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Exhaled NO and small airway function in asthma and cystic fibrosis

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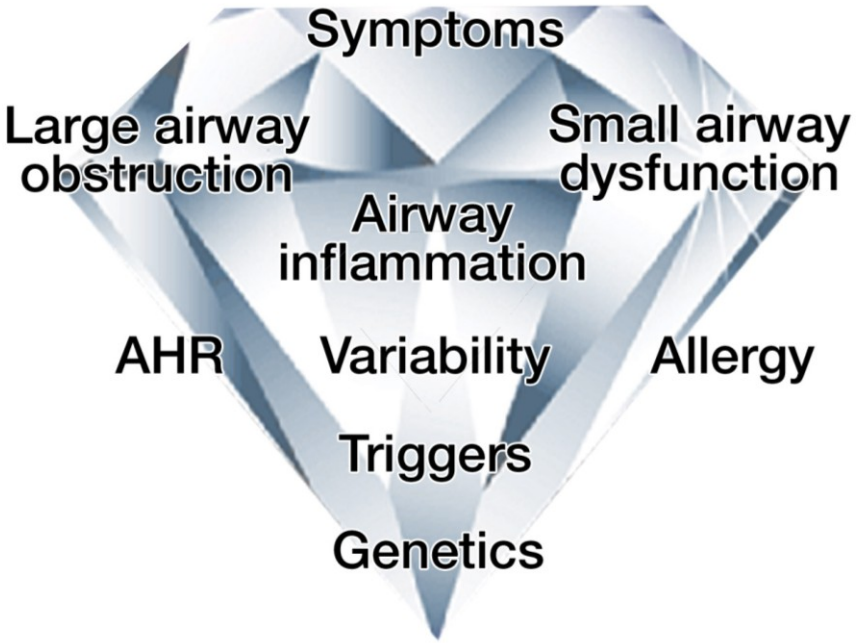

”The million dollar question”
How to monitor and treat subjects with multifaceted airway disease?

List of papers

This thesis is based on the following papers:

Paper I
C Keen, A-C Olin, Å Edentoft, E Gronowitz and B Strandvik**.** Airway nitric oxide in patients with cystic fibrosis is associated with pancreatic function, pseudomonas infection and polyunsaturated fatty acids. *CHEST*, 2007; 131(6):1857-1864

Paper II
C Keen, A-C Olin, S Eriksson, A Ekman, A Lindblad, S Basu, C Beermann and B Strandvik. Supplementation with fatty acids influences the airway nitric oxide and inflammatory markers in patients with cystic fibrosis. *Journal of Pediatric Gastroenterology and Nutrition,* 2010; 50(5):537-544.

Paper III
C Keen, P Gustafsson, A Lindblad, G Wennergren and A-C Olin. Low levels of exhaled nitric oxide are associated with impaired lung function in cystic fibrosis. *Pediatric Pulmonology*. 2010; 45(3):241-8.

Paper IV
C Keen, A-C Olin, G Wennergren, F Aljassim and P Gustafsson:
Exhaled NO, small airway function and airway hyper-responsiveness in paediatric asthma. (*Submitted)*

The papers will be referred to in the text by their Roman numerals.

Exhaled NO and small airway function in asthma and cystic fibrosis

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Background: Asthma and cystic fibrosis (CF) are chronic inflammatory airway disorders known to involve the peripheral airways. Non-invasive tests sensitive to peripheral airway function and inflammation are therefore of high priority. Multiple breath inert gas washout (MBW) is a test sensitive to small airway function and exhaled nitric oxide (NO) reflects airway inflammation in asthma.
Aim: To use exhaled NO in combination with MBW to assess the contribution of small airway inflammation and dysfunction in paediatric asthma and CF in order to potentially allow for earlier intervention and more successful management of these conditions in the future.
Results: CF subjects had reduced levels of nasal and exhaled NO. All but three children with CF had abnormally elevated LCI. Low levels of NO were associated with impaired airway function, chronic infection with *Ps. Aeruginosa,* severe genotypes and the fatty acid deficiency characteristic for CF subjects. Low levels of alveolar NO, albeit not lower in CF than in healthy controls, were associated with increased systemic inflammation and chronic bacterial airway colonisation.
LCI, S_{cond}, and S_{acin} were all significantly elevated in children with asthma and S_{cond} was the marker that most significantly differentiated the asthmatic children from the healthy controls. Increased S_{cond} was associated with increased levels of exhaled NO and airway hyper-responsiveness and S_{acin} correlated with alveolar NO.
Conclusions*:* This thesis provides further evidence of small airway involvement in both paediatric asthma and CF. The findings of clinically significant dysfunction of the small conducting airways in paediatric asthma and the associations between small airway dysfunction, increased levels of exhaled NO and airway hyper-responsiveness are novel findings. In CF, low levels of exhaled NO are linked to small airway dysfunction. These findings provide new exciting insights into the pathology and pathophysiology of paediatric asthma and CF and may allow for earlier and better targeted interventions.

Keywords: asthma, children, cystic fibrosis, flow independent exhaled nitric oxide, multiple breath inert gas washout, polyunsaturated fatty acids.

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Abbreviations

AA = arachidonic acid

ACT= Asthma Control Test

AHR = airway hyper-responsiveness

ALA = α-linolenic acid

ATS = American Thoracic Society

BMI = body mass index

cACT = child Asthma Control Test

CF = cystic fibrosis

$\mathbf{C_{alv}}$= alveolar NO concentration

cNOS = constitutive nitric oxide synthase

CFTR = cystic fibrosis transmembrane conductance regulator

$\mathbf{Daw_{NO}}$ = bronchial wall NO diffusion capacity

DHA = docosahexaenoic acid

eNOS = endothelial nitric oxide synthase

EDRF = endothelium dependent relaxing factor

EPA = eicosapentaenoic acid

ERS = European Respiratory Society

ESR = erythrocyte sedimentation rate

FA = fatty acid(s)

FENO = fraction of exhaled nitric oxide

$\mathbf{FENO_{50}}$ = fraction of exhaled nitric oxide at 50 ml/s

$\mathbf{FEV_{1.0}}$ = forced expiratory volume in one second

FRC = functional residual capacity

FVC = forced vital capacity

ICS =inhaled corticosteroid

IgG = immunoglobulin G

iNOS = inducible nitric oxide synthase

$\mathbf{Jaw_{NO}}$ = bronchial NO flux

LA = linoleic acid

LCI = lung clearance index

LLN = lower limit of normal

MBW = multiple breath inert gas washout

MMEF = maximum mid expiratory flow

nNO = nasal nitric oxide

nNOS = neuronal nitric oxide synthase

NO = nitric oxide

NOS = nitric oxide synthase

OA = oleic acid

ppb = parts per billion

PUFA = polyunsaturated fatty acids

$\mathbf{SF_6}$ = sulphur hexafluoride

$\mathbf{Sn_{III}}$ = normalized phase III slope

ULN = upper limit of normal

WBC = white blood cells

Vt = tidal volume

Contents

Introduction

Asthma and cystic fibrosis (CF) are chronic inflammatory airway disorders known to involve the peripheral airways[1-6]. Asthma and CF are like diamonds; multifaceted and expensive for the patient and the society. Safe, patient friendly, non-invasive tests sensitive to peripheral airway function and inflammation are therefore of high priority. A better understanding of the asthma spectrum could help to better target treatment to obtain full asthma control. Early detection of airway disease in CF is essential to start aggressive therapy, which might prevent irreversible lung function impairment.

The aim of this thesis is to use exhaled nitric oxide (NO) and multiple breath inert gas washout (MBW) to assess the contribution of airway inflammation and small airway dysfunction in paediatric asthma and CF, to allow for potential earlier intervention and treatment that is more successful in paediatric asthma and CF in the future. Exhaled NO and MBW are safe and non-invasive methods which are relatively easy to use, also in children.

Patients and society have much to gain if simple methods could be utilised to better understand the pathology and pathophysiology of airway disease, in order to target treatment. The ultimate objective of the studies would be to find ways to find the right treatment to avoid personal suffering and the use of expensive, sometimes ineffective or unnecessary interventions. This area of research is of importance and deserves further studies.

Airway inflammation

Asthma and CF are characterized by airway inflammation, excessive airway secretion and airway obstruction affecting people of all ages. Methods to assess airway inflammation therefore need to be feasible in young children as well as in older subjects (Table 1).

Bronchoscopy with biopsies and broncho- and bronchoalveolar lavage, has been the gold standard for studying airway inflammation but bronchoscopy is an invasive method requiring anaesthesia in children[7]. Induced sputum, also well validated, less invasive but rather unpleasant for the patient, is frequently used in research but difficult to use in clinical practice[8]. Several non-invasive methods for investigating inflammatory markers in exhaled air have been

described during the last twenty years and in the early 1990s it was discovered that NO was increased in exhaled air in asthmatics[9,10].

Table 1
Methods to measure airway inflammation

Method	***Advantages***	***Disadvantages***
Bronchial (transbronchial, endobronchial) biopsies[7]	Gold standard	Invasive Requires specialist care Results delayed Specimens from large airways
Bronchoalveolar lavage[7]	Validated	Requires bronchoscopy Requires anaesthesia Requires specialist care Results delayed Uncertain location
Induced sputum[8]	Possible to study several different markers of inflammation	Unpleasant for the patient Results delayed Samples requires expert handling
Exhaled NO[11]	Non-invasive Patient friendly Equipment for out patient clinic is available Immediate results	Uncertain value in non eosinophilic inflammation Influence of atopy Influence of smoking
Breath condensate[12]	Non-invasive	Insufficiently validated
Electronic nose[13]	Non-invasive	Insufficiently validated Not widely available
Exhaled particles[14]	Non-invasive	Not validated
Blood, urine[15]	Non-invasive	Indirect

Exhaled NO is validated and easy to use in children, but is it a useful marker of airway inflammation? There are conflicting data, but many studies have shown a correlation between exhaled NO and the eosinophil count in induced sputum and bronchoalveolar lavage and the number of eosinophils in bronchial biopsies in asthmatics[16-20]. Several authors are in favour of using exhaled NO as a marker of inflammation and steroid responsiveness in eosinophilic asthma [21-24], but this has been challenged by others, mainly due to the strong influence of

atopy and the conflicting data regarding the association between tissue airway inflammation and exhaled NO[25]. A recent Cochrane review concluded that exhaled NO can not routinely be recommended for tailoring the dose of inhaled corticosteroids in asthma[26].

In subjects with CF, exhaled NO is low in spite of often severe airway inflammation[27], and it has been suggested that *reduced* levels of exhaled NO could be associated with disease severity[28].

NO is produced all along the airway tree. More knowledge about the association between exhaled NO from different airway compartments and airway function could increase our understanding of the usefulness of exhaled NO for monitoring airway disease.

Exhaled nitric oxide

Background

Historically NO was regarded as a noxious environmental pollutant destroying the ozone layer but in 1992 NO was proclaimed the molecule of the year by the journal "Science". Why?

In 1980 R Furchgott and J V Zawadski showed that when strips of blood vessels, nurtured in an organ bath, were chemically stimulated, the muscles relaxed, a property that was lost if the inner layer of cells of an artery or vein, the endothelium, was absent[29]. This showed that a previously unrecognised substance must exist that regulated the tone of the smooth muscles of blood vessels. The mystery agent was referred to as endothelium dependent relaxing factor, EDRF.

