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**CLINICAL APPLICATION OF LUNG FUNCTION TESTING
IN CHILDREN**

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PREFACE

My work presented in this thesis is the achievement from my three years fellowship from October 2003 to February 2006 at the Division of Respiratory Medicine at the University Children's Hospital in Zurich, Switzerland.

During this period, as a member of the research group of PD Dr. med. Johannes Wildhaber at the Division of Respiratory Medicine, I focused my research under his supervision on the clinical application of lung function testing in children of different ages. Together with Vera Bernet and Daniel Straub I investigated the pulmonary long-term outcome in children after congenital diaphragmatic hernia repair. In addition, we reported as the first group on exhaled nitric oxide levels in this group of children. With Alexander Möller, Michael Friedt and Daniel Straub, I studied lung function and exhaled nitric oxide in children and adolescents with Morbus Crohn and Colitis ulcerosa and I have shown that exhaled nitric oxide is elevated in these patients. With Josué Sznitman, and Alexander Möller, I have been able to show that "eyeballing" of spirometry curves, translated in complex, computer-assisted, quantification of concavity of Flow/Volume curve includes important information about asthma severity. Finally, with Vera Bernet and Alex Möller, I measured repetitively exhaled nitric oxide in pulmonary healthy newborns to study diurnal variation and variation in nitric oxide production depending on feeding.

The work presented in this thesis would not have been possible with all those I have had the good fortune to be associated in close collaboration and friendship and who stimulated and motivated me.

I would like to thank the following already mentioned colleagues whose help with recruitment, data collection and statistical analysis made completion of the various studies possible: Vera Bernet, Michael Friedt, Alexander Möller, Josué Sznitman, and Daniel Straub.

My special thanks go to my mentors, PD Dr. med. Johannes Wildhaber, and Ass. Prof. Zdeněk Kokstein, for their understanding, help and expertise.

Finally, I would especially like to thank my wife Lenka and my son Vojta for their love, outstanding support, and in particular patience during preparation of this thesis, and to all the families with their sick children taking part in our studies.

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LIST OF ABBREVIATIONS

AHR	Airway hyperresponsiveness
AR	Airway responsiveness
BAL	Bronchoalveolar lavage
BHR	Bronchial hyperresponsiveness
ECP	Eosinophilic cationic protein
E_L	Total lung elastance
eNO	Exhaled nitric oxide
FEV _t	Forced expiratory volume time
FOT	Forced oscillation technique
FRC	Functional residual capacity
GER	Gastroesophageal reflux
IFN- γ	Interferon gamma
IgE	Immunoglobulin E
IL-1	Interleukin 1
ISAAC	The international study of asthma and allergies in childhood
LRTI	Low respiratory tract infection
Mch	Methacholine
NO	Nitric monoxide
O ₂	Oxygen
PEFV	Partial expiratory flow volume
P _{trans}	Transmission pressure
Raw	Airway resistance
R _L	Total lung resistance
RSV	Respiratory syncytial virus
RTC	Rapid thoracic compression technique
R _{ti}	Tissue resistance
RV	Residual volume
RVRTC	Raised volume rapid thoracic compression technique
TGF- β	Transforming growth factor beta
TNF- α	Tumor necrosis factor alfa
TLC	Total lung capacity
V'	Flow
V' _{max}	Maximal flow
V' _{maxFRC}	Maximal flow at functional residual capacity

INTRODUCTION

1.1 ASTHMA AND LUNG FUNCTION TESTING

Asthma is an immunologically and neurologically mediated chronic inflammatory disorder of the airways. Airflow obstruction with different grade of reversibility caused either directly by inflammation and/or by secondary bronchial hyperresponsiveness, is found in the most children. The etiology of asthma, including complex interaction among several cells, mediators and neural pathways ending in an inflammation is multifactorial, consisting of both hereditary and environmental factors.

Asthma is the most common chronic disease in childhood with a prevalence of 5 -10% in the most European countries, including Switzerland and Czech Republic.

Asthma is a major health burden, causing high costs due to use of medications, doctor's visits and hospital admissions and greatly affecting the quality of life of patients and their parents. Asthma in childhood has a high impact on the development of chronic pulmonary disease in adulthood. It has been shown that asthma in adulthood is the endpoint of a multiple step process, starting early in life and continuing during childhood, presenting with chronic pulmonary changes characterized by bronchial hyperresponsiveness, airway remodeling and irreversible airflow obstruction.

In order to improve quality of life, to reduce health costs and to avoid chronic pulmonary disease in later life, an early diagnosis of asthma in infancy is needed to establish appropriate early intervention strategies for prevention and therapy.

WHEEZING DISORDERS IN INFANTS

Wheeze, the clinical sign of asthma, is a noise produced by the bronchial system. The mechanism of production of wheeze has been shown to be related to flow limitation. It represents flutter in the airway wall which produces sound. The Wheeze is not a sign limited and specific to asthma, more than that, it is a sign of obstructed airflow in general, seen in a number of respiratory diseases in childhood. Therefore, it would be expected that the measurement of physiological parameters in lung function tests, showing obstruction to airflow, would not serve as diagnostic marker for asthma. However, measurements of pulmonary function in relation to wheeze, to smoke exposure and to a positive family history of atopy and/or asthma have shown some interesting findings. Low levels of lung function measured in early infancy by different methods are associated with an increased risk to develop recurrent wheeze in later infancy. Infants with a positive family history of asthma and/or smoke exposure have reduced pulmonary function in infancy. It might be that in combination with other physiological markers, such as genetic or inflammatory, a more precise diagnostic approach to the recurrently wheezy infant could be achieved. Some light has been shed on the subject of recurrent wheeze by a Tuscon longitudinal study. This large cohort study has followed lung function measurements associated with recurrent wheeze over a period of more than 20 years. It has been shown that infants can be divided into four groups: never wheezers, transient early wheezers, late onset wheezers and persistent wheezers. More usefully it was demonstrated that it may be possible by

means of PFTs to define these groups, at least in an epidemiological way. The pathophysiological basis of the obstruction of airflow in recurrently wheezy infants remains uncertain. There are several possible mechanisms. The infants may have classical asthma which would be defined in older children by intermittent and partly reversible airway obstruction, airway hyperresponsiveness and association with atopy (late onset and persistent wheezers). In contrast, there may be a general underdevelopment of the lung or disparity between airway and alveolar size (dysanaptic growth) in transient early wheezers. Alternatively, the transient, early wheezy infants may have normal airways and lung tissue and merely be unlucky to suffer from recurrent viral illnesses.

Measurement of lung function in co-operative individuals has added greatly to the scientific understanding of the nature and management of asthma. The measurements of pulmonary function would also be expected to be helpful in the assessment and management of recurrent wheeze in infancy. Pulmonary function testing in infancy is a changing field in which several different techniques have been developed over the last two decades. However, no single infant PFT has yet achieved the position of gold standard in the way that spirometry has in older children. Therefore unanswered questions remain with regard to the techniques.

Assessment of airflow obstruction in infants

Airway resistance may be measured by the single breath flow-volume technique, by pletysmography, either passively or dynamically and by FOT. More complicated and invasive techniques are required to separate these measurements into their lung and chest wall components, and to measure complex parameters such as R_{ti} and to measure airway wall properties. However, measurements of forced expiration prove more useful clinically, as the majority of respiratory illness encountered in infancy is obstructive in nature.

Rapid Thoracic Compression Technique

The most widely used infant PFT to measure flow-volume curves is the Rapid Thoracic Compression technique (RTC), which produces PEFV curves and gives maximal expiratory flow at FRC. RVRTC, variation of this technique was developed to allow production of the flow-volume curves over a larger proportion of expiration. Both methods use plastic inflatable jacket wrapped around the infant's chest and abdomen. Flow is measured at the mouth using a close fitting mask and a pneumotachograph with minimal dead space. Flow is integrated to volume and real time flow volume loops displayed. Once a stable tidal breathing loop has been established, the operator activates the compression jacket at the point of end inspiration. Most investigators use a valve to briefly occlude the airway to allow full jacket inflation. As mentioned, the maximal flow at FRC is the standard parameter produced by RTC. Flow is maximized by progressively increasing the jacket pressure until no further rise in V' occurs and V'_{max} has been reached. The maximal flow occurring at the previously established tidal FRC is then V'_{maxFRC} . Curves should be rejected if maximal flow is not achieved early in the curve, there is excessive noise on the expiratory curve, and inspiration has occurred before FRC, the curve is left shifted or a stable FRC had not been established.

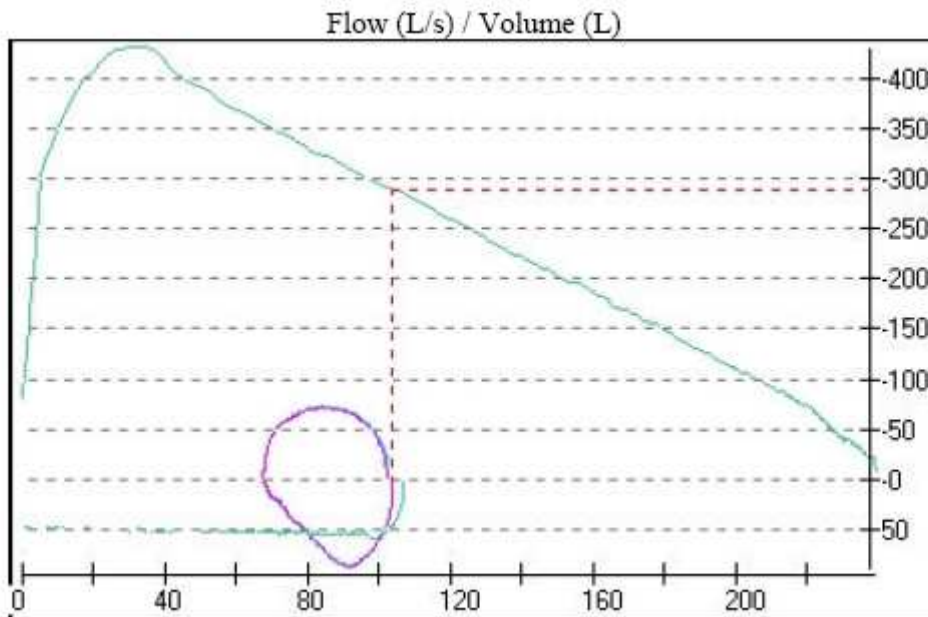
A major disadvantage of the RTC technique is the inherent instability of the FRC landmark in infants. As mentioned previously infants can set their own level of FRC and as a consequence of this it can vary during or between studies and thus degrade the information obtained from the test. Factors which can alter FRC include REM sleep, bronchoconstrictor agents, bronchodilator agents and disease state. This variability in FRC has been proposed as the explanation for the high variability of V'_{maxFRC} . The second major concern about the RTC technique is whether flow limitation occurs. Flow limitation ensures that the measurements reflect lung physiology. As mentioned previously, flow limitation is usually documented with iso-volume pressure flow curves. It is likely that flow limitation is easily achieved in diseased infants and indeed some are already flow limited at tidal breathing. The situation in healthy infants is much less certain.

Raised volume rapid thoracic compression technique

For the reasons outlined above, variable FRC and questionable flow limitation, many investigators were frustrated with the RTC technique and have looked to modify it to improve usefulness. Turner et al. modified the RTC method using a pump to rapidly raise lung volume prior to generation of a flow-volume curve. They recognized that their technique had several potential advantages: lung volume was

extended, inflation and deflation pressure could be standardized and FEV_t measurements would be available. In addition, flow limitation has been shown using this technique. At P_{trans} values between 20 and 25 cm H₂O, most outcome variables were pressure independent. This range has therefore been suggested to be the most suitable for use with the RVRTC technique.

Figure 1: Flow-volume curve obtained with the RVRTC technique.



Assessment of bronchodilator response in infants

There is a controversy regarding the effectiveness of bronchodilator agents in infants. Some authors have found no evidence of bronchodilator in infants with viral wheeze. Others have demonstrated a worsening of forced expiratory flows in the acute phase of the obstructive disease probably because of collapse of airway wall due to myorelaxation. Due to these controversial results, there exists still much debate as to the effects of bronchodilators on lung function in infants as does on their clinical usefulness. Results from studies using the RVRTC technique in symptomatic recurrently wheezy infants support the usefulness of the RVRTC technique as a tool to assess and to monitor bronchial reactivity.

ASTHMA AND LUNG FUNCTION IN PRE-SCHOOL AND SCHOOL-AGED CHILDREN

Based on the understanding of the pathophysiology it would make sense, that measuring the degree of airway obstruction, bronchial hyperreactivity, airway inflammation and airway remodeling would be the main measure to diagnose and to monitor asthma in children. However, despite the understanding of the pathophysiology the diagnosis and monitoring of asthma is still mainly based on symptoms.

The impact of airway remodeling on the natural course of the disease is still little understood. Assessment of airway inflammation has so far been proven to be too invasive, too expensive, not enough sensitive or specific and not enough validated and standardized to be used in clinical practice and hence, at the moment still remains mainly a research tool. In addition, despite there being some indications that the assessment of bronchial hyperreactivity is helpful not only in the diagnosis but also in the monitoring of the disease, these techniques are rarely used in clinical practice.

Therefore, despite asthma now mainly being recognized as a chronic inflammatory disease of the airways, measuring reversible airway obstruction is still the main objective outcome measure recommended by most guidelines for the diagnosis and monitoring of asthma in childhood. However, some of the lung function parameters used, such as PEF (peak expiratory flow) and FEV₁ (forced expiratory volume in one second) have been lately shown not to be as helpful in the care of children with asthma.

Whereas asthma is a well defined disorder in children older than five years of age, the diagnosis of asthma in younger children remains challenging. Children presenting with wheeze at an early age consist of a heterogenic group and little is understood about the pathophysiology of wheeze in young children and hence, the diagnosis and monitoring of wheezy disorders in young children are difficult. The tools available for the assessment of airway inflammation and/or reversible airway constriction and bronchial hyperreactivity in this young age group are not yet available for a wide clinical practice and remain mainly available for research purposes.

Pathophysiology of asthma in school-aged children

Asthma should be regarded as a syndrome and not as a single disease entity. Like other syndromes, asthma has its characteristic features, which are expressed to a variable extent in affected individuals.

The main pathophysiological characteristics are:

- Reversible airflow obstruction
- Bronchial hyperresponsiveness
- Airway inflammation
- Structural airway changes (airway remodeling)

Surprisingly, current strategies for the management of asthma as outlined in guidelines do generally not focus on the assessment of all of the above characteristics for diagnosing asthma and for monitoring the effectiveness of treatment.

Functional abnormalities

Increased resistance to airflow is the most important functional abnormality in asthma. It is the basis of the clinical manifestation of asthma, including dyspnea and wheeze. Increased airway resistance in asthma may be partly due to poor function of pulmonary surfactant. However, the main mechanisms leading to increased airway resistance are decreased airway diameter as a consequence of bronchial constriction, luminal narrowing due to airway wall edema and luminal obstruction resulting from hypersecretion of mucus. Normally, airflow limitation is reversible, however, there may be fixed airflow obstruction, which is mainly seen in later disease stage or may also be an expression of a specific asthma phenotype. The Childhood Asthma Management Program (CAMP) study looking at the effect of an inhaled steroid compared to nedocromil and placebo on the improvement of lung growth using post bronchodilator percent predicted FEV₁ as the primary outcome has shown an initial improvement. However, post bronchodilator percent predicted FEV₁ gradually diminished by the end of the treatment period over 4 to 6 years and did not differ from that in the nedocromil and placebo group. This finding may be indicative for pathophysiological processes (airway remodeling) other than airway inflammation, which are not influenced by an anti-inflammatory treatment with inhaled steroids.

While functional abnormalities of airflow in asthmatics have mainly been attributed to changes in resistance of large and medium sized airways, studies have shown that small airways and terminal airways contribute to airway resistance. Bronchial obstruction leads to air trapping and hence, to increased volume of gas remaining in the respiratory system at the end of tidal expiration (functional residual capacity=FRC) and at the end of forced expiration (residual volume=RV). Using capnometry and multiple-breath nitrogen wash-out technique it has been shown, that the obstruction of peripheral airways diminishes distribution of air during inspiration and cause ventilation/perfusion mismatch. Another functional abnormality is the hyperresponsiveness of the airways to stimuli to provoke airway narrowing. Children with asthma often have normal lung function and develop severe bronchial obstruction during an exacerbation. This finding is likely explained by an underlying bronchial hyperresponsiveness. Weiss et al. have shown a relationship in children with a normal FEV₁ of 94% predicted between severity and bronchial hyperresponsiveness. Thus, bronchial hyperresponsiveness may be helpful in the initial assessment of asthma severity as well as in the follow-up assessments of asthma control.

Assessment of resistance to airflow

The whole-body plethysmography serves as a reference method and the “gold standard” for measurement of airway resistance or airway conductance as its reciprocal value. Spirometry, the most widely used lung function test procedure, allows only indirect assessment of airflow resistance. Forced oscillation technique and impulse oscillometry allow measurement of impedance of respiratory system, which at frequencies 4-32 Hz reflects predominately airway caliber and hence allows estimation of airway resistance. The interrupter technique measures also a complex quantity rather than airway resistance only, because the influence of chest wall can not be neglected.

Assessment of lung volumes

The standard approach of lung volume measurement is direct determination of functional residual capacity (FRC) and then, when possible, calculation of total lung capacity (TLC) and residual volume.

The most widely used methods are whole-body plethysmography and gas-dilution techniques. In addition, there have been a number of attempts over the last decade to measure lung volume using imaging techniques. The high-resolution computer tomography (HR-CT) scan provides relatively precise and rapid assessment of lung volume, which correlated significantly with values, obtained using plethysmography.

Inflammation

Airway inflammation is the dominant feature of asthma and is present even at the earliest stage of mild disease. The inflammatory response is associated with the accumulation of chronic inflammatory cells, including lymphocytes, macrophages, and plasma cells abundant in the lamina propria in larger airways and in the adventitial connective tissue of the outer wall in smaller airways. So far, direct assessment of airway inflammation has been too invasive or not feasible for the use in children. However, detection of volatile substances in exhaled air; in particular nitric oxide represents new promising tools for non-invasive measurement of airway inflammation.

Airway remodeling

Airway wall remodeling refers to a variety of structural changes in the airway wall. Most prominent is the accumulation of collagens and other matrix components in the subepithelial region of the airway wall. In addition, there is smooth muscle hypertrophy and/or hyperplasia, epithelial proliferation and mucous cell hyperplasia/metaplasia, as well as increased vascularity of the mucosa. The alterations in airway wall thickness could have two opposite effects on airway mechanics. Firstly, when the increase in thickness predominantly affects the inner layers of the airway wall, pronounced narrowing of the airway with concomitant flow limitation will be the result. Secondly, when the outer layer of the airway enlarges, there will be increased stiffness without remarkable effect on airway resistance. Currently, bronchoscopy and bronchial biopsy are the most commonly used techniques for determining the extent of airway remodeling. However, apart from invasivity and risks, bronchoscopically obtained specimens do not permit an evaluation of the peripheral airways and their surrounding parenchyma. The high-speed interrupter technique allows studying the changes in airway wall mechanical properties, when the anti-resonance frequency (F_{ar1}) corresponds with airway wall compliance. The high-resolution CT scan, magnetic resonance imaging (MRI) and the single-photon emission computed tomography (SPECT) can be helpful in the assessment of the airway abnormalities in asthmatic patients.

The use of pulmonary function testing in asthmatic children older than five years of age

National and international guidelines recommend an initial evaluation followed by regular re-evaluations for the assessment of the severity and the control of asthma. The initial evaluation guides therapy based on the level of severity, whereas the regular re-evaluations assess the therapy response based on the level of control. Guidelines recommend a number of outcome measures for the initial evaluation and the regular re-evaluations. Pulmonary function tests, mainly spirometry, are generally considered the standard criterion for objective evaluation and re-evaluation.

Rationale to measure lung function

Despite the advantage of objective assessment of variable reversible airway obstruction by pulmonary function testing and the recommendation of most guidelines to perform lung function tests in the initial assessment as well as in the follow-up of asthma, lung function tests are not widely available in private practice. A doctor's diagnosis of asthma based on symptoms and clinical signs followed by a therapeutic trial is still common standard in the assessment of asthma in general practice. It was shown in the AIRE (Asthma Insights and Reality Europe) study, that only 29% of asthmatic children reported that their doctor had given them a lung function test in the past year, and over 50% of children with asthma had never undergone a lung function test. This is in agreement with epidemiological asthma studies, where the inclusion of patients was entirely based on a doctor's based diagnosis. On the other hand, spirometry, particularly FEV₁, is often used as the primary outcome variable for efficacy studies in asthma.

Diagnosis

The initial assessment of asthma as stated by guidelines, with FEV₁ being the only objective measure, serves to evaluate the severity of the disease in the initial evaluation. Children with mild persistent asthma have FEV₁ values of more than 80% predicted, children with moderate persistent asthma have values of 60-80% of predicted and children with severe persistent asthma have values of less than 60% of predicted. However, in the light that most asthmatic children have FEV₁ values in the normal range independent on disease severity, as discussed latter, it may be necessary to redefine asthma severity in future guidelines [34].

Monitoring

The regular monitoring of lung function may have some benefits for the patient as well as the doctor. The clinical assessment of asthma control by the doctor can be verified by an objective measure. Many asthmatics, especially children are poor symptom perceivers. An other rationale to regularly monitor lung function is, that poor lung function shown by airway obstruction, is a poor prognostic factor for the outcome of asthma later in life.

Poor perception of airway caliber in asthma

According to guidelines, children's and parent's reports on asthma symptoms are important in assessing asthma severity and control. Dyspnea, cough and wheeze, as well as exercise induced symptoms are helpful to guide treatment decisions. However, it is well known that many children and their parents do not adequately perceive or report asthma symptoms. There is evidence to support the hypothesis that poor perception of airways obstruction is a clinically relevant problem in children with asthma. Considerable airways obstruction may be present in asymptomatic patients with asthma. Children with long-standing airways obstruction are less likely to report dyspnea than children with acute onset of airways obstruction. Such poor perceivers are more likely to present with hypoxia during an acute exacerbation, predisposing to severe or life-threatening attacks. Reported symptoms do not reliably correlate with lung function results in asthmatic children and their parent's and correlation is dependent on the instrument used.

Reduced lung function as a poor prognostic factor of asthma outcome

A recent retrospective study in a large number of asthmatic children showed that children with significant airways obstruction were twice as likely to develop an asthmatic attack in the subsequent year as children with more or less normal lung function. However, many children with life-threatening asthma episodes have FEV₁ values on hospital admission >80% predicted. This implies that the number and severity of exacerbations should also be taken into account when assessing asthma severity. A large body of evidence shows that airways obstruction in children with asthma is associated with ongoing respiratory morbidity and reduced lung function in adulthood, both in general population based cohorts of children with mild disease and in hospital-based cohorts of patients with more severe asthma. These studies have demonstrated a small annual decline in lung function among patients with asthma, with a decline of approximately 1% FEV₁ of predicted per year. Thus, airways obstruction in children with asthma has both short-term and long-term prognostic significance. Ideally, lung function should be measured serially over time, with the understanding that the change in lung function over time may provide valuable information regarding the natural course of disease. However, it remains controversial, when and how often lung function has to be monitored in asthmatic children. Beside the initial assessment of asthma severity it is without any doubt indicated if symptoms deteriorate, are unexplained and/or do not respond to any anti-asthma therapy.

