Exhaled nitric oxide and hydrogen peroxide
as markers of airway inflammation in children

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Stikstofmonoxide en waterstofperoxide in uitademingslucht
als indicatoren van luchtwegontsteking bij kinderen

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PROMOTOR
Prof. dr. J.C. de Jongste

OVERIGE LEDEN
Prof. dr. J.M. Bogaard
Prof. dr. R. de Groot
Prof. dr. E.F.M. Wouters

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CHAPTER 1

General introduction and aims of the study

1.1   INTRODUCTION

Airway inflammation plays an important role in various respiratory disorders of childhood including recurrent wheezing, asthma, cystic fibrosis (CF), bronchopulmonary dysplasia (BPD), and respiratory distress syndrome (RDS) (1-4). In daily clinical practice, indirect indices of airway inflammation like symptoms and lung function measurements are routinely used for diagnosis and follow-up of inflammatory respiratory disorders. Especially in young children, these indirect indices of disease severity are less valuable than in adults; lung function studies in children are often not possible because of lack of cooperation. Furthermore, reporting of symptoms by others (parents) is much dependent on perception of symptoms with the potential risk for both under and overperception. Therefore, there is a need for objective and early criteria to detect and monitor airway inflammation in order to prevent or minimize the irreversible changes which are described in various chronic respiratory disorders including asthma, CF and BPD (5-9). The analysis of airway inflammatory cells and mediators has traditionally been performed by bronchoscopy on samples of bronchial mucosal biopsies or on bronchoalveolar lavage fluid (BALF) samples. Both methods are limited in their applicability because of the invasive procedure of bronchoscopy. However, these invasive procedures have greatly enhanced the understanding of the role of airway inflammation in various respiratory disorders (10-13). Bronchoscopy has its own pitfalls in the study of airway inflammation (14,15). All things considered, it is clear that in daily clinical practice, serial measurements of airway inflammation are not feasible with such invasive methods. A noninvasive method to assess the presence and activity of airway inflammation would be of great benefit for early diagnosis and monitoring of inflammatory airway diseases in children (16,17).

Inflammatory markers can be measured in blood or in urine (18,19). The noninvasiveness of these methods is an advantage, but their indirect nature is a limitation. Measurement of certain inflammatory markers in blood and urine represents whole body production and does not necessarily reflect production of these inflammatory markers in the respiratory tract. It is reasonable to assume that the composition of respiratory secretions might closer reflect airway inflammation than substances in blood or urine (20). Sputum is one of the respiratory secretions which can be used in the assessment of airway inflammation (21-23). Sputum can be obtained noninvasively, spontaneously or during chest physiotherapy. Sputum examination has been limited by the difficulty in obtaining adequate samples. When sputum can not be produced spontaneously it can be induced by inhalation of an aerosol of hypertonic saline (24). With this technique, sputum samples can be obtained in up to 75-100% of asthmatic and healthy adults (25). In children the success rate in obtaining adequate samples is definitely lower

(26). Other practical considerations of sputum induction include the inevitability of pretreatment with short-acting β_2-agonists in order to prevent an obstructive airway response provoked by inhalation of hypertonic saline, and the time-consuming procedure for inducing sputum and processing induced sputum samples. Furthermore, several studies have shown that repeated sputum induction in itself induces changes in airway inflammation within 24-48 hours after sputum induction, which limits the usefulness of this method for serial measurements (27,28). Other secretions from the respiratory tract include nasal and nasopharyngeal mucus. Nasal samples like nasal lavage fluid, mucosa brushing and -biopsies are easy to obtain and could represent a relatively noninvasive way of monitoring airway inflammation. However, whether nasal secretions can be used as a model for the assessment of lower airway inflammation is doubtful (29,30). Exhaled air is a recently rediscovered vehicle of substances from the respiratory tract, in which several potential markers of airway inflammation can be detected. In contrast with the other sampling methods mentioned above, exhaled air offers the advantage that it can be obtained completely noninvasively. Exhaled air may carry components from the lower airways, and does not disturb the airways, in contrast to bronchial biopsies, BAL and induced sputum, and it can be obtained with minimal risk and inconvenience from both adults and children.

1.2   HUMAN BREATH ANALYSIS

The diagnosis of diseases through analysis of human breath has a long tradition in medicine. In the past, diseases as diabetes mellitus, malfunction of kidneys and hepatic failure were diagnosed by means of the olfactory sense of volatile components, such as acetone, ammonia and sulphur compounds (31). In the last decades, the development of sensitive analytic methods for the detection of volatile substances has led to new developments in exhaled air analysis. In the meantime, approximately 200 different components have been identified in human exhaled air, some of which have been correlated to various diseases (31,32). Besides a potential diagnostic use for particular diseases, exhaled air analysis has been used in monitoring inhalation exposure to gaseous and volatile compounds (33). Furthermore, in daily life breath tests for the detection of ethanol in exhaled air are frequently used by the police for the sake of traffic safety. Diagnostic breath tests have been studied in intensive care (34), gastroenterology (35,36), infectiology (37), diabetes mellitus (38,39), uraemia (40) and oncology (41,42). Surprisingly the use of breath tests for the detection of disorders of the respiratory tract was until recently limited to lung cancer (43). However, in 1991 Gustafsson et al. (44) showed the possibility of measuring endogenous nitric oxide (NO) in the parts per billion (ppb) range in exhaled air of

humans and experimental animals. This observation marked the onset of an exciting recent development of noninvasive monitoring of inflammation markers in exhaled air. Exhaled air analysis has the potential of becoming a rapid, noninvasive diagnostic technique to assess airway inflammation.

Apart from low-molecular weight volatile substances, exhaled air may also contain nonvolatile macromolecules (45). These nonvolatile substances can be transported only as aerosol particles in the exhaled air. In contrast to the numerous studies on the composition and lung deposition of exogenous aerosols, the studies on the nature, composition and amount of endogenous aerosols excreted in exhaled air are scanty. It is generally known that aerosol particles are generated in the respiratory tract during forced exhalation and coughing. Two studies confirmed the existence of aerosol particles in exhaled air during quiet tidal breathing (46,47). By cooling exhaled air, water vapour in the gaseous phase, including the aerosol particles, can be condensed to a liquid phase. Collection of exhaled air condensate is another noninvasive method for obtaining secretions of the respiratory tract. The volatile and nonvolatile substances in human breath can potentially be used for noninvasive assessment of airway inflammation based on the assumption that aerosol particles or vapour in exhaled air reflect the composition of the lower airway fluids. By applying a cold trap setup, Scheideler et al. found proteins, carbohydrates and salts detectable in exhaled air condensate obtained during forceful breathing (45). Proteins in condensate were analysed by two-dimensional gel electrophoresis. Characterization is possible on the basis of their place in the electropherogram, or by molecular techniques. However, this study did not rule out contamination with saliva, which is a rich source of inflammatory molecules.

In summary, exhaled air and breath condensate are noninvasive methods to obtain samples from the lower respiratory tract. These methods are not disturbing to airways in contrast to induced sputum and BAL, and can be used in the determination of acellular markers of inflammation in exhaled air like nitric oxide (NO) and hydrogen peroxide (H_2O_2). This may prove valuable in the early diagnosis of inflammatory airway diseases in children.

1.3   EXHALED MARKERS OF INFLAMMATION

The ideal marker of airway inflammation should fulfil the following criteria: it should be a valid marker of inflammation which is sensitive enough to monitor early stages of airway inflammation and should preferably be specific for a particular respiratory disorder. It needs to be reproducible, simple and rapid to perform, inexpensive and acceptable, with minimal discomfort to the subject. It should be noninvasive and feasible for adults as well as children.

Inflammation markers should reflect the effect of anti-inflammatory treatment and be helpful in monitoring compliance with treatment.

1.3.1 NITRIC OXIDE

The measurement of exhaled nitric oxide (eNO) as a simple noninvasive means for the assessment of airway inflammation in the respiratory tract has excited considerable interest. NO is a reactive free radical gas which plays an important role in the regulation of many physiological and pathophysiological processes in various organ systems (48,49). In the past NO was regarded mainly as noxious air pollutant. In 1987, NO was characterized as the endothelium derived relaxing factor (EDRF), which acted through activation of guanylate cyclase and so increases cyclic GMP, resulting in relaxation of vascular smooth muscle cells (50,51). NO is now also recognized as a mediator of host defense, immune regulation, neural transmission, platelet inhibition and inflammation (48,49).

In the respiratory tract, endogenous NO may have various important roles in physiologic regulation of airway functions but it is also involved in the pathophysiology of inflammatory diseases (52-54). NO is a potent vasodilator of the bronchial circulation and plays an important role in regulating blood flow in the pulmonary circulation (52). Furthermore, NO acts as an endogenous neural bronchodilator and appears to be an important regulator of airway ciliary, immunological and inflammatory functions (53).

NO is formed from the semi-essential amino acid L-arginine during oxidation to L-citrulline by the enzyme NO synthase (NOS). Two functional classes of NOS can be identified: the constitutive form (cNOS) continuously producing small amounts of NO, and the inducible form (iNOS) producing high amounts of NO (figure 1). The constitutive isoforms are found in neuronal- (nNOS) and endothelial cells (eNOS) and are activated by a rise in intracellular calcium, in response to receptor stimulation or to various physiological stimuli. The inducible isoforms are induced in several cell types including endothelial cells, epithelial cells and macrophages by exposure to endotoxins and cytokines such as tumor necrosis factor-α, interferon-γ and interleukin-1β. Induction of iNOS is blocked by corticosteroids. All isoforms have been detected in the human respiratory tract (55,56). The relatively low concentrations generated by cNOS can lead to airway and vascular smooth muscle relaxation, whereas the relatively high concentrations generated by iNOS have pro-inflammatory and immunomodulatory effects.

Endogenous NO is detectable in exhaled air of healthy subjects, and appears to be elevated in asthmatic subjects who are not taking inhaled steroids (44,57,58). Furthermore, corticosteroids reduce eNO in asthmatic patients but not in healthy subjects (59), which is presumably because iNOS is the major source of
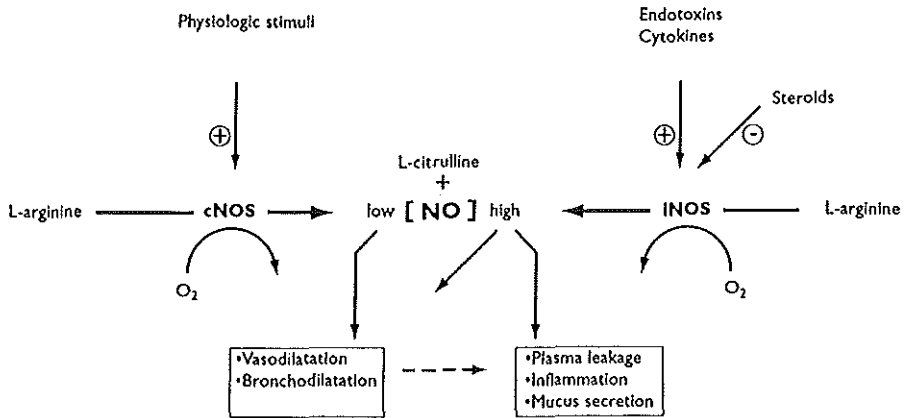
FIGURE 1. Nitric oxide (NO) synthesis by the different nitric oxide synthases (NOS)

elevated eNO values, whereas eNO in healthy subjects depends on cNOS which is not suppressed by corticosteroids. This suggests that eNO may be a useful marker of airway inflammation and that its noninvasive measurement may be useful in monitoring anti–inflammatory treatment. However, the production of NO in the respiratory tract is not only limited to the lower airways. NO is also generated in the nose, paranasal sinuses and nasopharynx (60,61). Nasal NO concentrations are high in comparison to the NO values detected in the lower respiratory tract. The high nasal NO values may have various physiologic roles, such as preserving sinus sterility and modulating ciliary motility (62,63). So, in the assessment of lower airway inflammation by eNO, contamination of orally exhaled air with much higher NO concentrations from the upper airways should be avoided.

The usual technique for measurement of NO in exhaled air is chemilumine-scence which has long been used in monitoring air pollution. The principle of this detection method is the reaction between NO in exhaled air and an excess of ozone (O_3), which leads to the formation of an unstable reaction product (NO_2^*). NO_2^* spontaneously disintegrates to the ground state of NO_2 while emitting a single photon. The photon is detected by a photomultiplier tube that proportionally converts the number of photons into an electrical signal from which the NO concentration can be calculated (64). There are three main approaches in sampling exhaled air for eNO measurement (65): 1) single breath constant flow method (on–line sampling); 2) single breath reservoir method (off–line sampling); 3) tidal breathing method (on– or off–line sampling). With on–line sampling, exhaled air is directly sampled by the NO–analyser at a fixed flow from a side port in the exhalation circuit. With off–line sampling, exhaled air
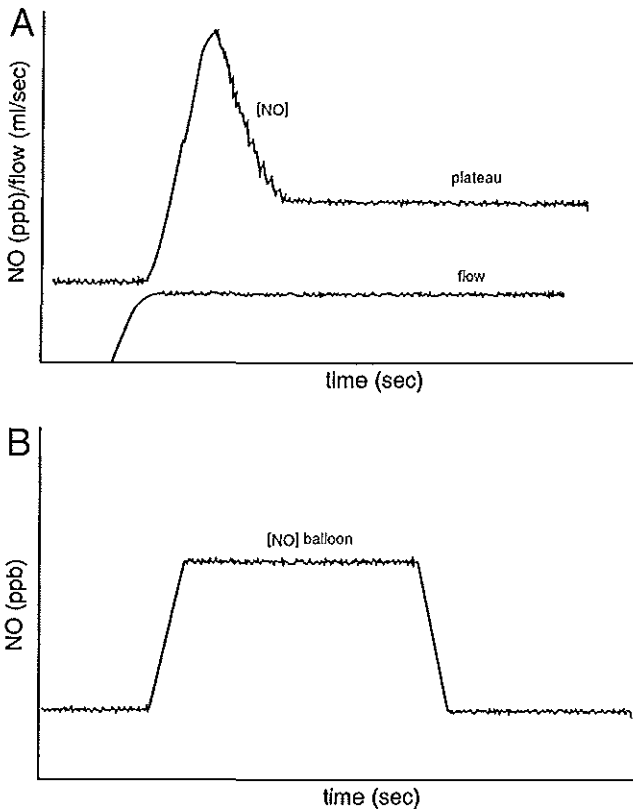
FIGURE 2. A) Exhaled NO measurement, obtained with the single breath constant flow method. B) Exhaled NO measurement, obtained with the single breath balloon method.

is collected in a NO-inert reservoir and later analysed for NO concentration. This has the advantage that it offers independence from the direct precence of an NO-analyser.

Using the single breath constant flow method, subjects perform one slow exhalation from total lung capacity (TLC) with a constant flow against an inline resistor. The resistor produces a positive pressure in the oropharynx which, by closing the soft palate, prevents contamination with nasal air. The initial part of the exhalation shows a peak in eNO, which corresponds with NO accumulated in the dead space air volume, followed by a NO plateau when there is a constant exhalation flow (figure 2A). Mean end-expiratory plateau values of eNO are measured (66). With the single breath balloon method, exhaled air is collected by asking the subject to inhale orally to TLC and then immediately perform a slow vital capacity manoeuvre against an expiratory resistance into a NO-inert balloon.

The NO concentration in the balloon is analysed later (figure 2B). The tidal breathing method can be performed on- and off-line. NO is measured in mixed expired air during tidal breathing over a period of time with or without a resistor in the expiration circuit. NO in exhaled air, recorded by a fast analyser, can be calculated as an average signal over several breaths. When recorded by a slow analyser, NO concentration is measured in a reservoir in which exhaled air during tidal breathing is collected.

In recent years, the measurement of NO in exhaled air has been studied for its potential as a noninvasive marker of airway inflammation in both diagnosis and monitoring of inflammation. Increased amounts of NO have been detected in exhaled air in humans with various inflammatory airway disorders such as stable and unstable asthma, respiratory tract infections, bronchiectasis, fibrosing alveolitis, pulmonary sarcoidosis and lung allograft rejection (57,58,67-72). On the other hand low NO values have been detected in respiratory disorders such as cystic fibrosis, immotile cilia syndromes, adult respiratory distress syndrome and smokers (73-76). Corticosteroids reduce eNO values in asthmatics but not in healthy subjects and this is because of an inhibitory effect on iNOS (77). Inhaled steroids will reduce eNO to normal levels even before an effect on symptoms is apparent (78). Furthermore, eNO increases during reduction of inhaled corticosteroids in asthmatic subjects. In asthmatic children, exhaled NO is reduced by leukotriene receptor antagonists which reduce airway eosinophilia (79,80). Another interesting finding is the positive correlation between eNO and eosinophils in induced sputum in asthmatic subjects, with eosinophil cationic protein (ECP) in induced sputum, and with eosinophil peroxidase (EPO) in BALF (81-83). This suggests that eNO may reflect eosinophilic airway inflammation. However, there was no correlation between eNO and direct indices of inflammation in airway biopsies (78). Anyhow, different research groups have reported widely varying levels of eNO values in similar patient groups and even in healthy subjects, probably due to differences in methodology.

1.3.2 HYDROGEN PEROXIDE

Oxygen derived free radicals are important mediators of cell and tissue injury during inflammation and are produced by several types of inflammatory cells and by airway epithelial cells (84,85). A free radical is a molecule or an atom with an unpaired electron in its outer orbit. The unpaired electron alters the chemical reactivity of a molecule or atom, usually making it more reactive than the corresponding non-radical (86). Free radicals react with a wide range of biological molecules. The partial reduction of O_2 by sequential single electron steps produces three intermediates: superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2), each more reactive than O_2 itself (figure 3). These three intermediates together with hypochlorus acid (HOCL) are called
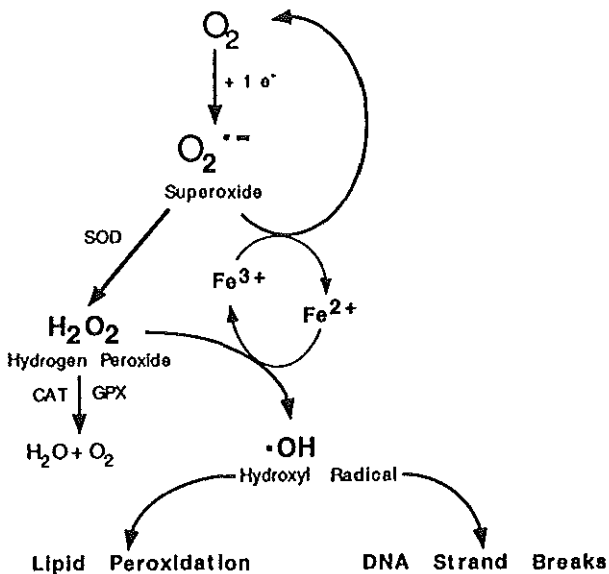
FIGURE 3. Reactive oxygen species, antioxidants and oxidative damage. SOD: superoxide dismutase; CAT: catalase; GPX: glutathione peroxidase; DNA: deoxyribonucleic acid.

reactive oxygen species (ROS). Antioxidant enzymetic systems are present for disposal of ROS. Superoxide dismutase, catalyzes the breakdown of $O_2^{\cdot-}$ to O_2 and H_2O_2, catalase converts H_2O_2 to H_2O and O_2, and glutathione peroxidase removes both H_2O_2 and lipid hydroperoxides. An excess of ROS, oxidative stress, has been implicated in the pathophysiology of a number of respiratory disorders such as chronic obstructive pulmonary disease (COPD), CF, and asthma (87-90). Activation of inflammatory cells such as eosinophils, neutrophils, and macrophages induces a respiratory burst resulting in the production of ROS. ROS may damage airway structural and functional components including lipids, enzymes and DNA (figure 3). H_2O_2 is a central precursor to $OH^{\cdot}$ and HOCL, which are both extremely potent oxidants (87). Bronchial and tracheal epithelial cells release H_2O_2 in response to inflammatory stimuli (85). In 1986 Baldwin et al. showed that H_2O_2 can be detected in exhaled air condensate of human subjects with respiratory distress syndrome (91). Increased levels of exhaled peroxide have been found in various respiratory disorders such as asthma, COPD, ARDS, smokers, and in bronchiectasis, mainly in adults (92-95). Therefore, exhaled H_2O_2 is a promising noninvasive marker of airway inflammation and, more specifically, oxidative stress.

1.3.3 OTHER EXHALED INFLAMMATORY MARKERS

NO is not the only inflammation marker that can be measured in exhaled air. However, data of those other exhaled inflammatory markers remains scanty, especially in children. Carbon monoxide (CO), a product of heme degradation by heme oxygenase 1 (OH-1) which is induced by inflammatory cytokines and oxidants, has been put forward as a potential marker of airway inflammation and oxidative stress. Increased levels of exhaled CO have recently been demonstrated in adult asthmatics as well as in asthmatic children (96,97). The value of exhaled CO in the assessment of asthmatic airway inflammation has not yet been established. A recent study showed no difference in exhaled CO in asthmatics and healthy controls (98). Elevated exhaled CO values are not specific for asthma because they are also found in association with various respiratory disorders such as CF, bronchiectasis, smoking and viral upper respiratory tract infections (99-102). A limited number of studies have evaluated exhaled volatile alkanes like pentane and ethane, products of lipid peroxidation, as markers of oxidative stress and airway inflammation, by means of gas chromatography (103). In adults, elevated levels of exhaled alkanes were found in smokers, asthmatics and patients with obstructive sleep apnea (104-106). In preterm infants pentane exhalation was related to the course of neonatal disease in a very limited number of infants (107).

Besides H_2O_2 other potential markers of airway inflammation can also be detected in breath condensate of adults and children, including oxidation metabolites of NO, nitrite and nitrate. Elevated levels have been shown in CF patients, even if eNO is not elevated in the gas phase, and in asthmatic patients (108,109). Exhaled air analysis of 8-isoprostane, is a new noninvasive approach to study oxidative stress in airway inflammatory diseases. Isoprostanes are free radical-catalyzed products of arachidonic acid oxidation that may reflect oxidative stress in the respiratory tract. Recently, isoprostane has been found to be increased in exhaled condensate in asthmatic subjects, CF and in adult respiratory distress syndrome (110-112). Exhaled isoprostanes have not yet been studied in pediatric respiratory disease. There are also a few reports of measurements of leukotrienes (LTs) (the chemotactic LTB_4 and the cysteinyl LTs, LTC_4, D_4 and E_4) and cytokines (TNF-α) in exhaled air condensate of adults (45,113). There is a lack of data relating to children, and further information is needed on normal values, reproducibility and validation of these potential markers.

1.4 AIMS OF THE STUDIES AND OUTLINE OF THIS THESIS

At the moment, there is no specific, noninvasive and objective method for the assessment of airway inflammation in children. The aim of the studies

was to develop noninvasive methods to assess airway inflammation in children. For this purpose, two approaches have been followed:
1.   Detection of NO in exhaled air of children.
2.   Detection of H_2O_2 in breath condensate of children.
In the studies in this thesis, eNO and H_2O_2 in breath condensate were measured, by means of chemiluminescence and fluorimetry, respectively, in children with asthma, cystic fibrosis and in healthy controls with and without upper respiratory tract infections. We hypothesized that those markers could be measured reproducibly and are sensitive enough to discriminate healthy children from those with airway inflammation and reflect the severity of the inflammatory process. This could be valuable in the early diagnosis and monitoring of airway inflammation in children, and could potentially be applied to guide anti-inflammatory drug treatment.

Values of exhaled NO as initially reported by various investigators differed greatly in similar patient groups and even in healthy subjects. This can be explained by differences in measurement techniques. Therefore, we first compared different methodological aspects of NO measurement in stable asthmatic and healthy children, and assessed the reproducibility of NO measurements (**chapter 2**).
Since NO continuously diffuses into the airway lumen, high concentrations will result when exhalation is slow, and low concentrations when exhalation is fast. It is unknown to what extent disease affects the flow-dependency of NO in exhaled air. Therefore, we examined the effect of varying the exhalation flow on the NO concentration in exhaled air of children, and examined the concentration of NO and the NO/flow relationship in healthy children, asthmatic children and children with CF (**chapter 3**).
In an attempt to standardize eNO measurements a European Respiratory Society (ERS) task force group recommended on-line eNO measurement. However, this method is, in contrast to off-line eNO measurement, rather difficult to perform on children. In **chapter 4** we established a reference range of eNO, obtained with the child friendly off-line sampling balloon method, in a large group of healthy school children and examined the influence of ambient NO, noseclip and breathholding on eNO.
The objective of the study in **chapter 5** was to validate an alternative off-line single breath, low flow eNO sampling method against on-line sampling according to ERS guidelines, in children old enough to perform both sampling methods and to establish a reference range of low flow off-line eNO measurement in healthy children.
In **chapter 6** reference values of H_2O_2 in exhaled air condensate in a large group of healthy school-aged children were established as well as the reproducibility of

repeated measurements and the stability of peroxide in the frozen condensate over a period of time.

In **chapter 7** we compared the H_2O_2 concentration in exhaled air condensate of stable asthmatic children versus healthy controls and determined the influence of inhaled steroids on exhaled H_2O_2 in the asthmatic study population.

As CF-patients characteristically have severe chronic airway inflammation due to bacterial infection, noninvasive markers of airway inflammation could be useful to guide anti-inflammatory treatment in CF-subjects. The objective of the study described in **chapter 8** was to assess whether serial measurement of exhaled H_2O_2 and NO can serve to monitor the anti-inflammatory effect of treatment with antibiotics in CF-children with acute infective pulmonary exacerbations. Exhaled H_2O_2 is elevated in various inflammatory disorders of the lower respiratory tract. However, exhaled H_2O_2 can potentially be confounded by inflammation of the upper airways. In **chapter 9** we studied whether upper respiratory tract infections influenced the concentration of H_2O_2 in exhaled air condensate of healthy subjects.

