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Towards Improved Diagnostics and Monitoring in Childhood Asthma

*Methodological and Clinical Aspects of Exhaled NO
and Forced Oscillation Technique*

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ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2016

ISSN 1651-6206
ISBN 978-91-554-9710-1
urn:nbn:se:uu:diva-304264

Dissertation presented at Uppsala University to be publicly examined in Robergsalen, Akademiska sjukhuset, Ingång 40, 4tr, Uppsala, Friday, 25 November 2016 at 13:00 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English. Faculty examiner: Lauri Lehtimäki (Faculty of Medicine, University of Tampere).

Abstract

Heijkenskjöld Rentzhog, C. 2016. Towards Improved Diagnostics and Monitoring in Childhood Asthma. Methodological and Clinical Aspects of Exhaled NO and Forced Oscillation Technique. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1262. 79 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-554-9710-1.

Background: Asthma is a heterogeneous disease. Diagnosis relies on symptom evaluation and lung function tests using spirometry. Symptoms can be vague. Spirometry is effort-dependent and does not reliably evaluate small airways. Allergic asthma in preschool children is not easily separated from episodic wheeze.

Exhaled NO (FeNO) is a marker of allergic Th2-cytokine-driven airway inflammation. However, FeNO is not feasible in preschoolers with current devices and algorithms. Alveolar NO is an estimate of small airway involvement. Forced oscillometry (FOT) is an effort-independent lung function test assessing both large and small airways.

Aims: To study clinical and methodological aspects of FeNO, alveolar NO and lung function indices by FOT.

Methods: Asthmatic children and young adults and healthy controls, were included in the studies. FeNO at 50 mL/s was performed in all studies (in study III with an adapted single-breath method with age-adjusted exhalation times). FeNO at multiple exhalation flow rates were performed in studies I, II and IV to calculate alveolar NO, as was spirometry. FOT indices were assessed in study IV.

Results: The exhalation time needed to reach steady-state NO was < 4 s in subjects aged 3-4 years, and was related to subject height. FeNO was higher in ICS-naïve asthmatic children than in controls. ICS-naïve asthmatic preschool children had FeNO < 20 ppb. The oral contribution to FeNO was similar in asthmatic and healthy youths. Multiple flow rates and modelling of alveolar NO were feasible in children aged 10-18 years. Alveolar NO correlated to asthma characteristics, though not when axial diffusion correction was applied. FOT resistance measures were associated with asthma diagnosis, and small airway FOT measures were associated with asthma control, in adolescents.

Conclusion: An adapted FeNO method is feasible from 4 years, and exhalation time is related to child height. Our findings emphasise the need to refine clinical cut-offs for FeNO in younger children. FOT variables discriminate between asthmatics and controls, much like spirometry. The information provided by FOT is additive to that from spirometry. Further studies of exhaled NO dynamics and FOT indices of small airways are warranted to evaluate new treatment options and possibly improve asthma control.

Keywords: asthma, children, exhaled NO, forced oscillation technique, airway inflammation, small airways, asthma diagnostics

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ISSN 1651-6206

ISBN 978-91-554-9710-1

urn:nbn:se:uu:diva-304264 (<http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-304264>)

bright eyes guiding thy fire

To our three diamonds

Arvid Erik Ärna

List of Papers

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

- I Heijkenskjöld-Rentzhog C, Alving K, Kalm-Stephens P, Lundberg JO, Nordvall L, Malinovski A. (2012) The fraction of NO in exhaled air and estimates of alveolar NO in adolescents with asthma: methodological aspects. *Ped Pulm*; 47(10): 941-949.
- II Heijkenskjöld-Rentzhog C, Nordvall L, Janson C, Borres M.P, Alving K, Malinovski A. (2014) Alveolar and exhaled NO in relation to asthma characteristics - effect of correction for axial diffusion. *Allergy*; 69(8): 1102-1111.
- III Heijkenskjöld-Rentzhog C, Kalm-Stephens P, Nordvall L, Malinovski A, Alving K. (2015) New method for single-breath fraction of exhaled nitric oxide measurement with improved feasibility in preschool children with asthma. *Ped All Immun*; 26(7): 662-667.
- IV Heijkenskjöld-Rentzhog C, Janson C, Berglund L, Borres M.P, Nordvall L, Alving K, Malinovski A. Overall and peripheral lung function assessment by spirometry and forced oscillation technique in relation to asthma diagnosis and control. *Submitted for publication*.

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Abbreviations

ACT	Asthma Control Test
ATS	American Thoracic Society
AUC	Area Under the Curve
Cal _{VNO}	estimated alveolar nitric oxide concentration
CHX	chlorhexidine
ERS	European Respiratory Society
FEF ₅₀	forced expiratory flow at 50 % of vital capacity
FEV ₁	forced expiratory volume in 1 second
FeNO	fractional exhaled nitric oxide
FOT	forced oscillation technique
F _{res}	resonance frequency
FVC	forced vital capacity
ICS	inhaled corticosteroid
IgE	immunoglobulin E
IOS	impulse oscillometry
iNOS	inducible nitric oxide synthase
J' _{awNO}	bronchial NO flux
LLN	lower limits of normal
LTRA	leukotriene receptor antagonist
MIDAS	Minimally-Invasive Diagnostics for Asthma and allergic diseaseS
NO	nitric oxide
PD ₂₀	cumulative methacholine dose causing 20 % fall in FEV ₁
R ₅	mean total respiratory resistance at 5 Hz
R ₁₉	mean total respiratory resistance at 19 Hz
R ₅ -R ₁₉	resistance difference between 5 Hz and 19 Hz
ROC	Receiver Operating Characteristic
Th2	T-helper cell type 2
TMAD	trumpet model and axial diffusion
X ₅	mean total respiratory recatance at 5 Hz

Introduction

Asthma; definition and burden of disease

Asthma is a heterogeneous disease, defined by a history of obstructive respiratory symptoms that vary over time and in intensity, and by variable expiratory airflow limitation. The underlying disease process is usually characterised by chronic airway inflammation (1).

Behind this description various asthma subtypes are found, with regard to clinical expression, inflammatory processes and response to treatment. In childhood asthma different classifications, based on age at onset, duration of disease and atopic status, have been suggested and these are partly overlapping. This emphasises the difficulty of diagnosis, treatment guidance and prognostic assessment, especially in preschool children (2-5).

In order to address these differences, asthma is suggested to be used as an umbrella term, like arthritis or anaemia (6).

Asthma is among the most common chronic diseases in childhood, and among the top ten chronic conditions in ages 10-19 years regarding disability-adjusted life years worldwide (7). In Sweden, studies have reported on a prevalence of 6-7 % in preschool to early school age (8, 9) and 11 % in 12-year-old children (10). Globally, the overall prevalence of current symptoms of asthma in both age groups is reported to be somewhat higher than the asthma prevalence in Sweden. There is a wide variability among countries, regions and sites in the prevalence reported (11).

Despite availability of effective medication, many children have inadequately controlled asthma (12, 13). On the other hand, significant and costly over-diagnosis of asthma needs to be minimised (14, 15).

Asthma diagnosis in childhood

The diagnosis of asthma is based on evaluation of symptoms and lung function tests (1). Physical examination often yields normal results, unless performed during exacerbation. Also, allergy testing is recommended. In lung function assessment, variable airflow limitation can be detected, either by reversibility with response to bronchodilation or day-day variation in lung

function, or sustained improvement after introduction of anti-inflammatory controller therapy (1).

The parameters used in standard spirometry are forced expiratory volume at 1 second (FEV_1) and the ratio FEV_1 / forced vital capacity (FVC). Peak expiratory flow is still used, but is a less reliable measure than FEV_1 (16). Recently, the Global Lung Initiative has presented reference equations that span all ages and the use of z-score is suggested instead of % predicted values for FEV_1 and FEV_1/FVC , to take age-related differences in variance into account (17, 18). These are yet to be clinically implemented.

In the diagnostic procedure of childhood asthma though, gold standard spirometry is a somewhat blunt tool. Children below 6 years of age are often unable to perform reliable forced exhalation manoeuvres, and short-term variability is relatively large in early school age, especially in spirometry test-naïve children (19). Asthmatic children often have normal lung function assessed by FEV_1 (20). FEV_1/FVC and also mid-expiratory flow rate are dependent of complete continuation of exhalation.

In older children assessment of bronchial hyperresponsiveness by pulmonary function provocation tests is useful when the initial lung function assessment is inconclusive.

Differential diagnoses of chronic respiratory symptoms in childhood, such as upper respiratory disease, foreign body, gastroesophageal reflux disease, cystic fibrosis, anatomical anomalies and ciliary dyskinesia, need to be considered in the diagnostic procedure.

Once the diagnosis of asthma is made, lung function assessment is regarded useful as an indicator of exacerbation risk (1, 21).

In monitoring the disease, asthma control is a central concept. The goal of asthma treatment is to achieve and maintain asthma control with regard to both symptoms and future risks, at the lowest needed treatment line. Symptom control, preferably assessed over 4 weeks, is defined by the extent of symptoms day and night, the need for reliever medication and the impact on daily activities (1). The long-term risk of asthma includes asthma attacks, impairment of lung function, and medication side effects.

Disease severity is expressed in retrospect and covers the level of medication needed to obtain asthma disease control. International recommendations on asthma monitoring in childhood are presented (1, 22).

Rationale for improved asthma diagnostics

In many cases, asthma can be easily diagnosed and controlled by standard clinical routine, but still under- and overtreatment both remain as problems (1, 14, 23). In preschool age, the diagnosis of asthma relies heavily on symptom evaluation and a confident diagnosis of asthma may be difficult. Many

patients with a diagnosis of asthma in primary care cannot be confirmed as having asthma (24, 25). On the other hand, asthmatic children might have normal lung function between exacerbations, as assessed through spirometry (1, 20).

Two aspects of the asthma syndrome that are incompletely covered by the recommended diagnostic routine are the determination of the underlying inflammatory subtype to complement lung function assessment, and also the assessment of small airway engagement in the disease.

A potential health economic gain might be accomplished with improved clinically useful diagnostic tools, both in childhood asthma standard care and for the subgroup of severe and treatment-refractory asthma. This subgroup composes a significant individual and socioeconomic burden, although it is relatively small.

Inflammatory subtypes related to treatment decision

Inflammation of the airways is a central aspect of asthma, and inhaled corticosteroid (ICS) treatment is the mainstay of controller treatment. By means of sputum inflammatory cell count, asthma can be divided into eosinophilic neutrophilic, mixed granulocytic and paucigranulocytic airway inflammation. However, inflammatory subtyping assessed by sputum analysis is not necessarily stable over time (26). The eosinophilic airway inflammation that is driven by T-helper cell type 2 (Th2) cytokines, such as interleukin 4 and 13, has been shown to respond well to inhaled corticosteroid treatment (27, 28). As for the Type 2-cytokine-low inflammatory subtypes (neutrophilic or paucigranulocytic), the advantage of anti-inflammatory treatment is not convincing (2, 29, 30). To complicate the picture, in some cases eosinophilic airway inflammation is not clearly allergen-driven, possibly systemic in origin and/or driven by another airway inflammatory signalling route than Th2-cytokines.

To determine the underlying inflammatory subtype before treatment decision would be helpful, especially in preschool age where most cases of suspected asthma are viral wheeze with an underlying neutrophilic inflammation. In order to evaluate treatment regimens and disease progress, an assessment of the actual underlying airway inflammation is also desirable as complementary information at least, in addition to symptom reporting and lung function test also in older children (31).

Clinical standard practice is, at best, guided by allergy screening in this matter. However, the presence of a positive allergy test, allergic sensitisation, does not necessarily prove that the allergen in question is actually causing airway inflammation and respiratory symptoms.

Easy and reliable inflammatory markers of presence, magnitude and subtype of underlying airway inflammation could also help to differentiate allergic airway inflammation from other possible causes of respiratory disease.

Inflammatory biomarkers in sputum, blood and urine have been investigated. A recent ERS Task Force review summarises available non-invasive markers of airway inflammation (exhaled nitric oxide, sputum analysis and exhaled breath condensate) for monitoring asthma in childhood (32).

Studies in asthma have investigated biomarkers such as, for example, sputum eosinophils (33, 34), blood eosinophils (35), sputum interleukin-13 and -26 (36, 37), eosinophil cationic protein (ECP) in serum and sputum (38), serum periostin (39) and urinary leukotriene E4 (40). Some of these measures provide information related to systemic inflammation, which is of value in understanding the asthma syndrome, but none of these methods have yet been proven useful in a broad clinical setting.

Lung function and small airways

Asthma was classically considered to be primarily a disease of the large airways (see Figure 1). Airway generations 4-8 account for most of the resistance to flow. Changes in this region such as constriction of airway wall, excessive mucus production and wall thickening due to inflammation can be mirrored as airflow limitation by spirometry measures.

The small airways, defined as non-cartilaginous airways with an internal diameter < 2 mm (i.e., from airway generation ~9 and below) (Figure 1), normally contribute around 10 % of the total airway resistance to flow in adults (41), perhaps slightly more in the first years of life (42). It has been estimated that 75 % of all small airways need to be obstructed before changes can be detected in FEV₁ through standard spirometry (43). An inflammatory obstructive process might exist in the small airways without being caught by standard spirometry. Mid-expiratory flow measures in spirometry are sometimes used to assess small airways, but this is discouraged by the ATS due to low reproducibility and the influence of large airway obstruction (44, 45).

From lung biopsy findings and through the use of endobronchial catheter technique, advanced imaging and immunohistochemical techniques, the distal lung region has been highlighted as an important site of pathophysiology in asthma (46-49). Evidence indicates that small airway processes is associated with asthma control (46, 50) and that small airway engagement is present in asthma of all severities (49).

Since most inhalation devices are unable to deliver particles as far down as the small airways (51-53), a peripheral disease process might not be controlled by conventional inhaled corticosteroid treatment. With the availability of new extra-fine drug formulations which can potentially reach farther into the airways (54-56), clinically useful markers of small airway inflammation and obstruction in asthma would be needed to guide treatment decisions.

If small airway disease can be estimated and controlled, a subset of individuals with uncontrolled disease might benefit.

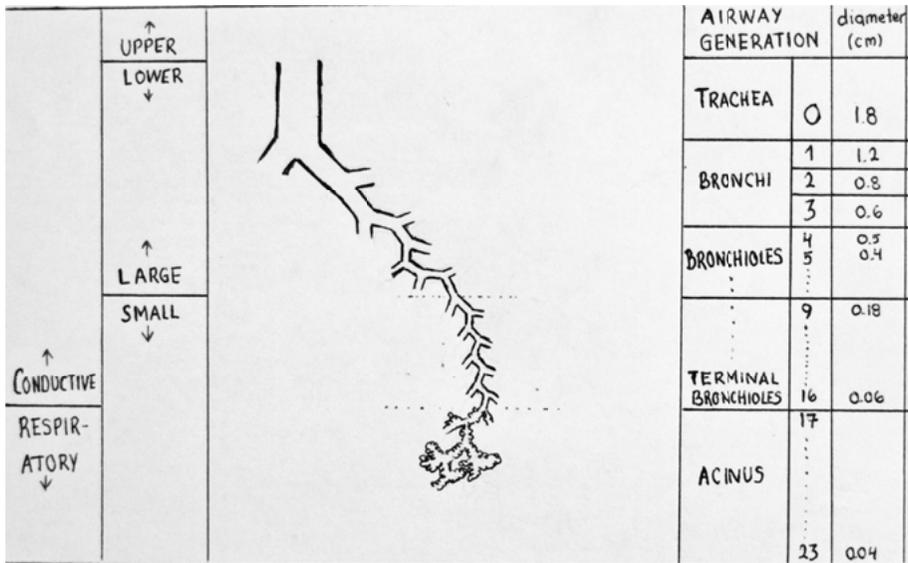


Figure 1. Lower airways; large and small airways, airway generations and approximated airway internal diameter at generation level. (Adapted from Weibel, 1963)

The recent ERS Task Force review also summarises methods for measuring of lung function (spirometry, resistance methodologies, inert gas wash-out tests) in childhood asthma (32).