Evidence for the usefulness of lung function measurements

Two recent reviews on the usefulness of monitoring lung function in asthma have clearly stated that despite there being a rationale for the recommendation to monitor lung function there is apparently no firm evidence. There has so far not been a single randomized trial looking at the usefulness of monitoring lung function on short and long term outcomes in asthmatic children.

Peak Flow Measurements

Portable PEF meter have been advocated as an objective measure of asthma control and included in guidelines for a more effective asthma management in order to reduce morbidity and mortality based on rationales outlined above, mainly the poor perception of symptoms by children. However, there are no or weak correlations reported between PEF and individual symptom scores and/or bronchial hyperresponsiveness in asthmatic children. It has also been shown that changes in PEF do not correlate with lung function measured by spirometry. Clinically significant falls in PEF were found to occur in the absence of changes in lung function, and significant falls in lung function occurred that were not reflected by a fall in PEF. In addition, it has been shown that the information provided in a PEF diary by apparently well motivated children with asthma and their families is unreliable [60]. Not only do patients cheat by inventing PEF values, but they also misreport the readings they have made. Asthmatic children and their parents are more likely to use PEF meters to get an objective measure during symptomatic times, however, daily use is likely to be an unrealistic expectation. In summary, there is no evidence to support the general use of the current PEF measurements for home monitoring in asthma management in childhood. Home monitoring may be beneficial in asthmatic children facing

extra challenges as a result of disease severity, sociodemographic or health care system characteristics. New portable electronic devices measuring PEF and FEV₁ may allow a more accurate and controllable objective measure for home monitoring in asthmatic children.

Spirometry

Fulhbrigg et al, performed an analysis of spirometry in more than 3000 children with asthma who were observed for up to 15 years and found >90% of all FEV₁ values to be >80% predicted. Over 50% of asthmatic children from the CAMP study with moderate persistent asthma based on frequency of symptoms had mean pre- and postbronchodilator FEV₁ values between 94% and 103% predicted. Bacharier et al. found that the mean FEV₁ was 95.1% of predicted in children with moderate persistent asthma, 90.2% in those with moderate persistent asthma, and 83.8% in those with severe persistent asthma. This general finding that most asthmatic children have FEV₁ in the normal range has major implications for its usefulness in the initial assessment of asthma severity as well as in the follow-up of asthma control. If the diagnosis and/or the management of asthma greatly rely on FEV₁ measurements, there is a risk of underdiagnosis and/or undertreatment of asthmatic children. On the other hand, a low FEV₁ seen in follow-up visits may likely be explained by gross undertreatment or unexplained disease progression. There is also a lack of correlation between FEV₁ and individual symptom scores in asthmatic children. Thus, asthma status may be better characterized by parent-reported symptoms, health care utilization, and functional health status measures than by FEV₁. In contrast to a baseline FEV₁, the measurement of a β -agonist response in FEV₁ by performing a pre- and postbronchodilator spirometry correlates with airway inflammation. As discussed earlier, small airways and terminal airways contribute to airway resistance in asthma. This may explain why pulmonary function parameters (PEF, FEV₁) thought to represent larger airways, do not well correlate with other asthma outcome parameters (symptoms, inflammatory parameters), whereas some recent studies found a better correlation between pulmonary function parameters (Maximal expiratory flow=MEF_{25, 50 and 25-75}) thought to represent smaller airways and other asthma outcome parameters. In summary, for the critical assessment of asthma severity and asthma control pre- and postbronchodilator response in FEV₁ should be obtained and analyzed together with other parameters (MEF_{25, 50 and 25-75}) on the expiratory loop. Brand et al. call the process of looking at the entire flow volume curve "eyeballing", where a concave pattern in the curve may be seen despite a normal FEV₁. The limitation of FEV₁ as inclusion/exclusion criteria for efficacy studies has to be stressed.

Other pulmonary function parameters

The most useful pulmonary function test in the assessment of acute asthma is pulse oxymetry. The importance of non-invasive assessment of gas exchange arises from the observation, that pulmonary gas exchange abnormalities resulting from alveolar hypoventilation and ventilation-perfusion mismatch are present even in the clinically mildest form of asthma and furthermore, the correlation with FEV₁ is only weak. This fact implies the importance of peripheral airway involvement.

It is known, that in mild or moderate asthma, at times when FVC and FEV₁ spirometry values are entirely normal, the measurement of lung volume can identify the presence of air trapping. The RV is the most sensitive parameter of airflow obstruction in children and the decrease in RV after

bronchodilator administration appears to be more specific for asthma diagnosis as an increase in FEV₁. Currently, there are not data supporting the clinical utility of static lung volumes measurement in management of childhood asthma.

The use of lung function tests in wheezy disorders in children less than five years of age

Due to the age-dependent lack of cooperation lung function measurements in young children are difficult to perform. Newborns and infants are not co-operative at all and therefore pulmonary function can only be assessed passively. Some techniques like tidal breathing measurements, interrupter techniques and gas-dilution measurements may be performed in non-sedated sleeping infants, whereas measurements of forced expiratory flows as well as body-plethysmographic measurements require sedation. For preschool children tidal breathing measurements and measurements using the interrupter or oscillation techniques can provide reasonable results while only minimal cooperation is required.

Pathophysiology

The pathophysiological aspects discussed previously also apply to children less than five years of age. The same is true for functional abnormalities like airflow limitation and altered lung volumes. However there are some functional characteristics specific to this age group: The chest wall is highly compliant and the balance between airway wall compliance, airway resistance and lung recoil differs from that of older children, resulting in age-dependent patterns of airflow limitation. The functional residual capacity (FRC) is not constant in this age group. Infants modulate expiratory flow in order to dynamically elevate FRC above the level passively determined by the outward recoil of the chest wall and the inward recoil of the lung, an important strategy to establish and maintain an adequate lung volume in the presence of a highly compliant chest wall. Furthermore FRC shows certain intra-individual variation due to changes with different sleep stages. Measurements of airway resistance are also influenced through the upper airways, especially when measured by using a facemask. About 50% of the total airway resistance is generated by the upper airways, mostly by the nose.

Rationale to measure lung function

Wheezy disorders in early childhood consist of a heterogeneous group. According to Martinez it would be important to distinguish between transient early, late onset and persistent wheeze. The majority of infants with wheeze do not have an increased risk of asthma later in life. In a substantial minority of infants, however, wheezing episodes as well as reduced lung function and increased airway hyper-responsiveness are probably related to persistent asthma [Because wheezy disorders are difficult to distinguish clinically, it would be helpful to have some objective measures for diagnosis. Asthma starts early in life, and hence, objective measures applicable for all age groups would be helpful to guide treatment from early childhood into adulthood.

Assessment of resistance to airflow

By assessing tidal breathing flows a range of physiological parameters related to respiratory control and pulmonary function can be studied. The tidal forced expiration (rapid thoraco-abdominal compression technique; RTC) and the raised volume rapid thoraco-abdominal compression technique (RVRTC) have been widely used in infants. The RTC-technique is suitable for measurements of maximal expiratory flows at functional residual capacity ($V'_{\max}\text{FRC}$); RVRTC allows measuring an enhanced spectrum of flow indices. A relatively high intra-subject variation limits the use of RTC whereas the intra-subject variation of RVRTC is much less. Additionally forced expiratory maneuvers allow judging the shape of flow-volume curves, which may reflect the degree of airway obstruction: convex curves being associated with better respiratory function than concave curves.

Measurement of lung mechanics

Mechanics of the respiratory system can be measured by the forced oscillation technique (see chapter), where sound waves of a certain spectrum of frequencies are applied to the respiratory system and the complex ratio of airway opening pressure (P_{ao}) to flow (V'_{ao}) is measured. At very low oscillation frequencies (< 1 Hz), the response to the pressure oscillation gives predominately the information about viscoelastic properties of the lung tissue and therefore low frequency forced oscillation (LFOT) seems to be an interesting technique to investigate peripheral lung mechanics. At frequencies above 4 Hz the contribution of airway resistance to total impedance is raised, and at very high frequencies (> 100 Hz) the impedance provides information about airway wall compliance, probably particularly important in evaluating airway remodeling.

Measurement of airway hyper-responsiveness

Several approaches have been used in infants to assess airway response to inhaled bronchoconstrictive or bronchodilator agents. These include the measurement of airway resistance, analysis of tidal breathing pattern and measurement of transcutaneous oxygen tension as example, but mainly a combination with measurements of forced expiratory flows has been used and found to be appropriate, whereas tidal breathing measurements are not discriminating enough.

Assessment of lung volumes

Like in older children lung volume measurements can be performed using gas dilution techniques or whole-body plethysmography. Body-plethysmography measures all thoracic gas including areas of trapped air and intestinal air, whereas gas dilution techniques measure only the lung volume communicating with the upper airways. Gas dilution techniques, such as the closed circuit helium dilution (FRC_{He}), the open circuit nitrogen washout technique (FRC_{N_2}), and also the multiple-breath inert gas washout using a gas mixture containing 4% sulphur hexafluoride (FRC_{SF_6}) are used in adults and have been adapted for the use in infants. Beside the measurement of the lung volume, indices of ventilation distribution such as the lung clearance index (LCI) can be calculated for estimation of the degree of peripheral obstruction [100]. A substantial advantage of these methods is that conclusions can be drawn on very peripheral obstructions, not detectable by flow measurements.

Evidence for the usefulness of lung function measurements in young children

The evidence of infant lung function measurements is limited by the lack of standardized equipment and a relatively large inter- and intra-subject variation of some techniques.

Tidal volume measurements

The ratio T_{PTEF}/t_E , calculated from tidal breathing flow curves, has been shown to predict the development of recurrent wheezing over the subsequent 3 years of life. This approach allows to distinguish between asthmatic and healthy preschool children and to evaluate the response to methacholine, histamine or bronchodilators.

Forced expiratory maneuvers

Rapid thoraco-abdominal compression technique, suitable for measurements of maximal expiratory flows at functional residual capacity ($V'_{max}FRC$), has been widely used to investigate aspects of lung development, and lung disease the RVRTC technique has been introduced relatively recently and therefore the number of studies published is still small. Expiratory flows obtained by RVRTC distinguish better between healthy and infants with recurrent wheeze than $V'_{max}FRC$ as produced by RTC technique, reliably detects differences in airway obstruction between healthy and recurrently wheezy infants and is suitable to assess airway hyper-responsiveness. Forced expiratory maneuvers have been used to show that asymptomatic infants with recurrent wheeze have lower $FEV_{0.5}$ than normal, to investigate the airway tone and the airway response to a beta-agonist in healthy young children and to demonstrate that in asymptomatic recurrently wheezing infant's beta-agonists as well as anti-inflammatory treatment cause no significant improvement in lung function. Furthermore the response to airway challenge tests has been assessed. The disagreement of maternal perception of the infants' health with objective lung function parameters and doctor's health assessment has also been demonstrated, stressing the importance of objective measurements. Regarding the longitudinal course, a reduced $V'_{max}FRC$ in the first month of life, correlating independently with airway hyper-responsiveness at the age of 11 years, could be demonstrated in wheezing infants.

Measurement of functional residual capacity by gas dilution techniques

There are only few studies in this age group. Using the multiple-breath N_2 and the SF_6 – washout technique in infants, the involvement of the peripheral airways in asthma up to airways close to the acinar entrance has been documented. Recently a new method has been published, which aims to solve the problem of measuring trapped gas.

Measurement of lung mechanics

With the low-frequency forced oscillation technique it has been shown that asymptomatic wheezy infants have altered respiratory tissue mechanics and that infants with a family history of asthma have elevated respiratory system resistance and reduced elastance. The low-frequency FOT was found to be a suitable method to study bronchodilator responsiveness in infants. Investigating airway reactivity to methacholine has shown conflicting results: in pre-school children FOT was found to be unreliable

when determining respiratory system resistance when using frequencies of 6 and 8 Hz. However, using a frequency spectrum of 0.5 – 20 Hz in infants, respiratory tissue mechanics were significantly altered after methacholine challenge correlating with a decline in FEV_{0.5}. Furthermore using a spectrum of high frequencies a dramatically change in airway wall mechanics after methacholine challenge could be demonstrated.

In preschool children with asthma the interrupter technique (Rint) may be helpful in monitoring lung function. It has been shown to correlate with FEV₁ but to underestimate airway resistance compared with body-plethysmography. Furthermore it has a good short-term repeatability and can therefore be used to examine bronchodilator response in children with asthma. Interrupter technique measured higher resistance values in children with persistent wheeze as compared with others. On the other hand some investigators mention a number of theoretical and technical problems, requiring further exploration, and call for studies standardizing the equipment and identifying the most appropriate analysis technique for application in infants.

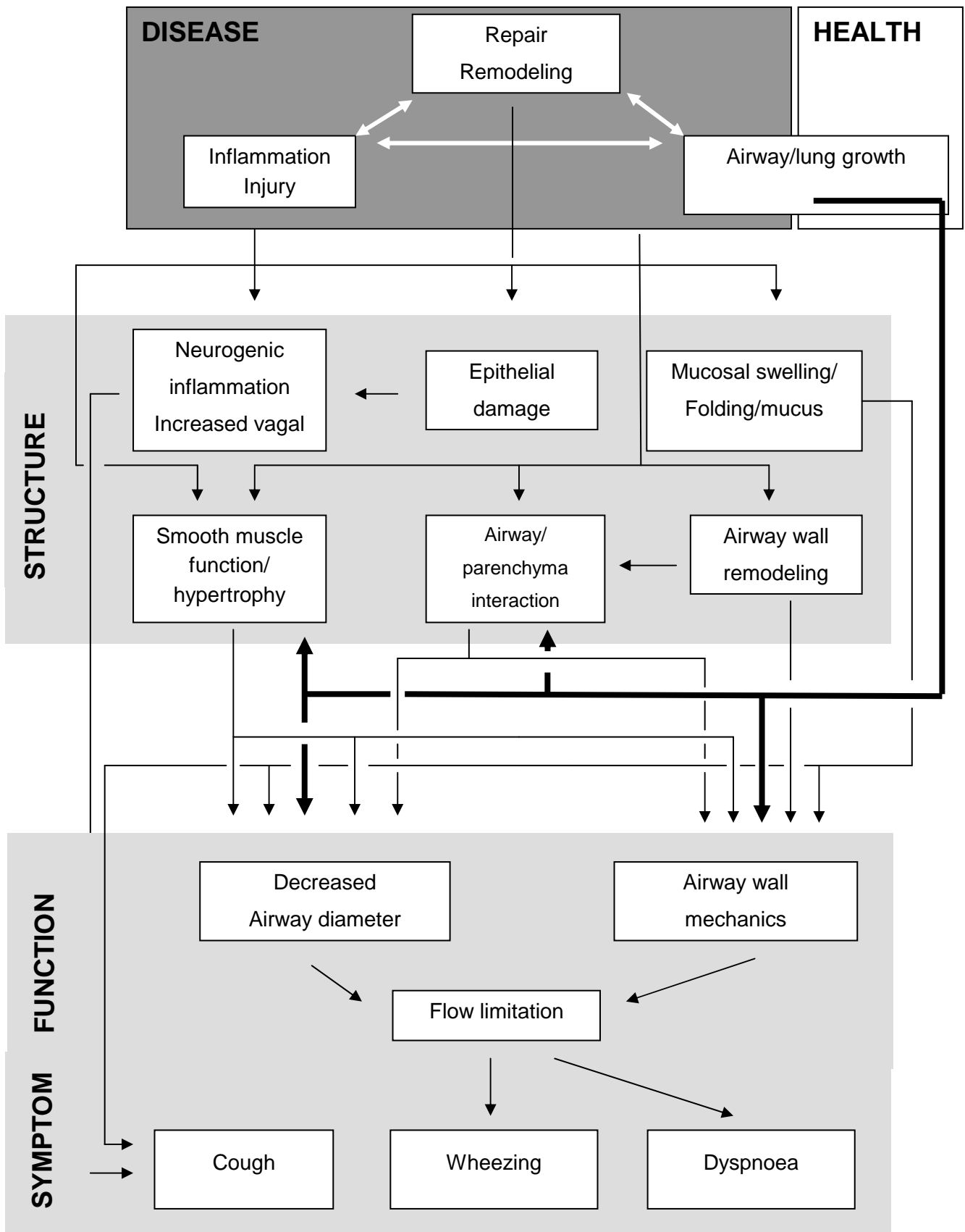
The high speed interrupter technique has been used for investigations in infants mainly by one study group who demonstrated altered airway wall mechanics in asymptomatic infants with a history of wheeze.

Airway responsiveness in infancy

Bronchial challenge tests have been found to be a safe and tolerable tool even in infants, when using appropriate monitoring. By assessing airway responsiveness, it could be demonstrated, that bronchial responsiveness in the neonatal period and male sex is a risk factor for wheezing in infancy. Increased airway responsiveness at the age of 1 month is associated with doctor-diagnosed asthma and decreased lung function at the age of six years.

Legend to illustration:

Complex interactions in early childhood asthma. (from reference: Frey U: Why are infants prone to wheeze? Physiological aspects of wheezing disorder in infants. Swiss Med Wkly 2001; 131: 400-406).



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NITRIC OXIDE IN HEALTHY AND PEDIATRIC RESPIRATORY DISEASES

During the past decade many hundreds of studies have disclosed the multiple roles of nitric oxide (NO) in physiology and pathophysiology of human body. NO, produced by a wide variety of cell types, is generated via oxidation of L-arginine. This reaction is catalyzed by the enzyme NO synthase (NOS). NOS exists in three distinct isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). NO derived from the constitutive isoforms of NOS (nNOS and eNOS) have been shown to be modulators of bronchomotor tone and an important mediator in neuronal transmission. NO derived from iNOS seems to be a potent proinflammatory mediator with immunomodulatory effects. The fractional exhaled NO is abnormal in different airway diseases and the repetitive measurement of fractional exhaled nitric oxide called 'inflammometry' has changed gradually the principles of asthma monitoring. Finally, the production of NO under oxidative stress conditions secondarily generates strong oxidizing agents (reactive nitrogen species) that may modulate the development of chronic inflammatory airway diseases and/or amplify the inflammatory response.

I. INTRODUCTION

In the 1980s several lines of research showed that NO is an essential molecule in the physiology of the human body. In 1987, the proof that NO is identical to endothelium derived relaxing factor (EDRF), was provided. Subsequently, the importance of NO and other nitrogen oxides in the regulation of various body functions, including platelet aggregation and neurotransmission emerged. Eventually, this set of observations was honored by the Nobel Prize in 1998. Shortly after the publication of landmark papers proposing EDRF to be NO, several investigators made observations suggesting that nitrogen oxides are relevant to respiratory biology. The group of Gustafsson measured as first the endogenous NO in the exhaled air of humans. A tremendous amount of research has subsequently been devoted to addressing the pulmonary NO biology: NO and NO donors relax human airway smooth muscle in vitro and a bronchodilatory effect of inhaled NO was demonstrated during methacholine-induced bronchoconstriction. The other way around, inhibition of NO formation increases airway responsiveness to contractile agents in asthmatic patients. In addition, NO acts also as a neurotransmitter of the inhibitory nonadrenergic noncholinergic (NANC) nerves. During the last few years several studies have been performed to assess the relationship between levels of exhaled NO and airway inflammation.

B. Bioactive Forms of NO

NO itself has a short half-life in vivo (1–5 s) because of its reactivity with hemoglobin. It has one unpaired electron, making it a free radical that promptly reacts with other molecules such as oxygen or superoxide radicals. NO is an ubiquitous messenger molecule that affects various biological functions, either at low concentrations as a signal in many physiological processes such as blood flow regulation, platelet reactivity, NANC neurotransmission or at high concentrations as cytotoxic and cytostatic defensive mechanisms against tumors and pathogens. Many studies demonstrated a significant role

for nitrogen oxides in modulating pulmonary function and in the pathogenesis of various pulmonary diseases. Reactions of NO ultimately lead to the nitration (addition of $-\text{NO}_2$), nitrosation (addition of $-\text{NO}$), and nitrosylation ($-\text{NO}$) of most classes of biomolecules. Another aspect of NO metabolism represent S-nitrosothiols (SNO) that appear to be important molecules signaling “NO” bioactivity in the lung. SNOs are products of NOS activation that are present in the airway lining fluid in micromolar concentrations, stored in specific cellular compartments to achieve bioactivity and metabolically regulated to deliver bioactivities both through transnitrosation reactions and through release of NO.

C. Regulation of NOS

NO is produced by a wide variety of residential and inflammatory cells in the airways. NO itself is generated via a NADPH-dependent oxidation of terminal nitrogen on the aminoacid L-arginine (Fig.1).

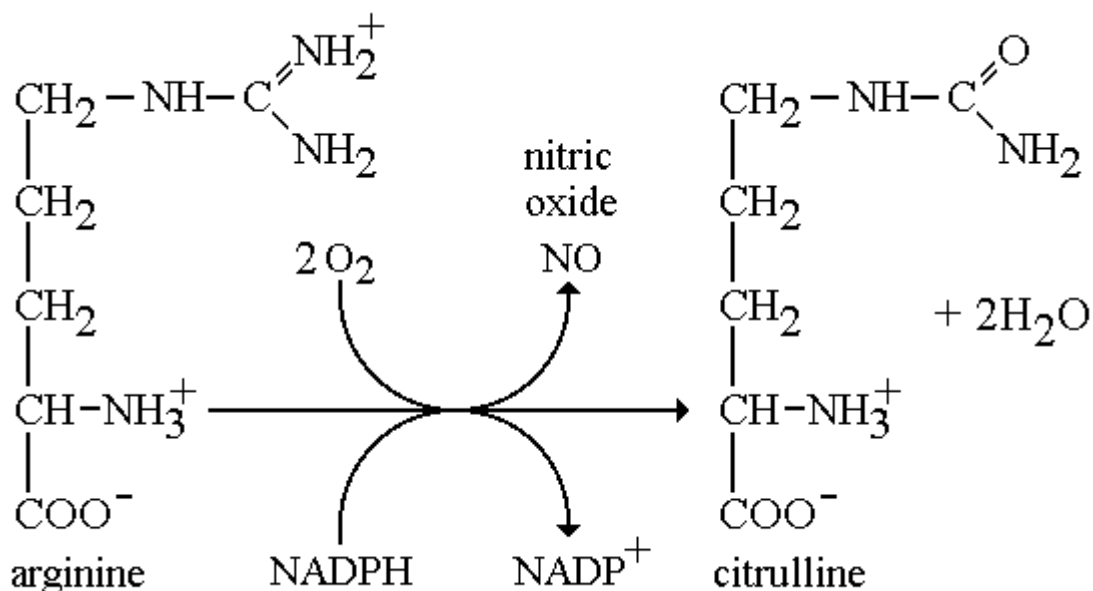


Fig. 1: Chemical pathway of nitric oxide

The enzyme system responsible for producing NO is NOS, which exists in three distinct isoforms: 1) constitutive neuronal NOS (NOS I or nNOS); 2) inducible NOS (NOS II or iNOS); and 3) constitutive endothelial NOS (NOS III or eNOS). nNOS, iNOS, and eNOS are products of distinct genes located on different human chromosomes (12, 17, and 7 chromosomes, respectively). All of the three NOS isoforms are expressed in the airways. Functionally, NOS exists in constitutive (cNOS) and inducible (iNOS) forms. cNOS is a Ca^{2+} and calmodulin- dependent enzyme and releases, within seconds, femtomolar or picomolar concentrations of NO upon receptor stimulation by selective agonists.

iNOS isoform can be induced by proinflammatory cytokines, such as tumor necrosis factor- α (TNF- α), Interferon- γ (IFN- γ), and interleukin (IL)-1. iNOS releases large quantities (nM concentrations) of NO several hours after exposure.

