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EXHALED NITROGEN OXIDES AND CARBON MONOXIDE IN ASTHMA AND CYSTIC FIBROSIS: MARKERS OF INFLAMMATION?

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ABSTRACT

Background: Exhaled markers of airway inflammation have attracted much interest as potential tools for monitoring the bronchial inflammation of respiratory disease. Exhaled nitric oxide (NO), its metabolites nitrite and nitrate in breath condensate (EBC), and exhaled carbon monoxide (CO), have been suggested for this purpose. However, their site of origin has not been fully investigated and the enterosalivary circuit of nitrate, with its possible off springs of nitrite and NO, could constitute a confounding factor. Exhaled NO is increased in asthma, but its levels are unexpectedly low in cystic fibrosis (CF). Exhaled CO and EBC nitrite are instead elevated in both these conditions, where the latter could reflect an increased NO activity also in CF.
Aims: The aim of study I was to compare the profiles and possible origins of exhaled NO and CO in children and adults with asthma and CF by the introduction of highly specific infrared technique and controlled flow rate for CO measurements. In study II we wanted to investigate the possible influence from salivary formation of NO on measurements in exhaled air. Study III was designed to evaluate the postulated link between nitrite and nitrate in EBC and exhaled NO in children with allergic asthma, also in relation to other disease markers. In study IV, these EBC metabolites were examined in relation to the salivary contents of the same in subjects with CF, to evaluate a possible influence from oral bacteria, which may reduce nitrate to nitrite.
Methods: In study I, 56 children and adults with asthma, 16 with allergic rhinitis, 9 CF patients and 30 age-matched controls performed exhaled CO and NO measurements with two different flow rates. Study II was performed on ten healthy adults who ingested 240 mg of nitrate on empty stomach for consecutive measurements of exhaled and nasal NO and salivary nitrate and nitrite, followed by a series of mouthwash experiments. In study III, 27 children with allergic asthma and 21 age-matched controls were examined with exhaled NO, EBC nitrite and nitrate, blood eosinophils, spirometry and methacholine challenge. Whereas EBC and salivary nitrite and nitrate, together with exhaled NO, were studied before and after mouthwash with the anti-bacterial solution of chlorhexidine in15 CF patients and 15 controls in study IV.
Results: Exhaled CO, was in contrast to NO, not elevated in asthma and allergic rhinitis, and both markers were negative in CF. The change of exhalation flow rate did, furthermore, not affect the levels of CO but gave a proportional change of NO. The intake of nitrate resulted in a 150% increase of exhaled NO after 2 h, whereas nasal NO was unaffected. This increase was largely abolished by chlorhexidine mouthwash, which also decreased baseline NO levels with 30%. EBC nitrite, but not nitrate, was significantly elevated in the children with allergic asthma, but no correlation was found to increased levels of NO or other disease markers. EBC nitrite was also significantly higher in the CF patients, as was salivary nitrite, but these levels were almost eradicated by chlorhexidine, which in addition reduced exhaled NO more in CF than in controls.
Conclusions: The flow independence of exhaled CO proves that it has its origin in the alveoli and is therefore not a suitable marker for bronchial inflammation. There is a substantial salivary contribution to exhaled NO from the non-enzymatic reduction of nitrite, which can be greatly increased by the intake of nitrate-rich foods. There is also a most prominent salivary contribution to EBC nitrite in CF, and probably even in asthma, which indicates an altered activity of oral bacteria in these conditions, rather than increased NO metabolism, as an explanation for their higher levels of EBC nitrite.

LIST OF PUBLICATIONS

I. **Zetterquist W**, Marteus H, Johannesson M, Nordvall SL, Ihre E, Lundberg JON, Alving K. Exhaled carbon monoxide is not elevated in patients with asthma or cystic fibrosis. *Eur Resp J* 2002; 20: 92-99.

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IV. **Zetterquist W**, Marteus H, Kalm-Stephens P, Näs E, Nordvall SL, Johannesson M, Alving K. Oral bacteria – the missing link to ambiguous findings of exhaled nitrogen oxides in cystic fibrosis. *Resp Med* (2008), doi: 10.1016/ j.rmed.2008.09.009. In Press.

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LIST OF ABBREVIATIONS

ATS	American Thoracic Society
CF	Cystic fibrosis
CO	Carbon monoxide
COPD	Chronic Obstructive Pulmonary Disease
EBC	Exhaled breath condensate
ECP	Eosinophilic cationic protein
EDRF	Endothelial Derived Relaxing Factor
EPO	Eosinophilic Peroxidase
EPX	Eosinophilic Protein X
ERS	European Respiratory Society
FENO	Fraction of exhaled NO
FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
GM-CSF	Granulocyte macrophage – colony stimulating factor
HO-1	Heme oxygenas -1
ICS	Inhaled corticosteroids
IFN-γ	Interferon-γ
IgE	Immunoglobulin E
IL	Interleukin
ISAAC	International Study of Asthma and Allergies in Childhood
NDIR	Non-disperse infrared (analyser)
NF-κb	Nucleotide factor –κb
NO	Nitric oxide
NOS	Nitric oxide synthase
ppb	Parts per billion
ppm	Parts per million
RSV	Respiratory Syncytial Virus
Th	T-helper cell
TNF-α	Tumour necrosis factor-α

1 BACKGROUND

CHRONIC AIRWAY INFLAMMATION

There are several conditions which could be sorted under the epithet chronic airway inflammation. However, apart from perhaps chronic obstructive pulmonary disease (COPD) in the elderly population, asthma is by far the most important of these conditions in terms of prevalence and health economics for the population in general. Asthma is together with rhinoconjunctivitis the most frequent manifestation of allergic sensitization. As allergy is the most common chronic disease among schoolchildren in the Western world, the management of asthma is of particular importance from a paediatric perspective. Swedish figures also show that obstructive airway symptoms are the most frequent cause for admission to paediatric emergency clinics. According to the latest survey from the International Study of Asthma and Allergies in Childhood (ISAAC) the prevalence of asthma symptoms is about 10% among Swedish schoolchildren, while the prevalence of doctors-diagnosed asthma is slightly less, about 7 %, which is in line with figures from several other current studies (1). The Swedish figures are quite representative for other European countries but an even higher prevalence is seen in the UK, as well as in other developed English-speaking countries, e.g. the US and Australia. Until the last decade the incidence of asthma has shown a steady increase, but it has now levelled out and even decreased for older children and teenagers in Sweden and neighbouring countries. In stead, there is a current increase in the incidence of asthma in developing countries, according to the ISAAC study.

Cystic fibrosis (CF), which is an inherited multisystem disorder with a severe chronic airway inflammation as a main characteristic, is a much less common cause for airway inflammation, but should be taken into consideration as a differential diagnosis in children with repeatedly difficult obstructive episodes. The incidence of CF varies widely with ethnicity but the highest figures are found among the white population where it is found in approximately 1 per 4000 live births. It is inherited as an autosomal recessive disease and in this population it is the most common life-limiting disorder with recessive trait. The airway inflammation in CF is supposedly due to the impaired salt secretion of the airway epithelium and thereby a markedly reduced content of water in the mucus on the epithelial surface. This mucus with high viscosity reduces ciliary clearance and makes the CF airway more susceptible to infections and chronic infestations of airway pathogens. The airways become obstructive from the poor mucus

clearance but also by the general inflammatory activity, causing subepithelial oedema, smooth muscle contraction, and airway remodelling.

Mechanisms of asthma development and sensitisation

The chronic airway inflammation in asthma has some features in common with those of the CF airways, as it is characterized by a mucous oedema, smooth muscle hypertrophy, increased mucus secretion, and epithelial damage. However, the inflammatory process of the asthmatic airways also results in hyperresponsiveness to different provocative agents, causing recurrent episodes of airflow obstruction. Between these obstructive periods the patients might have very limited symptoms of their asthma, or even lack manifestations completely, in spite of the persisting inflammatory activity.

The driving force of the asthmatic inflammation can be of various nature and the different mechanisms are not yet fully understood. This difference in asthma development is probably explained by different asthma phenotypes and a variable response to provoking stimuli with age and physical development. During infancy and early childhood this process can develop in response to respiratory infections, particularly with airway viruses, such as rhinovirus. Bronchiolitis in early life with respiratory syncytial virus (RSV) have also shown to increase the later incidence of childhood asthma. Others develop asthma which rather responds to physical stimuli, such as exercise, cold air and non-allergic irritants. However, the predominant mechanism for the development of more persistent childhood asthma is the sensitization to allergens.

The fundamental condition for the development of an allergic inflammation in the airways is an immune response to certain allergens resulting in the production of IgE antibodies. It is still unclear why this formation of IgE antibodies to different allergens, also referred to as atopy, is evoked. However, genetic predisposition is certainly a key factor but the increasing incidence of allergies in the last decades is also explained by a general shift in the immune response from the Th1- to the Th2-pathway. Allergens and some microbial antigens are presented to a type of T-lymphocytes, called T-helper cells, on the surface of different antigen presenting cells, particularly dentritic cells and macrophages. Antigen presenting cells that are infected by an intracellular pathogen release IL-12 which strongly stimulates the Th1 subset of T-helper cells. The Th1 cells produce among other things IFN-γ, which in turn helps the antigen presenting cell to kill the intracellular microbe by potentiating its microbicidal mechanisms. In the

allergic immune response there is instead an activation of Th2-cells upon allergen presentation by dendritic cells or macrophages. The Th2 cells release cytokines, e.g. IL-4, IL-5, IL-9, and IL-13, which stimulate B-lymphocytes to the production of IgE antibodies. The formation of IL-12 strongly promotes Th1 cell proliferation and IFN-γ from the Th1 cells effectively inhibits Th2 activation. The possible shift in our immune systems towards the Th2-pathway is a part of the hygiene hypothesis, as an attempt to explain the increased incidence of allergies. This suggests that our interactions with microbial antigens have decreased in modern society – e.g. from vaccinations, managing of foods, and increased personal hygiene – and, therefore, the immune system favours the Th2-pathway and the development of IgE-mediated allergies.

The allergic airway inflammation

In case of allergic sensitization IgE antibodies will be bound to the surface of mast cells which are found in abundance in the airway mucosa. When the individual is exposed to the specific allergen this interacts and cross-links with the IgE antibodies on the mast cell's surface, resulting in degranulation of the mast cell and the release of especially histamine and leukotrienes, which are largely responsible for the early-phase reactions of the allergic airway inflammation. These include bronchospasm, vasodilatation and increased vascular permeability – resulting in a mucosal oedema, and infiltration of inflammatory cells. Other substances released are, for example IL-4, TNF-α and eotaxins, which all play a role in the recruitment of additional inflammatory cells and, thus, for the development of the late-phase reaction. More recent research has shown that IgE-coated basophils also contribute to the early-phase reactions by performing in a fashion similar to the mast cell (2).

The late-phase reaction establishes the inflammatory activity and is largely responsible for the development of a chronic airway inflammation. This phase is characterized by the migration of inflammatory cells from the circulation and bone marrow to the tissues of the airways. There is a variety of cell types involved in the late-phase reaction, such as eosinophils, neutrophils, basophils, mast cells, dendritic cells, macrophages and Th2-lymphocytes. However, the infiltration of eosinophils to the inflammatory tissue represent the most typical and specific cellular change in the development of allergic asthma. They are recruited from the bone marrow as mature eosinophils or precursor cells, whose maturation is particularly stimulated by IL-5 and GM-CSF, and released to the blood. The degranulation of mast cells and other inflammatory cells elicits the formation of specific adhesion molecules on the

endothelial surface by the site of allergic inflammation, which guides the eosinophils (but also other participating cells) to the affected tissue. Thus, in allergic asthma increased numbers of eosinophils can be found in the target organ, by different methods of airway sampling, as well as in the peripheral blood. Activated eosinophils release a number of mediators, which further enhance and aggravate the inflammatory response. The most important of these are perhaps the eosinophilic granule proteins; eosinophilic cationic protein (ECP), eosinophilic peroxidase (EPO), and eosinophilic protein X (EPX). They have been shown to damage epithelial cells, induce airway responsiveness and cause additional degranulation of mast cells and basophils (3). The activation of eosinophils also yield substantial formation of leukotrienes, which increase bronchial constriction and vascular permeability, as stated above. In addition, there are several cytokines released from the recruited eosinophils, where perhaps IL-5 has the most profound impact on further eosinophilic activity and the proceeding of the allergic inflammation. It does not only stimulate the maturation and release of additional eosinophils from the bone marrow, but also stimulates their cytokine production and is capable of inducing the self-generation of eosinophil precursor cells in the allergic tissue.

The inflammatory cells are attracted and guided to the inflammatory site by different mediators, such as the cytokines IL-4, IL-5, TNF-α and GM-CSF. There are also chemokines released from eosinophils and other inflammatory cells which serve this specific purpose, e.g. eotaxin and RANTES. The former seems to act more exclusively on eosinophils and is stimulated by Th2 released cytokines, as IL-4 and IL-13 (4). In addition to eosinophils, the Th2 lymphocytes constitute a very important cell type for the inflammatory process in allergic asthma and other atopic conditions. As stated above, they initiate the allergic sensitisation, however, they also orchestrate the forth going inflammatory machinery by their continuous release of pro-inflammatory cytokines. Furthermore, the Th2 cytokines, e.g. IL-4, IL-5, IL-9, and IL-13, can delay the apoptosis of other inflammatory cells and thereby prolong and aggravate the inflammatory response. These cytokines also contribute to the remodelling and the structural changes of the airways that develop after more sustained allergic inflammation in the tissue. More specifically they have been shown to induce mucus production, stimulate fibroblast growth and subepithelial fibrosis. In addition to the effect on fibroblasts and mucus cells, there is an influence on the proliferation and function of epithelial cells and smooth muscle cells by the inflammatory process and its mediators, eventually leading to more comprehensive structural and physiological

changes of the airways. The remodelling of the airways in chronic allergic asthma normally includes thickening of basement membranes, submucosal fibrosis, hyperplasia of smooth muscle and mucus cells, and metaplasia of the airway epithelium. These structural changes cause a gradual decline in lung function and contribute, together with the vast presence of inflammatory cells, to the typical hyperreactivity of chronic asthma. The increased reactivity in the airways to different provocative stimuli, among these also infectious agents, maintains and reinforces the allergic inflammation.

The allergic airway inflammation is a very complex process where different cell types and their mediators interact and stimulate each other to an often increasingly active response. However, if there is no further exposure to the allergens in question and a limited burden of other stimuli there is a good chance that counter-mechanisms in our biological system can be successful and prevent the development of a chronic airway inflammation. Nevertheless, if the allergic spark is lit there are the prerequisites for the development of a more or less self-perpetuating process.

MARKERS OF AIRWAY INFLAMMATION

As long as the individual with asthma suffers from recurring episodes of asthmatic symptoms, there is a chronic inflammatory activity present in the lower airways. However, during periods of variable length there are often no evident signs of this ongoing disease activity. Management of asthma is therefore a challenging task as it might be difficult to be on target with the anti-inflammatory treatment. There is an evident risk of either insufficient regimes or excessive treatment with unwanted side effects as the major outcome. With insufficient treatment, the inflammatory process in the airways will be aggravated, leading to further tissue damage and remodelling and, thus, increased symptoms of hyperreactivity and airflow obstruction. Therefore, to optimize asthma control there is a need for sensible markers of airway inflammation, which can provide us with a proper status of current disease activity even when symptoms are scarce.

Different candidate markers, from variable means of sampling, have been suggested to serve this purpose. Since the eosinophil is a key cell of the allergic inflammation in asthma, several ways of assessing its activity have been explored. Increased numbers of eosinophils are found in peripheral blood, induced sputum, bronchoalveolar lavage and samples of bronchial tissue from patients with allergic asthma and their levels have further been demonstrated to correlate with disease

activity (5-9). Alternative markers of eosinophilic activity are the granule proteins, e.g. ECP, EPO and EPX, where ECP is the most extensively studied. This marker has also been shown to be elevated in similar ways of sampling and its correlation to asthma severity has been demonstrated (10-12), even though data on the latter are ambiguous (13-15). EPX is excreted in the urine and determining its levels in a urine sample can serve as a non-invasive alternative of eosinophil activity in asthma, but its specificity and sensitivity is rather low, as is its correlation to lung function parameters (16). Nevertheless, the eosinophil markers are in general informative and provide a good estimate of the current inflammatory activity in the airways. Apart from the inevitable delay between sampling and test result, their wider clinical use is, however, limited by their rather invasive means of sampling, which can be troublesome and painful for the patient, as well as time consuming and costly for the clinic. These shortcomings probably limit the clinical usefulness of some other soluble products of inflammatory activity as well, such as cytokines and leukotriene metabolites, for asthma monitoring.

Exhaled markers

Inflammatory markers that can be detected in the exhaled air, also with the possibility of getting an immediate result, seems as a more attractive alternative, in comparison with the eosinophil markers, for the development of a clinically applicable tool. Collecting the marker from exhaled air has the obvious advantage of being truly non-invasive, but it is also a simple way of obtaining samples from the actual site of inflammation, i. e. the conducting airways and the lung, which should be preferable in terms of specificity, as compared to sampling peripheral blood or urine.

This is where measurements of exhaled nitric oxide (NO) come in. This airborne molecule was first described in the exhaled air of humans by Gustafsson et al in 1991 (17). When Alving *et al* in 1993 (18) found increased concentrations of NO in the exhaled air of asthmatics, detected on-line with a chemiluminescence analyser, an entirely new and extremely active research field emerged to evaluate and develop this method. Today, there are more than 1550 articles published with the terms “exhaled nitric oxide” in their abstract! The NO production in the airway epithelium is mainly induced in allergic, i.e. eosinophilic, inflammation and the highest levels of exhaled NO are normally found in individuals sensitized to airborne allergens. Exhaled NO in patients with allergic asthma has repeatedly been shown to correlate with eosinophilic activity and other disease markers. It is now the most established marker of airway inflammation in clinical practice and in parallel with the development of smaller, less

expensive and more user-friendly analysers the use of exhaled NO is steadily increasing for asthma monitoring.