Curiosity provoked several laboratories to start searching among the body's complex bio molecules to find a candidate for EDRF. Ferrige and Moncada devised experiments to test whether NO could account for the actions of EDRF. Equipment developed for their studies included a highly sensitive, miniaturised version of an instrument used in the car industry to measure NO in the exhaust gas of petrol engines. When linked to endothelial cells, repeated measurements demonstrated that NO was indeed the relaxing factor released by these cells[30,31]. Moncada, Ignarro and Ferrige were awarded the Nobel Prize for this discovery in 1998.

Nitric oxide is a small molecule of only 30 Daltons, involved in many different biological functions in humans. Compared with the complexity of the hundreds

of other molecules that keep us ticking; the free radical form of nitric oxide is simplicity itself: just two atoms, one atom of oxygen and one of nitrogen.

Nitric oxide is synthesized from L-arginine by NO synthase (NOS). Three forms of the NOS enzyme have been described: endothelial NOS (eNOS), neuronal NOS (nNOS), and the inducible form (iNOS)[32] . These NOS have been differentiated based on their constitutive (eNOS and nNOS) vs. inducible (iNOS) expression[33,34] . Lately it has become clear that this is too simplistic and all three NOS can be induced, but by different stimuli[35]. All three NOS are expressed in the airways[36] (Table 2).

Table 2
NOS located in the airways[36]

NOS	***Where NOS is expressed in the airways***
eNOS	Endothelial cells in the pulmonary and bronchial vessels Bronchial epithelial cells Type II alveolar epithelial cells
iNOS	Respiratory epithelial cells Type II alveolar epithelial cells Endothelial cells Macrophages, neutrophils, mast cells chondrocytes lung fibroblasts Vascular smooth muscle cells
nNOS	Airway nerve fibres, innervating smooth muscle and submucosal glands

In addition to the enzymatic production of NO, a non-enzymatic production occurs consisting of the reduction of nitrite to NO in the urine, oropharyngeal and gastrointestinal tracts, and on the surface of the skin[37]. The importance of the NOS independent pathway for exhaled NO has been revealed by the observations that exhaled NO levels can be reduced either by administration of chlorhexidine mouthwash or by buffering the salivary pH[38,39].

Nitric oxide is an important signalling messenger in the cardiovascular system[30,40]. In inflammation, NO has multiple actions, both beneficial and harmful[41,42]. Constitutive low NO exerts protective effects against microcirculatory damage and oedema formation. NO has many documented anti

microbial properties[43] and the high NO levels prevailing in inflammation might exert cytotoxic effects not only against invading microorganisms but also against host cells[44].

Constitutive low levels of NO have important regulatory functions in the airways. NO is a potent dilator of blood vessels in the bronchial circulation and a bronchodilator [45]. NO also has a stimulatory effect on airway submucosal gland secretion and ciliary beat frequency, hereby helping clearing the airways of inhaled particles, including bacteria[45,46]. High levels of NO (and reactive nitrogen species), following upon increased iNOS expression may be associated with cytotoxicity and potentiating of many detrimental events including pro-inflammatory activities, such as vasodilatation and plasma extravasation of the bronchial circulation; increased airway secretions; impaired ciliary motility; promoting Th2 cell-mediated, eosinophilic inflammation; and necrosis and apoptosis (which may also be protective!)[45]. There is data supporting that the biological effects of NO in the airways could be mediated through the formation of S-nitrosothiols, which have a significant bronchodilating effect[47]. S-nitrosoglutathione is an endogenous bronchodilator regulated by S-nitrosoglutathione reductase and it has been suggested that S-nitro glutathione is of great importance to the NO signalling in the lungs[48]. In summary, there is a complex balance between the beneficial and harmful effects of NO in the airways.

Exhaled NO - how to measure?

Nitric oxide was first found in exhaled air by Gustafsson and co workers in 1991[49] and two years later Alving *et al.* reported that exhaled NO was increased in asthmatics[9]. The reported levels of NO in exhaled air varied considerably between different studies and it was later found that exhaled NO was very dependent on what technique and which exhalation flow rate that was used[50,51]. Joint guidelines on how to measure exhaled and nasal NO were therefore presented by the European Respiratory Society (ERS) and the American Thoracic Society (ATS) in 2005[52]. The recommendation is real-time measurement of NO during a single slow exhalation. An inspiration of NO-free air via a mouthpiece to total lung capacity should immediately be followed by a full exhalation at an even rate (recommended exhalation flow rate 50 mL/s) through the mouthpiece into the apparatus.

It was initially thought that exhaled NO derived mostly from the sinuses, which contain high levels of NO[53]. It has subsequently been shown that when exhaling against a positive pressure in order to close the velum the lower airways

contribute to most of the exhaled NO (Fig 1)[54]. This is the recommended method today.

The gold standard for detecting NO in exhaled air is the chemiluminescence method[55]. It is based on a reaction between NO and ozone (O_3), which is generated from the ozone generator in the analyzer. NO and ozone form nitrogen dioxide (NO_2), part of which is the excited form, NO_2*. When the excited form of NO_2 resumes its stable form, light is emitted and can be quantified by a photomultiplier. The amount of light emitted is proportional to the amount of NO in gas collected from the samples.

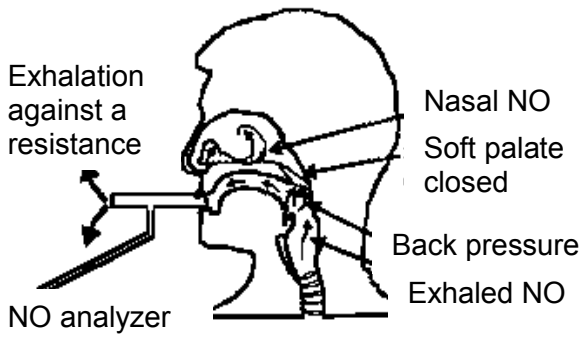


Figure 1
NO in exhaled air.
Figure adopted by Barnes.

Small handheld devices are widely used for measuring exhaled NO in the clinic, e.g. Niox Mino™. Niox Mino™ is using an electrochemical technique for measuring exhaled NO and has shown good repeatability and agreement with devices using the chemiluminescence method[56]. These small handheld devices are used for the recommended exhalation flow of 50 mL/s only, while the large, more expensive equipment using the chemiluminescence method can measure exhaled NO at different exhalation flow rates.

Fraction of exhaled NO (FENO) is the term used for exhaled NO and in this thesis $FENO_{50}$ is the term used for exhaled NO at the recommended flow rate of 50 mL/s.

Exhaled NO is easy to measure and reproducible and well accepted by both healthy and asthmatic subjects of most ages[51,57]. In young children below the age of 4-5 years, who can't perform a slow exhalation, NO measurements can be performed during tidal breathing[58], but this method is outside the scope of this thesis.

Data on reference values for $FENO_{50}$ are rapidly increasing (Table 3). When interpreting reference data it is important to take into consideration the method used and whether the population is relevant to the study in question.

Table 3
$FENO_{50}$ in healthy subjects

Author	*Subjects (n)*	*$FENO_{50}$ (ppb)*
Children		
Franklin et al.[59]	157	10.3*
Kharitonov et el.[57]	20	15.6±9.2*
Buchvald et al.[60]	405	9.7*
Malmberg et al.[61]	114	10.3*
Adults		
Olivieri et al.[62]	204	10.8±4.7*
Olin et al.[63]	1131	16.6**
Travers et al.[64]	193	17.9**
Dressel et al.[65]	514 (♀)	17.5**

* Data presented as mean (± SD)
** Data presented geometric mean

Several factors, other than the investigation procedures, influence the FENO levels and this could have implications on the interpretation of the results (Table 4). Atopy and smoking status are two main confounding factors when evaluating FENO in the clinic. Atopy is sometimes defined as the genetic predisposition to become IgE-sensitized to allergens commonly occurring in the environment[66]. However, in this thesis the word atopy is used synonymously with allergic sensitization, as this definition is commonly used in the literature.

Exhaled NO and allergic sensitization (atopy)

Children with allergic (atopic) asthma have higher levels of FENO than children with non-allergic asthma[67-69]. There are studies showing no difference in FENO levels between subjects with non-allergic asthma and healthy controls[61,70]. Moreover, there is evidence that some atopic individuals even without asthma have abnormally high NO levels[59,71-74] and that subjects with persistent rhinitis sensitized to pollen can have a seasonal variation in FENO[75,76].

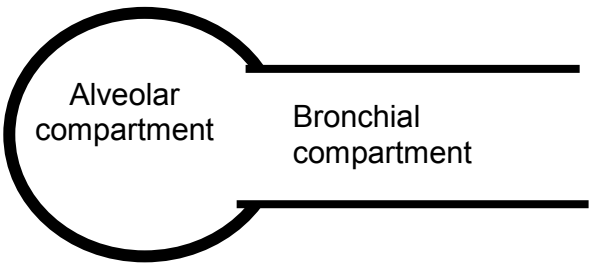
Table 4
Factors affecting the FENO levels

	Effect on FENO	*Clinical consequence*
Age[59-61,72]	↑	Different cut offs for normal levels in children and adults
Height[61,65,72]	↑	Se above. In adults no clinical consequence
Gender[57,62,64,65,71]	Males ↑	None. Many studies show no difference
Atopy[59-61,64,65,72,77]	↑	Risk of high FENO in non asthmatic atopic individuals
Current smoking[64,65,72,78,79]	↓	Uncertain value in smokers
Increased BMI[80-82]	↑↓	?
Oral pH[38,39]	↑↓	Refrain from eating one hour prior to the measurements
Nitrate rich meal[83]	↑	Refrain from nitrate rich meal several hours prior to measurements
Exercise[84,85]	↓	Refrain from exercise one hour prior to the measurements
Spirometry[84-86]	↓	Perform spirometry after the NO measurements
Respiratory tract infection[65,87]	↑	Uncertain value of measurement during infection.

Flow independent NO variables

The $FENO_{50}$ levels do not provide any information about the localisation of the NO production (or inflammation) in the airways. Mathematical models of NO dynamics suggest that the peripheral (alveolar) and the central (bronchial) airway contribution to the FENO value can be calculated on the basis of NO measurements at multiple exhalation flow rates[88-91] . There is a strong inverse relationship between the concentration of exhaled NO and the exhalation flow[92,93] . There is also a positive relationship between the elimination rate of NO (product of concentration and flow) and exhalation flow[93]. To explain these observations Tsoukias and George presented the two-compartment model, taking both peripheral and central airways into consideration[90] (Fig 2).

In the two-compartment model a flexible expansible alveolar compartment represents respiratory bronchiole and alveolar regions (generation 18 and beyond according to Weibel[94], see fig 10). A single rigid cylindrical tube bronchial compartment represents the conducting airways down to the respiratory bronchioles (from trachea to generation 17 according to Weibel). Exhaled NO originates from both these compartments.



Flowindependant NO variables

$\mathbf{C_{NO}}$= bronchial wall NO concentration
$\mathbf{C_{alv}}$= avleolar NO concentration
v= flow rate
$\mathbf{Daw_{NO}}$= bronchial wall NO diffusion capacity
$\mathbf{Jaw_{NO}}$= bronchial NO flux

Figure 2
The two-compartment model of the airways.

Nitric oxide is mainly produced in the airway wall and NOS is found in the airway wall along the entire airway tree, including the bronchi, bronchioles and alveoli[95,96].