D. Localization of NO in the Airways

1. eNOS (NOS III)

eNOS is constitutively expressed in human bronchial epithelium and in type II human alveolar epithelial cells. Abundant eNOS immunoreactivity was found in endothelial cells of pulmonary blood vessels and also in the epithelium of human nasal mucosa. Ultrastructural studies revealed that eNOS is localized at the basal membrane of ciliary microtubules, where it is thought to contribute to the regulation of ciliary beat frequency.

2. nNOS (NOS I)

nNOS (NOS I) is localized in airway nerves of humans and animals. In human airways, these nerve fibers are present in the airway smooth muscle, where NO is the major mediator for the neural smooth muscle relaxation. NOS-containing nerve fibers are also present around submucosal glands although their functional role for the regulation of glandular secretion is not clear yet. In the lamina propria, NO has potent dilatory effects on blood vessels and on the regulation of plasma extravasation. In sensory neurons, NO could act as a neuromediator both at the central ending and the periphery. In animals, nNOS is also present in nonneuronal tissues like the respiratory epithelium and in normal endothelial cells.

3. iNOS (NOS II)

iNOS (NOS II) has been identified as a separate, calcium-independent isoform, which could be detected in many different cells, including macrophages. In the respiratory tract alone, expression of iNOS has been reported in alveolar type II epithelial cells, lung fibroblasts, airway and vascular smooth muscle cells, airway respiratory epithelial cells, mast cells, endothelial cells and neutrophils. The stimuli that cause activation of iNOS in these cells varied widely and included endogenous mediators (such as chemokines and cytokines) as well as exogenous factors such as bacterial toxins, virus infection, allergens, environmental pollutants (ozone, oxidative stress), hypoxia, tumors, etc. In conclusion, all three NOS isoforms are localized in the respiratory system where they may cooperatively regulate airway smooth muscle tone and immunologic/inflammatory responses.

E. Molecular Action of NO

NO bioactivities are broadly classified as NO mediated/ cGMP dependent and cGMP independent. Many bioactivities, such as airway smooth muscle relaxation, appear to use both. Chemical features of NO radical include its rapid diffusion from the point of synthesis, the ability to permeate cell membranes, the interactions with intracellular molecular sites within both generating and target cells, and its intrinsic instability, all properties that eliminate the need for extracellular NO receptors or targeted NO degradation. The best-characterized target site for NO is the iron bound in the heme component of soluble guanylyl cyclase stimulating conversion of GTP to cGMP. Subsequently, cGMP exerts most of the intracellular actions by coupling to cGMP-dependent protein kinase (PKG). It is

generally accepted that cGMP triggers relaxation of smooth muscle by activating two molecular mechanisms: reduction of $[Ca^{2+}]$ and reduction of the sensitivity of the contractile system to the Ca^{2+} .

As mentioned before NO mediates other actions that are independent of guanylyl cyclase and cGMP. The high level of NO released by iNOS has an effect as immune effectors molecule in killing tumor cells, in hindering viral replication, and in eliminating various pathogens including fungi, parasites and *Mycobacterium tuberculosis*. The responding mechanism may involve, at least in part, inhibition of DNA synthesis. Finally, NO appears to signal through its reactivity with cysteine groups. One of the general mechanisms of antimicrobial defenses involving NO is S-nitrosylation by NO of cysteine proteases, which are critical for virulence, or replication of many viruses, bacteria, and parasites. Interaction of NO with many molecular targets also may represent a pathway for its breakdown and inactivation. The most important interaction is probably its reaction with superoxide anion (O_2^-) to yield peroxynitrite anion ($ONOO^-$), which is a potent cytotoxic molecule.

II. NITRIC OXIDE AND PHYSIOLOGY OF THE RESPIRATORY SYSTEM

A. NO and Lung Development

Spatial and temporal nNOS and eNOS expression patterns occur during development of the lung. It was speculated that the rise in fetal lung eNOS may contribute to the marked lung growth and angiogenesis that occurs during the same period of time. Shaul et al. suggested that the increase in nNOS and eNOS in the lung early in the third trimester in the primate may enhance airway and parenchymal function in the immediate postnatal period.

B. NO and Transcriptional Regulation in the Lung

S-nitrosylation reactions appear to be of particular importance to regulation of gene expression in the lung. For example, SNOs associated with hemoglobin deoxygenation appear to stabilize the α -subunit of hypoxia-inducible factor 1 (HIF-1). Through increased HIF-1 DNA binding activity, in turn, increase in expression of hypoxia-inducible genes such as vascular endothelial growth factor in the pulmonary vascular endothelium occurs. High levels of nitrosative stress can inhibit transcription factor NF γ -B through direct S-nitrosylation of γ -B kinase. This signaling mechanism may serve to control cytokine production under physiological conditions, while increasing cytokine production during periods of nitrosative stress.

C. NO and neural innervation of airway

Cholinergic and adrenergic systems control the bronchomotor tone together with the NANC system. Recent evidence has shown that NO, as a neurotransmitter of NANC, is involved in airway smooth muscle relaxation. NOS immunoreactive neurons are more prominent in proximal than in distal airways, in agreement with the distribution of iNANC functional responses. The human iNANC response in central and peripheral airways is completely mediated by NO and involves selective activation of K_{Ca} channels in airway smooth muscle. Recently, it has been shown that a NO-dependent component of noncholinergic parasympathetic nerves modulates airway smooth muscle tone at baseline, pointing out the spontaneous activity of noncholinergic nerves during tidal breathing

Furthermore, it has been noted that the circadian variations of the iNANC response may contribute to overnight bronchoconstriction in patients with nocturnal asthma

D. NO and Airway Smooth Muscle Relaxation.

The ability of NO to relax smooth muscle has been described in multiple models and muscle types, including airway smooth muscle. More than half a century ago, nitrates were supposed to induce bronchial relaxations. However, clinical studies regarding the bronchorelaxant effects of the nitrovasodilators were conflicting. Inhaled NO at a concentration of 80 ppm has no effect in healthy human subjects, but a small bronchodilator effect in asthmatic patients. NO-dependent airway relaxation is partially due to activation of KCa channels via guanylyl cyclase and due to inhibition of Ca²⁺ release.

One other metabolic pathway for NO also involved in airway smooth muscle relaxation is the reaction in the presence of thiol to form SNOs. SNOs are present in the airways of normal subjects at concentrations sufficient to influence airway tone and have a substantially greater half-life than NO. Recently, it has been found that severe asthma is associated with low concentrations of airway SNO, suggesting that the deficiency of such an endogenous bronchodilator mechanism is due to an accelerated degradation of SNO in the lungs of severe asthmatic individuals contributing to severe and refractory bronchospasm.

E. NO and Pulmonary Circulations

Nitrogen oxides can account for the biological activity of EDRF and are involved in the regulation of vascular tone. Release of NO from endothelial cells in the pulmonary circulation appears to regulate vascular basal tone and counteract hypoxic vasoconstriction. In the healthy human, eNOS isoform is present in the endothelium of pulmonary vessels, but its expression is down regulated in patients with primary pulmonary hypertension. This suggests that the pulmonary vasoconstriction and the increased smooth muscle layer in the pulmonary vessels, main features of this disease, are associated with impaired expression of eNOS. Interestingly, these abnormalities might be associated with smoking. An in vitro study of pulmonary artery endothelial cells incubated with cigarette smoke extract resulted in a time- and dose-dependent decrease in eNOS activity associated with a nonreversible reduction of eNOS protein content and eNOS mRNA. This indicates that chronic exposure of cigarette smoke may contribute to the risk of pulmonary endothelial dysfunction via impairment of eNOS expression. Polymorphisms of the eNOS gene have been associated with high-altitude pulmonary edema, suggesting that a genetic background may underlie the impaired NO synthesis in the pulmonary circulation of this disease contributing to its exaggerated pulmonary hypertension. Interestingly, recent evidence suggests ethyl nitrite is more potent as selective pulmonary vasodilators, and is associated with less withdrawal rebound hypertension, than NO itself. This observation suggests that the most relevant reaction leading to pulmonary vascular smooth muscle relaxation may involve S-nitrosylation chemistry.

F. NO and Mucus-Electrolyte Secretions

In the airways, endogenous NO stimulates submucosal gland secretion. Platelet activating factor, histamine, and TNF- α , enhance also release of mucin by tracheal epithelial cells, but the stimulatory effect of each is inhibited by preincubation of the cells with a competitive inhibitor of NOS. This indicates that these mediators provoke mucin secretion via a mechanism involving intracellular production of NO as a signaling molecule. Ciliary motility is an important host defense mechanism of airway epithelium. Interestingly, low levels of nasal and exhaled NO in patients with primary ciliary dyskinesia (PCD) are related to mucociliary dysfunction, and treatment with NO substrate L-arginine improves mucociliary transport in patients with PCD. Abnormal electrolyte transport produces changes in periciliary fluid volume and composition, inhibits mucociliary clearance, and leads to chronic infection of the airways, as occurs in cystic fibrosis (low-volume model). Modulation of ion channels by NO has emerged recently as a significant determinant of ion channel function. NO activates both apical anion channels and basolateral potassium channels via cGMP-dependent pathway. Thus NO is a physiological regulator of transepithelial ion movement, and alterations of its generation and action may play an important role in the pathogenesis of lung disorders characterized by abnormal secretion of airway surface liquid.

III. NITRIC OXIDE AND OXIDATIVE STRESS:

Reactive oxygen species (ROS) are generated by various enzymatic reactions and chemical processes. NO can interact with ROS to form reactive nitrogen species (RNS). ROS, NO, and RNS are essential in many physiological reactions and are important for the killing of invading microorganisms. However, when airway cells and tissues are exposed to oxidative stress elicited by environmental pollutants, infections, inflammatory reactions, or decreased levels of antioxidants, enhanced levels of ROS and RNS can have a deleterious effect within the airways. ROS and RNS can damage DNA, lipids and proteins leading to impaired cellular functions. In this way, ROS and RNS play a prominent role in the pathogenesis of various lung disorders such as acute respiratory distress syndrome (ARDS), interstitial lung disease, cystic fibrosis and asthma.

Because NO and superoxide are free radicals, both molecules rapidly react with many different molecules in a biological environment. Of particular interest is the interaction between the two molecules and their reactive downstream metabolites. Enhanced cytotoxicity is possible when NO and superoxide are released simultaneously, which is a likely event during inflammatory responses. The most direct interaction between NO and superoxide is their rapid reaction to form the potent oxidant peroxynitrite. ROS is a collective term that includes a large variety of free oxygen radicals (e.g., superoxide anion and hydroxyl radicals) but also derivatives of oxygen that do not contain unpaired electrons (e.g., hydrogen peroxide, hypochlorous acid, peroxynitrite, and ozone). Formation of ROS takes place constantly in every cell during normal metabolic processes. Cellular sites for production of ROS include mitochondria, microsomes, and enzymes (e.g., xanthine oxidase, *P*-450 monooxygenase, cyclooxygenase, lipoxygenase, indole amine dioxygenase, monoamine oxidase). Activated phagocytic cells (neutrophils, eosinophils, monocytes, and macrophages) produce large amounts of ROS. Besides the generation of reactive species via cellular pathways, formation of ROS and RNS in the lungs can

also take place after inhalation of exogenous compounds like ozone, nitrogen dioxide, cigarette smoke and other chemicals, and dust particles. In addition, such exposures lead to depletion of endogenous antioxidants that are present in the epithelial lining fluid. Due to the complex chemistry and often short half-life of RNS, the exact metabolic fate in vivo remains unclear. Nonetheless, some stable end products of RNS are detectable in body fluids and tissues. First, NO decomposes into nitrite and nitrate, and these metabolites can be measured in plasma and in exhaled breath condensates. Enzymes and chemicals are present within the airway cells and in the airway epithelial lining fluid to protect against the toxicity of generated ROS and RNS. The major enzymatic systems present in the airways are manganese and copper-zinc superoxide dismutases, which rapidly convert the superoxide anion to hydrogen peroxide, catalase that converts hydrogen peroxide into oxygen and water, and the glutathione redox system (GSH-peroxidase and GSH-reductase) that inactivates NO, hydrogen peroxide, and other hydroperoxides. Other non-enzymatic factors with scavenging properties for oxygen radicals that can be present within the airways are vitamin E (α -tocopherol), vitamin C (ascorbic acid), uric acid, β -carotene, flavonoids, taurine, lactoferrin, albumin, and bilirubin. A disadvantage of limiting RNS formation is of course a compromised defense against invading microorganisms. Moreover, nonspecific NOS inhibition may lead to a compromised function of NO as a paracrine messenger, for instance, leading to hypertension.

B. Airway Damage by “Nitrosative Stress”

The effects of RNS once formed in vivo, on tissues, cells, and biomolecules are diverse. Important targets of RNS in proteins are, for example, tyrosine residues, thiols, and heme groups. Furthermore, RNS alter lipid oxidation pathways, cause DNA damage, and inhibit mitochondrial respiration. Exposure of cells to RNS leads to both apoptosis and necrosis dependent on the severity of cell damage. Again, these detrimental effects may affect both an invading pathogen and the (infected) host.

IV. EXHALED NITRIC OXIDE

Exhaled air of humans contains detectable amounts of NO, in the ppb range as measured by rapid chemiluminescence's analyzers. The measurement of exhaled NO is critically dependent on expiratory flow, which requires careful standardization of the measurement. Such standardization has recently been accomplished by international guidelines on the methods of measurement of exhaled NO, both for adults and in children. The levels of NO in the exhaled air are determined by 1) NO production by various cells in the airways and/or lung parenchyma, 2) diffusion of NO into the capillary circulation, and 3) alveolar ventilation and bronchial airflow. It appears that the NO production and expiratory NO concentrations can be predicted by a two-compartment model of the lung, consisting of a non-expandable compartment representing the conducting airways and an expandable compartment representing the respiratory bronchioles and alveoli. The model predicts that both compartments contribute to NO in the exhaled breath and that the relative contributions of airways and parenchyma can be separated by analysis of the relationship between exhaled NO output (nl/s) against expiratory flow rate (ml/s). Interestingly, such analysis may indeed allow the discrimination of airway diseases,

such as asthma, from hypersensitive alveolitis or liver cirrhosis in patients with elevated levels of exhaled NO.

A. Exhaled NO and Bronchial Asthma

Children and adults with atopic asthma show increased levels of exhaled NO compared with healthy controls. In asthma, the increased levels of exhaled NO have a predominant bronchial origin and appear to be associated with increased expression of corticosteroid sensitive iNOS. However, there is recent evidence that exhaled NO levels in asthma are also associated with a known functional missense sequence variant in the eNOS gene. This indicates that both NOS II and NOS III are important in determining the NO detected in the exhaled air in patients with asthma. Recent studies documented, that exhaled NO reflects disease activity and, to a greater extent, clinical control of asthma. Exhaled NO has been used to monitor asthma exacerbations, both spontaneous and induced by steroid reduction, and the effect of anti-inflammatory treatment in asthma. It can be postulated that asthma treatment with corticosteroids results in a reduction of expired NO levels due to both reducing effects of steroids on the underlying airways inflammation in asthma and inhibitory effects on iNOS expression itself. Oral and inhaled corticosteroids have been shown to result in a rapid (after 6 h following a single corticosteroid treatment) and dose-dependent reduction. In patients with more severe persistent asthma, airway inflammatory processes may overcome this steroid sensitivity of NO, leading to increased levels of exhaled NO even during treatment with high doses of oral or inhaled corticosteroids. During the last few years several studies have been performed to assess the relationship between levels of exhaled NO and lung function parameters or other markers of airway inflammation. Exhaled NO in patients with asthma is correlated with airway hyperresponsiveness to methacholine, but no correlation was found between FeNO and classical spirometric parameters as FEV1. Furthermore, exhaled NO is associated with eosinophilic inflammation as determined in blood, urine, bronchoalveolar lavage, and sputum in asthmatics with varying disease severity. This indicates that exhaled NO is a novel noninvasive marker reflecting airway eosinophilic inflammation in asthma.

NO and airway remodeling

Airway smooth muscle hypertrophy and hyperplasia, features of airway remodeling, are important determinants of airway hyperresponsiveness in asthma. In vitro studies have recently demonstrated that DNA synthesis and proliferation of human airway smooth muscle cells (HASMC) are reduced by exogenous administration of NO donors. More recently, it has been demonstrated that NO inhibited HASMC proliferation in G1 phase via cGMP-dependent pathway, but the inhibition of HASMC proliferation in S phase was due to cGMP-independent inhibition of ribonucleotide reductase. These newly discovered antiproliferative effects of NO on airway smooth muscle might become an important clue for future strategies to prevent airway remodeling in chronic asthma and COPD.

B. Exhaled NO and Other Respiratory Disorders

Other disorders associated with increased exhaled NO levels include allergic rhinitis, bronchiectasis, pulmonary sarcoidosis, hypersensitive alveolitis, and acute lung allograft rejection. In contrast, low levels of exhaled NO have been reported in patients with Primary Ciliary Dyskinesia, cystic fibrosis, PiZZ phenotype-related α -1-antitrypsin deficiency, and pulmonary hypertension. Certain pulmonary infections, such as viral respiratory illnesses, increase exhaled NO values, while others, such as chronic colonization of the cystic fibrosis airway with denitrifying organisms, attenuate exhaled NO values. In particular, PCD, including Kartagener's syndrome, is a genetic disease characterized by defective motility of cilia, in which the levels of exhaled NO are very low compared with normal subjects. Such low levels of exhaled and nasal NO are not seen in any other condition and are therefore used as a screening test to detect PCD among patients with recurrent chest infections or chronic ENT symptoms. The mechanism of low NO production by nasal and airway mucosa in PCD is unknown, but it might be linked to abnormalities in iNOS gene expression.

VII. CONCLUSION

The discovery of the specific role of endogenous NO in the homeostasis of various cellular functions and the dynamic behavior of the airways has led to a new, rapidly progressing area of physiological science. The complexity of NO synthesis and the wide functional profile of its various bioactive forms have not been resolved in full detail yet. Endogenous NO is synthesized by various, independently controlled enzymatic pathways. These can be constitutively expressed as well as induced and regulated at the gene-transcriptional level by several cytokines, chemokines, and mediators. The bioactivity of NO is partially provided by S-nitrosothiols. Furthermore, NO can also be regarded as a free radical that interacts with reactive oxygen species, to form reactive nitrogen species. These include extremely bioactive products such as nitrite, nitrate, nitrotyrosine, and peroxynitrite. NO has a definite role during the embryological development of the lung. Based on its various bioactive forms and depending on a wide local concentration range, NO can have either protective or deleterious activities during states of airway damage, inflammation, and repair. The potentially protective effects of NO include neuromodulation by mediating inhibitory noncholinergic nonadrenergic nerve activity, smooth muscle relaxation, attenuating airway hyperresponsiveness to bronchoconstrictor stimuli, and the killing of invading microorganisms. The potentially deleterious effects of NO (and reactive nitrogen species) include pro-inflammatory activities, such as vasodilatation and plasma extravasation of the bronchial circulation; increased airway secretions; impaired ciliary motility; promoting eosinophilic inflammation; and necrosis and apoptosis.

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STUDIES

2.1 Lung function in children with congenital diaphragmatic hernia, long-term outcome

Introduction:

Congenital diaphragmatic hernia (CDH) refers to a defect in the diaphragm that allows abdominal organs to move into the chest cavity. Depending on the timing of the herniation and the volume of abdominal organs involved, this defect has the potential to disrupt the normal lung development. Delayed operative repair (1), inhaled nitric oxide (iNO) (2), high-frequency oscillation ventilation (HFOV) (3), gentle ventilation with permissive hypercapnea (4), and extracorporeal membrane oxygenation (ECMO) (5) have resulted in an increased survival. Chronic pulmonary disease, feeding problems including gastroesophageal reflux (6), orthopedic problems such as scoliosis (7), and neurological complications (8) represent the major causes of morbidity in CDH survivors today.

Aim of the study: In the presented study we aimed to evaluate the long-term pulmonary morbidity, particularly lung function, in children, who had undergone repair of a CDH during a ten years period in a tertiary centre in Switzerland.

Materials and Methods

Subjects

Clinical records of all children born between 1991 and 2001 with the diagnosis of CDH and referred to the University Children's Hospital Zurich were reviewed retrospectively. Data collected included gestational age, gender, birth weight, Apgar score, duration of assisted ventilation, age at surgery, and length of hospitalization. 5 patients with chromosomal aberration as well as 4 patients with diaphragmatic relaxation or Morgagni hernia were excluded.

The Medical Ethical Committee of the University Hospital Zurich approved the study and written informed consent was obtained prior to testing from all parents.

Long-Term Follow-Up

A questionnaire referring respiratory symptoms in the first three years of life, history of asthma and allergy, gastroesophageal reflux requiring pharmacological treatment or surgery at any time and parental smoking was given to patient's parents. A physical examination was performed in all children by the same investigator. Each patient's growth was assessed and compared with the standard growth curve of Swiss children. After the clinical examination, the patient underwent the pulmonary function testing in the following order: nitric oxide measurement, spirometry and body-plethysmography, measurement of airway impedance using forced oscillation technique and finally a methacholine bronchial provocation test.

Exhaled nitric oxide

Exhaled nitric oxide was measured on-line using the single-breath technique by means of a chemiluminescence's analyzer (CLD 88 EXHALYZER, ECO MEDICS AG, Switzerland). Measurements were made according to European Respiratory Society (ERS) guidelines.

Lung function

Spirometry and whole body-plethysmography were performed according to standardized criteria using the measurement unit MasterLabPro (Jaeger GmbH; Würzburg, Germany).

The following parameters were recorded: forced vital capacity (FVC), forced expiratory volume in first second (FEV₁), peak expiratory flow (PEF), FEV₁/FVC ratio, maximum expiratory flow at 75%, 50% and at 25% FVC (MEF₇₅, MEF₅₀, MEF₂₅) and MEF 75-25 ratio. The reference values from Zapletal et al. (9) were used for analysis.

Whole body-plethysmography was performed in the same laboratory (MasterLabPro, Jaeger, Würzburg, Germany). The following parameters were recorded:

Total lung capacity (TLC), residual volume (RV), vital capacity (VC), intra-thoracic gas volume (ITGV), total resistance (R tot), total specific airway resistance (SR tot), RV%/TLC ration, and ITGV%/TLC ratio.

Bronchial hyper-responsiveness

The bronchial provocation test with methacholine (MCH) was performed, when the baseline FEV₁/VC ratio was at least 0.7. MCH was given in doubling concentration of 0.01 mg/ml to 2 mg/ml using a calibrated Nebulizer and FEV₁ was measured after each dose-step until the FEV₁ value had fallen from baseline by at least 20%.

Forced oscillation

The impedance of total respiratory system was measured using an oscillometry system (Quark i2m Forced Oscillation Measurement system, Chess medical technology, NV). Three reproducible measurements were selected for the analysis, to calculate a mean value for resistance of respiratory system at 8 Hz (Rrs 8) considered highly associated with bronchial obstruction. The results were compared to the reference values supplied by manufacturer (10).