The introduction of exhaled NO and the concept of investigating the exhaled air for inflammatory products has also opened up for research regarding other possible markers and sampling methods. Carbon monoxide (CO) is another airborne molecule which can be detected in exhaled air and in 1997 Zayasu et al reported elevated concentrations of exhaled CO in asthmatics (19). This was followed by a number of studies where exhaled CO was found increased in various inflammatory airway conditions. Interestingly, exhaled CO was also reported elevated in patients with CF (20), in contrast to NO - which is surprisingly low given the intense inflammatory activity in the airways of these individuals (21, 22). The increase of CO in airway inflammation has been argued to reflect oxidative stress (23), which is a part of every inflammatory activity, allergic or not.

Another non-invasive method to sample the airways is by cooling down the exhaled air and obtain a liquid phase of the condensed water vapour and droplets. The latter are believed to be released from the surface of the airway epithelium and the content of the droplets could possibly reflect the composition of substances found on the epithelial surface, including different inflammatory products. However, inflammatory cells and even larger molecules are normally not found in the condensate, as they can not be carried in these water droplets. Several substances in exhaled breath condensate (EBC) have been found elevated in asthma and CF, as well as in other airway conditions. Given the large interest for exhaled NO, special attention has been paid to soluble NO metabolites - such as nitrite, nitrate and s-nitrosothiols – which in contrast to NO itself have been reported to be increased in both asthma and CF (24-28). Collection of EBC has consequently gained a general interest, as it might not only give further information on the role of nitrogen oxides in airway inflammation, but also provides a potential to study a wide range of substances taking part in the pathophysiology of airway disease.

NITRIC OXIDE (NO)

Nitric oxide is an inorganic gas molecule which for long was considered a merely exogenous substance, mainly generated from different types of combustion. There are substantial amounts of NO in, for example, car exhausts and cigarette smoke, and it was therefore primarily regarded as an air pollutant. It was not until 1987 that an endogenous formation and a physiological role of NO were discovered. This was when

researchers found that NO is responsible for the actions of the formerly known endothelium-derived relaxing factor (EDRF) and, thus, plays an important role in the regulation of vascular tone (29, 30). Further extensive research has shown that there is a substantial formation of NO in just about every tissue in the body and that it is an important actor in a large number of biological processes - where the complex machinery of inflammation is one. The initial discovery of NO as a potent endogenous vasodilator, which lit the spark to this entire research field, was awarded with the Nobel Prize in 1998.

Formation and actions of NO in the biological system

The formation of NO in mammalian cells is primarily enzymatic and provided for by different NO synthases (NOS), which convert L-arginine to NO. There are three known iso-forms of NO synthase, referred to as neuronal NOS (nNOS or NOSI), inducible NOS (iNOS or NOSII) and endothelial NOS (eNOS or NOSIII). The actions of nNOS and eNOS are Ca^{2+}-dependent and serve a constitutive function in the production of small amounts of NO for the regulation of nerve transmission and vascular tone (31). The activity of the iNOS enzyme is induced under inflammatory conditions and is rather regulated at the transcriptional level. Its induction results in the formation of relatively large amounts of NO, which might yield several-fold higher levels of NO compared to constitutive NO synthases. The expression of iNOS is stimulated by different cytokines, transcription factors (such as NF-κb), bacterial products and oxidative stress (32). This enzyme can be expressed in many different cell types, but has primarily been demonstrated in activated inflammatory cells - such as macrophages, monocytes and polymorphonuclear cells – and in epithelial cells (33). Increased amounts of iNOS has been recognized in the bronchial epithelium of asthmatics (34). It is, consequently, the induction of iNOS in the respiratory epithelium and in different inflammatory cells, which is believed to be responsible for the elevated levels of NO seen in the exhaled air of patients with asthma and some other inflammatory airway conditions.

There is no single explanation for the role of iNOS and NO formation in inflammation. NO has rather been demonstrated to have multiple actions in the inflammatory process, both beneficial and harmful. On a cellular level NO has well-documented bacteriostatic, antiviral, antiparasitic, and anti-tumoural properties and thereby contribute to the immune system’s combating of pathogens and tumour growth (33, 35). There is also evidence that NO can act as a scavenger for the free radicals

which appear by oxidative stress (36). However, in higher concentrations of certain oxygen free radicals there may be a reaction with NO forming even more reactive radicals, for example peroxynitrite (37, 38). In the inflamed airways it has a positive bronchodilating effect and it seems to be a potent dilator of the bronchial circulation, which could serve a protective role by clearing the tissues from antigens and inflammatory products (39).

The pro-inflammatory action of NO and the inducible NOS enzyme is also demonstrated by the strong inhibition of NO formation seen with the treatment of glucocorticoids (further referred to as steroids) (40). Their suppression of iNOS expression is believed to act through the down regulation of NF-κb (41). However, it seems as if this steroid induced inhibition of NF-κb goes through the suppression of pro-inflammatory cytokines and is not a direct effect on the iNOS transcription (42). Studies of asthma treatment with inhaled steroids have shown dose-dependent reductions of exhaled NO levels (43) and correlations to parallel reductions of eosinophil numbers in different airway samples (44).

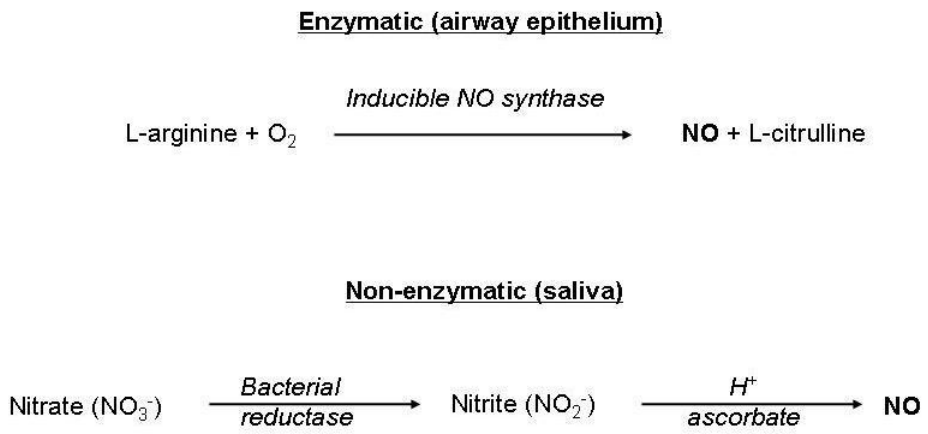

Fig 1. Nitric oxide formation pathways. NO is formed enzymatically by different isoforms of the NO synthase (NOS) enzyme, using L-arginine as a substrate. The inducible form (iNOS) constitute the majority of the NOS enzymes in the respiratory epithelium of the conducting airways and the paranasal sinuses. Its expression is markedly increased by inflammatory processes in the airways, particularly by eosinophilic inflammation, as seen in allergic asthma. NO can also be formed non-enzymatically through the reduction of nitrite. This occurs in the oral cavity and in the gut from salivary nitrite, which in turn has been formed from the bacterial reduction of nitrate in the saliva.

In addition to the enzymatic, NOS-dependent, production of NO there is also a non-enzymatic formation of NO from the direct reduction of nitrite. This reduction is enhanced in an acidic environment and by the presence of other reducing agents (45). The non-enzymatic NO formation has been demonstrated to occur in various organs and body fluids. There is, for example, a substantial reduction of nitrite in the ischemic myocardium which serves a protective role by increasing local blood flow (46). Salivary nitrite has been shown to generate NO in the oral cavity (47, 48) and the stomach (49), which has a documented antibacterial effect on local pathogens (50, 51). The host-defence mechanism of nitrite reduction has also been demonstrated to play a role in the urinary tract (52, 53) and on the skin (54, 55).

Chemistry and metabolism of NO

The small size and lipophilicity of the NO molecule allows it to diffuse easily through cell membranes. NO is fairly stable in the gas phase, whereas it is a very labile compound in solutions and different body fluids. The NO molecule therefore favours the gas phase and readily diffuses to luminal air in organs where this is possible. This diffusion to luminal air is the basis for measurements of exhaled NO, as the formation of the molecule mainly takes place on the inside of epithelial cells and various inflammatory cells. NO can also be detected in luminal air from the intestines, the urinary bladder, and the paranasal sinuses due to similar diffusion from various cell types in respective mucous tissue. It could here be mentioned, that highly increased levels of NO has, for example, been demonstrated in samples of intestinal air from patients with inflammatory bowel disease (56, 57) and in air samples from the urine bladder of patients with different types of cystitis (58).

The concentration of NO in gaseous phase is best determined with chemiluminescence technique. This method is based on the reaction between NO and ozone, which yields NO_2 and light. The amount of light emitted is directly proportional to the NO concentration in the gas sample. The concentrations of NO in luminal air from different body compartments vary, but in general they represent a very small part of the total particle content and are expressed as parts per billion (ppb) or parts per million (ppm).

In solution, NO is quickly converted to more soluble and stable metabolites and the direct measurement of NO in body fluids is therefore difficult. Assessment of NO activity in body fluid samples is rather obtained by indirect methods, such as measurements of soluble end products of NO. The complete scheme of possible

pathways and metabolic products of NO metabolism is quite complex and depends on the presence of different co-substrates. However, under normal conditions NO is mainly oxidized to the stable end products nitrite and nitrate. Alternative metabolites of NO that are fairly stable and can be measured in solution are s-nitrosothiols or nitrotyrosine, where the former is formed upon reaction with redox-activated thiols and the latter requires peroxynitrite (ONOO-) for its synthesis. Peroxynitrite is a product of NO plus the free radical superoxide (O^{2-}), which represents one of the scavenging actions of NO. Even peroxynitrite is, however, mainly oxidized to nitrate rather than reacting with tyrosine.

NO in the airways

There are at least two main sources of NO in the human airways, which both can contribute to the NO detected in exhaled air. In the use of exhaled NO as a marker of lower airway inflammation, it is the iNOS dependent NO formation from epithelial cells and activated inflammatory cells of the bronchial mucous tissue which is of main interest. The respiratory epithelium comprise a much larger total cell surface than the inflammatory cells and studies have also demonstrated that the epithelial iNOS is the major determinant of NO concentrations in exhaled air (59). Increased levels of NO is not only found in the exhaled air of asthmatics, but can also bee seen in patients with mere allergic rhinitis (60, 61). This is however probably due to a subclinical bronchial inflammation. In atopic subjects not currently exposed to the aeroallergen and not suffering from any respiratory symptoms exhaled NO has been found to be normal (62). Nevertheless, it seems as if the sensitization to airway allergens is a strong driving force for the induction of NO synthesis in the respiratory epithelium. This may be concluded from several reports where atopic subjects with asthma or mere allergic rhinitis display significantly higher levels of exhaled NO than non-atopic subjects with asthma and/or rhinitis (60, 63). Chronic obstructive pulmonary disease (64), bronchiectasis (65) and viral respiratory infections (66, 67) are other airway conditions where increased levels of exhaled NO have been reported. However, all individuals release NO to the exhaled air, even without any signs of airway inflammation, and whether epithelial iNOS is exclusively responsible for this low-rate constitutive NO formation is still unclear. The two other isoforms (eNOS and nNOS) are also found in bronchial tissue and eNOS is even expressed in respiratory epithelial cells and may contribute to the baseline output of NO from the lower airways. The fact that there is a constitutive NO formation in the bronchial epithelium indicates also a basic role for NO

in the local homeostasis. This could be in maintaining host defence mechanisms or regulating bronchial and vascular tone.

The other principle source of NO in the airways is the nose and the paranasal sinuses. In fact, the highest airway concentrations of NO in humans are found in air sampled from these sinuses. In human sinus epithelial cells there is a high constitutive expression of iNOS responsible for this substantial NO output, which possibly serves an antimicrobial effect to maintain a sterile milieu in the sinuses (68). The NO synthesized in the sinuses spread to the nasal cavity and mix with breathing air. Thus, NO concentrations in nasal air are comparatively high, as seen in a several-fold increase of the NO content in nasally versus orally exhaled air (69). However, the iNOS expression of the airway epithelium in the nasal cavity is moderate (68), as is probably the NO contribution from this tissue. Furthermore, the admixture of NO generated in the sinus epithelium to breathing air might serve physiological roles in modulating ciliary motility (70) and improve alveolar oxygenation (71). The former effect of NO is supported by the fact that patients with Primary ciliary dyskinesia present an almost total lack of nasal NO (72) and that CF patients, with their impaired mucociliary clearance, also have comparatively low nasal NO levels (21, 73).

In general, the formation of NO in the sinuses and the contribution from the nasal area seems fundamentally different from the one found in the lower airways. There is, as mentioned, a constant high NO output from constitutively expressed iNOS enzymes in the paranasal sinuses even in healthy controls, which appears to serve basic physiological functions. In the bronchial epithelium there is, in contrast, a very moderate expression of NOS enzymes in healthy individuals and the induction of iNOS and high-rate production of NO commence first with the presence of inflammatory activity. The similar induction of iNOS upon inflammatory activity in the sinuses and the nasal cavity, as could be expected in conditions such as allergic rhinitis, has not been clearly demonstrated. Increased levels of nasal NO has been reported in asthma and allergic rhinitis (74), but other results are more conflicting (75, 76).

Measurement of exhaled NO

The technique employed for analyzing the concentration of NO in exhaled air is predominantly the previously described chemiluminescence technique. In the early stages many different methods were used to measure exhaled NO. In some studies the NO concentration was determined from an impermeable reservoir which the subjects had filled with a fixed volume of exhaled air. However, on-line measurements, where

samples of the exhaled air are continuously fed into the chemiluminescence analyzer, soon became the procedure of choice. Continuous registrations of the NO concentration during tidal breathing can be employed. But since the bronchial output is of main interest, the single-breath procedure with a prolonged exhalation has evolved as the standardized method. During a single-breath maneuver there is an initial peak of NO, composed of accumulated NO and possibly even nasal and oral contamination, which is followed by a plateau phase with a fairly stable NO concentration. This plateau concentration represents the NO output from the conducting airways, i.e. bronchial NO. In current standard methods the NO concentration of exhaled air is determined from a fraction of this plateau phase.

There are two main factors which have to be considered for comparable results and for the standardization of the single-breath procedure. These are the avoidance of nasal contamination and flow-dependency of exhaled NO concentrations. Since nasal air contains higher and more variable concentrations of NO, admixture from the nose yields inconsistent and elevated levels which could be falsely interpreted as a sign of current bronchial inflammation. This is avoided by the employment of a positive pressure during the oral exhalation, which cause a closure to the nasal cavity by the soft palate. In the early stages this was solved by the application of different resistors, with fixed resistances, to the exhalation device, but modern NO-analyzers have solved this with one variable resistor which adjusts to the exhalation flow rate for an appropriate positive pressure. The flow-dependency of exhaled NO concerns mainly the NO concentration in air from the plateau phase of exhalation, which represents bronchial NO. The strict relationship between exhalation flow rate and the concentration of NO in the exhaled air is here explained by the fact that there is a continuous contribution of NO from the conducting airways to the air passing by (77). A low exhalation flow rate allows more NO per volume of air to diffuse from the bronchial epithelium than a high flow rate. Thus, low exhalation flows yield high NO concentrations and vice versa. In fact, if the more extreme flow rates are excluded, there is an almost linear relationship between flow rate and NO concentration, where a 2-fold increase of the flow rate gives a 50% reduction of NO concentration and so on (78, 79). Initially, different research groups used different flow rates, which obviously complicated the interpretation and comparisons of reported results. This could be partly remedied by converting the NO concentrations to NO “release rate” (pmol/s), where the ppb level of NO was multiplied with flow rate and transformed to pmol/L and (see Methods). Nevertheless, since this procedure is a bit complicated and it was not unanimously performed it became evident

that there was a strong need for the use of a standardized exhalation flow rate. It has been shown that reproducibility and discrimination between patients with asthma and controls gain from comparatively low flow rates, but for the very low exhalation flows the time to reach the plateau phase will be so prolonged that a single-breath manoeuvre is difficult to perform, especially for children (77, 79). Eventually, international researchers came to a consensus decision for the use of 0.05 l/s as the standardized exhalation flow rate, which was first stated in the American Thoracic Society's (ATS) guidelines for measurements of exhaled nitric oxide in1999 (80). This was also reinforced in the 2005 joint statement from ATS and European Respiratory Society (ERS) on the recommended procedures for measurements of exhaled and nasal NO (81).

Asthma and exhaled nitric oxide

Increased levels of exhaled NO in both adults and children with asthma, as compared with age-matched control subjects, have been reported in numerous studies (81) and measurements of NO in exhaled air has today become an approved method for asthma monitoring even in clinical practice. Exhaled NO has been shown to correlate with different eosinophil markers, such as eosinophil counts in peripheral blood (82), induced sputum (83), bronchoalveolar lavage (84) and bronchial biopsies (85). In some studies a correlation to bronchial reactivity and lung function have been established (86, 87), but these results are ambiguous (13, 88, 89) and in general there is a poor correlation between exhaled NO and lung function parameters (81). As earlier mentioned, there is a decrease in exhaled NO concentrations upon treatment with steroids (43, 44), but also with the administration of leukotriene antagonists (90).

The elevated bronchial output of NO is primarily seen in individuals with atopy and allergic asthma, whereas other phenotypes, such as the one of more physiological nature with mainly exercise-induced airway obstruction seems to display more normal levels of exhaled NO (60, 63). Nevertheless, some element of atopy is most frequently present in asthmatic subjects, especially among children with asthma. The use of exhaled NO as a marker of airway inflammation can therefore be well justified as a clinical tool in the management of asthma. It has been reported as superior to lung functional tests in diagnosing asthma (91) and it has shown useful in the prediction of asthma exacerbations (92). Monitoring exhaled NO has also been proposed to improve overall asthma control (81) and verify adherence to therapy (93), as well as response to anti-inflammatory treatment (94).

