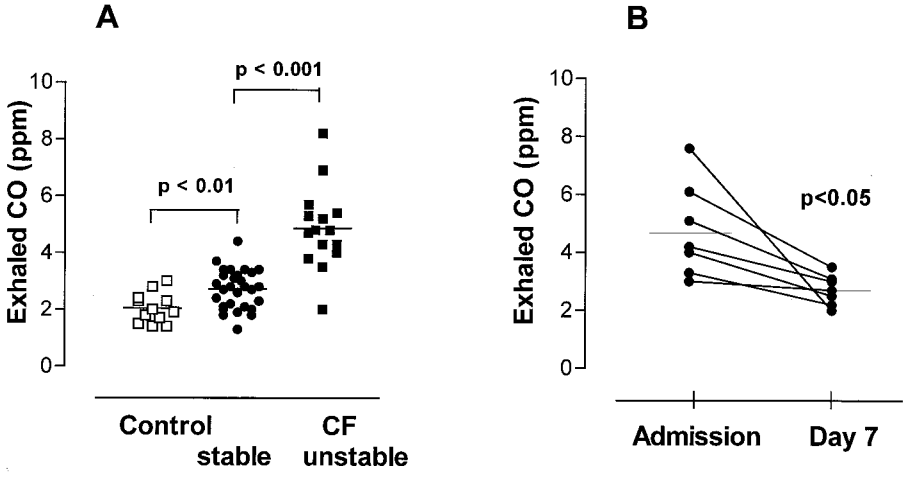

Figure 5. Exhaled CO in CF: effect of disease severity (A) and antimicrobial treatment (B) (from Antuni *et al.* 2000).

Infections and other conditions

HO1 is induced by many infectious agents and may provide protection to cells against attack by infectious agents. Upper respiratory tract viral infections may induce the expression of HO1, resulting in increased exhaled CO in adults (Yamaya *et al.* 1998) and children (Uasuf *et al.* 1999). Elevated exhaled CO levels might provide an early warning signal for an acute infective episode, which may lead to exacerbation of asthma and COPD. Elevated levels of CO have been measured in patients with lower respiratory tract infection in general practice, and were significantly reduced after 5 days' treatment with antibiotics (Biernacki *et al.* 1998).

Critically ill patients have a significantly higher CO concentration in exhaled air as well as total CO production compared with healthy controls (Scharte *et al.* 2000). Interestingly, the levels of exhaled CO in these patients are similar to the levels seen in severe asthma and may be a reflection of systemic rather than local oxidative stress. Exhaled CO levels are also increased in diabetes, and the level is significantly related to the level of hyperglycaemia (Paredi *et al.* 1999a). The mechanism is unclear, but hyperglycaemia and oxidative stress in uncontrolled diabetes may activate HO1.

Exhaled breath condensate

The detection of non-volatile mediators and inflammatory markers from the respiratory tract involves invasive techniques, such as BAL or induced sputum. They cannot be repeated within a short period of time because of their invasiveness, and because the procedures themselves may induce an inflammatory response. Exhaled breath condensate is collected by cooling or freezing exhaled air and is totally non-invasive (figure 6). The collection procedure has no influence on airway function or inflammation, and there is accumulating evidence that abnormalities in condensate composition may reflect biochemical changes in the airway lining fluid. Several non-volatile chemicals, including proteins, have now been detected in breath condensates. The first studies identifying the surface-active properties, including pulmonary surfactant, of exhaled condensate were published in Russia in the 1980s (Sidorenko *et al.* 1980, Kurik *et al.* 1987) and since then several inflammatory mediators, oxidants and ions have been identified in exhaled breath condensates.