In the two-compartment model, the final concentration of NO in exhaled air depends on two mechanisms:
1) NO concentration in alveolar air and
2) conditioning of alveolar air while it travels through bronchial compartments.

The accumulation of bronchial NO from the bronchial wall to exhaled air while it travels through the bronchial tree can be further modelled by dividing the bronchial compartment into infinitely short units. When entering the bronchial compartment the luminal air NO concentration equals the alveolar NO concentration. In the *first* unit NO diffuses from the bronchial wall to the luminal air and at the entry of the *second* unit the luminal NO concentration equals the alveolar NO concentration + NO diffused in the first unit and so forth. In *every unit,* the diffusion of NO from the bronchial wall to luminal air is driven by the NO concentration gradient between these two. The diffusion rate is determined by the bronchial diffusing capacity of NO, Daw_{NO}.
Conditioning of alveolar NO in the bronchial compartment thus depends on transit time of the air through the conducting airways, the airway wall concentration, C_{NO}, and Daw_{NO}. By forming a differential equation based on this model the final NO concentration in exhaled air (FENO) can be expressed as an exponential function of exhalation flow rate[88-90,97] (Fig 3).

$$FENO = C_{NO}(1-e^{-DawNO/v}) + C_{alv} \times e^{-DawNO/v}$$

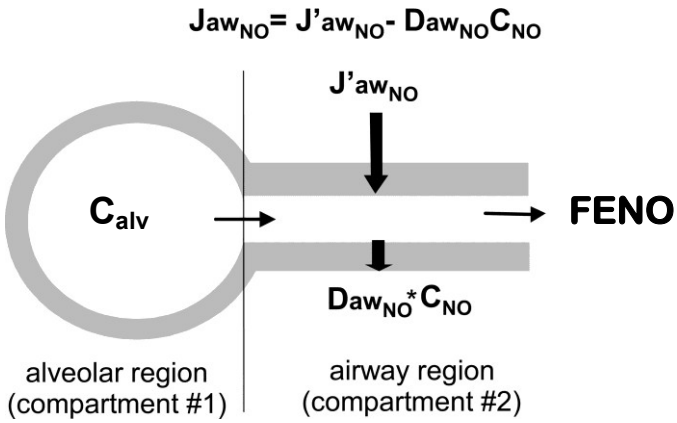


Figure 3
Schematic of 2-compartment model used to describe nitric oxide (NO) exchange dynamics. Adapted after George, S. C. et al. J Appl Physiol 2004; 96: 831-839

NO diffuses rapidly over the thin alveoli membranes and reacts much faster with the red blood cells than carbon monoxide, a property used in lung function testing. Diffusing capacity for nitric oxide can be used to directly describe pulmonary membrane diffusing capacity[98]. NO produced in the alveoli would therefore never reach the more proximal airways and the term alveolar NO is then misleading but it is used in this thesis to represent the exhaled NO coming from the bronchiole and alveolar region since it is the established term.

Different mathematical approaches are used to calculate the flow independent NO variables[88-90]. In this thesis two methods are used, the so called non-linear method presented by Högman *et al.*[89] and the linear method described by Tsoukias and George[90]. For the non-linear method, at least three exhalation flows are required, low, medium and high, initially 10, 100 and 300 mL/s. The low flow rate, 10 mL/s, is difficult to achieve for children and therefore others and we have used a somewhat higher flow rate as the low flow rate. Results from the measurements are plotted in the exponential equation below (Fig 4) and the flowindependant NO variables are calculated from the equation above.

When plotting the values into the curve one also gets the calculated $FENO_{50}$, which is used as an affirmation of quality if the calculated value is consistent with the measured value.

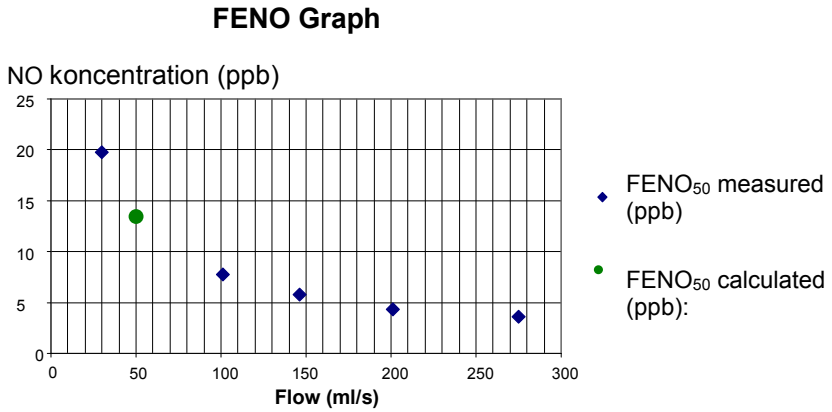


Figure 4
FENO values and exhalation flows plotted in the non-linear method according to Högman *et al.*[89]

At higher flow rates (>50ml/s) the exponential equation can be substituted with a linear approximation where NO output is plotted against flow rate, the slope, intercept model according to Tsoukias, or the linear method. The slope of the regression line is an approximate for alveolar NO concentration and the intercept is an approximate for bronchial NO flux, Jaw_{NO}[90] (Fig 5).

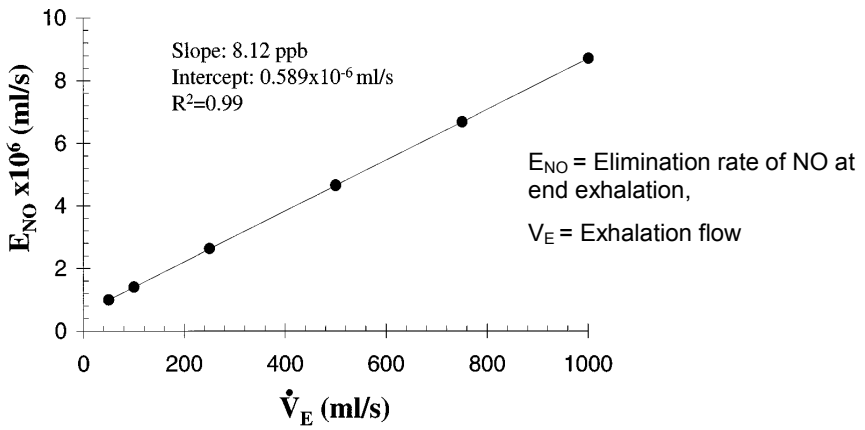


Figure 5
Example of the slope-intercept model (the linear method). Tsoukias et al. J Appl Physiol 85 (2): 653. (1998)

The two-compartment model is reproducible in healthy children[99] as well as in children with asthma[100] and CF[101]. The linear and the non-linear methods have

been compared in healthy children by Sepponen *et al*. and they found significant differences in the calculated values for alveolar NO and bronchial NO flux but the values were highly correlated[99]. Data presented below (Table 5).

Table 5
NO variables in healthy children presented as median (mean ± SD)[99]

NO variables	***Healthy children*** *(n=66)*
$FENO_{50}$ mL/s (ppb)	10.3 (11.7±5.4)
Alveolar NO (ppb)[1]	1.9 (2.0±0.8)
Bronchial NO flux (pL/s)[1]	400 (500±300)
Alveolar NO (ppb)[2]	1.4 (1.5±0.7)
Bronchial NO flux (pL/s)[2]	500 (600±300)
Bronchial wall NO conc. (ppb)[2]	49.6 (68±53.3)
Bronchial NO diffusion capacity (pL/s/ppb)[2]	10.1.(8±7.5)

[1]= Calculated according to (the linear method) Tsoukias [90]
[2]= Calculated according to (the non-linear method) Högman [89]

Several authors have suggested additional improvements of the two-compartment model. The airway tree has more of a trumpet shape (increasing surface area per unit volume) and Condorelli *et al.* suggested a trumpet shaped model. The importance of axial diffusion has been discussed by a few authors and different adjustments have been proposed[102,103]. The latest published improvement is correction for ventilation inhomogeneity in a multicompartment model[104]. So far, these different models are used in the research setting and there is no joint recommendation on which model to use.

Nasal NO

Nasal NO (nNO) is measured in a similar way to FENO and the ATS/ ERS guidelines provide recommendations also for nNO[52]. Nasal NO is measured by sampling nasal air from one nostril through at catheter with a constant sample rate of 50 ml/s, leaving the other nostril open. The measured NO concentration varies with the flow rate through the nose and there is still need for a more standardised method to measure nNO. Just as for FENO, a simultaneous exhalation is recommended with a positive pressure >5 cm H_2O in the mouth to ensure closure of the velum to prevent pollution by NO from the lower airways.

Exhaled NO and asthma

There is a large number of studies showing increased levels of FENO in children and adults with asthma, using first tidal breathing and later the recommended single breath technique[9,10,52,57,58,105-107]. High levels of FENO is highly suggestive of asthma[108] and a good predictor of response to treatment with corticosteroids[109] but maybe more so in adults than in children[11]. Increased FENO has been linked to enhanced expression of iNOS in the airway epithelium in asthmatic subjects [110,111]. iNOS expression can be down regulated by corticosteroids and there is substantial data showing that FENO decreases after treatment with inhaled corticosteroids (ICS)[112]. The reduction is rapid and dose-dependent[113-118] but so far there is not enough evidence of benefits using FENO compared to clinical symptoms in tailoring the dose of ICS to support the regular use of FENO for this purpose[26]. Treatment with leuokotriene receptor antagonists resulted in reduced levels of FENO in some studies[119-121], but in other studies no change in FENO was seen after treatment with leuokotriene receptor antagonists[122,123].

Asthma is a variable disease and FENO is a highly dynamic measurement in asthma with a great intra individual variation over time[124]. A single measurement is therefore of little value in asthma but examples of FENO results in asthmatic children are presented in Table 6.

Table 6
$FENO_{50}$ in asthmatic children and healthy controls

	Subjects	***$FENO_{50}$ (ppb)***	
Authors	*(n)*	*Asthmatic*	*Healthy controls*
Kharitonov et el.[57]	40	24.9±22.3	15.6±9.2
Malmberg et al.[106]	143	22.1±3.4	5.3±0.4
Silvestri et al.[107]	112	15.9± 4.3	7.6±1.6

Data is expressed as mean± SD

Many authors have shown that there is no correlation between FENO and different spirometry values but regarding airway hyper-responsiveness, there are contradictory results. Some studies have shown a correlation between FENO and bronchial provocation test[16,125-127], while other studies have shown no correlation between FENO and airway hyper-responsiveness after provocation test[107,128]. This suggests that airway hyper-responsiveness and FENO are only partly correlated and therefore could reflect different aspects of the asthma disease.

Studies regarding the association between FENO and asthma control also show contradictory results. Increased levels of FENO have been associated with a deterioration in asthma control in some studies[125,129] , while others found no correlation between asthma control and FENO[130,131].

The discrepancy in the correlation between FENO and other markers of airway disease could be due to different methodology used, or to the multifaceted nature of the asthma disease with many different phenotypes within the asthma population[132,133].

Flow independent NO variables in asthma

Many studies have shown that bronchial NO flux, (NO coming from the large airways) is increased in asthma (Table 7) and that treatment with ICS can reduce bronchial NO flux[100,134-136]. For alveolar NO the results are more contradictory, but there is data indicating that alveolar NO is increased in severe asthma but no different compared to healthy controls in mild to moderate asthma[100,134,135].