Cystic fibrosis and exhaled nitric oxide

In spite of the intense inflammatory activity present in the airways of patients suffering from cystic fibrosis (CF), their concentrations of exhaled NO are not elevated. In fact, several studies have shown subnormal levels of NO in the exhaled air from patients with CF (22, 95). Given the normal induction of NO formation in inflammatory processes this has been considered a puzzling paradox, which is not yet fully understood. One contributing factor could be that the inflammatory process in CF is characterised by a predominantly neutrophilic activity (96), whereas the iNOS expression and exhaled NO mainly increase by eosinophilic inflammation (97). Another often suggested explanation is attributed to the thick and characteristic mucus that lines the airway epithelium in CF and possibly prevents the diffusion of NO to the lumen (98). This has been proposed to rather cause an accumulation of soluble NO metabolites, such as nitrite and nitrate. Findings of elevated concentrations of nitrite in breath condensate from subjects with CF have been argued as evidence for this (25, 26, 98), as have reports of increased contents of nitrite/nitrate in saliva (99) and high concentrations of nitrate in sputum (100). Even concentrations of nitrotyrosine have been found elevated in EBC (101) and sputum (100) from CF patients. These findings of increased NO metabolites in CF have been argued to reflect a genuinely increased activity and turn-over of NO, however, not visible in the exhaled air because of the issued mucus trapping (98). However, there are also other findings which rather suggest that there is some fundamental dysfunction of the NOS enzymes in CF or an impaired regulation of their expression. Studies of iNOS from CF patients *in vitro* have for example shown a defect in their signalling system (102, 103) and other reports declare a decreased expression of iNOS in the CF bronchial epithelium (104). They even seem to have a reduced expression of NOS enzymes in their nasal mucosa (105), which is in line with their reportedly lower levels also of nasal NO (21, 73).

Non-enzymatic NO formation and airway sampling

Nitrate is formed in the human body through NO metabolism or it can be supplied through dietary sources. Ingested nitrate is largely absorbed to the blood where it is added to the pool of nitrate formed through nitrogen metabolism of the body. A substantial part of plasma nitrate is transported to the salivary glands, from where it is excreted with the saliva to the oral cavity. Salivary nitrate is then reduced to nitrite through the action of the bacterial enzyme nitrate reductase, which is found in different

species colonizing the oral cavity and pharynx, especially facultative anaerobic bacteria on the posterior surface of the tongue (47, 106). It has further been shown that this nitrite can be non-enzymatically reduced to NO in the pharyngo-oral tract of humans (47). However, a large proportion of the salivary nitrite is swallowed to the gut where the acidic environment enhances the chemical reduction to NO (49). This alternative pathway for NO synthesis has been demonstrated to serve an important function of host defence against microbial pathogens in both the oral cavity and the gut (50, 51). Whether the enterosalivary circulation of nitrate and its non-enzymatic NO formation could influence measurements of exhaled NO was not known by the time of planning for this thesis. That query, and, consequently, to decide whether the intake of nitrate rich foods could be a confounding factor for the determination of NO in exhaled air, was therefore set as one of the aims of the research project.

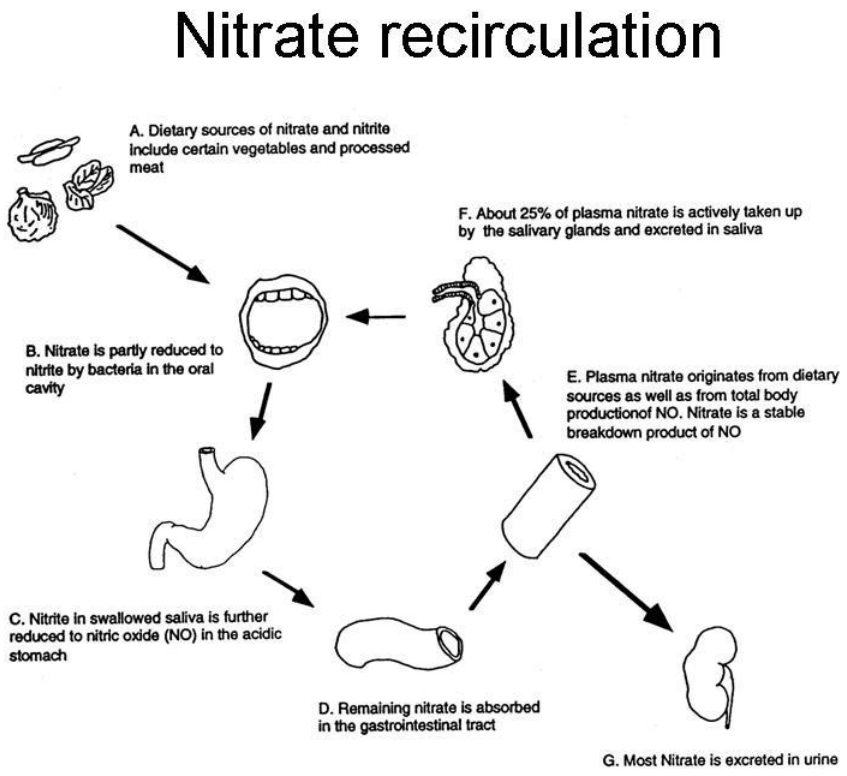

Fig 2. Enterosalivary cicuit. Dietary nitrate is absorbed to the circulation from the gastrointestinal tract. A substantial part of nitrate in plasma is actively taken up by the salivary glands and excreted with the saliva. In the oral cavity this nitrate will be reduced by bacteria to nitrite through the action of the bacterial enzyme nitrate reductase. The nitrite can, in turn, be non-enzymatically reduced to NO, which contributes to the host defence mechanism against different pathogens in the oral cavity and the stomach. Reproduced by the kind permission of Professor Jon Lundberg.

NITROGEN OXIDES IN EXHALED BREATH CONDENSATE

The idea of collecting exhaled breath condensate (EBC) for markers of airway inflammation has been around for more than a decade and one of the first reports on the subject is from 1993, where elevated levels of hydrogen peroxide is described in EBC from children with asthma (107). The findings of increased NO in exhaled air of asthmatics brought the attention to soluble NO metabolites in EBC and in 1995 elevated levels of nitrite was reported in the expired condensate of subjects with acute asthma (24). Subsequently, increased EBC nitrite have been proclaimed for both paediatric asthma, cystic fibrosis and COPD (25-27). Also nitrate, being the soluble end-product of NO metabolism, has been reported elevated in EBC from patients with asthma and acute pneumonia (28). In addition, increased levels of other putative NO metabolites, such as nitrosothiols and nitrotyrosine, have been reported in EBC from subjects with asthma, CF, and COPD (27, 101, 108).

As EBC is collected through normal tidal breathing it is yet the non-invasive method of airway sampling which requires the least of cooperation from the subjects. Therefore, it could have a specifically large potential for investigating inflammatory markers in smaller children and in other patient categories with a limited ability to cooperate. This, obviously, applies to measurements of NO metabolites in EBC as a promising alternative to determining exhaled NO, which requires a controlled single breath manoeuvre at a fixed flow rate. In addition, it seems as if measuring nitrite and other nitrogen oxides in EBC can provide additional information on the role of NO activity in airway inflammation as these metabolites are increased also in conditions where exhaled NO is low or normal, such as CF and acute pneumonia. Limited information is available on levels of nitrogen oxides in EBC in atopic versus non-atopic asthma. However, it appears as if they, in parallel with exhaled NO, decrease with steroid treatment (108, 109).

CARBON MONOXIDE IN EXHALED BREATH

A few years after the first discoveries of exhaled NO it was reported that also carbon monoxide (CO) is increased in the exhaled air of asthmatics (19). This was highly interesting, since CO concentrations in exhaled air are normally hundred-fold higher than NO and can be detected by less sophisticated, i.e. less expensive, analysing techniques. Measurements of CO in exhaled air are normally applied as an assisting tool for smoking cessation where small, hand-held, electrochemical analysers are used. These analysers measure CO in parts per million (ppm) and a healthy non-smoking

individual presents levels of 1-2 ppm in the exhaled air, as compared with the approximately 10-20 ppb of NO.

As exhaled CO promised to be a novel and more attainable marker of airway inflammation a series of consecutive studies were published with the similar message; if there is airway inflammation - there is increased CO. In addition to asthma, elevated levels of exhaled CO was reported in patients with CF (20) and bronchiectasis (110). Increased amounts of exhaled CO were also reported in less severe conditions, not associated with a profound lower airway inflammation, such as atopic subjects without asthma (111) and in subjects with mere upper respiratory tract infections (112).

The increased CO output in the inflamed airways was explained by the higher presence of oxidative stress, as CO is formed in the first step of oxidative degradation of heme to bilirubin through the action of the haeme oxygenase (HO-1) enzyme. This reaction might serve a protective role against cell injury, since bilirubin has antioxidant properties and CO has the ability to stimulate guanylate cyclase (113). A certain increase of the HO-1 expression has been described in airway macrophages of asthma (23), but for the bronchial epithelium and the airways in general no such convincing data has been presented (114).

The fact that CO is present in many-fold higher concentrations than NO in exhaled air is obviously to its advantage as it allows for less sophisticated analyzing tools. In the studies referred to above exhaled CO was measured with quite simple electrochemical sensors, intended for smoking detection. For measurements with those analysers a proper exhalation is required but exhalation flow rate is not monitored or regulated. By using that procedure, CO measurements are also less complicated than registrations of exhaled NO, however, it might reduce the congruence between CO readings and the actual airway formation. An evident disadvantage of using CO as a marker of airway inflammation is the possible influence from ambient CO exposure. Cigarette smoke, also as passive exposure, gives elevated levels of exhaled CO, which possibly applies even for other forms of air pollution, such as car exhausts.

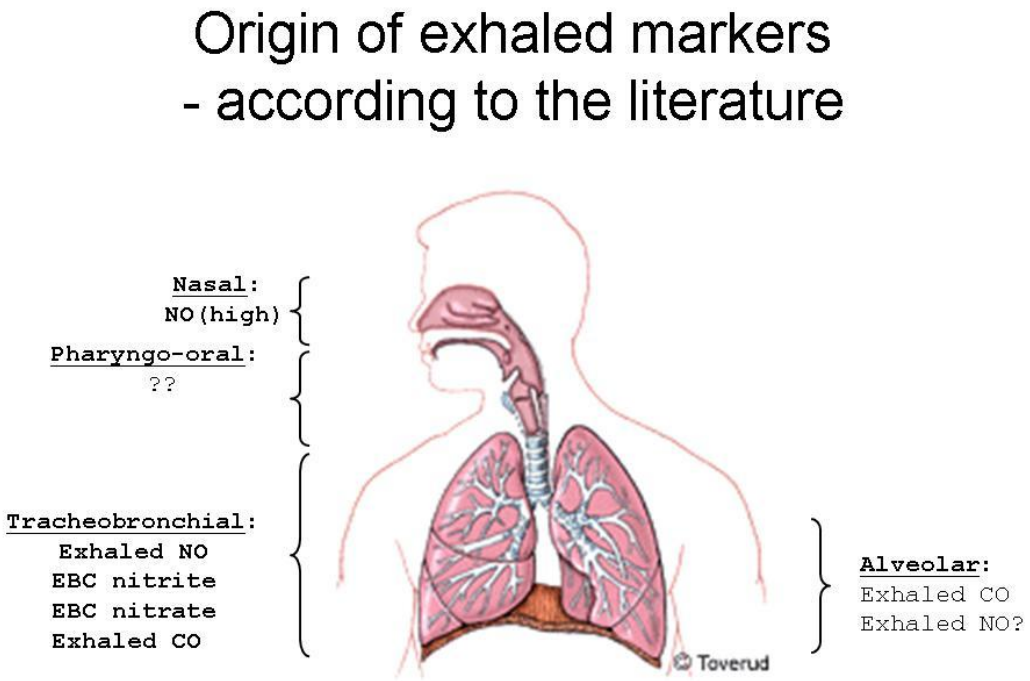

Fig 3. The origin of exhaled markers, as referred to in the literature. Exhaled NO and CO are enzymatically produced in the bronchial epithelium and their formation is induced by inflammation and oxidative stress, respectively. EBC nitrite and nitrate are the products of NO metabolism in the bronchi. Nasal NO levels are high due to constitutively high production of NO in the paranasal sinuses.

2 AIMS OF THE PRESENT STUDY

The overall aims of this thesis were to further study the origin of nitrogen oxides and carbon monoxide in the respiratory tract and their relation to airway inflammation and the possible influence on their presence in exhaled air from soluble nitrogen oxides in the enterosalivary circuit.

The specific aims were:

I. To evaluate differences in magnitude and airway origin of exhaled NO and CO in children and adults with asthma and cystic fibrosis by the introduction of highly specific infrared technique and flow rate-controlled measurements for CO.

II. To investigate the possible influence on exhaled NO from non-enzymatic reduction of salivary nitrite and bacterial conversion of nitrate.

III. To asses the relation of nitrite and nitrate in exhaled breath with exhaled NO, eosinophil activation, bronchial reactivity and lung function in children with allergic asthma.

IV. To evaluate the possible influence of bacterial reduction of nitrate to nitrite in the pharyngo-oral tract on exhaled nitrogen oxides in patients with cystic fibrosis.

3 MATERIALS AND METHODS

CHARACTERISATION OF THE SUBJECTS

Study I

The disease groups and the control group consisted of both children and adults in the comparative study of exhaled CO and NO. The measurements were performed on 32 steroid-naïve asthmatics (8-57 years, 19 females), 24 steroid-treated asthmatics (12-64 years, 11 females), 16 subjects with allergic rhinitis (10-61 years, 7 females), nine subjects with cystic fibrosis (7-32 years, six females), and 30 healthy controls (13-45 years, 12 females). All of the subjects were non-smokers.

The asthmatic subjects had, without exception, a doctor's diagnosed disease and a documented history of asthmatic symptoms. The children with asthma were recruited from the Paediatric allergy clinic of Uppsala University Hospital, and the adults with asthma were recruited either from the Lung and Allergy Clinic of Karolinska Hospital in Stockholm or from The Swedish Asthma and Allergy Association. No discrimination was done between allergic and non-allergic asthma. They were, however, divided into steroid-treated, i.e. under current treatment with inhaled corticosteroids (ICS), and steroid-naïve patients. The latter could have been prescribed ICS in the past but had not been treated with ICS in recent times. The patients with mere allergic rhinitis, but no history of asthma, were recruited from the two allergy clinics mentioned above, where they received specific immunotherapy against various airborne allergens. The nine subjects with CF were outpatients at the Paediatric Clinic of Uppsala University Hospital. They were all colonized with at least one opportunistic bacterial pathogen in their airways, but at the time of the study none of these received antibiotic treatment. Thus, they were not considered to suffer from any severe exacerbations upon examination.

Some additional experiments in this study - where the influence of exhalation flow rate, breath hold and cigarette smoke exposure on the levels of exhaled CO and NO was evaluated - were performed on eight of the healthy controls.

Study II

The ten subjects who participated in this study were all healthy non-smoking adults in the ages from 23 to 43, of which seven were males. One of the aims of the study was to examine whether the intake of nitrate could influence measurements of exhaled NO.

Therefore, they were given a standardized amount of potassium nitrate (400 mg) on an empty stomach. This meant that they had been fasting overnight prior to the experiments, which took place in the morning.

Study III

In this study the relationship of nitrite and nitrate in exhaled breath condensate (EBC) to exhaled NO, blood eosinophils, airway hyperresponsiveness and lung function in children with allergic asthma was evaluated. Thus, both the asthmatic subjects and the healthy controls were all children. The study included 27 children with allergic asthma (6-17 years, 14 females) and 21 healthy controls (7-17 years, 10 females). The children with asthma were recruited either from the Lung and Allergy clinic at Astrid Lindgren Children's Hospital in Stockholm or from the hospitals affiliated outpatient clinics. They were all IgE-positive for at least one aeroallergen. Their disease activity ranged from moderate to severe and they all had a regime for ICS, but a few claimed not to be on current treatment with steroids due to poor compliance or seasonal variation. Nevertheless, 23 of the 27 declared that they had been taking ICS regularly prior to the examination. The healthy controls had no history of asthma or allergic rhinoconjunctivitis. They were all also tested negative for IgE antibodies against aeroallergens. A few additional subjects participated in the experiments as potential healthy controls, but were excluded when the subsequent result of the IgE analysis was positive.

Each subject was tested on a single occasion, irrespective of season or ambient pollen levels. In the protocol, collection of EBC was followed by measurements of exhaled NO, spirometry, methacholine challenge, and blood samples, in that order. All recruited children were able to perform these measurements. To minimize interference with the methacholine challenge, they had been asked to refrain from anti-histamines for three days and β2-agonists and leukotriene receptor antagonists for two days prior to the study.

Study IV

The aim of this study was to examine the influence of salivary nitrite and nitrate on the outcome of exhaled nitrogen oxides, i.e. EBC nitrite plus nitrate and exhaled NO, in patients with cystic fibrosis. Fifteen patients diagnosed with CF (10-43 years, 7 females) and an equal number of age-matched healthy controls (9-44 years, 9 females) were included in this investigation. The examined subjects were predominantly

children and teenagers. The CF patients were recruited from the CF outpatient facility of the Paediatric Clinic at Uppsala University Hospital. All the individuals included were non-smokers. None of the CF patients or the controls had a history of atopy and the subjects with CF had all been tested negative for airway allergens, by either skin prick test or RAST. According to current sputum cultures were 12 of the CF patients colonized with opportunistic bacterial strains in their airways, predominantly Pseudomonas aeruginosa and Staphylococcus aureus. Ten of these subjects were under treatment with antibiotics, mainly inhaled tobramycin, at the time of the investigation. A few had mild airway symptoms, such as cough. However, like the subjects with CF in study I were none of these patients considered to have an acute exacerbation, which probably reveals a shift over time in terms of antibiotic treatment of CF patients, where the use of inhaled antibiotics have become more widespread. All 15 patients received regular treatment with bronchodilators and expectorants. Five of them were also treated with inhaled corticosteroids.