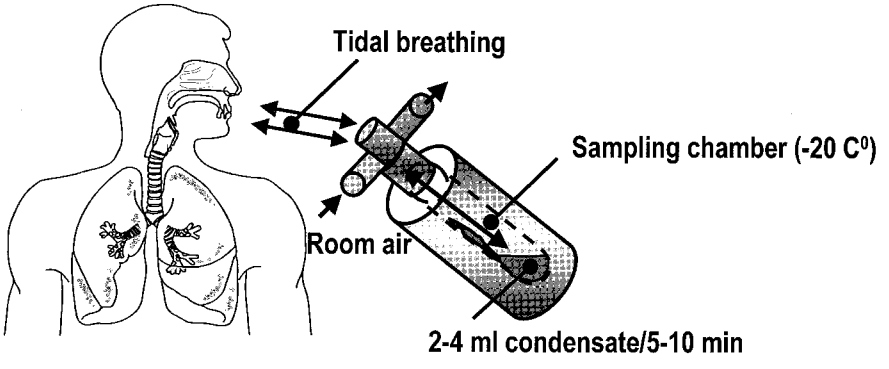

Figure 6. Exhaled breath condensate: diagram of the apparatus.

Origin

Potentially, condensate measurements reflect different markers and molecules derived from the mouth (oral cavity and oropharynx), tracheobronchial system and alveoli, and the proportional contributions of these different sources has not yet been sufficiently studied. It is assumed that airway surface liquid becomes aerosolized during turbulent airflow, so that the contents of the condensate reflects the composition of the airway surface liquid, although large molecules may not aerosolize as well as small soluble molecules. A strong correlation between the levels of CO_2 and O_2 in exhaled fluid and exhaled breath (von Pohle *et al.* 1992) suggests that aerosol particles exhaled in human breath reflect the composition of the bronchoalveolar extracellular lining fluid.

Factors affecting measurements

Several methods of condensate collection have been described. The most common approach is to ask the subject to breathe tidally via a mouthpiece through a non re-breathing valve in which inspiratory and expiratory air is separated. During expiration the exhaled air flows through a condenser, which is cooled to 0°C by melting ice (Montuschi *et al.* 1999) or to −20°C by a refrigerated circuit (Scheideler *et al.* 1993), and the breath condensate is then collected into a cooled collection vessel. A low temperature may be important for preserving labile markers such as lipid mediators during the collection period; it usually takes between 10 and 15 min to obtain 1–3 ml of condensate. Exhaled condensate may be stored at −70°C and is subsequently analysed by gas chromatography and/or extraction spectrophotometry, or using enzyme-linked immunosorbent assays (ELISAs).

Salivary contamination may influence the levels of several markers detectable in exhaled breath condensate. High concentrations of eicosanoids (thromboxane B_2, leukotriene B_4, $PGF_{2\alpha}$), but low levels of PGE_2 and prostacyclin have been found in the saliva of children with acute asthma (Mozalevskii *et al.* 1997). In addition, the presence of high concentrations of nitrite/nitrate in the diet may affect NO-related markers in condensate (Zetterquist *et al.* 1999). It is therefore important to minimize and monitor salivary contamination. Subjects should rinse their mouth before collection and keep the mouth dry by periodically swallowing their saliva. Salivary contamination, measured by the amylase concentration in the condensate, should be routinely monitored. In most of the reported studies, amylase has been measured in the condensate and no salivary contamination has been detected (Scheideler *et al.* 1993, Horvath *et al.* 1998a, Loukides *et al.* 1998a). Subjects should wear a nose clip in order to collect only mouth-conditioned exhaled air into the collection system. Flushing the nose with helium may help to reduce contamination of the exhaled breath with nasal air, which contains high levels of NO that may potentially influence the results of NO-related markers (nitrite/nitrate, S-nitrosothiols) (Ho *et al.* 1998). Another approach to exclude nasal contamination is to collect the condensate during a series of exhalations against a resistance (Ho *et al.* 1998). However, it has not yet been shown that nasal NO affects measurements in exhaled condensate. The quantity of exhaled condensate is dependent on the ventilation volume per unit time (minute volume), but this does not affect the concentration of mediators (Montuschi *et al.* 1999,

Reinhold *et al.* 1999). It is also dependent on exhaled air temperature and humidity (Paredi P *et al.*, unpublished observation).