Table 7
Flow independent NO variables in asthma.

		Alveolar NO (ppb)		***Bronchial NO flux (pL/s)***	
Authors	***age***	*Asthma*	*Controls*	*Asthma*	*Controls*
Robroeks et al. [137]	6–13	4.1±0.5		1093±251	
*Paraskakis et al.[100]	4–18	2.2 (0.4–6.6)	1.63 (0.44–3.0)	1230 (8204–9236)	480 (196–1913)
Kerckx et al. [102]	38±14	4.8±2.1	3.1±1.5	2254±1150	745±311
Lehtimäki et al. [138]	32±2	1.1±0.2	1.1±0.2	2500±300	700±100

values presented as mean ±SD
*values presented as median (range)

There is an ongoing discussion that inhaled corticosteroids (ICS) cannot reach the most peripheral airways and subsequently there would be no change in alveolar NO after treatment with ICS. Systemic corticosteroids could better reach the peripheral airways and thereby reduce alveolar NO. This is supported by studies showing a reduction of alveolar NO after oral corticosteroids, but not by ICS[135,138,139]. There are new small-particle formulations of ICS that target inflammation in the small airways, and there is data suggesting that treatment with one of these ICS could result in decreased levels of alveolar NO[140].

Exhaled NO and CF

Airway inflammation starts early in life in subjects with CF, but in spite of often marked airway inflammation, levels of FENO have either been reported not different[101,141,142] or decreased[28,143-146] compared to control subjects (Table 8).

Table 8
$FENO_{50}$ in children and young adults with CF.

	CF	***$FENO_{50}$ (ppb)***	
Authors	*subjects (n)*	*CF*	*Controls*
Suri et al.[146]	22	9.4 (2.7–26.9)*	10.4 (4.5–29.6)*
Robroeks et al.[28]	48	10±1.2**	15.4±1.4 **
Hubert et al.[147]	30	8.4 (6.2–16.2)***	

* values presented as median (range)
** values presented as mean± s.e.m
*** values presented as median (25^{th}–75^{th} percentile

Nasal NO has consistently been reported to be low in CF subjects[28,142,144,148]

Several possible explanations have been presented for the low levels of FENO and nNO seen in CF patients[27,149].

One explanation could be that the airway inflammation in CF is dominated by neutrophils as opposed to the most common eosinophilic inflammation in asthma which is associated increased FENO levels[16,22,150].

Second, there are studies showing polymorphisms in the genes coding for constitutive NOS[151,152] and decreased activity or expression of iNOS[153,154]. Inflammatory changes in the epithelial cells result in loss of epithelial cell integrity and the respiratory epithelium is an important site for iNOS[155]. Consequently NO production could be lower in CF due to less or inactive NOS. This is supported by a bronchoscopy study showing an inverse relationship between neutrophilic airway inflammation and iNOS expression in the airway epithelial cells and airway macrophages in children with CF, not seen in the healthy controls[156].

Arginine is the substrate for NO production, and low bioavailability of arginine has been shown in CF patients suggesting a third explanation for the low levels of FENO[157,158]. Grasemann and coworkers have shown an increase in FENO in

CF patients after giving arginine intravenously or orally[159,160] and an increase in both FENO and FEV_1 shortly after an inhalation with arginine[161] .

Mucus plugging and an asymmetric obstruction of the airways characterize CF airway disease. This could result in low levels of NO in exhaled air due to poor diffusion of NO into the gaseous phase[148,162] and/or degradation of the produced NO by bacteria found in the mucus, for example *Ps. aeruginosa*[163].

Low levels of nNO in CF subjects could be explained by blocked sinuses (where the highest concentration of NO is found) which has been a big problem in the CF population, but today blocked sinuses are much less prevalent due to better treatment.

Up till today, these explanations are just speculations and there is still a big uncertainty about the true cause and the clinical importance of the low levels of NO found in the respiratory tract in patients with CF.

Flow independent NO variables in CF

There are only a few studies with extended NO measurements in CF, most of them including a very small number of patients.

Table 9
Alveolar NO and bronchial NO flux in children and young adults with CF (linear model[90])

	Alveolar NO (ppb)		***Bronchial NO flux (pL/s)***	
Authors	*CF*	*Controls*	*CF*	*Controls*
Shin et al.[101]	1.96±1.18*	4.63±3.59*	607±648*	784±465*
Suri et al.[146]	2.2 (0.6–5.6)**	1.5(0.4–2.6)**	445 (64–1256)**	509 (197–1913)**
Hubert et al.[147]	3.3 (2.4–6.4)***		283 (150–500)***	

* values presented as mean ±SD
** values presented as median (range)
*** values presented as median (25^{th}-75^{th} percentile)

Shin *et al.* reported flow independent NO variables in 9 children with CF. Bronchial NO flux was reduced and alveolar NO was no different from that in healthy children[101]. The same results were reported from a study in adults (n=12)[164]. In contrast to these findings *Suri et al.* found increased levels of alveolar NO but no difference in bronchial NO levels in 22 children with CF compared to healthy controls[146] (Table 9).

Asthma

Asthma is a polygenetic disease where environmental factors are very important for the clinical picture. It is still incompletely understood and continues to be a significant management problem for clinicians, particularly as childhood disease develops into chronic airflow obstruction in adults irrespective of treatment[165]. Asthma is the most common chronic disease of childhood and accounts for a large proportion of paediatric hospitalisations, health care visits and absenteeism from day care and school[166]. There are many different asthma phenotypes where the characteristic features (Fig 6) (Table 10), airway obstruction, airway inflammation and airway hyper-responsiveness (AHR) are more or less evident[132,133,167].

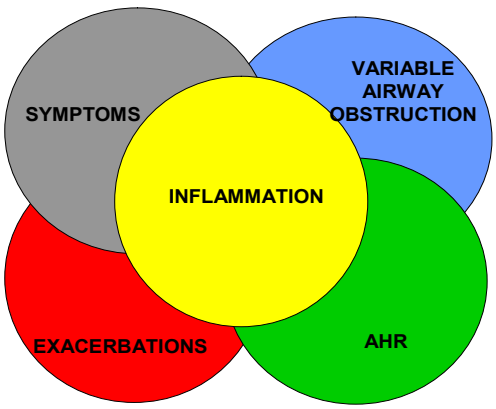


Figure 6
The different features in asthma: symptoms, airway obstruction in the small and large airways, airway inflammation, airway hyper-responsiveness (AHR) and exacerbations are only partly associated.

The treatment of choice today is ICS, used to treat the underlying airway inflammation and the advantageous effects of ICS on symptoms, airway function and inflammatory markers have been shown in a large number of studies in children[168-170]. However, epidemiological and clinical studies suggest that many asthmatic children do not achieve sufficient asthma control in spite of the availability of efficient drugs[171,172]. Lack of compliance is one important factor for not achieving asthma control. Another reason could be that the inhaled corticosteroids do not reach the small airways or that the airway inflammation is not steroid sensitive. Individuals with different asthma phenotypes might benefit from different types of treatment, emphasizing the need for a better understanding of the asthma disease and its underlying mechanisms[168].

How to monitor asthma?

Current guidelines recommend diagnosis and treatment based on symptoms and spirometry (FEV_1)[168]. FEV_1 is not sensitive to small airway dysfunction[173,174] and small airways could be a missing link in the monitoring of asthma today. There are several other possible methods to evaluate the different facets of the asthma disease (Fig 7).

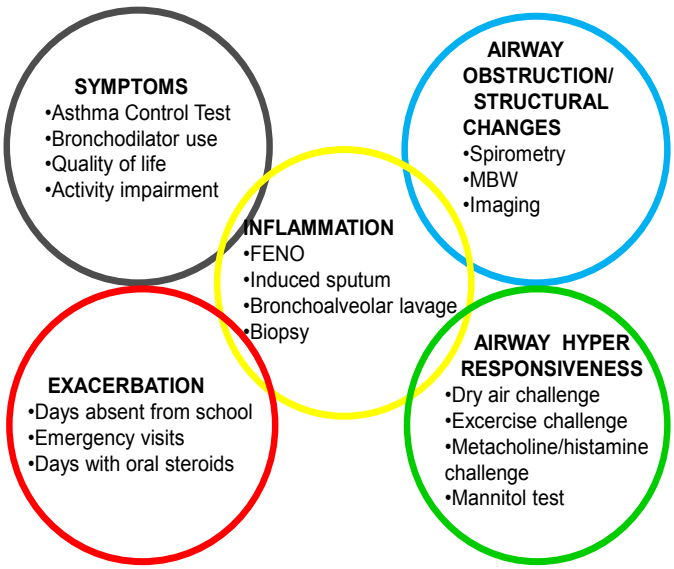


Figure 7
Possible asthma outcomes

Symptoms in children are often reported by the parents. Parents might over or under report symptoms or signs of airway disease. The typical reversible large airway obstruction may be absent during long periods and diagnosis can therefore be difficult, especially in children. There is often little or no correlation between symptoms and large airway obstruction (FEV_1) or between FEV_1 and airway inflammation[11,127,128,175]. Children with little or no symptoms and normal lung function can suddenly deteriorate[176]. To find these children we need to have a better understanding of the underlying mechanisms and pathophysiology.

Many new anti inflammatory drugs with more specific targets of inflammatory mechanisms than the inhaled corticosteroids have are under development, and also drugs targeting the small airways, emphasising the need to assess the response to anti-inflammatory pharmacological treatment also in the small airways.

Cystic fibrosis

CF is a rare, progressive disease, often leading to premature death. Airway inflammation and infection are known to start early in life, invoking a progressive decline in lung function, starting in the peripheral airways[6,177-181]. CF is one of the most common genetic diseases in the western world and in Sweden; the incidence is around 1/5000-6000 newborn. CF is an important differential diagnosis to asthma in young children with obstructive airway disease. Airway inflammation plays a central role in both asthma and CF and there are many similarities but also important differences (Table 10).

Thirty years ago, patients with CF were not expected to live into adulthood. Due to remarkable improvements in the care of CF patients, more than 60% of the Swedish CF population is today above 18 years of age[182]. The mean age for survival in Sweden is around 40-50 years. Nevertheless, CF is still a progressive disease that often leads to chronic respiratory failure.

CF is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene leading to dysfunction of the CFTR protein[183]. More than 1500 mutations have been identified (http//www.genet.sickkids.on.ca/cftr/) and they are referred to six classes based on the function of the defective protein[184,185]. dF508 is the most common CFTR mutation in Sweden and elsewhere[184,186].

The CF diagnosis is based on the following criteria[187].
1.
Sweat chloride concentration of > 60 mmol/L on two occasions.
Two genetic mutations causing CF
Disturbed chloride transport measured as an epithelial potential difference
2.
Sibling with CF
Positive result at newborn screening (not done in Sweden)

CF is characterized by a wide variability of clinical expression and CFTR is expressed in the epithelial cells in many different organ systems; the lungs and pancreas but also in the salivary glands, sweat glands, kidneys, intestines, gallbladder and uterus. Approximately 85 % of the CF subjects have pancreatic insufficiency at diagnosis and it is associated with increased resting energy expenditure and enzyme deficiency leading to fat malabsorption. Supplementation with enzymes, especially lipase, is routinely prescribed and a high energy intake is often recommended to children and adults with CF[188].