MEASUREMENT OF NITRIC OXIDE

Exhaled nitric oxide

These measurements were at large performed in accordance with the present international recommendations for on-line measurements of exhaled NO, as previously described and as provided in task force reports from the American Thoracic Society and the European Respiratory Society (80, 81, 115). The currently recommended exhalation flow rate of 0.05 L/s was applied in studies III and IV. However, at the time when studies I and II were conducted there was not yet any consensus to this and different exhalation flow rates were applied, see below. Even though the experimental set-up and the analysing equipment has somewhat varied from the first study till the more recent ones the fundamental physiologic and technical conditions of the single-breath procedure have remained equivalent. The subjects were seated and inhaled NO-free air, either from a non-diffusing gas collection bag connected to the inhalation limb of the analyser (study I-II) or from the NO scrubber of the analyser (study III-IV), and subsequently exhaled through a linear (study I-II) or dynamic resistor (study II-IV). Irrespective of exhalation flow rate the resistance applied have aimed to create an oral pressure of 8-12 cmH_2O to close the vellum and prevent admixture of nasal air. For the different set-ups of analysers a pneumotachymeter has registered oral pressure and flow

rate, which have been registered on-line on the computer screen to help the subjects to maintain the target flow rate throughout the required exhalation. Today, the standardised length of a single-breath exhalation is set to 10 seconds, which was applied in study I, III and IV. However, in study II the exhalation time was prolonged to 20 seconds. The registered NO values were normally calculated as the mean plateau concentration of three exhalations.

Study I

Measurements of exhaled NO were made as usual with a chemiluminescence analyser (CLD 77 AM; Eco Physics, Dürnten, Switzerland). In this study the registration of exhaled NO was integrated with parallel on-line measurements of carbon monoxide (CO). The subjects inhaled purified medical breathing air from the gas collection bag, which had been further purified from CO by passing through an electronic scrubber. Since previous studies on exhaled CO had applied a 15 s breath hold before exhalation, the subjects were here asked to do the same. Exhaled NO is normally not measured after a previous breath hold, but since this does not affect the plateau levels of NO (also shown in additional experiment of the study) the breath hold was applied on behalf of the simultaneous CO measurement. The exhaled air of the subjects was then simultaneously fed into both the NO and CO analyser. For the main part of the study all the subjects performed these measurements at two different flow rates; 0.075 L/s (with linear resistance of 100 cmH_2O) and 0.15 L/s (switching resistance to 50 cmH_2O).

In additional experiments on eight of the healthy controls, exhaled NO (and CO) was measured with variable lengths of preceding breath hold (0, 10, 20, and 40 seconds) and after smoking one cigarette (repeated after 1, 10, and 30 min).

Study II

This study was performed with the Eco Physics CLD 700 AL chemiluminescence analyser and with the exhalation flow rate of 0.15 L/s for all NO measurements, using the fixed linear resistance of 50 cmH_2O. The levels of exhaled NO were here registered as NO release rate, instead of NO concentration (ppb). The unit for NO release rate is pmol/s and this measure is obtained by first converting the concentration in ppb to pmol/L, which can be done from the fact that 1 ppb of NO equals 40.908 pmol/L in room temperature and at atmospheric pressure. The concentration in pmol/L is then multiplied with the exhalation flow rate (L/s): pmol/L x L/s = pmol/L. This calculation was here performed by a software program, which integrated the NO levels in ppb from

the analyser with the flow signal and presented the NO release rate in real time on the computer screen.

In this study exhaled NO was measured before and repeatedly after the ingestion of a 400 mg potassium nitrate load (mean of three measurements) and before and after the application of different mouthwash solutions (one measurement). In the nitrate load experiment, NO was registered at baseline and at 5, 15, 30, 60, 90, 120, 150, and 180 min after the ingestion.

Study III-IV

In these two studies exhaled NO was measured with the NIOX® analyser (Aerocrine AB, Solna, Sweden). Exhalation flow rate was set to the standardised 0.05 L/s, using the built-in dynamic resistor, which adjusts the resistance to keep a steady flow rate (within 8-20 cm H_2O oral pressure).

In study IV exhaled NO was measured before and after a chlorhexidine mouthwash.

Nasal nitric oxide

Study II

Nasal NO was determined as the NO release rate in nasal air, which was sampled by the NO analyser (Eco Physics CLD 700 AL) from each nostril through a nasal olive at a flow rate of 0.5 L/min. To avoid NO contamination from the oral cavity and the lower airways the subjects performed a simultaneous single breath manoeuvre for 20 s, as above, with the purpose of closing the velum. These 20 s were sufficient to obtain a stable plateau of nasal NO levels, from which NO release rate was registered. The registrations from both nostrils were used to calculate mean value for nasal NO. These measurements of nasal NO were performed before and repeatedly after the ingestion of 400 mg potassium nitrate, at the same time points as above.

MEASUREMENT OF EXHALED CARBON MONOXIDE

Study I

In all the previous reports on elevated levels of exhaled carbon monoxide (CO) in asthma, cystic fibrosis and other inflammatory conditions of the airways the Bedfont EC50 Mini-Smokerlyzer™ (Bedfont Scientific, Kent, UK) was used. This CO analyser registers the CO concentration by an electrochemical sensor and delivers the value in absolute numbers of ppm after considerable delay (up to 20 s). In this study we wanted to compare measurements with the Mini-Smokerlyzer™ to registrations with a more

CO specific fast responding non-disperse infrared (NDIR) analyser (UNOR 610, Maihak AG, Hamburg, Germany). The latter delivers the CO concentration in less than three seconds and its output signal was integrated with the software system for NO analysis and enabled the concentration curve for CO to be displayed on-line on the computer screen, together with curves for flow rate and oral pressure. Thus, with the NDIR analyser exhalation flow rate could be controlled. It also makes a more precise reading of the CO concentration and delivers a three decimal value. In the use of the Mini-Smokerlyzer™ exhalation flow rate or time for exhalation can not be controlled.

All the subjects with asthma, allergic rhinitis, cystic fibrosis, and the healthy controls performed CO measurements with both of the two analysers. The procedure for the Mini-Smokerlyzer™ was as described in previous reports and as in the guidelines from the manufacturer. Thus, the subjects inhaled room air to total lung capacity, held their breath for 15 s and made a full exhalation to residual volume. The mean value from two measurements was reported. For the CO measurements with the NDIR analyser the procedure was as described for NO in the same study; i.e. a full inhalation of CO-free air, 15 s breath hold, followed by a controlled 10 s exhalation at both 0.075 L/s and 0.15 L/s. Two measurements at each flow rate were performed and the mean value was registered for respective flow rate.

Additional experiments on exhaled CO, with the two different analysers, were performed on eight of the healthy controls analogous to the ones described for NO in the same study; i.e. with variable length of preceding breath hold and after cigarette smoking. Exhaled CO was also evaluated in a series of different flow rates (0.05, 0.1, 0.2, and 0.5 L/s) with the NDIR analyser in these eight subjects.

ANALYSIS OF NITRITE AND NITRATE

Study II, III, and IV

Nitrite and nitrate was analysed in saliva (study II and IV) and in exhaled breath condensate (study III and IV) with fluorometric technique. For these studies we have used a commercial spectrofotometric or fluorometric assay (Cayman Chemical Inc, Ann Arbor, MI, USA). Fluorescence after treatment with nitrate reductase gives the total concentration of nitrate and nitrite, whereas fluorescence without enzymatic priming represents the nitrite concentration. Thus, the nitrate concentration is obtained by subtracting the total concentration with the concentration of nitrite. These analysis were performed in batches on a separate occasion after having had the samples of saliva and/or condensate fluid stored in -20˚ C.

COLLECTION OF EXHALED BREATH CONDENSATE

Study III

The exhaled breath condensate (EBC) from the children with asthma and the controls was collected in a commercial breath condenser (EcoScreen, Jaeger, Germany) during five minutes of tidal breathing with a nose-clip. The collecting chamber was centrifuged for one minute to spin down the condensate fluid, whereupon the fluid was transferred to sample tubes and stored at -20°C for later analysis. The volume of collected condensate varied from about 0.5 to 2 ml. To protect from contamination of microorganisms and avoid salivary droplets in the condensate a sterile filter was placed between the mouthpiece and the condenser. The lack of salivary admixture in the EBC samples was also confirmed by negative assays for salivary amylase in random samples (EnzCheck Amylase Assay Kit, Molecular Probes Inc, Eugene, OR, USA).

Study IV

In an attempt to further standardise the procedure for EBC collection and improve its reproducibility the method was slightly adjusted for this study. Instead of setting the time for tidal breathing to five minutes the EBC collection was interrupted at a total exhalation volume of 60 litres, which was monitored by a spirometer (SpirPro+, Jaeger, Germany) attached to the condenser. The amount of EBC fluid collected by this method amounted to 0.5-1.0 ml. Furthermore, to avoid possible nitrite/nitrate contamination of the condenser tubes a plasma polymerisation was deposited on the existing Teflon-coating of the tubes (in-house method at Institute for Surface Chemistry, Stockholm, Sweden) and they were thoroughly cleaned in nitrogen-free disinfectant (Descogen; Jaeger, Germany) between each sampling. Apart from these changes the procedure was the same as in study III.

MOUTHWASHES

Different types of mouthwashes have been applied in study II and IV to examine the influence of salivary nitrate/nitrite on exhaled nitrogen oxides and to see how manipulation of the local milieu affects the same.

Study II

In these experiments the subjects rinsed their oral cavities with a broad variety of solutions to study their effect on measurements of exhaled NO. After registration of

baseline levels the 10 subjects rinsed with 30 ml of each solution for 30 seconds, whereupon exhaled NO was measured repeatedly. The solutions were distilled water, sodium bicarbonate (10%), ascorbic acid (3%), 10 mM potassium nitrite, and 0.2% chlorhexidine acetate. The rationale for applying sodium bicarbonate and ascorbic acid was that the non-enzymatic reduction of nitrite to NO is enhanced in an acidic environment and therefore we wanted to see whether mouthwash with a mild base or acid also affects levels of NO in exhaled air. Mouthwash with potassium nitrite was investigated as to see whether increased amounts of substrate for non-enzymatic NO formation would yield higher levels of exhaled NO. Chlorhexidine was chosen for its anti-bacterial effect, which consists of both bactericidal and bacteriostatic mechanisms, and for the fact that the bacterial nitrate reductase activity is particularly susceptible to its action (116, 117). Rinsing with distilled water was applied to control for a mere rinsing effect of the mouthwashes. In an additional set, measurements of exhaled NO after mouthwash with nitrite solutions of increasing concentrations (1, 10, and 100 mM) were performed. The subjects also rinsed with chlorhexidine 90 minutes after the ingestion of a nitrate load (400 mg of potassium nitrate) to see whether its potential suppression of exhaled NO would be more pronounced when there is more substrate present for nitrite and NO synthesis.

Study IV

Measurements of exhaled NO and the collection of EBC and saliva were here performed before and after a 30 seconds mouthwash with 30 ml of 0.2% chlorhexidine gluconate. The objective of this was mainly to see whether nitrite and nitrate in EBC, which have been reported to be elevated in cystic fibrosis, are influenced by a possible contribution from the saliva and the oral cavity.

METHACHOLINE CHALLENGE AND SPIROMETRY

Study III

Methacholine challenge was performed to evaluate airway hyperresponsiveness in the children with allergic asthma. The objective was to relate measures for bronchial hyperreactivity and lung function with the levels of nitrogen oxides in EBC. Lung function was here represented by conventional dynamic spirometry which is an integrated and repeated feature of the protocol for methacholine challenge. The challenge was performed with the hospital's standardised protocol, which is a modified version of the Mefar dosimeter protocol (118), where a step-wise cumulative dose of

methacholine is given to determine PD_{20}, i. e. the cumulative dose required to cause a 20% drop in FEV1. Before the challenge each subject performed a dynamic spirometry (Vitalograph Spirotrac IV®, Vitalograph Ltd, Buckingham, England) for baseline values of lung function – presented as FEV_1/FVC – and to ensure an FEV_1 value ≥ 65% of predicted, in order to safely start methacholine administration. The nebulised methacholine was delivered by a dosimeter (Spira® Elektro 2 Dosimeter, Spira Respiratory Care Center Ltd, Hämeenlinna, Finland), to provide an exact amount with every inhalation. The cumulative dose ranged from 0.06 to 29.7 µmol methacholine. Measurement of FEV_1 was done three minutes after each dose increase. The challenge was stopped when a decline of FEV_1 ≥ 20% from baseline was observed, whereupon the subjects were treated with salbutamol inhalation and PD_{20} was calculated.

4 RESULTS AND COMMENTS

PAPER I

CO and NO in airway disease

In contrast to previous reports there was no significant increase of exhaled CO in the subjects with asthma, neither for the steroid-naïve ($p=0.74$) or steroid treated ($p=0.75$) subjects, when the fast-responding NDIR analyzer was applied. There was no significant difference between the healthy controls and the subjects with allergic rhinitis ($p=0.46$) or cystic fibrosis ($p=0.82$) either, which also contrasts to previously published results with the electrochemical CO-analyser (Mini-Smokerlyzer™). However, somewhat to our surprise, we could not establish any significant increases of exhaled CO in any of the disease groups either by the Mini-Smokerlyzer™. Exhaled NO was, on the other hand, significantly elevated in the subjects with steroid-naïve asthma ($p<0.001$) and allergic rhinitis ($p<0.001$), but not in the steroid-treated group of asthmatics or in the patients with CF. For the latter there was rather a statistical trend towards lower NO concentrations than in the control group ($p=0.067$). Switching the exhalation flow rate from 0.15 L/s to 0.075 L/s did not alter the exhaled concentrations of CO, as measured with the NDIR analyzer (the flow rate of exhaled air could not be monitored with the setup for the electrochemical analyzer). The concentrations of exhaled NO were, however, roughly doubled by the 50% reduction of flow rate. The statistical relationship between the NO levels of the groups was the same for the two flow rates. See also Figure 4.

Bronchial or alveolar origin?

Measurements of exhaled CO at four different flow rates, ranging from 0.05 to 0.5 L/s, were also performed on eight of the healthy controls without any significant differences in CO concentrations. Exhaled NO was measured simultaneously with a clear flow-dependent pattern of the obtained concentrations, as previously described, but the latter was not presented as it was far from a novel finding. This flow-independency of exhaled CO clearly indicates that there is no substantial contribution of CO from the conducting airways, i.e. the bronchi, neither under normal conditions nor in states of airway inflammation. Thus, the concentrations in the exhaled air are unaffected by the flow of air through the airway lumens and a low flow rate yields no more CO particles per volume of air than a high flow rate. If exhaled CO is not

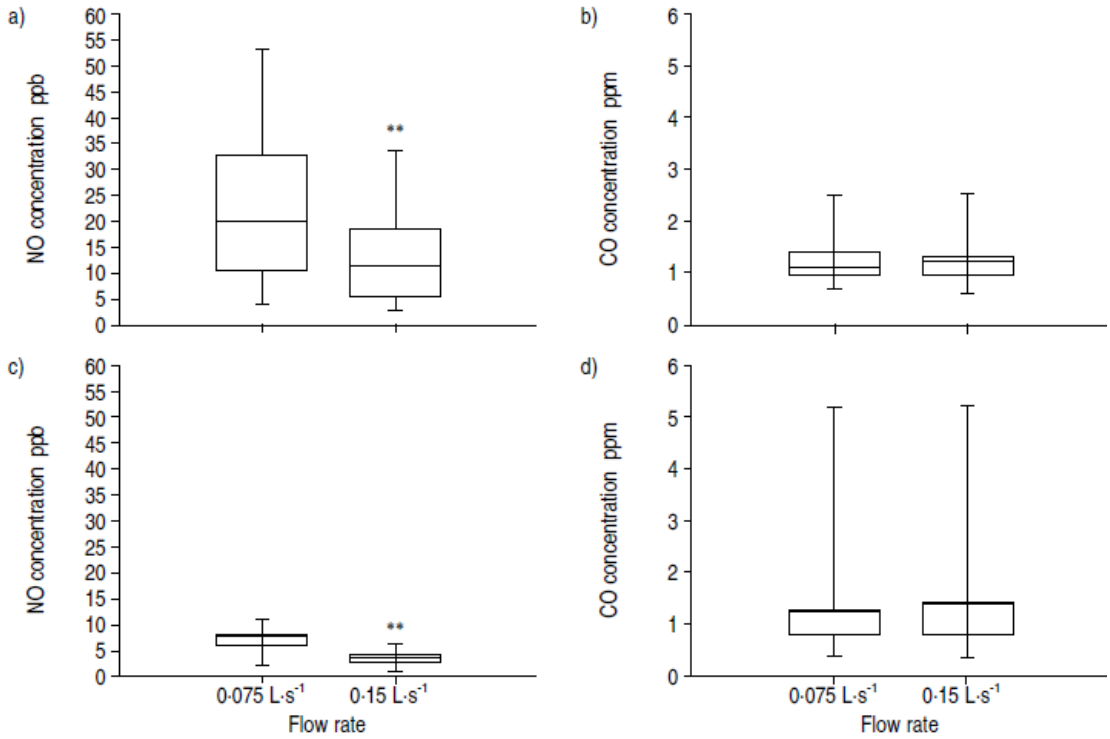

Fig 4. Relationship to exhalation flow rate for exhaled NO and CO. Panel a) and b) illustrates the concentrations for exhaled NO and CO at two different flow rates (0.075 and 0.15 L/s). In panel c) and d) the NO and CO concentrations at the same flow rates are illustrated for the patients with cystic fibrosis. The figure shows a marked flow dependence of the levels of exhaled NO, where a doubled exhalation flow rate yields about half the concentrations of NO in the exhaled air, whereas the concentrations of exhaled CO are unaffected by the change in flow rate. This clearly indicates an origin in the conducting airways for NO and an alveolar origin for CO. Asthmatics n=32, cystic fibrosis n=9. **=p<0.01.

generated in the conducting airways its site of origin must rather be the alveoli. This was also supported in the series of CO and NO measurements after variable lengths of preceding breath hold; 0, 10, 20, and 40 seconds. The plateau levels of CO increased about 80% ($p<0.01$) with a 10 s breath hold, as compared to exhaling without breath hold. No further increase of exhaled CO was seen with the 20 and 40 s breath hold. The concentrations of exhaled NO remained unchanged, regardless of whether there was previous breath hold or not (note, these are plateau concentrations; if one measures the NO concentration of the initial part of the exhalation, breath hold creates an initial peak of NO, which is increased with prolonged breath hold). The increase of CO plateau levels after breath hold implies that the alveolar air receives CO from the microcirculation, but it reaches equilibrium quite soon as prolonged breath hold did not result in any higher concentrations.