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EXHALED NITRIC OXIDE
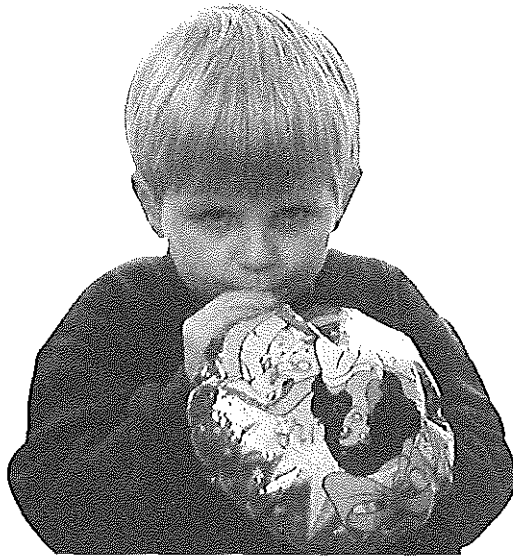
Off-line collection of exhaled NO (balloon method)

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CHAPTER 2

Sampling of exhaled nitric oxide in children: end-expiratory plateau, balloon and tidal breathing methods compared

Q. Jöbsis, S.L. Schellekens, A. Kroesbergen, W.C.J. Hop[1], J.C. de Jongste

Erasmus University Medical Centre/Sophia Children's Hospital, Department of Paediatrics, Division of Paediatric Respiratory Medicine and Department of Biostatistics[1], Dr. Molewaterplein 60, 3015 GJ Rotterdam, The Netherlands

ABSTRACT

The aim of this study was to compare exhaled nitric oxide (NO) concentrations obtained during controlled slow exhalation, presently considered as the method of choice, with two sampling methods that are easily performed by children: blowing air into a balloon and tidal breathing through a mouthpiece. One hundred and one well controlled, stable allergic asthmatic children (median age 11.7 yrs) performed the following tasks in duplicate: 1) exhalation from total lung capacity (TLC) through a mouthpiece against a resistor with a standardized flow rate of 20% of the subject's vital capacity per second, using a biofeedback system; 2) a single deep exhalation into an NO-impermeable Mylar balloon; and 3) tidal breathing through a low resistance mouthpiece over 2 minutes. NO was measured using a chemiluminescence analyser. Twenty-nine children (29%) were not able to perform a constant-flow exhalation of at least 3 sec. All children performed the balloon and tidal breathing methods without difficulty. NO concentrations (means±SEM) were 5.3±0.2 parts per billion (ppb) at the end-expiratory plateau, 5.2±0.3 ppb in balloons (intraclass correlation coëfficient (r_i)=0.73) and 8.0±0.4 ppb during tidal breathing (p<0.001, r_i= 0,53 compared to plateau values). Mean values of NO during tidal breathing increased significantly with time, suggesting increasing contamination with nasal air. It was concluded that, in asthmatic children, the end-expiratory plateau concentration of NO during exhalation at 20% of the vital capacity per second is similar to values obtained with the balloon method, with satisfactory agreement, but differs from values obtained during tidal breathing. The balloon method is cheap, simple and offers the interesting possibility to study exhaled NO in young children independently of the presence of an NO-analyser.

INTRODUCTION

Invasive procedures such as bronchoscopy and bronchoalveolar lavage (BAL) have greatly enhanced the understanding of the role of inflammation in asthma (1-3). In young children, BAL is generally not acceptable for research, diagnosis and monitoring purposes. A noninvasive method to assess the presence and severity of airway inflammation is important for this group (4). Gustafsson et al. demonstrated that nitric oxide (NO) can be detected in exhaled air of animals and humans (5). NO is produced by various cells within the respiratory tract, and plays an important role in the pathophysiology of inflammatory airway disease (6,7). In recent years, the measurement of NO in exhaled air has been studied for its potential as a marker of airway inflammation in both diagnosis and management. Increased amounts of NO have been detected in the exhaled air in humans with various inflammatory airway disorders (8-12). There is persuasive evidence that levels of NO are decreased by anti-inflammatory therapy (13-16). However, different groups have reported widely varying levels of exhaled NO in similar patient groups and in healthy subjects, probably owing to differences in methodology (17,18). In an attempt to standardize NO measurement in exhaled air a European Respiratory Society (ERS) task force report was recently published (18). In adults and children (from the age of 6 yrs), a slow exhalation from total lung capacity (TLC) through a mouthpiece with a constant flow against a resistance was recommended for the measurement of NO in exhaled air. It appears, however, that children may have difficulty maintaining a fixed flow. As recommended in the ERS task force report, a balloon method, a simple technique which is easily performed by children, was used in this study to measure exhaled NO. Others have proposed a tidal breathing method with measurement of NO in the mixed expired air during resting ventilation, with or without a fixed resistor in the expiration circuit (8,19,20). Comparison between these different sampling methods in subjects is needed. The aim of this study was to compare in subjects: 1) single exhalation from TLC with a constant flow against a resistance; 2) single uncontrolled deep expiration into a balloon; and 3) continous sampling of exhaled air during tidal breathing without expiratory resistor. Furthermore, the short term reproducibility of NO measurements in exhaled air with the different sampling methods was assessed.

PATIENTS AND METHODS

STUDY POPULATION

One hunderd and one stable asthmatic children (67 males and 34 females) attending the outpatient clinic for pediatric respiratory medicine at Sophia Chil-

dren's Hospital were included. Their median age was 11.7 years (range 7.0-17.6). Asthma was diagnosed on clinical grounds and according to international guidelines (21). All had bronchial hyperresponsiveness (a provocative dose of inhaled methacholine that produced a 20% fall in forced expiratory volume in one second (PD20) of <150 µg) documented in the past, and had allergy as documented by a radio allergosorbent test (RAST) class 2 or higher for at least one common airborne allergen. Appropriate therapy was prescribed by the patient's own physician, and had not been changed during the 3 months preceding the study. All subjects used an inhaled bronchodilator on demand, 90 (89%, 60 males and 30 females) used an inhaled corticosteroid (mean daily dose 400 µg budesonide or beclomethasone, range 100–1500 µg), and 23 used long-acting beta-agonists. All were lifelong nonsmokers and were clinically stable. None of the subjects reported symptoms of acute respiratory infection within the month before NO was measured. The study was approved by the medical ethics committee of the university hospital.

LUNG FUNCTION

All subjects underwent flow-volume measurements immediately before NO was measured. Flow-volume curves were obtained in triplicate, using a Lilly-type pneumotachograph (Masterlab Jaeger, Würzberg, Germany). Results of forced expiratory volume in 1 second (FEV $_1$) and forced expiratory vital capacity (FVC) were expressed as percentage predicted (22).

EXHALED AIR SAMPLING AND NITRIC OXIDE MEASUREMENT

All subjects performed 3 different expiratory manoeuvres in duplicate, always in the same order.

A single slow exhalation: children were instructed to perform a slow exhalation from TLC through a mouthpiece and a two way non-rebreathing valve (Rudolph Inc., Kansas City, MO, USA) into a wide bore teflon tube against an inline resistor (20 cm H_2O/L/sec, Rudolph Inc.) with an individually standardized flow rate of 20% of the subject's vital capacity (VC) per second. A biofeedback exhalation flow display provided visual guidance to help the subject maintain their exhalation flow at the desired level. The mean end-expiratory NO level was measured in duplicate from air sampled from exhalation at 200 mL/min. via teflon tubing during an end-expiratory flow plateau of at least 3 sec.

One single deep expiration into a balloon: subjects were asked to take a deep breath, not necessarily to TLC, and to perform one single deep exhalation via a plastic tube (length 5 cm, internal diameter 3.7 mm) into an NO-impermeable Mylar balloon (maximum capacity 1750 mL). Flow and pressure were not monitored, but with balloon filling in 3-5 sec, to a volume of 1250-1750 mL, flow rates would range 250 and 600 mL/sec. Preliminary measurements showed that,

during balloon blowing, oral positive pressure was always >6 cm H_2O, sufficient to close the velum and prevent nasal contamination of exhaled air. Gas was sampled from the balloon within 15 min after filling. The mean NO concentration was measured during 30 sec of sampling from the balloon (at 200 mL/min). This manoeuvre was performed in duplicate.

Tidal breathing: subjects performed two minutes of quiet tidal breathing through a mouthpiece connected to a two way non-rebreathing valve into a wide bore low resistance teflon tube, where gas was continuously sampled via a side port at 200 mL/min. The mean NO value of the first minute and of the second minute were calculated by computer.

All measurements were performed with the children seated. All children were allowed 2 min rest between the different expiratory manoeuvres to restore resting conditions of ventilation. They did not wear a noseclip. To exclude the effect of high ambient NO levels to the level of exhaled NO, all tests were performed with ambient NO concentrations <10 parts per billion (ppb). Preliminary experiments demonstrated that NO concentrations in Mylar balloons remain stable for at least 6 h. NO was measured with a chemiluminescence analyser (Sievers 280, Boulder, CO, USA) with a sampling flow rate of 200 mL/min and a response time of 200 milliseconds. The analyser was regulary calibrated according to the manufacturer's guidelines, using certified NO gases (100 ppb and 9 ppm) and certified NO-free gas (HoekLoos, Barendrecht, The Netherlands).

DATA ANALYSIS

Data of NO concentration and flow rates were entered into a computer at a sampling rate of 20 Hz. Mean values of NO concentrations at selected time intervals were calculated afterwards by means of a custom data processing program. Data are presented as mean ± standard error (SEM). End-expiratory NO during exhalation from TLC with continuous flow was taken as the gold standard. The agreement of the balloon method and the tidal breathing method with end-expiratory levels was assessed according to Bland and Altman (23). As these plots indicated that the outcome variability increased with increasing NO levels, further analysis was carried out using logarithmically transformed data to stabilize variances. Mean data from the different methods were compared using the Student t-test for paired samples. To characterize the agreement between methods, intraclass correlation coefficients (r_i) were calculated (24). The same methods were used to analyse within-subject reproducibilities. A two tailed p-value <0.05 was considered significant.

RESULTS

Lung function measurements showed near-normal mean values (mean ±
SEM): FVC 103 ± 2% predicted, FEV_1 94 ± 2% pred. Twenty-nine children
(29%) (mean age 11.0 yrs, range 7.3-16.3 yrs) were not able to sustain a stable
end expiratory flow plateau of at least 3 sec. In contrast, all children performed
the balloon method and the tidal breathing method without any difficulty. In
the 72 stable asthmatic children (mean age 12.1 yrs, range 7-17.6 yrs) who per-
formed the three different procedures successfully, NO concentrations (median
(range)) were 5.0 (2.3-12.8) ppb at the end expiratory plateau; 4.8 (2.2-17.2)
ppb from balloons; and 7.8 (2.7-20.0) ppb during tidal breathing (p=0.43 and
p<0.001, respectively, compared to plateau values). The within-method
short-term reproducibility of duplicate NO values obtained at the
end-expiratory plateau and from balloons was excellent (r_i = 0.90 and 0.91, res-
pectively). The reproducibility of the tidal breathing method was less (r_i=0.85).
With tidal breathing, consistently higher mean NO concentrations were found
during the second minute than during the first minute (8.5 ppb versus 7.5 ppb),
the difference being dependent on mean NO concentration. The mean of all
individual coefficients of variation of all NO values was 7% for the
end-expiratory plateau, 7% for the balloon method, and 22% for the tidal breat-
hing method. Ninety percent of all within-subject coefficients of variation were
<15% for the end-expiratory plateau, <12 % for the balloon method, and <81%
for tidal breathing. Comparison of the average NO (duplicate measurements)
obtained at end-expiratory plateau and from balloons showed a statisfactory ag-
reement between the two sampling methods (r_i = 0.73) (figure 1), and this was
similar if the first NO measurements only were considered (r_i=0.72). The geo-
metric mean ratio of the NO values obtained at the end-expiratory plateaus and
with balloons was 1.05 ppb with 2.5 and 97.5 percentiles of 0.60 and 1.60 ppb,
respectively (figure 2), indicating no systematic error and good agreement. In
contrast, a poor agreement was found between the NO values obtained with
the tidal breathing method and at the end expiratory plateau, with r_i=0.52 for
duplicate measurements, and r_i=0.53 for single measurements (figure 3).
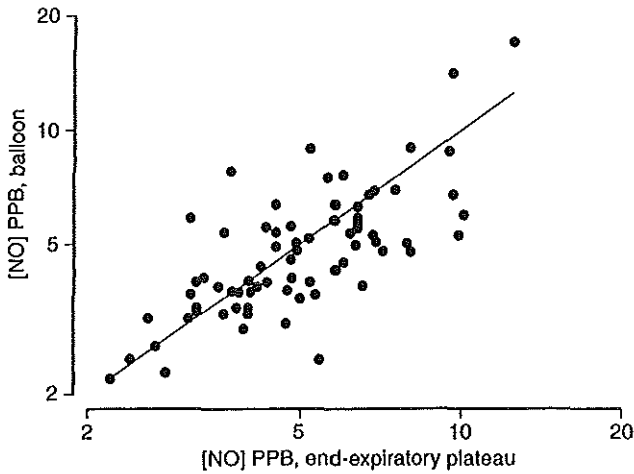
FIGURE 1. Comparison of exhaled NO concentrations obtained during a single exhalation at a fixed flow of 20% of vital capacity per sec (x-axis) and values measured in Mylar balloons filled in a single deep exhalation (y-axis). Data are from 72 out of 101 children who were capable of performing both methods. There is good agreement with an r_i of 0.73. The line represents line of identity. Note the logarithmic scale.

FIGURE 2. Ratios of exhaled NO concentrations obtained from Mylar balloons and at an end-expiratory plateau during exhalation with constant flow of 20% of vital capacity per sec (logarithmic scale), plotted against their means (x-axis). The average ratio is 1.05; horizontal lines indicate the 2.5 – 97.5 percentile range. A good agreement was noted with no systematic error. ppb: parts per billion.
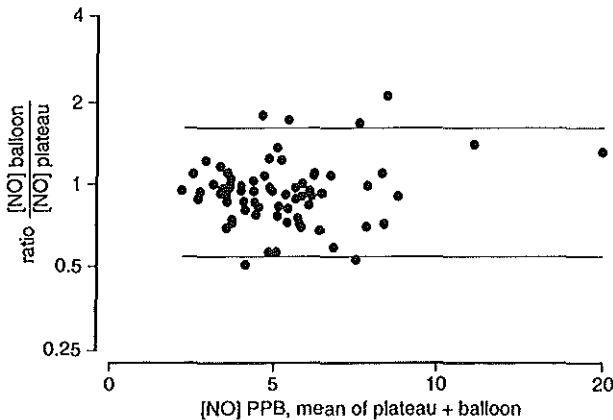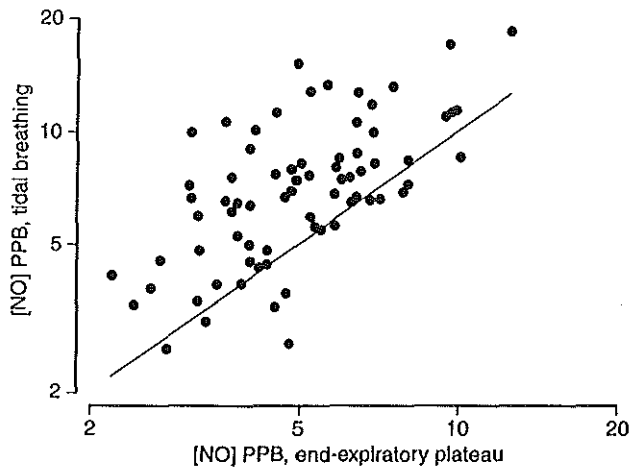
FIGURE 3. Comparison of exhaled NO concentrations obtained at the end-expiratory plateau during a single exhalation at a fixed flow of 20% of vital capacity per sec (x-axis) and the mean concentration of exhaled NO during 2 min of quiet tidal breathing (y-axis). Data are from 72 out of 101 children who could perform both methods satisfactorily. There is poor agreement with an r_i of 0.52; NO values during tidal breathing showed an increase over time. Note the logarithmic scale. ppb: parts per billion.

DISCUSSION

This is the first study in children that has compared different sampling methods for exhaled NO. Thirty percent of the children studied were not able to perform the recommended sampling procedure of single slow exhalation with a constant flow against a resistance. This underlines the importance of developing alternative methods for use in children. Until recently, measurement techniques for expired NO were not standardized. Values have been obtained for peak expired NO, end-expiratory plateau levels, mixed expired levels during tidal breathing and NO production over time. This study compared three different NO measurement methods in stable asthmatic children, using controlled exhalation with constant flow as a gold standard. Satisfactory agreement and no significant differences were found between the end-expiratory plateau value of NO during exhalation at 20% of VC/sec and NO values obtained from a balloon. However, there was a highly significant, time and concentration-dependent difference between NO values during tidal breathing and at the end-expiratory plateau, suggesting that sampling during tidal breathing without expiratory resistance has limited value in children.

Relatively low NO values were found in well controlled asthmatic children, most of whom used inhaled steroids. This is in agreement with a number of other studies showing that inhaled steroids normalize exhaled NO in asthmatics (11,13,15).

Regarding the feasibility of slow exhalation with constant flow, the present results are different from those of Balfour-Lynn et al. (25), who reported single slow exhalations against a resistance by 63 children with cystic fibrosis and 57 normal children aged 6-17 yrs. However, they did not control for the exhalation flow rate or pressure. As the concentration of NO depends on air flow (26,27), maintaining a constant expiratory flow is now considered important (18).

Other studies have measured NO in mixed exhaled air, collected in a reservoir during tidal breathing (19,20). In the present study, mean exhaled NO values obtained with the tidal breathing method were significantly higher than those obtained with the other two methods and increased with time. This could well be due to contamination from nasal NO. During tidal breathing, NO may diffuse from the upper airways and contaminate orally exhaled air, as the soft palate opens during inspiration (26,28). Although contamination of orally exhaled NO by nasal NO can be prevented by single exhalations against a resistance (26,29), an expiratory resistor was not used during tidal breathing. The palate will open during tidal inspiration regardless, thereby allowing for contamination with nasal NO.

No significant differences, and a good agreement were found between [NO] in balloons and [NO] during controlled single exhalations. Balloon filling through the resistor tube produced at least 6 cm of H_2O positive pressure in the mouth, which will close the soft palate. This has been previously demonstrated in studies using Argon gas, a tracer applied to the nose that was not retrieved in either the exhaled air or in balloons (30,31).

The balloon method collects air not only from the alveolar compartment, but also from the dead space. The potential effect of the dead space on the NO concentration in the balloon is a point of concern, as the concentration of NO in the dead space may be relatively high, causing an initial NO peak during slow exhalations. However, no systematic difference was found between the balloon method and single exhalations with constant flow. This may partly be explained by the fact that children have a smaller dead space than adults which, with a similar balloon volume, will have a smaller effect on mixed NO values. To prevent accumulation of NO in the airways and nasal NO entering the dead space compartment, care was taken that the NO in the ambient air was kept <10 ppb, that children inhaled through the mouth, and exhaled immediately without breath holding. Another point of concern in the balloon method is the lack of flow standardization. However, the short-term reproducibility of NO data obtained with the balloon method appeared excellent, and corresponded well with end-expiratory plateau

NO levels during exhalation at a constant flow. This may be explained by the relatively high flow rate used in both methods; it is evident that flow dependency is most important with very low flow rates of approximately ≤ 200 mL/min (26), whereas dependency is much less at higher flow rates such as that employed in the present study. It may be advantageous to adapt the balloon method for lower, controlled flow rates, although this would require a more complicated system and better cooperation and skills from the children (31,32).

Recently, Deykin et al. demonstrated that repeated spirometry may induce a slow onset, prolonged fall in exhaled NO (33). In the present study spirometry was performed immediately before NO sampling and may have introduced a bias. However, all measurements were always performed in the same order, mostly within 15 min, whereas the maximal fall in NO after spirometry reported by Deykin et al. (33) occurred after 30 min; furthermore, an excellent within-method short-term reproducibility of single exhalations and balloons, without a downward trend, was found in the present study. Therefore, it is believed that the possible systematic error induced by the spirometry does not invalidate the results from this study.

In conclusion, a simple and reproducible method of measuring exhaled NO in balloons was described, producing results that are similar to the values obtained at an end-expiratory plateau. The balloon method is feasible in young children and has the additional advantage that it can be carried out independent of the presence of an NO analyser.

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CHAPTER 3

Flow-dependency of exhaled nitric oxide in children with asthma and cystic fibrosis

A. Kroesbergen, Q. Jöbsis MD, E.H.D. Bel MD[1], W.C.J. Hop PhD[2], and
J.C. de Jongste MD PhD

Erasmus Medical Center/Sophia Children's Hospital, Department of Paediatrics,
Division of Paediatric Respiratory Medicine and [2]Epidemiology and Biostatistics,
Dr. Molewaterplein 60, 3015 GJ Rotterdam, The Netherlands; [1]Department of
Pulmonology, Academic Hospital Leiden, Albinusdreef 2, 2333 ZA Leiden, The
Netherlands

ABSTRACT

The concentration of nitrc oxide (NO) in exhaled air, a marker of airway inflam-
mation, depends critically on the flow of exhalation. Therefore, the aim of this
study was to determine the effect of varying the flow on end-expiratory NO
concentration and NO output in children with asthma or cystic fibrosis (CF) and
in healthy children. Nineteen children with stable asthma, 10 with CF, and 20
healthy children exhaled from TLC while controlling expiratory flow by means
of a biofeedback signal at approximately 2, 5, 10 and 20% of their vital capac-
ity per second. NO was measured in exhaled air with a chemiluminescence
analyser. Comparisons between the three groups were made by analysing the
NO concentration at the endexpiratory plateau and by calculating NO output at
different flows. Exhaled NO decreased with increasing flow in all children.
Children with asthma had significantly higher NO concentrations than healthy
children, but only at the lowest flows. Asthmatics using inhaled steroids
(n=13) tended to have lower median exhaled NO than those without steroids.
The slope of linearized (log-log transformed) NO/flow plots was significantly
steeper in asthmatics than in healthy controls. CF patients had a significantly
lower NO concentration and output over the entire flow range studied, com-
pared to asthmatics and control subjects, with a similar NO/flow slope as con-
trol subjects. In conclusion, the NO concentration in exhaled air is highly
flow-dependent, and the NO-flow relationship differs between asthmatics ver-
sus CF patients and control subjects. Assessment of the NO/flow relationship
may help in separating asthmatics from normal children.

INTRODUCTION

Nitric oxide (NO) has an important regulatory role in the lung and has been implicated in the pathophysiology of airway diseases (1). During the past few years, NO in exhaled air has been examined as a marker of airway inflammation. NO can be measured directly in exhaled air (2); increased levels have been documented in several inflammatory airway disorders, including asthma. However, patients with cystic fibrosis (CF) were found to have similar or lower levels compared to normal subjects, either in nasal or in oral air (3,4). Both an increase and a decrease in the level of NO in exhaled air may therefore be taken as potential markers of disease and exhaled NO may be clinically useful in diagnosing and monitoring airway diseases. Values of exhaled NO reported by various investigators differ greatly (5). This can be explained by differences in the methods of measurement. NO in exhaled air depends on airflow (6). Since NO is continuously released into the airway lumen, high concentrations will result when exhalation is slow, and low concentrations when exhalation is fast. It is unknown to what extent disease affects the flow-dependency of NO in exhaled air. Recently, a European Respiratory Society task force published guidelines on measurement of exhaled NO, emphasizing that normalization for flow is vital (7). Until now, studies on exhaled NO in children did not take flow-dependency into account. Therefore, the aim of this study was to determine the effect of varying the flow of exhalation on the NO concentration in exhaled air, and to examine the concentration of NO and the NO/flow relationship in normal subjects, asthmatic children and children with CF.

PATIENTS AND METHODS

STUDY POPULATION
Patients were recruited from the outpatient clinic for pediatric respiratory medicine of Sophia Children's Hospital, and healthy control subjects were selected from a school population according to the following criteria. Asthma was diagnosed according to international guidelines, based on a typical clinical history (8). In addition, all asthmatic children had bronchial hyperresponsiveness (provocative dose of inhaled methacholine that produced a 20% fall in forced expiratory volume in 1 sec of <150 μg) and allergy, as previously documented by radio allergosorbent test class ≥2 for at least one common airborne allergen. All asthmatics were clinically stable for at least 2 weeks before the measurement, and all were lifelong nonsmokers. Thirteen used inhaled corticosteroids (median daily dose 400 μg, range 100–1000 μg/day), all used bronchodilators on demand. CF was diagnosed on the basis of typical clinical symptoms,

abnormal sweat test and the identification of 2 CF mutations. All CF patients had been clinically stable for at least 2 weeks prior to the measurements. One CF patient used inhaled corticosteroids. Healthy children were selected on the basis of no history of allergic respiratory disease and eczema, as defined by negative answers to the International Study on Allergies and Asthma in Children (ISAAC) core questionnaires, no symptoms of respiratory infection during the past 2 weeks, never smoked and had no other chronic disease. All had a normal lung function. None used medication. All subjects underwent flow volume curve measurements, using a heated Lilly-type pneumotachograph (Jaeger, Würzburg, Germany); results were expressed as per cent predicted (9). The study was approved by the hospital ethics committee and informed consent was obtained from all subjects.

NITRIC OXIDE MEASUREMENT

NO was measured in exhaled air with a Sievers 280 chemiluminescence analyser (Sievers, Boulder, CO, USA) with a sensitivity of < 0.1 parts per billion (ppb) and a detection range of <0.1-500,000 ppb. The sampling flow was 0.2 L/min, the response time was 0.2 sec and data were displayed with a lag time of approximately 2 sec. The analyser was calibrated regularly according to the manufacturer's guidelines, employing certified calibration gases containing 0 ppb, 100 ppb and 9 parts per million (ppm) NO (HoekLoos, Barendrecht, the Netherlands). The measurement circuit consisted of a mouthpiece connected to a two-way non rebreathing valve (Rudolph Inc.,MO,USA) through which the subjects inhaled ambient air while seated and without wearing a noseclip. After inserting the mouthpiece the subjects inhaled to total lung capacity (TLC) and immediately exhaled for as long as possible into a wide bore tube, with an in-line flow resistance (model #7100 R-20, 20 cm H_2O/L/sec, Rudolph Inc.). This was performed at flows corresponding to 2, 5,10 and 20% of the subject's vital capacity (VC) per second. A fine bore teflon tube continuously sampled the exhaled air from a side-port directly after the mouthpiece at 0.20 L/min for the measurement of NO. Airflow was measured by a heated Lilly-type pneumotachograph (Jaeger), mounted after the resistance. Flow was displayed on a video screen as a moving block that could be seen by the subject and should be kept between two arrows. This biofeedback system made it possible to standardize exhaled flow to an individually set value. An end-expiratory flow-plateau of at least 3 s for the different flows (2 s for the highest flow) was the end-point of the measurement. A plateau was defined as a flow tracing which varies less than ±10% around the target flow. The test was performed in triplicate for every flow and average NO values at the flow plateau were calculated. Between the different manoevres, a resting period of 2-3 min was maintained for equilibrati-

on of resting ventilatory conditions. All tests were performed with ambient NO concentrations <5 ppb.

DATA ANALYSIS

Both the NO signal and the flow signal were fed into a computer at a sampling rate of 20 Hz. Values of NO and flow were obtained at each attempt by calculating the mean value in defined time intervals of 3 or 2 seconds, using custom-made software, and individual means were calculated from triplicate NO and flow (L/sec) values. NO output, defined as the product of mean NO and flow (L/sec), was calculated for each target flow, and expressed in nL/min. NO concentrations and NO output values were lognormally distributed and compared for each of four target flows between patient groups and healthy control subjects by means of the Mann-Whitney test. Plots of NO versus flow in L/sec were linearized by log-log transformation, and regression analysis for repeated measurements was used to compare NO/flow slopes for the 3 groups (10). The effect of normalizing flow for VC was evaluated by plotting individual NO values versus flow, either expressed as %VC/sec or as mL/sec, in log-log normalized plots. Standard deviations of the intercepts of individual regression lines in both types of NO/flow plots at an arbitrary flow of log(7%VC/sec) and log (300 mL/sec) were compared. The level of significance was set at p= 0.05 (two sided).