Table 10
Characteristics of the typical airway inflammation in asthma and cystic fibrosis

	Asthma[189-191]	***Cystic fibrosis***[177-179]
Genetics	Multiple genes	Single gene
Clinical presentation	Non productive cough Reversible obstruction Airway hyper-responsiveness Chest tightness Wheeze	Productive cough Irreversible obstruction Respiratory failure
Variability	Variable	Chronic
Progressive	Non progressive	Progressive
Triggers	Allergens Infections, viral Irritants	Infections, viral and bacterial
Signalling substances and mediators	IL4 IL5 IL13 IgE Leukotriens Prostaglandins	IL-8 Proteases Oxygen radicals Leukotriene B_4
Cells	Eosinophils Mast cells CD4+ lymphocytes	Neutrophils Macrophages
Typical characteristics	Variable inflammation triggered by typical triggers	Bacterial infection with intense inflammation Oxidative stress
Small airways involved	Conducting airways	Intra acinar airways
Consequences of airway inflammation	Remodelling and fixed airway obstruction	Respiratory failure
Exhaled NO	Increased	Decreased

Liver disease is present in approximately 5-10 % of the CF population[192] and there is an increased risk of osteoporosis[193,194] and diabetes with increasing age[195].

It is the pulmonary abnormalities causing the greatest morbidity and mortality in CF[177]. The degree of pulmonary involvement is not related to the genotype and therefore other modifying genes have been discussed, i.e. different genes encoding for the inflammatory response in the airways. The NOS genes have been suggested as possible disease modifying genes[196].

Persistent infection, often caused by *Pseudomonas aeruginosa,* and inflammation, involving the peripheral (small) airways, begin at a very early stage in patients with CF[197,198] and continues throughout life[199,200]. Chronic inflammation directly damages the airway wall, ultimately leading to bronchiectasis and progressive decline in pulmonary function, which often accelerates during adolescence. Monitoring and controlling the infection and inflammatory process early in the course of disease may limit the damaging effects of excessive inflammation, thus delaying progression of pulmonary deterioration and potentially decreasing morbidity and mortality[201].

Fatty acids in CF and fatty acids and inflammation

Fatty acid deficiency is an important feature in individuals with CF, who have an impaired fatty acid metabolism with increased release and high turnover of arachidonic acid (AA), and decreased levels of docosahexaenoic acid (DHA), in plasma, erythrocyte membranes, platelets and tissue biopsies[202-204]. The ratio of AA (20:4n-6) to DHA (22:6n-3) is therefore often elevated. The high turnover of AA results in low concentration of the essential fatty acid linoic acid (LA). Eicosanoids, which are important inflammatory mediators, are synthesized from AA and the high AA turnover results in a high eicosanoid production[205] . The fatty acid abnormality in CF is associated with the CFTR mutation[206] but the connection between the abnormalities in the fatty acids has not been satisfactorily explained and the reason for the high turnover of AA in CF is not known[203,207].

The fatty acid deficiency seen in CF could have implications on the airway inflammation because AA is mainly associated with an increase of pro-inflammatory eicosanoids, while DHA provide anti-inflammatory factors[208].

The fatty acids in our diet can be separated into three types; saturated, monounsaturated and polyunsaturated fatty acids (PUFA)[209]. Saturated fatty

acids do not contain any carbon double bonds, as the fatty acid is fully saturated with hydrogen. Monounsaturated fatty acids, as the name suggests contain fatty acids with one carbon double bond and likewise PUFA contain two or more carbon double bonds. PUFA are further classified as n-9 (omega 9), n-6 (omega 6) or n-3 (omega 3) PUFA.

The difference between the PUFA is the location of the first double bond; on carbon number 3 (n=omega-3), 6 (n=omega 6) or carbon number 9 (n=9) from the methyl end of the molecule (Fig 8).

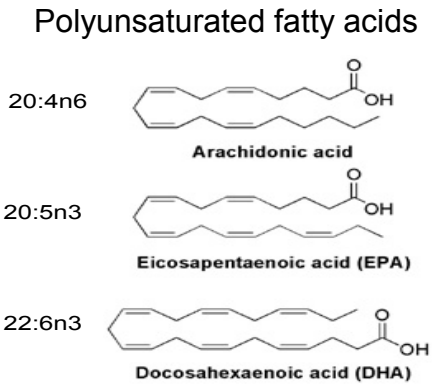


Figure 8
The carbon skeleton of the three main long chain polyunsaturated fatty acids (PUFA).

The chemical names and frequently used abbreviated names of the most important PUFA are shown in Table 11. The so-called essential fatty acids, LA and α-linolenic acids (ALA) have to come through our diet since humans cannot synthesize them. The other fatty acids are either synthesized in the human body from the essential fatty acids or ingested as part of our diet (Fig 9).

Table 11
N-3 and n-6 polyunsaturated fatty acids and their main food sources.

Name	***Chemical name***	***Abbreviation***	***Main food source***
α-linolenic	18:3 n-3	ALA	Flaxseeds, canola, walnuts
Eicosapentaenoic acid	20:5n-3	EPA	Fish and seafood, meat, eggs
Docosahexaenoic acid	22:6n-3	DHA	Fish and seafood, eggs
Linoleic acid	18:2 n-6	LA	Sunflower oil, corn, poppy seeds
Arachidonic acid	20:4n-6	AA	Meat, egg, dairy products

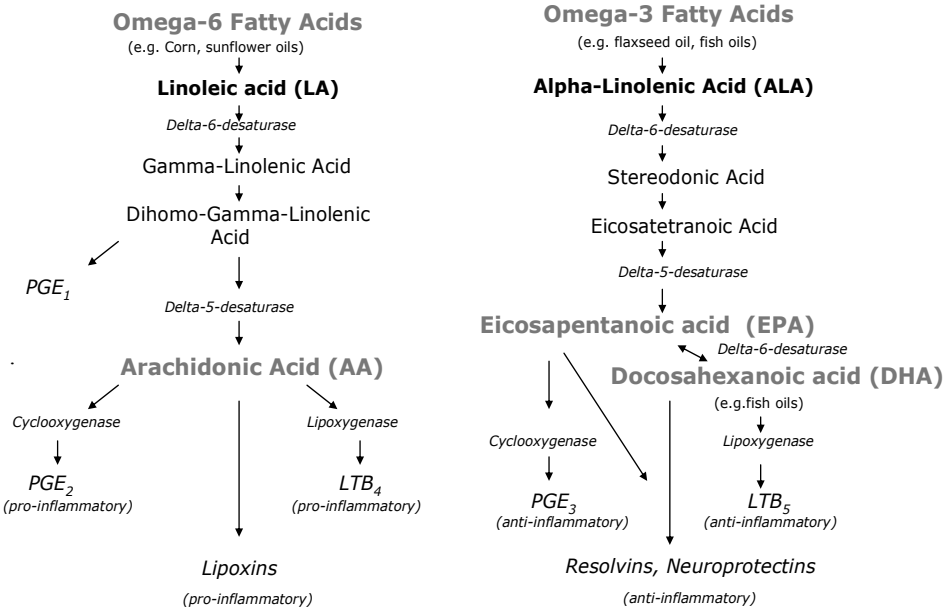


Figure 9

The long chain n-3 and n-6 polyunsaturated fatty acids

There is evidence suggesting that n-3 PUFA are associated with more health benefits, including anti-inflammatory properties than n-6 PUFA, especially in the western world where there is an unfavourably high n-6 to n-3 ratio in the diet[208]. An increased intake of n-3 PUFA, especially the long chain DHA and EPA, may therefore have a beneficial role in the prevention and treatment of inflammatory disorders[208,210,211].

There is a complicated cross talk between PUFA and NO production, where PUFA have been shown to both increase and decrease NO production, partly through influencing the different NOS[212,213]. The changed fatty acid metbolism in CF subjects could therefore influence the NO production in the airways.

There are several studies with n-3 PUFA substitution in different diseases, but according to two recent reviews, so far not enough evidence for a beneficial role to support broad clinical use of PUFA substitution in all CF patients[214,215].

Small airways

In the 1960s Weibel proposed a structural model of the airways[94].The airways are designed to optimize the gas exchange which is done over an inner surface area the size of a tennis court. This is achieved by attaching the gas exchange units at the end of a branched tree of conducting airways. A new airway "generation" starts at each branching and there are around 23-26 generations from the trachea down to the alveoli, which in adults corresponds to approximately 25-26 cm[216]. The first 16 generations are conducting airways where the air is transported downwards. The acinus (generation 17 onwards) contains airways designed for both air transport and gas exchange, and alveoli primarily designed for gas exchange (Fig 10).

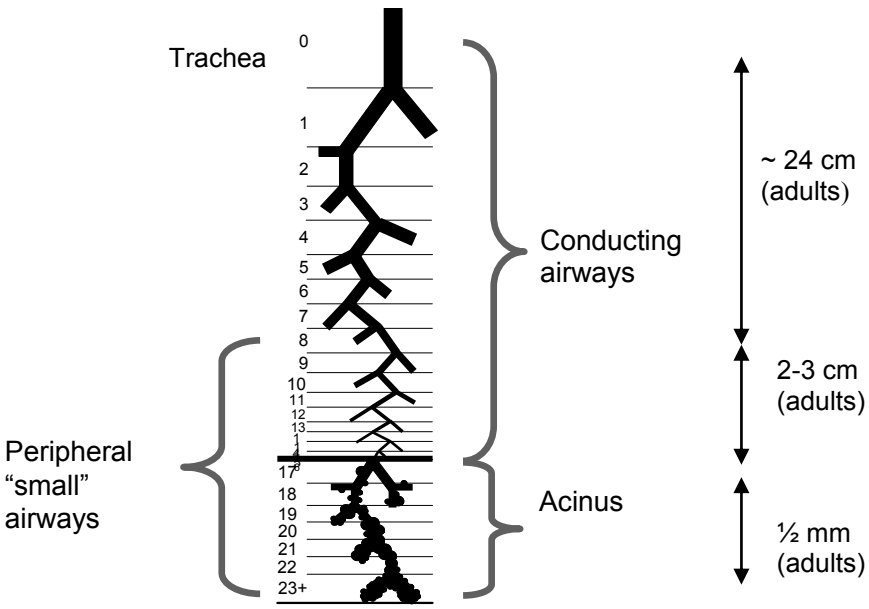


Figure 10
Schematic drawing of the airways with approximate distances in adults. Adapted from Henrik Ljungberg.

The small airway zone (airways < 2mm inner diameter in adults), also referred to as the peripheral airways, starts around the eighth generation. The large airways are kept open by an outer wall of cartilage. The small airways have no cartilage in the airway wall but instead the small airways are held open by the balance between the elastic fibres and smooth muscle in the airway wall and the lung's elastic recoil and the lining fluid consisting of low surface tension

surfactant. In disease, these mechanisms might be in imbalance, resulting in small airway obstruction.

Post-mortem lung specimens and in vivo transbronchial biopsies have demonstrated that both large and small airways are engaged in asthma[2,217] and CF[218]. Later supported by imaging studies showing airway wall thickening, luminal obstruction, bronchiectasis and gas trapping even early on in the course of CF airway disease[219]. In asthma a direct challenge study has shown increased airway resistance in the small airways measured directly through bronchoscopy[220]. These studies provide evidence of small airway involvement in both asthma and CF, but the methods used are invasive or cause radiation, making them less useful in the every day clinic. Different methods to assess the small airways in airway disease are discussed in Table 12. Multiple breath inert gas washout is one example of a non-invasive, relatively easy to perform method to evaluate lung function even in the peripheral airways.