Forced oscillation technique can provide measures of lung mechanics and distal lung pathophysiology. Inert gas wash-out tests can give estimates of closing volume (i.e., the volume at which small airway close) and the regional heterogeneity of airflow that may add valuable information with regard to small airway status (57, 58). For these tests, the methodology and standardisation still needs to be developed (59). Another method used to measure airway resistance is the interrupter technique. This method does not provide variables reflecting peripheral airway mechanics and ventilation inhomogeneity (60).

Other methods used to assess the small airways are body plethysmography measurements and imaging techniques. In body plethysmography, airway resistance can be measured, and a measure of air trapping can be extracted from lung volumes, which can reflect ventilation inhomogeneity. Advanced imaging techniques which can be used, such as magnetic resonance studies and high resolution computational tomography, are highly restricted not only by lack of standardisation and high cost, but also for computational tomography due to the use of ionising radiation.

Exhaled nitric oxide

A marker of airway Th2-cytokine-driven inflammation

Nitric oxide (NO) was found in exhaled air of humans in 1991 (61), and soon thereafter it was discovered that the fraction of exhaled NO was higher in atopic asthmatic individuals than in healthy controls (62). Since then, several thousands of publications have been presented, and the technique of measuring this small molecule in exhaled air has been standardised for older children and adults.

The origin of exhaled NO has been thoroughly investigated. Endogenous NO is formed by NO-synthases (NOS) when L-arginine and oxygen form L-citrulline and NO. NO in exhaled air, measured with a standardised technique where the contribution of nasal NO is eliminated by velum closure, is proposed to be derived mainly from lower airway epithelial cells, in which inducible NO synthases (iNOS) is expressed (63, 64). A constitutive isoform, endothelial NO synthase is also present, but produces orders of magnitude lower amounts of NO and therefore its contribution to exhaled NO is believed negligible in the context (64, 65). In the pharyngo-oral tract, there is a non-NOS-related production of NO contributing to exhaled NO levels, which is based on bacterial reduction of nitrate to nitrite, which is subsequently further reduced to NO (66).

Data from early experimental studies suggested a peripheral airway source to exhaled NO in healthy subjects (67) and more recent modelling studies further proposed distal airway NO production to be a considerable contribution of exhaled NO (68). However, presence of NOS mRNA likely to contribute to exhaled NO has not been confirmed so distal in the airway tree (69, 70). Below the conductive airway generations, NO in the alveolar airway compartment normally diffuses easily across the alveolar-capillary membrane, and binds rapidly and strongly to haemoglobin.

In addition to the sources contributing to exhaled NO, there are production sites in lung tissue further away from the airway lumen, such as vascular endothelial NO synthases and neuronal NO synthases. A reason why these sites do not contribute substantially to the fraction of exhaled NO is that the small, lipophilic and easily cell membrane diffusing NO molecule avidly reacts in aqueous solution and is rapidly converted when not produced close to the airway epithelial luminal border (71, 72).

The allergic inflammation involves mast cells, Th-2 cells, eosinophils and signalling pathways including IL-4, 5 and 13. Studies have shown that IL-4 and IL-13 can induce iNOS expression in human airway epithelial cells (73, 74). The basal NO formation by epithelial iNOS, and possibly endothelial NOS, is unaffected by the Th2-cytokines. This NO production is concerted by signal pathways other than those responsible for the iNOS upregulation

associated with an allergic airway inflammation, and may involve the normal formation of interferons in the airways (65).

Allergens can work as trigger factors and upregulate the expression of iNOS in sensitised individuals. Several studies have confirmed a link between iNOS expression and asthma (75, 76). It has also been shown that iNOS expression in airway epithelia, as well as exhaled NO, is higher in allergic asthmatics than in healthy controls and that the expression of iNOS increases in allergic asthmatics after bronchial provocation (77).

NO airway dynamics and standardisation of measurement

NO that is produced in the airway wall diffuses radially into the airway lumen and then follows the airstream towards the mouth, where it can be measured as a fraction in exhaled air. An important feature of airway NO dynamics is that the NO measurement is dependent on exhalation flow. During the earlier phases of experimental studies on exhaled NO it was discovered that higher exhalation flow results in much lower fractions of exhaled NO, see Figure 2. Also, the total NO output (i.e., exhalation flow times concentration of NO) is higher at higher than at lower exhalation flow rates (Figure 2).

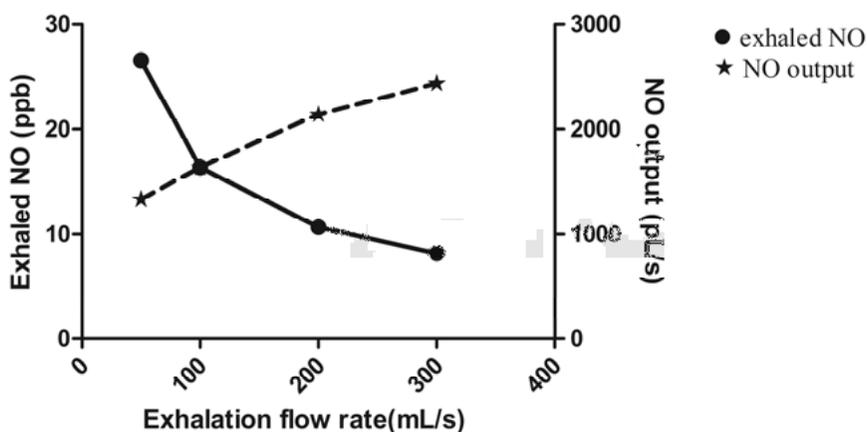


Figure 2. Exhaled NO (left axis) and NO output (right axis) at different exhalation flow rates in an asthmatic subject.

The subsequent standardisation of NO measurement meant that the procedure is performed at an exhalation flow rate of 50 mL/s (78), which is easily performed by most individuals. In this text, this standardised measurement is from here on referred to as exhaled NO, or FeNO, if not otherwise stated.

Interference of NO produced in other compartments than lower airways must be avoided during the measurement procedure. Large amounts of NO

are produced in the paranasal sinuses. To avoid nasal cavity air to mix with exhaled air, the exhalation is performed against a pressure of $> 5\text{cmH}_2\text{O}$ to achieve closure of the soft palate. In the standardised procedure, the patient also inhales to near total lung capacity, and immediately exhales without a breath hold.

To gain a reproducible NO measurement, the single breath method, which is the preferred one, further offers the possibility of measurement at the time/volume window during exhalation where a NO steady-state in the exhaled air is reached. When an exhalation is initiated, airway as well as apparatus dead space must be flushed before a stable concentration is achieved and reliable measurement can be performed. Guidelines (78) for chemiluminescence measurements recommend a wash-out phase before entering NO plateau phase of at least 4 s in children < 12 years, and at least 6 s for older individuals, followed by a 3 second (2 second in younger children) measurement phase. For children below 6 years of age, guidelines suggest a total exhalation time of at least 4 seconds, but little data exists regarding time to NO plateau in children.

In commercial electrochemical devices, the standard exhalation time is pre-set to measure in the late phase of a 6 (to 10) second exhalation in children and 10 (to 12) second exhalation in adults (79).

Instead of a single-breath manoeuvre, methods using tidal breaths for measurements of exhaled NO have also been used, especially in children, to overcome the above mentioned criteria for flow rate and duration. The single breath measurement is still the preferred method in all children who can cooperate with regard to the above mentioned requirements for control over exhalation flow and plateau-phase, in order to increase the reliability of measurement and for reflection of the lower airway contribution (78).

The development and standardisation of methods to measure exhaled NO has provided a reproducible and non-invasive means to obtain information about Th2-cytokine-driven inflammatory activity in the airways applicable from 6-7 years of age in clinical routine. However, for preschool children, a feasible and clinically useful technique needs to be developed, and standardised. Since the reproducibility is dependent on measurement during the plateau phase, and feasibility of the exhalation manoeuvre strongly depends on exhalation time in children, the question of total exhalation time needs to be clarified.

Further, the non-NOS-related production of NO, based on the reduction of nitrite by facultative anaerobe oral bacteria in the pharyngo-oral tract, contributes to exhaled NO and may interfere with the signal reflecting inflammation in the airways. The oral contribution has been studied in healthy controls and it was reported to constitute up to 20 % of measured NO levels at the standard exhalation flow rate (80-82), but no studies have quantified this contribution in asthmatic children. Elevated levels of nitrite or nitrogen oxides have been reported in induced sputum (83) and exhaled breath con-

densate (84, 85) of asthmatics, suggesting, at least, that asthmatic subjects might have a higher non-NOS related production of NO. This issue was not addressed by any studies neither in adults nor children.

Subject characteristic determinants of exhaled NO

Several studies have shown correlations between exhaled NO and height and age in both children and adults (86-90). In the hitherto largest, population-based study including more than 13, 000 participants with measurements of exhaled NO (89), a positive association was found between exhaled NO and height, and non-white ethnicity in children 6-11 years of age, and the same associations were found for older children and adults, as was also a positive association for age. Exhaled NO was negatively associated with body mass index in children 6-11 years of age, and to female gender in subjects 12 years and older.

With regard to bronchial-bronchiolar tonus and exhaled NO, studies have shown that exhaled NO can in different subjects be either elevated or reduced after bronchodilation (91-94). Exhaled NO has been shown to decrease shortly after bronchial provocation (95). Physical exercise can shortly after cessation alter baseline exhaled NO, with different effects in asthmatic subjects and healthy control. Therefore, it is advised that study subjects refrain from physical exercise shortly before measurement (78).

Environmental determinants of exhaled NO

Airborne allergens are probably the most important environmental agents that affect exhaled NO, but the effect is restricted to atopic subjects (96, 97).

Respiratory tract infections also influence exhaled NO, where rhinovirus infection increases airway NO formation (98), but the NO response to rhinovirus infection seems to be reduced in patients with atopic asthma (99). Influenza virus and respiratory syncytial virus infection slightly reduces exhaled NO (100, 101).

Current cigarette smoking reduces exhaled NO in both healthy individuals and asthmatic subjects by 40-60% (102, 103). Passive smoking has also been shown to be negatively associated, but to a much lesser extent (89, 104, 105).

Air pollution (especially particulate matter and ozone) has also been associated with increased exhaled NO and this increase seems to be more pronounced in asthmatic subjects (106-110).

Ingestion of a nitrate-rich meal leads to a transient increase in exhaled NO with about 150 % increase at 2 h in NO-release as a maximum after an equivalent of 200g spinach in healthy fasting controls (81).

Factors known to affect exhaled NO are listed in Table 1.

Table 1. *Factors affecting exhaled NO.*

Method-related	Individual-related	Environment-related
Exhalation flow	Age. Height	Allergen
Nasal air contamination	Gender	Rhinovirus infection
NO-plateau	Ethnicity	Smoking. Air pollution
Oral contribution	Airway obstruction	Dietary nitrate

Exhaled NO measurements techniques

Different techniques for measuring exhaled NO exist. Chemiluminescence measurement was the first method used. This technique is very sensitive and allows for measurements down to 1 ppb and is considered the gold standard (78). However, analysers are relatively expensive and bulky. The development of devices based on electrochemical sensors allows for lower expenses and portable analysers for clinical use. The electrochemical method has been validated (79, 111). In research settings laser-based measurements have also been used.

Measurements are usually performed online, simultaneous with the exhalation, but can also be performed with offline techniques, where exhaled air is collected in containers and analysed subsequently.

Exhaled NO in asthma diagnostics

The findings that exhaled NO is associated with asthma, especially in allergic subjects, have been shown repeatedly (62, 75, 112). Exhaled NO is higher in asthmatic subjects and decreases with ICS-treatment in asthma (91, 113).

Prediction equations have been proposed, accounting for known determinants of exhaled NO, though with an explanatory power of 10-25 % of the variation in the general population. Under these circumstances, threshold levels are the rational way of using exhaled NO in clinical practice.

According to ATS guidelines (114), the clinical use of exhaled NO is supported for children and adults in asthma management:

- to determine eosinophilic airway inflammation,
- to determine likelihood for corticosteroid response,
- as a complementary marker in asthma diagnosis, and
- to follow up on airway inflammation, and possible non-adherence to therapy.

International guidelines for interpretation of measurements recommend cut-off points for exhaled NO. In children < 12 years of age, an exhaled NO < 20 ppb is to be considered a normal value or with low likelihood of steroid re-

sponsiveness, and > 35 ppb is indicative of eosinophilic and steroid responsive airway inflammation. In older children and adults, the corresponding cut-off levels are < 25 ppb and > 50 ppb respectively (114).

Several recent systematic reviews of studies in both children and adults support the idea that exhaled NO is a useful complement to the basic assessment of symptom reporting and lung function tests, especially since these basic assessment steps, at least in mild asthma, are often inconclusive, and would require bronchial provocation tests, which are time-consuming and uncomfortable for the patients (115, 116) (117). A recent review also suggests exhaled NO for specifically targeting the steroid-sensitive eosinophilic subtype of asthma, and a predictive value of high exhaled NO for treatment response in steroid-naïve asthmatic subjects (118).

The method offers an opportunity to estimate the underlying Th2-cytokine-driven inflammatory subtype which could prove valuable both in order to avoid unnecessary controller treatment and to actually find the subset of preschool age children with early allergen-driven airway inflammation in need of accurate and optimally dosed ICS controller therapy for short- and long-term disease control.

The clinical use of exhaled NO in childhood asthma diagnosis and management could be further clarified with more precise knowledge of subject-related determinants in lower ages. Methodological aspects also need to be dealt with in order to allow for reliable measurements in preschool age.

Estimation of NO in the distal airway- Alveolar NO

As mentioned above, early experimental observations on exhaled NO revealed that higher exhalation flows created lower fractions of exhaled NO while at the same time higher total NO output was gained (see Figure 2). In order to interpret and understand these experimental findings and the NO dynamics of the airways, theoretical models of the lung were developed.

The two-compartment model presented by Tsoukias and George was most widely adopted, and offered a simplified description of the dynamical processes of NO production and transport in the airway tree, where exhaled NO can be described as alveolar NO concentration increased by the transport through the conductive airway during exhalation (119), see Figure 3.

The model consists of the conductive airway compartment (generations 0-16), and the alveolar compartment (generations 17-23) surrounded by the alveolar membrane. The NO produced in airway cells either reacts directly, diffuses towards the circulation where it instantaneously and irreversibly reacts with the haemoglobin of the blood, or diffuses toward the airstream. It was proposed that the fraction of exhaled NO would depend on two basic mechanisms, i.e., the exchange of NO in the alveolar compartment and the conditioning of the alveolar gas with NO produced in the airway wall as the

airstream moves through the airway compartment. An estimate of alveolar NO ($Calv_{NO}$) would thus describe the distal “steady-state” of alveolar NO concentration, given knowledge of the efficient diffusion of NO over the alveolar membrane to the “infinite sink” of the vascular bed, see Figure 3. The estimate of bronchial flux was also gained, describing the conditioning of luminal air with NO produced in the conductive airways. The estimate of bronchial flux has been shown to closely relate to exhaled NO.

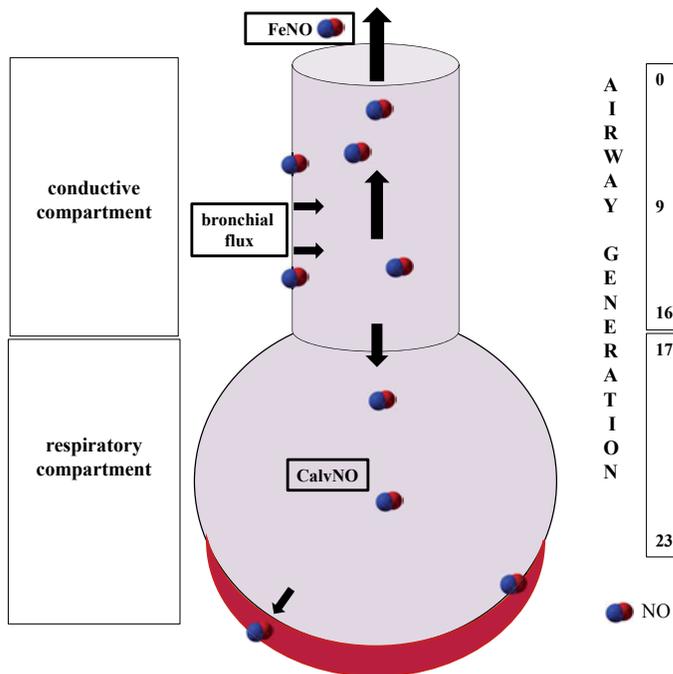


Figure 3. Schematic two-compartment model of the airway. Adapted from Tsoukias and George. Red area illustrates the “infinite sink” of the vascular bed.