Statistical Analysis

Results are expressed as mean (SD) for continuous data or median with range. Continuous data from CDH survivors and non-survivors as well as questionnaire data taken at follow-up visit were tabulated and described as numbers. Lung function data were described as percent predicted. The relationship of the most important data is shown using scatter plots; corresponding coefficient of correlation is also given.

Results

Patient Presentation and Initial Management

During the ten years period, 46 patients with CDH were admitted to the University Children's Hospital Zurich. Demographic data of all patients are shown in table 1.

Thirty (65 %) children survived to discharge from the hospital. Median age at surgery was 4 days [2 and 21 days]. In the group of survivors, 17/30 (57%) children were operated within 96 hours of presentation. The median for duration of artificial ventilation in the group of survivors was 12 days [1 and 162 days]. Neither gestational age nor Apgar score at five minutes correlated with intubations length (Fig.1).

Follow-up

From the 30 survivors, eleven children were either lost for follow up or did not consent for the study. The remaining 19 children (63%) were recruited to attend the long-term follow-up. Mean (SD) age at follow-up was 8.2 (2.8) years. The patient characteristics of survivors, those how died and the children included in the follow up study are presented in table 1.

Questionnaire

A review of questionnaires revealed that respiratory problems occurred commonly in the first three years of life. At least one wheezy episode requiring bronchodilators was present in 8/19 (42%) children during this period. Recurrent wheezing episodes ($\geq 3/\text{year}$) in the first three years of live were reported by parents of 4/19 (21%) children. The duration of artificial ventilation, ICU-hospitalization length and total hospitalization length did not correlate with recurrent respiratory symptoms later in childhood. In two children was the diagnosis of allergic asthma established. Smoking occurred in 6/19 (30 %) of the households. 5 of 6 CDH patients, which were postnatal exposed to tobacco smoke have been using bronchodilators in the last year before follow-up ($r^2= 0.49$)

The family history (first degree) for an atopic disease was positive in 8/19 (42%) cases.

GER required pharmacological treatment was present in 3/19 (16%) children, whereas one child underwent Nissen fundoplication. The Questionnaire data are summarized in table 2.

Clinical examination

Mild to moderate developmental delay was present in two children. These patients were not able to perform reliably the lung function tests.

Height values were within normal range in all children, and only 2 children present with body weight bellow the 3rd percentile.

Lung function

16 children performed reproducible and acceptable forced vital capacity maneuver, whereas acceptable whole body plethysmography tests were obtained only in 11 children, aged 6 to 13 years.

As a whole group, the CDH patients showed a mild reduction in the majority of measured parameters compared to predicted values (Table 3).

The individual analysis of measured parameters showed normal values in only 7/16 (44%) subjects. 4 children had restrictive pulmonary disease, 4 had evidence of airflow limitation and one child showed a mixed pattern of pulmonary disease.

Correlation between lung function results and patient characteristics

The spirometric parameters (FVC, FEV1, FEV1/FVC, MEF 75, MEF 50, MEF 25 and MEF 75/25), did not correlate with either gestational age, birth weight, or 5 minute Apgar score (for example, Fig. 2).

Neither duration of assisted ventilation nor the ICU-hospitalization length and total hospitalization length showed any significant correlation with spirometric parameters (Fig.3).

Similar figures, i.e. no significant correlations, were found for plethysmographic parameters.

Positive familial history of atopy or asthma and tobacco smoke exposition did not affect significantly the measured lung function parameters.

Measurement of exhaled nitric oxide (eNO)

Mean value of eNO for the whole group was 11 (12) ppb. The highest eNO value, 58 ppb, was obtained from a child with bronchial asthma and allergy.

The remaining patients had eNO levels between 2 and 15.2 ppb, which were considered within normal limits. No significant correlation was found between eNO on one side and family history of asthma or allergy, tobacco smoke exposition, history of GER or recurrent wheezing episodes in the first year of live on the other side. Similarly, the duration of assisted ventilation and total hospitalization time did not correlate with eNO. (Fig.4)

Methacholine bronchial provocation test

Only 8 patients correctly completed the bronchial challenge test. Inhalation of methacholine resulted in a 20% or more decrease of FEV1 in 6/8 (75%) children.

All the patients with positive methacholine bronchial provocation test had eNO values between 1.8-11.1 ppb.

Measurement of resistance using forced oscillation technique

15 children successfully performed the FOT measurement. 9/15 (60%) children showed increased resistance measured by 8 Hz (Rrs 8). The duration of artificial ventilation, ICU-hospitalization time and total hospitalization time did not correlate with the Rrs 8 (Fig.5).

Similarly, no significant correlation was found for Rrs 8 and family history of allergy or asthma, recurrent respiratory symptoms or eNO. Six children with increased Rrs 8 had also an abnormal lung function in spirometry. Two other children with increased Rrs 8 but normal spirometry were already prenatal exposed to tobacco smoke and received bronchodilator therapy in the last year before follow-up visit. We found no correlation between oscillatory resistance and the specific airway resistance measured by whole body plethysmography.

Discussion

The overall mortality rate of 35 % in our study is similar to that reported from other tertiary care-based studies (11). It is well known, that some live-born infants with CDH die already before referral to a tertiary centre. Consequently, the population of infants reaching a tertiary care centre commonly represents only 40 -50 % of total cases of CDH. This disparity reflects the "hidden mortality" of CDH, as first described by Harrison et al. (12). Because of well-developed prenatal screening program in Switzerland, it can be assumed that the overall mortality of CDH patients calculated on behalf of a population-based study would be twice as high (13).

The limited number of patients (63% of all survivors) who underwent follow-up assessment is a potential weakness of the study. As shown in Table 1, there was only minimal difference in the most descriptive parameters compared to CDH survivors, whose were lost from follow-up. Recent studies have shown that gastroesophageal reflux is commonly found in survivors of CDH (14). The reported incidence of GER depends partially on the diagnostic methods used. Using a questionnaire, we found symptoms consistent with GER requiring pharmacological or surgical therapy beyond the neonatal period in four patients. Recurrent bronchitis, aspiration pneumonia, and worsening pulmonary function were reported as respiratory complications of GER. Anti-reflux surgery is reserved for pathologic GER that persists despite maximization of medical therapy. In our study group, only one patient required Nissen Fundoplication. Failure to thrive is frequently described in CDH survivors. The relative low incidence of GER and of severe pulmonary impairment, could explain the low incidence of failure to thrive in our group of patients.

Many studies report a high incidence (>60%) of chronic lung disease initially (15, 16), but with improvement in most children with CDH repair during the first year of life. The same trend was noted in our study. 8/19 (42%) followed children presented with wheezy episodes early in life. Respiratory symptoms become less common and milder as patients grow up, nevertheless 8/19 (42%) patients received bronchodilator therapy in the last year before the follow up. 2/19 (10%) children were diagnosed of having asthma bronchiale. This corresponds well with the overall asthma incidence in childhood in Switzerland (8%).

The followed group of CDH patients in our study represented 63% of all survivors. It is not unknown phenomenon that parents of more severe ill children more likely than others refuse to participate in studies. As shown in table 1, the CDH survivors, whose were lost from follow-up, have a slightly prolonged course of artificial ventilation and prolonged hospitalization time also. It may be possible, that after including these patients in the analysis, we would be seen even higher incidence of respiratory problems.

In the last years, several studies looking at lung function outcomes in children surviving congenital diaphragmatic hernia were performed (17, 18). In the present series, we found various degrees of obstructive or restrictive ventilatory impairment in 50 % of the patients, a figure comparable with results from other studies (19, 20). We typically observed reduced functional lung volumes combined with hyperinflation. Interstitial emphysema (21), increased airway collapsibility and, decreased compliance of chest wall or diaphragm may be responsible for these functional changes.

In our study sample, lung function parameters were not predicted by perinatal factors. Contrary to other, larger studies (20), we did not find any significant correlation for lung function parameters and duration of artificial ventilation. The only weak trend showing increasing lung function impairment, i.e. smaller FEV₁, depending on the length of intubations could be simple result of small sample size in our study. We are aware that results of our study are limited due to the small number of patients who correctly performed the methacholine provocation test. In consequence, we did not attempt to draw any conclusion on the basis of presented data from this test and present the data in a descriptive way only.

This is the first follow-up study in CDH including the measurement of respiratory system resistance using forced oscillation technique. Our aim was to assess the ability of the FOT to detect the alternations in the respiratory mechanics in CDH patients. There are many studies addressing respiratory mechanics in lung disease in childhood (22, 23) which suggested that FOT detects abnormal airway resistance more peripherally in the lung, hence may give more information about the peripheral airways as compared to spirometry. The fact, that 9/15 (60%) of CDH patients had an increased resistance measured by 8 Hz and 6 from this children had also an abnormal spirometry pattern, may suggest a rather symmetrical involvement of small and large airways. This type of abnormal lung mechanics would be explainable due to bronchial tree hypoplasia.

There are no data on exhaled nitric oxide (eNO) at long-term follow-up in children with CDH. Epithelial cells of the bronchi were determined as the main source of exhaled nitric oxide and abnormal values of eNO were reported in many respiratory conditions. Patients with bronchopulmonary dysplasia (BPD) were studied in regard to eNO. Baraldi et al. (24) found four times lower eNO values in school-age survivors of BPD compared with a group of patients with asthma with a comparable airflow limitation. Others have shown normal values of eNO in non-atopic schoolchildren with a history of BPD (25). Duration of artificial ventilation and oxygen therapy in CDH patients is comparable with BPD patients and dysplasia of bronchial tree was commonly described in CDH survivors. Hence, CDH survivors represent a group of patients with potentially abnormal airway nitric oxide production. Analyzed as a whole group, we found normal values of exhaled nitric oxide. Exhaled NO is presently recognized as a marker of airway inflammation and is related to bronchial hyper-responsiveness in asthmatic patients (26). Therefore one may hypothesize other reasons than airway inflammation to be responsible for the bronchial hyper-responsiveness seen in many of the CDH survivors. The reduced spirometry parameters in context with normal eNO values seen in our group of CDH survivors, may suggested that the lung function impairment has rather a structural and not inflammatory origin.

In conclusion, the presented results of follow-up assessments are in agreement with other published studies and show a good general prognosis of CDH patients. Children, who survived CDH repair, have reduced functional lung volumes but normal TLC. Exhaled nitric oxide is in non-atopic CDH survivor's normal. FOT can be used as a screening method to detect CDH survivors with abnormal spirometry parameters. Even in the absence of clear clinical significance, the incomplete functional recovery of the lung in CDH survivors and risk of neurodevelopmental and nutrition morbidity require a long-term follow-up in a pediatric centre. This follow-up should include a multidisciplinary team formed by pediatric surgeons, neonatologists, pediatric pulmonologists, pediatric orthopedics, gastroenterologists, dieticians and cardiologists.

Table 1. Patient characteristics and initial management according to outcome and to participation in the study. Continuous variables are summarized by either the mean (SD) or the median [range].

	Died	CDH survivors as a whole group	CDH survivors participated in the study	CDH survivors lost from follow-up
Number	16 (11 male)	30 (16 male)	19 (11 male)	11 (5 male)
Gestational age, weeks	38 [33, 42]	38 [30, 42]	38 [30, 42]	39 [33, 41]
Birth weight, g	3100 [1700, 4250]	3000 [2250, 3900]	3000 [2400, 3900]	3000 [2250, 3900]
5 minute Apgar score	6 (2)	7 (1)	7 (1)	6 (1)
Side of CDH				
Right	2	2	1	1
Left	16	30	19	11
Day of surgery	*	5 [2, 21]	5 [2, 21]	5 [2, 17]
Duration of artificial ventilation, days	3 [1, 195]	12 [2, 120]	7 [2, 33]	15 [2, 120]
Length of ICU Hospitalization, days	3 [1, 195] †	16 [2, 133]	13 [2, 133]	18 [4, 128]
Total Hospitalization Length, days	3 [1, 195] †	30 [10, 156]	30 [12, 145]	30 [10, 156]

* 13/16 of died CDH patients succumbed to respiratory failure before a surgical intervention could be performed

† All patients died on ICU

Table 2. Data from questionnaire regarding the respiratory problems and gastroesophageal reflux as reported by parents of the CDH survivors (N= 19) at follow-up visit

	N	%
Family history of Atopy	9	47
Family history of Asthma	5	26
Current Asthma	2	10
At least 1 episode of obstructive breathing	9	47
Recurrent wheezing or persisted cough	4	21
Tobacco smoke exposition	6	32
Smoking (mother) during pregnancy	1	5
Past history of GER* in early childhood	4	21
Fundoplication	1	5
GER symptoms in last 12 months	1	5
Bronchodilator therapy in last 12 months	9	47

* Gastroesophageal reflux requiring pharmacological or surgical therapy

Table 3. Pulmonary Function Testing at follow-up visit

Body-plethysmography (N=11)	
Total Lung Capacity (TLC)	93 (20)
Vital Capacity	75 (18)
Intrathoracic Gas Volume (ITGV)	107 (23)
Residual Volume (RV)	131 (67)
RV/TLC	136 (63)
ITGV/TLC	110 (41)
Resistance total	175 (47)
Specific Resistance total	227 (102)
Spirometry (N=16)	
Forced Vital Capacity (FVC)	67 (19)
Forced Expiratory Volume in 1 st second (FEV1)	74 (21)
FEV1/FVC	109 (8)
Peak Expiratory Flow	67 (24)
Mean Expiratory Flow at 75% of Vital Capacity (MEF 75)	66 (27)
Mean Expiratory Flow at 50% of Vital Capacity	68 (34)
Mean Expiratory Flow at 25% of Vital Capacity (MEF 25)	69 (48)
MEF 75-25	68 (37)

Values shown are means of percent-predicted (SD) except for RV/TLC, ITGV/TLC and FEV1/FVC, which are defined as percent.

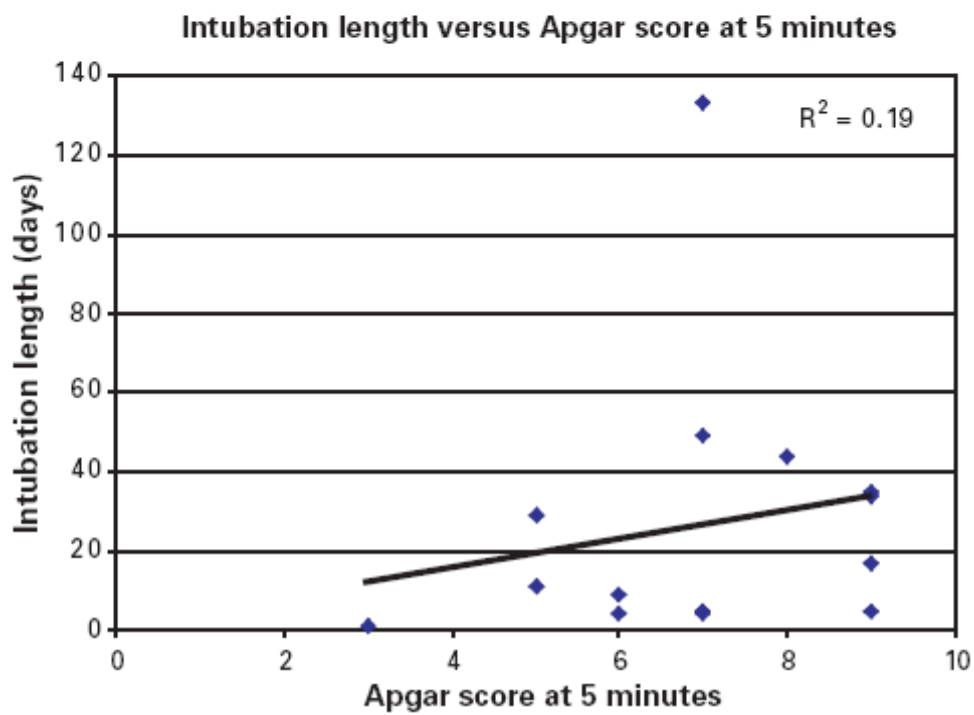
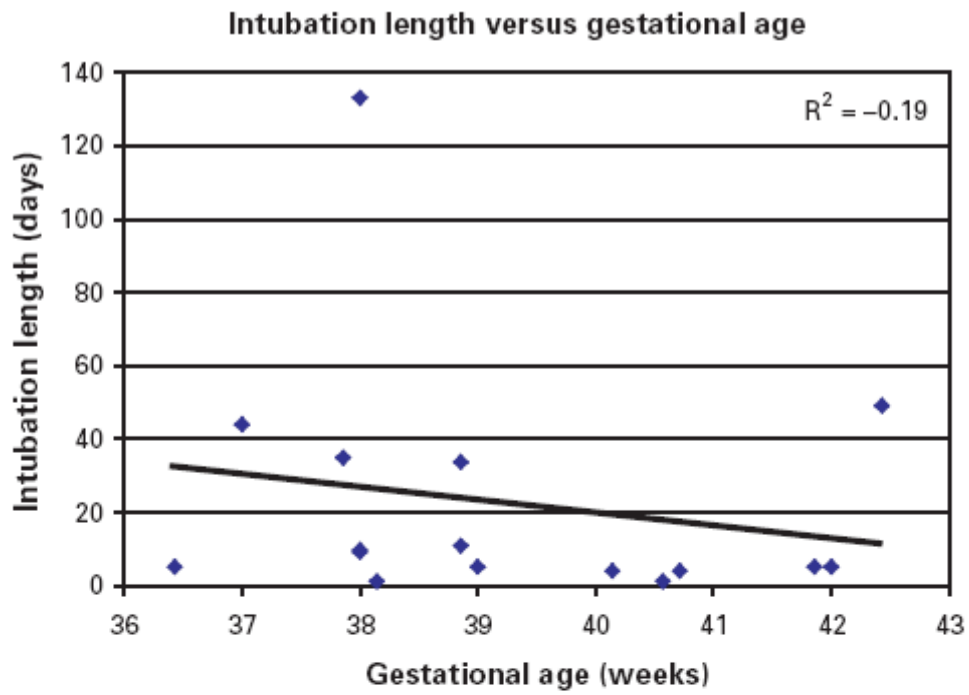


Figure 1: Intubation length versus gestational age and Apgar score at 5 minutes.

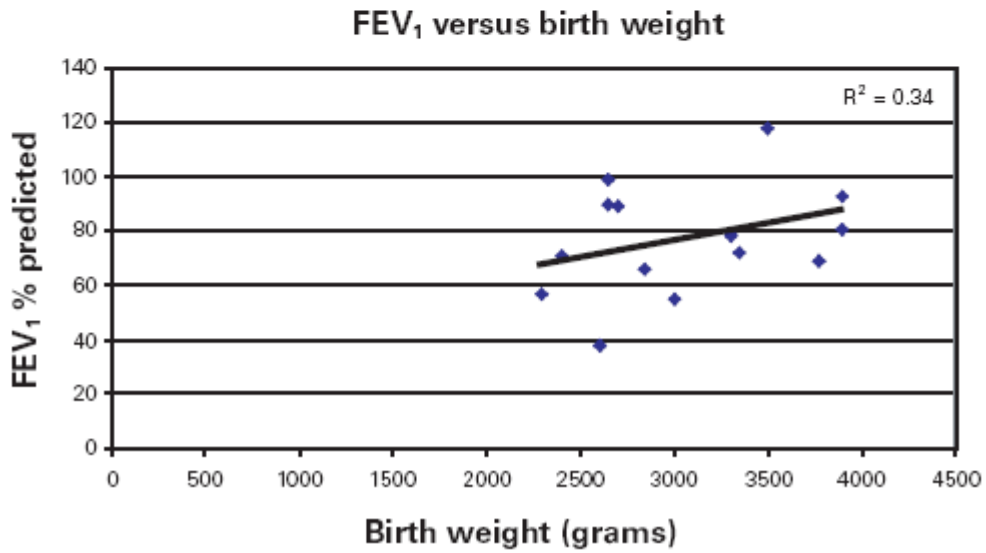
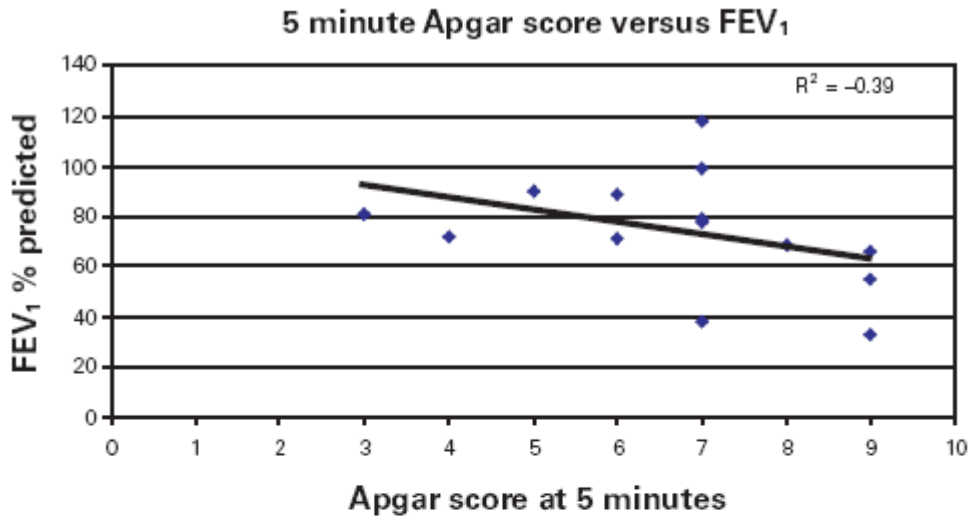


Figure 2: Intubation length versus gestational age and Apgar score at 5 minutes.