Hydrogen peroxide and thiobarbituric acid-reactive products

Activation of inflammatory cells, including neutrophils, macrophages and eosinophils, results in increased production of O_2^-, which by undergoing spontaneous or enzyme-catalysed dismutation lead to formation of H_2O_2. As H_2O_2 is less reactive than other reactive oxygen species, it has the propensity to cross biological membranes and enter other compartments. Because it is soluble, increased H_2O_2 in the airway equilibrates with air (Dohlman *et al.* 1993), and has potential as a marker of oxidative stress in the lungs.

Asthma. H_2O_2 has been detected in exhaled condensate in healthy adults and children, with increased concentrations in asthma (Dohlman *et al.* 1993, Jöbsis *et al.* 1998). Asthmatic patients also exhale significantly higher levels of thiobarbituric acid-reactive products (TBARs), which indirectly reflect increased oxidative stress (Antczak *et al.* 1997).

COPD. Cigarette smoking causes an influx of neutrophils and other inflammatory cells into the lower airways, and five-fold higher levels of H_2O_2 have been found in the exhaled breath condensate of smokers compared with non-smokers (Nowak *et al.* 1996). Levels of exhaled H_2O_2 are increased compared with normal subjects in patients with stable COPD, and are further increased during exacerbations (Dekhuijzen *et al.* 1996, Nowak *et al.* 1998).

Other lung diseases. Increased H_2O_2 levels in exhaled breath condensate have been found in ARDS (Baldwin *et al.* 1986, Heard *et al.* 1999) bronchiectasis (Loukides *et al.* 1998a) and following lobectomy/pneumonectomy in patients with lung carcinoma (Lases *et al.* 2000), indicating increased oxidative stress in these conditions, and are significantly reduced during antibiotic treatment in patients with infective exacerbations of CF (Jobsis *et al.* 2000).

Eicosanoids

Eicosanoids are potent mediators of inflammation and are responsible for vasodilatation/vasoconstriction, plasma exudation, mucus secretion, bronchoconstriction/bronchodilatation, cough and inflammatory cell recruitment.

Prostanoids. There is an increased expression of inducible cyclo-oxygenase (COX2), which forms prostaglandins and thromboxane in asthma, COPD and CF, and exhaled PGE_2 and $PGF_{2\alpha}$ are markedly increased in patients with COPD, whereas these prostaglandins are not significantly elevated in asthma (Kharitonov and Barnes 2001). In contrast, thromboxane B_2 (TxB_2) is increased in asthma but is not detectable in normal subjects or in patients with COPD.

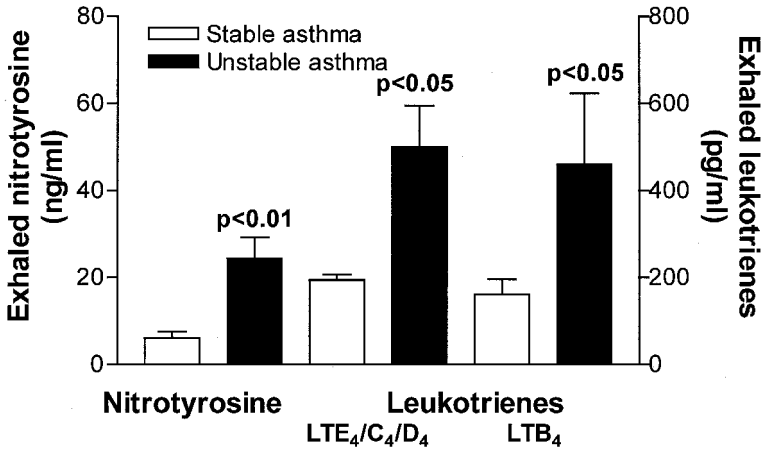

Figure 7. Exhaled nitrotyrosine and leukotrienes before and after steroid withdrawal in patients with moderate asthma (from Hanazawa *et al.* 2000a).