PAPER II

The influence of nitrate intake on exhaled and nasal NO

After baseline measurements of exhaled and nasal NO the ten healthy subjects ingested 400 mg potassium nitrate (KNO_3), which equals 240 mg of pure nitrate, on an empty stomach, where after they were asked to thoroughly rinse their mouth with tap water. The subsequent measurements - at 5, 15, 30, 60, 90, 120, 150, and 180 min after the intake - gradually revealed a marked increase in the levels of exhaled NO. As seen in Figure 5, the NO release rate reached a maximum at 120 min after the nitrate load. At that stage the mean value for exhaled NO had increased with almost 150% compared to baseline ($p<0.01$). However, nasal NO levels were not at all affected by the intake of nitrate during the monitored 180 min. This indicates that the elevated NO seen by conventional oral exhalation has another explanation than increased formation in the respiratory epithelium.

In the same experiment saliva was collected at baseline and after 60 and 120 min for analysis of nitrate and nitrite content. The mean nitrate concentration showed a 10-fold increase at 60 min ($p<0.01$) compared to baseline, with a slight decline of the levels after 120 min. The peak concentrations of salivary nitrite were, however, found in the samples collected at 120 min, with a 4-fold increase from baseline of the mean value ($p<0.01$). Thus, the observed peak of salivary nitrite paralleled the maximum levels of exhaled NO. Given the fact that NO can be formed through non-enzymatic reduction of nitrite, this observation clearly indicates a causal link between the two.

To confirm the formation of NO from nitrite in saliva we performed an in vitro experiment where a salivary sample from the subjects was placed in two separate syringes with a head space of NO-free air. One syringe was added nitrite to 2mM (equivalent to salivary concentration after nitrate load) and the two syringes were incubated at 37˚ C for 15 min. The mean NO concentration of the aspirated head space air from the syringe with un-spiked saliva was 60 ppb, whereas the addition of 2 mM nitrite resulted in a good 500% increase of NO ($p<0.01$). An evidence for oral NO formation from nitrite was also provided in vivo by a series of measurements after previous mouthwash with nitrite solutions of 1, 10, and 100 mM. The mouthwash of 1mM gave only a slight increase of exhaled NO, whereas the 100 mM solution yielded a 40-fold increase of the NO release rate as compared to baseline ($p<0.01$).

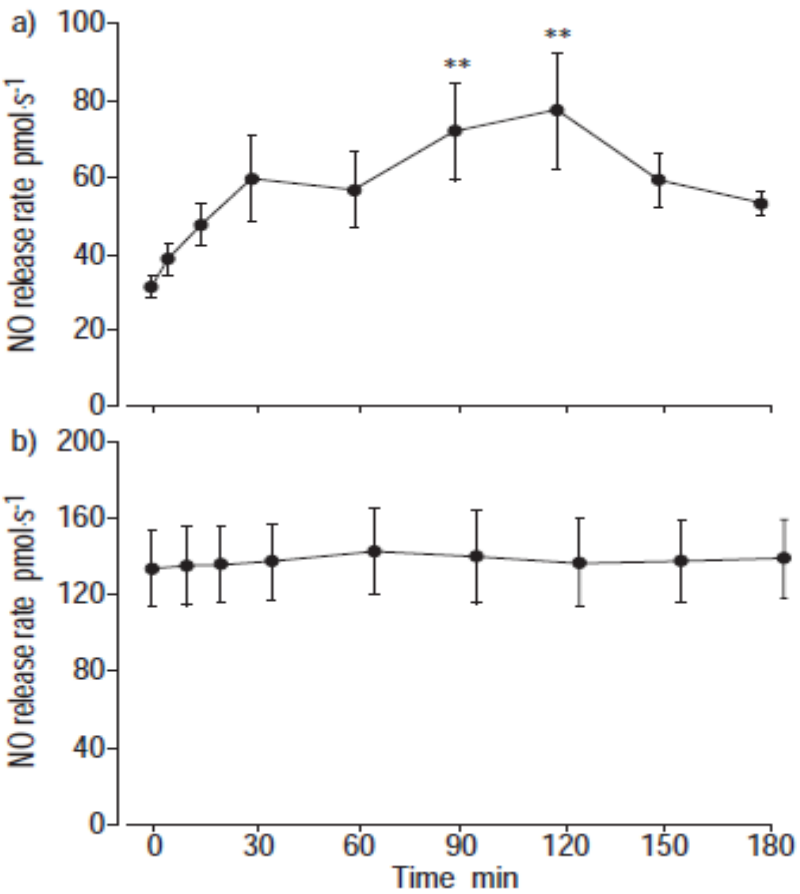

Fig 5. The effect of nitrate intake on exhaled and nasal NO. After baseline measurements, 240 mg of nitrate was ingested and consecutive measurements of exhaled NO and nasal NO were thereafter performed. a) Exhaled NO showed a gradual increase and peaked after 120 min, which was also the time point when the highest concentrations of salivary nitrite were obtained. b) Nasal NO levels were not affected by nitrate intake. N=10. **=p<0.01.

Mouthwashes

The ten subjects volunteered for additional mouthwash experiments, both with and without previous nitrate loading.

The kinetics of the reduction of nitrite to NO was examined by quickly repeated measurements of exhaled NO after mouthwash with 10 mM of nitrite solution. Immediately after the mouthwash exhaled NO showed a 2-fold increase ($p<0.001$) and after 1 min the increase was 4-folded ($p<0.001$). The following measurements showed, however, a steady decline and the levels were back to baseline after 4 min.

Mouthwash with the 3% ascorbic acid solution (pH 2.5) also created a marked but short-lasting increase of exhaled NO. The levels increased with 400% ($p<0.01$) immediately after the mouthwash but were back to baseline already after 2 min. The effect of ascorbic acid on exhaled NO is explained not only by creating an acidic environment, which enhances the reduction of nitrite to NO, but also by ascorbic acid's reducing properties.

The effect of sodium bicarbonate (pH 7.85) mouthwash was also transient but meant instead a decrease of about 25% of exhaled NO ($p<0.01$). After two minutes, the levels begun to return to baseline. The inhibitory effect of sodium bicarbonate was more pronounced, especially in absolute numbers, after nitrate loading. The ten subjects performed a mouthwash with sodium bicarbonate at the end (after 180 min) of the previously described nitrate load experiment, and then there was more than a 30% reduction of NO release (36.2±4.2 vs 52.7±4.8 pmol/s, $p<0.01$). The effect of sodium bicarbonate should be explained by the temporary increase of pH in the oral cavity, which inhibits the ability of nitrite reduction.

The antibacterial solution of chlorhexidine acetate proved to have a more long-lasting effect than sodium bicarbonate and was also more efficient in decreasing the levels of exhaled NO. Under normal conditions, i. e. without a preceding nitrate load, the chlorhexidine mouthwash created an immediate 30% decrease of NO release rate ($p<0.05$) and the effect could be seen throughout the measurement period of 20 min. In the separate nitrate load experiment (400 mg potassium nitrate), where the ten subjects performed a chlorhexidine mouthwash 90 min after the nitrate ingestion, the immediate decrease of exhaled NO levels were close to 50% (34.8±4.2 vs 60.8±6.4 pmol/s, $p<0.001$) and the effect remained throughout the measurement period of 30 min, when the decrease was still highly significant. See Figure 6.

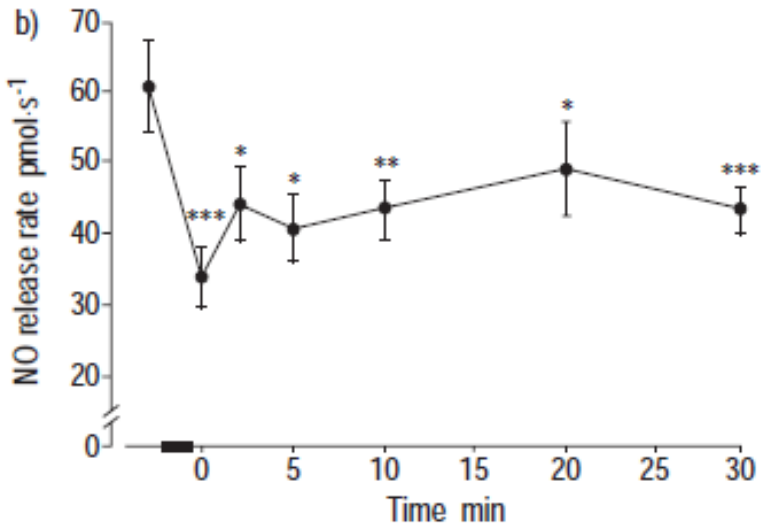

Fig 6. The effect of chlorhexidine mouthwash on exhaled NO. Chlorhexidine, which prevents the bacterial reduction of nitrate to nitrite in the pharyngo-oral tract, markedly reduces the levels of NO in exhaled air, illustrating the salivary contribution to exhaled NO through the further reduction of nitrite. The figure shows chlorhexidine application 90 min after nitrate intake. N=10. ***=$p<0.001$, **=$p<0.01$, **=$p<0.05$.

PAPER III

Nitrite and nitrate in exhaled breath condensate

The median level of nitrite in exhaled breath condensate (EBC) from the children with asthma was significantly higher than in EBC from the age-matched controls (8.9 vs 3.6 µmol, p=0.002; see Fig 7a). However, for EBC nitrate there was no significant difference between the asthmatic children and the control group (p=0.597). Since nitrite is a soluble metabolite of NO one could expect a correlation between EBC nitrite and exhaled NO to be indicated, but there was, in fact, a complete lack of any statistical relationship between the two (r=0.046; see Fig 8a). In addition, EBC nitrite showed no signs of correlation to any of the other disease markers in this study; i. e. blood eosinophils, IgE levels, methacholine reactivity (PD_{20}), and airway function (FEV_1/FVC). EBC nitrate, on the other hand, did correlate to exhaled NO (r=0.40, p<0.05), even though there was a discrepancy between the two among many of the asthmatics. As for nitrite, there were no statistical relation to be found between EBC nitrate and the other parameters.

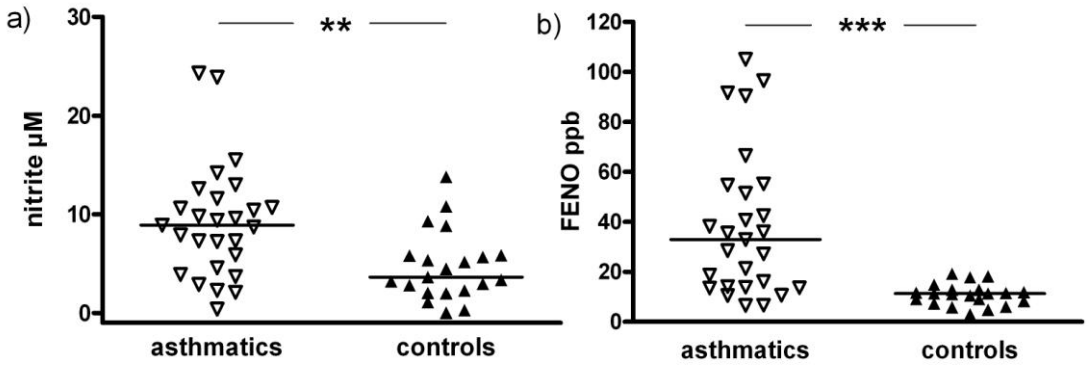

Fig 7. EBC nitrite and exhaled NO in children with allergic asthma. a) EBC nitrite and b) exhaled NO are elevated in the children with asthma as compared to the healthy controls. EBC nitrate, however, was not different in the asthmatic group (not shown). Asthmatics n=27, controls n=21. ***=p<0.001; **=p<0.01.

Inflammatory and functional markers

Exhaled NO and eosinophils in peripheral blood were the two parameters used as reference markers for allergic airway inflammation. The children with allergic asthma had significantly higher levels of both exhaled NO (median 32.9 vs 11.3 ppb, p<0.001;

see Fig 7b) and eosinophils (median 0.5 vs 0.2 x 10^9/L, p<0.001). There was also a significant correlation between these two markers (r=0.43, p<0.05) suggesting an interlinked mechanism of NO and eosinophils in the pathophysiology of allergic asthma. See Figure 8b.

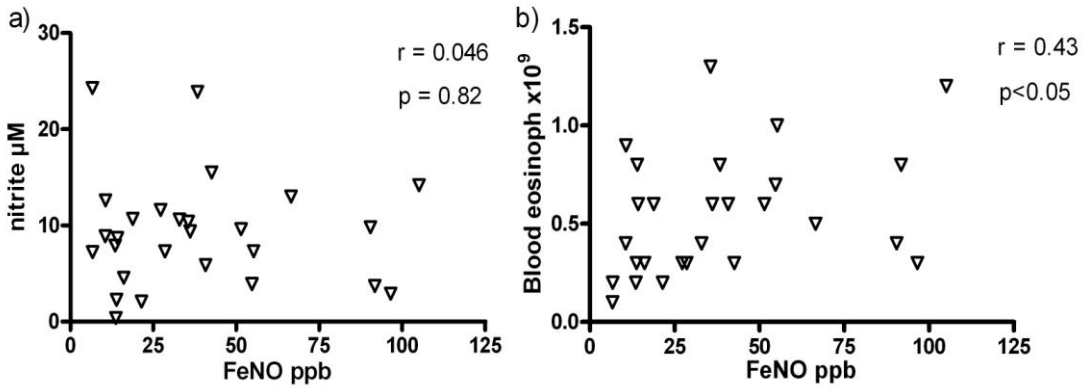

Fig 8. Correlations between exhaled NO (FENO) and a) EBC nitrite and b) blood eosinophils in children with allergic asthma. There is no correlation at all between levels of exhaled NO and EBC concentrations of nitrite, in spite of the fact that they were both increased in the asthmatic children compared to controls. A significant correlation between exhaled NO and eosinophils in peripheral blood could be established. N=27.

Airway function, which was assessed by measuring the forced expiratory volume in relation to vital capacity (FEV_1/FVC), was decreased in the children with asthma (median 86.8 vs 91.4 %, p<0.01) as compared to the control subjects. The methacholine challenge also revealed markedly lower PD_{20} levels in the asthmatic children (median 0.40 vs 5.19 µmol methacholine, p<0.001), which means they had a more pronounced reactivity in their airways. The PD_{20} levels of the asthmatic subjects correlated significantly to their FEV1/FVC (r=0.42, p<0.05). Even though functional parameters and inflammatory markers represent different aspects of the disease activity, there was also a significant inverse correlation between eosinophils and FEV_1/FVC (r=-0.53, p<0.01), as well as a trend (p<0.1) towards significant correlations between FENO and PD_{20} and between FENO and FEV_1/FVC. The figures for FEV_1/FVC and PD_{20} also correlated with the IgE levels for perennial allergens (p<0.001 and p<0.05, respectively).

PAPER IV

Nitrite and nitrate in EBC and saliva

The concentrations of nitrite in EBC were significantly higher in the subjects with CF than in the healthy controls (median 3.6 vs 1.3 µM, $p<0.05$). Chlorhexidine mouthwash greatly reduced the levels of nitrite in both CF patients (3.6 – 1.4 µM, $p<0.01$) and controls (1.3 – 0.5 µM, $p<0.01$). The difference between the two groups decreased by 60%, from 2.2 to 0.9 µM, by the mouthwash and the inter individual spread was markedly reduced. For further details, see Fig 9a.

The simultaneously collected samples of saliva showed, in parallel to EBC, elevated levels of nitrite in the CF group (median 219.6 vs 55.0 µM, $p<0.01$). The concentrations in saliva were equally decreased by the chlorhexidine mouthwash in both CF patients and controls (219.6 – 63.5 µM, $p<0.001$; and 55.0 – 19.8 µM, $p<0.01$). The interlinked relationship between salivary and EBC nitrite concentrations was also verified by a statistically strong correlation ($r=0.60$, $p<0.001$; see Fig 9b). Therefore, it is evident that the majority of EBC nitrite originates in the saliva and the elevated levels in the CF patients clearly seem to be explained from an altered condition in the pharyngo-oral area. However, the EBC levels of nitrite were still somewhat higher in the CF patients also after the mouthwash and this could obviously be explained by yet another mechanism, even though such an alternative source of nitrite in the CF patients would be of minor importance in terms of actual quantities. This remaining difference is more likely explained by an incomplete effect of the chlorhexidine mouthwash to eradicate all nitrate reducing bacteria since also the salivary levels of nitrite remained higher in the subjects with CF after the manoeuvre.

The concentrations of EBC nitrate were, in contrast to nitrite, not at all elevated in the CF group, either before (median 7.8 vs 8.2 µM, $p=0.80$) or after chlorhexidine (8.8 vs 9.3 µM, $p=0.59$). The salivary levels of nitrate were more affected by the mouthwash than the concentrations in EBC and increased considerably (CF: 189.8 – 964.3 µM, $p<0.01$; controls: 31.0 – 226.8 µM, $p<0.05$). Thus, an over all elevated content of salivary nitrate was observed in the CF patients, which became statistically significant after chlorhexidine application (964.3 vs 226.8 µM, $p<0.05$).

These results indicate that EBC nitrate levels are not particularly related to salivary concentrations, which was also illustrated by a rather poor correlation between the two ($r=0.28$, $p=0.15$). Therefore, it seems as if nitrate in EBC, to a much larger extent than nitrite, actually derives from the lower airways.