Table 12
Methods to assess the small airways.

Method	***Disadvantages***
Histopathology of organ specimens[221]	Requires surgery
Bronchoscopy with biopsy, lavage[222]	Invasive
Bronchoscopy with ultrasound[223]	Invasive
CT scan[224, 225]	Radiation
MRI[225]	Availability, cost, patient collaboration
Spirometry (MMEF)[226]	Not sensitive, non specific , large interindividual variability
Frequency dependence of Cdyn[227]	Invasive
MEFV-curves +/- Heliox[228]	Indirect, only suggestive
Forced oscillation (FOT)[229]	Dependant on operator technique
Alveolar NO[230]	Different models, conflicting results
Inert gas washout[231]	Cost, Time consuming

Lung function tests

Spirometry

Spirometry (meaning *the measuring of breath*) is the most commonly used pulmonary function test. It measures the amount (volume) and/or speed (flow) of air that can be inhaled and exhaled. The most commonly used indices are forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC) and maximum mid expiratory flow (MMEF) reflecting the mechanical properties in airway function. Disease causing obstruction of the larger airways will easily affect the spirometry variables. Spirometry is, however, not sensitive enough to detect early small airway involvement since the small airways are pathways of very low resistance and only contribute to about 10% of the total airway resistance[174]. Small airway obstruction, however, affect how inspired air mixes with the gas in the lungs and reduced efficiency in gas mixing is called ventilation inhomogeneity. Many school age children with CF have FEV_1 within the normal range even though they have signs of ventilation inhomogeneity as evidence of residual airway disease[180]. Early changes in the small airways are also seen in mild asthma[5,232].

Multiple breath inert gas washout

Inert gas washout was first described over 60 years ago[233]. It measures ventilation inhomogeneity and accordingly it provides information about the small (peripheral) airways[173]. Inert gas washout is performed during a single vital capacity breath or over a series of tidal breaths - the so-called MBW method. This method requires minimal cooperation apart from maintaining an adequate mouthpiece/face-mask seal and regular breathing pattern and MBW can be successfully obtained in infants from very young age as well as in older children where also within test repeatability is good[234,235]. The single breath method requires more cooperation from the subject. Recent reports suggest that indices derived from MBW may be more sensitive than spirometry in detecting lung disease in children with CF[180].

Inert gas washout is performed with different gases and most commonly used is the N_2 washout using 100% O_2. In this thesis an inert gas mixture with 4% SF_6, 4% He, 21% O_2, balance N_2 is used. Each test consists of two phases: a wash in phase during which a dry gas mixture containing the gas, i.e. 4% SF_6, 4% He, 21% oxygen (O_2), and balance nitrogen (N_2), is administered, and a washout phase when the subject breaths normal air again in order to washout the inert gas mixture (Fig 11). Wash in is continued until the inspiratory and expiratory SF_6 concentrations are stable and equal, plus another 30 s.

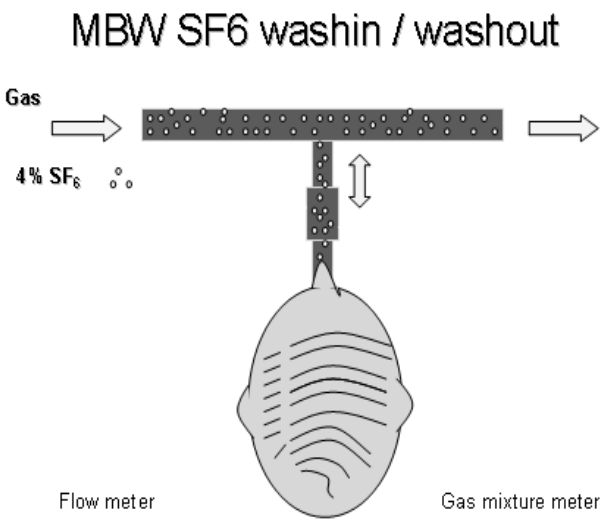


Figure 11
During the wash in phase, the patient breathes the gas mixtures and during the washout, the patient is switched to room air. Cartoon adapted from Per Gustafsson.

At this moment, the bias flow is stopped during expiration by disconnecting the T-piece and the washout phase starts. The washout phase continues until the end-tidal SF_6 concentration is below 0.1% (i.e. 1/40th of the starting concentration) (Fig 12).

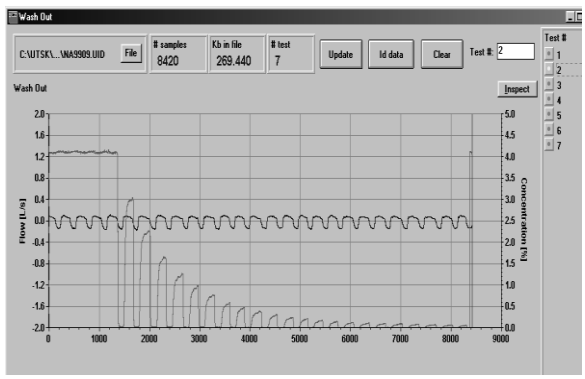


Figure 12
Computer screen readout from SF_6 multiple breath washout.

Multiple breath inert gas washout provides data about the resting lung volume, functional residual capacity (FRC, the volume of air left in the lungs after a tidal expiration) and indices of ventilation distribution inhomogeneity. FRC is calculated from the net amount of marker gas exhaled during washout (reinspired gas subtracted), divided by the difference in marker gas concentration at start and end of washouts[235]. Several markers of ventilation inhomogeneity have been reported in the literature[236]. They all reflect

differences in specific ventilation (ventilation per unit volume) between lung regions, which result in delayed washout of the marker gas from the more poorly ventilated regions.

Lung clearance index

The lung clearance index (LCI) is the variable of ventilation inhomogeneity that is the easiest to calculate and easiest for the patient/ parent and examiner to understand. LCI is calculated as the number of lung volume turnovers that the child must breathe to clear the lungs of the marker gas (to 1/40th of the starting concentration) and it is calculated from the cumulative expired volume during a washout divided by the FRC[237]. An increase in LCI indicates increased ventilation inhomogeneity. See Table 13 for LCI in healthy subjects.

Table 13

LCI in healthy controls and in subjects with asthma and CF.

			LCI		
Authors	*n*	*age (years)*	*Healthy*	*Asthma*	*CF*
Gustafsson et al.[180]	71	3–18	6.33 (0.43)		8.33 (2.48)
Aurora et al.[234]	55	6–16	6.45 (0.49)		11.53 (2.86)
Horsley et al.[238]	45	6–49	6.3 (0.5)		13.1 (3.8)
Gustafsson [239]	44	6–29		8.7 (1.3)	11.5 (3.3)
Macleod et al. [240]	60	5–16	6.24 (0.47)	6.69 (0.91)	
Downie et al. [241]	40	18–66		8.16 (1.1)	

Data presented as mean (±SD)

Phase III slope analysis from MBW

LCI from MBW reflect overall ventilation inhomogeneity and does not provide any information about the location of the pathology.

More sophisticated analysis of the so called phase III (alveolar phase) slopes generated during MBW has been proposed to provide more information about the localisation of the ventilation inhomogeneity in the airway tree[242,243]. This method is based on the understanding that gas mixing in the lungs is based on two mechanisms:

-convection which is predominant in the conducting airways

-diffusion which occurs in the intra-acinar airways

Convection

Convection is the net transfer of gas molecules from areas of high pressure to areas of low pressure with a pressure gradient.

Diffusion

Diffusion - the process by which gas molecules spread from areas of high concentration, to areas of low concentration with a concentration gradient

The zone in between where convection and diffusion occurs to the same extent is called the diffusion-convection front. Ventilation inhomogeneity that takes place proximal from the diffusion-convection front is called convection dependant inhomogeneity and when it occurs in the vicinity of the diffusion-convection front, it is called diffusion-convection interaction-dependent inhomogeneity. The evolution of the phase III slope (Fig 13) from each breath during the MBW enables the determination of the relative contribution of these two mechanisms[244,245].

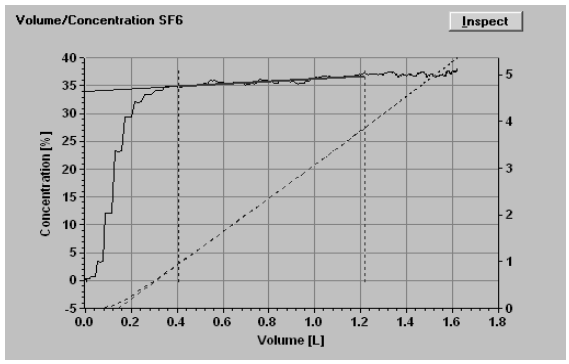


Figure 13
Phase III slope

Verbanck introduced S_{cond} and S_{acin} (Fig 14) to reflect convection dependent inhomogeneity and diffusion-convection interaction-dependent respectively[245].

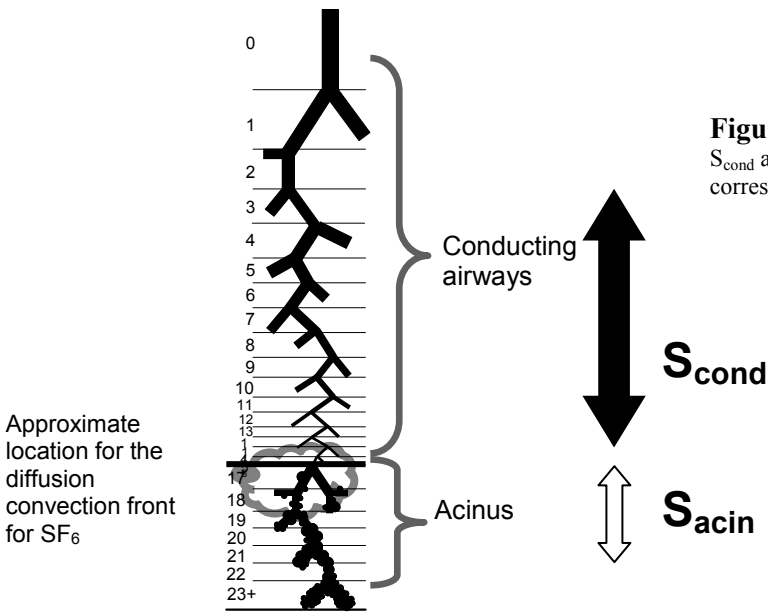


Figure 14
S_{cond} and S_{acin} and the corresponding airways

The phase III slope of each breath is analysed to calculate S_{cond} and $S_{acin.}$ To compare phase III slopes from subsequent breaths, the effect of the dilution of the marker gas is compensated for and the result is called the concentration normalized phase III slope (Sn_{III}). The Sn_{III} for each breath is then plotted against responding lung volume turnovers and S_{cond} is defined as the calculated Sn_{III} increase between lung volume turnover 1.5 and 6.0. S_{acin} is defined as the first breath Sn_{III} value minus the convection dependant inhomogeneity contribution to this value and constitutes the diffusion-convection interaction-dependant contribution to the Sn_{III} of the first washout breath.