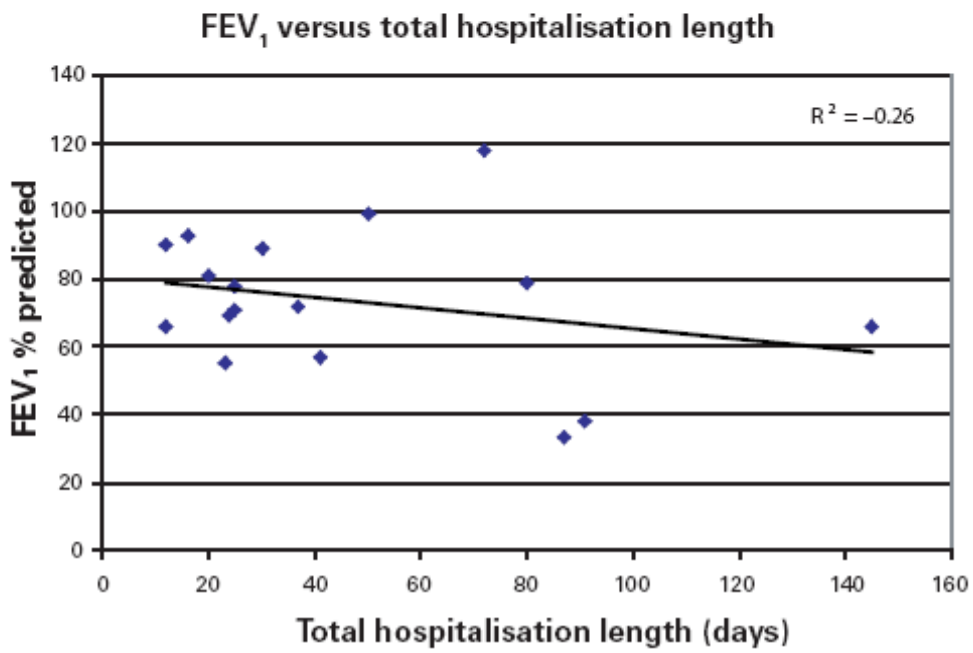
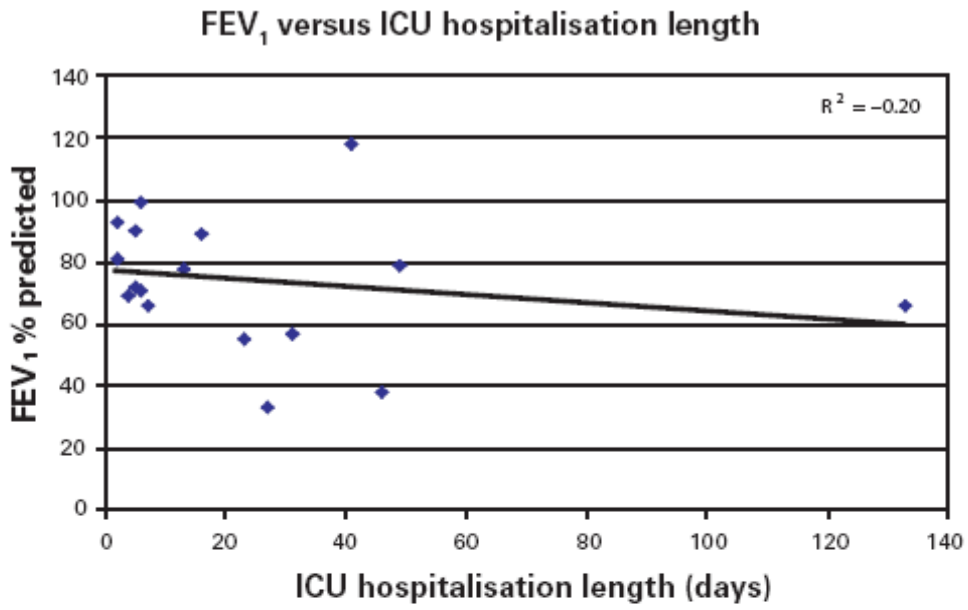


Figure 3: FEV₁ versus ICU hospitalisation length and total hospitalisation length.

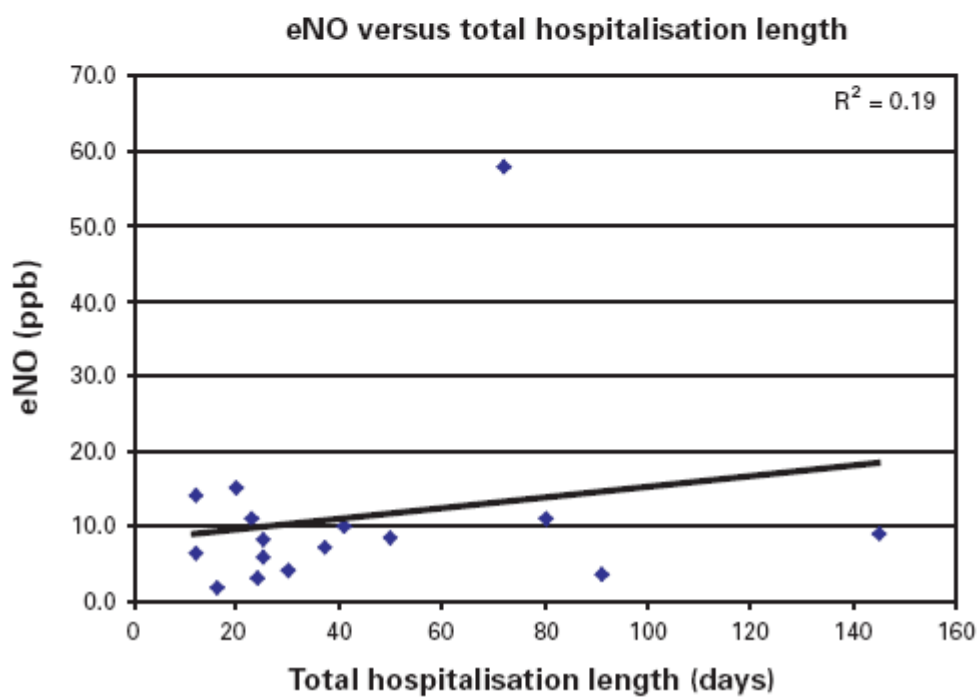
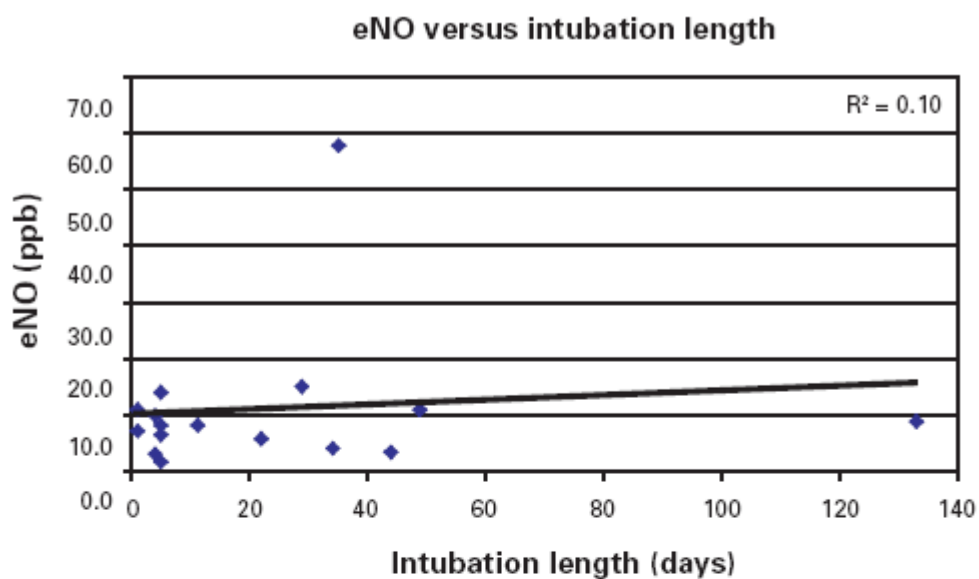


Figure 4: Exhaled nitric oxide (eNO) versus intubation length and total hospitalisation length.

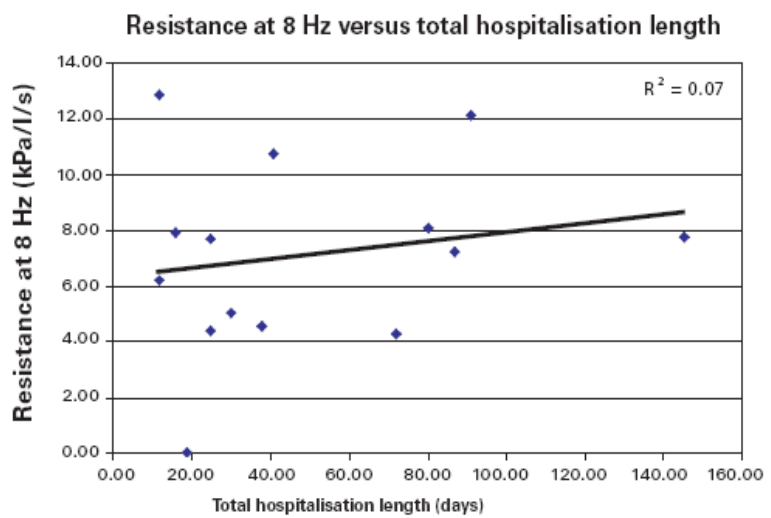
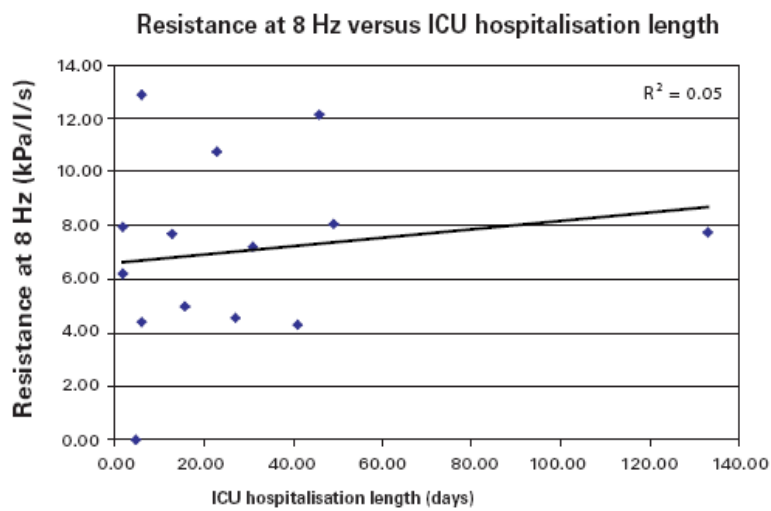
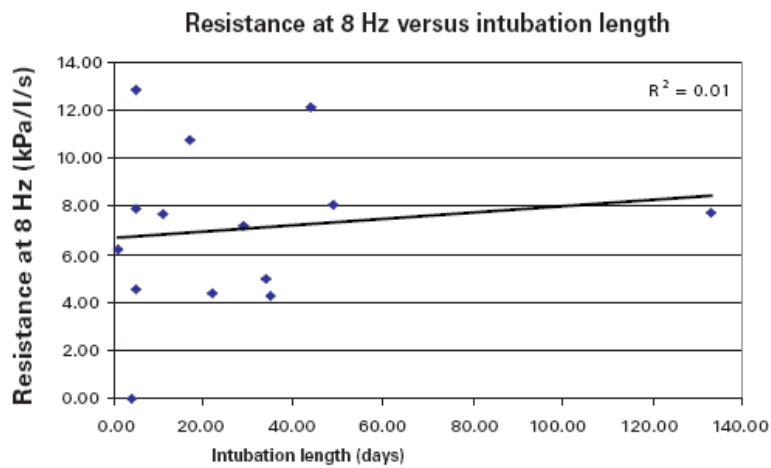


Figure 5: Resistance at 8 Hz versus intubation length, ICU hospitalisation length and total hospitalisation length.

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2.2 Lung function and markers of inflammation in exhaled air in paediatric patients with inflammatory bowel disease

Introduction

The inflammatory bowel diseases (IBD), e.g., Crohn's disease and ulcerative colitis are chronic idiopathic inflammatory disorders of the intestine and/or colon. A growing body of experimental and clinical data suggests that chronic gut inflammation may result from a deregulated immune response to normal bacterial antigens (1). This uncontrolled immune system activation results in the overproduction of reactive metabolites of oxygen and nitrogen (2 - 4). Effect of these reactive species leads thereafter to mucosal injury, reduced perfusion; poor wound healing, and maintenance of chronic inflammation (5 - 6). In the early 1990s, a burst of research activity occurred in the area of airway inflammation and in examining the potential role of nitric oxide (NO) as mediator in mucosal inflammation.

Crohn's disease and ulcerative colitis are associated with a variety of systemic manifestations affecting skin, joints, eyes, kidney, pancreas and liver involvement. Different bronchopulmonary involvement has been reported, including bronchiolitis obliterans, interstitial lung fibrosis, granulomatous lung disease, and bronchiectasis and bronchopulmonary suppuration (7-10). In adults with IBD, the most frequent observed lung function abnormality is impaired lung transfer factor (T_{lco}) suggesting the presence of an interstitial pulmonary process. Higher prevalence of bronchial hyperreactivity suggesting persistent airway inflammation is another frequently described pathological finding. Respiratory function abnormalities including bronchial hyperreactivity have been reported in asymptomatic children also. Ceyhan et al. (7) has shown that the allergic symptoms and skin test positivity are more prevalent in IBD patients than in normal population. Nitric oxide pathway is actively involved in the colon epithelium damage in the course of Crohn's disease.

In patients with chronic inflammatory respiratory diseases such as asthma bronchiale or bronchiectasis the level of exhaled nitric oxide (eNO) was found to correlate proportionally with intensity of airway inflammation (11 - 15). Increased levels of nitric oxide metabolites (nitrite) were found in exhaled breath condensates (EBC) in asthmatic patients (16).

We hypothesized that exhaled nitric oxide is elevated in children with IBD as a marker for subclinical lung involvement.

Aim of the study: The primary aim of the presented study was to measure level of eNO and nitrite concentration in EBC in pediatric patients with clinical stable IBD. Furthermore, we have screen for pulmonary and allergic symptoms and using skin prick test we have evaluated the prevalence of atopy in our group of patients with IBD. Standard lung function tests were performed to screen for obstructive or restrictive/interstitial lung disease.

Patients and Methods

Twenty children with IBD, 17 with Crohn's disease and 3 with ulcerative colitis, were studied after parental informed consent. For the comparison of values of exhaled nitric oxide and values of nitrite in exhaled breath condensates we have used two age-matched control groups: The first one control group comprised 19 non-smoking volunteers, without history of lung disease or allergy. 20 children with mild persistent, currently untreated, asthma represented the second one.

The study group and both control groups were similar in age and sex (Table 1). The mean age in IBD patients was 13.9 years (range 10.1 – 18 years), and the mean age in control subjects was 13.7 years (range 10.3 –18.5 years) and 13.3 years (10.1 –17 years), respectively.

The mean duration of disease in IBD patients was 20 months (range 7 – 60 months).

1 patient was receiving methotrexat, 1 patient infliximab, 3 patients were on sulphasalazine, and 12 patients were receiving azathioprine at the time of study visit. The remaining 3 patients did not use any special medication at the time of study visit. Corticosteroid therapy was terminated at least 4 wks before the study visit in each patient. None of the IBD patients had previously undergone bowel surgery. The clinical stability of bowel disease was assessed by experienced gastroenterologist concerning general well-being, abdominal pain, and occurrence of blood in stool, stool frequency and CRP values. Only these patients, selected by the gastroenterologist as patients with IBD in remission, were included in the study. Other inclusion criterion was the absence of on acute respiratory tract infection 4 weeks before the study visit. Parents of all subjects gave their informed written consent and the ethics committee of the university hospital approved the study.

Questionnaire

A standardized pediatric questionnaire was administered to patients with IBD and their parents to obtain personal and family history of respiratory disease and allergic symptoms. A full clinical examination was performed and blood samples were collected for determination of CRP and hemoglobin levels.

Investigation

After the clinical examination and completion of questionnaire, the patient underwent the following tests in order: skin-prick testing, nitric oxide measurement, spirometry and bodyplethysmography, measurement of TLCO and sampling of exhaled breath condensates.

FeNO

Exhaled nitric oxide was measured using the single-breath technique by means of the rapid chemiluminisence analyzer (CLD 88 EXHALYZER, ECO MEDICS AG, Duernten, Switzerland) adapted for online recording. Measurements were made according to European Respiratory Society (ERS) guidelines (17) and the mean of values obtained from three reproducible recordings was referred.

Lung function measurement

Spirometry (forced maximum expiratory flow-volumes curve) and whole bodyplethysmography were performed according to standardized criteria using the measurement unit MasterLabPro (Jaeger GmbH; Würzburg, Germany). Before recordings, each child was trained on the technique of the forced vital capacity maneuver. Tests were performed with the children sitting and wearing nose clips. The forced expiratory variables were determined from the best of three reproducible forced maneuvers and expressed as percentages of predicted values for height. The following spirometric parameters were recorded: forced vital capacity (FVC), forced expiratory volume in first second (FEV₁), peak expiratory flow (PEF), FEV₁/FVC ratio, maximum expiratory flow at 75, 50% and at 25% FVC (MEF₇₅, MEF₅₀, MEF₂₅) and MEF 75-25 ratio. The reference values from Zapletal et al. (18) were used for analysis. Whole body plethysmography was performed after the spirometry in the same laboratory (MasterLabPro, Jaeger, Würzburg, Germany). The following parameters were recorded: Total lung capacity (TLC), residual volume (RV), vital capacity (VC), intra-thoracic gas volume (ITGV), total resistance (R tot), total specific airway resistance (SR tot), RV%/TLC ratio, and ITGV%/TLC ratio. All variables obtained from bodyplethysmography were expressed as percentage of predicted values for height. The pulmonary carbon monoxide transfer factor (TLCO) was assessed using the steady state method. The values of TLCO were expressed as the percentage of the normal value for gender, age and height after correction for hemoglobin according to reference equation (19).

Skin prick test

In all patients and controls, the presence of atopy was evaluated by skin prick test (SPT) using nine standard antigen solutions of common aeroallergens (house dust mite, grass and tree pollen, cat, dog, molds) obtained from ALK (Denmark). The standard antigen solutions were applied before pricking the skin of the volar aspect of the forearm with a special lancet having 1 mm tip. Histamine was used as positive control. All of the tests were performed by the same experienced health technician. Wheal diameters (mm) were measured after 15 minutes. The mean diameter larger than three mm was regarded as positive. "Atopy" with SPT was defined as a positive reaction to any one of the allergens.

Nitrite in EBC

Exhaled breath condensates were collected using EcoScreen device (ERICH JAEGER GmbH, Germany), which allows non-invasive sampling of nongaseous substances contained in the expired air by cooling it to about 14°F (-10°C). The subjects were breathing through a mouthpiece and a two-way valve, which included a saliva trap which keeps the collection amylase free. They breathed at a normal frequency with a controlled volume for a period of 10 minutes. The condensate, at least 500 ul, was collected as ice at -20° C and immediately stored at -70°C. Nitrite concentration in the breath condensate was measured by a colorimetric assay based on the Griess reaction where 100 ul of breath condensate were reacted with 25 ul of Griess reagent (0.1% naphthylethylene diamine dihydrochloride, 1% sulphanilamide, 3% H₃PO₄) and measured at absorbance of 570 nm with a microplate reader.

Statistical Analysis

Questionnaire data taken at study visit were tabulated and described as numbers and percents. Lung function data were also tabulated and described as percent predicted. Results are expressed as mean \pm SD if not indicated otherwise. Data were tested for normal distribution. Unpaired data from IBD and controls were compared using Student's t-test. All analyses were performed using the statistical package Sigmasat. P value of less than 0.05 was considered as significant.

Results

Questionnaire and atopy testing

A review of questionnaires revealed that respiratory problems occurred uncommonly in young patients with IBD. Only one child suffers from asthma bronchiale and an other one has had episode of viral wheeze in the pre-school age. The family history (first degree) for an atopic disease was positive in 6/20 (30%) cases and 9 children with IBD was consider as atopic on behalf of positive skin prick testing. The Questionnaire data are summarized in table 1.

Clinical examination

Normal cardiopulmonary status was present in all studied children. Height values were within normal range in all children, and only one child with IBD presents with body weight below the 3rd percentile.

Lung function

All 20 subject with IBD and all control children performed reproducible and acceptable forced vital capacity maneuver and acceptable whole body plethysmography tests. As a whole group, the IBD patients showed a normal lung function parameters compared to predicted values (Fig.1, Table 2). In the individual analysis, 6 children with IBD (32%) showed at least one pathological (< 80% of predicted value) pulmonary function test. In 5/6 was the type of pulmonary dysfunction described as obstructive. In one case, there was a mixed, obstructive-restrictive pattern of pulmonary dysfunction. TLCO after correction for Hb was normal in all IBD patients. The means of lung function data are provided in table 2.

FeNO and nitrites in exhaled breath condensates

Fractioned exhaled nitric oxide levels in patients with IBD, healthy controls and asthmatic children are depicted in Fig.2. The median of FeNO in IBD group was 13.9 ppb (3.6 -78.3), the median of FeNO in the group of healthy children was 6.2 ppb (3.4 -17.1), and the median of FeNO in the group of asthmatics was 28.4 ppb (4.2 – 89). No significant difference in FeNO was found in atopic versus nonatopic IBD children (Fig.3). The concentration of nitrites in EBC did not differ significantly in children with IBD compared with healthy; the group of asthmatic patients had significantly higher values as the group of healthy controls (Fig. 4). Regarding FeNO, the group of IBD patients with CRP <4 mg/l did not differ from the group of IBD patients with CRP \geq 4 mg/l (Fig.5).

Discussion

Crohn disease and Colitis ulcerosa should be regarded as multisystem disorders that involve mainly the gastrointestinal tract. Although the overall prevalence of concomitant bronchopulmonary manifestations in adults has been evaluated as low as 0.4%, subclinical involvement in at least half of adults with Crohn disease has been demonstrated by some authors (20) suggesting underlying bronchial inflammation. Bronchial hyperresponsiveness and reduced lung function parameters were described repetitively in adult patients with IBD (21-22) and in adolescents (23). For example, Herrlinger et al. (24) reported reduced FEV1 in 39% of adults with Crohn disease and in 45% of patients with Colitis ulcerosa. As in other studies, the impairment of pulmonary function tests was more pronounced in patients with active disease.

Bronchopulmonary manifestations of inflammatory bowel disease in young children have been rarely described up to now (25). Early involvement of the lung in children with Crohn disease was observed in the study from Munck et al. (26). He investigated the incidence of pulmonary abnormalities in 26 children with acute or quiescent Crohn's disease in terms of clinical pulmonary symptoms, chest roentgenograms and pulmonary function tests, including lung transfer factor for carbon monoxide (TLCO). Only TLCO (% predicted) was significantly decreased during the active phase of the disease, and the authors suggested that latent pulmonary involvement is also present in a pediatric population with active Crohn's disease.

In our study group, at the time of investigation, only one child with IBD suffered on respiratory problem and received inhaled therapy for its asthma. The conventional spirometric and bodyplethysmographic parameters as well as DLCO were in all IBD patients in normal range. As the group, children with IBD had significantly lower VC compared with the group of healthy children. The clinical consequence of this phenomenon is unclear.

In respect to different published reports, there is no consensus if atopy is associated more frequently with inflammatory bowel disease. Louis et al. (27) observed a positive skin test in 42% of adult IBD patients and 21% of controls. In contrast, Troncone et al. (28) did not find any effect of IBD on prevalence of atopy. In our study, we observed at least one positive skin test to common aeroallergen in 45% of our patients with IBD and in 21% of the controls, even though this did not result in a statistically significant difference.

Although nitric oxide is considered as very important mediator/effector in pathophysiology of gut mucosal damage in inflammatory bowel disease, there is only single one study describing FeNO values in patients with IBD. Koek et al. (29) measured FeNO in 31 adult patients with Crohn disease and found a higher FeNO levels in patients with active disease.

In our study, children with IBD had significantly higher FeNO compared to healthy children, but the concentration of nitrites in exhaled breath condensates did not differ significantly between healthy and IBD patients. As many studies with asthmatic patients shown, regarding elevated FeNO levels, presence of atopy is an important compounder factor. In aspect of atopy presence, the FeNO levels did not differ significantly in our study, so we hypothesized that condition of inflammatory bowel disease alone or in association with therapy, predispose to higher FeNO.

Level of nitrites did not differ significantly in IBD patients compared to healthy. However, a statistical significant difference was found in asthmatic patients compared to healthy children, when the level of nitrites in EBC of asthmatics was higher as in healthy. We interpreted this result in terms of inferior capability of analysis of exhaled breath condensates as a method to detect specifically nitric oxide metabolites.

It is important to consider whether therapy with sulphasalazine or azathioprine (15/19 IBD subjects in our study) may have been responsible for the pulmonary disease and elevated FeNO levels. This distinction may be difficult regarding lung involvement due to medications and IBD disease. None of the patients in this study had clinical or functional findings indicating interstitial lung disease or bronchiolitis obliterans, known adverse pulmonary reactions of sulphasalazine and azathioprine (30). However, there are no data about possible induction of NO synthesis in gut/airway mucosa by the aforementioned medication.