RESULTS

Patient characteristics are given in Table 1. The measurements were well tolerated. Two or three reproducible NO values for each flow rate were always obtained within five attempts. The NO level at the end-expiratory plateau decreased with increasing flows in all children. Median values of NO concentration and NO output, as well as the median absolute flows at which these were obtained, are given in table 2. Individual NO-flow relationships for the three groups are shown in figure 1. Asthmatics had higher median NO concentrations than control subjects, but a significant difference was only present at the lowest end of the flow spectrum. Likewise, median NO output was higher in asthmatics than in control subjects at the lower end of the flow range, but the differences were not significant. Strikingly, the variation in exhaled NO was much larger in asthmatics than in healthy children. Median NO values were higher in asthmatics without corticosteroids than in those with steroids (12.1, 6.1, 4.2 and 2.7 ppb versus 16.7, 17.1, 11.1, and 6.8 ppb at a flows of 2, 5, 10 and 20% of VC, respectively), but none of the differences reached statistical significance. CF patients had a significantly lower median NO concentration and output

TABLE 1. Patient characteristics

	Healthy controls	Asthmatic children	CF patients
Number	20	19	10
Sex (m/f)	12/8	9/10	5/5
Age (months)	165 ± 10 (149-180)	154 ± 22 (116-198)	143 ± 22 (114-183)
FVC %	101 ± 12 (82-122)	101 ± 12 (76-121)	94 ± 13 (78-126)
FEV$_1$ %	103 ± 12 (85-126)	88 ± 16 (54-116)	84 ± 17 (52-110)

Data are mean ± standard deviation (ranges). CF: cystic fibrosis; m: male; f: female.

TABLE 2. Exhaled NO in children with and without asthma or cystic fibrosis

	Healthy controls (n=20)	Asthmatic children (n=19)	CF patients (n=10)
Exhaled NO (ppb)			
at 2 %VC/sec	8.8 (5.4-23.6)	12.1 (1.7-75.1)*	3.5 (0.4-9.9)**
at 5 %VC/sec	5.9 (3.6-13.9)	6.4 (1.0-36.8)	1.9 (0.6-4.3)**
at 10 %VC/sec	4.8 (2.6-10.0)	4.6 (1.1-21.3)	1.5 (0.2-3.6)**
at 20 %VC/sec	4.4 (3.2-6.6)	3.2 (0.9-18.5)	1.4 (0.4-2.6)**
NO output (nL/min)			
at 2 %VC/sec	39 (17-107)	64 (6-271)	11 (1-35)**
at 5 %VC/sec	65 (35-195)	72 (7-320)	15 (3-40)***
at 10 %VC/sec	102 (60-241)	109 (15-357)	27 (5-45)***
at 20 %VC/sec	190 (113-368)	151 (24-650)	43 (16-83)***
Actual flows (mL/sec)			
at 2 %VC/sec	70 (40-120)	60 (30-300)	52 (30-200)
at 5 %VC/sec	170 (100-300)	150 (70-270)	123 (90-210)
at 10 %VC/sec	341 (200-610)	283 (160-540)	272 (160-410)
at 20 %VC/sec	716 (420-1210)	570 (340-1070)	556 (360-740)

All data are median (range). *: p<0.05; **: p<0.001, ***: p<0.0001 compared to control subjects and asthmatics.

than healthy control subjects and asthmatics over the entire flow range tested. Double logarithmic transformation of flow and NO resulted in a linear NO/flow relationship for each individual in all three groups within the flow range studied (figure 1). Using regression analysis of log NO versus log flow, with flow expressed as mL/sec, it appeared that the average decrease of NO per doubling of flow in asthmatics was 33% (95% confidence interval (CI) 29-37%). For healthy children and CF patients, these figures were 21% (95% CI 18-24%),
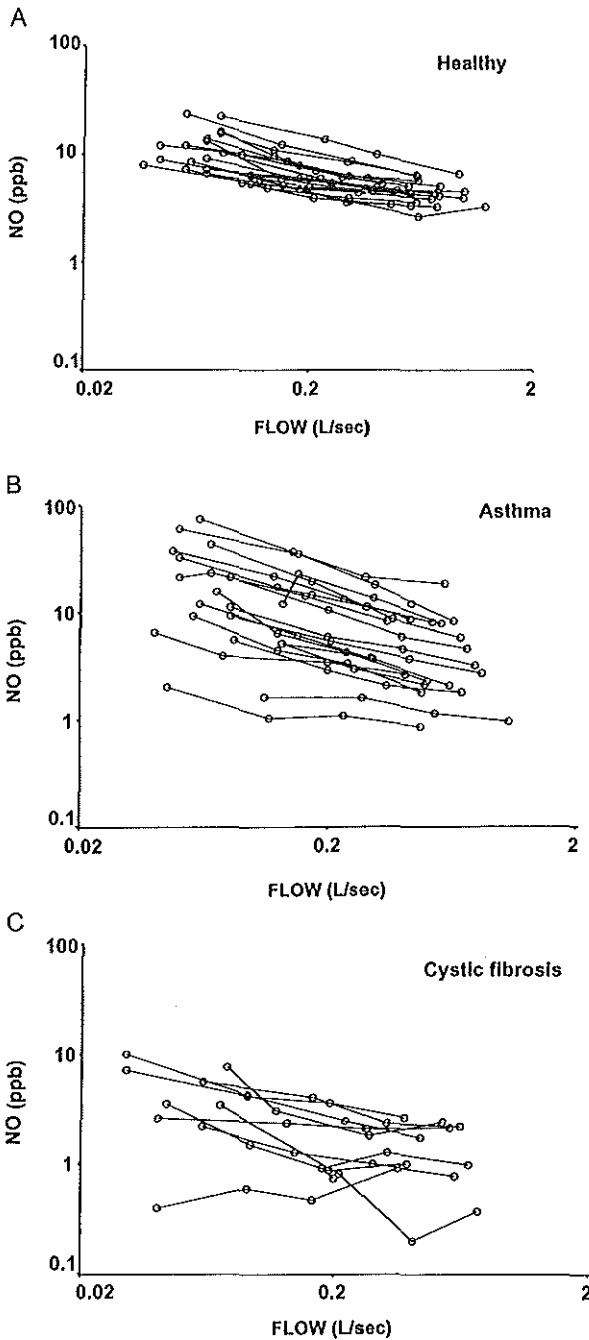
FIGURE 1A, B, C. Exhaled NO versus flow end-expiratory plateaus in A) healthy children (n=20), B) children with asthma (n=19) and C) children with cystic fibrosis (n=10). Each line represents one child. Both exhaled NO and end-expiratory flow were measured on a logarithmic scale. ppb:parts per billion.
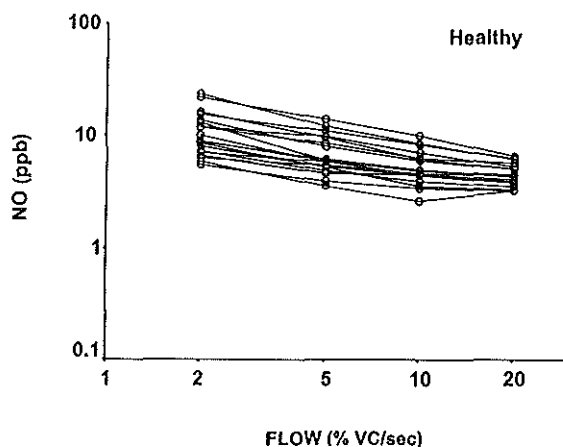
FIGURE 2. Log-log transformed plots of exhaled NO of 20 healthy children versus flow normalized as a percentage of the patients' vital capacity (VC) per sec (X-axis). Comparison with fig. 1A shows that normalization of flow for lung size did not result in a reduced variation, confirmed by statistical analysis, showing a similar standard deviation of the intercepts of individual regression lines at arbitrary flows of 7% of VC and 300 mL/sec (see Results section).

and 24% (95% CI 10-36%), respectively. The slope of NO versus flow was significantly steeper for the asthmatic patients than for the control subjects (p=0.003). These results were the same, irrespective of whether flow was expressed as mL/sec or as %VC/sec.

For each group, whether baseline lung function influenced the outcomes was investigated. It appeared that the NO concentration, -output and slopes were not significantly affected by either forced expiratory volume in one second (FEV_1) or forced vital capacity (FVC). The asthmatic patients who used steroids (n=13) had NO levels that were slightly lower, but not significantly different from those without steroids. Within groups, age did not correlate with exhaled NO values.

Normalizing flow for lung and airway size by expressing it as a percentage of VC/sec instead of mL/sec did not reduce the standard deviation of the intercepts of the individual regression lines at an arbitrary flow of 7%VC/sec or 300 mL/sec within groups (fig. 2).

DISCUSSION

The present study confirms that the NO concentration in exhaled air rises with decreasing flow, and shows that the NO/flow relationship differs between children with asthma on the one hand, and CF patients and healthy children on the other hand. The clinical implication of these results is that the measurement of NO clearly distinguished between the different disease states at low flow rates, and that not only NO concentration at a fixed flow rate, but also the slope of the NO/flow relationship discriminates between asthmatics and healthy subjects or CF patients.

This study is the first to quantify the effect of varying the flow rate on the NO concentration in exhaled air in children and to establish the effect of different disease states on the NO/flow relationship. Silkoff et al.(6) recruited a group of healthy adult volunteers (age 16-50 yrs) for their study of the flow–dependency of end-expiratory NO in exhaled air. Sato et al.(11) studied the effect of the duration of exhalation on the NO levels in exhaled air in healthy and asthmatic adults. Exhaled air was collected in a reservoir and the subjects wore a noseclip. Hence, not only the plateau level of NO, but also the initial peak level, and probably contamination with nasal air contributed to the results of this study. Byrnes et al. (12) studied the mean peak concentration of NO at different flow rates in adults. However, peak NO concentrations reflect the NO concentration in the anatomical dead space, contaminated by nasal NO; noseclips may have enhanced nasal contamination. The increase of NO in exhaled air with decreasing flow observed in the current study matches the results by Silkoff et al. (6) and Byrnes et al. (12), and confirms that the NO levels in exhaled air in well-controlled asthmatics are only slightly higher than in healthy subjects.

The results of the present study might have been biased by methodological aspects. Firstly, it could be argued that the flow resistance used was not high enough to close the soft palate, leading to nasal contamination. In a study in adults, no contamination of NO from the nasal cavity was observed with a flow resistance of at least 3 cm H_2O/L/sec (13). No similar studies have been published for children. The flow resistance used in the current study was 20 cm H_2O/L/sec, and it is therefore likely that nasal contamination of the NO concentration in exhaled air was effectively prevented at least at flow rates >150 mL/sec, corresponding to oral pressures >3 cm H_2O/L/sec. However, the mean flows at 2%, and sometimes at 5% of VC/sec were below this value, and therefore some nasal contamination at the lowest flows cannot be excluded. This has not necessarily introduced a bias, as it would affect results in asthmatic and control subjects similarly.

Secondly, the study assessed whether normalizing expiration flow as a percentage of the patient's VC has advantages above comparing fixed flows, which do not

take differences in lung and airway size into account. The results showed that normalization of expiration flow for the VC is not very important when comparing exhaled NO in children of different sizes. It may still be that in larger groups within-group variation is smaller with measurement at a lung size-dependent flow, but this remains to be shown. Alternatively, standardizing for the lung volume at which NO is measured may increase the accuracy of NO measurements. However, the present study did not explicitly define the lung volume at which results were obtained.

A final source of error may be the repeated performance of expiratory manoevres. All children performed a number of deep inspirations and expirations and, since this study, it has been shown that forced manoevres cause a decreased NO output (14). The NO levels may therefore have been underestimated. It is thought that such an effect may have been small, as no decreasing trend was obvious for the repeated measurements at a given target flow.

The group of children with asthma had a higher median level of NO in exhaled air, which may be due to residual chronic airway inflammation despite steroid treatment, and to the fact that a number of children did not use steroids and, indeed, tended to have higher NO values than those who did.

CF patients have lower exhaled NO than healthy subjects. As inflammation is invariably present in CF airways (15), it has been speculated that the increased amount of thick bronchial mucus in patients with CF may favour retention and metabolism of NO in airway liquids (4). That increased NO production takes place in CF airways is supported by data demonstrating increased nitrite levels in breath condensate, despite normal NO in exhaled air, in a group of older CF patients (16). The results of the present study confirm that increased NO levels in inflamed CF airways are not reflected by an increase in exhaled NO (3,4,16). In contrast to earlier reports, significantly lower orally exhaled NO values in CF than in control subjects were found. This may partly be due to important differences in methodology: no expiratory resistance (4), tidal breathing versus single controlled expiration (3) and older, more severe patients (16) in previous studies.

In conclusion, this study shows that differences in exhaled NO between asthmatics, CF patients and healthy children are greatest at low flows; the linearized NO/flow relationship is steepest in asthmatics. These results indicate that accurate standardization of low flow rates is necessary; a convenient target flow could be 100 mL/sec. For firm recommendations, larger groups should be studied and a cutoff level determined for a given purpose, e.g. detection of disease. For diagnostic purposes in children, NO measurements in exhaled air at different flow rates may prove an additional new tool for the differential diagnosis of airway diseases, especially asthma and cystic fibrosis.

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CHAPTER 4

Off-line sampling of exhaled air for nitric oxide measurement in children: methodological aspects

Q. Jöbsis[1,3], S.L. Schellekens[1], A. Kroesbergen[1] W.C.J. Hop[2] and J.C. de Jongste[1]

[1] Department of Paediatrics, division Paediatric Respiratory Medicine, and
[2] Department of Epidemiology and Biostatistics, Erasmus University and University Hospital/Sophia Children's Hospital, Rotterdam and [3] Dept. of Paediatrics, University Hospital Maastricht, The Netherlands

ABSTRACT

Measurement of nitric oxide (NO) in exhaled air is a non-invasive method to as-sess airway inflammation in asthma.This study was undertaken to establish the reference range of exhaled NO in healthy school-aged children and to deter-mine the influence of ambient NO, noseclip and breathholding on exhaled NO using an off-line balloon sampling method. All children attending a primary school (age range 8-13 years) underwent NO measurements on two occasions with high and low ambient NO. Each time, the children performed 4 expiratory manoeuvres into NO-impermeable balloons, with and without 10 secs of breathholding and with and without noseclip. Exhalation flow and pressure were not controlled. NO was measured within 4 h after collection, by means of chemiluminescence. All children completed a questionnaire on respiratory and allergic disorders, and performed flow-volume curves. With low ambient NO, the mean exhaled NO value of 72 healthy children with negative questionnaires and normal lung function was 5.1 ± 0.2 ppb, versus a mean of 6.8 ± 0.3 ppb in the remaining 49 children with positive questionnaires for asthma and allergy, and/or recent symptoms of cold (p=0.001). Exhaled and ambient NO were sig-nificantly related, especially with ambient NO > 10 ppb (r=0.86, p=0.0001 ver-sus r=0.34, p=0.004 for ambient values < 10 ppb). The use of a noseclip, with low ambient NO and without breathholding, caused a small decrease in exhaled NO values (p=0.001). The effect of breathholding on exhaled NO depended on ambient NO. With ambient NO > 10 ppb exhaled NO decreased, whereas with ambient NO < 10 ppb, exhaled NO increased after 10 sec breathhold. We con-clude that off-line sampling in balloons is a simple and, hence, attractive method for exhaled NO measurements in children, which differentiates be-tween groups with and without self-reported asthma, allergy and colds, when ambient NO is < 10 ppb. Wearing a noseclip and breathholding affect measured values and should therefore be standardized or, preferably, avoided.

INTRODUCTION

Nitric oxide is a reactive free radical gas, produced by various cells within the respiratory tract. It plays an important role in the pathophysiology of inflammatory airway disease and can be detected in exhaled air (1-4). There is now convincing evidence that NO in exhaled air (eNO) is a non-invasive marker of airway inflammation in various airway disorders (1,5,6). Recently, slow exhalation from TLC with a constant flow against a resistance was recommended for measurement of eNO (6). However, this manoeuvre is difficult to perform for many children (7), and requires the presence of an NO-analyser and equipment to measure flow with a visual feedback control. We have developed a simple off-line single breath balloon sampling method to measure eNO. Blowing air in a balloon is feasible even for young children, and produces values similar to those of end-expiratory plateaus (7,8). Off-line sampling has the advantage that children may be studied at home or school, and that it can be used for large epidemiological studies, independent from the direct presence of an NO-analyser. The aim of this study was to establish a reference range of eNO sampled in balloons, of a large group of healthy school-aged children. Furthermore, we examined the influence of ambient NO, the effect of 10 sec of breathholding and of using a noseclip on measured values.

PATIENTS AND METHODS

All pupils of a primary school (n=129) participated. They were interviewed with validated questionaires on asthma, allergy, eczema and rhinitis translated from the core questionnaires on wheezing, rhinitis and eczema of the international study of asthma and allergies in childhood (ISAAC) (9), and were in addition asked for recent symptoms of cold (stuffy or running nose, sneezing, coughing, sore throat, with or without fever). Measurement of eNO was done on two occasions in the same children. On the first occasion, the ambient NO was high (median 35 ppb, range 15-188 ppb). At that time, 91 children (mean age 10 yrs) had negative questionnaires on asthma, allergy and eczema (all questions answered negatively) and did not report symptoms of cold within the 3 weeks before the measurements. On the second occasion 3.5 months later, ambient NO was low (median 7.0 ppb, range 1.5-9.0 ppb). At that time, 8 children were absent, 72 of those 91 who initially had negative ISAAC questionnaires reported no symptoms of cold, and 49 had either positive ISAAC questionnaires or reported a recent cold. Measurements took place during several sessions at each occasion, always between 14.00 and 16.00 h pm. Ambient NO was drawn and stored for later analysis before and after each session, and the mean of these

two samples was taken as the ambient NO concentration at that session. All children were self-reported never smokers, never had had any other chronic illness, and used no medication. The study was approved by the hospital ethical committee.

EXHALED AIR SAMPLING AND NO MEASUREMENTS

The children were asked to take a deep oral breath and exhale via a piece of plastic tubing into a mylar balloon with a maximum content of 1750 mL. These balloons are impermeable to- and nonreactive with NO (10). The plastic tube (internal diameter 3.7 mm and length 5 cm) worked as a fixed flow restrictor, causing a positive mouth pressure during the exhalation manoeuvre. Preliminary experiments showed that with a flow of 250 mL/min, this pressure is 6 cm H_2O. At the school, exhalation flow and mouth pressure were not monitored. Subjects filled the balloon within 3-5 s. The range of expiratory volumes which were collected was 250-1750 mL, the extrapolated expiratory flows thus ranged between 250-600 mL/s.

All subjects performed expiratory manoeuvres into 4 balloons: 1) immediate exhalation with a noseclip; 2) immediate exhalation without a noseclip; 3) exhalation after breath-holding for 10 seconds with a noseclip; 4) exhalation after breathholding for 10 seconds without a noseclip. All measurements were performed with the children seated. The NO concentration in balloons was measured within 4 h after collection. Air was drawn out of the balloons at 200 mL/min by a chemiluminescence analyser (Sievers 280, Boulder, CO, USA) with a response time of 200 msec. The NO profile showed a rapid rise to a steady NO plateau, at which eNO was calculated. The analyser was regulary calibrated according to the manufacturer's guidelines, with two different certified NO gases (100 ppb and 9 ppm) and a certified NO free air mixture (HoekLoos, Barendrecht, The Netherlands).

LUNG FUNCTION

All subjects performed maximal expiratory flow–volume measurements immediatly after eNO was collected in the balloons on the first occasion. Flow–volume curves were obtained in triplicate, using a calibrated single breath electronic screening spirometer (Vicatest P2A, Mijnhardt, Zeist, The Netherlands). Results of expiratory forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV_1) were expressed as percentage predicted (11).

STABILITY OF ENO IN BALLOONS

Five subjects filled each 1 balloon with orally exhaled air and 1 with nasally exhaled air. These balloons were sealed and stored at room temperature.

During a period of 6 h, air was sampled for NO measurements with hourly intervals.

DATA ANALYSIS

Data of NO measurements were fed into a computer at a sampling rate of 20 Hz. Mean values during 30 sec sampling of the plateau were calculated. Correlation between eNO and other variables were analysed in a multiple regression model. In this model, eNO was entered as the dependent variable and ambient NO, gender, age and FEV_1 were entered as independent variables. Differences between healthy and "diseased" groups were evaluated with Student's t-test for independent samples. Differences between eNO values at high and low ambient NO within groups were tested using a paired sample t-test. The relation between eNO and ambient NO concentrations was tested with Spearman's rank test. Data are reported as means $\pm$ 1 standard error (SEM). In all statistical comparisons a two-tailed p value of <0.05 was considered significant.

RESULTS

Characteristics of the study population are given in table 1. There were no differences in age, male/female ratio, anthropometric data and lung function between healthy and "diseased" groups at both sampling moments.

Reference values. All children performed the sampling procedures without difficulty. Values obtained in 72 healthy children, not wearing noseclips, without breathhold, and while ambient NO was below 10 ppb are considered as reference values and show a normal, symmetric distribution, with a mean level of 5.1 ± 0.2 ppb (95% reference interval 1.7-8.5 ppb). Exhaled NO was similar in boys and girls (means 5.1 ppb and 4.9 ppb respectively, p=0.15). There was no significant correlation between eNO and FVC or FEV_1 (p=0.56). We found a significant positive correlation between age and eNO; for every 10 months of increasing age eNO increased 0.3 ± 0.1 ppb (p=0.013). Individual data and 95% reference interval are given in figure 1. The children of the 'diseased' group (N=49) with positive questionnaires on asthma, rhinitis, and/or eczema, and/or symptoms of cold within 3 weeks before eNO measurements, with ambient NO<10 ppb, showed a significantly higher mean eNO than the corresponding healthy group (6.8 ± 0.3 ppb, p=0.001). The mean eNO values of the healthy and 'diseased' groups of children under the different measurement conditions, with ambient NO < 10 ppb, are given in table 2.

Effect of ambient NO. High concentrations of ambient NO led to significantly higher eNO values in healthy children: 19.2 ± 1.6 ppb with ambient NO > 10

TABLE 1. Patient characteristics at two points in time, with high and low ambient NO. Data are presented as means and 1 standard error.

	Ambient NO < 10 ppb.		Ambient NO > 10 ppb.	
	healthy	'diseased'*	healthy	'diseased'*
N	72	49	91	38
Sex M/F	31/41	28/21	46/45	19/19
Age (months)	123.8 ± 1.8	123.3 ± 2.2	125 ± 1.6	120.2 ± 2.5
Heigth (cm)	144.3 ± 1.2	144.4 ± 1.6	144.6 ± 1.1	142.8 ± 2.5
Weight (kg)	37.8 ± 1.0	38.8 ± 1.2	38.0 ± 0.9	38.0 ± 1.5
FVC % pred.	98 ± 1	99 ± 1	98 ± 1	100 ± 2
FEV_1% pred.	99 ± 1	99 ± 1	99 ± 1	99 ± 2

*'Diseased': a positive questionnaire for asthma and allergy, and/or recent or current symptoms of cold.

TABLE 2. NO values of schoolchildren with and without reported symptoms of asthma, allergy or colds (indicated as 'healthy' and 'diseased', respectively), with and without noseclip or breath-holding, measured when ambient NO was < 10 ppb. Means, SEM and ranges are given for each condition.

	n	noseclip - breathholding -	noseclip + breathholding -	noseclip + breathholding +	noseclip - breathholding +
Healthy	72	5.1 ± 0.2	4.5 ± 0.2**	6.2 ± 0.4**	6.4 ± 0.4***
		(2.1 – 10.9)	(1.1 – 9.5)	(2.2 – 20.5)	(2.7 – 21.6)
'Diseased'	49	6.8 ± 0.3*	5.9 ± 0.4*	8.9 ± 1.1*	8.3 ± 0.9*
		(2.4 – 15.3)	(1.8 – 14.3)	(2.0 – 46.2)	(2.3 – 32.1)

* p = 0.001 compared to healthy children, same measurement conditions. ** p = 0.001 compared to healthy children without noseclip and breathhold. *** p < 0.0001 compared to healthy children without noseclip and breathhold.

ppb, versus 5.1 ± 0.2 ppb with ambient NO < 10 ppb (p<0.0001). Exhaled NO values and ambient NO concentrations were significantly related (Figure 2). With ambient NO levels >10 ppb this relation was stronger than with levels < 10 ppb (r=0.86, p<0.0001 versus r=0.34, p=0.004, respectively). Between 0 and 10 ppb, eNO increases with 0.2±0.2 ppb per 1 ppb increase of ambient NO. Wearing a noseclip had no influence on the observed relation between eNO and ambient NO.

Effect of noseclip. The noseclip significantly reduced eNO values when ambient NO was < 10 ppb. In 72 healthy children, the mean eNO value was 4.5 ± 0.2 ppb, with- and 5.1 ± 0.2 ppb without noseclip (p=0.001). There was no significant effect of a noseclip on eNO values under all other measurement conditions:
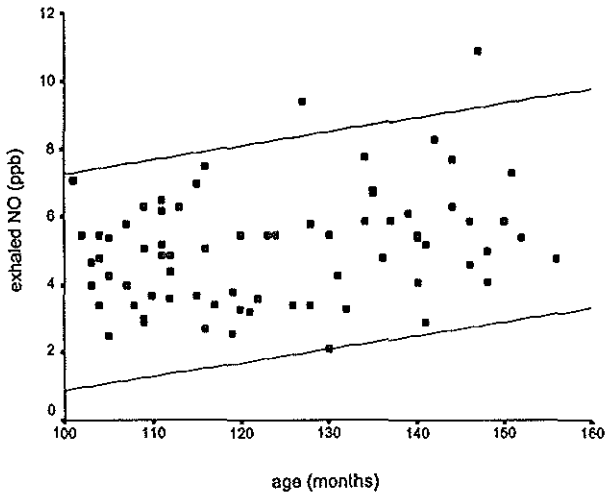
FIGURE 1. Exhaled NO (in ppb, vertical axis) of 72 healthy school children versus age (in months, horizontal axis). Values are from children without reported asthma, allergy or recent cold, and were measured when ambient NO was <10 ppb. All measurements were done without breathhold or noseclip. There is a small but significant increase in NO with increasing age, corresponding to 0.3 ± 0.1 ppb per 10 months ($r = 0.3$, p=0.013). Lines indicate the 95% reference interval.

FIGURE 2. Exhaled NO (in ppb, vertical axis) versus ambient NO on days with ambient values > 10 ppb (horizontal axis) in 91 healthy children. Note that both axes are logarithmical. There is a significant correlation (Spearman rho = 0.86, p=0.0001).

FIGURE 3. NO concentrations in mylar-balloons, stored for 6 h at room temperature. Vertical axis depicts NO in ppb, the horizontal axis shows time after balloon filling in h. The upper curve is the mean [NO] from 5 balloons containing nasal air, the lower curve the mean [NO] from 5 balloons with orally exhaled air. Concentrations were stable in time.
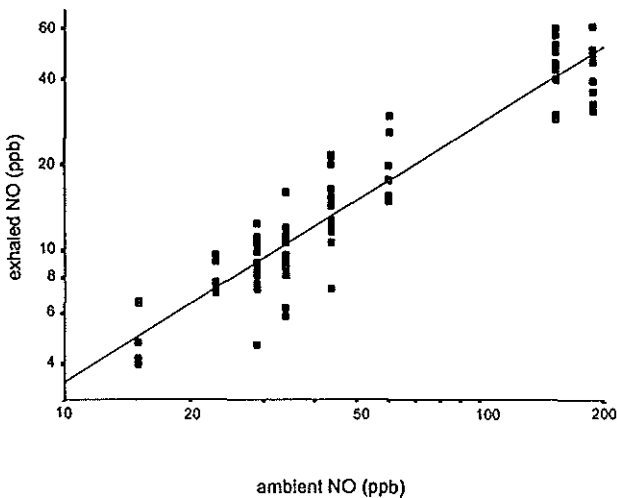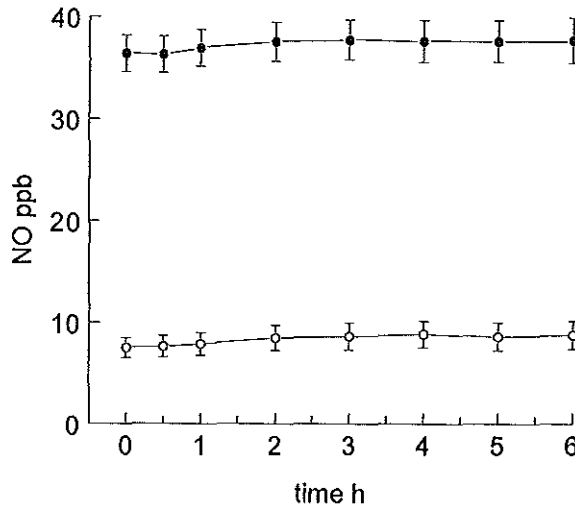
high ambient NO levels with (p=0.2) or without breathholding (p=0.9) and low ambient NO levels with breathholding (p=0.4).