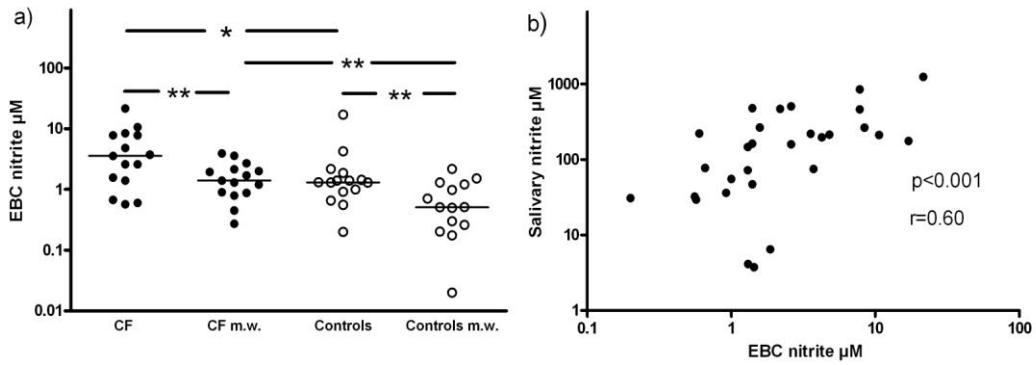

Fig 9. EBC nitrite and its correlation to salivary nitrite in patients with cystic fibrosis (CF) and controls. a) Concentrations of nitrite in EBC were elevated in the patients with CF as compared to the healthy controls. There was a marked decrease in the concentrations after the 30 s mouthwash with chlorhexidine (m.w.). b) A strong correlation was observed between the concentrations of nitrite in EBC and saliva. Illustrated here are the baseline values. Thus, salivary nitrite levels were elevated in parallel with the EBC nitrite in the CF patients. Note log-scale for nitrite concentrations. CF n=15, controls n=15. **=p<0.01; *=p<0.05.

Exhaled NO

At baseline there was a statistical trend towards lower levels of exhaled NO in the CF patients compared to controls (median 9.5 vs 10.6 ppb, p=0.081). After the mouthwash with chlorhexidine exhaled NO decreased in both the CF group (9.5 – 4.4 ppb, p<0.001) and the controls (10.6 – 8.4 ppb, p<0.001), but the decrease was more marked in the subjects with CF and illustrated a significantly lower bronchial contribution of NO in this group (5.4 vs 8.4 ppb, p<0.05). See also Figure 10.

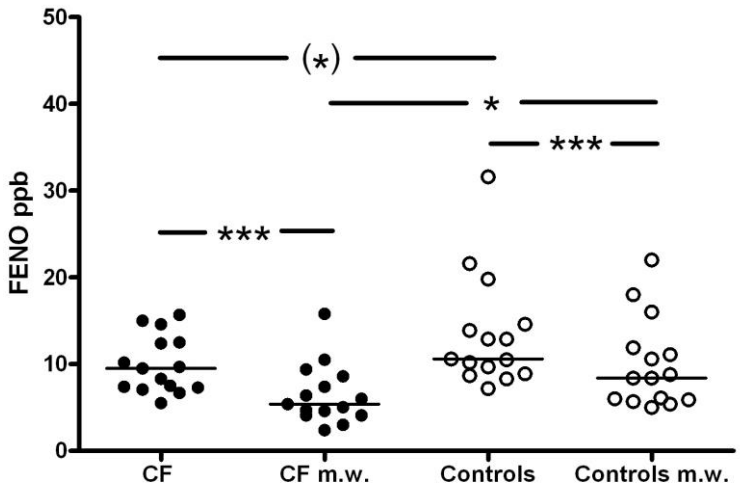

Fig 10. Exhaled NO in CF patients and controls before and after mouthwash with chlorhexidine. Exhaled levels of NO are low at baseline but decrease to a larger extent than corresponding values for the controls with chlorhexidine application, making the NO concentrations from the CF patients significantly lower. Given their simultaneously higher levels of salivary nitrite this illustrates a proportionally large salivary contribution to exhaled NO in the CF group; or reversed, a proportionally small contribution from the lower airways. CF n=15, controls n=15. ***=p<0.001; *=p<0.05; (*)=p<0.1.

5 GENERAL DISCUSSION

The main objective of this thesis was to further examine the role and formation of NO and other nitrogen oxides in the airways and to evaluate their roles as exhaled markers of inflammation and disease activity. A part of this mapping has included assessments of how soluble nitrogen oxides (nitrate and nitrite) - normally present in the physiological enterosalivary circulation - might influence what is measured in the exhaled air. Furthermore, we have investigated exhaled NO in relation to carbon monoxide (CO), another monoxide present in the exhaled air of humans and reported to be elevated in conditions of airway inflammation.

ALVEOLAR CO AND BRONCHIAL NO

Regarding study I and the evaluation of exhaled CO and NO we introduced flow-controlled measurements of exhaled CO with a fast responding (highly CO-specific) NDIR analyser. We could here demonstrate that the CO concentrations were completely independent of the exhalation flow rate, both in the patients with inflammatory airway conditions and in the healthy controls. This characteristic of CO, which contrasts the strong flow-dependency of NO, together with the observed influence of a breath hold clearly suggests an alveolar origin of CO. However, CO does most certainly not have its original formation in the alveoli, but rather diffuses from the alveolar vessels and is hence a product from the blood stream. Other groups have claimed that exhaled CO can be elevated in various conditions of airway inflammation, such as asthma (19, 23, 119, 120) and CF (20, 121), and that it would serve as a marker of oxidative stress in the airway mucosa (23). This has been explained by the formation of CO from the action of the enzyme haeme oxygenase (HO-1) and its oxidative degradation of haeme to bilirubin (113). The HO-1 enzyme of the airway mucosa has been argued to be induced in conditions of airway inflammation, but such an induction has, in fact, not been demonstrated in the inflamed airway epithelium (114). However, one study has, even though not convincingly, shown increased expression of HO-1 in airway macrophages from the respiratory tract of subjects with asthma (23). These findings contrast the current knowledge of exhaled NO and the NOS-enzymes, responsible for the enzymatic production of NO in the conducting airways. The expression of iNOS is markedly increased in both the airway epithelium and in various inflammatory cells of the mucosa from subjects with asthma (33, 34). Yet, exhaled

concentrations of NO do not amount to more than parts per billion (ppb) in the exhaled air. Given this, it also seems unlikely that an enzyme which can not be demonstrated as increased in the airway mucosa of subjects with respiratory disease, i. e. HO-1, could be responsible for the formation of several-fold increases of parts per million (ppm) of CO. These facts together with our conclusions of a predominant alveolar origin of CO, strongly questions the role of exhaled CO as a marker of bronchial inflammation.

CO – end of story?

On the further negative side for exhaled CO as a disease marker of the conducting airways, was the fact that we could not demonstrate any elevated concentrations of CO in the exhaled air from either the subjects with asthma, allergic rhinitis or CF. Previous reports of increased CO in patients suffering from these conditions can, however, not be neglected. It could be argued that our patients were not as severely affected by their conditions as in the comparative studies. That is not a very likely explanation since exhaled CO has been reported to be elevated in patients with mere atopy, without asthma, and in patients with mild steroid-naïve asthma. Furthermore, CF is a condition where an ever on-going airway inflammation is “mandatory” and yet exhaled CO was normal in the nine CF patients in our study, in spite of uniform colonisation with opportunistic bacterial strains. Also in the asthmatic groups, where some were quite affected by their disease and for example presented markedly increased levels of NO, exhaled CO was shown to be normal.

Another possible explanation could be different exposition to ambient CO. The two research groups responsible for the early studies on exhaled CO in asthmatics, CF patients and others are operating in high density city areas (i.e. London and Tokyo) with normally high levels of ambient CO. In our setting ambient CO was always low; never more than 1 ppm. Exposure to ambient CO and for example occasional smoking cause elevated levels of exhaled CO for a considerable time period. In this study we observed markedly increased levels of exhaled CO 30 min after smoking one cigarette, and the levels had not decreased much from the measurement performed immediately after the cigarette. Off the record, we also observed elevated levels of exhaled CO in a few control subjects who had been exposed to tobacco smoke in a bar the night before the experiments, which caused us to postpone further measurements to another day. Thus, exposure to CO in the ambient air is a major confounding factor for measurements of exhaled CO and it might be that this affects the outcome from individuals with airway disease differently. Perhaps it could be that individuals with

asthma, CF or other airway conditions are less capable of ventilating ambient CO and possibly other air pollutants out of their system. There is nothing yet published which could answer this hypothesis, but it would certainly be an interesting study to take on in the future. However, since our results out ruled exhaled CO as a marker of bronchial inflammation - but confirmed this role for exhaled NO - the further studies for this thesis have rather been focused on nitrogen oxides, whereas the unresolved questions regarding CO were not prioritised. In fact, the general interest for exhaled CO as a disease marker cooled off dramatically after we published our data in 2002. The topic was very hot after the initial reports of increased exhaled CO in asthmatics and a number of studies on exhaled CO as marker of airway inflammation were published between 1997 and 2002. The publication of our study in European Respiratory Journal resulted in a further published correspondence with a couple of the responsible authors for the reference studies. However, after that the field of exhaled CO rapidly died out and very few studies with main focus on CO as a marker of airway inflammation has been published since. Even less is exhaled CO today seriously discussed as a possible marker of airway inflammation.

EXHALED NO AND SOLUBLE NITROGEN OXIDES

The further studies in this thesis have examined exhaled NO in relation to the soluble nitrogen oxides nitrite and nitrate, which can be formed through the oxidative metabolism of NO or exist in various body fluids and tissues, where they comprise forms of the body's reservoir of nitrogen. As previously described, there is a physiological entero-salivary circulation of nitrate which, according to our results, seems to influence both measurements of exhaled NO and levels of particularly nitrite in exhaled breath condensate.

Salivary contribution to NO

In study II we could clearly demonstrate that there is a contribution from the saliva to NO detected in exhaled air, through a non-enzymatic reduction of salivary nitrite. Firstly, this was demonstrated by the marked decrease in the levels of exhaled NO by the application of chlorhexidine and sodium bicarbonate mouthwashes, where the former prevents the bacterial formation of nitrite from nitrate by blocking the enzyme nitrate reductase and the latter decreases the reduction of nitrite to NO by providing a more alkaline milieu. The oral formation of NO as a phenomenon and its influence on the concentrations of exhaled NO was also visualised by the dramatic increase in NO

levels by the application of both nitrite solutions, which of course provide more substrate for NO formation, and by the mouthwash with ascorbic acid, which facilitates the non-enzymatic reduction of nitrite to NO.

Influence from food intake

The influence from nitrate in the enterosalivary circulation was demonstrated as a nitrate load caused markedly increased levels of exhaled NO, which peaked about two hours after the nitrate ingestion. A gradual increase of the salivary concentrations of nitrate and nitrite was observed in parallel to the successively increasing levels of exhaled NO. Thus, the ingested nitrate load is eventually excreted from the circulation to the salivary glands and causes an elevated content of nitrate in the saliva which creates an increased formation of nitrite in the oral cavity, catalysed by the bacterial enzyme nitrate reductase (106). Since nitrite in turn can be non-ezymatically reduced to NO (47), this explains the observed effect on exhaled NO by the intake of nitrate. The amount of nitrate ingested was here 240 mg, which is equivalent to the nitrate content in no more than roughly 100 g of spinach or 50 g of lettuce, and yet there was a dramatic increase of about 150% in the levels of exhaled NO for several hours after the intake. Such an increase in the concentrations of exhaled NO would normally amount to levels which are to be interpreted as if there is an elevated inflammatory activity in the airways. Thus, the intake of nitrate-rich food stuff prior to a measurement can cause falsely “positive” registrations of exhaled NO, where measurements on healthy individuals or well-controlled asthmatics can be misinterpreted and lead to inappropriate medical decisions. Therefore, it is vey important to consider food intake in relation to measurements of exhaled NO. The most optimal solution to avoid this would be to perform measurements on an empty stomach, but that is obviously not very practical and it would limit the clinical use of the method. A more pragmatic approach is to have the patients refrain from eating approximately three hours prior to the examination, or in case of elevated levels of exhaled NO, ask the patient about recent food intake and if there is any suspicion of influence from foods re-examine the patient on a more empty stomach.

Our results regarding nitrate intake as a confounding factor for exhaled NO measurements have been appreciated by the international expertise and resulted in a recommendation to refrain from food intake prior to an examination in the current guidelines for measurements of NO in exhaled air from ATS and ERS (81). However, in the latest updated version from 2005 a minimum 1 hour (!) food- and beverage-free

interval is recommended, which is probably a well pragmatic concession to not discourage the use of the method. Nevertheless, a general awareness of the possible influence from food intake on the concentrations of exhaled NO and to let the patients inform about recent intake is also recommended.

Standardised mouthwash?

Another way of avoiding this confounding factor could be the general application of a suitable mouthwash before the NO measurement. The most efficient agent we have tried for the purpose of eliminating a salivary contribution to exhaled NO is the chlorhexidine solution. In study II and IV mouthwash with chlorhexidine decreased levels of exhaled NO with about 30% in control subjects and with 44% in CF patients, where the larger effect among the latter is explained by our finding of higher salivary concentrations of nitrite. After nitrate loading, as in study II, the decreasing effect of chlorhexidine on exhaled NO was about 50%. Chlorhexidine has since long been the most efficient yet essentially harmless agent used for local antibacterial treatment in dental practice (116). It has both bactericidal and bacteriostatic properties and bacterial growth has been shown to be radically decreased in cultures taken from saliva and other oral locations after the application of chlorhexidine (122-124). Furthermore, the action of the bacterial nitrate reductase activity has been demonstrated to be particularly susceptible to the application of chlorhexidine (117). It is also clear that the decrease of exhaled NO by chlorhexidine mouthwash is explained by this antibacterial mechanism rather than a mere rinsing effect, since mouthwash with pure water, as demonstrated in study II, did not affect the levels of NO substantially.

Salivary origin of EBC nitrite

The enterosalivary circulation of nitrate does not only influence concentrations of exhaled NO, but as demonstrated with the chlorhexidine application in study IV it also affects the levels of soluble nitrogen oxides in EBC. Breath condensate is believed to represent the composition of substances found on the epithelial surface of the conducting airways since the water droplets which precipitate into the condensate are mainly released from there due to turbulent air flow. A possible contribution from the oral cavity has in general been dismissed, as for example detection for salivary amylase in EBC fluid has shown to be negative (125). Previous reports have presented elevated concentrations of EBC nitrite in pediatric (25, 126) and adult asthma (24, 27), as well as in CF (25, 26). Nitrate in EBC is less studied but has been reported increased in

asthma (28). These findings have been interpreted as a result of an induced formation and turn-over of NO in the bronchial respiratory epithelium (24-28), since nitrite and nitrate are the main metabolites of NO (31). However, in a study on tracheostomised patients by Marteus *et al* EBC nitrite has been shown to largely originate in the pharyngo-oral tract, as nitrate intake caused a many-fold higher increase of nitrite concentrations in EBC collected through oral breathing compared to EBC collected through the tracheostomy (127). This is also confirmed by the results in study IV, where we clearly show that the lion part of nitrite in EBC has its origin in the pharyngo-oral tract and that the increased concentrations of EBC nitrite in the patients with CF are explained by a higher content of nitrite in their saliva. The salivary origin of EBC nitrite is concluded from the data obtained before and after mouthwash with chlorhexidine, which effectively prevents nitrite formation in the oral cavity. The baseline concentrations of EBC nitrite decreased with more than 60% in both controls and CF patients after the mouthwash but the reduction was much larger in absolute amounts for the subjects with CF, and the difference between the two groups was also largely abolished by chlorhexidine. The link between nitrite in EBC and saliva was illustrated by a very strong correlation between the two, where salivary concentrations of nitrite were equally elevated at baseline and showed a parallel reduction after chlorhexidine application. Therefore, our results deny the former idea that elevated nitrite in EBC from CF patients represent an increased formation and metabolism of NO in the lower airways.

Altered microflora in the oral cavity?

With its salivary origin we rather suggest that the increased EBC nitrite is caused by an altered bacterial activity in the pharyngo-oral tract of the CF patients. This since salivary nitrite is formed through bacterial reduction of nitrate. In fact, nitrite in the saliva has no other origin than this nitrate reduction through the action of the bacterial enzyme nitrate reductase (47). Thus, as previous studies have demonstrated, there is a complete lack of nitrite in saliva collected directly from the salivary ducts (128) as well as in saliva from germ-free rats (47). The bacterial formation of nitrite is believed to represent a symbiotic relationship which provides host defence against microbial pathogens in the oral cavity and the gut through the further non-enzymatic reduction of nitrite to NO (50, 51). Nitrate reductase is predominantly found in facultative anaerobics on the posterior surface of the tongue (129). The comparatively higher levels of nitrite in both EBC and saliva from CF patients indicate that they have an

altered activity of these nitrate-reducing bacteria, perhaps even of bacteria in general, in the oral cavity.

The results and conclusions from study IV suggest a similar explanation for our findings of EBC nitrite in study III, where the nitrite concentrations in the children with asthma also were markedly increased as compared to the levels in the control group. In this study we failed to establish any correlations between these levels of EBC nitrite and the simultaneously increased levels of exhaled NO in the children with asthma. In fact, the increased concentrations of EBC nitrite did not even show a tendency of correlation to any of the other disease markers. Since the metabolism of NO should generate both nitrite and nitrate and the nitrate levels in EBC were normal in the asthmatic children it is also unlikely that an exclusive increase of nitrite would represent an induced NO formation and turn-over in the bronchial epithelium. In addition, a quite recent study by Erpenbeck *et al* has rather shown that induction of NO synthesis in the airways, by segmental allergen challenge, primarily generates local accumulation of nitrate (130). Given these absent links between EBC nitrite and exhaled NO it is therefore reasonable to assume that the increased levels of EBC nitrite in the children with asthma also originate in the oral cavity, as demonstrated for the subjects with CF in study IV. To argue that CF patients would have an altered bacterial composition in their pharyngo-oral tract, in parallel with their vast colonization or even infection of opportunistic bacteria in the airways does not seem very far fetched, but if this also applies to asthmatics it raises some interesting questions. Could this be a primary condition, which contributes to the development of asthma, or is it secondary to the ongoing eosinophilic inflammation or the anti-inflammatory treatment? What are the characteristics of the pharyngo-oral microflora of subjects with asthma and how does it compare with the microflora of subjects with CF and healthy controls? If the microflora is substantially altered in the children with allergic asthma, how does it apply to early wheezers and early childhood asthma? To follow this trace further, those questions call for answers by the pursuit of further studies. The first step then needs to be a mapping of the bacterial composition in the pharyngo-oral tract, with emphasis on nitrate-reducing bacteria, for subjects with asthma and determine whether it truly differs from the microflora of healthy controls.