There is increasing evidence that IBD-associated respiratory disease represents a significant problem also in the pediatric age. Exhaled nitric oxide was found to be elevated in adults, and as shown in our study in pediatric patients with IBD. Although the clinical relevance and therapeutic consequences of this result are unknown, we can speculate that airway inflammation co-exists in patients with Crohn disease and Colitis ulcerosa and nitric oxide pathway plays an important role in gut and airway mucosa damage. The most conclusive data about a role of nitric oxide in pulmonary involvement in IBD patients would be acquired by means of bronchoscopy, bronchoalveolar lavage and, probably, using biopsy of bronchial mucosa. Because of relative low prevalence of pulmonary disease in children with Crohn disease and Colitis ulcerosa, and in particularly because of concern about invasivity and safety of bronchoscopy in pediatric population, new elucidated data about exhaled nitric oxide will be reported in the future from adult respiratory specialist.

Table 1. Questionnaire data

	Subjects N = 20	Healthy control N = 19	Asthmatic children N = 20	
Age, years *	13.9 (10.1, 17.7)	13.7 (10, 18)	14.1 (9.9, 17)	NS
Sex				
Male	15	14	14	NS
Female	5	5	6	
Duration of disease, months	16 (7, 60)			
Recurrent wheezing or persisted cough	1 (5%)	0		NS
Family history of atopic disease	6 (30%)	4 (21%)	8 (40%)	NS
Current Asthma	1 (5%)	0	20	NS
Hayfever	3 (15%)	2 (11%)	9 (45%)	
Skin test positivity	9 (45%)	4 (21%)	20 (100%)	p= 0.06

* Mean and range

Table 2. Selected lung function data

	Subjects	Healthy	Asthmatics	
FEV1 predicted, %	96 ± 12	99 ± 9	86 ± 17	NS
FVC predicted, %	91 ± 12	97 ± 10	95 ± 16	NS
FEV1/FVC, %	105 ± 9	102 ± 10	92 ± 10	NS
MEF 50 predicted, %	87 ± 24	91 ± 10	71 ± 31	NS
MEF 25 predicted, %	82 ± 30	88 ± 10	59 ± 26	NS
TLC predicted, %	95 ± 18	100 ± 11	100 ± 12	NS
VC predicted, %	90 ± 16	102 ± 12	97 ± 14	p=0.01
TLCO, %	93 ± 19	100 ± 11	96 ± 15	NS

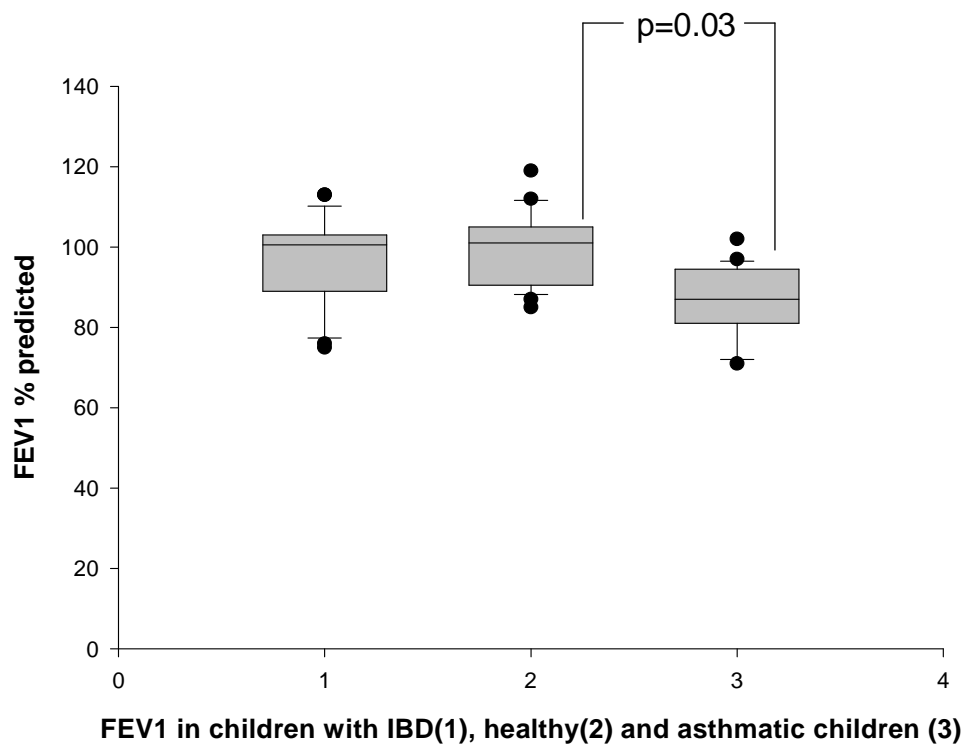


Figure1: Not significant difference in FEV1 between patients with IBD and healthy controls.

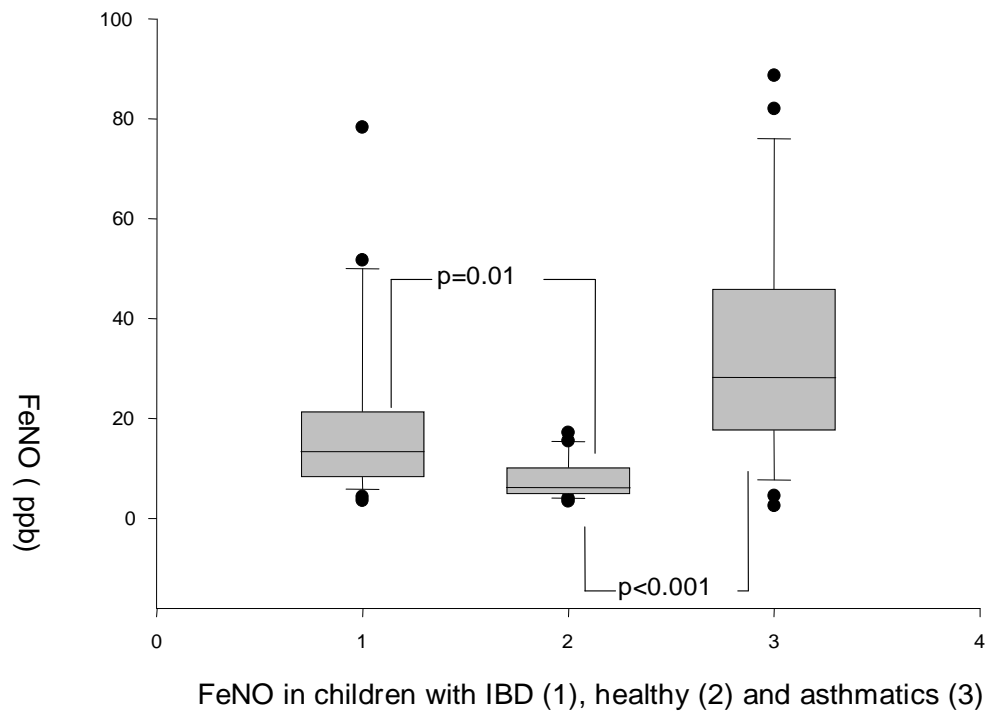


Figure 2: FeNO in children with IBD, asthmatic children and healthy.

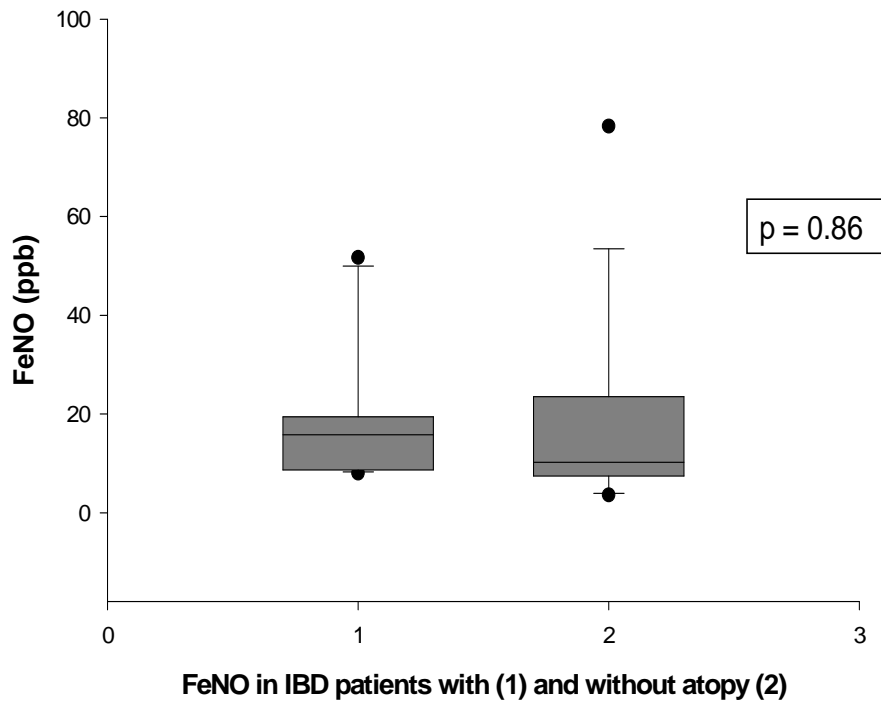


Figure 3: Comparison of FeNO values in atopic IBD patients versus IBD patients without atopy.

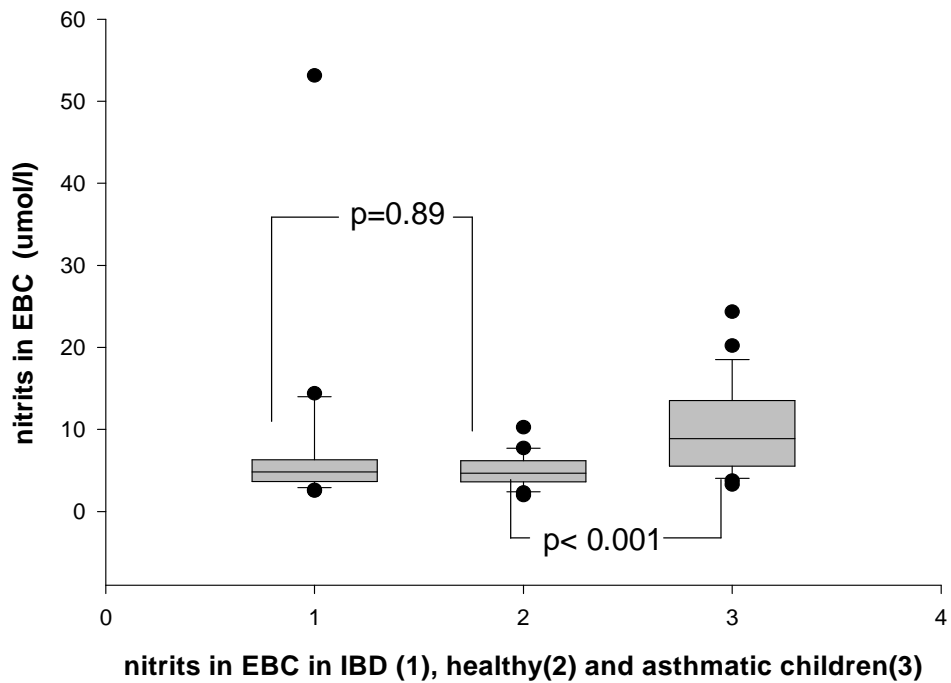


Figure 4: Concentration of nitrites in exhaled breath condensates in children with IBD, asthmatic children, and healthy. Significant difference was found only in group of asthmatic children compared with healthy.

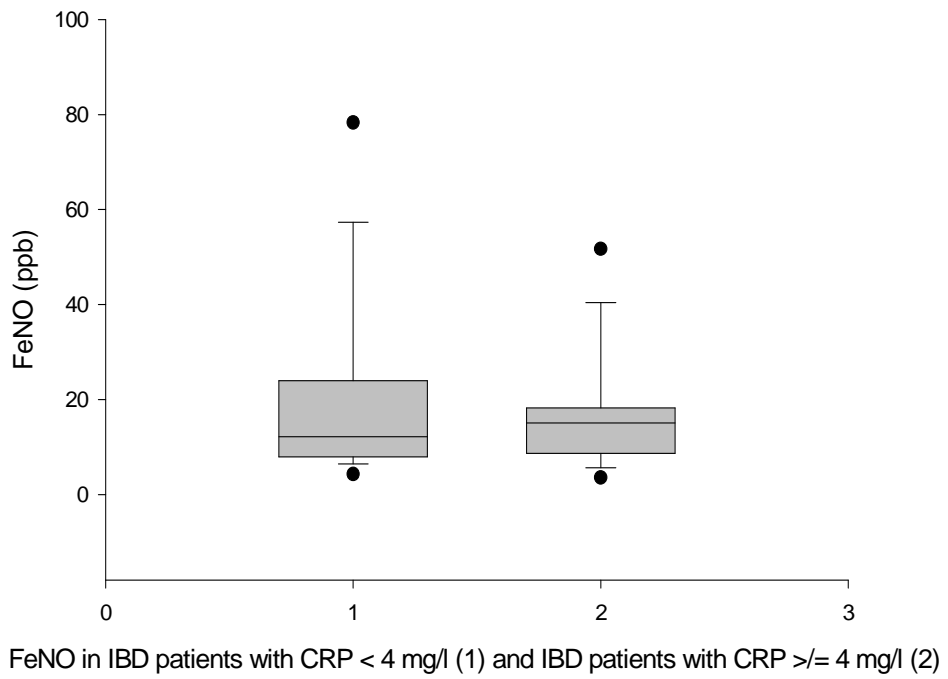


Figure 5: No difference in FeNO comparing IBD subjects with CRP <4 mg/l and IBD subjects with CRP \geq 4 mg/l.

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2.3 Novel Approach in Childhood Asthma Assessment:

Quantitative Determination of Curvature of Flow-Volume Curves

Introduction

Asthma guidelines recommend an initial evaluation of lung function for the assessment of disease severity followed by regular re-evaluation for the assessment of disease control (1, 2). Spirometry is considered the standard tool for objective assessment of lung function in asthma. In particular, FEV1 (Forced Exhaled Volume in 1 second) is used not only in the assessment of disease severity and disease control but also as the primary outcome in clinical studies (3). However, most asthmatic children have FEV1 values in the normal range independent of disease severity and/or disease control (3–5). Moreover, there exists a lack of correlation between FEV1 measures and individual symptom scores in asthmatic children (6, 7)]. This fact is not surprising as asthma is considered mainly a disease of the small airways and FEV1, thought to represent larger airways, therefore only reflects limited information on disease activity (8).

It is hypothesized that some other spirometry parameters (Maximal Expiratory Flow at 50% and 25%, MEF50 and MEF25, respectively), thought to represent smaller airways, correlate better with asthma symptoms (6, 9–12). In current practice however, given the lack of correlation between lung function parameters, mainly FEV1 and symptoms, it is generally recommended to assess asthma severity and asthma control using multiple lung function parameters jointly (FEV1, MEF25 and MEF25–75) (13, 14). Furthermore, clinicians will “eyeball” the flow-volume curve to assess asthma severity, where a concave pattern may be observed despite possibly normal PEF and FEV1 values (11, 15).

Aim of the study: The primary aim of our study was to evaluate the usefulness of single lung function parameters (FEV1, MEF50 and MEF25), as well as post-bronchodilator changes in these parameters, in the assessment of childhood asthma severity. We then evaluated the usefulness of the subjective “eyeballing” method by introducing a novel quantitative approach. Using raw data from the flow-volume curves, we numerically computed average curvatures of the concave pattern of the expiratory loops to quantitatively assess asthma severity.

Methods

35 asthmatic patients (16 females; mean [\pm SD] age, 10.2 \pm 2.7 years) were recruited for the present study. Spirometry was performed using a MasterLab spirometry (Jaegger, Germany) unit and symptom scores were obtained using a previously published questionnaire (16). Following such questionnaire, a symptom score of 0 corresponded to no asthma symptoms (normal). Three levels of asthma severity were characterized with respect to symptom score values: values in the range of 1 to 5 reflected mild asthma, values in the range 6 to 10 moderate asthma and values between 11 and 16

characterized severe asthma. Lung function parameters were obtained from the expiratory loop of the flow-volume curves (FEV1, MEF50 and MEF25) as well as post-bronchodilator changes in these parameters (change in FEV1 and MEF50 respectively). In addition, the expiratory loop of the flow-volume curves were subjectively qualified as normal, mild, moderate or severe “obstructed” by three independent pediatric pulmonologists, by “eyeballing” the curve with respect to its concavity (11, 15).

Numerical Algorithm

The “eyeballing” method consists in a clinician’s subjective assessment of the severity of the concave pattern when observing the flow-volume curve beyond PEF (Peak Expiratory Flow). A mathematical translation of the “eyeballing” method then simply corresponds to the quantitative evaluation of the average curvature of the concave pattern under investigation. The following steps are undertaken: We first determine the region of the flow-volume curve to be quantitatively “eyeballed”. Raw data is obtained from the spirometer and is characterized with uniform equidistant discrete points (measures are obtained with uniform 0.04L volume incremental). The expiratory loop of flow-volume curve may then be described by a discrete function $f(V)$ in [L/s], consisting of n points, where V is the volume in liters [L]. Based on Taylor series expansion, we approximate first and second derivatives, denoted f' and f'' respectively, using second order difference schemes (17). Looking at the behavior of f'' indicates that the expiratory loop, $f(V)$, largely consists of two distinct regions (Figure 1). The first region on the left hand side, which contains PEF, is characterized with a concave (i.e. concave downward) behavior, i.e. $f'' < 0$. The region of interest on the right-hand side is convex (i.e. concave upward) with $f'' > 0$. Concave and convex regions of the flow-curve are separated by an inflection point given by $f'' = 0$ (18). At each discrete point of the convex region of the curve, we compute the mathematical definition of curvature, $\kappa = |f''| / (1 + (f')^2)^{3/2}$. To obtain a description of the overall curvature over the convex region, which coincides with the “subjective” eyeballing method, we compute the average curvature, $\kappa^- = \int \kappa ds/L$, over the length, L , of the convex portion of the curve, where ds describes the arc length of the convex curve (19). Raw data from the flow-volume curve is intrinsically noisy (coughing, lack of child compliance, etc.) and an objective measure of the average curvature, κ , would require a smooth function $f(V)$. For such reason, the raw data is first filtered with a running average (20). Looking at the behavior of f'' over the convex region, our results for all patients suggests that the second derivative remains approximately constant over the convex region. This important result implies that the convex portion of the flow-volume curve may adequately be described with a quadratic model. Thus, we approximate the convex portion with a smooth least-square quadratic fit and then compute the average curvature.

Statistical Analysis

The data were entered in our computer base and analyzed with MATLAB software (21). Data on demographic characteristics and pulmonary function were summarized descriptively for our patient group (Table 1). Variables were expressed as means \pm SD unless indicated otherwise. To assess the usefulness of lung-function values and average curvatures in relation to symptom scores, we computed correlation coefficients, r (Pearson’s correlation), as obtained from the zero lag of the

normalized covariance function. A P value of less than 0.005 was considered to indicate statistical significance. Finally, we used a linear least-square regression method using a Trust-Region algorithm (22) to fit our scatter plots and computed the coefficients of determination, r^2 .

Results

Means \pm SD of the lung function parameters (FEV1, MEF50 and MEF25) as well as post-bronchodilator changes in parameters (changes in FEV1 and MEF50) are summarized in Table 1. According to GINA-guidelines (23), asthma severity in pediatric patients was considered as mild intermittent for $FEV1 \geq 80\%$, mild persistent for $60\% < FEV1 < 80\%$ and severe persistent for $FEV1 < 60\%$. No correlation was found between FEV1 ($r=-0.16$, $P=0.36$, Figure 2), MEF50 ($r=-0.04$, $P=0.82$) and MEF25 ($r=-0.19$, $P=0.28$) respectively and symptom scores. In addition, no correlation was found in changes in FEV1 ($r=-0.19$, $P=0.37$) and MEF50 ($r=0.04$, $P=0.87$) respectively and symptom scores. The subjective “eyeballing” method leads to significant discrepancy in the results obtained from each pulmonologist. Observer 1 distinguished 5 normal, 15 mild, 12 moderate and 3 severe cases of asthma. Observer 2 distinguished respectively, 15 normal, 14 mild and 2 moderate cases of asthma. Observer 2 found no severe cases of asthma and could not conclude on asthma severity from “eyeballing” the flow-volume curves in 4 patient cases. Observer 3 distinguished respectively, 14 normal, 19 mild, 2 moderate cases of asthma and no severe cases. To test the usefulness of the subjective “eyeballing” method, we then associated each asthma group (normal, mild, moderate, severe) with a quantitative value representing increasing asthma severity such that normal=1, mild=2, moderate=3, and severe=4 (we set the “normal” group at an arbitrary starting value of 1 and each subsequent group followed with an incremental of 1). We tested for possible correlation between the sets of subjective asthma groups, as determined respectively by each observer, and symptom score values (Figure 3). A weak correlation pattern was found for each observer ($r= 0.27$, $P= 0.11$; $r=0.28$, $P= 0.12$; $r= 0.22$, $P= 0.21$, respectively for observer 1, 2 and 3). Means \pm SD of the average curvature, κ^- , of the expiratory loop of the flow-volume curves are summarized in Table 1. Significant correlation was found between numerically determined average curvatures, κ^- , and symptom scores ($r= 0.53$, $P=0.001$; Figure 4). Note that values of κ^- may be negative, $\kappa^- < 0$, suggesting that the pattern of the flow-volume curve is concave (i.e. concave downward) rather than convex (i.e. concave upward). We subsequently compared results obtained from the subjective “eyeballing” method with results obtained from our quantitative “eyeballing” scheme (i.e. numerical average curvature). We found significant correlation between the sets of subjective asthma groups, as determined respectively by each observer, and the average curvature values ($r=0.61$, $P= 0.0001$; $r= 0.52$, $P= 0.003$; $r=0.48$, $P= 0.003$, respectively for observers 1, 2 and 3).

Discussion

It is surprising that measurement of airway obstruction by lung function parameters in childhood asthma, defined as a disease of reversible airway obstruction, do not correlate with asthmatic symptom scores. Such results may be explained in part by the fact that asthma is not solely an obstructive

disease, as described by Salter over a century ago (24), but rather, asthma is a more complex disease, in which inflammation plays a key role and observing functional changes may not be enough. On the other hand, it may well be that the way we currently look at and measure airway obstruction in asthmatic children may not be appropriate.

With our study, we have proven this point and shown that single lung function parameters (FEV1, MEF50 and MEF25) do not sufficiently correlate with asthma symptom scores to be used in the initial evaluation and in the follow-up for the assessment of disease severity and disease control. In contrast, we have shown that the “eyeballing” of the concave pattern of the flow-volume curve and in particular the quantitative assessment of the average curvature, κ^- , does correlate with symptom scores. We therefore conclude that the quantitative assessment of the average curvature of the expiratory loop should be used as an objective parameter in the assessment of asthma severity and asthma control. Based on our findings, we have made a preliminary attempt to categorize asthma groups with respect to average curvature values: no asthma (normal) would correspond to an average curvature approaching zero ($-0.05 < \kappa^- < 0.05$) suggesting that there is no concavity in the expiratory loop; mild asthma may be found in the range $0.04 < \kappa^- < 0.1$; moderate asthma may be found in the range $0.09 < \kappa^- < 0.18$; for values of $\kappa^- > 0.2$, one may expect severe asthma condition.