The slope-intercept model to calculate flow-independent NO estimates

Different models have been used in order to calculate alveolar NO and other so called flow-independent parameters of NO dynamics and exchange in the airways. The most used is the linear slope-intercept model, which enables for calculation of alveolar NO and bronchial flux from the relation between the exhalation flow and NO output (i.e., the NO fraction at a certain exhalation flow times the actual exhalation flow) (Figure 2), generally at exhalation flow rates above 100 mL/s.

NO measurements are performed at multiple exhalation flows. The NO output is plotted against the exhalation flow and the regression line for NO

output versus flow provides estimates of alveolar NO derived from the slope, and bronchial flux, derived from the y-intercept.

A non-linear model yields markers of airway wall concentration of NO and airway NO transfer factor, but this requires also FeNO measurements at exhalation flow rates < 50 mL/s and is not further discussed in the present work (120).

The optimal exhalation flow rates range for modelling the flow-independent parameters was previously inadequately studied in children; both too low and too high flow rates can impose values that do not fit the linear model (121-123).

Trumpet shape of the airways and axial diffusion adjustments

Any model that describes physiological processes suffers inherently from simplification. The awareness of a possible overestimation in the two-compartment model of estimated alveolar NO, due to axial “downstream” diffusion of NO molecules, led to further modelling. With the starting point that NO from a higher concentration in the bronchial airway lumen diffuses into the alveolar zone during exhalation, trumpet shape of the airway and axial diffusion (TMAD) adjustment algorithms were developed (124, 125). Especially in situations with raised bronchial NO flux, the back diffusion process would lead to falsely high levels of alveolar NO (126). Such an adjustment algorithm has been proposed to be added to the original slope-intercept model. Few studies have studied this correction algorithm in children, however.

Alveolar NO and asthma assessment

The estimate of alveolar NO has been validated as a marker of alveolar inflammation in allergic alveolitis (127). Before the introduction of TMAD adjustments, studies have also presented association between higher alveolar NO and certain asthma subgroups in adults and children, such as severe asthma (128), nocturnal asthma (129) and treatment refractory asthma (130).

With introduction of TMAD adjustments, more recent studies have presented variable results on associations between alveolar NO and asthma characteristics and severity indices (131-134).

Forced oscillation technique

In the 1950s, Dubois and co-workers first described the forced oscillation technique (FOT) as a means to study lung mechanics (135). During the last decades technological improvements have made it possible to use this method in clinically oriented research (136-139). By forced oscillometry, the

overall behaviour of the airways can be reflected in measurements of the mechanical input impedance of the lung (140). The technique may provide unique insights into structure-function relationships and pathophysiology in asthma, chronic obstructive lung disease and acute lung injury (141).

An important advantage of this technique over other pulmonary function tests is that it requires minimal patient cooperation. This makes it an interesting method especially in a paediatric setting and with older patients otherwise incapable of the forced exhalation manoeuvre required in spirometry.

Forced oscillometry provides measures of distal airway processes, and by assessing the airways during tidal breathing instead of forced exhalation, it is interesting also as complementary lung function test, for cooperative older children and adults.

Forced oscillometry and impedance measures

The principle of forced oscillometry is application of small-amplitude oscillations to the lungs via the airway opening (mouth) during normal tidal breathing, while the flow and pressure oscillations generated are measured. From the measures of pressure and flow, respiratory impedance is calculated. The physiological interpretation rests on advanced lung mechanic function modelling.

The respiratory impedance (the ratio of pressure to flow) for each oscillation frequency represents the sum of all forces which oppose the oscillation propagation, and is a complex measure that comprises the vector sum of the respiratory resistance (R) and reactance (X). Resistance is derived from the component of impedance, where flow changes are in phase with pressure changes. Reactance is derived from the component where flow changes are out of phase with pressure changes.

A central aspect in forced oscillometry is that oscillations of low frequencies travel farther than those of higher frequencies. In terms of the airway system, oscillations below ~8 Hz are believed to travel all the way to the distal airways. Oscillations above ~15Hz are regarded to be reflected in the proximal airways.

Respiratory resistance (R) derived by forced oscillometry includes the resistance of the oropharynx, larynx, large and small airways, and lung and chest wall tissue. At low frequencies, this resistance parameter is very comparable to airway resistance (R_{aw}) derived from whole body plethysmography, but slightly higher due to contributions from the chest wall (142) (143) At higher frequencies, the resistance measured by forced oscillation technique tends to underestimate R_{aw} , especially at higher resistance values, likely due to upper airway shunting.

Respiratory resistance can be understood as the airway luminal forces opposing oscillation waves, thus reflecting airway calibre. In healthy adult

subjects the resistance is thought to be nearly independent of oscillation frequency in the frequency range between roughly 4 and 30 Hz (144, 145).

As a consequence of the fact that different oscillation frequencies travel differently in the airway tree, a large airway obstruction increases the resistance at both low and higher frequencies. With obstructive processes in small airways, only lower frequency resistance is affected, which produces a pattern of frequency dependency of the resistance (see Figure 4). This frequency dependence of resistance is often expressed as the difference in resistance between low frequency (5Hz) and higher frequency (~ 20Hz) resistance. This measure is thought to also reflect ventilation inhomogeneity which is a consequence of the disrupted airway patency.

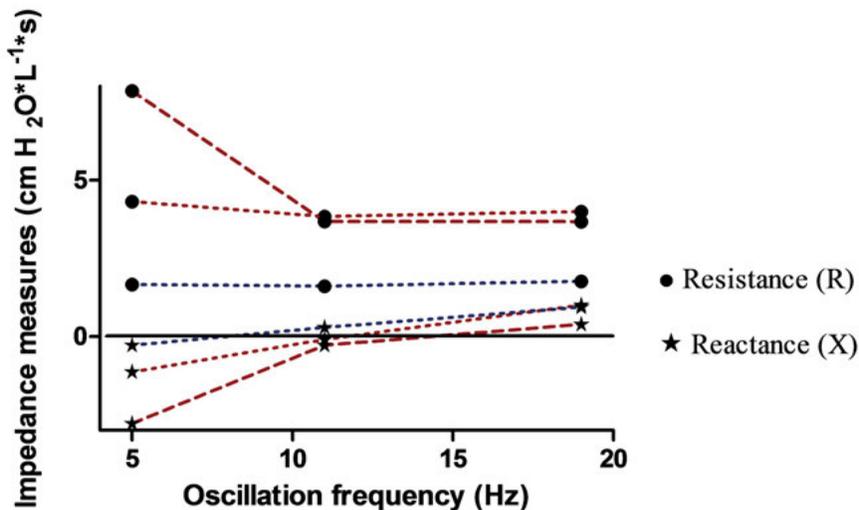


Figure 4. Impedance measures at 5, 11 and 19 Hz in two asthmatic subjects (red lines), one with distal obstruction (striped red lines) and one with proximal obstruction (dotted red lines), and a healthy control (blue lines). Respiratory resistance presented as circles and reactance presented as stars. The resonance frequency for each subject is found where the reactance is approximated to 0.

Respiratory reactance (X) is determined by the elastic and inertive properties of the respiratory system which are counteracting. The elastic properties of the lung dominate at the lower oscillation frequencies, and the inertive properties dominate at higher frequencies. Thus, reactance is always dependent on oscillation frequency (see Figure 4). At a certain frequency the magnitude of elasticity and inertia are equal. Since they are opposite in sign, the total reactance at this frequency is zero. This is called the resonance frequency (F_{res}), and indicates where impedance is due only to resistive losses.

Different instrumentation for forced oscillometry

Numerous techniques and oscillatory signal types have been developed. Originally, single sinusoidal waves were used for forced oscillation (FOT). Further, a technique with pseudorandom noise, which means simultaneously applying several frequency waves, was introduced. Thereafter, a technique has also been developed using square wave impulses, from which a range of frequencies can be derived.

Forced oscillometry can thus be performed with multiple (or single) frequency sinusoidal waves (FOT method), or with impulse waves (Impulse Oscillometry, IOS). Most recent studies have used the latter methods. Importantly, FOT and IOS techniques measure similar, but not equal values of impedance.

Forced oscillometry performance

To minimise artefact imposed by extra-thoracic forces, which would disturb the interpretation of flow and pressure measures, the patient should be comfortably seated with legs uncrossed, neck in neutral position, cheeks supported by hands and with a tight seal between the mouthpiece and the lips to prevent air leaks. In addition to the cheek support to minimise the upper airway artefact, the tongue position also has to be controlled (otherwise raised resistance is produced over all frequencies). Further, air leaks, swallowing and breath-hold may distort the measurements and need to be controlled for during the measurement procedure.

The feasibility of forced oscillometry measurement has been reported to be more than 80 % in preschool children, and was reported to be even higher (96 %) in a study by Malmberg *et al.* (146) in children 4-7 years of age.

The short-term intra-individual coefficient of variation of FOT variables is 5-15 % in children and healthy adults (144).

Assessment of distal lung mechanics by forced oscillometry

As outlined above, forced oscillation technique offers estimates that are proposed to mirror distal lung mechanics (60). Specifically, reactance at low frequencies (X_5) is thought to reflect the elastic properties of the respiratory system/distal airways. A raised resonance frequency (F_{res}) is suggested to reflect distal mechanics, as is also the negative part of the reactance curve-area calculated for IOS. Finally, the resistance difference between low and high frequencies (R_5-R_{19}) is interpreted as a measure of disturbances affecting small airway regions, either ventilation inhomogeneity or increased peripheral airway resistance.

Forced oscillometry in asthma diagnostics

The subject's height, age, gender and, less consistently, body mass index have been reported as predictors of resistance and reactance. Regression equations in children and adults have been reported, mostly for IOS (147-150). Regarding sinusoidal FOT methodology, reference equations have been presented for some devices and some oscillation frequencies (151, 152). Since different techniques produce different resistance and reactance values, and the reference values offered with FOT methodology do not cover all oscillations frequencies, predicted values for one device cannot be applied to another.

Forced oscillometry has been used to demonstrate response to bronchodilation and to discriminate adult mild asthmatic from healthy subjects (153). Studies have further presented that forced oscillometry indices are more sensitive to detect changes with bronchoconstriction and bronchodilatation than spirometry measures in asthma (154-156).

In a clinical trial among children 6-14 years of age, forced oscillometry measures reflected ongoing clinical improvement with treatment, which was not shown by spirometry (157). Data also indicate a relation between forced oscillometry measures at preschool age and lung function in adolescence (158). The possibility to detect lung function abnormalities at pre-school age, when spirometry is not yet applicable in a clinical setting is important. Results have been presented which support the use of forced oscillation technique in diagnosis of childhood asthma (159).

Studies have also presented data on associations between forced oscillometry values thought to reflect distal airway pathology and clinical asthma characteristics and asthma control, in both children and adults (160-166), and also association to markers of small airway inflammation in adult asthma (167, 168).

To summarise, forced oscillometry might offer:

- an alternative lung function method for assessing patients that cannot perform the spirometric forced exhalation manoeuvre,
- a complementary method when spirometry is not informative but clinical suspicion remains, and
- a tool for evaluation of small airways.

Further research must determine whether the method might contribute clinically with measures of distal airway pathophysiology in the light of the growing interest in small airway contribution to asthma. With several different equipment and measurement procedures (IOS and FOT) that produce measures not equal, more studies are needed on methodological matters and also for normative data, before the clinical implications of this method outside the research site, and beyond specific devices (169), can be determined.

Aims

The overall aim of this thesis was to study methodological and clinical aspects of measurements of exhaled NO, estimates of alveolar NO, and lung function indices by forced oscillation technique in asthma.

Paper I: To investigate the oral contribution to exhaled NO in young people with asthma and its potential effects on alveolar NO. To investigate the effect of different proposed adjustments for trumpet model and axial diffusion (TMAD), and also the effect of choice of different exhalation flow rates, on estimated alveolar NO.

Paper II: To investigate the clinical signal provided by primarily alveolar NO and secondarily exhaled NO, by evaluating their relation to various asthma characteristics, with the estimate of alveolar NO calculated with and without correction for back diffusion (TMAD adjustment).

Paper III: To assess the feasibility in children aged 3-10 years of an adapted single-breath online method for exhaled NO measurement and to test the hypothesis that the total exhalation time needed to achieve a steady-state NO concentration is a function of the child's height or age. Secondarily we examined the clinical value of exhaled NO as a signal of airway inflammation in this age group.

Paper IV: To compare lung function parameters obtained by spirometry and FOT, with respect to asthma diagnosis and level of control, and specifically to assess the likelihood of diagnosis and uncontrolled disease for different lung function parameters.

Materials and methods

Study subjects and clinical asthma assessment

Study I was based on 29 individuals with asthma in the age range 10-19 years, and 29 healthy, non-atopic control subjects in the age range 10-20 years. We recruited patients with physician-diagnosed asthma, on continuous ICS therapy and with allergy to furred animals, consecutively during regular follow-up visits at the Department of Paediatrics, Uppsala University Hospital. Subjects/parents responded to questions regarding present medication and reported allergies, and any current cold symptoms were noted.

None of the participants reported current smoking, and the asthmatic patients were mostly well-controlled. The subjects in asthma and control group had similar age and height distribution. All subjects in the asthmatic group were IgE-sensitised against furred animal, as was one subject in the control group.

Studies II and IV were based on subjects that participated in the Minimally-Invasive Diagnostics for Asthma and allergic diseaseS (MIDAS) asthma cohort.

MIDAS asthma cohort

A total of 413 children and young adult asthmatic patients, aged 10-35 years, were recruited within the framework of an industry-academy collaboration from both primary and secondary care in Uppsala, Sweden. Inclusion criteria were physician-diagnosed asthma, self-reported use of daily anti-inflammatory treatment during at least three of the preceding 12 months and written consent. Further, 118 non-asthmatic age- and gender-matched individuals were randomly chosen from the population registry as control subjects. At baseline, asthmatic subjects responded to questions regarding asthma symptoms and exacerbations during the last 12 months and the asthmatic subjects' use of anti-inflammatory treatment was recorded in the interview. The prescribed daily dose of ICS at baseline visit was also collected from the subjects' medical record. At a follow-up visit, conducted 3-4 years later, 257 asthmatic subjects and 63 healthy controls participated. The previous asthma characteristics were reassessed, and lung function measurement with forced oscillation technique was performed in 234/257 asthmatic subjects and 60/63 healthy controls.

Smokers were not excluded, but were only a small proportion (5 %) of all subjects. Most asthmatic subjects were IgE-sensitised and reported current symptom control. A total of 64 % of asthmatic subjects at the first visit, and 46 % at the second visit, though reported having an asthma attack at least once during the preceding 12 months.

Study II was based on asthmatic individuals at baseline visit.

Study IV was based on data from the follow-up visit for both asthmatic subjects and healthy controls. In study IV, healthy controls were somewhat taller than asthmatic subjects, but there were no other differences in age, gender, weight, body mass index, or smoking status.

Study III was based on 63 subjects, aged 3-10 years, mostly patients with physician-diagnosed asthma (n = 54) who were recruited during ordinary visits at a primary or secondary asthma paediatric office in Uppsala, Sweden, and 9 healthy children without asthma or allergy. Parents were asked about allergic symptoms to aeroallergens and food allergens and ongoing respiratory infections, in the child.

No significant differences between control group and asthmatic patients were found regarding age, sex, or height. Children with asthma reported more ongoing respiratory infections than healthy controls. In asthmatic children, allergic symptoms to aeroallergens were commonly reported; in 79% of the children with ongoing ICS and in 68% of asthmatic children without ICS. Subjects with no approved exhalation manoeuvre were mainly below age 4 years (ten out of 12).