Effect of breathholding. In healthy children, 10 secs of breathholding resulted in an increase in eNO from 5.1 ± 0.2 ppb to 6.4 ± 0.4 ppb (p<0.0001) with ambient NO<10 ppb. In contrast, 10 secs of breathholding produced a significant reduction of eNO from 19.2 ± 1.6 ppb to 9.6 ± 0.8 ppb (p<0.0001) with ambient NO >10 ppb.

Stability of eNO in balloons. The mean NO concentration in 5 mylar-balloons with nasal air was 36.4 ± 1.8 ppb and with oral air 7.5 ± 0.9 ppb. Nasal and oral NO concentrations in the balloons were stable for at least 6 h (figure 3).

DISCUSSION

In this study, we define a reference range of eNO obtained off-line with balloon sampling in a large group of healthy school-aged children. The mean eNO concentration of healthy children in our study is in agreement with eNO concentrations of healthy children from a number of previous studies (12-16). Furthermore, we show that a number of methodological factors influence eNO

collected off-line in balloons, including ambient NO, the use of a noseclip and breathholding. We detected significant differences between children with and without self-reported asthma, allergy and colds, suggesting that this method is sufficiently sensitive to detect minor degrees of airway inflammation in groups otherwise healthy school-aged children.

There are a number of factors that should be considered when comparing our simple off-line sampling technique with more complicated on-line sampling techniques where flow is controlled and the NO signal displayed during exhalation.

We previously showed that, despite lack of flow standardization, off-line sampling in balloons produces eNO values that are reproducible and similar to those of end-expiratory plateaus measured on-line with constant flow (7). We and others have shown that eNO is flow dependent, but mainly at flows of ≤ 150 mL/s (17-19), whereas flow dependency is much less at higher flow rates. The children in our study blew relatively high flows of at least 250 mL/s. This could explain why eNO in balloons is reproducible despite a lack of flow standardization (7).

In a previous study we showed an excellent within-subject short-term repeatability of eNO with an intraclass correlation coefficient (r_i) of 0.91, using the same technique without noseclip (7). Small alterations of this original sampling technique, like the use of a noseclip or 10 sec breathholding, could potentially influence the repeatability. We and others previously showed that short-term repeatability in clinicallly stable allergic asthmatic children was also excellent when a noseclip was used (r_i=0.96) (7,8,14,21). We did not specifically investigate the influence of 10 secs breathholding on the repeatability of eNO obtained with the off-line balloon method, and no such data are available in the literature.

Contamination with nasal NO is another potential problem with off-line sampling. An expiratory resistance is recommended to isolate the lower airway gas from gas with much higher NO concentrations produced in the nose and paranasal sinuses (6). In our study, balloons were filled via a plastic tube during a single oral exhalation. The tube worked as a fixed flow restrictor which causes a positive mouth pressure during the exhalation manoeuvre. It has been shown in adults that a mouth pressure of 4 cm H_2O is sufficient to keep the soft palate closed, thus preventing contamination with NO from upper airways (22). Studies on mouth pressure and velum closure were done in adults; it may well be that lower pressures close the velum in children. The lowest estimated mean exhalation flow of 250 mL/sec in this study produces a mouth pressure of 6 cm H_2O. This should be sufficient to close the velum, and we are therefore confident that contamination with NO from the upper airways, despite our simple setup without monitoring of flow or pressure during exhalation, did not occur to an important degree.

The potential effect of NO in the dead space air volume on eNO in the balloon is another point of concern. Children have a smaller dead space volume than adults which, in a similar balloon volume, will cause a smaller effect on NO values. We speculate that in children the effect of the dead space air on the eNO value will be minimal when ambient NO is < 10 ppb, and when subjects inhale orally before exhaling into the balloon, as was done in the present study.

Previous studies on the effect of ambient NO on eNO vary remarkably. Some authors found no influence of ambient NO on eNO values (23-27), others found important effects (28-32). We found a strong positive relation between ambient NO concentrations and eNO in balloons. The variation of sampling techniques used in the different studies probably explains the differences. As an example, Corradi et al (32) found no relationship between ambient NO and eNO in balloons, with ambient NO < 35 ppb, but the expiration in their study in adults took at least 20 s compared to 3-5 sec in our study. This may explain the difference, as we found that breathhold significantly reduces the effect of high ambient NO on exhaled values. The positive relation between ambient and eNO was more pronounced with high ambient NO levels, but there was also a positive correlation at ambient NO levels below 10 ppb. The magnitude of this effect, a mean rise of eNO 0.2 ppb for each 1 ppb rise in ambient NO, is small compared to the differences in eNO between healthy and 'diseased' children. To minimize the effect of ambient NO on eNO, we recommend that eNO should not be measured when ambient NO is above 10 ppb. Ambient NO should be recorded, or sampled in balloons and analyzed later. Alternatively, NO-free air may be inhaled before sampling, but this makes the method more complicated and formal comparisons of these approaches have not been done.

We found a significant correlation between increase in eNO concentrations and increasing age in healthy children. A possible explanation is the influence of the different dead space air volumes on eNO values in balloons. One could also speculate that this correlation is caused by increasing nasal contamination of the eNO with age, due to development and pneumatization of the paranasal sinuses in childhood. Indeed, nasal NO increases with age reaching levels similar to those in adults at the age of approximately 10 years and this corresponds roughly with the period of sinus development (33). However, oral pressures were probably sufficient to close the velum and prevent contamination by nasal NO (22). Furthermore, Franklin et al recently described a positive correlation between eNO and age, in a comparable group of healthy children.using an on-line single breath plateau technique at a mouth pressure of 15 cm H_2O (34). Other eNO studies in children have not been able to demonstrate a relationship of eNO with age (14,15,30). However, a number of methodological differences of the different studies makes it difficult to compare results. For instance, different ages, differ-

ences in sampling methods with eNO values obtained in different ways and differences in dealing with the ambient NO.

It has been stated that using a noseclip enhances nasal contamination of eNO, although there are no data to support this (2,10,17). Several investigators routinely require subjects to wear noseclips during NO measurements (10,26, 31,35), while others do not (17,21,36). Without a noseclip, diffusion of NO through the nares as well as into the posterior nasopharynx is possible; with a noseclip only diffusion towards the pharynx can occur, and this would increase contamination with high nasal NO levels. Using the present off-line single breath balloon sampling method with an expiratory resistance, we found that a noseclip in combination with low ambient NO levels causes a small but significant decrease in exhaled NO values. A possible explanation is that a noseclip prevents a minimal nasal inspiration, thus preventing contamination of eNO with nasal NO. Although a noseclip only minimally affected eNO, our data do not argue against the ERS task force recommendation not to use noseclip during NO measurement (6).

Prior studies have shown that breathholding increases eNO in asthmatics and in healthy subjects in a time dependent way (10,17,37,38). However, others showed that when 40 ppm is inhaled, a breathhold of several seconds reduced NO in exhaled air to 1–3 ppm (25). This decrease may be caused by rapid uptake of NO by haemoglobin in the lung capillaries (17,25,39). We found that the effect of breathholding on eNO depends on ambient NO; with high levels, breathholding reduces NO, whereas with low levels it increases eNO. It is not likely that these results are influenced by nasal contamination during breathholding or in the oral expiration phase, as shown earlier with an inert tracer gas (22).

We conclude, that off-line measurement of NO sampled in balloons is a simple and feasible method of measuring eNO in children. Off-line sampling has the important advantage that it offers independence from the presence of an NO analyser. Off-line balloon sampling without use of a noseclip and without breathhold, and with ambient NO < 10 ppb, shows differences between healthy children and those with self-reported asthma, allergy or cold, confirming that eNO may be used as a surrogate marker of airway inflammation. The simplicity of this method makes it attractive for studies in larger populations, where differences between groups can be detected.

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CHAPTER 5

Controlled low-flow off-line sampling of exhaled nitric oxide in children

Q. Jöbsis[1,3], H.C. Raatgeep[1], W.C.J. Hop[2] and J.C. de Jongste[1]

[1]Dept. of Paediatrics, div. of Paediatric Respiratory Medicine, [2]Department of Epidemiology and Biostatistics, Erasmus University and University Hospital / Sophia Children's Hospital, Rotterdam and [3]Dept. of Paediatrics, University Hospital Maastricht, The Netherlands

ABSTRACT

The aim of this study was to validate exhaled NO (eNO) values obtained with an alternative off-line, single breath, low flow balloon sampling method against on-line sampling according to ERS guidelines, in children old enough to perform both methods. One hunderd and twenty seven children (median age 14.1 yrs), all pupils of a secondary school, participated. They performed the 2 different sampling techniques at three different flows of 50, 100, 150 mL/sec. Additional measurements were done in a random subgroup of 76 children to determine the influence of the dead space air on eNO values obtained off-line, by excluding the first 220 mL of exhaled air. All children completed a questionnaire on respiratory and allergic disorders, and underwent spirometry.

Results: the off-line eNO values were significantly higher than the on-line values at all flows. At 100 mL/sec, median off-line eNO was 12.9 ppb (range 6.4-104.4 ppb) and on-line eNO was 9.6 ppb (3.2-128.8 ppb) (p<0.0001). However, when dead space air was discarded, off-line and on-line values were similar at all flows; at 100 mL/sec, off-line eNO was 8.8 ppb (3.3-32.5 ppb) and on-line eNO 8.7 ppb (3.2-35.0 ppb). There was a highly significant linear relation between off-line values, especially without dead space air, and on-line eNO (r=0.93, p<0.0001). Off-line eNO at 100 mL/sec of 80 children with negative questionnaires for asthma, rhinitis and eczema was 10.7 ppb (6.4-50.0 ppb) versus 23.9 ppb (8.3-104.4 ppb) in the remaining 47 children with positive questionnaires on asthma and allergy and/or recent symptoms of cold (p<0.0001). We conclude that, in children, off-line assessment of eNO values using constant low flow sampling and excluding dead space air are similar to values obtained on-line with the same exhalation flow. Both sampling methods are sufficiently sensitive to differentiate between groups of otherwise healthy school children with and without self-reported asthma, allergy and or colds.

INTRODUCTION

Nitric oxide (NO) is produced by various cells in the respiratory tract, and has been implicated in the pathophysiology of airway diseases (1,2). NO can be detected in human exhaled air (3). Exhaled NO (eNO) has been proposed as a non-invasive marker of airway inflammation, especially in asthma (4). Recently, attempts have been made to standardize eNO measurement procedures (5,6). An ERS task force recommended on-line single breath collection of eNO in adults and children from the age of 6 yrs (5). An alternative method is off-line sampling, which has the advantage of independence from the presence of an analyser. Here, subjects perform a single deep expiration against a resistance into an NO inert and -impermeable balloon (7). The content of the balloon is analysed for eNO later. Subjects may be studied outside the laboratory, so that this method is potentially useful for large epidemiological studies or home monitoring of asthmatic airway inflammation.
Measurements conditions, including expiratory flow rate and profile, ambient NO, breath holding, previous forced expiratory manoeuvres and contamination by nasal NO, have important effects on eNO values (5,6,8). NO in exhaled air is flow dependent, an effect that is most pronounced at low flows (9-11). Until now, most off-line measurement techniques for children did not take flow dependency into account. We previously showed in children that, despite lack of flow standardization, off-line sampling in balloons produces eNO values that are reproducible and similar to those measured on-line, with high flows (7). Furthermore, we showed that eNO separates better between different disease states, when measured on-line with low flows (11). Therefore, the aim of this study was to compare a modified off-line single breath, low-flow method with on-line single breath measurements in children, and to establish a reference range of low-flow, off-line eNO in healthy schoolchildren.

PATIENTS AND METHODS

We recruited 127 non-smoking children (median age 14.1 yrs, range 12.0-16.1 yrs) from a secondary school. They were interviewed with questionnaires on asthma, eczema and rhinitis, translated and validated from the core questionnaires of the International Study of Asthma and Allergy in Childhood (ISAAC)(12), and were asked for recent symptoms of cold. Of these 127 children, 80 had negative questionnaires and did not report symptoms of cold within the 3 weeks before the study. Those 80 children were regarded as healthy. The remaining 47 children had positive questionnaires and/or recent colds, and

will be referred to as 'diseased'. The study was approved by the hospital ethics committee and informed consent was obtained.

EXHALED AIR SAMPLING

Exhaled air was sampled on-line and off-line. Both sampling methods were performed by all subjects with 3 different flows: 50 mL/sec, 100 mL/sec and 150 mL/sec in random order. All measurements were performed with the children seated and without a noseclip. Between the different manoeuvres, a resting period of 2 min was maintained for equilibration of resting ventilatory conditions. After the exhaled air sampling procedures all subjects underwent flow-volume curve measurements, using a heated Lilly-type pneumotachograph (Jaeger, Würzberg, Germany). Results of forced expiratory vital capacity (FVC) and forced expiratory volume in one second (FEV_1) are expressed as percentage of predicted values (13).

On-line measurement: the measurement circuit consisted of a mouthpiece connected to a two-way nonrebreathing valve (Rudolph Inc., Kansas City, MO, USA) through which the subjects inhaled ambient air when the ambient NO concentration was below 10 ppb, and NO-free medical air when the ambient NO was higher. The subjects inhaled to total lung capacity (TLC) and immediately exhaled, with a constant flow, for as long as possible into a wide bore tube with an in-line flow resistance (20 cm H_2O/L/sec, Rudolph Inc.). A fine bore teflon tube continuously sampled the exhaled air from a side-port directly after the mouthpiece at 200 mL/min for measurement of eNO. This manoeuvre was performed with three different expiratory target flows of 50, 100 and 150 mL/sec. This produced mouth pressures of respectively 4, 7 and 10 cm H_2O. Airflow was measured by a heated Lilly-type pneumotachograph (Jaeger, Würzberg, Germany) mounted after the resistance. A biofeedback display provided visual guidance to help the subject maintain their exhalation flow at the desired level. An end-expiratory flow-plateau of at least 3 s for the different flows was the end-point of the measurement. A plateau was defined as a tracing where flow varies less than $\pm$ 10% around the target flow. All subjects performed this test in triplicate for each target flow. Both the NO signal and the flow signal were fed into a computer at a sampling rate of 20 Hz. Values of NO and flow were obtained by calculating the mean value in defined time intervals of at least 3 seconds, using custom-made software. Individual means of exhaled NO for each target flow were calculated from at least 2 acceptable manoeuvres.

Off-line measurement: The collecting device consisted of a mouthpiece connected to a rigid perspex tube with a fixed flow restrictor, which contained an upstream pressure transducer (14). The signal from the transducer was used to feed a LED-display, mounted on the tube, to enable the subjects to maintain a constant flow (figure 1). Subjects were asked to take a deep breath and to perform one
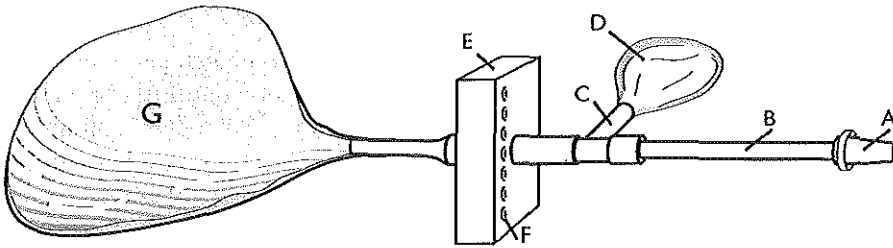
FIGURE 1. Sampling unit used in the off-line collection of eNO. The mouthpiece (A) is connected to a rigid perspex tube (B) which has a side port (C) with low resistance by which a non-compliant balloon (D) first fills with dead space air. Next, air passes through a small box (E), containing the flow transducer with LED display (F) and an in-line flow resistance through which a second balloon (G) fills while oral pressure is elevated.

single exhalation with a constant flow into an NO inert and –impermeable mylar balloon (maximum capacity 1750 mL). Children inspired ambient air, or NO-free medical air when the ambient NO was 10 ppb or higher. This manoeuvre was performed with three different target flows of 50, 100 and 150 mL/sec, leading to mouth pressures of respectively 7 cm H_2O, 20 cm H_2O and 35 cm H_2O, sufficient to close the velum and prevent contamination with nasal NO (15). The mean NO concentrations in the balloons was measured during 30 s sampling within 3 h after collection, and were fed into a computer at a sampling rate of 20 Hz. We formerly found that NO is stable for at least 6 hours in mylar balloons (16).

To investigate the influence of the exhaled dead space volume on eNO in balloons we performed additional measurements in a random subgroup of children (n=76) where we adapted the off-line collecting device with a low-resistance, noncompliant small NO impermeable balloon (capacity 220 mL) connected to the exhalation tube, upstream of the fixed flow restrictor. The first 220 mL of exhaled air was thus discarded in this small balloon, after which the large balloon was inflated with the remaining part of the exhaled volume. Short-term reproducibility of the off-line sampling method was assessed in a random subgroup of 15 children who repeated the manoeuvre after a 5 min interval.

NO MEASUREMENT

NO was measured with a Sievers 280 chemiluminescence analyser (Sievers, Boulder, CO, USA) with a sensitivity of <0.1 ppb and a detection range of <0.1–500,000 ppb. The sampling flow was 200 mL/min, the response time 200 ms and data were displayed with a lag time of approximately 2 sec. The analyser was calibrated regularly using certified NO gases (100 ppb and 9 ppm) and certified NO-free gas (HoekLoos, Barendrecht, The Netherlands).

DATA ANALYSIS

Results of eNO concentrations are expressed as median and range. Because eNO was log-normally distributed, comparison of individual eNO levels obtained on-line and off-line with the same target flow, and of eNO at different target flows within the same sampling method, was carried out on logtransformed data by using Wilcoxon's matched-pairs signed rank test. Differences between the healthy and 'diseased' groups were assessed by the Mann-Whitney test. Reference intervals of eNO for the different target flows in the healthy group are expressed as median and 95% reference intervals. The relation between age and FVC on the one hand and eNO on the other hand in healthy children was assessed by Spearman's rank correlation coefficient. To analyse the within-subjects short-term reproducibility, intraclass correlation coefficients (r_i) were calculated (17). A two tailed p-value of <0.05 was considered significant.

RESULTS

Characteristics of the two groups are shown in table 1. Mean values of FVC and FEV_1 were normal for both groups. Both eNO sampling methods were well tolerated and performed by all children. No significant difference was observed in eNO between girls and boys. In both groups, there was no significant correlation between eNO and age or lung function data under all different sampling conditions. The off-line sampling method was succesfully performed by all subjects. With on-line measurement, some children failed to blow an acceptable plateau (6-10 % of all children for various flows). The median eNO concentrations at the on-line end-expiratory plateau are shown in figure 2. Exhaled NO fell significantly with increasing flows under all sampling conditions in both groups (all p<0.0001) (figure 2).

TABLE 1. Subject characteristics (mean, SEM)

	healthy (n=80)	"diseased" (n=47)*
sex m/f	44/36	23/24
age (months)	166 ± 1.15	170.9 ± 1.91
length (cm)	162.9 ± 1.24	166.2 ± 1.25
weight (kg)	51.4 ± 1.26	55.5 ± 1.62
FVC % pred	98.5 ± 133	98.6 ± 1.81
FEV_1 % pred	100.4 ± 1.37	99.4 ± 1.75

* "diseased" means self-reported asthma, eczema or hay fever and/or recent colds

TABLE 2. eNO values of 100 school-aged children measured at 3 different flow rates, on- and off-line
(in ppb), values are medians; ranges of at least 2 successful attempts

n = 100	on-line eNO (ppb)**	off-line eNO (ppb)**
50 mL/sec	12.7 (3.9 - 160.3)**	15.6 (7.4 - 152.8)
100 mL/sec	9.6 (3.2 - 128.8)**	12.9 (6.4 - 104.4)
150 mL/sec	8.3 (2.9 - 82.7)**	11.8 (6.1 - 98.4)

** all differences between-methods and between-flows are significant (p<0.001)

Comparison of on-line and off-line methods: 100 children performed the two different
samplings methods at the three expiratory flows successfully. Their on-line eNO
was significantly lower than their off-line eNO at the same target flows (figure 3).
The median (range) eNO values for on-line and off-line data at the three different
target flows are given in table 2.
Influence of dead space air on off-line eNO: 53 children performed off-line measure-
ments with and without discarding the first 220 mL of exhaled air, with an exhala-
tion flow of 50 mL/sec. The eNO in balloons including dead space air were
significantly higher than eNO without dead space air: 16.4 (8.0-97.8) ppb and
13.7 (6.8-63.9) ppb, respectively (p<0.0001). In another 23 subjects who exhaled
at a controlled exhalation flow of 100mL/sec, eNO in the balloons including the
dead space was 10.5 (7.2-34.6) ppb, compared to 8.8 (3.3-32.5) ppb when the
dead space was discarded (p=0.001).
Off-line eNO without dead space air with an exhalation flow of 50 or 100 mL/sec
was not significantly different from on-line measured eNO values obtained with
the same target flows: 13.7 (6.8-63.9) ppb versus 14.7 (4.9-73.2) ppb at 50
mL/sec (p=0.07, n=53) and 8.8 (3.3-32.5) ppb versus 8.7 (3.2-35.0) ppb at
100mL/sec (p=0.6, n=23) (figure 4).
Reference range of eNO in normal children, obtained on-line and off-line: eNO values
obtained with the different sampling methods and target flows in the healthy
group can be used as reference values. The eNO concentrations showed a
log-normal distribution. Median and 95 % reference intervals are given in table
3A. Under all different measurement conditions, the children of the diseased
group (n=47) with positive questionnaires on asthma, eczema and/or rhinitis,
and/or symptoms of cold within 3 weeks before the eNO measurements, showed
a significantly higher eNO than the healthy group (p<0.0001). (table 3B)
Reproducibility of off-line eNO: The within-method short-term reproducibility of
duplicate NO values obtained off-line with exhalation flow of 100 mL/sec was
excellent (r_i=0.94). The mean of all individual coefficients of variation of all eNO
values was 6 %. Bland and Altman analysis showed that differences between
repeated eNO measurements were small and independent of mean eNO within
the measured range (figure 5).
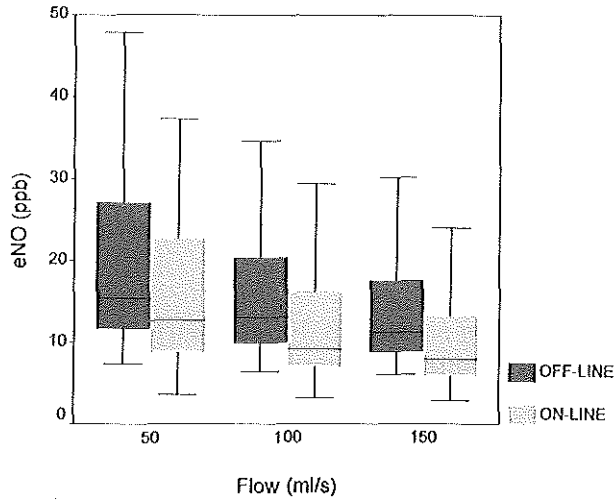
FIGURE 2. Boxplots showing median and quartiles of eNO values of the whole study population at flows of 50, 100 and 150 mL/sec obtained off-line and on-line. All differences between on-line and off-line values at corresponding flows, and all within-method differences at different flows are significant.
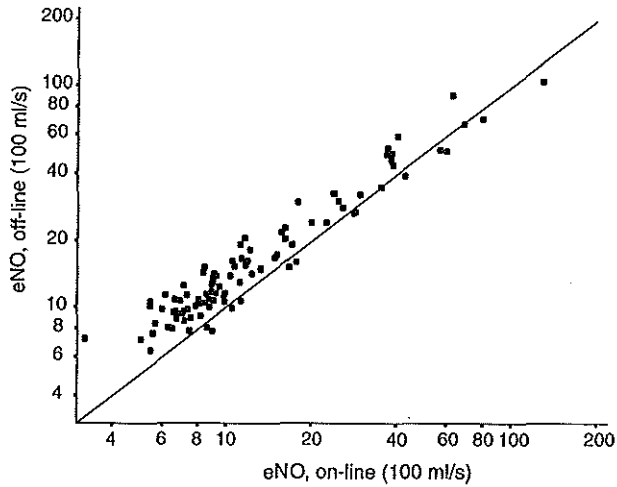
FIGURE 3. Scatterplot showing individual eNO values obtained on-line (x-axis) and off-line (y-axis) at a target flow of 100 mL/sec (N=100). Values obtained off-line were significantly higher than those obtaine on-line.
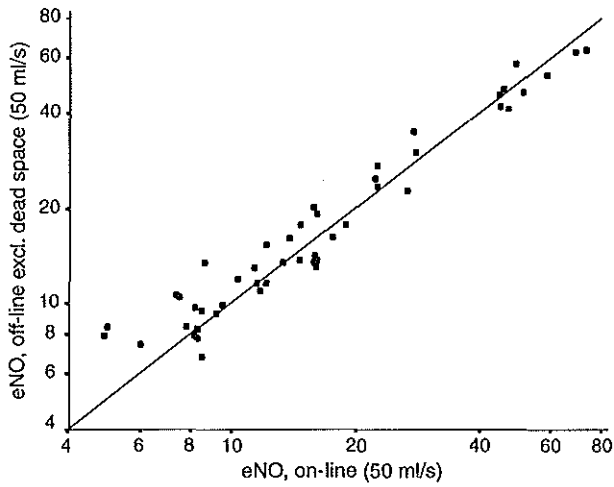
FIGURE 4. Scatterplot showing individual eNO values obtained on-line (x-axis) and off-line excluding dead space at a target flow of 50 mL/sec (n=53).

FIGURE 5. Reproducibility of the adapted off-line sampling method. Difference of two eNO measurements 5 min apart, in ppb (y-axis) against their mean (x-axis), according to Bland and Altman, in 15 subjects.