We briefly comment on the results obtained for the average curvature, κ^- , and discuss the limits and weaknesses of the numerical algorithm currently implemented. Despite significant correlation between κ^- and symptom score values, we observe a weak goodness in the linear fit ($r^2=0.28$; Figure 4). Such result suggests that using a linear regression scheme may not be an appropriate model to describe the dependence of symptom scores on average curvature. Indeed, symptom scores and average curvature, κ^- , do correlate but further investigation is needed to reach an appropriate model (e.g. quadratic, etc.) to predict their interdependence. In addition, the numerical computation of κ^- is directly dependent on two governing factors: the goodness of the quadratic fit of the concave pattern of the flow-volume curve (for all patients, our fits yield $r^2 > 0.98$) as well the correct determination of the location of the inflection point separating the two main regions of the expiratory loop. Both the goodness of the quadratic fit and the determination of the inflection point are themselves contingent upon the smoothness of the raw data describing the curve. Flow-volume curves are intrinsically dependent on patients, such that noisy curves (due to patient coughing, lack of child compliance, etc.) cannot be excluded. Therefore, noisy data may significantly deteriorate the objectiveness of the computed average curvature, κ^- , and compromise accurate assessment of asthma severity. More robust algorithms need further development with the aim of integrating them within spirometric hardware to obtain real-time measures of average curvature as opposed to a current post-processing approach.

In summary, based on our recent findings, quantitatively determined average curvature is likely to provide a marker to look retrospectively or prospectively at the outcome of clinical studies and is prone to be used in the assessment of disease severity and disease control in pediatric asthma.

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Table 1: Base-Line Characteristics of the Patient Group

Characteristic ^a	Patients (N=35)
Male sex (% of children)	55
Age (yr)	10.17 ± 2.72
Height (cm)	137.14 ± 23.52
Weight (kg)	35.4 ± 11.09
FEV ₁ (% of predicted value)	91.47 ± 14.84
Change in FEV ₁ (% points) ^b	6.8 ± 7.14
MEF ₅₀ (% of predicted value)	65.83 ± 27.11
Change in MEF ₅₀ (% points) ^c	9.7 ± 11.84
MEF ₂₅ (% of predicted value)	58.4 ± 34.32
Symptom score	3.46 ± 3.57
Average curvature $\bar{\kappa}$	0.083 ± 0.071

^aPlus-minus values are means ± SD.

^bN=24

^cN=24

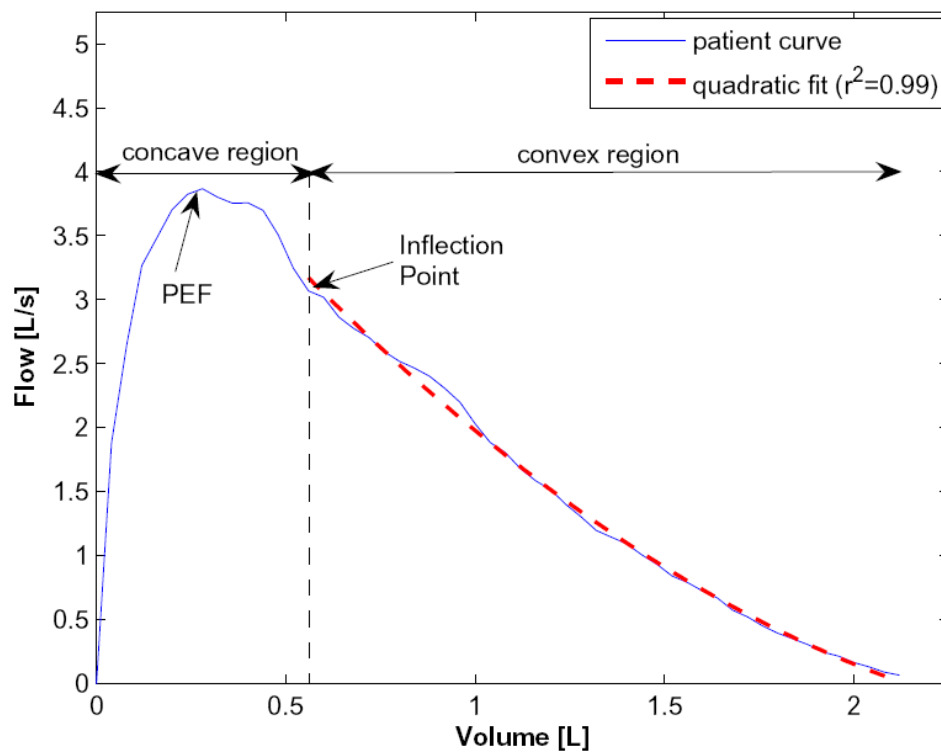


Figure 1: Typical patient expiratory loop of flow-volume curve

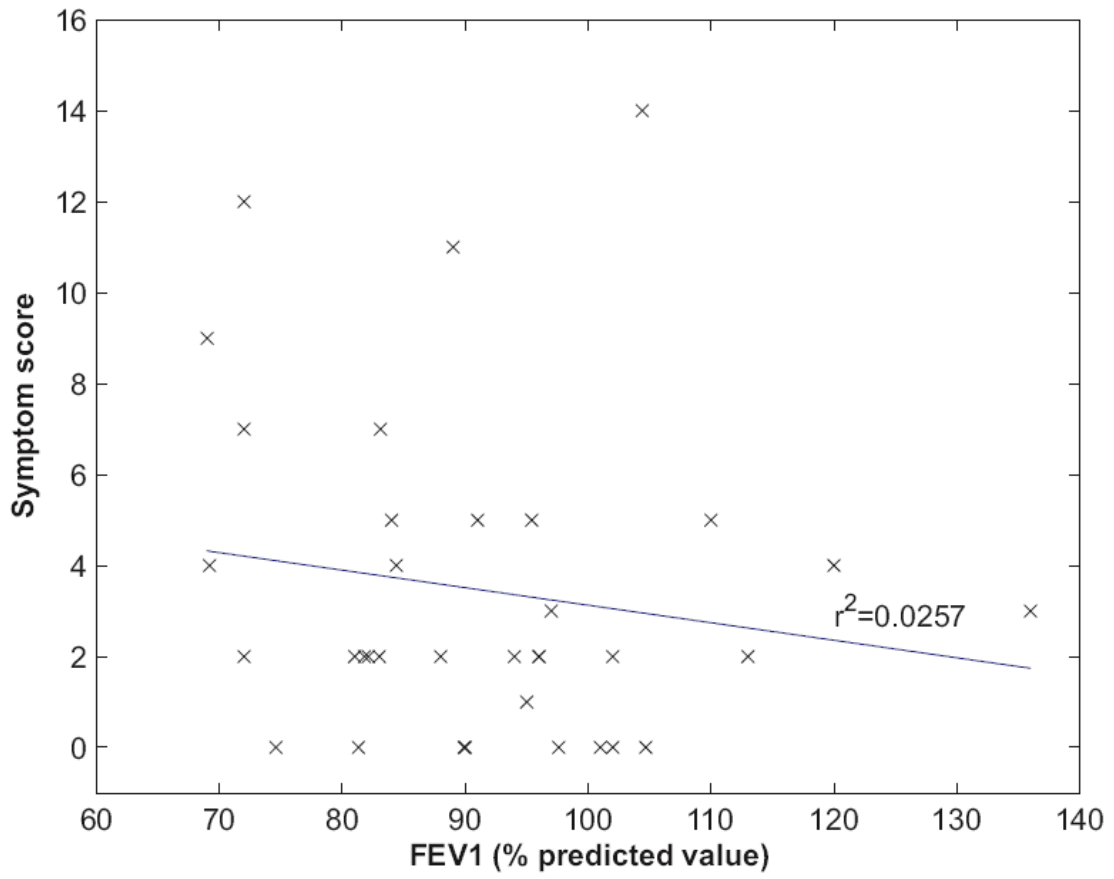


Figure 2: Correlation scheme: symptom score vs. FEV₁ values.

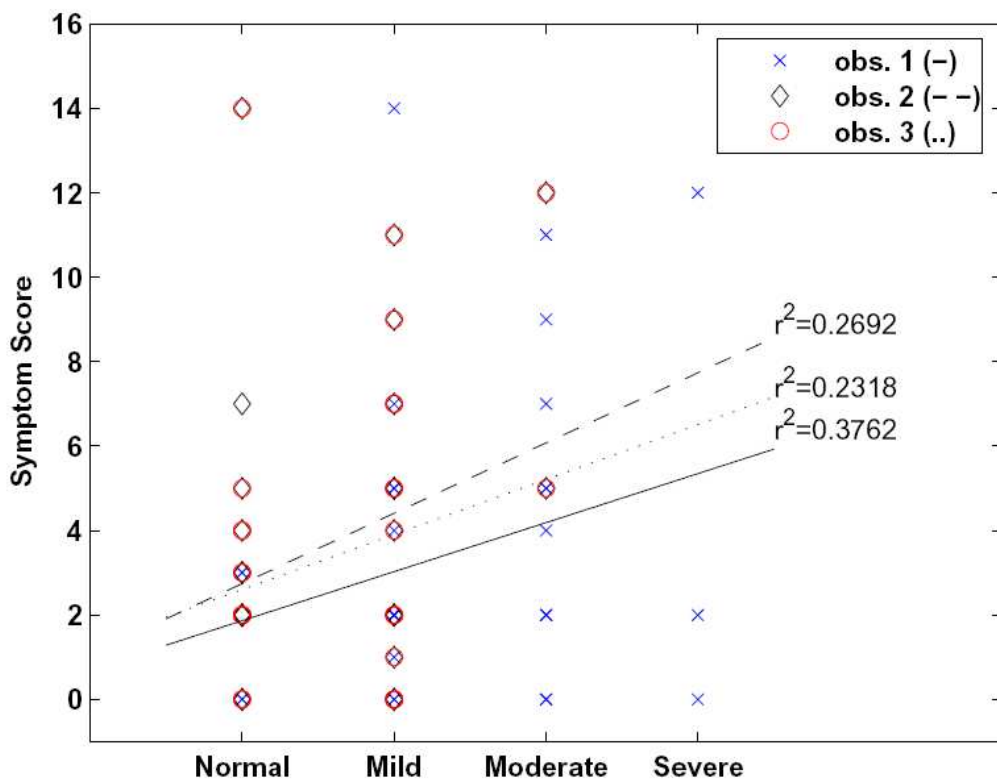


Figure 3: Correlation scheme: symptom score vs. subjective asthma group determined from the “eyeballing” method.

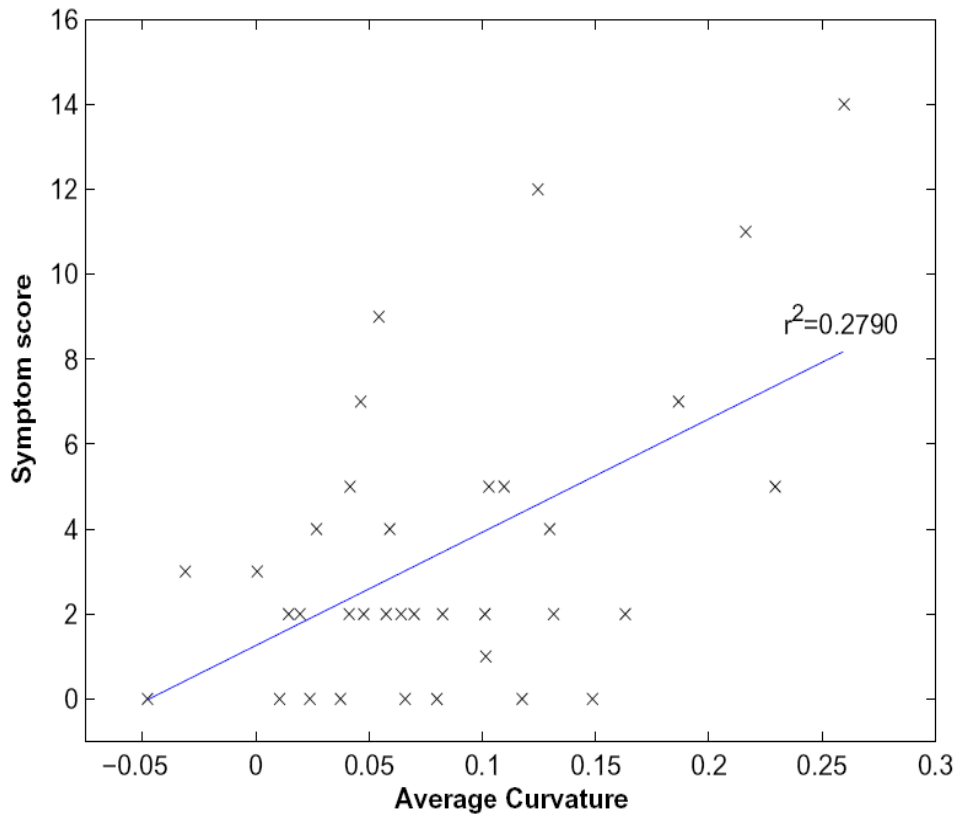


Figure 4: Correlation scheme: symptom score vs. average curvature, $\bar{\kappa}$, values.

2.4 Exhaled nitric oxide in newborns measured by multiple-breath method. Is there any diurnal rhythm?

Introduction:

Nitric oxide (NO) is produced by various cells in the respiratory tract of human and can be measured directly in exhaled gas using a rapid chemiluminescence analyser. The fraction of nitric oxide in exhaled air (Fractional concentration of exhaled nitric oxide, FeNO) is increasingly used as a marker of eosinophilic airway inflammation in diagnosis and management of childhood asthma (1-4).

In addition, NO plays an important role also in pathogenesis of other inflammatory disorders as cystic fibrosis or non-CF bronchiectasis (5). Furthermore, exhaled NO (eNO) is altered during both oxidative stress and neutrophilic inflammation of airway.

In the last few years there has been an increasing interest in measuring exhaled nitric oxide also in infants, as it might provide a useful tool to study inflammation in airway diseases early in life (6-9).

Some studies suggest a role for NO under various pathophysiological conditions in newborn, such as bronchopulmonary dysplasia or persistent pulmonary hypertension of the newborn (10, 11).

FeNO is critically dependent on exhalation flow. In school-age children ATS/ERS guidelines(12) recommends measurement during a single, slow exhalation from total lung capacity at a constant flow of 50 mL.s⁻¹. This method is unsuitable in young uncooperative children and newborn. Consequently, eNO measurements in infants have been tried during quiet, regular tidal breathing.

Franklin et al. (13) have shown that factors like male sex, ambient nitric oxide and different environmental factors influence eNO as well. No data has been published about possible diurnal pattern of NO production in newborn.

Aim of this presented study was to measure repetitively mixed (oral and nasal) tidal exhaled NO (tFeNO) and NO-output (V'NO) in a group of newborn without lung disease during 24 hours. In the analysis, we studied diurnal changes as well as the influence of feeding on eNO and V'NO levels.

Patients and Methods

In a prospective cohort study 10 newborns without lung disease were enrolled. The study was conducted at the Department of Intensive Care and Neonatology, University Children's Hospital, Zurich, Switzerland. The study protocol was approved by local ethical committee and written consent was obtained from the parents. Data regarding mother's history, pregnancy, maternal drug use and smoking as well as gestational age (GA), birth weight (BW) and Apgar score at 1 and 5 minutes of life were recorded. Exclusion criteria included gestational age less than 36 weeks, history of assisted ventilation or prolonged oxygen therapy (> 24 hours), and known chronic respiratory or cardiac disease.

Measurement of exhaled NO

All the infants were studied in a supine position, mostly during unsedated quiet sleep, with the head in the midline. Heart rate monitoring and pulse oxymetry were performed throughout the study in each child. A soft silicon mask (Infant mask, size 0 or 1; Homedica, Cham, Switzerland) was placed over the nose and mouth, and flow–volume loops were inspected for leaks prior to commencing the measurement. NO filter ensured that all infants inhaled NO-free air. Tidal gas flow, tidal volume, tidal eNO (tFE_{NO}) and CO₂ levels were registered for approximately 2 minutes (100 breaths) using commercially available prototype infant lung function equipment (Exhalyser; EcoMedics, Duernten, Switzerland) as previously validated and described in detail elsewhere (14).

This measurement was performed repetitively, at least every hour in each baby for a total of 24 hours. Each child received minimally one reliable tFE_{NO} measurement one hour before as well as one, two and three hours after each feeding. All measurements were done by the same experienced person.

Analysis

The representative example of online measured tidal flow, tFE_{NO} and NO output shows a steep increase of eNO at the beginning of expiration, which achieves a plateau towards the end of this phase. During inspiration, eNO rapidly returns to zero, indicating negligible re-breathing of NO from the equipment dead space. NO output is calculated by multiplying tidal flow by tFE_{NO}. Flow rapidly approximates to zero at the end of expiration. These rapid flow changes result in variable NO output during the fourth quartile of the breath duration. tFE_{NO} and NO output have, therefore, been measured in the third quartile of expiration, since this shows the lowest breath-to-breath variability and corresponds approximately to the phase III slope (14) (Fig 1). In order to ensure consistency of analysis between infants, only the first 100 breaths in the tidal breathing data were analysed using Spiroware software (EcoMedics). tFE_{NO} was analyzed by using an algorithm previously reported (ref.)*. NO output ($\dot{V}' \text{ NO}$) was calculated as follow: $\dot{V}' \text{ NO} = \text{FeNO} \times \dot{V}'$.

Statistical analysis

Fractional exhaled NO values were log-transformed prior to the analysis in order to obtain a near-normal distribution and analyzed by means of parametric tests. Then tFE_{NO} values were back transformed and presented as geometric mean. The unit of analysis was each exhaled gas collection. One-way repeated measures analysis of variance was used for evaluation of influence of time of day on tFE_{NO}. Regression analysis was used to evaluate the relation between tFE_{NO} and feeding. Tukey-Kramer adjustments were used for multiple comparisons with an alpha level of 0.05.

Results

The mean gestational age of studied newborns was 38.1 weeks and the mean birth weight was 2820 grams. More demographic data are presented in Table 1. The fluctuation of individual tFE_{NO} during the 24 hours are demonstrated in Figure 2. Coefficient of variation between the measurements of the individual patients ranged between 12.3 and 33.3%, mean CV was 20.2%. Mean tFE_{NO} ranged between 11.0 and 29.2 ppb. The mean of all tFE_{NO} and $V'NO$ measurements in all patients was 15.6 (5.1) ppb and 47.6 (9.8) η /min, respectively. There was no influence of the measurement time point on the tFE_{NO} ($p=0.62$) (Fig. 3) and $V'NO$ values ($p=0.97$) (Fig. 4). within the 24 hours time period. However, when analysed tFE_{NO} , tidal flows and NO-output in 4 different 'day time zones' (zone I: 10pm-3am, zone II: 4-9am, zone III: 10am-3pm, and zone IV: 4pm-9pm), there were significant differences. Tukey test showed significantly higher tFE_{NO} levels ($p=0.027$) and NO-output values ($p=.019$) in time period from 10am to 3pm compared to 4am to 9am. No significant difference was found for tFE_{NO} values when nitric oxide was measured within 1 hour, between 1 and 2 hours, between 2 and 3 hours, or more than 3 hours after feeding ($p=0.79$) (Fig. 5). For $V'NO$ values, there was a statistic significant difference with lowest $V'NO$ values measured 1 hour after feeding (Fig. 6). In our study, tFE_{NO} and $V'NO$ mean values were independent of gestational age ($p=0.60$) birth weight ($p=0.69$), intrauterine nicotine exposure ($p=0.25$) and parental history of allergy/asthma ($p=0.17$).

Discussion

Aim of the presented study was to identify a possible diurnal rhythm of tFE_{NO} in healthy newborns and to analyse the influence of feeding on tFE_{NO} . Our main finding was that we could not find any diurnal rhythm when analyzing tFE_{NO} over 24 hours. However, we demonstrated an increased tFE_{NO} level during 10 am and 3 pm compared to an early morning period (time period 4am to 9am). There was no systemic effect of feeding on the tFE_{NO} measurements, the nitric oxide output was higher short after feeding (<1 h).

Few recently published studies suggest an existence of circadian rhythm in nitric oxide metabolism. Borgonio et al (22) shown that nitric oxide metabolites in urine are subject of circadian rhythm. O'Hearn et al. (23) described significantly reduced exhaled nasal NO output at night during sleep. Oppositely, ten Hacken et al. (24) measured orally exhaled NO in awake normal and asthmatic subjects and found no significant temporal pattern over a 24 h period. The simultaneously registered tidal flows and NO output values showed the same trend allowing speculation, that exhaled mixed NO concentration and nitric oxide output are decreased at early morning. Interestingly, in the same period when the tFE_{NO} levels in our study reached their nadir, the secretion of cortisol physiologically peaks. Alveolar hypoxia can lead to decreases in NO production in the lung. However, our subjects were considered pulmonary healthy and none of them have significant apnea or decrease in oxygenation based on pulse oxymetry during the study.

Analogous to the group of de Jongste (16) we could not demonstrate any systemic effect of feeding on tFE_{NO} values in healthy infants. This issue has been previously investigated and guidelines recommend refraining from eating and drinking before NO analysis. An increase in FeNO measured by single-

breath technique has been found after the ingestion of nitrate or nitrate-containing foods, such as lettuce (17, 18). In our study, infants after feeding were more likely to cooperate and to maintain a tidal quiet breathing during the tFE_{NO} measurement, especially if awake. However, after feeding, the breathing pattern of studied babies changed in most cases as follow: the respiratory frequency and tidal flow raised and NO output consequently also elevated.

Analysing our data with regards to family history of atopy or asthma we did not find any effect of paternal atopy on mean tFE_{NO} values. This might be due to the small sample size. Two larger studies reported higher $FeNO$ levels in infants from atopic mothers (15, 21). In addition, Wildhaber et al. (20) using single-breath technique found significantly higher levels of exhaled NO in infants with both maternal and paternal atopy. Analysed for effect of prenatal tobacco exposure there was as well no effect on tFE_{NO} and NO-output values, which fact is again most likely explained by the small sample size. Two recent studies (14, 21) found that prenatal tobacco exposure was associated with significantly lower tFE_{NO} in infants.

Limitation of our study represents the relatively small number of infants, which make a presence of alpha-type error possible. Because the EEG signal was not registred during tFE_{NO} measurement, we could not separately analyse for tFE_{NO} and NO-output at sleep, at awakening in the night, compared to daytime awake levels.

Conclusion

In the present study, tFE_{NO} values were independent of feeding. Analyse of diurnal profile revealed elevated tFE_{NO} levels between 10am and 3pm. Together with gestational age, birth weight, intrauterine nicotine exposure and parental history of allergy/asthma, this has to be taken into account, when tFE_{NO} and or $V'NO$ are measured in newborns.