Asthma Control Test

Asthma Control Test (ACT), a well- validated instrument to assess asthma symptoms control (170), was used in studies I, II and IV; more specifically the questionnaire for children older than 12 years. This questionnaire encompasses five questions regarding daytime and night-time obstructive symptoms, usage of reliever medication, influence on everyday life, and the patient's own opinion on disease control, on average during the last 4 weeks. ACT scores range from 5-25, with a lower score reflecting more poorly controlled asthma. Uncontrolled asthma was defined as an ACT-score < 20.

Exhaled NO measurements

In studies I, II and IV, exhaled NO was measured according to the recommendations of the American Thoracic Society/European Respiratory Society regarding use of an online single breath technique. A chemiluminescence analyser (NIOX Flex; Aerocrine AB, Solna, Sweden) was used and measurements were always performed before spirometry. In addition to the standard flow rate of 50 mL/s, three exhalation flow rates (100, 200, and 300

mL/s) were used, in order to calculate flow-independent NO estimates in the slope-intercept model. Measurements of exhaled NO were performed in duplicate, in random order with regard to exhalation flow rate, and starting from near-total lung capacity by inhalation of NO-free air from a scrubber. The mean value was automatically calculated, and if the difference between individual measurements exceeded 10 %, additional measurements were performed. The analyser was calibrated every 14 days with certified NO/N₂ gas of 200 ppb according to the manufacturer's recommendations.

Subjects were instructed to avoid intense physical exercise and eating large quantities of green-leaved vegetables on the day of the measurement. In study II, they were also instructed not to eat or drink during 30 min prior to measurements, as well as to avoid smoking and oral tobacco. In studies I, II and IV, subjects were asked to report intake of large quantities of green-leaved vegetables and physical exercise on the day of and prior to the measurement. In study I, three subjects (one with asthma) reported intake of large amounts of vegetables on the day of the measurement, though none of these three had exhaled NO exceeding 20 ppb. In study II, one subject (control) reported nitrate-rich intake. In study IV, three subjects (of which one asthmatic) reported nitrate-rich food intake within 4 h prior to measurements. None of these subjects had exhaled NO >20 ppb. Only one subject (asthma) reported having performed strenuous exercise within 1h prior to measurement. These subjects were all included in analyses.

In study I, NO measurements were performed before, and 5 min after, a 30 s mouth wash with anti-bacterial solution containing chlorhexidine 0.2 %.

In study III, a prototype instrument was used, consisting of a handheld unit incorporating an electrochemical sensor (City Technology), and a newly developed flow-control unit (NIOX NOVA; Aerocrine AB, Solna, Sweden). The children inhaled ambient air instead of inhalation through the mouthpiece, to maximise success for the small children. Ambient air was controlled for level of NO, which never exceeded 8 ppb (median 0.9 ppb, interquartile range 0.1-1.3). The flow-control unit allowed an oral pressure range of 5 cmH₂O to approximately 35 cmH₂O, while still keeping exhalation flow rate within 45-55 mL/s. An acceptable NO measurement was defined as i) the ability of the child to create an oral pressure with a minimum of 5 cmH₂O through the exhalation and ii) acceptable flow control defined as a mean of 45-55 mL/s during the last 2 s of the exhalation. The exhaled air was led through a buffer chamber (85 ml) in the device, from which air was continuously sampled (1.8 mL/s) to the electrochemical sensor. Exhalation time was pre-adapted to age and set to 4 s for the age of 3-4 years, 6 s for the age of 5-6 years, 8 s for the age of 7-8 years, and 10 s for the age of 9-10 years. This methodology was validated by comparison with a fast-response chemiluminescence device (NIOX Flex; Aerocrine AB) as air was simultaneously sampled at 5 mL/s from the buffer chamber during exhalation into the chemiluminescence device.

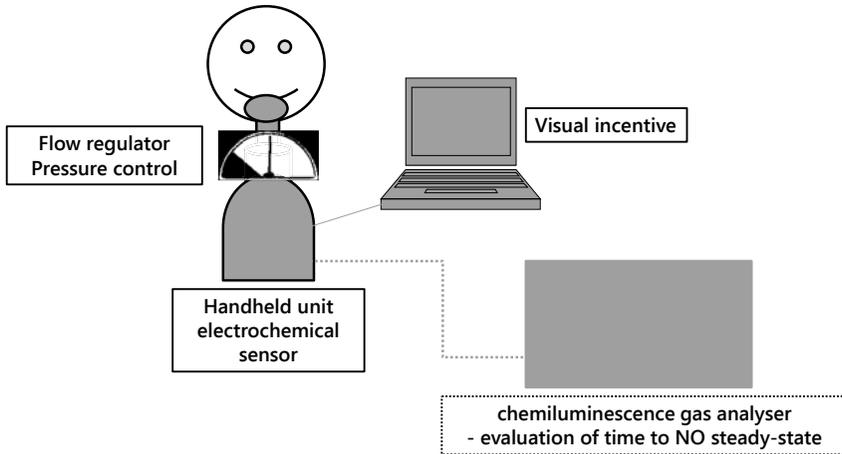


Figure 5. Measurement setup, study III. A handheld unit with an electrochemical sensor was used for recording the exhaled NO values. Simultaneous sampling to NIOX Flex enabled both registration of time to steady-state concentration and chemiluminescence NO analysis for validation of the electrochemical sensor.

Determination of time to steady-state NO concentration

During the exhalation manoeuvre into the electrochemical device, air was simultaneously sampled at 5 mL/s from the buffer chamber into a fast-response chemiluminescence device (see Figure 5). The chemiluminescence device was used in the Nasal mode, and the measurement was started by the operator when the child started to exhale into the handheld device. The NO signal (data points at 20 Hz) was exported from the chemiluminescence device, and the time to steady-state NO concentration in the buffer chamber and the mean NO concentration during steady-state were calculated using a computer algorithm in Excel. The time to steady-state was determined by iterative linear regression analyses of 2-s windows, starting at the first NO data point, until a slope within $\pm 10\%$ was achieved. This analysis was also reviewed visually.

Alveolar NO

In studies I, II and IV, the estimate of alveolar NO was calculated using the slope-intercept model and the NO measurement at 100-300 mL/s, except in the specific context in study I where we compared the estimates obtained with 50-200 mL/s and 100-300 mL/s. As previously described, the slope and intercept refer to the regression line of NO output against exhalation flow rate where the slope corresponds to the alveolar NO concentration and the

intercept with the Y-axis to the bronchial NO flux. The Pearson correlation coefficient (r) between NO output and exhalation flow was automatically calculated.

In studies II and IV, subjects were defined as (slope-intercept) model compliers when $r \geq 0.8$, as previously described by Mahut (133).

In study II, 29 out of 410 asthmatic subjects were thus non-compliant for calculations of alveolar NO, whereof four individuals also yielded negative alveolar NO. Non-compliers were excluded from analyses in study II, and also in study IV (14/234) in the analyses of associations to alveolar NO. In study I, no such model-compliance criterion was used. One negative alveolar NO was obtained and excluded.

Trumpet shape of the airways and axial diffusion adjustments

In study I, estimated alveolar NO was adjusted for the trumpet shape of the airways and axial diffusion (TMAD) from conductive airways, with two different equations: TMAD-adjusted alveolar NO according to Kerckx = $(0.08 \times \text{FeNO})/0.92$ (124) and TMAD-adjusted alveolar NO according to Condorelli = $\text{Cal}_{\text{VNO}} - J'_{\text{awNO}}/860$ (125). These adjustments were applied only to the 100-300mL/s set of exhalation flow-rates, since no such correction coefficients are described for 50-200mL/s. After TMAD- adjustment, negative alveolar NO values were replaced with 0.

In study II, the Condorelli method was chosen rather than the Kerckx method because the Condorelli method gave fewer negative TMAD-adjusted alveolar NO (20 versus 47). Negative TMAD-adjusted alveolar NO values were given an arbitrary value of 0.01 to allow for logarithmic transformation.

In study IV, only unadjusted alveolar NO was used in analysis due to our previous conflicting findings on the relevance of existing adjustment algorithms, in asthmatic subjects with varying disease severity and control.

Spirometry

Flow-volume curves were obtained in accordance with the American Thoracic Society recommendations (16) using a Masterscope spirometer (Jaeger Master, Germany). For subjects <18 years of age, Zapletal reference values for lung function were used (171), and for subjects ≥ 18 years of age Hedenström reference values were used (172, 173).

In studies I and II, values are presented as % predicted, with the exception of the ratio $\text{FEV}_1 / \text{FVC}$ in study II that was presented as the lower limit of normal (LLN) according to Hankinson (174). In study II, subjects were subdivided according to normal or impaired lung function values ($\text{FEV}_1 < \text{or} \geq$

80%, FVC < or \geq 80%, FEV₁/FVC < or \geq LLN, forced expired flow at 50% of vital capacity (FEF₅₀) < or \geq 50%).

In study IV, FEV₁/FVC ratio is expressed as a percentage, and the values for volume and flow are presented in (L) and (L/s).

Forced oscillation technique

In study IV, FOT measurements were performed with Resmon Pro device (Restech, Italy). A specialised mouthpiece was used to position and hold down the tongue during measurement, in order to minimise artefacts due to tongue positioning. Measurements were done prior to spirometry. A nose clip was used and subjects were placed in a comfortable sitting position with firm support for the cheeks. A bacterial filter was used as recommended.

The first five breaths were always automatically discarded in order to avoid any large variations in breathing pattern during the first breaths. After these, the device sampled ten accepted tidal breaths. The acceptance is based on an internal quality check, built on the values of resistance, reactance, breath duration and amplitude for breaths outside physiological appearance. The mean values of total respiratory impedance from these ten accepted breaths were calculated.

In the first 27 recruited asthmatic individuals, a mono-frequency (5 Hz) signal was applied, while for the rest of the studied population a multi-frequency (5, 11 and 19 Hz) signal was applied. Resistance at 5 Hz (R₅), 19 Hz (R₁₉), resistance difference between 5 and 19 Hz (R₅-R₁₉), reactance at 5 Hz (X₅) and resonance frequency (F_{res}) were obtained. In three cases, negative values of resonance frequency were found. These were excluded from variable specific analysis.

Other methods for assessment

Bronchial responsiveness

In study II, methacholine provocation was performed with Aerosol Provocation System (Viasys Healthcare GmbH) in accordance with a simplified protocol described in detail elsewhere (175). Bronchial responsiveness was defined as normal with methacholine cumulative dose causing a fall in FEV₁ (PD₂₀) > 1.0 mg, borderline-to-mild with PD₂₀ 0.3-1.0 mg, and moderate-to-severe with PD₂₀ < 0.3 mg, in accordance with Schulze *et al.* (176).

IgE sensitisation and blood eosinophils

In study I, blood samples were collected for measurement of IgE antibodies to the most important (regional) airborne allergens using immunoCAP (Phadia, Uppsala, Sweden). Subjects were defined as sensitised if they had the respective IgE antibodies ≥ 0.35 kU/L.

In studies II and IV, IgE against a mix of aeroallergens (grass, tree & weed pollen, furry animal, mite and mould allergens) (Phadiatop, Immundiagnostics Thermofisher Scientific, Uppsala, Sweden) (177) were measured in all subjects except three. Subjects were defined as atopic if they had IgE antibodies against Phadiatop ≥ 0.35 kU/L.

In studies II and IV, blood eosinophils were counted at the Department of Clinical Chemistry at Uppsala University using a routine method (Cell-Dyn 4000, Abbott, Illinois, USA). In study II, subjects were divided into three groups according to level of blood eosinophils: normal ($< 0.3 \times 10^9$ /L), intermediate ($0.3\text{-}0.5 \times 10^9$ /L) and high ($> 0.5 \times 10^9$ /L).

Salivary nitrite

Two millilitres of unstimulated saliva were collected from all subjects in study I. The samples were immediately frozen at -80°C for later analyses. Nitrite in saliva was measured after methanol precipitation of proteins (1:1 v/v) by using a dedicated high-performance liquid chromatography system (ENO-20; EiCom, Kyoto, Japan) (178). All saliva samples were analysed on the same occasion.

Ethics

Written informed consent for participation in the studies was obtained from the parents of the participants or from the participants themselves, if they were at least 18 years, before inclusion in any of the studies. The protocols were approved by the Uppsala Regional Ethical Review Board.

Statistical analyses

In Study I, nonparametric statistical analyses (Mann-Whitney U-test for comparisons between groups, Wilcoxon signed-rank test for paired comparisons and Spearman's rank correlation) were performed using the GraphPad Prism 5.0 (GraphPAD Inc, San Diego, CA, USA). Data are presented as median values (interquartile range).

In studies II-IV, statistical analyses were performed with STATA/IC 12.1 (StataCorp LP, College Station, Texas, USA) and log-transformed NO pa-

rameters were used to enable parametric statistical analyses. NO parameters are presented as geometric means (95 % confidence interval). Lung function values are presented as means \pm SD.

In studies II and III, unpaired t-tests were used to compare NO parameters in dichotomised subject groups. Linear regression models were applied for associations between bronchial flux and alveolar NO in study II, for analysis of the relation between time to steady-state NO concentration and height in study III, and for analysis of associations between NO parameters and lung function values in study IV. Multiple linear regression models were used for differences in NO parameters of asthma characteristics adjusted for age, sex, height and smoking in study II. In study IV, linear regression analyses were performed with each lung function parameter as outcome, to present mean differences for asthma and level of control. Height, gender, age, weight, and age², known determinants of lung function, were added into these multivariate linear regression models. Logistic regression analyses were performed in study IV to study the likelihood of asthma and uncontrolled disease in relation to lung function indices from spirometry and FOT. In these analyses, standardised lung function variables (the difference from the mean of the original variable in number of standard deviations of the original variable) were used. In multivariate logistic regression models, height, age, gender and weight were added. Odds ratio (OR), and also area under the curve (AUC) values from receiver-operating characteristic (ROC) curves, were calculated.

In Study III, the FeNO values are based on the individuals' mean levels of all approved measurements (range 2-5 measurements), except for subjects with only one approved measurement (n = 8). Analysis of the correlation between time to steady-state NO level and height and age was done using the first approved NO measurement with confirmed steady-state level (n = 45/51). All FeNO values presented are from the electrochemical prototype analyser.

In study IV, sub-analyses were done in adult individuals (age \geq 18 years), since the low number of children did not allow for separate sub-analyses.

A p value of < 0.05 was considered significant throughout.

Results

Methodological investigation of exhaled NO

Oral contribution to exhaled NO

In study I, we found that exhaled NO was reduced after the chlorhexidine (CHX) mouthwash in a similar manner in asthmatic subjects and healthy controls, see Figure 6. The relative reduction of exhaled NO at 50 mL/s after CHX mouthwash was of the same magnitude in both groups (8.8 % (2.6-15.9) vs. 9.8 % (3.4-17.8), $p = 0.49$).

Salivary nitrite levels at baseline were similar in asthmatic and healthy subjects (56.4 μM (34.9-83.0) vs. 78.4 μM (40.4-143.2), $p = 0.25$) and CHX mouthwash resulted in significantly decreased salivary nitrite levels in asthmatic subjects and healthy controls ($p < 0.01$ for both). The relative reduction of nitrite levels was of the same magnitude in both groups (67.7% (51.7-79.8) vs 69.8% (42.1-81.0)).

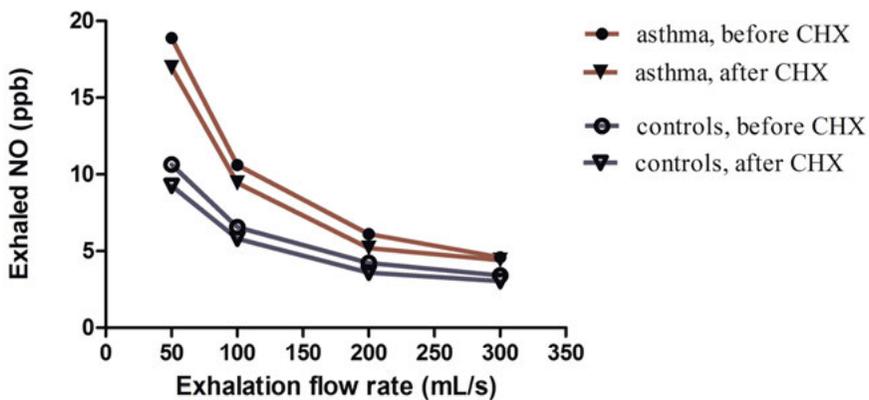


Figure 6. Exhaled NO (median values) before and after chlorhexidine mouthwash in asthmatic subjects (red line) and healthy controls (blue line).