TABLE 3A. Reference values of eNO (ppb) obtained with the different sampling methods (median and 95% reference interval)
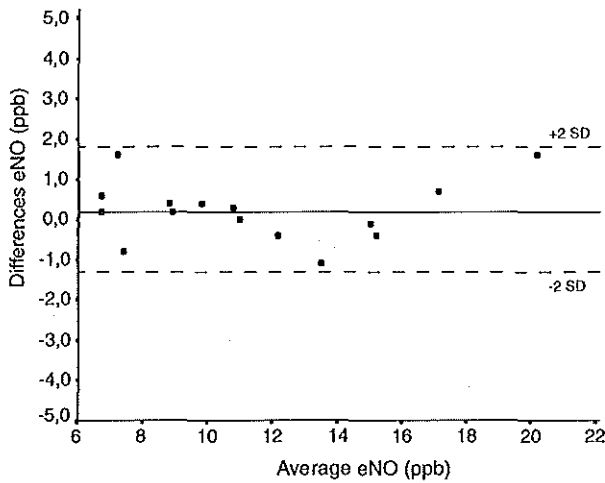
| Healthy group | Off-line | | | | On-line | |
| | with dead space | | without dead space | | | |
	median	Ri 95%	median	Ri 95%	median	Ri 95%
50 mL/sec	12.8	8.4-33.8	11.0	7.1-52.4	9.9	4.7-24.2
100 mL/sec	10.7	7.6-22.6	8.5	3.3-16.2	8.1	4.3-17.6
150 mL/sec	9.9	7.1-17.6	—	----	6.6	4.1-13.1

TABLE 3B. eNO values of healthy and "diseased" children measured at 3 different flow rates, on- and off-line (in ppb, values are medians and ranges)

| | healthy | | "diseased" | |
	median (range)	n	median (range)	n
on-line				
50 mL/sec	9.9 (3.6-69.1)	73	29.6 (8.4-160.3)	41
100 mL/sec	8.1 (3.2-59.4)	74	22.5 (5.9-128.8)	45
150 mL/sec	6.6 (2.9-42.4)	73	19.1 (4.7-82.7)	41
off-line (dead space air included)				
50 mL/sec	12.8 (8.0-70.1)	80	30.9 (7.4-152.8)	47
100 mL/sec	10.7 (6.4-50.0)	80	23.9 (8.3-104.4)	47
150 mL/sec	9.9 (6.1-37.1)	80	21.3 (6.9-98.4)	47

DISCUSSION

This study shows that on-line eNO was lower than off-line eNO, measured with a controlled low-flow balloon sampling technique. However, when dead space air was discarded, agreement was excellent. Furthermore, we found significant differences of eNO between children with and without self-reported asthma, allergy and colds, suggesting that our off-line method is sufficiently sensitive to detect minor degrees of airway inflammation in groups of otherwise healthy school-age children.

The concentration of NO in exhaled air depends critically on the flow of exhalation, an effect that is most pronounced at low flow rates (11). Therefore, it is desirable to measure eNO at a constant, low expiratory flow. Recommendations about the preferred flow rate are not uniform. Silkoff et al. recommend to measure eNO at very low flow rates of 10 to 40 mL/sec (9). Högman et al. propose a flow rate of 150 to 300 mL/sec (10). The ERS task force report recommends flow rates of 167 to 250 mL/sec, independent of age (5). An ATS task force

recommends for on-line measurements 50 mL/sec for adults as well as for children. For off-line measurements in adults and children a target flow rate of 350 mL/sec is recommended (6). Low flows may allow for a better detection of various disease states (9,11). Because of the non-uniform flow recommendations, we used three different target flows (50, 100 and 150 mL/sec) to compare two sampling methods, and we found a significant flow dependency of eNO in children with both methods. Off-line measurement studies of eNO in children previously did not take flow dependency into account (7,18-20).

We found that on-line eNO values were lower than off-line values under all measurement conditions. This seems in contrast with results from our previous eNO study in younger children, which showed similar eNO values on-line and off-line with a balloon sampling procedure (7). We can think of three explanations for this discrepancy. First, the latter study employed high flows of at least 250 mL/sec. There is much less flow dependency of eNO with high flows, and less time for contamination. Secondly, the children in the former study were younger and had a smaller dead space volume than the school children in the present study. The influence of a smaller dead space volume will be less important in a large exhaled volume. Thirdly, the nasal contribution to the dead space air might be less important in young children. With the development and pneumatization of the paranasal sinuses in childhood, nasal NO increases with age. For instance, it was recently shown in a limited number of young children that mixed oral/nasal eNO values collected off-line via a face mask in a balloon were similar to eNO values obtained after tracheal intubation (21).

The concentration of NO in the airway dead space represents a mixture of ambient NO and upper and lower airway NO which may influence eNO values with off-line sampling (5). Therefore, it is desirable to exclude dead space air with off-line sampling. In adults, Paredi et al. (22) compared on-line eNO with off-line, flow- and pressure controlled sampling excluding dead space air, and found good agreement. The present study extends these observations and shows that exclusion of dead space volume improves the agreement of on-line and off-line eNO measurements in children, in the recommended low flow range.

The on-line eNO measurement technique proposed by the ERS (5) is rather difficult for young children (7,23). Canady et al. (23) showed that 24 % of children were unable to perform this manoeuvre. In a previous study we found that nearly 30 % of children were not able to sustain a stable end expiratory flow plateau, employing relatively high flows (7). In the present study, only up to 10 % of school children had difficulty in obtaining a stable end-expiratory plateau at various flows. The expiratory flow of 100 mL/sec with a corresponding mouth pressure of 7 cm H_2O had the lowest failure rate in this study. A likely explanation of the higher success rate of on-line sampling in this study compared to our previous study (7) is the difference in age of the study populations; median age and

range in this study 14.1 yrs and in the previous study 11.7 yrs. Especially for younger children the on–line sampling method remains difficult to perform and for those an alternative methodology should be developed.

A source of error in the present study may be nasal contamination, especially with the lowest mouth pressures, e.g. 4 cm H_2O during on–line sampling at 50 mL/sec. One could argue that this pressure was not high enough to close the soft palate, potentially leading to nasal contamination. However, a study in adults found no contamination of NO from the nasopharynx with a mouth pressure of at least 3 mm Hg (= 4 cm H_2O) by using Argon as a tracer gas which was applied to the nose and not retrieved in the exhaled air (15). No similar studies have been published for children. Baraldi et al. observed in children no correlation between nasal and exhaled NO values using a low expiratory resistance providing a mouth pressure of 3-4 cm H_2O (20). Furthermore, in the present study eNO at flows of 100 and 150 mL/sec obtained on- and off-line, and employing higher pressures, suggest no important contamination at lowest flows, selectively. This suggests that contamination with nasal air has not introduced a bias.

Interestingly, we found significantly elevated eNO in children who reported atopic symptoms or recent colds. This suggests that eNO differentiates between healthy children and those who are likely to have minimal airway inflammation but no actual symptoms and normal lung function. We speculate that subclinical airway inflammation may already produce significant elevation of eNO.

In conclusion, off-line measurement of eNO is a simple and feasible method of measuring eNO in school children. The off-line eNO values with constant low flow, excluding dead space volume, show good agreement with on-line values, and are feasible in school-aged children. Both sampling methods discriminate between groups of children with and without self reported asthma, allergy and colds. Off-line sampling offers the possibility to study eNO independently of the presence of a NO analyser, which could be useful for epidemiologic studies and home monitoring of asthma.
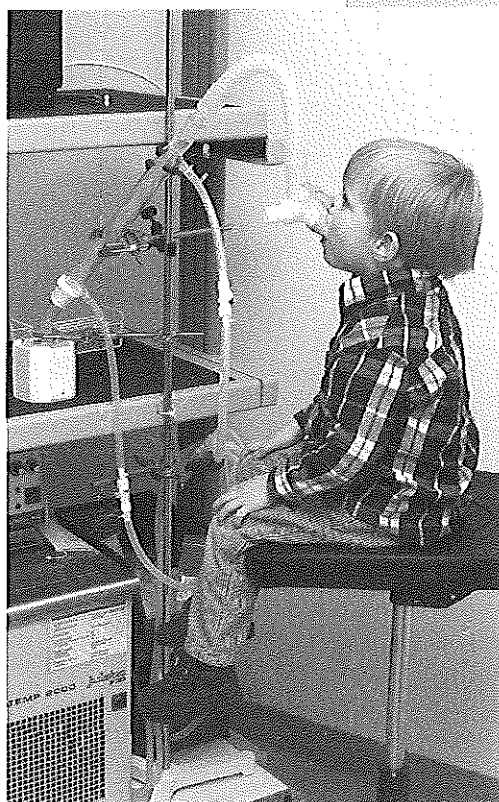
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EXHALED HYDROGEN PEROXIDE

Collection of exhaled air condensate

CHAPTER 6

Brief communication

Hydrogen peroxide in exhaled air of healthy children: reference values

Q. Jöbsis, H.C. Raatgeep, S.L. Schellekens, W.C.J. Hop[1], P.W.M. Hermans, J.C. de Jongste

Erasmus University and University Hospital/Sophia Children's Hospital, Department of Paediatrics, Division of Paediatric Respiratory Medicine, and Department of Biostatistics[1], Dr. Molewaterplein 60, 3015 GJ Rotterdam, The Netherlands

ABSTRACT

An increased content of hydrogen peroxide (H_2O_2), a marker of inflammation, has been described in the condensate of exhaled air from adults and children with inflammatory lung disorders, including asthma. However, the normal range of $[H_2O_2]$ in exhaled air condensate from healthy children has not been established. Therefore, the aim of this study was to determine the reference range of exhaled $[H_2O_2]$ in healthy school-aged children. Ninety-three healthy nonsmoking children (48 female and 45 male, mean age 10 yrs, range 8-13 yrs), with a negative history for allergy, eczema or respiratory disease and with a normal lung function, participated. Exhaled air condensate was examined fluorimetrically for the presence of H_2O_2. In addition, the reproducibility of $[H_2O_2]$ within-subjects and between days and the stability of $[H_2O_2]$ during storage at -20°C were assessed. The median $[H_2O_2]$ in the exhaled air condensate of all children was 0.13 µM, with a 2.5-97.5% reference range of <0.01-0.48 µM. No significant difference existed between males and females. There was no correlation between exhaled $[H_2O_2]$ and age or lung function. Repeated $[H_2O_2]$ measurements on two consecutive days showed satisfactory within-subject reproducibility and $[H_2O_2]$ in stored samples remained stable for at least 1 month at -20°C. In conclusion, this study provides reference data for exhaled H_2O_2 in a large group of healthy children. The observed levels were lower than those reported previously for healthy adults and were independent of age, sex and lung function.

INTRODUCTION

A noninvasive method to assess the presence and activity of airway inflammation would be valuable in the early diagnosis and monitoring of inflammatory airways diseases (1). Exhaled air condensate can be collected with minimal risk and inconvenience and its content may reflect the composition of the lower airway fluids (2-3). Hydrogen peroxide (H_2O_2) in the exhaled air condensate is a potential marker of airway inflammation (4-10). The only two studies to suggest elevated levels of exhaled H_2O_2 in asthmatic children have used small numbers of healthy adults as controls (4,10). The aim of this study was, therefore, to establish reference values of [H_2O_2] in the exhaled air condensate of a large group of healthy school-aged children.

PATIENTS AND METHODS

One hunderd and twenty-nine school children, pupils of a primary school, were interviewed with questionnaires on asthma, rhinitis and eczema, translated and validated from the core questionnaires of the "International Study of Asthma and Allergy in Childhood" (ISAAC). Of these children, 93 had negative questionnaires and were included. Their mean age was 10 years (range 8-13 yrs) and 45 were male (table 1). All were term born, Caucasian lifelong nonsmokers, within the normal range for height, and used no medication. None of the subjects reported symptoms of acute respiratory infection within the previous month. Maximal expiratory flow-volume measurements were performed in all children (Vicatest P2A, Mijnhardt, The Netherlands); forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1) were expressed as percentage predicted (11). Condensate was collected while the children, wearing a nose-clip, breathed quietly through a mouthpiece and a two-way non-rebreathing valve (Rudolph, Kansas City, MO, USA), which also served as a saliva trap. At least 1.5 mL of condensate was obtained by passing expired air through a 50 cm double jacketed glass tube cooled to 0°C, and collected on ice. The concentration of H_2O_2 in the condensate was determined in duplicate with a fluorimetric assay based on the reaction of H_2O_2 with horseradish peroxidase to form a compound which oxidizes p-hydroxyphenylacetic acid to a fluorescent product, as described previously (4,12). As saliva is a source of H_2O_2, the equipment was designed to avoid such contamination, as verified previously by measuring amylase in saliva and breath condensates. Amylase was present in saliva in high concentrations (20.000-300.000 U/L), and could never be demonstrated in condensate (invariably <10 U/L, unpublished data); therefore, it could be stated with confidence that no contamination with saliva had

TABLE 1. Characteristics of the study population. Healthy children (n = 93)[1]

age yrs (mean, range)	10 (8-13)
sex male/female	45/48
FVC % pred (mean ± SD)	98 ± 12
FEV$_1$ % pred (mean ± SD)	100 ± 12

[1] All were lifelong nonsmokers, had no symptoms of asthma, eczema or rhinitis, used no medication, and had no symptoms of respiratory infection in the 4 weeks before the study.

taken place. To examine the reproducibility of repeated H_2O_2 measurements within subjects, condensate was collected from 10 healthy subjects on two consecutive days in the morning. To assess the stability of $[H_2O_2]$ in the frozen condensate, 4 mL of condensate was collected from 5 subjects. These were devided into 1 mL aliquots, in which H_2O_2 concentrations were determined immediatly after collection, after 48 hours, one week and one month of storage at -20°C.

ANALYSIS OF DATA

Results of H_2O_2 measurements are expressed as median and range because of an asymmetrical distribution and because some values were below the detection limit. Least squares regression analysis was used to determine possible effects of age or lung function on exhaled H_2O_2. Differences between males and females were evaluated with the Mann–Whitney test. Reproducibility was assessed by calculating the mean within-subject difference of two H_2O_2 measurements obtained on separate days from 10 subjects and the standard deviation (SD) of these differences. Stability of H_2O_2 in frozen samples was estimated by comparing mean concentrations using a two sided paired t-test, and by calculating mean differences and SD of the differences of immediately determined values and values after a given period of storage. Significance was assumed at $p<0.05$.

RESULTS

All subjects had a normal lung function with a mean FVC of 98% (SD 12%) and an FEV$_1$ of 100% (SD 12%). The H_2O_2 concentration in the exhaled air condensate ranged from below the detection limit of 0.01 µM to 0.50 µM, with a median value of 0.13 µM. The 2.5 and 97.5 percentiles were <0.01 and 0.48 µM, respectively. Median $[H_2O_2]$ from males and females were similar (0.13 µM and 0.14 µM, respectively, p=0.98). Individual data of all children are shown in figure 1. There was no effect of age on $[H_2O_2]$ (r=-0.04, p=0.68). Likewise, no correlation was found between absolute values of FEV$_1$ and
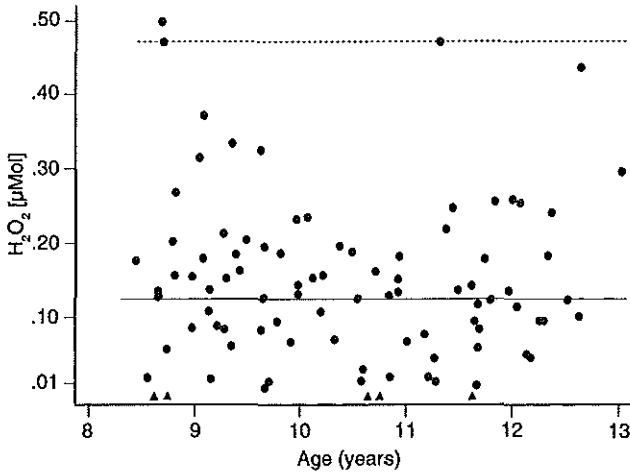
FIGURE 1. Concentration of H_2O_2 in the exhaled air condensate in µMol (y-axis) of 93 healthy children. The results were independent of age (x-axis). The dotted line represents the 97.5 percentile upper reference limit (0.48 µM); and the solid line represents the median (0.13 µM). Each symbol represents one subject. Triangles indicate values below the detection limit (0.01 µM).

$[H_2O_2]$ in the condensate (r=0.07, p=0.48). Repeated measurements on two consecutive days showed a mean within-subject difference of 0.02 µM (SD 0.04 µM). The stability of $[H_2O_2]$ in frozen condensate was satisfactory, with no significant difference between the $[H_2O_2]$ (mean, SD) immediately after collection (0.13, 0.03 µM) and after storage at -20°C for 48 hours (0.11, 0.02 µM; p=0.14), one week (0.12, 0.05 µM; p=0.55) and one month (0.13, 0.02 µM; p=0.59).

DISCUSSION

In this study the reference range of H_2O_2 in exhaled air condensate obtained from a large group of healthy school-aged children has been defined. The methodology was noninvasive and well-tolerated and gave reproducible results. Samples could be stored for at least one month and analyzed later without changes in $[H_2O_2]$.

Until now, published data on normal values of H_2O_2 concentration in exhaled air were obtained from small numbers of adults (4-6,8-10). Peroxide concentrations were observed previously in healthy adults that were higher than the upper limit of the reference range in the present study (4). This suggests either that healthy

children produce less peroxide than adults or that subclinical airway inflammation may be more common in asymptomatic adults than in healthy children. On the basis of these results it can be stated that earlier observations in asthmatic children indeed have shown H_2O_2 concentrations in exhaled air that were substantially higher than those found in healthy children (4,10).

In conclusion, this study defined the reference range of H_2O_2, a putative marker of airway inflammation, in exhaled air condensates from a large group of healthy school-aged children. Longitudinal studies are needed to examine the potential value of this inflammatory marker in the management of childhood asthma.

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CHAPTER 7

Hydrogen peroxide in exhaled air is increased in stable asthmatic children

Q. Jöbsis, H.C. Raatgeep, P.W.M. Hermans, J.C. de Jongste

Department of Paediatrics, Division of Paediatric Respiratory Medicine, Erasmus University and University Hospital/Sophia Children's Hospital, Rotterdam, The Netherlands

ABSTRACT

Exhaled air condensate provides a noninvasive means of obtaining samples from the lower respiratory tract. Hydrogen Peroxide (H_2O_2) in exhaled air has been proposed as a marker of airway inflammation. We hypothesized that in stable asthmatic children the H_2O_2 concentration in exhaled air condensate may be elevated as a result of airway inflammation. In a cross-sectional study, 66 allergic asthmatic children (of whom, 41 were treated with inhaled steroids) and 21 healthy controls exhaled through a cold trap. The resulting condensate was examined fluorimetrically for the presence of H_2O_2. All subjects were clinically stable, non-smokers and without infection. The median H_2O_2 level in the exhaled air condensate of the asthmatic patients was significantly higher than in healthy controls (0.60 and 0.15 μmol, respectively; $p<0.05$), largely because of high values in the stable asthmatic children who did not use anti-inflammatory treatment (0.8 μmol; $p<0.01$ compared to controls). We conclude that H_2O_2 is elevated in exhaled air condensate of children with stable asthma, and may reflect airway inflammation.

INTRODUCTION

Our present understanding of asthma as a disease of airway inflammation has been greatly enhanced by studies using bronchoalveolar lavage (BAL) (1). In young children, BAL is generally not acceptable for diagnostic and monitoring purposes. A noninvasive method to assess airway inflammation is important for this group (2). Exhaled air condensate has been proposed as a noninvasive means of obtaining samples from the lower respiratory tract, based on the hypothesis that aerosol particles excreted in breath may reflect the composition of the lower airway fluids (3). Several investigators have analysed exhaled air condensate for different mediators of inflammation (3-5); however, data on children are scanty (5). Hydrogen peroxide (H_2O_2) is a putative marker of airway inflammation in exhaled air: Inflammatory cells produce H_2O_2 (6), which causes lung inflammation and damage (7-9); stimulated alveolar macrophages from asthmatics generate more reactive oxygen species than alveolar macrophages from healthy subjects (10). Indeed, an increased content of H_2O_2 has been described in various inflammatory lung disorders: in exhaled air of cigarette smokers (11); patients with adult respiratory distress syndrome (ARDS) (12,13); and patients with acute hypoxemic respiratory failure (14). Dohlman et al. described an increased H_2O_2 concentration in exhaled air condensate in a small group of asthmatic children, mainly in those with acute disease (5). As inflammation is also likely to be present in symptom-free asthmatics (15), we hypothesized that the hydrogen peroxide concentration may also be elevated in exhaled air condensate of children with stable asthma, and may serve as a marker of airway inflammation.

PATIENTS AND METHODS

STUDY POPULATION
Sixty-six asthmatic children (23 females and 43 males) attending the out-patient clinic of Sophia Children's hospital were included in the study. Asthma was diagnosed on clinical grounds following international guidelines (16). Appropriate therapy was prescribed by the patient's own physician, and had not been changed during the 3 months preceding the study. All 66 patients used an inhaled bronchodilator on demand, and 41 (15 females and 26 males) used an inhaled corticosteroid daily. The median age of those without steroids was 12.6 yrs, and of those receiving steroids 11.1 yrs. All were lifelong non-smokers, and were clinically stable. All had bronchial hyperresponsiveness (provocative dose of inhaled metacholine that produced a 20% fall in forced expiratory volume in one second, PD20 of < 150 µg) documented in the past, and

had allergy as documented by radio allergosorbent test (RAST) class 2 or higher for at least one common airborne allergen. Control subjects were healthy, non-smoking young adult volunteers (median age 25 years), with no history of allergy and respiratory disease, nor of any other chronic illness, and used no medication. None of the subjects had had symptoms of acute respiratory infection within the month before the condensate was collected. The study was approved by the Hospital Ethical Committee.

COLLECTION OF EXHALED AIR CONDENSATE

Exhaled air condensate was obtained by passing expired air through a 50 cm double jacketed glass tube cooled to a temperature of 0°C by means of countercurrent circulating iced water. The subjects breathed through a mouthpiece and a two-way nonrebreathing valve (Rudolph, Kansas City, MO, USA), which also served as a saliva trap. They were asked to breath at a normal frequency and tidal volume, wearing a noseclip, for a period of 10-15 min. The condensate, at least 1 ml, was collected on ice.

LUNG FUNCTION

All subjects underwent flow-volume measurements immediatly after collection of the condensate. Flow-volume curves were obtained in triplicate, using a Lilly-type pneumotachograph (Masterlab Jaeger, Würzberg, Germany) before and after inhalation of 1 mg of terbutaline powder (Turbohaler®). Results were expressed as percentage predicted (17).

HYDROGEN PEROXIDE

The concentration of H_2O_2 in exhaled air condensate was determined with a fluorimetric assay based on the reaction of H_2O_2 with horseradish peroxidase to form a compound which oxidizes p-hydroxyphenylacetic acid to a fluorescent product (18). Briefly, 400 μl of condensate was mixed with 1.5 mM (10 μl) p-hydroxyphenylacetic acid and 100 μg/ml (4 μl) horseradish peroxidase (both Sigma Chemical Co., St. Louis, MO, USA) immediately after collection, and frozen at -20°C. The fluorescent product of the condensate and of standard solutions of H_2O_2 were measured with a fluorimeter (model 3000; Perkin-Elmer, Norwalk, USA) at an excitation wavelength of 295 nm and an emission wavelength of 405 nm. The lower limit of H_2O_2 detection was 0.1 μM. Concentrations of H_2O_2 in condensate were obtained by linear interpolation of a standard curve. Preliminary observations in a limited number of young healthy adults indicated that, with this method, the within-subject between-days variations were within 0.1 μmol.

DATA ANALYSIS

Results are expressed as median and range because of a nonsymmetrical distribution and because some values were below the detection limit. Since the anti-inflammatory action of inhaled steroids could be a confoundig factor influencing H_2O_2 in exhaled air condensate as marker of airway inflammation, subgroups with and without steroids were analysed separately. Comparisons between groups were made by the Mann-Whitney test for two unpaired independent samples. A two tailed p-value less than 0.05 was considered significant. There were insufficient data to perform a formal power calculation, but we reasoned that with a group size of 20 subjects we should be able to detect a 1 SD difference (our own preliminary observations suggested this to be about 20%) at a two-sided alpha of 0.05 with a power of 90%.

RESULTS

All subjects completed the protocol without difficulty. The time to collect at least 1 mL of condensate varied between 10 and 15 minutes.

Lung function measurement in asthmatic children without steroids showed near-normal values mean forced vital capacity (FVC) 93% pred, forced expiratory volume in one second (FEV_1) 100% pred, increase in FEV_1 after 1 mg inhaled terbutaline 7%). Asthmatics with steroids were slightly better (FVC 100% pred, FEV_1 107% pred, increase in FEV_1 after terbutaline 6%), but not significantly different from the children without steroids. Controls all had a normal lung function (mean FVC 108% pred, FEV_1 111% pred, increase in FEV_1 after terbutaline 3%).

The median H_2O_2 concentration in condensates of all asthmatic children was significantly higher than in condensate from healthy control subjects (0.60 and 0.15 µmol, respectively; $p < 0.05$). The median H_2O_2 concentration in the asthmatics who used steroids (0.45 µmol) was higher than in healthy control subjects, but the difference was not significant. There was a highly significant difference in median H_2O_2 concentration between asthmatics without anti-inflammatory treatment and healthy controls (0.8 µmol and 0.15 µmol, respectively; $p < 0.01$). Individual data are shown in figure 1. There was no correlation between the concentration of exhaled H_2O_2 and the change in FEV_1 before and after bronchodilatation in control subjects or in asthmatic patients. Furthermore, no correlation was found between the daily dose of inhaled steroids and H_2O_2 concentration in the condensate (data not shown).
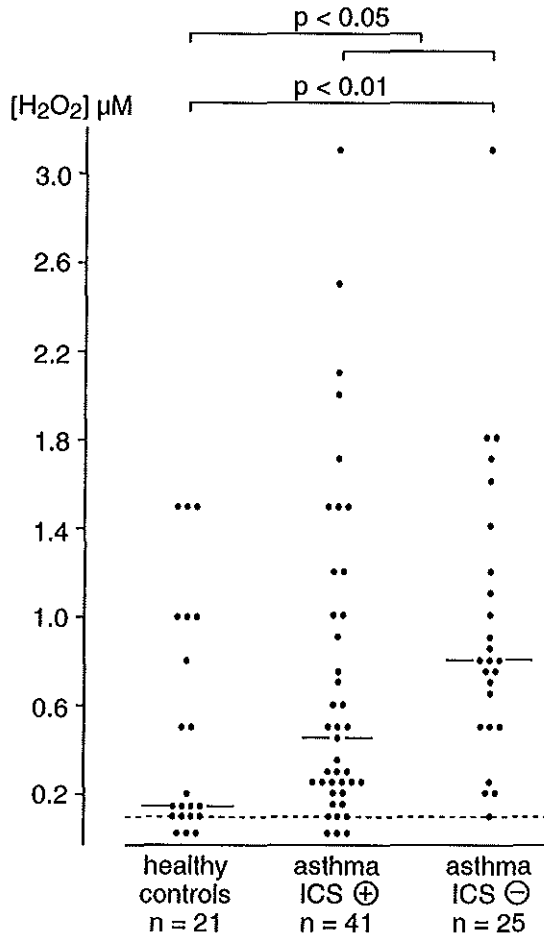
H_2O_2 concentration in exhaled air condensate

------- detection limit

— — median

FIGURE 1. Concentration of H_2O_2 in exhaled air condensate of stable asthmatic children and healthy control subjects. The asthmatics are divided into a subgroup using inhaled corticosteroids (ICS+), and a group without anti-inflammatory treatment (ICS-). Each dot represents one patient. Median values are indicated by a horizontal line and the detection limit by a horizontal broken line. The differences between median values of healthy subjects and all asthmatic subjects was significant (0.15 and 0.60 μmol, respectively; p<0.05), the difference between healthy subjects and the asthmatics without steroids is highly significant (0.15 μmol and 0.80 μmol respectively; p<0.01).