To compensate for the subjects size and breathing pattern S_{cond} and S_{acin} are calculated from Sn_{III} multiplied by tidal volume for each breath[246]. Normative data for S_{cond} and S_{acin} has recently been published and the levels are similar for children, teenagers and adults (Table 14)[246].

Table 14
S_{cond} and S_{acin} in healthy subjects. Corrected for tidal volume.

				MBW	
Authors	***n***	***age** (years)*	***Inert gas***	***S_{cond} (/L)***	***S_{acin} (/L)***
Gustafsson (unpubl)	44	41.1± 10.3	SF_6	0.017 (0.006)	0.086 (0.026)
Horsley et al.[238]	17	31.1± 6.0	SF_6	0.0101 (0.015)	0.112 (0.055)
Macleod et al. [240]	28	11.1 (5–16)	SF_6	0.017 (0.020)	0.120 (0.06)
Aljassim et al. [247]	18	18.0 (17–18)	SF_6	0.018 (0.006)	0.086 (0.025)
Verbanck et al. [248]	63	31.1± 1.0	N_2	0.028 (0.001)	0.072 (0.003)

Values presented as mean (SD)

Airway challenge testing

Bronchial hyper-responsiveness, often resulting in exercise-induced bronchocontriction, is considered one of the key features in paediatric asthma[249-251] . The effect of exercise on ventilatory function in the child with asthma was discussed already in the early 60s by Jones *et al*.[252].
The mechanism of action is thought to be respiratory heat and water loss with transient hyperosmolarity in the respiratory mucosa causing the airways to narrow. Whether the magnitude of airway obstruction is determined by airway cooling alone or by evaporation of water from the respiratory mucosa irrespective of cooling, is a matter of debate[117,253-255].

Isocapnoic hyperventilation with dry air mimics the drying of the airway mucosa during exercise and has been used as a marker of airway hyper-responsiveness and exercise induced airway narrowing in asthma[256]. It is simple to perform and easier to standardize than exercise testing even in young children[257]. The reaction probably reflects the pathophysiology of asthma better than a pharmacological bronchoprovocation with histamine or metacholine[255] .

Isocapnoic dry air hyperventilation challenge test is used in this thesis to evaluate airway hyper-responsiveness. A 10% reduction in FEV_1 is outside the range for healthy subjects without asthma and this is therefore used as a cut off level for a positive test for airway hyper- responsiveness[256].

Asthma Control Test

There are several validated patient reported outcomes developed for asthma. Asthma Control Test (ACT) is a short questionnaire, which is validated in many languages. ACT is a five-item tool for identifying patients with uncontrolled asthma developed by Quality Metric, Inc.and GlaxoSmithKline. Responses for each of the five items are summed to yield a score ranging from 5 (poor control of asthma) to 25 (complete control of asthma). Validity, reliability, and responsiveness have been established in subjects 12 years of age and older[258,259]. A paediatric version, the Childhood Asthma Control Test (C-ACT has been developed for children 4-11 years old[260] . C-ACT is a seven-item test where one part is filled in by the parents and one part filled in by the children themselves. The ACT and cACT were designed primarily as discriminatory tools and a score of 19 or lower identifies patients with poorly controlled asthma in both tests. C-ACT is complementary to, but not a substitute for, other markers of disease control in asthmatic children, especially in the context of follow-up visits[261]. Patient reported outcomes, such as ACT, are recommended by the GINA and other guidelines for monitoring subjects with asthma[168].

Aims

The overall aim of this thesis is to use exhaled NO in combination with MBW to assess the contribution of airway inflammation and small airway dysfunction in paediatric asthma and CF. This could potentially allow for earlier intervention and more successful management of paediatric asthma and CF in the future.

Specific aims in paper I-IV

Paper I

The aim of study I was to assess $FENO_{50}$ and nNO in subjects with CF and healthy controls and to examine if low levels of $FENO_{50}$ and nNO in CF are associated with the CFTR genotype, pancreatic insufficiency, chronic colonization with *Ps. aeruginosa* and the typical essential fatty acid imbalance seen in CF patients.

Paper II

Study II is a randomized double blind placebo controlled study aiming at evaluating the influence of different blends of n-3 or n-6 PUFA compared to placebo on $FENO_{50}$, nNO and systemic markers of inflammation in subjects with CF.

Paper III

The aim of study III was to explore if there is an association between flow independent NO variables, possibly reflecting airway inflammation in different segments of the airways, and early signs of impaired airway function in children with CF.

Paper IV

The aim of study IV was to assess NO variables, indicating airway inflammation, and LCI, S_{cond} and S_{acin}, reflecting airway dysfunction, at different depths of the airways, to search for an association between inflammation and small airway dysfunction in children with allergic asthma. A second objective was to explore if inflammation and impaired lung function in the small airways is associated to airway hyper-responsiveness, or if the presence of airway hyper-responsiveness is independent of small airway dysfunction and airway inflammation and reflect another type of asthma.

Study concept

Four studies are reported in this thesis. Study design, inclusion and exclusion criteria for study I-IV are reported in Table 15.

Table 15
Inclusion, exclusion criteria and study design in paper I-IV.

	Study I	***Study II***	***Study III***	***Study IV***
Patient category	CF + controls	CF	CF + controls	Asthma + controls
Patients (n)	59	35	45	47
Healthy controls (n)	114		35	74
Age	≥ 7 years	≥ 7 years	6 – 20 years	5 – 20 years
Study design	Cross sectional Consecutively enrolled	Double blind placebo controlled study	Cross sectional Consecutively enrolled	Cross sectional Explorative
Inclusion criteria	Pathological sweat test	Pathological sweat test Pancreas insufficiency	Pathological sweat test	Doctors diagnosed asthma with allergic sensitization
Exclusion criteria	Acute exacerbation Smoking	Intake of n-3 capsules Acute exacerbation FEV1 ≤ 40% of predicted Severe liver disease Smoking	Acute exacerbation Smoking	Acute airway infection Smoking

Study II is a double blind placebo controlled study where CF-subjects with pancreas insufficiency were consecutively randomized into three parallel groups (A, B and C). Each group was assigned to a fat blend with a different composition of FA. Group A was given an experimental fat blend enriched by n-3 fatty acids, group B a blend rich in saturated fatty acids (FA), corresponding

to an ordinary diet and used as placebo. Blend C was enriched by n-6 FA, especially AA to see whether this could act as a feedback mechanism and inhibit further transformation from LA. Blend C also contained more EPA and DHA than the control, blend B, but less than blend A (Table 16).

Table 16
Fatty acid composition (mol %) of the three experimental fat blends used in the different groups.

	Group A	***Group B***	***Group C***
12:0	1.04	4.43	1.20
14:0	5.60	11.55	3.99
16:0	15.20	31.02	18.21
18:0	3.27	7.73	7.13
14:1n-5	0.43	1.18	0.32
16:1n-7	3.51	1.60	0.55
18:1n-9 (OA)	18.45	25.11	18.68
18:2n-6 (LA)	16.74	15.57	21.52
18:3n-3 (ALA)	5.71	1.28	0.39
18:3n-6	0.13	0.02	4.03
20:3n-6	0.09	0.05	1.59
18:4n-3	1.85	0.10	0.15
20:4n-6 (AA)	0.80	0.06	18.03
20:5n-3 (EPA)	21.27	0.06	1.95
22:6n-3 (DHA)	6.99	0.03	0.52
SFA	25.69	55.0	31.99
MUFA	22.47	27.91	19.76
PUFA	53.69	17.19	48.41
n6/n3	0.50	10.69	15.08

The study subjects received 50 mg/day/kg body weight of the FA blend and they increased their pancreatic enzymes by 10-20% to keep “normal” stools.

Forty-five subjects were included but two subjects had to be excluded right away due to acute exacerbations, thus forty-three patients were randomized. Eight patients did not finish the study (distributed in all three groups) due to low compliance, stomach pains and/or weight gain. Thus, thirty-five patients completed the study and all results are based on these subjects.

The study subjects were examined at inclusion and after three months of supplementation.

Materials

Subjects in study I-IV

The CF diagnosis was based on a pathological sweat test with sweat chloride concentrations >60 mmol/L and genetic testing. Clinical characteristics and baseline measurements of all CF subjects are reported in Table 17.

Table 17
Characteristics and baseline measurements of the CF subjects in study I-III. Values are median (range) or numbers (proportions).

	Study I *(n =59)*	***Study II*** *(n = 35)*	***Study III*** *(n = 45)*
Age (yrs)	22.5 (7–55.3)	20.2 (8.1–41.2)	13.4 (6.2–18.3)
F/M	27/32	18/17	17/28
BMI (Z–score)	-0.1 (-1.1–2.0)	-0.24 (-1.61–1.0)	-0.22 (-1.44–1.44)
Pancreas sufficiency (n [%])	12 (20)	0 (0)	5 (11)
CF–related liver pathology treated with ursodeoxycholic acid. (n [%])	9 (15)	6 (17)	9 (20)
CF associated diabetes (n [%])	9 (15)	6 (17)	3 (7)
Chronic Ps. aeruginosa colonization (n [%])	29 (49)	15 (43)	16 (36)
FVC (% predicted)	98 (34–132)	97 (69–142)	100 (73.4–132.1)
$FEV_{1.0}$ (% predicted)	84 (13–127)	85 (47–129)	91 (54–122)
Current use of inhaled corticosteroids (n [%])	17 (29)	10 (28)	12 (27)
Long term antibiotics (azitromycin or flucloxacillin) (n [%])	38 (64)	22 (63)	22 (49)
Atopy (n [%])	22 (37)	9 (26)	22 (49)

Pancreatic function was determined by measuring pancreatic enzymes in duodenal secretions after stimulation with cholecystokinin and secretin or by determining fecal elastase.

Chronic *Ps. aeruginosa* infection was defined as consecutive positive sputum cultures during 6 months and raised serum titers of pseudomonas exotoxin A antibodies[262].

CF subjects in ***study I*** were classified into four groups according to the CFTR genotype were the patients in group 4 were pancreas insufficient and patients in all other groups were pancreas insufficient (Table 18).

Table 18
Genotypes in the different groups in study I

Group	***Genotypes***
1	homozygotes for dF508
2	heterozygotes for dF508 with one other "severe" mutation (394delTT, 3659delC, E60X, or 1112 delT) or homozygotes for that other mutation
3	compound heterozygotes for dF508 and V603F, R560T, or 621+1G-T
4	heterozygous for dF508, 3659del C or 394delTT and a mutation linked to a "mild" phenotype (N1088D, R117C, R117H, R75Q, R658X, S945L, 1154insTC, or T338I)

Atopy (allergic sensitization) was in ***study I and II*** based on a clinical question whether the patient had symptoms of allergic rhinitis after allergen exposure. In ***study III-IV,*** atopy was based on a positive Phadiatop™ test (Phadia, Uppsala, Sweden) which determines the presence of raised specific serum IgE antibodies to common inhaled allergens (dog, cat, horse, timothy, birch, mugwort, house dust mite and *Cladosporium*). It is expressed as positive or negative with a cut-off level at 0.35kU/l.

Asthma was defined according to current guidelines based on clinical symptoms of asthma and demonstrated reversible airway obstruction or a positive bronchial challenge test[168]. Clinical characteristics of the asthma subjects in ***study IV*** are reported in Table 19.

Table 19
Characteristics and baseline measurements of study subjects in study IV. Values are mean (SD) or numbers (proportions).