Determination of time to [NO] steady-state in children

This question was addressed in study III by the simultaneous sampling to a connected chemiluminescence apparatus from which the calculations of exhalation time to obtain a steady-state NO concentration in the buffer cham-

ber were made (see Figure 5 for measurement set-up). For all approved FeNO measurements where a steady-state NO concentration could be simultaneously confirmed in the chemiluminescence device, the exhalation time needed to reach steady-state was within the pre-set time for each age group. The steady-state for NO was obtained within 4 seconds for children age 3-4 years, and within 5.2 seconds for children age 5-6 years, see Table 2.

Table 2. *Time to steady-state [NO] in all subjects with approved measurements and data on time to steady-state.*

Age (years)	Subjects (n)	Time to steady-state [NO] (s), median (IQR range)	Maximum time to steady-state [NO] (s)
3-4	5	2.5 (2.4-3.5)	3.9
5-6	13	3.5 (2.7-3.8)	5.2
7-8	10	4.6 (4.2-5.0)	6.7
9-10	17	3.7 (3.1-4.8)	5.9

A positive association between height and time to steady-state was found ($r^2 = 0.13$, $p = 0.02$). Also we found an association between time needed to obtain steady-state NO concentration and the steady-state concentration itself, by comparing highest exhaled NO quartile (24.7 ppb (95 % CI 19.3-31.5), $n = 11$) and lowest quartile (5.9 ppb (4.8-7.3), $n = 12$) groups (4.3 ± 0.7 s vs. 3.1 ± 1.5 s, $p < 0.01$) with no difference in age or height between the upper and lower FeNO quartiles. The relationship between time to steady-state and height remained after adjustment for FeNO quartile groups.

Feasibility of an adapted single-breath online FeNO method

In study III, 81 % of the children, aged 3-10 years, managed at least one approved exhaled NO measurement with an adapted single-breath method and a newly developed flow-control unit. Five out of six (83 %) of the children aged 4 years succeeded with at least one measurement. No child aged 3-5 years had previous experience of exhaled NO or spirometry measurements. Sixty-eight percent of all the subjects achieved at least two approved measurements, and the success rate improved with age, being 100 % from 8 years and up.

Clinical aspects of exhaled NO

Effect of height/age on exhaled NO

With data from study I, the association between exhaled NO and height and age in healthy non-infected controls aged 10-20 years was additionally analysed by linear regression for log-transformed exhaled NO (data not published). In 26 healthy non-infected controls, a positive association was found

between exhaled NO and height ($r^2 = 0.21$, $p = 0.02$), and the results remained after gender adjustment. The same association was found between exhaled NO and age ($p = 0.02$).

In our sample of children aged 3-10 years, a positive trend was seen when plotting exhaled NO values and height for all subjects with one accepted exhaled NO measurement ($n = 51$) (see Figure 7), but in linear regression this was not significant ($p = 0.05$), (data unpublished).

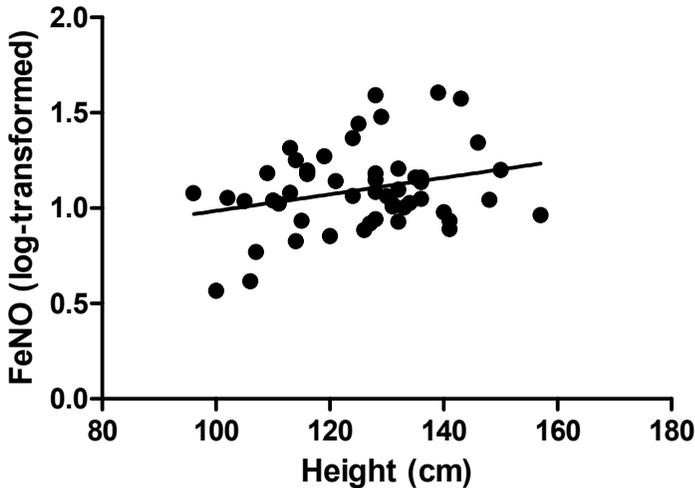


Figure 7. Scatter plot of exhaled NO (log-transformed) and subject height in a mixed sample of asthmatic and healthy children age 3-10 years.

Clinical signal of exhaled NO in children 3-10 years of age

In study III, among children aged 3-10 years, a higher exhaled NO was found in the asthmatic children without ICS treatment (15.9 ppb (95 % CI 12.2-20.9)) compared with healthy controls (9.1 ppb (6.6-12.4)) and also compared with asthmatic subjects with ongoing ICS treatment (11.5 ppb (9.7-13.6)), see Figure 8. In a subgroup analysis where subjects with symptoms of ongoing upper respiratory tract infection were excluded, we found that the abovementioned differences were strengthened ($p = 0.001$ and $p = 0.003$, respectively). The findings between asthmatic groups and controls remained consistent after adjustment for children's height or age.

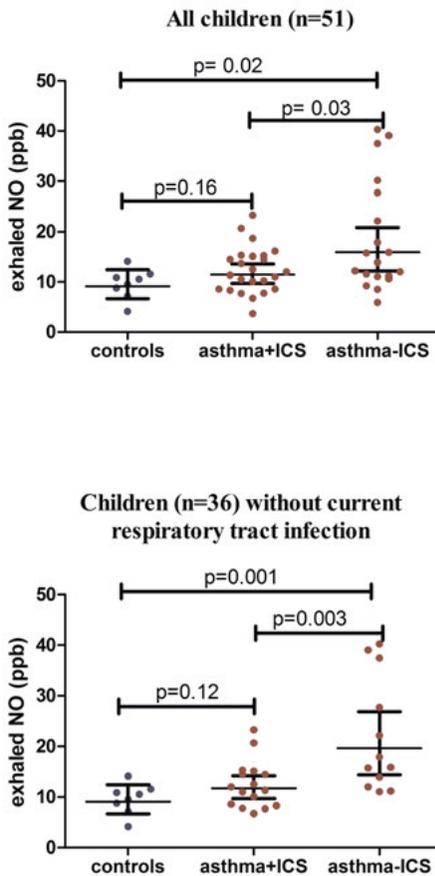


Figure 8. Exhaled NO in clinical groups of children aged 3-10 years. Bars indicate geometric means and whiskers 95% CI.

Relation between exhaled NO and asthma characteristics

In study II, based on asthmatic subjects at baseline MIDAS-visit, exhaled NO was higher in subjects with reported daytime wheeze in the preceding 12 months (18.2 ppb (95 % CI 16.5-20.0)) compared with those with no reported daytime wheeze in the preceding 12 months (12.5 ppb (11.1-14.0)), $p < 0.001$. It was also higher in subjects with reported awakening by dyspnoea (20.9 ppb (16.4-26.6)) compared with subjects without awakening by dyspnoea (15.7 ppb (14.5-17.1)), $p = 0.01$, in the preceding 12 months. However, exhaled NO was not associated with ACT score ($p = 0.17$). Further, higher exhaled NO was found in subjects with moderate-to-severe bronchial hyperresponsiveness versus normal bronchial responsiveness ($p < 0.001$) and in subjects with raised levels of blood eosinophils compared with normal levels ($p < 0.001$), and also in atopic subjects compared with non-atopic (17.9 ppb (16.4-19.5) vs. 11.3

ppb (9.6-13.2), $p < 0.001$). Exhaled NO was lower in asthmatic subjects on higher inhaled corticosteroid dose compared with ICS-naïve asthmatic subjects, see Table 3. These results were consistent after adjustment for age, sex, height and smoking.

Table 3. *Exhaled NO, and alveolar NO, in asthmatic subjects on different ICS- dose. P-values indicate difference from treatment-naïve asthmatic subjects. Data presented as geometric means (95 % confidence intervals)*

	ICS naïve	<500 µg	500-800 µg	>800 µg
n=376	71	227	49	29
Exhaled NO (ppb)	20.5 (16.5-25.5)	16.4 (14.9-18.0) $p = 0.03$	14.7 (11.7-18.4) $p = 0.02$	11.4 (8.9-14.7) $p = 0.01$
Alveolar NO (ppb)	3.2 (2.7-3.8)	2.8 (2.6-3.0) $p = 0.07$	2.3-2.7 $p < 0.01$	2.1 (1.8-2.5) $p < 0.01$

Methodological investigation of alveolar NO

Slope-intercept model for estimation of alveolar NO

A Pearson correlation coefficient (r) > 0.80 between NO output and exhalation flow rates was used as a prerequisite for slope-intercept model fit in calculations of alveolar NO (133). In study II, 376 subjects out of 410 (91.7 %) were slope-intercept model compliers by this definition. Non-compliers to the model ($n = 29$) were younger, shorter, had a higher FEF₅₀ and a lower exhaled NO compared with compliers (all $p < 0.05$). In study IV, 14 (6.1 %) asthmatic subjects were not compliant with the slope-intercept model by this prerequisite. Here, the non-compliers had a lower % predicted FEV₁, FEV₁/FVC and FEF₅₀ (all $p < 0.05$).

Similar alveolar NO was calculated when flow rates between 50 and 200 mL/s and between 100 and 300 mL/s were used in study I, for both the asthmatic group (2.0 ppb (IQ range 1.4-2.9) vs. 2.1 (1.5-2.6) $p = 0.34$) and the control group (1.7 ppb (1.4-2.8) vs. 1.8 ppb (1.2-2.1) $p = 0.90$). The goodness of fit (r) for the slope-intercept model was also similar for 50-200 mL/s vs. 100-300 mL/s in both the asthmatic group (0.995 vs. 0.988, $p = 0.78$) and the control group (0.988 vs. 0.99, $p = 0.65$).

Feasibility and effect of different TMAD adjustments

In study I, TMAD adjustment could be used with negative alveolar NO values obtained in 4 asthmatic subjects and 3 controls (Condorelli), and 6 sub-

jects from each group (Kerckx), respectively. In study II, using the algorithm by Condorelli, 20 asthmatic subjects out of 376 obtained negative alveolar NO after TMAD-adjustment.

In study I, a positive association in asthmatic subjects was found between unadjusted alveolar NO and bronchial NO flux, both before ($\rho = 0.64$, $p < 0.001$) and after chlorhexidine mouthwash ($\rho = 0.38$, $p = 0.04$). With TMAD adjustments according to Condorelli, no association was found ($\rho = -0.26$, $p = 0.18$). In healthy subjects, a negative association was found before mouthwash ($\rho = -0.50$, $p = 0.006$). With TMAD adjustments, this association was strengthened ($\rho = -0.77$, $p < 0.001$ before mouthwash, and $\rho = -0.71$, $p < 0.001$ after mouthwash). The CHX mouthwash did not significantly affect TMAD-adjusted alveolar NO levels, neither with using the Condorelli nor with the Kerckx correction.

In study II, a positive relation was again found in asthmatic subjects between unadjusted alveolar NO and bronchial flux ($\beta = 0.22$, $p < 0.001$ and $r = 0.38$, 95 % CI 0.29-0.46), see Figure 9. With TMAD-adjusted alveolar NO on the other hand, a negative correlation with bronchial flux was found ($\beta = -0.38$, $p < 0.001$ and $r = -0.26$, 95 % CI ((-0.35)-(-0.16))), see Figure 9. These results remained when subjects were divided into two groups, < 18 years of age or ≥ 18 years of age.

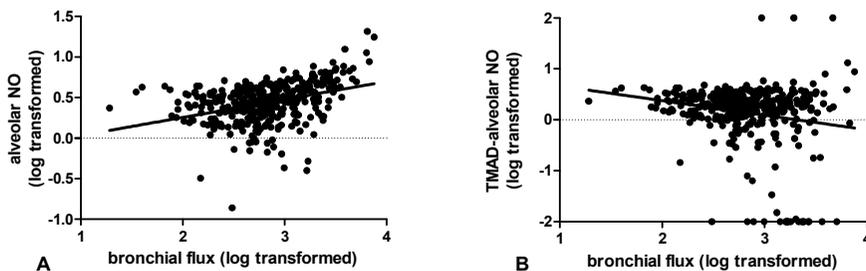


Figure 9. Regression plot of log-transformed data: bronchial flux and A. alveolar NO unadjusted, and B. TMAD-adjusted alveolar NO.

Oral contribution to alveolar NO

Unadjusted alveolar NO was not affected by the chlorhexidine mouthwash in asthmatic subjects (median with interquartile range: 2.1 ppb (1.5-2.6) vs. 1.9 ppb (1.4-2.6) $p = 0.30$) and in healthy controls (1.8 ppb (1.2-2.1) vs. 1.5 ppb (1.4-2.2), $p = 0.78$), in study I.

Clinical aspects of alveolar NO

In study I, no differences in alveolar NO, unadjusted ($p = 0.11$), or TMAD-adjusted according to Condorelli ($p = 0.56$) or Kerckx ($p = 0.27$), were found between asthmatic subjects and healthy controls.

In study IV, unadjusted alveolar NO was higher in asthmatic subjects (2.9 ppb (95% CI 2.7-3.1)) than in healthy controls (2.4 ppb (2.1-2.7)), $p=0.02$, and remained after adjusting for height.

Relation between alveolar NO and asthma characteristics

In study II, asthmatic individuals were grouped with regard to reported asthma symptoms in the year preceding the study, grading of bronchial responsiveness, grading of blood eosinophil count, level of inhaled corticosteroid treatment, normal or low lung function parameters, atopy and level of asthma control.

We found that TMAD-adjusted alveolar NO was similar for all subgroup analyses with regard to asthma characteristics as above. As a total, in asthmatic individuals, alveolar NO after TMAD adjustment was approximately ~1 ppb (1.3 ppb (95 % CI 1.1-1.5)).

On the other hand, unadjusted alveolar NO was higher in subjects who reported symptoms and asthma attack in the preceding 12 months than among those who did not, and also in subjects with moderate-severe bronchial responsiveness compared with normal to borderline bronchial responsiveness. Also, higher alveolar NO was found in subjects with higher level of blood eosinophils than in subjects with lower levels, and in atopic subjects (all $p < 0.05$, Table 4). Unadjusted alveolar NO was lower in asthmatic subjects on higher inhaled corticosteroid dose compared to ICS-naïve asthmatic subjects, see Table 3. These results were consistent after adjustment for age, sex, height and smoking.

Table 4. *Alveolar NO in relation to asthma characteristics, dichotomised. Data presented as geometric mean values and 95 % confidence intervals.*

Asthma characteristics	Yes	No	p-value
Daytime wheeze in the preceding 12 months	2.9 (2.7-3.1)	2.3 (2.1-2.6)	<0.001
Asthma attack in the preceding 12 months	2.9 (2.7- 3.1)	2.5 (2.3- 2.7)	0.03
Exercise-induced asthma in preceding 12 months	2.8 (2.7-3.0)	2.4 (2.1-2.8)	0.02
Bronchial hyperresponsiveness (PD ₂₀) < 0.3 (mg)	3.0 (2.7-3.2)	2.4 (2.3-2.6)	<0.001
Blood eosinophils $\geq 0.3 \times 10^9/L$	3.3 (3.0 -3.7)	2.4 (2.3-2.6)	<0.001
Atopy	2.8 (2.7-3.0)	2.3 (2.0-2.7)	0.01

Lung function by FOT versus spirometry in asthma

In study IV we investigated lung function variables obtained through standard spirometry and forced oscillometry, in relation to asthma diagnosis and disease control, in 234 asthmatic subjects and 60 healthy controls, at follow-up MIDAS visit. We analysed the relations with measures supposed to reflect both mostly proximal lung function and measures proposed to reflect distal airway processes (see lung function indices overview, Table 5).

Table 5. Lung function variables used in study IV by spirometry and FOT, and suggested airway zone mirrored.