Nitrate vs nitrite

The nitrite concentrations in EBC were significantly increased in the children with asthma as well as in the subjects with CF, whereas the levels of EBC nitrate not

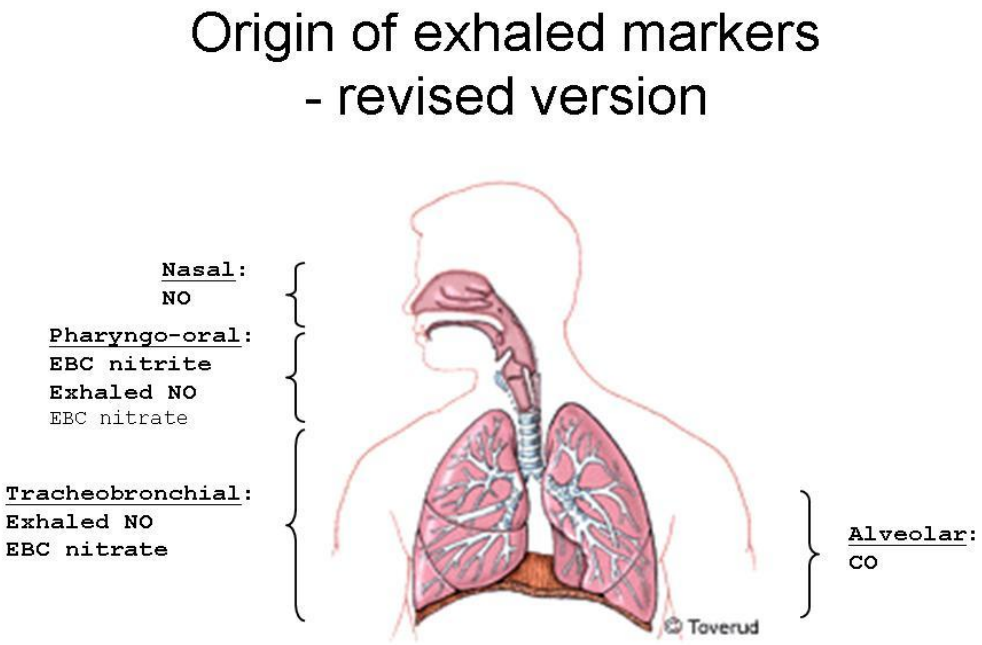

Fig 11. The origin of exhaled markers as according to our results. We have demonstrated a substantial salivary contribution to exhaled NO, which can also be greatly increased by the intake of nitrate rich foods. In addition, we have shown that EBC nitrite has its main origin in the oral cavity and that the elevated levels of EBC nitrite seen in patients with asthma and CF most likely also are of pharyngo-oral origin. Exhaled CO originates exclusively in the alveoli and is not a suitable marker for bronchial inflammation.

differed from the ones registered in the control groups. The concentrations of nitrate are normally several-fold higher than nitrite in EBC, while they are more levelled in saliva. Since we now know that most of the EBC nitrite comes from the saliva it is not very likely that the same condition applies for nitrate, because then we would not see so much higher concentrations of nitrate in EBC. Thus, one can assume that a much larger proportion of the nitrate found in EBC really originates in the lower airways. In the previously mentioned study by Marteus *et al* it was also shown that EBC collected through oral breathing contained equal concentrations of nitrate as EBC collected through the tracheostomies. The intake of nitrate caused an increase in EBC nitrate which was more pronounced in the orally collected condensate, but the difference to the concentrations in the EBC collected through tracheostomal breathing was far from as dramatic as it was for nitrite (127). In study IV a significant increase of salivary nitrate was seen after mouthwash with chlorhexidine, but the mouthwash did not cause a statistically verified increase of EBC nitrate – however a slight increase was discerned.

These data confirms a predominant bronchial origin of EBC nitrate, even though a certain contribution from the saliva seems to occur.

Exhaled NO as inflammatory marker

The usefulness of exhaled NO as a marker of eosinophilic inflammation and disease activity in allergic asthma is widely documented and the method is today gaining its way into clinical practice on a broad front. Our results in study I and III, where significantly increased levels of exhaled NO were found in asthmatics and subjects with mere allergic rhinitis, are thus well in line with what is reported in the literature. However, it can be noted that the steroid-treated asthmatics in study I, as opposed to the steroid-naïve asthmatics, did not present increased levels of exhaled NO, whereas the NO levels in the children with asthma in study III - whom with only a few exceptions were on regular treatment with inhaled corticosteroids – were markedly increased. This could be due to the fact that the asthmatics in study I comprised a more heterogeneous group, both in terms of asthma phenotype and age. In this study we did not limit the study population of asthmatics to subjects with allergic asthma, but it was rather composed of a mix of different asthma phenotypes where for example a substantial part suffered from mere exercise-induced asthma, with a lesser degree of eosinophilic inflammation and therefore lower levels of exhaled NO – irrespective of steroid treatment. In study III were all the asthmatic subjects children and IgE-positve allergic asthma was a criterion for enrollment. The eosinophilic character of their asthma was also illustrated by their significantly elevated levels of eosinophils in peripheral blood and the established correlation between these levels and exhaled NO. In addition, it is quite likely that this group of children with allergic asthma was comprised of individuals who generally suffered from a more severe and persistent type of asthma than many of the subjects in study I.

When it comes to CF and exhaled NO there is a puzzling paradox of normal or even low NO concentrations in spite of the intense airway inflammation. Our results confirmed this picture in both study I and IV. The baseline levels of exhaled NO were in both studies slightly lower in the CF patients than in the healthy controls, however, after chlorhexidine rinsing in study IV, the levels of exhaled NO were significantly lower in the CF group. From their simultaneously higher concentrations of salivary nitrite and their more pronounced decrease of exhaled NO by chlorhexidine, we can conclude that the subjects with CF had a higher degree of salivary contribution to their

baseline levels of exhaled NO. Thus, the bronchially derived proportion of exhaled NO in CF patients is probably even smaller than previously assessed.

The reason for the discrepancy between the low levels of exhaled NO and the intense airway inflammation in CF has, as previously described, been quite extensively discussed and argued. The explanation of the predominantly neutrophilic activity of CF (96), as opposed to the primarily NO-inducing eosinophilic inflammation of atopic asthma, is not fully satisfactory as for example ordinary respiratory viral infections, without any particular eosinophilic engagement, also go with increased levels of exhaled NO (66, 67, 131). The mucus theory, which suggests that the paradox of low levels of exhaled NO depends on a trapping of NO on the epithelial surface behind the thick mucus of CF, has often been referred to in previous reports of increased EBC nitrite and other NO metabolites in different airway samples from CF patients (25, 26, 98-101, 132). Our results in study IV strongly question the idea of this mucus trapping as not only the increased EBC nitrite proved to be derived from the saliva, but also as the EBC nitrate levels were not increased and bronchial NO was decreased. Since nitrate and nitrite are the main soluble metabolites of NO there is really nothing left which indicates an induced formation and turn-over of NO in the lower airways. The findings in study IV then rather support the previously mentioned studies which report of an impaired function or regulation of the NOS enzymes in CF, where for example in vitro studies have shown a defect in the signaling system of iNOS in this condition or a decreased expression of iNOS has been found in the CF bronchial epithelium (102-104). Since NO has several beneficial properties in the inflamed airway tissue, such as host-defence against microorganisms and dilatation of bronchi and increase of bronchial blood flow, a genuinely impaired NO formation might substantially contribute to the pathology of the CF lung.

6 CONCLUSIONS

- Exhaled CO has its origin in the alveoli and not in the conducting airways. It is therefore not suitable as an inflammatory marker for asthma and CF, which are conditions characterised by primarily bronchial inflammation. Furthermore, increased levels of exhaled CO could not be detected in subjects with asthma, allergic rhinitis or CF with a specific infra-red analysing technique, whereas exhaled NO is increased in asthma and allergic rhinitis, while rather low in CF.

- There is a substantial contribution to exhaled NO from salivary nitrite. The intake of nitrate-rich foods can greatly increase levels of exhaled NO for several hours. This goes through the enterosalivary circuit of dietary nitrate, where plasma nitrate is excreted through the salivary glands and in the oral cavity is reduced to nitrite by bacterial nitrate reductase. The anti-bacterial solution of chlorhexidine can largely prevent this nitrate reduction and a mouthwash with chlorhexidine prior to measurements of exhaled NO substantially reduces the salivary contribution of NO.

- The soluble NO metabolite nitrite is elevated in EBC from patients with asthma and CF. However, this is not a result of increased NO formation in their bronchi, but it rather originates in the saliva and from bacterial activity in the pharyngo-oral tract. Nitrate in EBC seems to mainly derive from the lower airways but was not elevated in asthma or cystic fibrosis.

- Salivary nitrite levels are elevated in subjects with CF, in parallel with EBC nitrite. Mouthwash with chlorhexidine markedly decreases the concentrations of nitrite in both EBC and saliva in these subjects, but also reduces their levels of exhaled NO to a larger extent than in healthy controls. Thus, the bronchial contribution of exhaled NO is probably even lower than previously assessed.

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8 REFERENCES

1. Pearce N, Ait-Khaled N, Beasley R et al.: Worldwide trends in the prevalence of asthma symptoms: phase III of the International Study of Asthma and Allergies in Childhood (ISAAC). Thorax 2007; 62:758-766.
2. Falcone FH, Haas H, Gibbs BF: The human basophil: a new appreciation of its role in immune responses. Blood 2000; 96:4028-4038.
3. Kariyawasam HH, Robinson DS: The role of eosinophils in airway tissue remodelling in asthma. Curr Opin Immunol 2007; 19:681-686.
4. Conroy DM, Williams TJ: Eotaxin and the attraction of eosinophils to the asthmatic lung. Respir Res 2001; 2:150-156.
5. Horn BR, Robin ED, Theodore J, Van Kessel A: Total eosinophil counts in the management of bronchial asthma. N Engl J Med 1975; 292:1152-1155.
6. Virchow JC, Jr., Kroegel C, Walker C, Matthys H: Cellular and immunological markers of allergic and intrinsic bronchial asthma. Lung 1994; 172:313-334.
7. Hargreave FE, Popov T, Kidney J, Dolovich J: Sputum measurements to assess airway inflammation in asthma. Allergy 1993; 48:81-83; discussion 84-86.
8. Robinson D, Hamid Q, Bentley A, Ying S, Kay AB, Durham SR: Activation of CD4+ T cells, increased TH2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. J Allergy Clin Immunol 1993; 92:313-324.
9. Synek M, Beasley R, Frew AJ et al.: Cellular infiltration of the airways in asthma of varying severity. Am J Respir Crit Care Med 1996; 154:224-230.
10. Venge P: Monitoring of asthma inflammation by serum measurements of eosinophil cationic protein (ECP). A new clinical approach to asthma management. Respir Med 1995; 89:1-2.
11. Barck C, Lundahl J, Hallden G, Bylin G: Total eosinophil cationic protein levels in induced sputum as a marker of changes in eosinophilic inflammation in a patient with allergic asthma. Ann Allergy Asthma Immunol 2005; 95:86-92.
12. Bousquet J, Chanez P, Lacoste JY et al.: Indirect evidence of bronchial inflammation assessed by titration of inflammatory mediators in BAL fluid of patients with asthma. J Allergy Clin Immunol 1991; 88:649-660.
13. Wilson NM, James A, Uasuf C et al.: Asthma severity and inflammation markers in children. Pediatr Allergy Immunol 2001; 12:125-132.
14. Park JW, Whang YW, Kim CW, Park YB, Hong CS: Eosinophil count and eosinophil cationic protein concentration of induced sputum in the diagnosis and assessment of airway inflammation in bronchial asthma. Allergy Asthma Proc 1998; 19:61-67.
15. Marguet C, Dean TP, Basuyau JP, Warner JO: Eosinophil cationic protein and interleukin-8 levels in bronchial lavage fluid from children with asthma and infantile wheeze. Pediatr Allergy Immunol 2001; 12:27-33.
16. Wennergren G: Inflammatory mediators in blood and urine. Paediatr Respir Rev 2000; 1:259-265.
17. Gustafsson LE, Leone AM, Persson MG, Wiklund NP, Moncada S: Endogenous nitric oxide is present in the exhaled air of rabbits, guinea pigs and humans. Biochem Biophys Res Commun 1991; 181:852-857.
18. Alving K, Weitzberg E, Lundberg JM: Increased amount of nitric oxide in exhaled air of asthmatics. Eur Respir J 1993; 6:1368-1370.
19. Zayasu K, Sekizawa K, Okinaga S, Yamaya M, Ohrui T, Sasaki H: Increased carbon monoxide in exhaled air of asthmatic patients. Am J Respir Crit Care Med 1997; 156:1140-1143.
20. Paredi P, Shah PL, Montuschi P et al.: Increased carbon monoxide in exhaled air of patients with cystic fibrosis. Thorax 1999; 54:917-920.

21. Lundberg JO, Nordvall SL, Weitzberg E, Kollberg H, Alving K: Exhaled nitric oxide in paediatric asthma and cystic fibrosis. Arch Dis Child 1996; 75:323-326.
22. Grasemann H, Michler E, Wallot M, Ratjen F: Decreased concentration of exhaled nitric oxide (NO) in patients with cystic fibrosis. Pediatr Pulmonol 1997; 24:173-177.
23. Horvath I, Donnelly LE, Kiss A, Paredi P, Kharitonov SA, Barnes PJ: Raised levels of exhaled carbon monoxide are associated with an increased expression of heme oxygenase-1 in airway macrophages in asthma: a new marker of oxidative stress. Thorax 1998; 53:668-672.
24. Hunt J, Byrns RE, Ignarro LJ, Gaston B: Condensed expirate nitrite as a home marker for acute asthma. Lancet 1995; 346:1235-1236.
25. Formanek W, Inci D, Lauener RP, Wildhaber JH, Frey U, Hall GL: Elevated nitrite in breath condensates of children with respiratory disease. Eur Respir J 2002; 19:487-491.
26. Ho LP, Innes JA, Greening AP: Nitrite levels in breath condensate of patients with cystic fibrosis is elevated in contrast to exhaled nitric oxide. Thorax 1998; 53:680-684.
27. Corradi M, Montuschi P, Donnelly LE, Pesci A, Kharitonov SA, Barnes PJ: Increased nitrosothiols in exhaled breath condensate in inflammatory airway diseases. Am J Respir Crit Care Med 2001; 163:854-858.
28. Corradi M, Pesci A, Casana R et al.: Nitrate in exhaled breath condensate of patients with different airway diseases. Nitric Oxide 2003; 8:26-30.
29. Palmer RM, Ferrige AG, Moncada S: Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 1987; 327:524-526.
30. Ignarro LJ, Byrns RE, Buga GM, Wood KS: Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. Circ Res 1987; 61:866-879.
31. Lundberg JO, Lundberg JM, Alving K, Weitzberg E: Nitric oxide and inflammation: the answer is blowing in the wind. Nat Med 1997; 3:30-31.
32. Guo FH, Comhair SA, Zheng S et al.: Molecular mechanisms of increased nitric oxide (NO) in asthma: evidence for transcriptional and post-translational regulation of NO synthesis. J Immunol 2000; 164:5970-5980.
33. Kroncke KD, Fehsel K, Kolb-Bachofen V: Inducible nitric oxide synthase in human diseases. Clin Exp Immunol 1998; 113:147-156.
34. Hamid Q, Springall DR, Riveros-Moreno V et al.: Induction of nitric oxide synthase in asthma. Lancet 1993; 342:1510-1513.
35. Moncada S, Palmer RM, Higgs EA: Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991; 43:109-142.
36. Benz D, Cadet P, Mantione K, Zhu W, Stefano G: Tonal nitric oxide and health--a free radical and a scavenger of free radicals. Med Sci Monit 2002; 8:RA1-4.
37. Royall JA, Kooy NW, Beckman JS: Nitric oxide-related oxidants in acute lung injury. New Horiz 1995; 3:113-122.
38. Pryor WA, Squadrito GL: The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. Am J Physiol 1995; 268:L699-722.
39. Middelveld RJ, Zetterquist WC, Bergman D, Alving K: Nitric oxide synthase inhibition augments acute allergic reactions in the pig airways in vivo. Eur Respir J 2000; 16:836-844.
40. Moncada S, Palmer RM: Inhibition of the induction of nitric oxide synthase by glucocorticoids: yet another explanation for their anti-inflammatory effects? Trends Pharmacol Sci 1991; 12:130-131.
41. Kleinert H, Euchenhofer C, Ihrig-Biedert I, Forstermann U: Glucocorticoids inhibit the induction of nitric oxide synthase II by down-regulating cytokine-induced activity of transcription factor nuclear factor-kappa B. Mol Pharmacol 1996; 49:15-21.
42. Donnelly LE, Barnes PJ: Expression and regulation of inducible nitric oxide synthase from human primary airway epithelial cells. Am J Respir Cell Mol Biol 2002; 26:144-151.