Leukotrienes. Leukotrienes (LTs), a family of lipid mediators derived from arachidonic acid via the 5-lipoxygenase pathways, are potent constrictors and pro-inflammatory mediators that contribute to the pathophysiology of asthma. The cysteinyl-leukotrienes (cys-LTs) LTC_4, LTD_4 and LTE_4 are generated predominantly by mast cells and eosinophils, and are able to contract airway smooth muscle, cause plasma exudation and stimulate mucus secretion, as well as recruiting eosinophils (Leff 2000). In contrast, LTB_4 has potent chemotactic activity towards neutrophils (Larfars *et al.* 1999).

Detectable levels of LTB_4, LTC_4, LTD_4, LTE_4 and LTF_4 have been reported in the exhaled condensate of asthmatic and normal subjects (Becher *et al.* 1997, Hanazawa *et al.* 2000a). In mild asthmatic patients levels of LTE_4, LTC_4 and LTD_4 in exhaled condensate are increased during the late asthmatic response to allergen challenge (Hanazawa *et al.* 2000b). The levels of LTE_4, LTC_4 and LTD_4 in breath condensate are elevated significantly in patients with moderate and severe asthma (Hanazawa *et al.* 2000a). Steroid withdrawal in moderate asthma leads to worsening of asthma and a further increase in exhaled NO and the concentration of LTB_4, LTE_4, LTC_4 and LTD_4 in the exhaled condensate (Hanazawa *et al.* 2000b) (figure 7). LTB_4 concentrations are increased in exhaled breath condensate of patients with COPD (Kharitonov and Barnes 2001) and in moderate and severe asthma (Hanazawa *et al.* 2000a). This suggests that LTB_4 may be involved in exacerbations of asthma and may contribute towards neutrophil recruitment.

Isoprostanes. Isoprostanes are a novel class of prostanoids formed by free radical-catalysed lipid peroxidation of arachidonic acid. They are initially formed esterified in membrane phospholipids, from which they are cleaved by a phospholipase A_2. They circulate in plasma, are excreted in urine and can also be detected in exhaled breath condensate and BAL.

Asthma. Exhaled 8-isoprostane levels are approximately doubled in mild asthma compared with normal subjects, and increased by about three-fold

in those with severe asthma, irrespective of their treatment with corticosteroids (Montuschi *et al.* 1999). The relationship to asthma severity is a useful aspect of this marker, in contrast to exhaled NO. The relative lack of effect of corticosteroids on exhaled 8-isoprostane has been confirmed in a placebo-controlled study with two different doses of inhaled steroids (Kharitonov *et al.* 2000a). This provides evidence that inhaled corticosteroids may not be very effective in reducing oxidative stress. Exhaled isoprostanes may a better means of reflecting disease activity than exhaled NO.

COPD. The concentration of 8-isoprostane in exhaled condensate is increased in normal cigarette smokers, but to a much greater extent in COPD patients (Montuschi *et al.* 2000a). Interestingly, exhaled 8-isoprostane is increased to a similar extent in COPD patients who are ex-smokers as in smoking COPD patients, indicating that the exhaled isoprostanes in COPD are largely derived from oxidative stress from airway inflammation rather than from cigarette smoking.

Interstitial lung disease and CF. 8-Isoprostane is detectable in BAL fluid of normal subjects and is increased in patients with sarcoidosis, cryptogenic fibrosing alveolitis and fibrosing alveolitis associated with systemic sclerorosis, suggesting a higher level of oxidant stress and greater lung injury in these patients than in sarcoidosis (Montuschi *et al.* 1998). Elevated levels of 8-isoprostane have been detected in plasma (Collins *et al.* 1999). Concentrations of 8-isoprostane in the breath condensate of patients with stable CF are increased about three-fold compared with normal subjects (Montuschi *et al.* 2000b).