Spirometry			FOT		
Proximal	Total	Peripheral	Proximal	Total	Peripheral
FEV ₁	FEV ₁ /FVC	FEF ₅₀	R ₁₉	R ₅	R ₅ -R ₁₉ , X ₅ , F _{res}

Asthma diagnosis and overall lung function measures

Lower FEV₁/FVC and FEF₅₀ and higher R₅ and R₁₉, related to a higher likelihood of belonging to the group of asthmatic subjects when adjusted for age, height, gender and weight (see Table 6), analysing standardised lung function indices. Also, mean differences for each lung function variable between asthmatic individuals and healthy controls were analysed, adjusted for age, height, gender, weight and age² (see Table 7).

Table 6. Odds ratio (OR) for having an asthma diagnosis and for having uncontrolled asthma, for standardised lung function indices.

	Adjusted OR (95% CI) for having	
	Asthma diagnosis	Uncontrolled asthma
FEV ₁	0.83 (0.53-1.30)	0.62 (0.35-1.09)
FEV ₁ /FVC	0.47 (0.32- 0.69)	0.58 (0.40- 0.84)
FEF ₅₀	0.62 (0.46- 0.85)	0.66 (0.42- 1.03)
R ₅	3.31 (1.95- 5.62)	1.35 (0.93- 1.95)
R ₁₉	2.54 (1.65- 3.91)	1.22 (0.81-1.84)
X ₅	0.79 (0.54- 1.17)	0.69 (0.49- 0.97)
F _{res}	1.71 (0.99- 2.94)	1.41 (1.02- 1.95)
R ₅ -R ₁₉	1.71 (1.07- 2.73)	1.57 (1.05-2.35)

Table 7 Mean differences in lung function indices between asthmatic individuals and healthy controls, and between uncontrolled and controlled asthmatic subjects.

	Adjusted mean differences (95% CI) between	
	Asthma vs Control	Uncontrolled vs Controlled Asthma
FEV ₁ (L)	-0.05 (-0.19-0.10)	-0.12 (-0.29- 0.05)
FEV ₁ /FVC (%)	-4.4 (-6.6- -2.2)	-3.8 (-6.3- -1.3)
FEF ₅₀ (L/s)	-0.43 (-0.73- -0.13)	-0.35 (-0.71- 0.01)
R ₅ (cmH ₂ O/ L*s ⁻¹)	0.60 (0.31-0.89)	0.25 (-0.11- 0.60)
R ₁₉ (cmH ₂ O /L*s ⁻¹)	0.47 (0.24- 0.71)	0.13 (-0.18- 0.43)
X ₅ (cmH ₂ O /L*s ⁻¹)	-0.04 (-0.14- 0.05)	-0.13 (-0.25- -0.01)
F _{res} (Hz)	1.21 (-0.31- 2.73)	2.18 (0.03- 4.33)
R ₅₋₁₉ (cmH ₂ O/L*s ⁻¹)	0.12 (-0.002-0.23)	0.17 (0.01- 0.33)

Additive information by FOT in relation to asthma

FEV₁/FVC and R₅ or R₁₉, being the best discriminative measures, were concomitantly added in the same multiple logistic regression model on discriminative capacity for asthma diagnosis. An association to asthma diagnosis was found independently for higher resistance at 5 Hz (R₅) (OR 2.79 (95 % CI 1.62-4.81)) and lower FEV₁/FVC (OR 0.59 (0.39-0.88)) when age, height, gender and weight were added. Independent associations were found with R₁₉ (p = 0.001) and FEV₁/FVC (p = 0.003) in a similar model.

Asthma control and distal airway measures

We found that lower FEV₁/FVC and altered peripheral FOT measures (X₅, F_{res} and R₅-R₁₉) were associated with uncontrolled asthma (p-values < 0.05) in multiple logistic regression models for standardised lung function indices, with adjustments for age, height, gender and weight (see Table 6). Additionally mean differences for each lung function variable between uncontrolled and controlled asthmatic subjects were analysed, and adjusted mean differences are presented in Table 7. To investigate possible differences in outcome with regard to patient age, a sub-group analysis was performed in adults, and in doing this the associations between asthma control and peripheral FOT measures were lost.

Lung function and airway inflammation in asthma

In study II, higher exhaled NO was associated with impaired FEV₁ ($p = 0.03$) and FEF₅₀ ($p < 0.01$), when asthmatic subjects with impaired (FEV₁ % predicted < 80 and FEF₅₀ % predicted < 50 respectively) and normal lung function were compared. Also, a negative association was found between alveolar NO and FEF₅₀ ($p = 0.02$) when asthmatic subjects with normal or impaired lung function were compared.

In study IV, higher exhaled NO was weakly associated with lower X₅ ($\beta = -0.18$ ((-0.30)-(-0.05)), $r = 0.24$) in adjusted analysis (age, gender, height, weight) in asthmatic subjects. No association was found between exhaled NO and spirometry measures or for any other FOT measure.

Discussion

Exhaled NO

We have reported several important findings regarding exhaled NO measurements with implications for practical use of these measurements. The oral contribution to exhaled NO does not have an important impact on the evaluation of a lower airway Th2-cytokine-driven inflammation in asthmatic children and therefore no mouthwash is needed before clinical measurements. The minimal exhalation time needed in pre-schoolers in order to achieve a reliable exhaled NO measurement could be shortened to 4 sec in children 3-4 year old and less than 5.5 sec in children 5-6 year old. This would make these measurements more feasible in younger ages. The adapted single-breath method for exhaled NO was highly feasible from 4 years of age. However, an association between time needed to obtain steady-state NO concentration and the steady-state NO concentration itself was also found, suggesting that high levels of exhaled NO might require longer exhalation times. The level of exhaled NO, measured using this method, in untreated asthmatic subjects aged 3-10 years was higher than in healthy subjects and asthmatic children with ongoing anti-inflammatory treatment. However, a large proportion of the steroid-naïve asthmatic children in our study had FeNO levels below 20 ppb, which is the proposed lower cut-off point in children, and values below this threshold are usually regarded not to signal steroid-sensitive airway inflammation.

We found, in line with previous findings (179, 180), that asthma characteristics such as symptoms day or night during the preceding year, bronchial hyperresponsiveness, and blood eosinophil count are associated with exhaled NO. One might argue that the relation to long-term symptom reporting rather reflects an association between exhaled NO and a wheezing phenotype, in line with a large previous study (179).

In our study, exhaled NO was not correlated with current asthma control defined by ACT score, i.e., subjective disease activity during the last four weeks, which is in line with previous findings (133).

In our material of healthy youths aged 10-20 years, an association between exhaled NO and height could be found, in line with large studies (86, 89, 181, 182). As for preschool children, Malmberg *et al.* previously presented an association between exhaled NO and subject height in healthy children 4-7 years of age (146). We presented in study III, investigating the

association between exhalation time needed to obtain steady-state NO concentration and subject height, no difference in height between the highest and lowest exhaled NO quartile groups (thereby analysing 23 subjects). However, in post-hoc analysis looking at all 51 subjects aged 3-10 years, there was a trend for a higher exhaled NO with increasing child height, in this mixed sample. This relation was not our aim in the study and the material comprised mainly ICS-naïve or treated asthmatic children, which limits the interpretation.

Oral contribution to exhaled NO

Previously the oral contribution to exhaled NO was studied in adults, but no specific data on oral contribution was reported with regard to exhaled NO measurements in children. We have found the oral contribution to be approximately 10 % in youths age 10-20 years, which is lower than previously reported in adults (80, 82). We also found that the reduction in exhaled NO after anti-bacterial mouthwash was no different in atopic asthmatic subjects than in healthy controls. The levels of salivary nitrite in the present study are similar to the ones previously detected in healthy adults when using the same technique to measure nitrite (183). It has been shown that children have higher salivary pH than adults (184, 185) and this might explain the lower oral contribution to exhaled NO in children, since chemical reduction of nitrite to NO is enhanced in acidosis (186). A similar level of oral NO in asthmatic and healthy adults was suggested in a study that isolated exhaled NO from the oral cavity (187). Combining these data with our findings, this would also imply a similar nitrite-derived NO contribution from the pharyngo-oral tract to exhaled NO in asthmatics and controls.

Exhalation time needed for FeNO analysis at steady-state in children

We could demonstrate a relation between the exhalation time needed to obtain steady-state for exhaled NO and height and the FeNO level itself.

Only a few previous studies have addressed the question of time to steady-state NO concentration in children (122, 188), and to our knowledge no previous information on the relation between time to steady-state and height or FeNO level has been presented. Our study validated exhalation times of 4 seconds in children aged 3-4 years and 6 seconds in children aged 5-6 years. An association between time to steady-state NO concentration and subject height was found. This finding seems plausible, as dead space volume is related to height in children (189). We also found that the exhalation time needed to obtain a steady-state level was associated with the steady-state NO concentration itself.

To increase the feasibility of exhaled NO measurement in preschool children the shortest exhalation time possible is a great advantage.

Methods for measurement of exhaled NO in pre-school children

The single breath online method is the gold standard for analysis of FeNO in subjects from 6 years and upwards and provides control over physiological and physical factors known to influence FeNO (78). Specifically, the interference of the high concentrations of NO in the nasal cavity is avoided by exhaling against a resistance to keep oral pressure above 5 cmH₂O, which will ensure closure of the soft palate. Further, the variation in FeNO due to variation in exhalation flow rates is overcome by a standardised flow rate of 50 mL/s. However, young children have difficulty adhering to these two requirements when using the current available instruments, and if they do adhere to them, can only manage short exhalation times.

Measurements without reaching NO steady-state, such as tidal breath manoeuvres, cannot control for exhalation flow rate and are also at risk of nasal contamination, thereby decreasing the reproducibility. The limitations of tidal breathing measurements have been shown earlier, in relatively large studies (190, 191). Highly variable NO levels (1-30 ppb) have been shown in healthy children below 6 years of age, indicating nasal contamination. In another study, no difference between clinical groups of children with transient wheezing and asthma, or any effect of ICS treatment was found (191), indicating a poor sensitivity of the tidal breathing method.

The advantage of single-breath manoeuvres over tidal breathing would thus be a greatly enhanced reproducibility and FeNO levels that are more representative of the lower airways. However, the feasibility of exhaled NO measurement with the standard online single-breath method is problematic in younger children. Buchvald *et al.* found in a multicentre study a success rate of 40 % in 4-year-old children (88). Baraldi *et al.* reported success rate of 49% for the standardised single breath online method in children 4-8 years of age, and with hands-on operator-regulated flow control success rate was 93%, but the number of 4-year-old children in the study was not stated. Malmberg *et al.*, allowing slighter larger variation in exhaled flow, found that 71% of children 4-7 years of age managed acceptable single breath FeNO measurement.

We found high feasibility for children from age 4 years and upwards with an adapted single breath exhaled NO method, following established standardisation to minimise nasal contribution and flow-dependence, when using exhalation times adjusted according to age. The success rate achieved in our study was comparable to the success rate obtained using another electrochemical device in children 4-8 years old, where a steady-state NO concentration was not confirmed (192). It should be stressed that the relatively high success rate among our pre-school children was achieved in spite of the fact that these children had no prior experience from FeNO measurement or spirometry.

Clinical correlation for exhaled NO in children 3-10 years of age

In study III, higher exhaled NO was found in untreated asthmatic children, compared with both healthy subjects and ICS-treated asthmatic subjects.

Sub-analyses in subjects without upper respiratory tract infections were performed, as exhaled NO is increased by certain viral respiratory tract infections, particularly rhinovirus, by a mechanism that may not be corticosteroid-sensitive (193). In infection-free children, the relationship between exhaled NO and asthma diagnosis and ICS treatment was strengthened.

Our data are in line with those by Malmberg *et al.*(146), where it was shown that exhaled NO had a high discriminative capacity for probable asthma in children 4-7 years of age.

In steroid-naïve asthmatic individuals aged 3-10 years, mean exhaled NO in our study was found to be below 20 ppb, which is the previously proposed lower cut-off in children. The fact that our results are in line with previous studies (146, 192) emphasises the need to further refine these cut-offs in younger children.

Alveolar NO

In studies I, II and IV, the estimates of alveolar NO could be calculated satisfactorily in the majority of subjects. Less than 10 % of all measurements did not fit the linear slope-intercept model. This is in line with previous data (194). Our results showed further that both flow rates 50-200 mL/s and 100-300 mL/s offered similar goodness-of-fit for the slope-intercept model when investigating older children and adolescents, with similar alveolar NO regardless of flow rate interval used, in both asthmatic subjects and healthy controls, corroborating a previous study in children (123). This suggests that the flow range 50-200 mL/s for determination of flow-independent parameters is an adequate range when measurements are performed in children, especially since the flow duration for the highest flow rates is more difficult to manage in children, when standard exhalation time windows are used (194).

The unadjusted values of alveolar NO we obtained in asthmatic individuals and healthy controls were in the same range as those reported by previous investigators (129, 195, 196). In study I, there was no effect of reducing the oral contribution on the estimated alveolar NO either in asthmatic subjects or healthy controls.

Clinical signal of alveolar NO

Alveolar NO has been validated as a marker for alveolar inflammation in patients with allergic alveolitis (127), and was thereafter reported to be associated with uncontrolled and severe asthma (128-130). After refinement of

modelling, taking the trumpet shape of the airways and axial diffusion into account, clinical correlations were diverging.

We investigated the clinical signal of alveolar NO with regard to different asthma characteristics. In study II, when TMAD adjustments were adopted as proposed, alveolar NO was not associated with any asthma characteristics. This is also in line with previous reports (133, 197, 198) and in a large study Mahut *et al.* did not find any association between TMAD-adjusted alveolar NO and reported level of disease control in a similar population. However, Pucket *et al.* presented an association between worse asthma control and higher TMAD-adjusted alveolar NO in children 6-17 years (194).

On the other hand, unadjusted alveolar NO in our study did relate to several asthma characteristics. Symptom reporting of exercise-induced dyspnoea during the preceding year was associated with alveolar NO, despite no association between FeNO and exercise-induced dyspnoea. An association between alveolar NO and bronchial hyperresponsiveness, and blood eosinophils was also found.

Whether or not these findings should simply be dismissed and attributed only to the modelling artefact of trumpet model and axial diffusion will be further discussed.

Concerns regarding TMAD adjustments in calculating alveolar NO

NO in exhaled air, measured with a standardised technique, was proposed to be derived mainly from lower airway epithelial cells in healthy subjects (199). Based on these findings, which used intra-bronchial catheter measurement of NO, exhaled NO has also been suggested to be mainly derived from the most proximal lower airways, generations 0-1. However, early studies also pointed towards the most distal airways as substantially contributing to exhaled NO in healthy subjects (67).

To account for the experimental findings with flow dependency for exhaled NO and the inverse correlation to flow for NO concentration and output, the two-compartment model was described (Figure 3). In this, dual sources for exhaled NO, from the conductive zone and from the respiratory zone, were described, and further flow-independent NO parameters were calculated using the slope-intercept model.

Thereafter, the TMAD adjustments have been used in an attempt to compensate for back diffusion and trumpet shape of the conductive airways. The TMAD algorithms were based on healthy individuals or well-controlled non-obstructive asthmatic patients (124, 125).

The estimates of alveolar NO and bronchial flux were in previous studies shown to be correlated, and this association could be extinguished by TMAD correction (124, 133, 194). As proposed, a positive correlation would indicate falsely estimated high alveolar NO with higher bronchial flux, and a simultaneous underestimation of the bronchial flux, due to back diffusion of NO from higher concentrations in the conductive airways towards the infi-

nite sink behind the alveolar-capillary membrane. We therefore expected a similar pattern in our study. In study II, we found a positive association between the alveolar NO and bronchial flux in the asthmatic subjects, before TMAD adjustments. However, this association turned instead negative after TMAD adjustments, indicating a possible over-adjustment in our population. Our data corroborates recent findings from a large study in school-children, using multiple exhalation flow rate NO measurements for calculation of flow-independent parameters in different models (195) In this study Linn *et al.* reported a positive association between unadjusted alveolar NO and bronchial flux in an unselected population, while a weak negative correlation was found after TMAD adjustment.

In healthy controls, somewhat intriguingly, we found a negative association between bronchial flux and alveolar NO with unadjusted values, which was strengthened after TMAD-adjustment.