DISCUSSION

This study is the first to demonstrate a significantly increased concentration of H_2O_2 in exhaled air condensate from stable asthmatic children compared to healthy controls. That the H_2O_2 concentrations are lower in asthmatics who use anti-inflammatory medication supports the hypothesis that exhaled H_2O_2 reflects the presence of airway inflammation.

The present results seem different from those of an earlier study by Dohlman et al, describing increased H_2O_2 concentration in exhaled air condensate in a small group of asthmatic children, with acute respiratory disease, whereas exhaled H_2O_2 from stable asthmatics was not different from control subjects (5). Both studies used similar control groups. We think that much larger groups in the present study, and the separation of asthmatics with and without anti-inflammatory treatment explain the different outcomes.

The present study shows that inhaled steroids are associated with lower exhaled H_2O_2 concentrations in asthmatics. A study in ARDS patients treated with corticosteroids showed a tendency towards lower levels of H_2O_2 in the exhaled air condensate as compared to ARDS patients not receiving corticosteroids (14). The present cross-sectional study cannot demonstrate a causal relationship between steroid use and lower exhaled peroxide; prospective controlled intervention studies are needed for that purpose.

An increased content of H_2O_2 has also been reported in other inflammatory respiratory diseases (11–14). This means that exhaled H_2O_2 is probably not a specific diagnostic test for asthma. Rather than a diagnostic test, exhaled peroxide may be used to guide anti-inflammatory treatment and estimate disease severity over time within-subjects.

It can be argued that the control subjects in the present study were not age-matched, and that age-dependent changes in exhaled peroxide might partly explain the results. This seems unlikely: there are no data suggesting that healthy children produce more reactive oxygen species in their airways than healthy young adults. However, we do feel that, in the absence of such data, establishing normal values over a wide age range is an important goal for the near future.

In conclusion, this cross-sectional study has shown that the concentration of H_2O_2 in exhaled air condensate is increased in stable asthmatic children, and lower in patients receiving anti-inflammatory treatment, suggesting that airway inflammation increases exhaled peroxide. Correlation of H_2O_2 in exhaled air condensate with invasive measures of airway inflammation, such as bronchial biopsies, is needed to validate the hypothesis that exhaled peroxide reflects airway inflammation. On the basis of the present findings, we believe that the concentration of H_2O_2 in exhaled air is a potentially useful marker of airway inflammation in asthmatic children, especially since the procedure is quick and easy to

perform. Further studies should explore its value as a noninvasive test, e.g. for monitoring effects of anti-inflammatory therapy.

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CHAPTER 8

Hydrogen peroxide and nitric oxide in exhaled air of children with cystic fibrosis during antibiotic treatment

Q. Jöbsis[1,2], H.C. Raatgeep[1], S.L. Schellekens[1], A. Kroesbergen[1], W.C.J. Hop[3] and J.C. de Jongste[1]

[1]Dept. of Paediatrics/Respiratory Medicine, [3]Department of Biostatistics, Erasmus University and University Hospital/Sophia Children's Hospital, Rotterdam and [2]Dept. of Paediatrics, University Hospital, Maastricht, The Netherlands

ABSTRACT

Cystic fibrosis (CF) patients characteristically have severe chronic airway inflammation due to bacterial infection. A noninvasive marker of airway inflammation could be useful to guide treatment of CF lung disease. The aim of this study was to assess whether measurement of hydrogen peroxide (H_2O_2) and nitric oxide (NO) in exhaled air can serve to monitor the effect of treatment with antibiotics in CF-children with acute infective pulmonary exacerbations. Sixteen CF-patients (mean age 12.3 yrs) with exacerbation of their lung infection were treated with intravenous antibiotics. During treatment, H_2O_2 in exhaled air condensate was measured twice a week. In addition, serial NO measurements were performed in 9 patients. During antibiotic treatment the median H_2O_2 concentration in exhaled air condensate decreased significantly from 0.28 µM (range 0.07-1.20 µM) to 0.16 µM (range 0.05-0.24 µM) (p=0.002) and the mean FEV_1 significantly increased from 55 % of predicted value to 75 % (p=0.001). In individual subjects, changes of H_2O_2 and FEV_1 between pairs of serial measurements correlated weakly (p=0.08). Data on exhaled NO were inconclusive; eNO did not change systematically during treatment. We conclude that CF-patients with an acute pulmonary exacerbation have abnormally high concentrations of H_2O_2, but not of NO, in exhaled air, which decrease during i.v. antibiotic treatment. Further studies should establish if exhaled H_2O_2, may serve as a noninvasive parameter of airway inflammation to guide antibiotic treatment in CF lung disease.

INTRODUCTION

Invasive procedures such as bronchoscopy, bronchoalveolar lavage (BAL) and bronchial biopsies have greatly enhanced the understanding of the role of airway inflammation in various respiratory disorders like asthma and cystic fibrosis. Lung disease in patients with cystic fibrosis (CF) is characterized by recurrent exacerbations of respiratory symptoms due to chronic bacterial infection of the airways. Shortly after birth, patients with CF acquire respiratory infection that incites an inflammatory response (1,2). The continuous presence of bacteria in the lung induces a strong immunological response resulting in the release of inflammatory cytokines and mediators (3,4) which can be measured in bronchoalveolar lavage (BAL) fluid or sputum, even in young and stable CF-patients with clinically mild lung disease (1,5,6). In cystic fibrosis lung disease the inflammatory response leads to massive neutrophil recruitment (5,6). Activated neutrophils and macrophages are major sources of oxygen free radicals including hydrogen peroxide (H_2O_2). Indeed, lung infection in CF leads to increased oxygen free radical generation (7,8). Furthermore, sputum of CF-patients has been shown to prime neutrophils towards enhanced release of oxygen radicals (9). The airways in patients with CF are exposed to increased oxidative stress which appears to be a result of lower airway inflammation (10). The current available methods to obtain material from the lower respiratory tract are in general inappropriate for routine use in the assessment of lower airway inflammation in children because of the invasive nature of these procedures. A simple, noninvasive test to monitor airway inflammation would be important for the diagnosis of airway inflammation and to evaluate current and new therapeutic options. Exhaled air (condensate) has been proposed as a noninvasive means of obtaining samples from the lower respiratory tract (11). H_2O_2, a reactive oxygen radical, is a putative marker of airway inflammation in exhaled air condensate. An increased content of H_2O_2 has been described in exhaled air of patients with various inflammatory lung disorders (12–15). Exhaled nitric oxide (NO) has been put forward as another noninvasive marker of airway inflammation (16,17). However, in stable CF-patients orally exhaled NO levels are not elevated (18,19). Data in children on NO and H_2O_2 during acute infectious respiratory tract exacerbation in CF are lacking. The aim of this study was to assess whether exhaled H_2O_2 and NO can serve as noninvasive markers of airway inflammation during treatment of cystic fibrosis patients with an acute infective pulmonary exacerbation.

METHODS

STUDY POPULATION

Children with CF were recruited from the CF-centre of the Sophia Children's Hospital. CF had been diagnosed on the basis of typical symptoms, two mutations in the CF-gene, and an abnormal sweat test (a sweat sodium concentration > 70 mmol/l). Sixteen CF-patients with an acute infective pulmonary exacerbation, successively admitted for a course of in-patient intravenous antibiotic treatment, were included. Patients characteristics are shown in the table. Exclusion criteria were inability to perform pulmonary function tests, concomitant diagnosis of asthma, current oral steroid therapy and a sputum culture containing Burkholderia cepacia. Disease severity of the subjects varied widely when they entered the study, with predicted forced expiratory volume in one second (FEV_1) ranging from 42 to 75 percent (mean 55%) . Nine subjects used nebulized recombinant human dornase alpha (DNase). All subjects were infected with either *Pseudomonas aeruginosa* (n=10) and with *Staphylococcus aureus* (n=2) or a combination of the two organisms (n=4). All patients received a standard treatment protocol, including intravenous antibiotics, based on bacterial sensitivities, and chest physiotherapy. The duration of the intravenous antibiotic therapy ranged from 2 to 5 weeks (median 3 weeks). Informed consent for the study was obtained from the patients and parents on the day of admission. The study was approved by the medical ethical committee of the Erasmus University Medical Centre.

COLLECTION OF EXHALED AIR CONDENSATE

The subjects breathed through a mouthpiece and a two way non-rebreathing valve (Rudolph, Kansas City, MO, USA) which also served as as saliva trap. They were asked to breath at a normal frequency and tidal volume, wearing a noseclip. Exhaled air condensate was obtained by passing expired air through a 50 cm double jacketed glass tube cooled to a temperture of $0°$ C, by means of counter-current circulating ice water. The resulting condensate was collected on ice and frozen immediately at $- 20°$ C until analysis. The first collection of exhaled air condensate took place before the beginning of the intravenous antibiotics. Further samples were obtained twice a week at the same time of day during the intravenous antibiotic treatment period.

HYDROGEN PEROXIDE MEASUREMENT

The concentration of H_2O_2 in exhaled air condensate was measured in duplicate with a fluorimetric assay based on the reaction of H_2O_2 with horseradish peroxidase to form a compound which oxidizes p-hydroxyphenylacetic acid to a fluorescent product, as described in detail previously (12,20). The fluo–

TABLE 1. Patient characteristics before start of i.v. antibiotics

CF-patients (n=16)	
Sex (male/female)	4/12
Age (months, median, range)	148.0 (83-226)
Height (cm, mean, SEM)	145.3 ± 3.9
Weight (kg, mean, SEM)	33.7 ± 2.5
FVC (% pred, mean, SEM)	68 ± 3
FEV$_1$ (% pred, mean, SEM.)	55 ± 3
Inhaled steroids (n)	6

rescent product of the condensate and of a standard solution of H_2O_2 were quantified fluorimetrically. This assay showed a statisfactory within subject reproducibility of exhaled hydrogen peroxide values of healthy subjects obtained on 2 consecutive days and with 1 week interval (21, 22). Concentrations of H_2O_2 in condensate were obtained by linear interpolation of a standard curve. The lower limit of H_2O_2 detection was 0.01 µM. The equipment was designed to avoid contamination of the condensate with saliva, as saliva is a source of H_2O_2. We excluded saliva contamination, as described previously (21), by measuring amylase in all breath condensates which is a sensitive marker to detect contamination of the condensate with saliva (22, 23).

NITRIC OXIDE MEASUREMENT

In 9 subjects NO was measured in exhaled air, following the exhaled air condensate collection. NO was measured with two different sampling methods, each in duplicate. Firstly, off–line measurement via one single deep expiration into a NO–impermeable balloon: subjects were asked to exhale, without breath holding, via a plastic tube, which acts as a flow restrictor and causes elevation of oral pressure sufficient to avoid nasal contamination, into an NO–inert Mylar balloon. This off–line sampling method, including the advantages and disadvantages of this technique, was described earlier in detail (24). The NO concentration was measured from the balloons. Secondly, in children able to perform more complicated manoeuvres, NO was measured on–line during a controlled slow exhalation from total lung capacity (TLC) through a mouthpiece and a two way non–rebreathing valve against an in–line resistor (20 cm $H_2O/L/sec$, Rudolph Inc., Kansas City, MO, USA) with an individually standardized flow rate of 20% of the subject's vital capacity (VC) per second, as described previously in detail (24,25). A biofeedback display provided visual guidance for the subject to maintain the exhalation flow at the target level. The mean end–expiratory NO level was measured during an end–expiratory flow plateau

of at least 3 sec. Between the different expiratory manoeuvres, all children were allowed 2 min rest, to restore resting conditions of ventilation. To exclude the effect of high ambient NO levels on the exhaled NO values, children breathed NO-free air when the ambient NO was above 5 ppb. During NO measurements, subjects did not wear a noseclip. NO was measured with a chemiluminescence analyser (Sievers 280, Boulder, CO, USA) with a sampling flow of 200 mL/min and a response time of 200 msec. The analyser was regulary calibrated according to the manufacturer's guidelines, employing certified NO gases (100 ppb and 9 ppm) and certified NO-free gas (Hoek-Loos, Barendrecht, The Netherlands).

LUNG FUNCTION
All subjects underwent flow–volume measurements immediately after collection of the condensate and measurement of NO. Flow–volume curves were obtained in triplicate (Masterlab Jaeger, Würzberg, Germany). Results of forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) were expressed as percentage predicted (26).

DATA ANALYSIS
Results of H_2O_2 and NO measurements are expressed as median and ranges because of a nonnormal distribution. Lung function data are presented as mean $\pm$ SEM. Comparison of exhaled H_2O_2, NO and spirometric values before and after antibiotic therapy was done with the Wilcoxon signed ranks test for paired samples. A two tailed p-value of less than 0.05 was considered significant. Spearman correlation tests were performed to detect a correlation of changes of FEV_1 on the one hand, and changes of NO or H_2O_2 on the other hand between the first and last measurements in each child. Correlation between changes of H_2O_2 and changes of FEV_1 for the individual subjects between all subsequent pairs of measurements was investigated with regression analysis (27).

RESULTS

During i.v. antibiotic treatment, FEV_1 improved from 55 $\pm$ 3 % to 75 $\pm$ 4 % (p=0.001). Figure 1 shows the individual FEV_1 data at the start and at the end of i.v. treatment. All subjects completed the condensate collections without difficulty. Of 82 collected condensate samples, 3 were discarded because of contamination with saliva, as shown by detectable amylase. In 2 condensate samples, the H_2O_2 concentration was below the detection limit. Serial exhaled H_2O_2 measurements during i.v. antibiotics showed a decreasing trend with time. The median H_2O_2 concentration in exhaled air condensate just before the start of
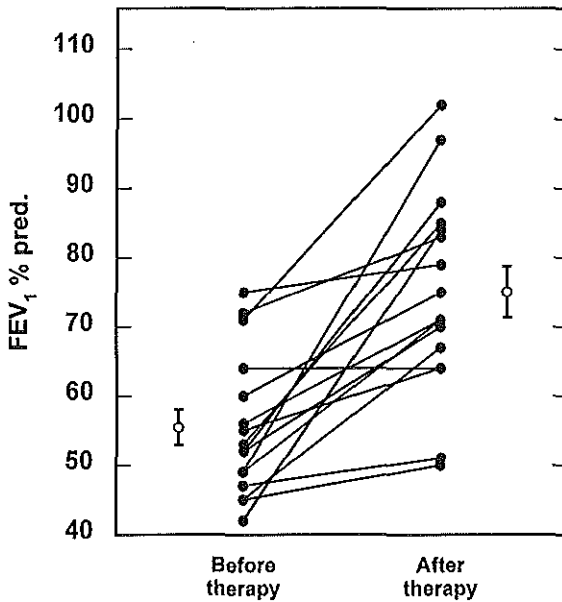
FIGURE 1. Changes in FEV_1 (expressed as percentage of predicted normal value) before and after i.v. antibiotic treatment of respiratory tract infectious exacerbations in 16 CF-patients. Closed dots indicate values of individual patients, open dots are mean values and SEM. The increase is statistically significant (p=0.001).

i.v. antibiotics was significantly higher than the concentration at the end of the treatment period: 0.28 µM (range 0.07-1.20 µM) and 0.16 µM (range 0.05-0.24 µM) respectively (p=0.002). The individual data are shown in figure 2. During treatment, 9 subjects showed decreases in exhaled H_2O_2 together with an improvement in lung function, 3 subjects showed decreases in exhaled peroxide with no consistent improvement in lung function and 4 subjects exhibited no consistent change in peroxide concentrations with (n=2) or without (n=2) improvement in lung function. Figure 3 shows, for the individual subjects, the negative correlation between changes of H_2O_2 and changes of FEV_1 of all different pairs of measurements during the treatment period. The overall correlation, corrected for the number of measurements per child, is of borderline significance (p=0.08). At the onset of each treatment, exhaled peroxide levels did not correlate with exhaled NO levels nor with baseline FEV_1 or FVC (p>0.1 for each). There was no significant difference in level of exhaled H_2O_2 between CF-patients on inhaled steroids (n=6) and those not on inhaled steroids.
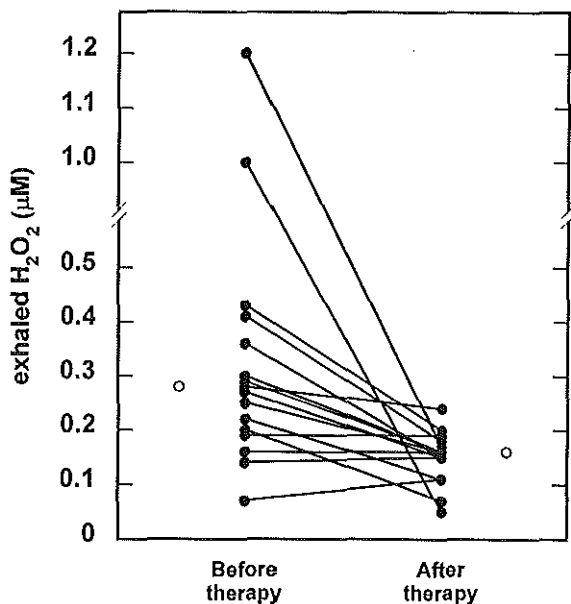
FIGURE 2. Changes in exhaled H_2O_2 in 16 CF-patients before and after i.v. antibiotic treatment of respiratory tract infectious exacerbations. Closed symbols are data from individual children, open dots are median values. The change is statistically significant (p=0.002).
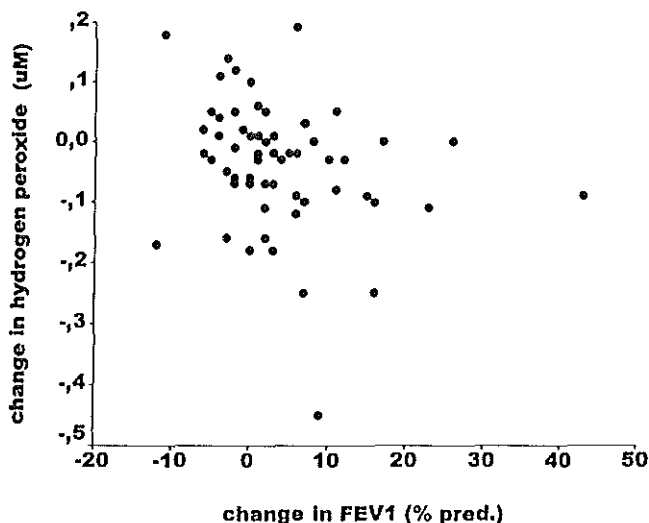
FIGURE 3. Scatterplot of changes in exhaled H_2O_2 versus changes in FEV$_1$ in paired observations made during antibiotic treatment of 16 children with CF treated with antibiotics for respiratory exacerbations. Regression analysis, correcting for the numbers of observations per patient, showed a weak correlation of borderline clinical significance (p=0.08).

Off-line eNO measurements by means of balloons were obtained without difficulty in all 9 children. In contrast, 3 out of 9 children were not able to sustain a stable end-expiratory flow plateau of at least 3 sec for on-line measurements of NO with constant flow. Serial exhaled NO measurements during i.v. antibiotics showed no consistent trend with time. For both sampling methods, the mean NO levels in exhaled air before treatment were not significantly different from those after treatment. Initial values of NO obtained with the balloon method and with slow controlled exhalation were 3.5 ppb (2.3-6.5 ppb) and 4.2 ppb (3.1-6.1 ppb), respectively. At the end of treatment exhaled NO was 3.4 ppb (2.2-6.8 ppb) (p=0.6) and 4.0 ppb (2.4-5.6 ppb) (p=0.4) for balloon- and on-line sampling, respectively. The correlation between FEV_1 and NO was not significant. No conclusion can be drawn about the effect of inhaled steroids on eNO, because only 2 of the 9 CF-children, in which we measured eNO, used inhaled steroids.

DISCUSSION

In this study we found that exhaled H_2O_2 was elevated and decreased significantly in CF-children with acute infective pulmonary exacerbations who were treated with i.v. antibiotics. Their lung function (FEV_1) improved significantly. Data on exhaled NO were inconclusive and showed no trend.
Careful monitoring of CF lung disease is important, especially in early stages of the disease where infection and the associated inflammatory response and oxidative stress are frequently insidious and asymptomatic (28). However, conventional methods to assess airway inflammation are complicated (bronchoscopy, broncho-alveolar lavage), and sputum may be difficult to obtain in young children. Hence, markers of inflammation like peroxide and NO that can be obtained easily and repeatedly are potentially important for monitoring CF lung disease. Exhaled H_2O_2 levels in adult CF-patients were investigated earlier and appeared not significantly different from those of healthy controls (22,29). In our study in CF-children with acute infective pulmonary exacerbations we found higher levels than in healthy children (21), and a significant decrease of serial exhaled H_2O_2 levels in the course of treatment with antibiotics. The observed H_2O_2 concentrations at the end of the treatment period are similar to those we found previously in healthy children (21), and are in agreement with the results of stable CF-adults (22,29). Thus, exhaled H_2O_2 may not be a suitable marker of airway inflammation in stable CF-patients, but is of potential value to monitor the effect of anti-inflammatory treatment for exacerbations.
That exhaled H_2O_2 is not elevated in stable CF-patients is possibly due to an increase of scavenging by for example glutathione or catalase which may balance the increased oxidative stress (29). In that case, our results suggest that infective

pulmonary exacerbations may disturb such a balance, and this could be due to various mechanisms. For example, increased oxidative stress; bacterial infection could contribute to oxidative stress by recruitment and activation of phagocytic cells in the lung (30), and by inactivating oxygen radical scavenging molecules. McGrath et al. recently showed that serum inflammatory markers during acute respiratory exacerbations in CF-patient were significantly elevated and showed improvement with antibiotic treatment (8). Furthermore, alveolar macrophages obtained by BAL fluid from subjects with a recent lower respiratory tract infection released more H_2O_2 (31). On the other hand a decreased antioxidant status; Range et al. recently demonstrated in 17 CF-subjects with infective exacerbations that treatment with intravenous antibiotics resulted in increased plasma levels of antioxidants (32).

There was no significant difference in median level of exhaled H_2O_2 between CF-patients on inhaled steroids and those not on inhaled steroids. This observation contrasts with results in stable asthmatic children where inhaled corticosteroids were associated with lower exhaled H_2O_2 (12). On the other hand, earlier studies in CF and bronchiectasis also showed no significant influence of inhaled steroids on exhaled H_2O_2 (13,22). A possible explanation is that corticosteroids do not alter the neutrophilic inflammation in CF as effectively as they affect eosinophilic inflammation in asthma (13); alternative, the relatively low levels in CF leave less room for change than the higher levels in untreated asthmatics.

Exhaled NO in CF-children was measured with two different sampling methods: on-line single slow flow controlled exhalation method and off-line via a single exhalation into a balloon. In contrast to the off-line sampling method the on-line NO sampling method recommended by the ERS (17) is rather difficult to perform in children. In this study the on-line constant flow method had a failure rate of 33%. In a previous study in a larger study population of 101 stable allergic asthmatic children we found a similar failure rate of 30% (24). Canadey et al. showed that 24% of their studied children (n=33) were unable to perform the on-line measurement at constant flow (33). Both NO sampling methods were used in this study; exhaled NO levels showed no consistent trend with time in both sampling methods. This result is in agreement with an earlier study by Ho et al. who found no change in exhaled NO levels during 7 days of treatment in 8 adults with infectious exacerbation of CF (34). This suggests that eNO may not be a suitable marker to monitor airway inflammation and to evaluate anti-inflammatory treatment in CF-patients with an infective pulmonary exacerbation. However, for a definitive conclusion on this subject further studies with larger study populations are needed. The initial NO value obtained with the balloon method in the present study was slightly lower than values obtained previously in healthy children with the same sampling method (35). Other studies also reported

exhaled NO levels in CF-subjects that were not higher than in control subjects (18,19,34). Whether this finding reflects reduced diffusion and metabolism of NO within the viscous airway secretions or a genuine reduction in airway mucosal NO synthesis is not clear. However, nitrite levels in exhaled air condensate were found higher in CF-patients than in normal subjects (36). This suggests that exhaled NO may not reflect the total NO production in the airways.
The relationship between initial values of exhaled H_2O_2 or NO and lung function was not significant. Probably, measurement of lung function and of exhaled NO and H_2O_2 give different information about the inflammatory process of the airways. Airway inflammation is only one of the many determinants of airflow limitation. Nevertheless, figure 3 shows a weak negative correlation between changes of H_2O_2 and changes of FEV_1 between the different serial measurements in individual subjects, and this does suggest that generation of peroxide is related to the inflammatory process which impairs lung function in CF.

In conclusion, our study shows that the levels of exhaled H_2O_2 are elevated in CF-children with an infective pulmonary exacerbation. Furthermore, in contrast to exhaled NO, exhaled H_2O_2 significantly decreases during serial measurements in CF-patients during i.v. antibiotic treatment. This suggests that exhaled H_2O_2 may be a helpful marker to monitor oxidative stress due to airway inflammation in CF-subjects with an acute infective pulmonary exacerbation.

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CHAPTER 9

Elevated hydrogen peroxide in exhaled air during upper respiratory tract infection

Q. Jöbsis[1,2], S.L. Schellekens[1], A. Kroesbergen[1], H.C. Raatgeep[1] and J.C. de Jongste[1]

[1] Dept. of Paediatrics, div. of Paediatric Respiratory Medicine, Erasmus University and University Hospital/Sophia Children's Hospital, Rotterdam and [2] Dept. of Paediatrics, University Hospital Maastricht, The Netherlands

ABSTRACT

Hydrogen peroxide (H_2O_2) is a potential marker of airway inflammation in ex-
haled air condensate, which is elevated in various inflammatory disorders of
the lower respiratory tract. We hypothesized that upper respiratory tract infec-
tions may increase H_2O_2 in exhaled air condensate of healthy subjects. There-
fore, we examined exhaled H_2O_2 in 20 normal subjects after symptomatic upper
respiratory tract infection and during recovery 2 weeks later, and similarly, in
10 healthy controls without recent or current symptoms of infection. Exhaled
air condensate was obtained by mouth breathing through a cooled glass tube.
The concentration of H_2O_2 in condensate was measured with a fluorimetric as-
say. At the time of infection, the median (range) H_2O_2 level in condensate was
0.20 μM (0.03-1.2 μM), and this decreased to 0.09 μM (<0.01-0.40 μM) after
recovery (p=0.006). There was no significant difference in FVC and FEV_1 during
and after infection. In the healthy control subjects, exhaled H_2O_2 values were
reproducible over a 2 week period: 0.10 μM (0.01-0.30 μM) initially, compared
to 0.08 μM (<0.01-0.25 μM) after 2 weeks (NS). We conclude that the H_2O_2
concentration in exhaled air condensate is significantly elevated during a
symptomatic upper respiratory tract infection, and returns to normal within 2
weeks of recovery in healthy subjects. This implies that, when using exhaled
H_2O_2 as a marker of chronic inflammatory airway disease such as asthma, mea-
surements should not take place within 2 weeks of symptomatic upper respira-
tory tract infection.