Products of lipid peroxidation

Significantly higher concentrations of primary (diene conjugates) and secondary (ketodienes) products of lipid peroxidation have been found in exhaled condensate and in bronchial biopsy samples from patients with COPD and chronic bronchitis compared with normal subject (Khyshiktuev *et al.* 1996, Ignatova *et al.* 1998). Increased levels of free fatty acids, including linoleic and arachidonic acids, have been measured in exhaled condensate and sweat in children (Prokhorova *et al.* 1998) and adults (Komar *et al.* 1996) with acute pneumonia and lung oedema (Gichka *et al.* 1998). In contrast, the level of lipid peroxidation in cancer patients was significantly reduced compared with healthy controls (Khyshiktyev *et al.* 1994). Exhaled condensates may be used in the prenatal diagnosis of fetal hypoxia, as significantly higher levels of diene conjugates and malonic dialdehydes have been found in pregnant women who gave birth to babies with severe fetal and neonatal hypoxia (Khyshiktueva *et al.* 1998). Recent studies suggest that the increased permeability in patients with interstitial lung disease results in an increase in alveolar-to-vascular leakage of surfactant proteins A and D (Takahashi *et al.* 2000). The system of clearance of these proteins from the circulation is unknown at present, but if they are detectable in exhaled breath condensate this may be the best practical examination for this disease.

Vasoactive amines

Elevated levels of acetylcholine, serotonin and histamine, which were related to the severity of airway inflammation, airway obstruction and airway hyper-responsiveness, have been reported in exhaled breath condensate in asthma (Goncharova *et al*. 1989) and acute bronchitis (Goncharova *et al*. 1996). High levels of acetylcholine, catecholamines, histamine and serotonin, and low levels of cortisol and thyroxine, have been reported in exhaled condensate in coal miners with the early stages of silicosis (Dzhangozina *et al*. 1999).

NO-related products

NO reacts with superoxide to yield peroxynitrite, which can be trapped by thiol-containing biomolecules such as cysteine and glutathione to form S-nitrosothiols or can be oxidized to nitrate and nitrite (Stamler 1995). Nitrogen intermediates such as peroxynitrite can induce a number of covalent modifications in various biomolecules, such as nitroso- and nitro-adducts. One such modification yields 3-nitrotyrosine, and detection of this adduct in proteins is now commonly used as a diagnostic tool to identify the involvement of NO-derived oxidants in many disease states (van Der *et al*. 1999).

Asthma. High levels of nitrite have been found in the exhaled breath condensate (Hunt *et al*. 1995) of asthmatic patients, especially during acute exacerbations (Hunt *et al*. 1995).The ratio of airway wall thickness to lumen diameter measured by high resolution computed tomography is significantly correlated with the sputum concentration of nitrite/nitrate (Gabazza *et al*. 2000). In fact, we have shown that nitrotyrosine, a stable product of peroxynitrite decomposition, in exhaled breath condensate is increased in mild steroid-naive asthma and is reduced in patients with severe asthma receiving steroid therapy (Hanazawa *et al*. 2000a). However, increased levels of nitrotyrosine in exhaled breath condensate are associated with worsening of asthma symptoms and deterioration of lung function during inhaled steroid withdrawal in moderate asthma (Hanazawa *et al*. 2000b), suggesting that nitrotyrosine may be not only a predictor of asthma deterioration, but may play a key role in the pathogenesis of airway remodelling.

The levels of S-nitrosothiols in exhaled breath condensate are reduced after 3 weeks of treatment with a high (400 μg daily) but not a low dose (100 μg daily) of inhaled budesonide (Kharitonov *et al*. 2000a). In contrast, there is a rapid and dose-dependent reduction in nitrite/nitrate in exhaled breath condensate in the same mild asthmatics, suggesting that nitrite/nitrate are more sensitive to anti-inflammatory treatment.

COPD. Chronic oxidative stress presented to the lung by cigarette smoke may decrease the availability of thiol compounds and may increase decomposition of nitrosothiols, explaining elevated levels of S-nitrosothiols in exhaled condensate in healthy smokers that are related to smoking history (Corradi *et al*. 2001). Levels of exhaled nitrite/nitrate are increased in COPD (unpublished observation). A significant negative correlation between FEV_1 and the amount of nitrotyrosine formation has been demonstrated in patients with COPD, but not in those with asthma or in normal subjects (Ichinose

et al. 2000), suggesting that NO produced in the airways is consumed by its reaction with superoxide anion and/or peroxidase-dependent mechanisms and that reactive nitrogen species play an important role in the pathobiology of the airway inflammatory and obstructive processes in COPD.