43. Silkoff PE, McClean P, Spino M, Erlich L, Slutsky AS, Zamel N: Dose-response relationship and reproducibility of the fall in exhaled nitric oxide after inhaled beclomethasone dipropionate therapy in asthma patients. Chest 2001; 119:1322-1328.
44. Lim S, Jatakanon A, John M et al.: Effect of inhaled budesonide on lung function and airway inflammation. Assessment by various inflammatory markers in mild asthma. Am J Respir Crit Care Med 1999; 159:22-30.
45. Weitzberg E, Lundberg JO: Nonenzymatic nitric oxide production in humans. Nitric Oxide 1998; 2:1-7.
46. Webb A, Bond R, McLean P, Uppal R, Benjamin N, Ahluwalia A: Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. Proc Natl Acad Sci U S A 2004; 101:13683-13688.
47. Duncan C, Dougall H, Johnston P et al.: Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. Nat Med 1995; 1:546-551.
48. Zetterquist W, Pedroletti C, Lundberg JO, Alving K: Salivary contribution to exhaled nitric oxide. Eur Respir J 1999; 13:327-333.
49. McKnight GM, Smith LM, Drummond RS, Duncan CW, Golden M, Benjamin N: Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans. Gut 1997; 40:211-214.
50. Doel JJ, Hector MP, Amirtham CV, Al-Anzan LA, Benjamin N, Allaker RP: Protective effect of salivary nitrate and microbial nitrate reductase activity against caries. Eur J Oral Sci 2004; 112:424-428.
51. Bjorne H, Weitzberg E, Lundberg JO: Intragastric generation of antimicrobial nitrogen oxides from saliva--physiological and therapeutic considerations. Free Radic Biol Med 2006; 41:1404-1412.
52. Lundberg JO, Carlsson S, Engstrand L, Morcos E, Wiklund NP, Weitzberg E: Urinary nitrite: more than a marker of infection. Urology 1997; 50:189-191.
53. Carlsson S, Wiklund NP, Engstrand L, Weitzberg E, Lundberg JO: Effects of pH, nitrite, and ascorbic acid on nonenzymatic nitric oxide generation and bacterial growth in urine. Nitric Oxide 2001; 5:580-586.
54. Weller R, Pattullo S, Smith L, Golden M, Ormerod A, Benjamin N: Nitric oxide is generated on the skin surface by reduction of sweat nitrate. J Invest Dermatol 1996; 107:327-331.
55. Weller R, Price RJ, Ormerod AD, Benjamin N, Leifert C: Antimicrobial effect of acidified nitrite on dermatophyte fungi, Candida and bacterial skin pathogens. J Appl Microbiol 2001; 90:648-652.
56. Lundberg JO, Hellstrom PM, Lundberg JM, Alving K: Greatly increased luminal nitric oxide in ulcerative colitis. Lancet 1994; 344:1673-1674.
57. Herulf M, Ljung T, Hellstrom PM, Weitzberg E, Lundberg JO: Increased luminal nitric oxide in inflammatory bowel disease as shown with a novel minimally invasive method. Scand J Gastroenterol 1998; 33:164-169.
58. Lundberg JO, Ehren I, Jansson O et al.: Elevated nitric oxide in the urinary bladder in infectious and noninfectious cystitis. Urology 1996; 48:700-702.
59. Lane C, Knight D, Burgess S et al.: Epithelial inducible nitric oxide synthase activity is the major determinant of nitric oxide concentration in exhaled breath. Thorax 2004; 59:757-760.
60. Franklin PJ, Turner SW, Le Souef PN, Stick SM: Exhaled nitric oxide and asthma: complex interactions between atopy, airway responsiveness, and symptoms in a community population of children. Thorax 2003; 58:1048-1052.
61. Cardinale F, de Benedictis FM, Muggeo V et al.: Exhaled nitric oxide, total serum IgE and allergic sensitization in childhood asthma and allergic rhinitis. Pediatr Allergy Immunol 2005; 16:236-242.
62. Olin AC, Alving K, Toren K: Exhaled nitric oxide: relation to sensitization and respiratory symptoms. Clin Exp Allergy 2004; 34:221-226.
63. Gratziou C, Lignos M, Dassiou M, Roussos C: Influence of atopy on exhaled nitric oxide in patients with stable asthma and rhinitis. Eur Respir J 1999; 14:897-901.

64. Corradi M, Majori M, Cacciani GC, Consigli GF, de'Munari E, Pesci A: Increased exhaled nitric oxide in patients with stable chronic obstructive pulmonary disease. Thorax 1999; 54:572-575.
65. Kharitonov SA, Wells AU, O'Connor BJ et al.: Elevated levels of exhaled nitric oxide in bronchiectasis. Am J Respir Crit Care Med 1995; 151:1889-1893.
66. Kharitonov SA, Yates D, Barnes PJ: Increased nitric oxide in exhaled air of normal human subjects with upper respiratory tract infections. Eur Respir J 1995; 8:295-297.
67. de Gouw HW, Grunberg K, Schot R, Kroes AC, Dick EC, Sterk PJ: Relationship between exhaled nitric oxide and airway hyperresponsiveness following experimental rhinovirus infection in asthmatic subjects. Eur Respir J 1998; 11:126-132.
68. Lundberg JO, Farkas-Szallasi T, Weitzberg E et al.: High nitric oxide production in human paranasal sinuses. Nat Med 1995; 1:370-373.
69. Kimberly B, Nejadnik B, Giraud GD, Holden WE: Nasal contribution to exhaled nitric oxide at rest and during breathholding in humans. Am J Respir Crit Care Med 1996; 153:829-836.
70. Jain B, Rubinstein I, Robbins RA, Leise KL, Sisson JH: Modulation of airway epithelial cell ciliary beat frequency by nitric oxide. Biochem Biophys Res Commun 1993; 191:83-88.
71. Lundberg JO, Settergren G, Gelinder S, Lundberg JM, Alving K, Weitzberg E: Inhalation of nasally derived nitric oxide modulates pulmonary function in humans. Acta Physiol Scand 1996; 158:343-347.
72. Lundberg JO, Weitzberg E, Nordvall SL, Kuylenstierna R, Lundberg JM, Alving K: Primarily nasal origin of exhaled nitric oxide and absence in Kartagener's syndrome. Eur Respir J 1994; 7:1501-1504.
73. Thomas SR, Kharitonov SA, Scott SF, Hodson ME, Barnes PJ: Nasal and exhaled nitric oxide is reduced in adult patients with cystic fibrosis and does not correlate with cystic fibrosis genotype. Chest 2000; 117:1085-1089.
74. Kharitonov SA, Rajakulasingam K, O'Connor B, Durham SR, Barnes PJ: Nasal nitric oxide is increased in patients with asthma and allergic rhinitis and may be modulated by nasal glucocorticoids. J Allergy Clin Immunol 1997; 99:58-64.
75. Wilson AM, Dempsey OJ, Sims EJ, Lipworth BJ: Subjective and objective markers of treatment response in patients with seasonal allergic rhinitis. Ann Allergy Asthma Immunol 2000; 85:111-114.
76. Palm JP, Alving K, Lundberg JO: Characterization of airway nitric oxide in allergic rhinitis: the effect of intranasal administration of L-NAME. Allergy 2003; 58:885-892.
77. Silkoff PE, McClean PA, Slutsky AS et al.: Marked flow-dependence of exhaled nitric oxide using a new technique to exclude nasal nitric oxide. Am J Respir Crit Care Med 1997; 155:260-267.
78. Kroesbergen A, Jobsis Q, Bel EH, Hop WC, de Jongste JC: Flow-dependency of exhaled nitric oxide in children with asthma and cystic fibrosis. Eur Respir J 1999; 14:871-875.
79. Pedroletti C, Zetterquist W, Nordvall L, Alving K: Evaluation of exhaled nitric oxide in schoolchildren at different exhalation flow rates. Pediatr Res 2002; 52:393-398.
80. Recommendations for standardized procedures for the on-line and off-line measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide in adults and children-1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. Am J Respir Crit Care Med 1999; 160:2104-2117.
81. ATS/ERS Recommendations for Standardized Procedures for the Online and Offline Measurement of Exhaled Lower Respiratory Nitric Oxide and Nasal Nitric Oxide, 2005. Am J Respir Crit Care Med 2005; 171:912-930.
82. Silvestri M, Spallarossa D, Frangova Yourukova V, Battistini E, Fregonese B, Rossi GA: Orally exhaled nitric oxide levels are related to the degree of blood eosinophilia in atopic children with mild-intermittent asthma. Eur Respir J 1999; 13:321-326.

83. Mattes J, Storm van's Gravesande K, Reining U et al.: NO in exhaled air is correlated with markers of eosinophilic airway inflammation in corticosteroid-dependent childhood asthma. Eur Respir J 1999; 13:1391-1395.
84. Warke TJ, Fitch PS, Brown V et al.: Exhaled nitric oxide correlates with airway eosinophils in childhood asthma. Thorax 2002; 57:383-387.
85. Payne DN, Adcock IM, Wilson NM, Oates T, Scallan M, Bush A: Relationship between exhaled nitric oxide and mucosal eosinophilic inflammation in children with difficult asthma, after treatment with oral prednisolone. Am J Respir Crit Care Med 2001; 164:1376-1381.
86. Silvestri M, Sabatini F, Sale R et al.: Correlations between exhaled nitric oxide levels, blood eosinophilia, and airway obstruction reversibility in childhood asthma are detectable only in atopic individuals. Pediatr Pulmonol 2003; 35:358-363.
87. Strunk RC, Szefler SJ, Phillips BR et al.: Relationship of exhaled nitric oxide to clinical and inflammatory markers of persistent asthma in children. J Allergy Clin Immunol 2003; 112:883-892.
88. Silvestri M, Spallarossa D, Battistini E, Brusasco V, Rossi GA: Dissociation between exhaled nitric oxide and hyperresponsiveness in children with mild intermittent asthma. Thorax 2000; 55:484-488.
89. Haley KJ, Drazen JM: Inflammation and airway function in asthma: what you see is not necessarily what you get. Am J Respir Crit Care Med 1998; 157:1-3.
90. Ghiro L, Zanconato S, Rampon O, Piovan V, Pasquale MF, Baraldi E: Effect of montelukast added to inhaled corticosteroids on fractional exhaled nitric oxide in asthmatic children. Eur Respir J 2002; 20:630-634.
91. Smith AD, Cowan JO, Filsell S et al.: Diagnosing asthma: comparisons between exhaled nitric oxide measurements and conventional tests. Am J Respir Crit Care Med 2004; 169:473-478.
92. Harkins MS, Fiato KL, Iwamoto GK: Exhaled nitric oxide predicts asthma exacerbation. J Asthma 2004; 41:471-476.
93. Beck-Ripp J, Griese M, Arenz S, Koring C, Pasqualoni B, Bufler P: Changes of exhaled nitric oxide during steroid treatment of childhood asthma. Eur Respir J 2002; 19:1015-1019.
94. Yates DH, Kharitonov SA, Robbins RA, Thomas PS, Barnes PJ: Effect of a nitric oxide synthase inhibitor and a glucocorticosteroid on exhaled nitric oxide. Am J Respir Crit Care Med 1995; 152:892-896.
95. Elphick HE, Demoncheaux EA, Ritson S, Higenbottam TW, Everard ML: Exhaled nitric oxide is reduced in infants with cystic fibrosis. Thorax 2001; 56:151-152.
96. Ratjen F, Doring G: Cystic fibrosis. Lancet 2003; 361:681-689.
97. Taylor DR, Pijnenburg MW, Smith AD, De Jongste JC: Exhaled nitric oxide measurements: clinical application and interpretation. Thorax 2006; 61:817-827.
98. de Winter-de Groot KM, van der Ent CK: Nitric oxide in cystic fibrosis. J Cyst Fibros 2005; 4 Suppl 2:25-29.
99. Grasemann H, Ioannidis I, Tomkiewicz RP, de Groot H, Rubin BK, Ratjen F: Nitric oxide metabolites in cystic fibrosis lung disease. Arch Dis Child 1998; 78:49-53.
100. Jones KL, Hegab AH, Hillman BC et al.: Elevation of nitrotyrosine and nitrate concentrations in cystic fibrosis sputum. Pediatr Pulmonol 2000; 30:79-85.
101. Balint B, Kharitonov SA, Hanazawa T et al.: Increased nitrotyrosine in exhaled breath condensate in cystic fibrosis. Eur Respir J 2001; 17:1201-1207.
102. Meng QH, Springall DR, Bishop AE et al.: Lack of inducible nitric oxide synthase in bronchial epithelium: a possible mechanism of susceptibility to infection in cystic fibrosis. J Pathol 1998; 184:323-331.
103. Zheng S, Xu W, Bose S, Banerjee AK, Haque SJ, Erzurum SC: Impaired nitric oxide synthase-2 signaling pathway in cystic fibrosis airway epithelium. Am J Physiol Lung Cell Mol Physiol 2004; 287:L374-381.
104. Kelley TJ, Drumm ML: Inducible nitric oxide synthase expression is reduced in cystic fibrosis murine and human airway epithelial cells. J Clin Invest 1998; 102:1200-1207.

105. Dotsch J, Puls J, Klimek T, Rascher W: Reduction of neuronal and inducible nitric oxide synthase gene expression in patients with cystic fibrosis. Eur Arch Otorhinolaryngol 2002; 259:222-226.
106. Tannenbaum SR, Weisman M, Fett D: The effect of nitrate intake on nitrite formation in human saliva. Food Cosmet Toxicol 1976; 14:549-552.
107. Dohlman AW, Black HR, Royall JA: Expired breath hydrogen peroxide is a marker of acute airway inflammation in pediatric patients with asthma. Am Rev Respir Dis 1993; 148:955-960.
108. Hanazawa T, Kharitonov SA, Barnes PJ: Increased nitrotyrosine in exhaled breath condensate of patients with asthma. Am J Respir Crit Care Med 2000; 162:1273-1276.
109. Kharitonov SA, Donnelly LE, Montuschi P, Corradi M, Collins JV, Barnes PJ: Dose-dependent onset and cessation of action of inhaled budesonide on exhaled nitric oxide and symptoms in mild asthma. Thorax 2002; 57:889-896.
110. Horvath I, Loukides S, Wodehouse T, Kharitonov SA, Cole PJ, Barnes PJ: Increased levels of exhaled carbon monoxide in bronchiectasis: a new marker of oxidative stress. Thorax 1998; 53:867-870.
111. Horvath I, Barnes PJ: Exhaled monoxides in asymptomatic atopic subjects. Clin Exp Allergy 1999; 29:1276-1280.
112. Yamaya M, Sekizawa K, Ishizuka S, Monma M, Mizuta K, Sasaki H: Increased carbon monoxide in exhaled air of subjects with upper respiratory tract infections. Am J Respir Crit Care Med 1998; 158:311-314.
113. Choi AM, Alam J: Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. Am J Respir Cell Mol Biol 1996; 15:9-19.
114. Lim S, Groneberg D, Fischer A et al.: Expression of heme oxygenase isoenzymes 1 and 2 in normal and asthmatic airways: effect of inhaled corticosteroids. Am J Respir Crit Care Med 2000; 162:1912-1918.
115. Kharitonov S, Alving K, Barnes PJ: Exhaled and nasal nitric oxide measurements: recommendations. The European Respiratory Society Task Force. Eur Respir J 1997; 10:1683-1693.
116. Jones CG: Chlorhexidine: is it still the gold standard? Periodontol 2000 1997; 15:55-62.
117. van Maanen JM, van Geel AA, Kleinjans JC: Modulation of nitrate-nitrite conversion in the oral cavity. Cancer Detect Prev 1996; 20:590-596.
118. Chinn S, Burney P, Jarvis D, Luczynska C: Variation in bronchial responsiveness in the European Community Respiratory Health Survey (ECRHS). Eur Respir J 1997; 10:2495-2501.
119. Uasuf CG, Jatakanon A, James A, Kharitonov SA, Wilson NM, Barnes PJ: Exhaled carbon monoxide in childhood asthma. J Pediatr 1999; 135:569-574.
120. Yamaya M, Hosoda M, Ishizuka S et al.: Relation between exhaled carbon monoxide levels and clinical severity of asthma. Clin Exp Allergy 2001; 31:417-422.
121. Antuni JD, Kharitonov SA, Hughes D, Hodson ME, Barnes PJ: Increase in exhaled carbon monoxide during exacerbations of cystic fibrosis. Thorax 2000; 55:138-142.
122. Pilloni AP, Buttini G, Giannarelli D et al.: Antimicrobial action of Nitens mouthwash (cetyltrimethylammonium naproxenate) on multiple isolates of pharyngeal microbes: a controlled study against chlorhexidine, benzydamine, hexetidine, amoxicillin, amoxicillin-clavulanate, clarithromycin, and cefaclor. Chemotherapy 2002; 48:168-173.
123. Paquette L, Legner M, Fillery ED, Friedman S: Antibacterial efficacy of chlorhexidine gluconate intracanal medication in vivo. J Endod 2007; 33:788-795.
124. Koeman M, van der Ven AJ, Hak E et al.: Oral decontamination with chlorhexidine reduces the incidence of ventilator-associated pneumonia. Am J Respir Crit Care Med 2006; 173:1348-1355.
125. Horvath I, Hunt J, Barnes PJ et al.: Exhaled breath condensate: methodological recommendations and unresolved questions. Eur Respir J 2005; 26:523-548.

126. Straub DA, Ehmann R, Hall GL et al.: Correlation of nitrites in breath condensates and lung function in asthmatic children. Pediatr Allergy Immunol 2004; 15:20-25.
127. Marteus H, Tornberg DC, Weitzberg E, Schedin U, Alving K: Origin of nitrite and nitrate in nasal and exhaled breath condensate and relation to nitric oxide formation. Thorax 2005; 60:219-225.
128. Granli T, Dahl R, Brodin P, Bockman OC: Nitrate and nitrite concentrations in human saliva: variations with salivary flow-rate. Food Chem Toxicol 1989; 27:675-680.
129. Doel JJ, Benjamin N, Hector MP, Rogers M, Allaker RP: Evaluation of bacterial nitrate reduction in the human oral cavity. Eur J Oral Sci 2005; 113:14-19.
130. Erpenbeck VJ, Jorres RA, Discher M et al.: Local nitric oxide levels reflect the degree of allergic airway inflammation after segmental allergen challenge in asthmatics. Nitric Oxide 2005; 13:125-133.
131. Proud D: Nitric oxide and the common cold. Curr Opin Allergy Clin Immunol 2005; 5:37-42.
132. Morrissey BM, Schilling K, Weil JV, Silkoff PE, Rodman DM: Nitric oxide and protein nitration in the cystic fibrosis airway. Arch Biochem Biophys 2002; 406:33-39.