INTRODUCTION

Exhaled air condensate can be collected with minimal risk and inconvenience and provides a means for obtaining samples from the lower respiratory tract (1,2). Hydrogen peroxide (H_2O_2) in exhaled air condensate is a potential marker of airway inflammation (1,3). An increased content of H_2O_2 has been described in exhaled air of patients with various inflammatory lung disorders (1,4-7). Therefore, H_2O_2 in breath condensate may be a simple and noninvasive marker to diagnose and monitor chronic airway inflammation in the lower respiratory tract. However, H_2O_2 in exhaled air condensate could be potentially confounded by intercurrent acute infection, especially common cold, which causes transient inflammation of the upper respiratory tract. It is, therefore, important to know the effect of upper airway infection on exhaled H_2O_2, and such data is lacking. The aim of this study was to assess to what extent upper respiratory tract infections affect orally exhaled H_2O_2 in otherwise healthy subjects.

METHODS

STUDY POPULATION
We recruited by advertisement 20 normal subjects with acute (less than 48 hours) symptoms of upper respiratory tract infection: stuffy or running nose, sneezing, sore throat, with or without fever. Similarly, we recruited 10 healthy age-matched control subjects without symptoms of upper respiratory tract infections. All subjects of both groups were lifelong nonsmokers, had no history of allergy, sinusitis and respiratory or cardiovasculair disease, and had no symptoms of asthma or eczema and used no medication. Healthy control subjects had no symptoms of respiratory tract infection in the 4 weeks before, or during, the measurements. Characteristics of the study population are shown in table 1. The study was approved by the medical ethical committee of the Erasmus University Medical Centre.

COLLECTION OF EXHALED AIR CONDENSATE
Condensate was collected twice in all subjects: at inclusion and 2 weeks later, when symptoms had dissapeared. The subjects breathed through a mouthpiece and a two way non-rebreathing valve (Rudolph, Kansas City, MO, USA) which also served as as saliva trap. They were asked to breath at a normal frequency and tidal volume, wearing a noseclip. Exhaled air condensate was obtained by passing expired air through a 50 cm double jacketed glass tube cooled to a temperture of $0°$ C, by means of counter-current circulating ice water. The resulting condensate was collected on ice and frozen immediately at $-20°$ C until analysis.

TABLE 1. Patient characteristics, lung function and H_2O_2 values of all subjects

	Upper Respiratory Tract Infection		Controls	
N	20		10	
Sex (male/female)	7/13		5/5	
Age (years; median, range)	25.6 (19.8-51.2)		26.0 (20.1-48.4)	
	t=0 wks	t=2 wks	t=0 wks	t=2 wks
	(acute infection)	(recovered)		
FVC (% pred, mean ± SEM)	108 ± 1.8	109 ± 1.8	101 ± 2.8	98 ± 3.1
FEV_1 (% pred, mean ±SEM)	106 ± 1.9	107 ± 1.9	101 ± 3.3	100 ± 3.1
H_2O_2 (median, μM)	0.20	0.09	0.10	0.08
(range, μM)	(0.03-1.20)	(<0.01-0.40)	(0.01-0.30)	(<0.01-0.25)

HYDROGEN PEROXIDE MEASUREMENT

The concentration of H_2O_2 in exhaled air condensate was measured in duplicate with a fluorimetric assay based on the reaction of H_2O_2 with horseradish peroxidase to form a compound which oxidizes p-hydroxyphenylacetic acid to a fluorescent product, as described in detail previously (1,8). The fluorescent product of the condensate and of standard solutions of H_2O_2 were quantified fluorimetrically. Concentrations of H_2O_2 in condensate were obtained by linear interpolation of a standard curve. The lower limit of H_2O_2 detection was 0.01 μM.

The equipment was designed to avoid contamination of the condensate with saliva, a source of H_2O_2. We excluded saliva contamination, as described previously (9), by measuring amylase in all breath condensates.

LUNG FUNCTION

All subjects performed flow-volume measurements immediately after collection of the condensate. Flow-volume curves were obtained in triplicate, using a Lilly-type pneumotachograph (Masterlab Jaeger, Würzberg, Germany). Results of forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV_1) were expressed as percentage predicted.

DATA ANALYSIS

Results of H_2O_2 are expressed as median and range because of a nonsymmetrical distribution and because some values were below the detection limit. Lung function data are expressed as mean±SEM. The difference, over a 2 week interval, in H_2O_2 levels and lung function parameters within each group was assessed after normalization by logtransformation with Student's paired t-tests, whereas differences between groups were tested with unpaired t-tests. A two tailed p-value of less than 0.05 was considered significant.

RESULTS

All subjects performed the condensate collections without difficulty. In none of the 60 collected condensate samples amylase was detected, excluding contamination with saliva. In subjects with upper respiratory tract infection H_2O_2 levels were 0.20 μM (0.03-1.20 μM). After recovery, H_2O_2 was significantly lower: 0.09 μM (<0.01-0.40 μM) (p=0.006). Decreases in exhaled peroxide were seen in 17 out of 20 subjects. In the control group the exhaled H_2O_2 levels were stable and reproducible over a 2 week period: 0.10 μM (0.01-0.30 μM) and 0.08 μM (<0.01-0.25 μM) (NS). The individual H_2O_2 data are shown in figure 1 and 2.

There was no significant change in FVC and FEV_1 during and after upper respiratory tract infection: FVC 108 $\pm$ 8 %, FEV_1 106 $\pm$ 9 % compared to FVC 109 $\pm$ 8%, FEV_1 107 $\pm$ 9 % after 2 weeks (fig. 3). Likewise, lung function was stable in all controls. There was no correlation between exhaled H_2O_2 levels and lung function in both groups.
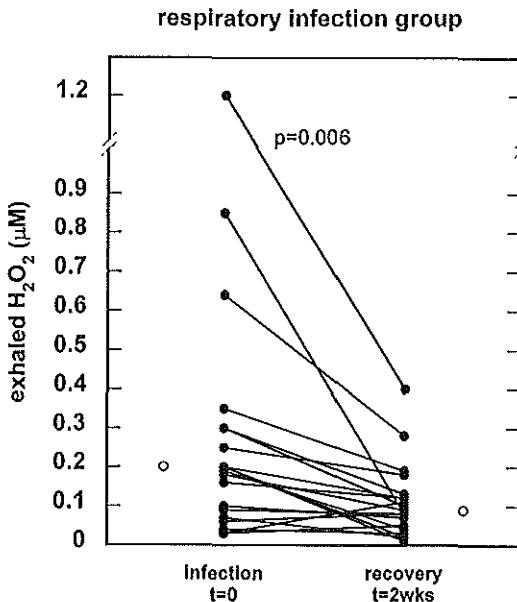
FIGURE 1. Individual values of exhaled H_2O_2 in 20 normal subjects during a symptomatic upper respiratory tract infection and 2 weeks afterwards when asymptomatic. Open dots are median values.

Control group

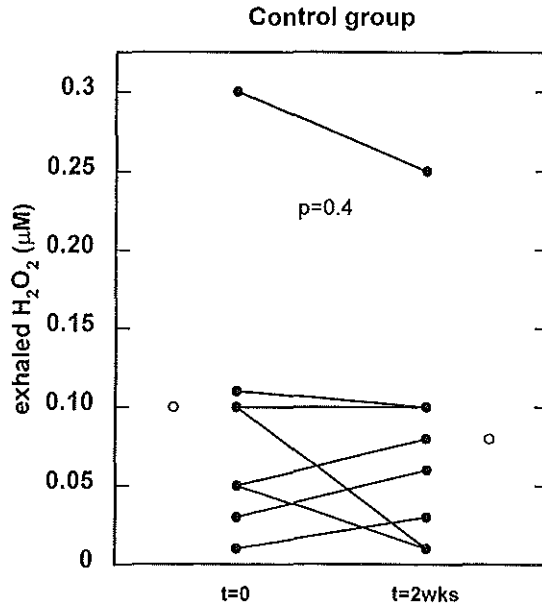FIGURE 2. Individual values of exhaled H_2O_2 in 10 healthy subjects, without symptoms of a upper respiratory tract infection, with a 2 week interval. Open dots are median values.
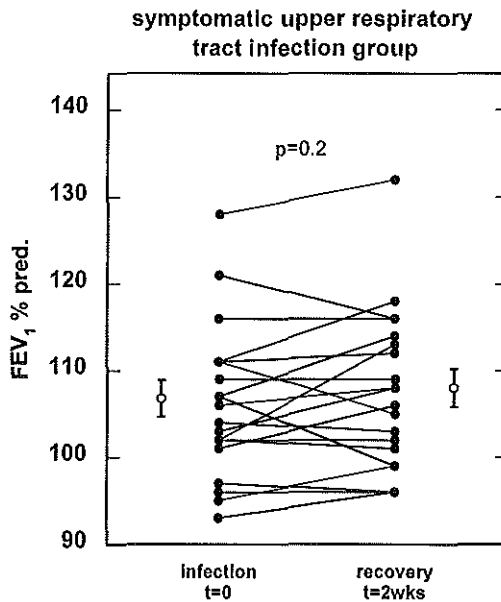
symptomatic upper respiratory tract infection group

FIGURE 3. Changes in FEV_1 during and after respiratory tract infection (N.S.) Closed dots indicate values of individual patients, open dots are mean values and SEM.

DISCUSSION

The results of this study show that upper respiratory tract infections are associated with transiently elevated levels of exhaled H_2O_2 in nonsmoking normal subjects. After recovery, 2 weeks later, exhaled H_2O_2 had decreased to values similar to those of an age-matched healthy control group. Lung function data in subjects with upper respiratory tract infection showed no significant change. These findings suggest that symptomatic upper respiratory tract infection increases production of peroxide in the respiratory tract, and may thus act as confounder in studies using exhaled H_2O_2 as a marker of chronic lower airway inflammation, especially in asthma.

Our present understanding of asthma as a disease of chronic lower airway inflammation has been greatly enhanced by studies using bronchoalveolar lavage (BAL) and bronchial mucosal biopsies (10-12). The invasive nature of those diagnostic procedures limited their use in the assessement of airway inflammation in children. Current treatment guidelines for asthma relies on symptoms and lung function for monitoring disease severity. Based on these guidelines, sufficient suppression of airway inflammation may not be achieved, potentially leading to irreversible changes in airway function (13,14). To trace patients with ongoing airway inflammation and to monitor the effects of therapy, assessment of lower airway inflammation in a more direct way would be valuable. H_2O_2 is a potential marker of airway inflammation, released by inflammatory cells such as eosinophils, neutrophils and macrophages. H_2O_2 damages DNA, proteins and lipids, resulting in airway epithelium damage (15,16), and mediates the release of inflammatory mediators (17). An increased content of H_2O_2 has been described in exhaled air condensate of patients with various noninfectious inflammatory disorders of the lower respiratory tract, including asthma (1,4), adult respiratory distress syndrome (ARDS) (5), chronic obstructive pulmonary disease (COPD) (6), bronchiectasis (7) and is elevated in exhaled air of cigarette smokers (18).

We based the diagnosis of upper respiratory tract infection on typical clinical symptoms. We did not attempt to identify respiratory viruses by means of culture, serology or other tests. However, the nature and time course of upper airway symptoms and the season of the year in which this study took place (autumn) makes it likely that respiratory viruses were involved. We have no indication that the lower airways were involved in the infectious process, as there was no significant change in FVC and FEV_1 or flow volume curve configuration during and after upper respiratory tract infection. Furthermore, none of the subjects of the infected group mentioned lower respiratory symptoms like bringing up sputum or dyspnea. Chest auscultation was always normal.

The elevated exhaled H_2O_2 levels (median 0.20 μM) during upper respiratory tract infection in this study were below levels reported previously by different

groups in stable steroid-naive asthmatic children and adults (1,3) and were in the same range as those in stable COPD patients (6). Our baseline values are in the same range as those reported in previous studies in normals (9, 19).

One could argue that increased H_2O_2 during common cold comes from the upper rather than the lower airways. We excluded saliva contamination and air was collected by mouth breathing with use of a noseclip which should prevent or limit contamination with nasal air.

Therefore it is likely that increased H_2O_2 was due to oral, pharyngeal or lower airway inflammation. Indeed, subclinical lower airway inflammation has been documented in viral upper respiratory tract infections and may go without significant changes in lung function (20-22).

What are the clinical implications of our findings? When using exhaled H_2O_2 as a marker of chronic airway inflammation in inflammatory lower airway disorders, an intercurrent symptomatic upper respiratory tract infection will likely contribute to exhaled peroxide levels and thereby act as a confounder. To avoid this, exhaled H_2O_2 measurements for monitoring lower airway inflammation should not take place during a symptomatic upper respiratory tract infection. An interval of 2 weeks appears sufficient for return to baseline, at least in normals.

In conclusion, we found that H_2O_2 concentration in exhaled air condensate is significantly elevated during symptomatic upper respiratory tract infections, and returns to normal within 2 weeks. Thus, when evaluating exhaled H_2O_2 as a marker of chronic lower airway inflammation in asthma, measurements during or soon after symptomatic upper respiratory tract infection should be avoided.

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GENERAL DISCUSSION AND SUMMARY
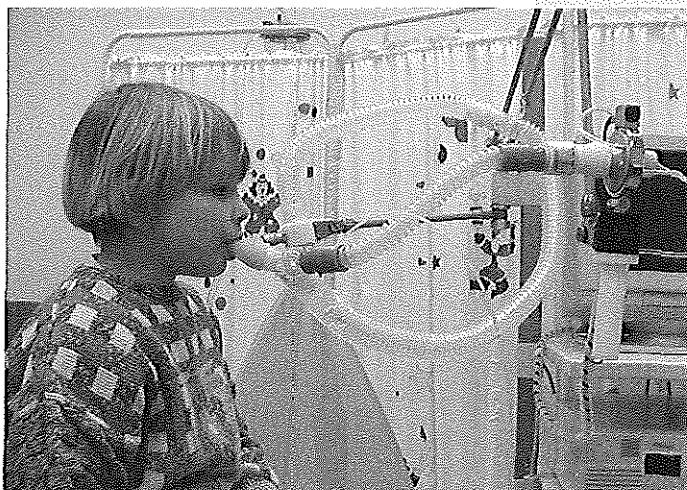
On-line measurement of exhaled NO as recommended by the ERS and ATS: single breath with a constant low flow against a resistance (anno 2000).

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CHAPTER 10

General discussion

A noninvasive test for the detection of airway inflammation promises to be far more practical in the clinical setting than currently available methods, such as bronchoscopy and BAL, and analysis of induced sputum. Potentially, these noninvasive tests will allow early and appropriate anti-inflammatory treatment, which hopefully will at least partly prevent later development of chronic respiratory diseases, and will help to avoid such treatment on patients whose symptoms are not related to airway inflammation, and who are unlikely to benefit.

10.1 NITRIC OXIDE IN EXHALED AIR

Since the beginning of eNO measurements, different methods of air sampling have been used, making it difficult to compare data. Thusfar, the differences in sampling techniques have limited the value of eNO measurements in the assessment of airway inflammation. Major methodological concerns, related to all sampling techniques, are the possible contamination with NO from the upper airways and from ambient air and the flow dependency of eNO. We and others have shown that eNO is flow dependent (1-3). Exhaled NO decreases with increasing flow, but mainly at flows of ≤150 mL/s, whereas flow dependency is much less at higher flows. NO is formed in the upper and lower respiratory tract and diffuses from the walls of the airways into the lumen by gaseous diffusion down a concentration gradient, thus conditioning exhaled air with NO (4). Since NO is continuously released into the airway lumen, high concentrations will result when exhalation is slow, and low concentrations when exhalation is fast. So, low flow rates amplify the NO signal. We also showed that differences in eNO between asthmatics, CF and healthy children are greatest at low flows (1). This means that to be able to compare eNO results of different studies and to maximize the sensitivity of eNO in discriminating between healthy subjects and those with airway inflammation, a standardized low exhalation flow rate must be agreed upon. Although several international guidelines for the measurement of NO in exhaled air have been published there is still no uniformity in the recommended expiratory flow rate (1-3,5,6). Not only the absolute eNO concentration at a constant low flow rate, but also the slope of the NO/flow relationship discriminates between asthmatics and healthy subjects or CF patients (1). For diagnostic purposes, eNO measurements at different flow rates may prove an additional new tool for the differential diagnosis of airway diseases.
Methods to control nasal NO contamination are important for standardization of the exhaled air sampling procedures. Exclusion of nasal NO is important in view of the high nasal NO values compared to the NO values of the lower airways. So, with nasal contamination the orally exhaled air may not adequately reflect the NO production in the lower airways. Exhalation against a resistance will create

positive mouth pressure. A positive mouth pressure of at least 4 cm H_2O is sufficient to close the soft palate and avoids nasal contamination, as validated by nasal Argon insufflation (7). The single breath profile shows in the initial part of the exhalation a peak in eNO, which corresponds with ambient NO accumulated in the dead space air volume, followed by a NO plateau. The effect of elevated ambient NO on on-line single breath end-expiratory plateau NO values is probably minor. However, high ambient NO values will prolong the exhalation time necessary to reach a plateau, which requires a manoeuvre that is difficult to perform, especially for children. If exhaled air is collected in a reservoir, during tidal breathing or single exhalation, the dead space air volume including ambient NO is also collected which may influence the measured eNO values. Indeed, we found a strong positive relation between ambient NO concentrations and eNO values in balloons, especially with ambient NO > 10 ppb (8). Therefore, during eNO measurements the ambient NO should be recorded, eNO should not be measured if ambient NO is above 10 ppb or alternatively, NO-free air may be inhaled before sampling. Comparison of these approaches has not yet been done.

There is a need for standardization of eNO measurement procedure in both adults and children if eNO measurements are to be applied in daily clinical practice and when comparing results from different research groups. In an attempt to standardize NO measurement in exhaled air, a European Respiratory Society (ERS) task force report was published (5). The on-line single breath method, exhalation from TLC with a constant flow against a resistance, was recommended for adults and children from the age of 6 years. However, we and others showed that children had problems in maintaining a constant exhalation flow, resulting in failure rates of up to 30% of the on-line single breath method in children (9-11). In contrast to the high failure rate of the on-line single breath method, the off-line single breath balloon method was performed without any difficulty by children (9,10). The off-line method is attractive when subjects are studied outside the hospital, where exhaled air samples can be obtained without the direct presence of a NO-analyser, which could be useful for epidemiologic studies and home monitoring of asthma. Balloons can be transported to the analyser and measured later. We found that NO remained stable in NO-inert Mylar-balloons for at least 6 hours (8). The flow during exhalation into the balloon can be standardized, by including a manometer and fixed resistor in the exhalation channel, or by a progressive resistance, or can be uncontrolled (9,12,13). We previously showed that off-line single breath sampling with uncontrolled high flows produces reproducible eNO values that are similar to those measured on-line single breath with constant flow (8). In the off-line balloon method, sufficient resistance should be present in the exhalation channel to create a positive mouth pressure of at least 4 cm H_2O to assure closing of the soft palate to avoid nasal contamination. By discarding the first part of the exhaled air volume in a separate balloon, it is

possible to exclude contamination with dead space air which consists of a mixture of ambient NO and upper and lower airway NO. In children we showed that the off-line eNO values with constant low flow, excluding dead space air volume, show excellent agreement with on-line values obtained with the same constant low exhalation flow (13). The off-line single breath balloon method with uncontrolled flow is a simple and hence attractive method for exhaled NO measurements with children older than 4 years. It differentiates between groups with and without self reported asthma, allergy and colds but is less suited for diagnostic use on individuals (8,13,14). In contrast, the controlled low flow off-line technique as described in chapter 5 is a more sensitive tool which should be able to detect meaningful within-subject changes of eNO.

There are only preliminary studies with small numbers regarding eNO measurements in infants and young children who have no ability to co-operate (15-18). However, especially in infants there is a need for developing noninvasive methods for the assessment of airway inflammation and to evaluate the effect of anti-inflammatory treatment. Wheezing in infants is a very common symptom, affecting 30 to 40% of all infants. However, wheezing infants are a heterogeneous group and different patterns of wheezing disorders exist (19). If eNO is validated as a reliable marker of airway inflammation in infancy, it might help to distinguish children with early-onset asthma from recurrent transient wheezing disorders without inflammation, and may be helpful to identify risk groups for airway inflammation in wheezing infants for appropriate early intervention with anti-inflammatory treatment. Exhaled air sampling in young unco-operative children and infants has been tried by using forced expiration and by collecting mixed exhaled air during quiet tidal breathing. With both sampling methods it was possible to measure NO in exhaled air. In recurrently wheezing infants, elevated eNO values, which decrease after corticosteroid therapy, were detected (15,16). It was recently shown, in a limited number of infants, that mixed expired eNO values collected off-line via a face mask in a reservoir were similar to eNO values obtained in the same children after tracheal intubation suggesting that contamination from the nose may not be as important as in older subjects (16). However, more data on reproducibility, normal ranges, nasal NO production and contamination are necessary to validate reliable sampling methods which can be used for the assessment of lower airway inflammation in infants who are unable to co-operate.

In children, we found that stable, treated asthmatics had significantly higher eNO values than healthy subjects, but only at low exhalation flows (1). Asthmatics using inhaled steroids tended to have lower eNO values than those without steroids. We found no significant difference in eNO values, obtained off-line with high flows, between stable asthmatic children using inhaled steroids and healthy children (8,9). These results are in agreement with eNO studies on adults,

where an increase in eNO in asthmatics has been reported in several studies and eNO has not increased in stable asthmatics treated with inhaled steroids. However, there is an increase in eNO values during asthma exacerbations and when the maintenance dose of inhaled steroids is reduced in well controlled asthmatic patients; eNO increased before an increase in symptoms or a fall in FEV_1 was seen (20-22). In conclusion, exhaled NO reflects airway inflammation, especially in asthma, and may therefore be useful as a noninvasive diagnostic method of asthmatic inflammation in the respiratory tract, but eNO may also be used as an early predictor of relapse after reducing anti-inflammatory treatment or as a measure of compliance with inhaled steroid treatment.

As stated in the general introduction of this thesis the ideal marker of airway inflammation should fulfil certain criteria. To what extent does exhaled NO meet the criteria of an ideal marker? Increased eNO values are not disease specific. There is persuasive evidence that eNO values are increased in association with (eosinophilic) airway inflammation and are decreased by anti-inflammatory treatments. Furthermore, there is now preliminary evidence that an increase in eNO may precede the increase in symptoms and the deterioration in lung function in asthmatics and thus may be one of the earliest signs of disease activity. However, validation against more direct (invasive) measurements of inflammation is needed. Despite the ongoing technical debate on how to measure NO in exhaled air, it is obvious that eNO measurement is an acceptable, simple, quick, reproducible, and a completely noninvasive method. Without any discomfort it can be performed repeatedly on children and adults, where more invasive techniques are impractical. Chemiluminescence NO-analysers are still expensive, but when using eNO in mass screening the costs are relatively low. In asthmatics, eNO is a sensitive and rapid marker of the anti-inflammatory effect of (inhaled) steroids and may so be useful in monitoring compliance with treatment.

10.1.2 DIRECTIONS FOR FUTURE RESEARCH

A rapidly growing body of literature supports the use of NO as an inflammatory marker in various respiratory disorders but especially in asthma. It may be useful in the assessment of airway inflammation and monitoring (compliance) anti-inflammatory treatment. Surprisingly the mechanisms underlying increased NO output from inflamed airways have not been studied in detail. Studies linking increased NO output to increased expression of iNOS in human airways are lacking. Therefore, it is important to validate the concept of increased iNOS expression in inflammation or to consider alternative explanations for increased NO output during airway inflammation. Also the clinical relevance of eNO remains to be validated. There is a need to further standardize the sampling technique, if eNO measurements are to be applied in the clinical setting, and for comparison of results from different laboratories. At the moment eNO

measurements are still a research tool. One can speculate that in the future hand–held portable NO–analysers may be available for home use as an easy, fast, and noninvasive way of daily monitoring airway inflammation; possibly leading to better individual adjustment of anti–inflammatory treatment dosage. But, before eNO measurements can be routinely applied in daily clinical practice the following issues have to be addressed:

- Standardization of on–line and off–line sampling techniques in adults and children, regarding mouth pressure, exhalation flow and dealing with ambient NO.
- Collection of normal reference values of eNO with the (different) standardized sampling techniques in different age groups.
- Examination of the influence of circardian rhytm on eNO is needed.
- Further development and standardization of sampling techniques in young unco-operative children and infants. Development of dynamic flow restrictor, and elimination of nasal NO contamination.
- Validation of eNO against more direct measurements of inflammation in the airways, such as obtained by bronchoalveolar lavage, bronchial biopsies, induced sputum, in different patient groups and healthy subjects is needed.
- Do markers of airway inflammation improve the management of various respiratory inflammatory diseases? What is the benefit of monitoring eNO as a response to treatment on top of clinical symptoms, lung function and hyperresponsiveness in terms of prevention of exacerbations and long-term reduction in chronicity of disease? These questions require long-term prospective studies with repeated measurements in individual subjects and correlation with clinical parameters.
- Predictive value of eNO with respect to benefits from anti–inflammatory treatment, monitoring compliance with treatments, and relapse of inflammation after reduction or termination of anti–inflammatory treatment.
- The sensitivity and specificity of eNO in the detection of different inflammatory diseases, including non–respiratory disorders.

10.2 HYDROGEN PEROXIDE IN EXHALED AIR CONDENSATE

The collection of exhaled air condensate is simple and noninvasive to perform. There is a variety of methods to collect exhaled air condensate, although they are fundamentally similar, based on condensation of exhaled air on a cold surface. A limitation is that the amount of condensate necessary for duplicate determination requires at least 10 minutes of quiet tidal breathing

through a mouthpiece, which is not feasible for most children younger than about 4 years. A point of concern is the potential possibility of contamination of the condensate by saliva, a rich source of proteins and inflammatory mediators such as reactive oxygen species and cytokines. As we showed, saliva contamination of the condensate sample can be verified by determining amylase concentration in the condensate (23). Amylase is present in saliva in high concentrations, whereas no traces could be detected in condensate samples and high amylase values in deliberately spiked condensate samples (23,24).

Hydrogen peroxide is a marker of oxidative stress in exhaled air condensate. We found elevated H_2O_2 values in exhaled air condensate of children with stable asthma, in CF-children with an acute infective pulmonary exacerbation, and in adults with symptomatic upper respiratory tract infections (25-27). In healthy children we found statisfactory within-subject reproducibility of repeated H_2O_2 measurements on 2 consecutive days (23). Furthermore, increased amounts of H_2O_2 have been described in various other respiratory disorders such as unstable asthma, COPD, bronchiectasis, and cigarette smokers (28-31). This means that elevated exhaled H_2O_2 values are not disease specific. Rather than a diagnostic test, exhaled H_2O_2 may be used to guide anti-inflammatory treatment and estimate inflammation severity over a period of time within subjects. Exhaled H_2O_2 values of asthmatic patients were correlated with induced sputum eosinophils and airway hyperresponsiveness (28). We found that in CF, exhaled H_2O_2 falls during antibiotic treatment, and that in asthmatics inhaled corticosteroids were associated with lower exhaled peroxide values (25,27). So, exhaled H_2O_2 may potentially serve as a noninvasive parameter of inflammation to guide anti-inflammatory treatment in inflammatory respiratory diseases. Different methods of measurement of peroxide in exhaled air condensate have been used by various research groups. Methods of H_2O_2 measurement are based on its ability to react with suitable reagents leading to colour, or fluorescence (32). Different reagents such as homovanillic acid (HVA), p-hydroxy phenylacetic acid (pHPAA) and tetramethylbenzidine (TMB), have been used. The measured H_2O_2 values are often at the lower limit of detection of the employed method in the different studies. Each method has a different lower limit of detection, by using different substrates, which makes direct comparison of results obtained with different methods difficult (33). The storage of condensate samples is also a point of concern. H_2O_2 will rapidly degrade at room temperature. Condensate samples should be collected on ice and after collection immediately frozen below -20°C until analysis. We found that the H_2O_2 concentration in frozen condensate samples remained stable for at least 1 month (28). The importance of collecting and storing the condensate sample in the dark to avoid break-down of H_2O_2 and the influence of the collecting surface of the sampling device on H_2O_2 concentration has not been evaluated. Another point which has not yet been evaluated, and

was not standardized in the different peroxide studies, is exhalation flow. This could critically influence the exhaled H_2O_2 concentration as it does in eNO measurement, and some preliminary evidence suggest that this indeed might be the case (R.A. Jörres, personal communication).