CF and other lung diseases. Elevated levels of nitrite and nitrate (Ho *et al*. 1998) and nitrotyrosine (Balint *et al*. 2001) have been found in exhaled condensate of patients with CF during both stable periods and exacerbations. In children with CF and normal lung function, however, the nitrite/nitrate concentrations in BAL are normal and the concentrations of S-nitrosothiols are reduced (Grasemann *et al*. 1999). Nitrite and nitrate concentrations are increased in exhaled breath condensate of patients with active pulmonary sarcoidosis (O'Donnell *et al*. 1997).

Ammonia

Ammonia (NH_3), a product of urease hydrolysis of urea to ammonia and carbamate, is one of the key steps in the nitrogen cycle. Ammonia in the respiratory tract may be able to neutralize inhaled acid vapours and aerosols, mitigating the pulmonary effects of pollution (Norwood *et al*. 1992), and has the potential to regulate NOS activity. Thus, plasma of patients with uraemia has an inhibitory effect on NOS3 in a human endothelial cell line and NOS2 in murine macrophages (Arese *et al*. 1995).

The first measurements of exhaled NH_3 were used to assess different food supplements given during space flights in the 1970s (Vysotskii 1975). Recently, using selected ion flow tube mass spectrometric techniques, the levels of alveolar exhaled ammonia (in the range from 200 to 1750 p.p.b.) have been detected from single exhalations in healthy volunteers who have ingested a liquid protein meal (Spanel *et al*. 1998).

Exhaled breath ammonia may be an important counteracting agent in a variety of respiratory conditions, as a low pH in exhaled breath condensate has recently been reported in asthma (Hunt *et al*. 2000). Exposure to ammonia gas in the workplace is significantly associated with an increase in respiratory symptoms and asthma (Ballal *et al*. 1998). It has been shown that elevated levels of urea can be used to predict oxidative stress, as the levels of urea in saliva are significantly increased after chronic hyperbaric oxygen exposure (Volozhin *et al*. 1998). The fact that acidic rinsing results in a considerable (90%) reduction in exhaled ammonia lasting for 1 h in normal subjects (Norwood *et al*. 1992) should be considered when ammonia is measured in exhaled condensate.

Ammonia is an important pathogenic factor for certain bacteria such as *Cryptococcus neoformans*, which is a significant human pathogenic fungus that produces large amounts of urease (Cox *et al*. 2000). Exhaled ammonia levels measured by chemiluminescence are not different between normal subjects and patients with stable CF, but are significantly higher in asthma and in normal subjects with upper respiratory tract infections (Kharitonov and Barnes 2000b). It is possible that measurements of exhaled ammonia might differentiate between viral and bacterial infections in a variety of lung diseases.

Electrolytes

Increased airway fluid osmolality in the lower airways as a result of exercise may activate mast cells and cause subsequent bronchoconstriction in a subset of asthmatics. A deficiency in magnesium and an elevation in calcium concentrations in exhaled breath condensate have been reported in atopic asthma (Emel'ianov *et al*. 1995), although a histamine-induced decrease in plasma magnesium levels occurs regardless of the diagnosis of asthma (Zervas *et al*. 2000). We have recently demonstrated that exhaled Na^+ and Cl^- are elevated in exhaled condensates of patients with CF, and correlate with the sweat test and the disease severity (Balint *et al*., unpublished observations). Recently a strong negative correlation between sputum Cl^- concentrations and exhaled NO has been demonstrated in patients with PCD (Tamaoki *et al*. 2000), suggesting that airway mucociliary clearance impairment might be monitored by exhaled/nasal NO and exhaled Cl^- levels.