There is no final consensus on whether the correction for trumpet shape of the airways and axial diffusion should be adopted or not. Our investigation of the clinical signal provided by alveolar NO in asthmatic individuals was the largest study to date, and we included both children and adults. Our data raise concerns regarding over-adjustment with the proposed correction algorithms.

Airway obstruction and NO dynamics

Despite the unrewarding situation of contradictory findings on the clinical importance of alveolar NO, and no final conclusions with regard to the question of back diffusion, the modelling and the questioning of the same can lead to further understanding of the core question, i.e., what goes on in the small airways.

A possible clue for increased understanding and application is the question of varying small airway obstruction which would lead to differing need for axial diffusion correction in different settings. Interesting data has been presented, implicating different patterns of airway generation obstruction being related to different patterns of NO dynamics. It has been shown that alveolar NO can increase after anti-inflammatory treatment in a subgroup of asthmatic patients (200). This might reflect an increase in back diffusion with the treatment effect on peripheral obstruction. Thus the need for TMAD correction would be smaller in case of pronounced peripheral small airway obstruction, as TMAD correction equations are defined based on healthy or unobstructed mild asthmatics.

Elevated exhaled NO in asthma was traditionally attributed mostly to large airway inflammation (65), although the relative contribution of large and small conductive airways in asthmatic subjects has not been determined. After findings of lowered exhaled NO shortly after bronchial provocation (201) (95, 202), further studies suggested that NO production occurred not only most proximally and most distally, but throughout the conductive air-

way generations in subjects with asthma (203). Van Muylem *et al.*, using assumptions of different effects of direct bronchial challenge (impact on generation down to ~10) and indirect bronchial challenge (impact on generation down to ~16), found with direct and indirect bronchial challenge in a cross-over design among newly diagnosed asthmatic subjects, a correlation between airway region of bronchoconstriction, and the percentage of exhaled NO decrease after challenge. This supports the hypothesis that NO production is spread throughout the entirety of the airways. Studies have shown that exhaled NO can, in different subjects, be either elevated or reduced after bronchodilation (91-94). A way to visually evaluate the impact of back diffusion has been suggested by plotting bronchial flux versus alveolar NO, and a zone of normality for the relation between alveolar NO and bronchial flux has been defined (204).

In the original two-compartment model, the alveolar region compartment composes a distal source of NO in exhaled air, where normally an instant uptake to the haemoglobin in the circulation of locally produced or back-diffused NO occurs. The theoretical explanations for a raised alveolar NO would thus be raised production (true alveolar/ alveolar-close interstitial inflammation), impaired transport over the alveolar-capillary membrane (impaired membrane diffusion), or back diffusion (diffusion from the most distal small airways).

A bold suggestion would be that most of the fluctuations in estimated alveolar NO when asthma is suspected or proven, and when basic slope-intercept modelling criteria are fulfilled, are working in concert with small airway patchy constriction in an intricate fashion. In a situation where iNOS and/or endothelial NOS activity are raised above normal in the small airways, and dependent on concomitant luminal obstruction at site, both estimated alveolar NO and exhaled NO will be affected, in a not easily transparent way and this might be difficult for the model to account for. Thus, the suggested TMAD-adjustment algorithms might diminish or extinguish the signal we are looking for.

Another theoretical possibility would be that alveolar NO reflects a systemic or alveolar/interstitial inflammation. This needs to be considered when alveolar NO is interpreted. In our study, we found an association between unadjusted alveolar NO and blood eosinophil count. These findings corroborate those of others (195, 205).

Forced oscillation technique and lung function

We found in study IV that lung function indices by FOT gave at least as much information as did spirometry measures in discriminating, on a group level, between mainly well-controlled asthmatic individuals and healthy controls. Among the FOT variables, resistance measures were most strongly

associated with asthma diagnosis. Our results on the relation between FOT measures and asthma diagnosis are in line with previous studies comparing forced oscillometry to spirometry in children (159, 206) and also corroborates previous studies on the discriminative capacity in asthma diagnosis (153, 207). Further we found that FOT measures reflecting peripheral engagement were associated with asthma control, corroborating previous results in children (208) and in adults (161, 164, 209).

FOT measures also offered information that was additive to that from spirometry and this will be further discussed.

Loss of airway patency is the basal feature of airway obstruction, and can be caused by airway constriction, airway wall thickening and/or excessive mucus. Airway structure and pulmonary function is interlinked and in order to understand pathophysiological processes of the airway, more or less advanced models are required.

Airway resistance is strongly dependent on airway cross-sectional area. This relation offers a basic understanding of how airway resistance is distributed throughout the airway generations according to Weibel (210), but also how to interpret lung mechanics and different patterns of impedance measures in obstructive airway disease (211).

Keeping in mind that the distinction between markers of large and small airway obstruction may be somewhat artificial we discuss our findings in terms of resistance at 5 Hz (R_5) to reflect overall lung function and the reactance at 5 Hz (X_5), the resistance difference between 5 and 19 Hz (R_5-R_{19}), and resonance frequency (F_{res}), suggested to reflect small airway engagement.

In standard spirometry, measures of lung volumes and flow are indirect measures of flow resistance. The parameters used are FEV_1 for mostly proximal lung function, and the ratio FEV_1/FVC which indicates airflow limitation and is considered to mirror, to some extent, also more peripheral airways. A mid-expiratory-flow measure such as FEF_{50} is sometimes used as a marker of small airways, but its use is discouraged in guidelines since it is highly variable (44). Limitations of spirometry exist with regard to feasibility in a clinical setting in children below 6 years of age and also because no reliable measure of small airways is offered. Standard spirometry is also based on measurement of airflow during forced expiration; thus, the basal respiratory function during tidal breathing is not mirrored.

In a recent study, improvement in FOT peripheral measures, but not in spirometry measures, was found adding extra-fine particles bronchodilation to ongoing ICS in adults (212), results which indicates small airway response mirrored by forced oscillometry but not by spirometry. Previous studies found modest associations between indices from spirometry and forced oscillometry (153), and information from these two different methods of assessing lung function being complementary rather than interchangeable

(157, 164, 209). Our findings of independent associations for lung function measures from FOT and spirometry in relation to asthma diagnosis may thereby be plausible.

Since no cross-device and cross-population based reference values exist for FOT as yet, we aimed only at investigating the variables of lung function obtained using standard spirometry and our FOT device in an explorative way.

In our study only subgroup analysis in adults was possible, due to the small number of children. When we excluded individuals aged 18 years and below, we found that the association between asthma control and peripheral FOT indices no longer was significant. This might partly be explained by loss of power, although it could be argued that the number of children was relatively small. It is also possible that there are larger differences in FOT measures between controlled and uncontrolled asthma groups in children. The literature provides some support for such a difference, as Shi *et al.* (208) presented in uncontrolled asthmatic children of similar clinical asthma characteristics as those in our study: AUC values > 0.80 in ROC-analyses for X_5 , F_{res} and R_5-R_{19} in univariate analysis, compared with AUC values from ROC-analyses of around 0.60 in our study. Takeda *et al.* found, in clinically stable adult asthmatics (mean age 55 years), an association of asthma control only with X_5 and this was low-moderate (161).

It could also be speculated that in children, distal airway changes are a sign of symptomatic disease, while in adults some of these changes might be due to longer duration of disease and airway remodelling and without a corresponding association to present symptom control. Thus, a possible explanation for the loss of association in adults in our study could be the relatively stable and well-controlled asthma in young adult patients with FEV_1 within normal range.

With regard to associations between exhaled NO parameters and forced oscillometry indices, studies have previously reported weak associations between alveolar NO and FOT measures thought to reflect peripheral processes (131, 167, 168). No associations were reported between exhaled NO and FOT measures (167, 168).

In our material, no convincing associations, or only weak associations, were found between exhaled NO parameters and lung function indices by FOT. This needs to be considered in light of the proposed complex interaction between small airways obstruction, airway surface area available for NO output, and airway NO dynamics. At very least, this highlights the fact that the interpretation of markers of small airway dysfunction is not easy and that these inflammatory and functional markers cannot be used interchangeably.

Strengths and limitations

Our investigation of the clinical signal provided by alveolar NO in asthmatic individuals was the largest study to date, and we included both children and adults, finding no interaction with age group regarding the main outcomes.

Further strengths are that we, to the best of our knowledge, for the first time investigated the oral contribution to exhaled NO in specifically a youth population. Also, the height/age dependence and the association between FeNO concentration itself and exhalation-time needed to establish a NO steady-state concentration for single-breath measurement in children was not previously reported.

Throughout the study samples in this thesis, asthma was defined as a physician's diagnosis, which might be regarded as both a strength and a limitation. Asthma defined by GINA diagnostic criteria (1) includes a confirmation of variable airway obstruction in lung function tests. Thus, the accuracy of asthma diagnosis in our studies might be questioned. On the other hand, in line with the earlier discussed limitations of standard spirometry for evaluation of lung function in children, the indices of spirometry may not always mirror the actual airway obstructive picture.

In study I, regarding the generalisability of the results on the oral contribution to exhaled NO in children, different populations of the same age may consume nitrate-rich foods differently. This would possibly affect the magnitude of oral contribution, but is unlikely to affect asthmatic and healthy children differently. Those few participants who did report large quantities of nitrate-rich foods prior to the measurements however had FeNO in the normal range.

Studies II and IV were based on the MIDAS asthma cohort, which was constructed to include asthmatic individuals from both primary care and specialist care (paediatric asthma and adult asthma outpatient clinics, Uppsala University hospital), covering the county of Uppsala. Losses to follow-up and due to study design should be mentioned. Out of a total of 413 asthmatic subjects in the MIDAS asthma cohort, we used in study II data from 376 subjects in whom the NO flow independent parameters were compliant with the mathematical model. The non-compliers have been described, and what we might have lost were patients with slightly more impaired lung function. At the follow-up 3-4 years later, 156 asthmatic subjects declined participation in the second visit of the MIDAS asthma cohort, and another 23 had no FOT measurement. No differences between asthmatic participants (n=234) and non-participants at follow-up were found with regard to age, height, weight, FEV₁, FEV₁/FVC, exhaled NO or atopy at first enrolment. When analysing symptom report in the preceding 12 months, recall bias must be considered.

In study III, parents were asked about allergic symptoms to aeroallergens and food allergens in their child, but no allergy sensitisation data was collected. The combined information on allergic sensitisation and reported clinical allergic symptoms would have been optimal. The lack of allergy test was due to the fact that the evaluation of a clinical signal was a secondary aim.

Also, a limitation in the study set-up for study IV might have been the lack of separate repeated measurements, as is suggested with the more standardised method of IOS. According to the FOT manufacturer's instruction, repeated separate measurements were not required. A common interference with FOT/IOS-registration is the tongue position upper airway artefact, which was minimised with our specialised mouthpiece.

Due to the lack of reference values for both children and adults for our FOT method, and for comparative reason, we analysed our data in study IV on lung function measures as numeric values for mean difference analyses, and as standardised variables of numeric values for logistic regression analyses. Thus, the best performance of spirometry indices might not have been appreciated, since nonlinear reference equations are stated for spirometry measures. We therefore compared our results on discriminative capacity gained in our modelling with % predicted values for spirometry measures, and the AUCs of the ROC-curves were similar. Also we received consistent findings in both multivariate linear regression and in logistic regression models.

Clinical implications, future perspectives

When a child presents with acute wheeze that responds well to a bronchodilator, there is little doubt that reversible airway pathology is at hand. When combined with a history of a characteristic pattern of respiratory problems, no lung function test is needed for diagnosis.

Asthma is more difficult to diagnose when symptoms are vague and the physical examination is normal. In such circumstances, objective markers of an underlying pathophysiology are needed. Spirometry with bronchodilator reversibility testing applies to this in many circumstances and helps to identify airway obstructive processes, at least down to airway generations 8-9. However, many children with asthma have normal lung function as assessed through standard spirometry between exacerbations. A predominant small conductive airway process, i.e., in generations ~9-16, could possibly go undetected with standard spirometry.

With the first case scenario with obvious reversible airway obstruction in a preschool child, we cannot be certain that the underlying process is ICS-responsive, or that the long-term effects of treatment or non-treatment with ICS would differ.

In Sweden, many children with recurrent obstructive respiratory symptoms are given ICS treatment during the cold season (3-6 months a year) for several years during the pre-school period. The evaluation of an underlying allergic airway inflammation is aided by the child's atopic history, and allergy testing. This information constitutes an indicator, but not evidence of a present allergic, steroid-responsive airway inflammation.

Could we improve the rationale for initiating ICS treatment? Optimally, we would perform an assessment of the underlying airway inflammation type before making the decision to start controller treatment, even with frequent episodes of obstructive airway symptoms in preschool children. It is also possible that an ongoing airway inflammation which would profit from ICS treatment could go undiagnosed in a child due to slow progressive disease with vague symptoms that the child adjusts to. In light of both over- and under-treatment in childhood asthma, assessment of the Th2-cytokine-driven eosinophilic, steroid-responsive inflammation could contribute to a more rational treatment. Exhaled NO is proposed as such a marker.

The adapted single-breath online method for NO measurement suggests a way to improve feasibility and reliability for NO measurement in preschool children. Our findings in study III, of highly feasible measurements from 4 years and upwards with this method, and at confirmed NO steady-state, may contribute to further establishing determinants of exhaled NO and to determining the clinical signal of exhaled NO in preschool age in a larger study. With knowledge of the total exhalation time needed to obtain steady-state FeNO, measurements with age- or height-adjusted exhalation times with an electrochemical device could be implemented, if repeated studies confirm our findings.

Our findings of mean exhaled NO in steroid-naïve asthmatic individuals aged 3-10 years being below 20 ppb, which is in line with previous studies, emphasises the need to further refine the cut-offs in children.

A practical implication of our findings regarding the oral contribution in study I is that an anti-bacterial mouthwash is not necessary before measurement of exhaled NO in clinical practice.

FOT measurement could possibly add clinical value in assessment of small airway processes in situations where a clinical suspicion is not confirmed by spirometry, and also when treatment control is not achieved despite consideration of possible environmental disease triggers and non-compliance issues. The results in study IV suggest that forced oscillometry might offer additive information to spirometry. Our results also indicate that valuable information might be obtained with regard to assessment of small airway processes.

Our knowledge on the role of small airways in asthma is restricted due to the relative inaccessibility of the small airways (213). Airway modelling is im-

plied in order to increase our understanding of the distribution of airway pathophysiology throughout the airway tree, and with regard to small airway pathology in asthma over all clinical disease ranges. Associations between different markers of small airway disease and symptom control (46, 161), exacerbation risk (50) and fine-particle treatment response (56, 214) have been reported. The indices from forced oscillometry and modelling of exhaled NO are together with inert gas washout variables and static spirometry measures of air-trapping the “best guess” as yet. However, the exact location of obstruction reflected by different methods is not completely mapped. Neither is it determined if small airway involvement should be defined by several of the functional methods. A possible alternative way to overcome the issue of correctly addressing back diffusion with regard to different small airway obstructive patterns might be to further study exhaled NO before and after change in bronchial-bronchiolar muscle tone.

Future research areas

- Repeated studies on the height/age dependency of exhaled NO steady-state in preschool and early school age children to establish suggested methodological exhalation-time adjustments for single-breath measurement in these age groups.
- If this method described above is developed, this would warrant studies on healthy children from 3-4 years and upwards, with exhaled NO, combined with forced oscillometry assessment, in order to define normal values in preschool and early school-age children.
- Further studies of methods of small airway assessment in older children, as forced oscillometry, exhaled and alveolar NO and inert gas washout tests, to investigate their relation and their clinical use in childhood asthma diagnostics and monitoring.
- Studies on new-onset/suspected childhood asthma and healthy controls for discriminative power of exhaled NO and forced oscillometry measures in childhood asthma.