In contrast to NO, exhaled H_2O_2 is a far less studied marker of airway inflammation. Therefore, for H_2O_2 measurement in exhaled air condensate some criteria of the ideal marker of airway inflammation, mentioned in the introduction of this thesis, have not yet been evaluated. Collection of exhaled air condensate is a simple, feasible, and completely noninvasive method which can be performed repeatedly with minimal discomfort, by adults as well as co-operative children. The determination of peroxide in condensate is reproducible and can be done at relatively low costs. Compared to eNO techniques, the collection of condensate as well as the measurement of H_2O_2 in condensate takes considerably more time. An increased content of H_2O_2 in condensate has been reported in various respiratory disorders, which means that H_2O_2 is not a disease specific diagnostic test. Rather than a disease specific diagnostic test, H_2O_2 can be used in the noninvasive assessment of airway inflammation within subjects, and to monitor the effect of anti-inflammatory treatment. Whether exhaled peroxide values are sensitive enough to detect early stages and small changes of airway inflammation is unclear. Anyhow, validation of this marker against more direct (invasive) measurements of inflammation is necessary.

10.2.2 DIRECTIONS FOR FUTURE RESEARCH

Inflammatory markers in exhaled air condensate, such as H_2O_2, for the noninvasive assessment of lower airway inflammation may become useful when the following issues are addressed:

- Standardization of the collecting method of condensate, with exclusion of saliva contamination.
- Influence of the breathing pattern and exhalation flow on the concentration of the various potential markers of airway inflammation.
- Influence of the upper airways on the concentrations of the exhaled markers.
- Influence of the collecting surface (glass, teflon, plastic,metal) of the sampling device on marker detectability and concentration.
- Development of sampling technique of exhaled air condensate that is suitable for young children and infants.
- Standardization of the measurement procedures of inflammatory markers in condensate.
- Correlation of markers against direct measurements of airway inflammation (biopsies) in groups with various inflammatory respiratory disorders and in healthy subjects.

- Reproducibility, normal variation (interday and intraday) and reference ranges (age dependent) of the inflammatory marker.
- Evaluation of the sensitivity and specificity of the various markers in differentiating inflammatory airway disorders.
- Predictive value of the various exhaled inflammatory markers in condensate as a response to anti-inflammatory treatment, relapse of lower airway inflammation after reducing or stopping anti-inflammatory treatment, and monitoring compliance with treatment.
- Existance of specific patterns of different markers concentrations in the detection of different respiratory disorders.

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Summary

Airway inflammation plays a central role in various respiratory disorders. In daily clinical practice, assessment of airway inflammation is based on clinical symptoms and lung function measurements. Especially in young children, these indices of airway inflammation are less reliable and feasible than in adults. Anyhow, these indirect methods do not specifically measure degree of inflammation which may potentially lead to insufficient or unnecessary treatment. Bronchial biopsy, BAL, and induced sputum can be used to assess the presence of airway inflammation. These methods are invasive, making their routine use impractical. Thus, there is a need for noninvasive measurement of airway inflammation which will allow early and appropriate anti-inflammatory treatment in order to reduce morbidity and prevent or limited the development of chronic respiratory diseases. Exhaled air and breath condensate contain substances which can potentially be used in the assessment of airway inflammation. The use of exhaled air (condensate) for the assessment of airway inflammation has the great advantage of being noninvasive and thus feasible and suitable for repeated measures in adults and children. It offers the prospect of relating airway inflammation to clinical features, abnormalities of lung function, and treatment response in various respiratory disorders.

This thesis contains a number of studies exploring the relevance of exhaled air and breath condensate as vehicles of inflammatory markers, and addresses a number of methodological issues of their use.

Chapter 1 contains a general introduction to the thesis, with particular reference to the exhaled inflammatory markers NO and H_2O_2, and the aims of the studies and outlines this thesis.

In **chapter 2** we compared three different NO measurement methods in 101 stable allergic asthmatic children. The on-line single breath constant flow method recommended by a ERS task force was used as the gold standard and compared against an off-line uncontrolled high flow single breath balloon method and a tidal breathing method without expiratory resistance. Almost 30% of the study population was not able to perform the recommended on-line single breath method. All children performed the off-line single breath balloon method and tidal breathing method without any difficulty. Statisfactory agreement and no significant differences were found between the eNO values obtained with the on-line single breath method and the off-line single breath balloon method. In contrast, a poor agreement was found between the eNO values obtained with the on-line single breath method and the tidal breathing method, which was prob-

ably due to nasal NO contamination in the tidal breathing method. The within-method short term reproducibility of duplicate NO measurements obtained with the three different sampling methods was good. The off-line uncontrolled high flow single breath method is a simple and feasible method of measuring eNO in children, which has the important advantage that it offers independence from the presence of an NO-analyser.

The study in **chapter 3** tries to quantify the effect of varying the flow rate on the NO concentration in exhaled air in children and to establish the effect of different disease states on the NO/flow relationship. The eNO values were obtained with an on-line single breath constant flow method at four different flows in children with stable asthma (n=19), CF (n=10), and in healthy children (n=20). Exhaled NO decreased with increasing flow in all children. Children with asthma had significantly higher eNO concentrations than healthy children, but only at the lowest flows. Asthmatics using inhaled steroids tended to have lower eNO values than those without steroids. CF-patients had a significantly lower NO concentration over the entire studied flow range, compared to asthmatics and control subjects, with similar NO/flow slope as healthy subjects. The slope of NO/flow plots was significantly steeper in asthmatics than in healthy controls. The clinical implication of these results is that eNO measurement clearly distinguished between the different disease states at low flow rates, and that not only NO concentration at a fixed flow rate, but also the slope of the NO/flow relationship discriminates between asthmatics and healthy subjects or CF patients.

In **chapter 4** we studied methodological aspects of an off-line balloon method which could potentially influence eNO measurement. Wearing a noseclip or holding one's breath affect measured eNO values and should therefore be standardized or, preferably, avoided. We found a significant positive correlation between ambient NO concentrations and eNO values in balloons, especially with ambient NO > 10 ppb. This suggests that off-line balloon eNO sampling should not be done when ambient NO is above 10 ppb, or NO-free air may be inhaled before sampling. The stability of NO in Mylar-balloons was assessed and NO showed to remain stable for at least 6 hours. Furthermore, we defined a reference range of eNO obtained off-line with this balloon sampling method in a large group of healthy school-aged children (n=72). We detected significant differences between children with and without self-reported asthma, allergy and colds, suggesting that this sampling method of eNO measurement is sufficiently sensitive to detect minor degrees of airway inflammation in groups of otherwise healthy school-aged children.

We previously showed (chapter 2) that off-line sampling in balloons with high uncontrolled flows produces eNO values that are similar to those measured on-line with high flows. However, we also found (chapter 3) that low exhalation flow rates amplify the measured NO concentrations and allow a better separation

between asthmatics, CF-patients and healthy children. Therefore, we validated in **chapter 5** an alternative off-line single breath controlled low-flow balloon sampling method against on-line single breath controlled low flow sampling according to ERS guidelines. The off-line eNO values with constant low flow, and without dead space air, show excellent agreement to values obtained on-line with the same low exhalation flow, and are feasible in school-aged children. Both sampling methods discriminate between groups of children with and without self reported asthma, allergy and colds.

In **chapter 6** we defined the reference range of H_2O_2 in exhaled air condensate obtained from a large group of healthy school-aged children. The observed H_2O_2 levels were independent of age, sex and lung function. Furthermore, some methodological aspects were examined: the fluorimetric assay gave reproducible H_2O_2 results, the H_2O_2 concentration in frozen condensate samples remained stable for at least 1 month, and the determination of amylase in breath condensate is a sensitive marker to detect contamination of the condensate with saliva.

In **chapter 7** a cross-sectional study of 66 allergic asthmatic children (of whom 41 were treated with inhaled steroids) and 21 healthy controls showed that the concentration of H_2O_2 in exhaled air condensate is significantly increased in the asthmatic children, and the exhaled H_2O_2 values are lower in asthmatics who used inhaled steroids. These results support the hypothesis that exhaled peroxide reflects airway inflammation.

In the study described in **chapter 8** we monitored the exhaled inflammatory markers NO and H_2O_2 in CF-children (n=16) with an acute infective pulmonary exacerbation during intravenous (i.v.) antibiotic treatment. We found that the levels of exhaled H_2O_2 are moderately elevated in CF-children with an exacerbation of their lung infection and decrease significantly during i.v. treatment. The lung function of those CF-children improved significantly during antibiotic treatment. In individual subjects, there was a weak negative correlation between changes of H_2O_2 and FEV_1. Exhaled NO did not change systematically during antibiotic treatment. So, exhaled H_2O_2 may be a helpful marker to monitor oxidative stress due to airway inflammation and may serve as a noninvasive parameter of inflammation during antibiotic treatment in CF lung disease.

The study of **chapter 9** showed that symptomatic upper respiratory tract infections are associated with elevated levels of exhaled peroxide in otherwise healthy subjects. After recovery, exhaled H_2O_2 had decreased to values similar to those of an age-matched control group. There was no significant change in lung function data during and after upper respiratory tract infection. Thus, upper respiratory tract infections increases production of H_2O_2 in the respiratory tract, and may be misleading in studies using exhaled peroxide as a marker of lower airway inflammation e.g. in asthma. Two weeks was sufficient for peroxide levels to fall to normal values after upper respiratory tract infection.

The measurement of exhaled NO and H_2O_2 may provide a simple, noninvasive means of measuring inflammation and oxidative stress in the respiratory tract. These markers may be useful in the assessment of airway inflammation and monitoring of anti-inflammatory treatment. Indeed increased exhaled NO and H_2O_2 values are associated with various respiratory disorders, and reduced by anti-inflammatory treatment. However, levels of these exhaled inflammatory markers varied widely in similar patient groups and even in healthy subjects, probably due to differences in methodology used by the different research groups. So there is a need for standardization of the measurement procedures of NO and H_2O_2, to enable accurate comparison of results from different research groups, and if these markers are going to be applied in daily clinical practice. Furthermore, the clinical relevance of both exhaled markers needs to be established in longitudinal studies in various respiratory disorders, with repeated measurements in individual patients. Correlation with clinical parameters and more direct, invasive, measurements of airway inflammation is needed.

Samenvatting

Luchtwegenontsteking wordt aangetroffen bij verschillende respiratoire aandoeningen en kan leiden tot diverse klachten en symptomen. In de dagelijkse praktijk wordt een ontstekingsproces in de luchtwegen vermoed op grond van anamnese, symptomen, en longfunktie onderzoek. Bij kinderen die te jong zijn voor longfunktie-onderzoek is de (waarschijnlijkheids)diagnose derhalve alleen te stellen aan de hand van symptomen en (hetero)anamnese. Alvorens een langdurige ontstekingsremmende therapie, bijvoorbeeld met inhalatie-steroïden, bij (jonge) kinderen gestart wordt zou de aanwezigheid van luchtwegontsteking moeten worden geobjectiveerd. Hiertoe zou het ontstekingsproces in de luchtwegwand direct moeten worden aangetoond, hetgeen in de praktijk problematisch is daar dit bronchoscopie en biopsie van de mucosa of bronchoalveolaire lavage vereist. Met name door de invasiviteit zijn deze onderzoek-methodieken dusdanig belastend dat ze niet routinematig in de dagelijkse praktijk toepasbaar zijn. Er is derhalve behoefte aan een eenvoudige en niet invasief te bepalen maat voor luchtwegontsteking, enerzijds als diagnosticum voor een ontstekingsproces, en anderzijds om de behandeling met ontstekingsremmende medicatie te sturen. Hiermee kan tijdig een gerichte ontstekingsremmende therapie gestart worden, met de veronderstelling dat zodoende morbiditeit en ontwikkeling van chronisch respiratoire aandoeningen afnemen, en dat onnodige ontstekingsremmende behandeling voorkomen wordt. Een nieuwe benadering voor het aantonen van luchtwegontsteking is onderzoek van uitgeademde lucht, waarin kleine hoeveelheden voorkomen van stoffen die vrijkomen bij ontsteking en derhalve potentieel zouden kunnen fungeren als indicatoren van luchtwegontsteking. Voor (jonge) kinderen lijkt uitademingslucht een bijzonder geschikt medium te zijn voor het bestuderen van luchtwegontsteking, daar het zonder bijzondere coöperatie op niet invasieve wijze te verkrijgen is en verder niet afhankelijk is van de techniek van longfunctieonderzoek.

Het onderzoek in dit proefschrift is verricht met het oogmerk een niet-invasieve methode te ontwikkelen, die potentieel bij jonge kinderen toepasbaar is, waarmee de aanwezigheid van een ontstekingsproces in de bronchiaalboom kan worden aangetoond. Twee benaderingen zijn bestudeerd:

1. Meting van stikstofmonoxide (NO) in uitademingslucht.
2. Bepaling van waterstofperoxide (H_2O_2) in uitademingsluchtcondensaat.

Hoofdstuk 1 plaatst het onderwerp van het proefschrift in perspectief, en richt zich met name op de potentiële rol van NO en H_2O_2 in uitademingslucht als indicatoren van luchtwegontseking op de kinderleeftijd. Verder worden in dit hoofdstuk de doelstellingen en een overzicht van de verschillende onderzoeken, zoals beschreven in de daarop volgende hoofdstukken, vermeld.

In de studie beschreven in **hoofdstuk 2** werden bij 101 kinderen met stabiel allergisch astma, 3 verschillende uitademingslucht-opvangtechnieken, die gebruikt worden om NO in uitademingslucht te meten, vergeleken. De door een ERS werkgroep aanbevolen directe opvangtechniek, één uitademing met een constante uitademingssnelheid (flow) direct in het NO-meetapparaat, werd vergeleken met de ballon methode, waarbij de lucht van één uitademing opgevangen wordt in een ballon waarna in een later stadium analyse van de NO concentratie in de ballon plaats vindt, en met de rustademhaling methode, waarbij de gemiddelde NO concentratie in uitademingslucht opgevangen in een omschreven tijdsinterval gemeten wordt. Bijna 30% van de kinderen van de studiepopulatie was niet in staat de door de ERS aanbevolen techniek op een juiste wijze uit te voeren terwijl de andere 2 opvangtechnieken in de uitvoering geen problemen opleverden. De NO waarden in uitademingslucht gemeten middels de door de ERS aanbevolen directe methode vertoonden een goede overeenkomst, zonder significante verschillen, met de NO waarden verkregen via de ballon methode met een hoge ongecontroleerde flow. Een slechte overeenkomst, met significante verschillen, werd gevonden tussen de NO waarden van de directe (ERS) methode en die van de rustademhaling methode, waarbij de NO waarden van de rustademhaling methode konsekwent hoger waren. Dit berust naar alle waarschijnlijkheid op bijmenging van nasale lucht, die een evidente hogere NO concentratie heeft, aan de orale uitademingslucht hetgeen tijdens rustademhaling, door wisselende monddruk, makkelijk plaats vind. De korte termijn reproduceerbaarheid van herhaalde NO metingen van de drie afzonderlijke opvangmethodieken was goed. De ballon methode is een eenvoudige en makkelijk uitvoerbare techniek om NO in uitademingslucht van kinderen te meten, en heeft als bijkomend voordeel dat de meting niet gebonden is aan de plaats van de NO meetapparatuur.

In **hoofdstuk 3** wordt, met de door de ERS aanbevolen directe methode met vier verschillende flows, de invloed van de flow op de uitgeademde NO concentratie bestudeerd bij kinderen met astma (n=19), cystic fibrosis (CF) (n=10) en bij gezonde kinderen (n=20). Bij alle onderzochte kinderen blijkt de uitgeademde NO concentratie flow afhankelijk te zijn; met toenemende flow neemt de uitgeademde NO concentratie af. Verder werd gevonden dat, bij lage flows, de kinderen met astma significant hogere uitgeademde NO waarden hadden dan de gezonde kinderen. Binnen de groep kinderen met astma was er een trend zichtbaar waarbij de kinderen met inhalatiesteroïden lagere NO waarden hadden dan

degene zonder inhalatiesteroiden. De kinderen met CF hadden over het hele bestudeerde flow traject significant lagere NO waarden in vergelijking met de gezonde groep, echter de NO/flow helling van beide groepen was vergelijkbaar. De NO/flow helling van de astma groep liep significant steiler dan van de gezonde groep. De klinische relevantie van de resultaten van deze studie is dat bij lage flows het onderscheidend vermogen van uitgeademde NO, tussen verschillende aandoeningen, het grootste is en dat niet alleen absoluut uitgeademde NO waarden maar ook de NO/flow helling een onderscheid kan maken tussen kinderen met astma enerzijds en gezonde of kinderen met CF anderzijds.

Hoofdstuk 4 is gewijd aan NO-metingen middels de kindvriendelijke ballon methode. Met deze methode werd in een grote groep gezonde schoolkinderen (n=72) normaalwaarden bepaald. Verder werden een aantal methodologische aspecten van deze specifieke methode bestudeerd. We vonden significante verschillen in uitgeademde NO waarden tussen gezonde schoolkinderen en schoolkinderen met zelf gerapporteerd astma, allergie en of verkoudheids verschijnselen, hetgeen aangeeft dat deze methode gevoelig genoeg is om onderscheid te maken tussen geringe verschillen in inflammatie binnen een verder gezonde groep kinderen. Het gebruik van een neusklem alsmede het inhouden van de ademhaling beïnvloedt de uitgeademde NO waarden en dient derhalve vermeden te worden of anders ten minste gestandaardiseerd te worden. Er bestaat een significant positieve correlatie tussen de uitgeademde NO concentraties en de NO concentratie van de ingeademde omgevingslucht, met name bij omgevingslucht met NO concentraties boven de 10 ppb. Dit impliceert dat NO metingen via de ballon methode in principe niet goed bruikbaar zijn tijdens perioden met NO concentraties boven de 10 ppb in de buitenlucht. Een mogelijkheid om dit probleem te omzeilen zou het inademen van lucht met een lage NO concentratie kunnen zijn alvorens de NO concentratie in uitademingslucht te bepalen. De NO concentratie was gedurende ten minste 6 uur stabiel in de door ons gebruikte Mylar-ballonnen.

Een eerdere studie (hoofdstuk 2) toonde dat de ballon methode met hoge ongecontroleerde flow geen significant verschillende NO waarden geeft ten opzichte van de directe (ERS) methode. Echter, uit een andere studie (hoofdstuk 3) bleek dat met name bij lage flows het onderscheidend vermogen van uitgeademde NO, tussen verschillende aandoeningen, het grootste is. Derhalve werd in **hoofdstuk 5** een alternatieve ballon methode met drie verschillende gestandaardiseerde laag gecontroleerde flows, in- en exclusief dode ruimte lucht, vergeleken met de directe (ERS) methode. De ballon methode met lage gecontroleerde flow zonder dode ruimte is een techniek waar de onderzochte kinderen geen moeite mee hadden en leverde gelijkwaardige NO waarden in uitademingslucht op in vergelijking met de directe (ERS) methode bij gelijke flow. De NO waarden van beide vergeleken technieken maakte een goed signifi-

cant onderscheid tussen enerzijds gezonde kinderen en anderzijds kinderen met zelf gerapporteerd astma, allergie en of verkoudheid tijdens de meting.

In **hoofdstuk 6** wordt een methode beschreven om H_2O_2 in uitademingslucht-condensaat te meten. Verscheidende methodologische aspecten van deze methodiek werden verder onderzocht. Gevonden werd dat de korte termijn reproduceerbaarheid van de H_2O_2 bepaling goed is, dat contaminatie van condensaat met speeksel eenvoudig aan te tonen is middels het bepalen van amylase in het condensaat en dat in diepgevroren condensaatmonsters de H_2O_2 concentratie gedurende ten minste 1 maand stabiel blijft. Verder werd met deze methode in een grote groep gezonde schoolkinderen normaalwaarden bepaald. De H_2O_2 waarden waren in deze groep onafhankelijk van leeftijd, geslacht en longfunktie.

In het cross-sectioneel onderzoek van **hoofdstuk 7** werd bij 66 kinderen met allergisch astma, waarvan 41 inhalatie-steroïden gebruikten, en bij 21 gezonde controles de H_2O_2 concentratie in de uitademingslucht gemeten. De H_2O_2 concentratie was bij de kinderen met astma significant verhoogd in vergelijking met de gezonde controle groep. Dit verschil was het meest uitgesproken bij de kinderen met astma die geen inhalatie-steroïden gebruikten. Deze resultaten steunen de veronderstelling dat H_2O_2 in uitademingslucht gebruikt zou kunnen worden om op een niet invasieve wijze ontsteking in de luchtwegen aan te tonen. Naast het aantonen van een ontstekingsproces in de luchtwegen zouden zowel H_2O_2 als NO in uitademingslucht ook een rol kunnen spelen bij het in de tijd vervolgen van het effect van behandeling op een ontstekingsproces. In **hoofdstuk 8** vervolgden we het beloop van uitgeademde NO en H_2O_2 concentraties bij 16 kinderen met CF, die waren opgenomen vanwege een pulmonale exacerbatie op basis van een bacteriële infectie voor een intraveneuze behandeling met antibiotica. De H_2O_2 concentratie in uitademingsluchtcondensaat daalde significant gedurende de intraveneuze behandeling met antibiotica terwijl de longfunctie significant verbeterde. Binnen personen bestond er een zwak negatieve correlatie tussen veranderingen in H_2O_2 en FEV_1. In tegenstelling tot H_2O_2 toonde de uitgeademde NO concentraties geen systematische veranderingen gedurende de behandeling met antibiotica. Derhalve heeft NO geen plaats bij het op een niet invasieve wijze vervolgen van het effect van antibiotica op het ontstekingsproces bij CF patiënten.

In **hoofdstuk 9** toonden we aan dat tijdens een symptomatische bovenste luchtweginfectie bij normaal gesproken gezonden, de H_2O_2 concentratie in uitademingsluchtcondensaat significant verhoogd is in vergelijking met een gezonde controle groep. Twee weken na herstel waren de H_2O_2 waarden weer gedaald tot het zelfde niveau als van de gezonde controle groep, terwijl in de gezonde controle groep geen significante verandering optrad in uitgeademde H_2O_2 waarden over een periode van twee weken. De longfunktiewaarden waren in

beide groepen vergelijkbaar en zonder significante verandering over de twee weken. Een bovenste luchtweginfectie beinvloedt dus de H_2O_2 concentratie in uitademingslucht en derhalve dient hiermee rekening te worden gehouden bij het gebruik van H_2O_2 als indicator van ontsteking in de onderste luchtwegen.

Zowel bij kinderen als bij volwassenen is het meten van NO en H_2O_2 in uitademingslucht een in potentie eenvoudige en niet invasieve methode om luchtwegontsteking vast te stellen. Naast het diagnosticeren van ontsteking zouden deze merkstoffen ook een rol kunnen hebben bij het vervolgen en beoordelen van het effect van ontstekingsremmende medicatie. Het blijkt inderdaad dat bij verschillende respiratoire aandoeningen de NO en H_2O_2 concentraties in uitademingslucht verhoogd zijn en dat ontstekingsremmende medicatie de uitgeademde waarden significant laat dalen. Echter resultaten van verschillende onderzoeksgroepen laten zowel voor uitgeademde NO als voor H_2O_2, waarden zien die zelfs bij gezonden uiteenlopen. Dit berust waarschijnlijk op methodologische verschillen, zoals onder andere verschillen in opvangtechniek van uitademingslucht. Om uitkomsten van verschillende studies met elkaar te kunnen vergelijken dient standaardisatie van de opvang- en meettechniek plaats te vinden. Voordat deze niet invasieve methode in de dagelijkse praktijk gebruikt kan worden zal verdere validering van deze markers plaats moeten vinden door correlatie met symptomen en longfunktiewaarden maar met name met uitkomsten van invasieve metingen van luchtwegontsteking zoals mucosa biopsie. De exacte klinische waarde van de bepaling van beide markers in uitademingslucht op lange termijn is nog onvoldoende duidelijk, hetgeen in longitudinale studies met herhaalde metingen binnen personen met verschillende luchtwegaandoeningen verder zal moeten worden vastgesteld.

.

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Curriculum vitae

De schrijver van dit proefschrift werd op 29 januari 1961 geboren te Oegstgeest. In 1979 behaalde hij het VWO-diploma aan het Snellius lyceum te Amstelveen. In hetzelfde jaar startte hij met de studie Geneeskunde aan de Universiteit van Amsterdam, alwaar hij in 1984 het doctoraalexamen en in 1987 het artsexamen behaalde. In 1988 was hij werkzaam als arts-assistent op de afdeling neurochirurgie van het Slotervaart ziekenhuis te Amsterdam (hoofd: dr. B. Matricali). In 1989 werd hij arts-assistent kindergeneeskunde in het Sophia kinderziekenhuis te Rotterdam, alwaar hij vanaf oktober 1989 in opleiding was tot kinderarts (opleider: Prof.dr. H.K.A. Visser). Het laatste jaar van de opleiding werd in het Juliana kinderziekenhuis te Den Haag volbracht (opleider: Prof.dr. A.J. van der Heijden). Van 1993 tot en met 1997 was hij medisch coördinator en begeleidend (kinder)arts van het kinderoncologisch skikamp "Winterkolder". In oktober 1994 werd hij als kinderarts geregistreerd. In de periode oktober 1994 tot eind 1997 volgde, middels een fellowship van het Nederlands Astma Fonds, verdere subspecialisatie in de kinderlongziekten op de afdeling kinderlongziekten van het Sophia kinderziekenhuis te Rotterdam (hoofd: Prof. dr. J.C. de Jongste). In deze periode werd tevens het onderzoek verricht dat de basis vormde voor dit proefschrift. Sinds 1998 is hij werkzaam als kinderarts-pulmonoloog bij de vakgroep kindergeneeskunde van het academisch ziekenhuis Maastricht. Hij is gelukkig getrouwd met Debby Vosse en is de trotse vader van Noor (5 jaar) en Huygen (3 jaar).

Quirijn Jöbsis [kinderarts-pulmonoloog]

Werkadres:
academisch ziekenhuis Maastricht (azM)
afdeling Kindergeneeskunde
Postbus 5800
6202 AZ Maastricht
E-mail: rjo@skin.azm.nl
Website: www.jobsis.nl