Hydrogen ions

An acidic microenvironment upregulates NOS2 in macrophages through the activation of nuclear factor-κB (Bellocq *et al*. 1998), making NO release moderately pH dependent (Sheu *et al*. 2000). Elevated levels of lactic acid have been found in exhaled condensate in patients with acute bronchitis (Goncharova *et al*. 1996), and exhaled condensate with a low pH is reported in patients with acute asthma (Hunt *et al*. 2000). Exhaled pH is free of salivary, nasal and gastric contamination and is not influenced by either airflow obstruction or by inhaled albuterol, but is increased by corticosteroid therapy.

Proteins and cytokines

Measurement and identification of proteins in exhaled condensate is controversial. It has been reported that the amount of protein in the breath condensate of eight healthy individuals ranged from 4 µg to 1.4 mg, originating from the nasopharynx, oropharynx and lower airways (Scheideler *et al*. 1993). The same group has also reported the presence of interleukin (IL)-1β, soluble IL2 receptor protein, IL6 and TNFα in exhaled breath condensate of patients with a variety of respiratory conditions (Scheideler *et al*. 1993). Recently, higher concentrations of total protein in exhaled condensate have been found in young smokers compared with non-smokers, whilst the levels of IL1β and TNFα were not different (Garey *et al*. 2000). We have found that IL8 levels in exhaled condensate are mildly elevated in stable CF, but are more than doubled in unstable CF patients compared with normal subjects.

Summary and future directions

Exhaled breath analysis has enormous potential as a non-invasive means of monitoring airway inflammation, oxidative stress and other conditions (for example metabolic disorders, bacterial and viral infections). The technique is simple for patients to perform and may be applied in neonates and patients with severe disease. Because the techniques are non-invasive it is possible to make repeated measurements without disturbing the system, in contrast to the invasive procedures currently used.

There is a pressing need for the evaluation of these techniques in long-term clinical studies (Kharitonov and Barnes 2000a, 2001). Whether repeated measurements of exhaled markers will help in the clinical management of lung diseases needs to be determined by longitudinal studies relating exhaled markers to other measurements of asthma control. This is most advanced with the measurement of exhaled NO (Kharitonov and Barnes 2000a), but it is still uncertain whether routine measurement of exhaled NO will improve the clinical control of asthma in a cost-effective way.

Profiles of mediators

We have reviewed a large body of data on exhaled volatile gases and exhaled breath condensate (Kharitonov and Barnes 2001), which demonstrate different patterns of change in different pulmonary diseases. At the moment single exhaled markers are usually evaluated in isolation but, as indicated above, markers are affected differently in different diseases, and different markers vary in their sensitivity to certain manoeuvres, such as the effect of therapy. For example, asthma is characterized by a large increase in exhaled NO, a modest increase in CO and a moderate increase in exhaled 8-isoprostane, whereas COPD is characterized by little or no increase in exhaled NO and by larger increases in exhaled CO and 8-isoprostane. In contrast, CF patients typically have low exhaled NO concentrations and high levels of exhaled CO and 8-isoprostane. Exhaled NO appears to be sensitive to inhibition by low doses of inhaled corticosteroids in asthma, whereas exhaled CO and 8-isoprostane are much less sensitive to inhibition by corticosteroids. These differences may be exploited in the future as more markers are characterized, with each disease having a characteristic profile or fingerprint of different markers that may be diagnostic. Treatments may also impose a characteristic effect on these markers and this may improve the specificity of treatment in the future, particularly as more potent and specific treatments become available.

New markers

It is likely that the possibilities for measurement of markers in exhaled breath are far greater than currently realized. It is clear that exhaled breath condensates contain many different molecules, including proteins. In fact, the application of proteomics, with high resolution two-dimensional gel electrophoresis and microanalysis of protein spots, may allow the recognition of particular protein patterns in different diseases and may result in the recognition of new diagnostic proteins or therapeutic targets. New and more sensitive assays may also allow the detection of many other markers of inflammation and even specific fingerprints of activation of particular cell types within the respiratory tract, such as eosinophils, neutrophils, epithelial cells and macrophages. This could have far-reaching potential for the diagnosis and treatment of many airway diseases.

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