Conclusions

To summarise the methodological findings in this thesis on exhaled NO and estimation of alveolar NO:

- An adapted single-breath online method to measure exhaled NO was highly feasible from 4 years and upwards. In preschoolers, the total time needed to NO steady-state during standard exhalation flow rate 50 mL/s was < 4 s at 3-4 years of age, < 5.5 sec at 5-6 years of age, and is dependent on height or age, and on the exhaled NO level itself (paper III).
- The oral contribution to exhaled NO is similar in atopic asthmatic youths and in healthy individuals of same age, and no effect on alveolar NO was found. No antibacterial mouthwash is needed before measurement of exhaled NO in clinical practice (paper I).
- An exhalation flow range of 50-200 mL/s allows for similar estimates of alveolar NO as a flow range of 100-300 mL/s (paper I). We suggest to refrain from routine application of TMAD corrections in estimation of alveolar NO in patients with asthma (paper II).

To summarise the clinical findings in this thesis on exhaled NO, estimated alveolar NO and forced oscillation technique:

- Exhaled NO is higher in ICS-naïve asthmatic children than in healthy children aged 3-10 years. In our study ICS-naïve asthmatic children had mean exhaled NO level below 20 ppb, implying a need for refined clinical cut-offs for exhaled NO in preschool children (paper III).
- Unadjusted alveolar NO is related to long-term symptom reporting, bronchial responsiveness, blood eosinophils and ICS-treatment in older children and adults. When TMAD correction is applied, no associations between alveolar NO and asthma characteristics are found (paper II).
- FOT resistance measures are associated with asthma diagnosis, much like spirometry. FOT offers lung function information which is additive to that from spirometry. Small airway measures by FOT, as well as FEV₁/FVC, are suggested to be related to asthma control (paper IV).

Sammanfattning på svenska

Astma är en av de vanligaste kroniska sjukdomarna hos barn och ungdomar. Sjukdomen kan sammanfattas som en benägenhet till varierande grad av luftvägssammandragning, som ger varierande andningssvårigheter, med en underliggande kronisk luftvägsinflammation. Många barn med astma har en bakomliggande luftvägsallergi, som kan hålla igång och förvärra sjukdomen. Hos förskolebarn är det dock vanligare att allergi inte förekommer, och då uppstår luftvägsbesvären vanligen av förkylningar.

Sjukdomen kan hos många kontrolleras så att dagliga aktiviteter, sömn och tillväxt inte påverkas, och astmaattacker undviks. En viss andel av barn med astma når inte sjukdomskontroll, trots möjlighet till underhållsbehandling med inflammationsdämpande läkemedel. Inhalationssteroider har god effekt på allergisk luftvägsinflammation, oftast redan i låga doser. Däremot fungerar inte den typen av behandling särskilt bra vid enbart virusorsakad luftvägsträngsel. En tydlig skillnad i behandlingseffekt för inhalationssteroider kan alltså påvisas för barn med- eller utan en bakomliggande allergisk luftvägsinflammation. Underhållsbehandling med inhalationssteroid kan idag bli aktuell för flertalet barn med misstänkt astma och uttalade symptom eller svåra försämringar. Därmed vore det önskvärt att enkelt och säkert kunna fastställa huruvida ett barn med misstänkt astma skulle vara hjälpt, på kort och lång sikt, av inhalationssteroider. **Utandad kväveoxid**, FeNO, har föreslagits som en metod som skulle möjliggöra detta. Nuvarande utrustning och metodik kan dock inte tillämpas på barn som är yngre än 6 år och därför behövs en anpassning av tekniken för yngre barn.

För att säkert ställa diagnosen astma skall en föränderlig luftvägsträngsel kunna påvisas med objektiva mått. Standardmetoden för att påvisa en s.k. variabel luftvägsobstruktion är en spirometri-undersökning. Man kan då mäta lungfunktionen före och efter att luftrörsvidgande läkemedel givits. Man kan också mäta lungfunktionen flera gånger och upptäcka förändringar från dag till dag, eller efter det att underhållsbehandling påbörjats i utredningssyfte. Barn kan tekniskt klara av spirometri från 6 år. Däremot är mätresultaten hos barn i tidig skolålder inte tydligt kopplade till faktisk bakomliggande sjukdom, och många barn klarar inte av tekniken att kraftfullt blåsa ut under en längre stund. Många barn som får astmaförsämringar har också helt normal lungfunktion när de testas mellan attacker. **Oscillationsteknik** är en annan metod för att mäta lungfunktion som är mindre krävande för patienten. Metoden baseras på att ljudvågor med olika frekvenser skickas in i

luftvägarna under lugn andning. Denna metod är möjlig redan från ca 4 års ålder. Möjligen kan man med oscillationsteknik bättre än med spirometri spegla även de minsta luftvägarna.

Syftet med denna avhandling var att undersöka dessa två metoder för diagnostik av astma hos barn. Målsättningen har varit att analysera ett antal metodologiska frågeställningar, och även frågeställningar som är kopplade till användbarheten av metoderna i det kliniska arbetet att diagnosticera och följa barn med astma.

Delstudie I

En del av det NO som utandas kommer inte från luftrören, utan från munhålan. Här kan NO bildas genom bakteriell omvandling av nitrat från maten till nitrit som utsöndras i spottkörtlar. Nitrit kan sedan omvandlas till NO av bakterier i munhålan. Inga tidigare resultat fanns för att bestämma om detta munhålebidrag är olika hos barn med astma jämfört med friska barn. Vi ville också studera hur olika modeller som delar upp bidraget till utandat NO från större luftvägar samt de mest perifera luftvägarna fungerade hos barn och ungdomar. Genom modellering erhålls alveolärt NO, som mått på bidraget från de mest perifera luftvägarna. Beräkning av alveolärt NO har föreslagits med eller utan inräkning av bakåtdiffusion av NO i luftvägarna ned mot luftblåsorna och blodet.

Metod: Vi mätte standardiserat utandat NO (50 ml/s), före och efter antibakteriell munsköljning. Vi uppmätte även utandat NO vid olika andningshastigheter i syfte att beräkna alveolärt NO, hos 29 individer med astma, och 29 friska kontroller i åldern 10-20 år.

Resultat: Munhålebidraget till utandat NO var ~10% hos både individer med astma och friska kontroller. De olika modellerna som föreslagits i litteraturen för att beräkna alveolärt NO fungerade på barn samt gav jämförbara resultat, och vi fann även att modellen fungerade utan att barnen behövde blåsa mer än 200ml/s, vilket kan underlätta genomförbarheten.

Slutsats: Ingen munhålesköljning är nödvändig vid uppmätning av utandat NO för att skilja mellan barn med astma och friska kontroller. Munhålebidraget hos barn var mindre än vad som tidigare beskrivits hos vuxna. Modeller för beräkning av alveolärt NO går att tillämpa på barn.

Delstudie II

Syftet med denna studie var att studera kopplingar mellan dels alveolärt NO, dels utandat NO, och olika astma egenskaper, för att utvärdera hur dessa två mått skall kunna användas för att bättre förstå astmasjukdomen.

Med fokus på alveolärt NO ville vi också kartlägga vilken typ av modell som bäst kunde spegla det mest perifera luftvägsbidraget till utandat NO. De modeller vi ställde mot varandra var med- eller utan korrektion för bakåtdiffusion ned mot lungblåsor och blodcirkulationen.

Metod: Data analyserades från 376 patienter med astma som deltagit i MIDAS-studien, genomfört utandad NO, och erhållit godkända beräkningar av alveolärt NO. Forskningsdeltagarna rekryterades från barn- och lungklinikerna vid Akademiska sjukhuset, samt primärvården i Uppsala. Astmadiagnos och uppgifter om aktuell behandling hämtades ur patienternas journaler. Spirometri, test av luftvägarnas retbarhet, och blodprov för analys av luftburen allergisensibilisering och blodeosinofiler genomfördes. Individerna skattade aktuell astmakontroll med AKT (Astma Kontroll Test), samt svarade på frågor om astmasymptom under det senaste året.

Resultat: Beräknat alveolärt NO var inte kopplat till någon astma karaktäristik, om föreslagen modell med korrektion för bakåtdiffusion användes. För beräknat alveolärt NO utan nämnda korrektion, sågs koppling till rapporterade astmasymptom vid ansträngning senaste året, bronkiell hyperaktivitet, blodeosinofiler, atopi och nivå av inhalationssteroidbehandling. Utandad NO var kopplat till symptom under det senaste året, bronkiell hyperaktivitet, blodeosinofiler, atopi samt nivå av inhalationssteroidbehandling.

Slutsats: Utandad NO är kopplat till större sjukdomsburden hos unga personer med astma. I vårt material sågs tecken till att den föreslagna korrekturen för bakåtdiffusion ledde till en överjustering i modellen, vilket ger belägg för att avråda från att schablonmässigt använda denna korrektion när alveolärt NO beräknas.

Delstudie III

I standardmetoden för uppmätning av utandad NO görs mätningen efter ett 10 sekunder långt utblås, eller 6 sekunder för barn < 12 år. Vårt syfte var att studera användbarheten för en vidareutveckling av metoden att säkert mäta utandad NO utan inblandning av luft från bihålorna, med ett jämnt flöde, och när koncentrationen av NO i utandningsluften är stabil. Vår hypotes var att tiden för att uppnå jämviktskoncentration av NO i utandningsluft vid ett givet flöde var kopplat till kroppsstorlek, och vi anpassade utandningstiden efter barnets ålder.

Metod: Totalt 63 barn i åldrarna 3-10, varav 54 barn med astmadiagnos och 9 friska kontroller, undersöktes. Barnen fick blåsa genom ett munstycke, och utandningstryck samt flödes hastighet kontrollerades. Den elektrokemiska apparaturen sammankopplades med en kemiluminescens-analysator för att möjliggöra analys av tidsåtgång till uppnådd stabil NO-koncentration under utandningen.

Resultat: Med denna metod lyckades 81 % av barnen med minst ett godkänt blås, och tiden för att uppnå stabil NO-koncentration i utblåset var < 4 sekunder för 3-4-åringar, och < 5.5 sekunder för 5-6-åringar. Tiden för att uppnå stabil NO-koncentration i utblåset var kopplat till barnens längd, men även till aktuellt utandad NO-värde. Utandad NO för barn med obehandlad astma var 15.9 ppb (95 % konfidensintervall 12.2- 20.9) och för friska barn 9.1 ppb (6.6- 12.4), $p = 0.03$.

Slutsats: Uppmätning av utandad NO med en utvecklad mätteknik hos förskolebarn, där utandningstiden anpassades efter barnets ålder, var möjlig att genomföra och > 80 % av 4-åringarna klarade ett godkänt blås. Barnen med obehandlad astma hade högre utandad NO-värde jämfört med friska, dock med medelvärden för barnen med obehandlad astma som låg under den nu föreslagna nedre cut-off-nivån på 20 ppb.

Delstudie IV

Vi analyserade mått på lungfunktion, dels med vanlig spirometri, och dels med oscillationsteknik (FOT), hos äldre barn och unga vuxna med kontrollerad och ej kontrollerad astma, samt hos friska kontroller. Huvudsyftet var att jämföra hur väl de olika lungfunktionsmått kunde särskilja mellan astma och frisk kontroll, samt mellan okontrollerad och kontrollerad astma.

Metod: Data insamlades från 234 patienter med astma samt 60 friska kontroller, vilka deltog i ett andra besök i MIDAS-studien, där undersökning med oscillationsteknik, utöver tidigare uppmätningar (se delstudie II), genomfördes.

Resultat: Beträffande FOT variabler var högre luftvägsresistens (R_5 , R_{19}) och större skillnad mellan resistensen för låga och högre oscillationer kopplade till en större sannolikhet att tillhöra astmagruppen. Beträffande spirometri var lägre FEV_1/FVC och FEF_{50} kopplat till en högre sannolikhet för att tillhöra astmagruppen. Information från FOT undersökning gav adderad information till spirometri, kopplad till astmadiagnos. FOT mått som avser att spegla små luftvägar (X_5 , F_{res} , R_5 - R_{19}), liksom FEV_1/FVC , var kopplade till astmakontroll.

Slutsats: Våra resultat ger stöd för att FOT-mätning kan ge kompletterande information om lungfunktion i samband med astmautredning och uppföljning.

Sammanfattningsvis har flera viktiga fynd rapporterats avseende uppmätning av utandad NO hos barn. En bakteriell munsköljning behöver inte genomföras före mätning av utandad NO. Utandad NO är kopplat till mera sjukdomsbörda hos unga personer med astma. Beräknat alveolärt NO kan ge information kopplad till astmaegenskaper, och våra data ger belägg för att inte schablonmässigt korrigera för bakåtdiffusion. Utandningstiden kan kortas för de mindre barnen med en vidareutveckling av standardmetoden för NO-mätning. Våra resultat ger stöd för att cut-off nivån för utandad NO för de mindre barnen kan behöva anpassas. Lungfunktionsmätning med FOT kan ge information som kompletterar den från spirometri. Vidare studier får ytterligare klargöra hur utandad NO och FOT kan användas för att belysa sjukdom i de små luftvägarna vid utredning och uppföljning av astma, vilket skulle kunna ha betydelse för optimerad behandling och astmakontroll.

Acknowledgements

This work was carried out at the Department of Women's and Children's Health, Uppsala University, Uppsala, Sweden. Financial support was kindly provided by Swedish Heart and Lung Foundation, Hesselman Foundation, Bror Hjerpstedt Foundation, Uppsala County Research Funding (FoU-medel), Agnes and Mac Rudberg's Foundation, Gillberg Foundation, and Swedish Association against Asthma and Allergy.

In the making of this thesis great many people have contributed invaluable, in different ways. In particular, I would like to express my sincere gratitude to:

All children and adults, who participated with effort and time in the studies. Without you this work would not have been possible.

Andrei Malinovschi, my main supervisor from 2012 and onward, for your highly skilled duly support, scientific guidance and clarity, and for expecting things to run as fast as your own mind. Thank you for your patience and all focused help when needed.

Lennart Nordvall, my main supervisor in the start, who opened the door for this journey of learning to begin, for your encouragement and inexhaustible enthusiasm for scientific thinking in the clinical field of asthma and allergy. Importantly, your expression "det här blir kul" always pinpointed the delight of the endeavor.

Kjell Alving, co-supervisor, for scientific clarity, indispensable contribution in the field of airway inflammation, for sharing your expertise and your exquisite clarity in presentation.

Christer Janson and **Magnus Borres**, co-writers of the articles, for your great knowledge, scientific advice, and all the constructive comments during writing.

Pia Kalm Stephens, whom I have been working with in many different ways during these years not only as co-writer, for your patience and accuracy in data collection, for your much appreciated patient contacts and for your professional skills.

Lars Berglund for valuable contribution as a co-writer, and practical guidance in statistics.

All the teachers at the doctoral courses, especially **Ulf Eriksson** at Scientific Presentation, Uppsala University for enthusiastic, wise and memorable talks, and **Werner Vach**, Regression models in medical research, Freiburg Universität, who managed to make statistics intelligible through web-based learning.

Linnea Holmen, and **Janet Holmen**, for appreciated help in linguistic revision.

My fellow PhD-students at the Lung Research Group, for interesting meetings.

My colleagues, both past and present, at the University children's hospital, Uppsala, especially at the newly combined Pediatric Allergy- and Children's Care and Health Clinics. To heads **Christophe Pedroletti**, **Tina Dahlgren** and **Monica Mäki Karlstrand** for allowing time-out from clinical work.

Cia, Lena, Dennis, Eva-Lena, Maja, Sanna and colleagues at Vretens förskola, for making value each day in a safe, yet lively way for Å among other children, which made it possible to be a mother, PhD-student and pediatrician without overwhelmingly bad conscience.

Dear old friends for great laughs, for being you and knowing me, and for support during times of trouble; **Annika, Lottie, Jill, Johanna W, Johanna S, Jenny O**. I hope to see you all soon.

Sweet neighbour friends for valuable pep-talks; **Pamela, Linda, Helena**.

Mother and father, for your love.

Darling brother **Jonas**, and sisters **Malin** and **Lena**, with families. **Eva**. Every dear member of my extended **Rentzhog** family.

The next billion or four letters reach towards the man making everything possible. **Sven**, as in bright eyes, belief and joy, trust and encouragement, all-in dårfink and tenderness, PATIENCE and your wisdom of life. With you there is no limit.

Arvid, Erik and **Ärna**. Hopefully your recollection of the most intense work period during the making of this thesis somehow may contribute to keep your own curiosity and quest for knowing more respectfully sparkling.

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