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Table 1.: Patients characteristics

N	Sex	GA at birth (weeks)	Age at measurement (days)	weight at birth (g)	actual weight (g)	actual length (cm)	diagnosi s	T	FH asthma	mean tFE _{NO} (ppb)	mean V'NO (nL/min)
1	M	36	23	2450	2750	46	icterus	0	0	28.8	54
2	M	41	18	3860	3900	50	infection	0	0	15.2	67.8
3	M	37	12	2570	2640	48	fracture	0	0	15.9	45.8
4	F	36	56	2860	4330	48.5	infection	0	0	12.7	44.4
5	F	38	23	3120	3880	49	pt	1	0	16.6	43.4
6	M	39	12	2450	2600	47	pt	1	1	11.2	38.6
7	F	40	14	3210	3450	50	infection	0	0	13.3	43.8
8	M	40	33	2890	3710	50	infection	0	0	17	52.3
9	F	37	24	2600	2960	46	hypogly	1	1	13.7	53.4
10	F	37	24	2200	2720	45	hypogly	1	1	11.5	32.4

M = male; F = female; infection: pyelonephritis (N 2 and 8), viral meningo-encephalitis (N 7), skin infection (N 4); hypogly: hypoglycemia (N 9 and 10)pt = poly-toxycomania of the mother; T: tobacco smoke exposure; FH: family history; tFE_{NO}: tidal fractional exhaled nitric oxide; V'NO: NO output

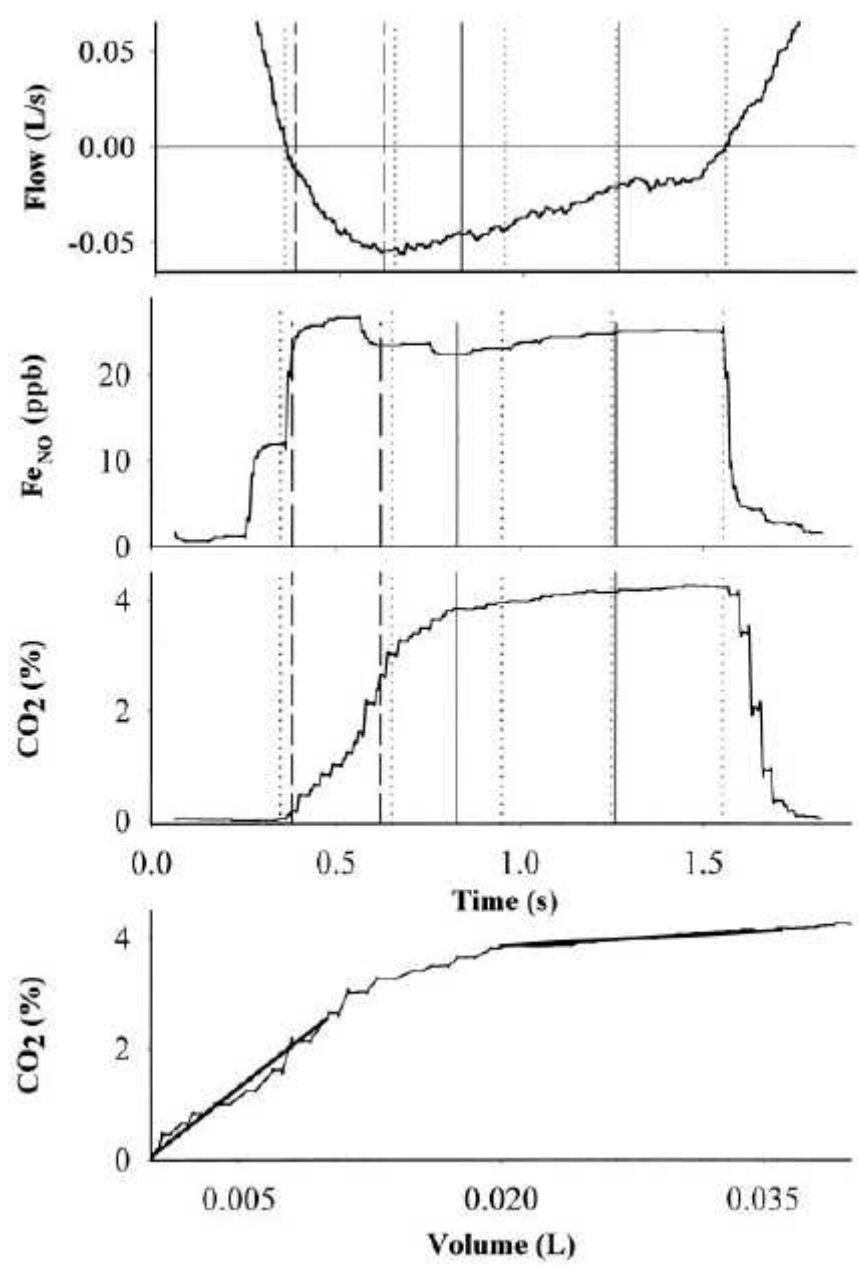


Figure 1.: Analysis of tFeNO at plateau of capnometry curve

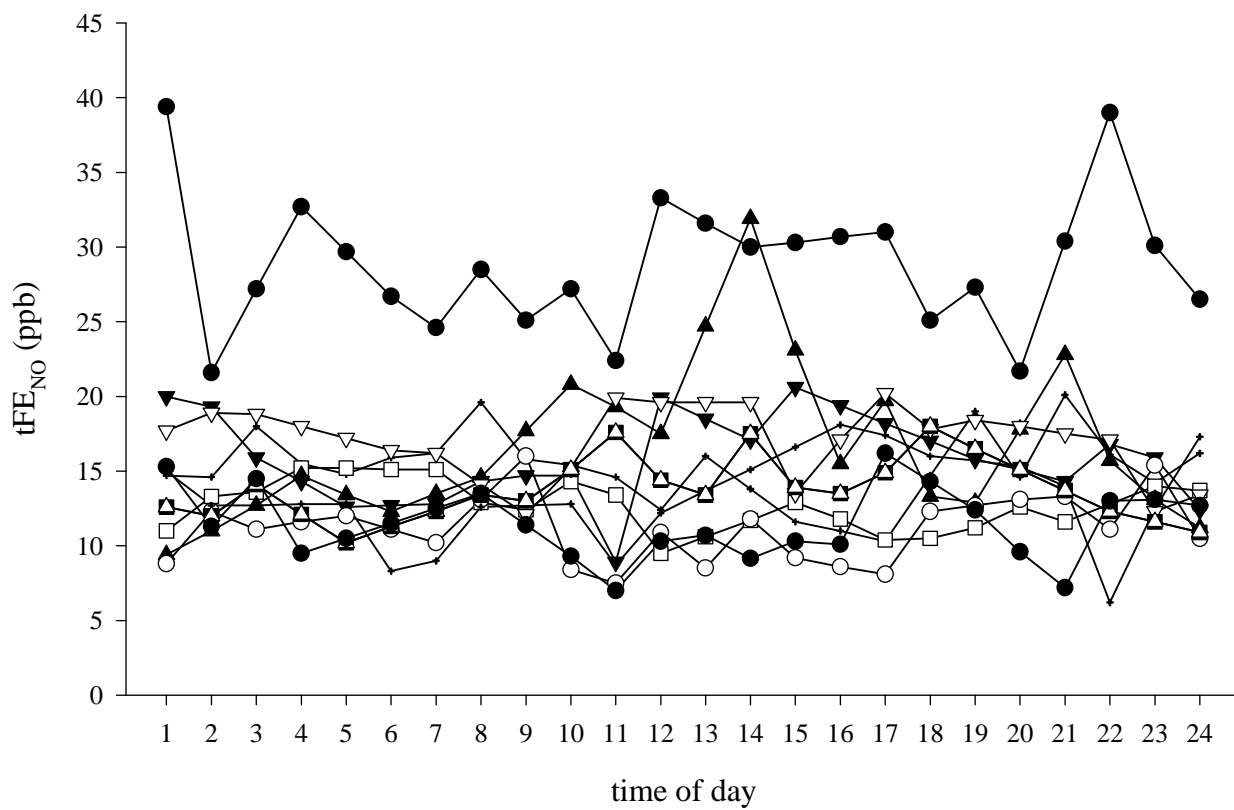


Figure 2: Individual tFE_{NO} values over 24 hours

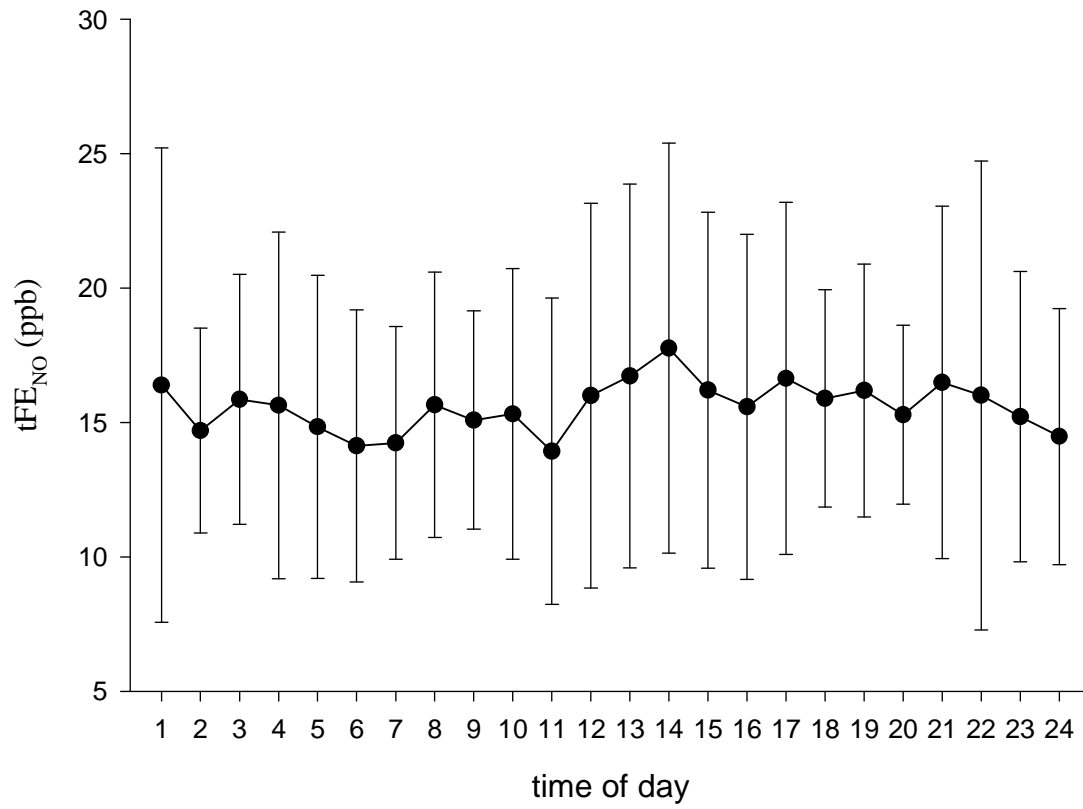


Figure 3: No significant diurnal pattern in tFE_{NO} values

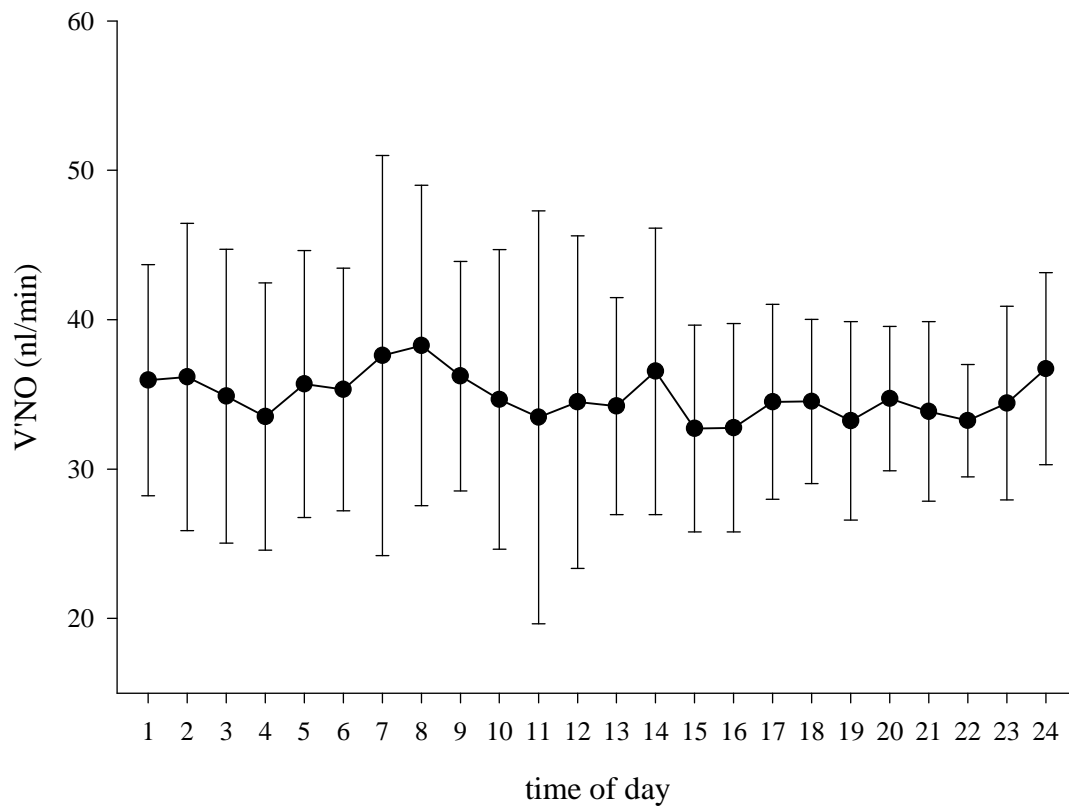


Figure 4: No significant diurnal pattern in nitric oxide output (V'NO)

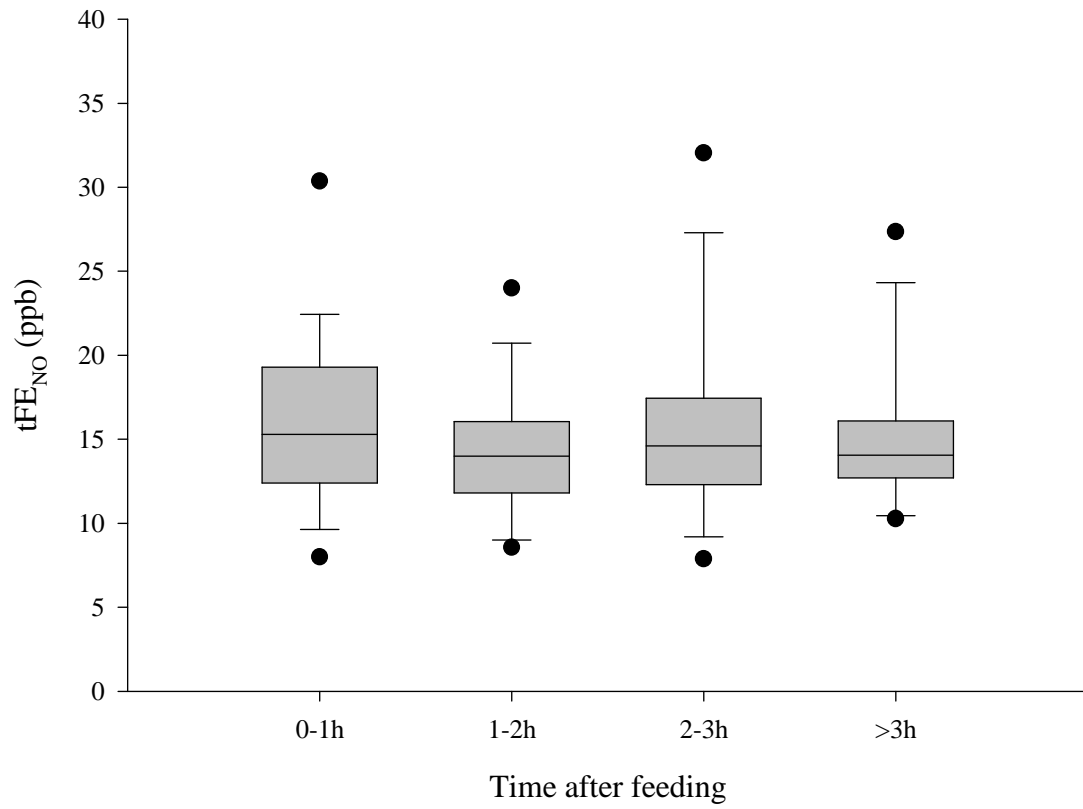


Figure 5: tFENO levels in regard to time after feeding. The median is the line bisecting the box, the box limits represent 25th and 75th percentiles and whiskers extend to the 5th and 95th percentile.

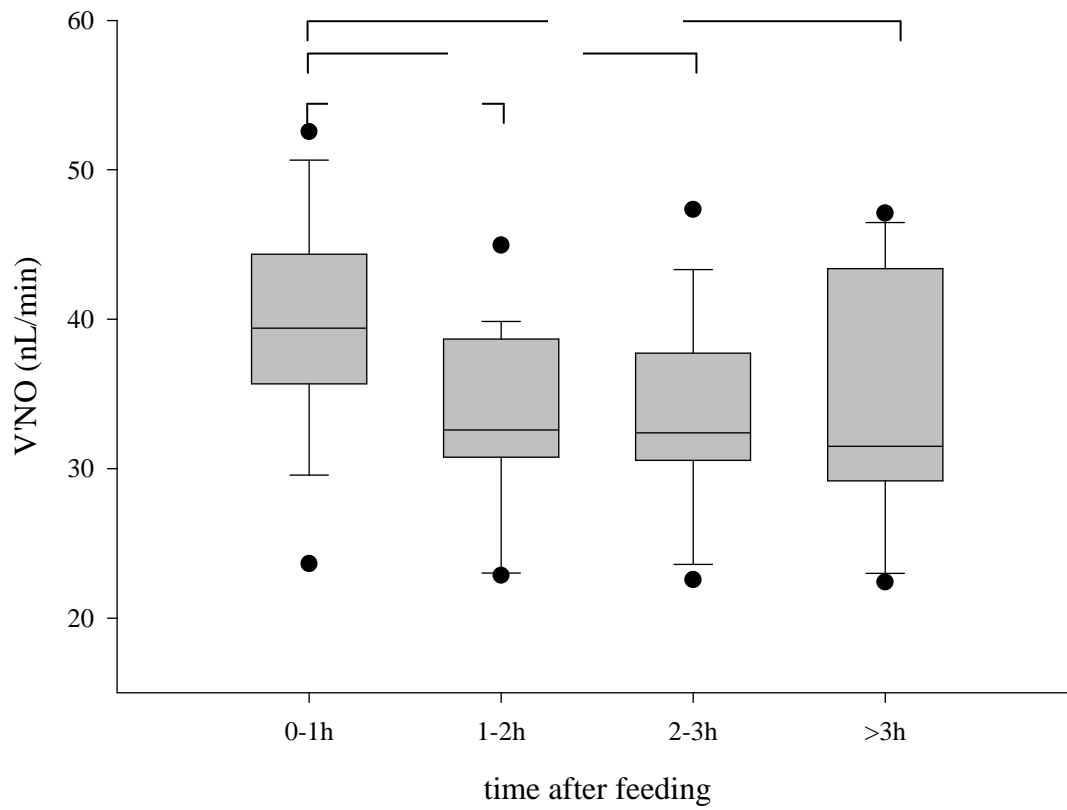


Figure 6: V'NO values in regard to time after feeding. The median is the line bisecting the box, the box limits represent 25th and 75th percentiles and whiskers extend to the 5th and 95th percentile.

CLOSING REMARKS

In summary, in my thesis I have been able to show important results from the clinical application of lung function testing in young children.

With improved survival over the last 20 years, increasing attention has been paid to the long-term outcomes of infants with congenital diaphragmatic hernia. The University Children's Hospital Zurich (called internally 'Kispi') represents a tertiary referring center for fetal and neonatal surgery in Switzerland, and therefore, has the most extensive experience with babies, who suffer from congenital diaphragmatic hernia, in the country. Our study summarized a ten years period, with about five children with CDH being born and consequently treated at 'Kispi' each year. The complex therapy of CDH and all the associated complications changed dramatically during this period. However, neither inhaled nitric oxide nor high frequency oscillation or ECMO changed significantly the prognosis of babies with CDH. The 'Kispi' population of children with CDH, as reported in our study, has a comparable prognosis as children born with the same defect in the largest centers in the US or Canada. Similarly, as children treated in Boston or Toronto, many survivors of CDH in Zurich have significant respiratory problems for many years after discharge from the hospital. The continual growth of airways and lung, however, corrects the initial significant pulmonary function deficit. In our study, we reported about exhaled nitric oxide in CDH survivors and found normal, non-elevated levels. At the time of study visit, all tested children were cardiopulmonary stable, without any sign of pulmonary hypertension. It would be interesting to measure exhaled nitric oxide early in the course of CDH, when a significant pulmonary hypertension frequently subsists.

The common embryological origin of the gut and airway tree and the report about high concentrations of nitric oxide in aspirated colonic gas, were the initial impulse for our study in children with Crohn disease and Colitis ulcerosa. Unlike, as a single published study in adults indicated, we found elevated exhaled nitric oxide also in children with non-active inflammatory bowel disease. Except one boy with Crohn disease and concurrent asthma, all tested children did not have any respiratory symptoms and did show normal lung function parameters. The next logical step, which would be of help to understand the role of nitric oxide in assumed airway mucosal damage, consists of direct bronchoscopy evaluation of the airway tree with consecutive evaluation of biotically sampled tissue.

The fact, that conventional analysis of spirometric parameters failed in predicting the clinical course of asthma in children was for me (at the beginning of my training in respiratory medicine) one of the most frustrating experiences. For the majority of respiratory specialists, the 'eyeballing' of the expiratory limb of the spirometric flow-volume curve is the first step in evaluating possible airway obstruction. It was an intriguing observation, that the subjective analysis of clinical severity of obstructive lung disease can be correlated to the measurement of curvature by using a mathematical equation.

There is a need for more studies focused on these phenomena, and today analysis of exhaled nitric oxide called 'inflammometry' dominates the general effort to improve the evaluation of asthma in respect to control and risk of exacerbation.

Nitric oxide is an important mediator of inflammation in several pathological conditions. Its role in pulmonary circulation and airway-tonus homeostasis is not completely understood to date, but the tendency to measure exhaled nitric oxide under different conditions has reached the neonatal medicine also. The multiple-breath method allows sampling of exhaled air via side stream during tidal breathing in unsedated infants. In our study, we asked two practice-relevant methodological questions: Is there any diurnal rhythm in nitric oxide production, and is there any effect of feeding?

After we analyzed our data set, we concluded that beside an effect of atopy and passive tobacco exposition, an effect of daytime on the levels of exhaled nitric oxide can be measured.

Similarly to an other group, we could not show any systemic effect of feeding on exhaled nitric oxide.

The 'Swiss Adventure', how I called familiarly the period of my two and half years as a Research Fellow at 'Kispi', was a very intense and fascinating period of my life. I had the unique opportunity to work with and to learn from experienced specialists in respiratory medicine not only from Switzerland, but due to the opportunity to travel to and to participate in international congresses, from people from allover the world. Looking at the questions which have arisen from my research, I understand that one of the most rewarding parts of a medical career is continuous learning. The personal contact, communication and friendship between medical specialists from various cultural backgrounds are essential not only for career opportunities but also for personal development and hence, advance of each physician. In this way, I would like to thank all the people from 'Kispi' and outside the Hospital, which I met in Zurich and generally in Switzerland who opened a new door for me.

My major career objectives are to continue a full-time career in pediatric respiratory medicine not only as a clinician and researcher, but also as a teacher. In this way, I would like to go back to my home country and use my training and experience to supervise and train new fellows in pediatric respiratory medicine.