	Controls (n = 74)	***Asthma*** (n = 47)	***p-value***
Age (yrs)	13.6 (3.2)	13.2 (3.0)	0.542
Sex (F/M)	36/38	12/35	
Height (cm)	160 (16)	159 (15)	0.725
ACT score< 20 (n [%])	n.a.	17 (38)*	
Current asthma controller medication			
Inhaled corticosteroids (n [%])	0	37 (79)	
Combination therapy (ICS and LABA) (n [%])	0	23 (49)	
Montelukast (n [%])	0	710 (21)	
FVC (% predicted)	102 (11)	100 (10)	0.216
FEV_1 (% predicted)	101 (11)	92 (12)	0.0001
MMEF (% predicted)	99(21)	74 (22)	0.0001
FEV1/FVC (%predicted)	98 (6)	96 (8)	0.0001

* Missing data for three subjects.
ACT=Asthma Control Test; ICS=inhaled corticosteroids; LABA=long acting β_2 agonist

All study subjects in ***study I-IV*** were never smokers and free of ongoing acute airway infection.

Healthy controls

In ***paper I***, subjects examined within the scope of the European Community Respiratory Health Survey II (ECRHSII)[263], were used as healthy controls (Table 20).

In ***study III***, healthy children were recruited from nearby schools, and they were matched for age and atopy with the CF subjects (Table 20). These healthy controls and a further thirty-nine children who only performed the MBW and spirometry were in the control group in ***study IV*** (Table 19). Controls and patients were examined with the same equipment and procedures in respective study.

Table 20
Clinical characteristics of the controls in study I and III. Values are median (range) or numbers (proportions)

	Controls study I *(n=104)*	***Controls study III*** *(n = 35)*
Age (yrs)	39.5 (30–54)	13.2 (7.7–18.5)
F/M	51/53	22/13
Height (cm)		159.0 (125–188)
Body mass index BMI (Z–score)		–0.1 (–1.27–1.6)
Atopy (n [%])	0 (0)	16 (43)

Methods

Exhaled and nasal NO

Exhaled NO

$FENO_{50}$ was measured according to the ATS/ERS recommendations for on-line NO measurements, as described earlier[52]. The subjects were told not to eat or drink and to avoid hard exercise four hours ahead of the NO measurements.

In ***study I*** and ***II*** nNO and $FENO_{50}$ were measured with a chemiluminescence analyzer (Ecophysics Breath Analyzer CLD 77 AM; Switzerland) with a detection limit of 0.5 ppb. A visual feedback helped the subjects tested to keep expiratory flow steady and close to target flow during the recordings (Exhalation Breath Analyzer TM; Aerocrine A, Stockholm, Sweden). In ***study III*** and ***IV*** the Eco Medics system was used and nitric oxide was measured with a chemiluminescence analyzer (ANALYZER CLD 88 AM; Eco Medics AG, Duernten, Switzerland) with a detection limit of 0.1 ppb. A two-point calibration of the analyzer was performed weekly with a certified NO calibration gas.

$FENO_{50}$ was measured in triplets at a plateau of 3 seconds during a slow single exhalation for 10 seconds. The mean $FENO_{50}$ from three recordings within 10 % was reported.

In s***tudy III*** and ***IV,*** exhaled NO was measured at several exhalation flow-rates, 30, 50, 100, 150, 200 and 250 mL/s and in ***study IV*** in some patients 300 mL/s. Different exhalation flows were obtained by using flow resistors. The exhalation times varied between 6 and 15 seconds depending on the flow-rate. Two exhalations that varied less than 5 % or three exhalations that varied less than 10% were obtained at each flow-rate.

NO flow-independent variables

In ***study III*** and ***IV*** extended NO analysis was used, which have been described and validated before[90,134,264]. Two different models, the slope intercept (the linear model)[90] and the non-linear model were assessed[134]. From the linear model the two variables, alveolar NO and bronchial NO flux are obtained and

from the non-linear model the same two variables plus the airway wall NO concentration and the airway NO diffusing capacity were calculated. Flow-rates above 50 mL/s were used for the linear method and for the non-linear method, all flow-rates were used. Two published methods were applied to correct for the back diffusion of NO into the alveolar space when calculating alveolar NO with the linear method.

A. **Alveolar NO corr = Alveolar NO – bronchial NO flux/740** as proposed by Condorelli *et al.*[103] and
B. **Alveolar NO corr = (Alveolar NO- 0.08 × $FENO_{50}$) / 0.92** as proposed by Kerckx *et al.* [102].

Nasal NO

Nasal NO was recorded in ***study I*** and ***II*** with the Aerocrine system. Nasal NO was measured according to guidelines by sampling nasal air from one nostril at a constant sample rate of 50 ml/s, leaving the other nostril open[52]. To ensure closure of the velum a simultaneous exhalation was performed with a positive pressure >5 cm H_2O in the mouth. Both nostrils were examined in ***study I***. The mean level of nasal NO was determined during a plateau lasting more than three seconds and the mean value calculated from three recordings was recorded.

Lung function tests

Spirometry

Spirometry was performed after the NO measurements and according to the ATS/ERS standards[265] using a Jaeger Masterscope spirometer (Erich Jaeger AG, Würzburg, Germany). Results were expressed as percentage of predicted values[266,267]. In ***study IV*** results were expressed as percentage of predicted values using the new global "all ages" reference values recently presented by Stanojevic *et al.*[268].

Multiple breath inert gas washout

MBW was performed as described in the Introduction section. The children were investigated in the sitting position and the younger subjects watched a

video while the older subjects watched a tidal volume trace on a computer screen and were instructed to keep breathing regular with a tidal volume (VT) between 10–15 mL/kg body weight. All subjects used a nose clip and breathed through a Fleisch no.1 pneumotachometer (Metabo SA, Lausanne, Switzerland) via a mouthpiece. A sampling tube from a mass spectrometer was introduced in the middle of the air stream between the mouthpiece/mask and the pneumotachometer through a short connecting piece. The external dead space was 15 mL for the mouthpiece system and 30 mL for the mask system.

Single breath inert gas washout was also performed but a large proportion of the children were not able to perform this according to the protocol and results are therefore only reported from the MBW.

The dry gas mixture used for MBW in ***study III and IV*** contained 4% SF_6, 4% He, 21% oxygen (O_2), and balance nitrogen (N_2). The different gas concentrations were measured by the mass spectrometer as dry gas concentrations. Recorded inspiratory and expiratory flows and volumes were converted to body temperature and ambient pressure, and saturated with water vapour conditions. Gas samples and flow signals were aligned in time. The sample flow of the mass spectrometer was 20 mL/min and the gas concentration signals were updated at a rate of 33.3 Hz. All signals were recorded at 100 Hz by a computer using software that corrected the flow signal sample-by-sample for changes in dynamic viscosity caused by the variations in gas composition.

MBW calculations

LCI was calculated as the number of lung volume turnovers needed to lower the end-tidal tracer gas concentration to 1/40th of the starting concentration. The mean value, calculated from three recordings, was recorded.

The phase III slope of each breath during MBW was analysed to calculate S_{cond} and S_{acin}. To compensate for the subjects size and breathing pattern S_{cond} and S_{acin} were calculated from Sn_{III} multiplied by tidal volume for each breath as suggested by Robinson *et al*.[246]. Quality criteria for Sn_{III} analysis included regular breathing with a tidal volume between 10 and 15 mL/kg body weight. All asthma patients fulfilled this but less than half of the controls managed to do so. LCI results are therefore reported from all the controls (n=74) while S_{cond} and S_{acin} are reported only from those individuals who fulfilled the quality criteria (n=36).

Airway challenge

Airway challenge was performed by four minutes hyperventilation of isocapnoic dry air from the Ailos Astmatest™ (Ailos Medical B, Karlstad, Sweden). Flow was set at 75 of predicted maximum voluntary ventilation, which was approximated as 26 x subject's baseline FEV_1. Hyperventilation was guided by a target balloon on the air-administering arm of the apparatus. Spirometry was measured at 2, 5 and 10 minutes post challenge. The response was measured as the maximum percentage of fall in FEV_1. AHR was defined as maximum fall in $FEV_1 \geq 10\%$. The bronchodilator response was measured as the percentage rise from baseline FEV_1 after inhalation of four puffs of salbutamol (0.1 mg/dose) from a metered dose inhaler via an anti-static holding chamber. Short acting bronchodilator treatment was withheld for 12 hours prior to testing and long acting bronchodilators, inhaled corticosteroids and montelukast were withheld for 24 hours.

Asthma Control Test

Symptoms in the asthmatic children were recorded using ACT and cACT in children over and under twelve years of age respectively. The number of children with an ACT score of 19 or less, the cut off value for well-controlled asthma, is reported.

Fatty acids

Fatty acids in serum were analysed as previously described[269]. Total lipids of serum were extracted and serum phospholipids were fractionated and transmethylated and analyzed by capillary gas-liquid chromatography.

Urinary analysis

8-iso-$PGF_{2\alpha}$ (marker for oxidative stress) and 15-keto-dihydro-$PGF_{2\alpha}$ (major metabolite of cyclooxygenase catalyzed $PGF_{2\alpha}$) in urine were analyzed by radioimmunoassay (RIA) as previously described[270,271].

Systemic inflammatory variables

Inflammatory markers in blood, used in the routine care, were analyzed at Sahlgrenska University Hospital (Gothenburg, Sweden). In ***study II*** cytokines (interleukin [IL]-10, IL-1b, IL-8, IL-6, and tumour necrosis factor [TNF]-a) were analyzed in serum by enzyme-linked immunosorbent assay (Peli-Kine Compact ELISA kits, Sanquin reagents, Amsterdam, the Netherlands).

Statistics

The statistical analyses were performed with the SPSS software package, version 11 and version 15 (Chicago, IL, USA) and SAS (version 9.1, SAS Institute, Cary, NC, USA, procedure Proc Mixed).

Results are given as median and range or mean (SD) if not otherwise stated.

Non-parametric tests were applied as the distributions of NO variables were not normal distributed. Ln NO, which had a normal distribution, was used for the multiple regression analysis. *Mann-Whitney's U-test and Kruskall-Wallis* were used to assess differences between groups and Student's *t-test* was used for lung function variables showing a normal distribution. Nominal data were compared using the Yates corrected *chi2 test.*

Wilcoxon signed rank test was used to assess differences within one individual. *Spearman's rank correlation and multiple linear regression analysis* were performed to assess the strength of the relationships between different variables. The two-tailed significance level was set at $p<0.05$. Upper limit of normal is defined as (mean + 1.96x SD) for reference population (healthy controls).

Ethics

The Ethics Committee of the University of Gothenburg approved the study protocols for all four studies. Informed consent was obtained from all patients and healthy controls and from the parents of children under 18 years old.

Results

Paper I

Summary

CF subjects with pancreatic insufficiency have lower nNO and $FENO_{50}$ than pancreas sufficient subjects. Low $FENO_{50}$ and low nNO are associated with chronic colonization with Ps aeruginosa, and with the lipid abnormalities often seen in CF subjects.

NO and genotype

Nasal NO and $FENO_{50}$ were significantly lower in the CF subjects than in healthy controls. There was no difference in nNO between the nostrils. CF subjects homozygous for dF508 and other severe mutations had lower levels of nNO and $FENO_{50}$ compared to those with mutations associated with mild disease (Fig 15).

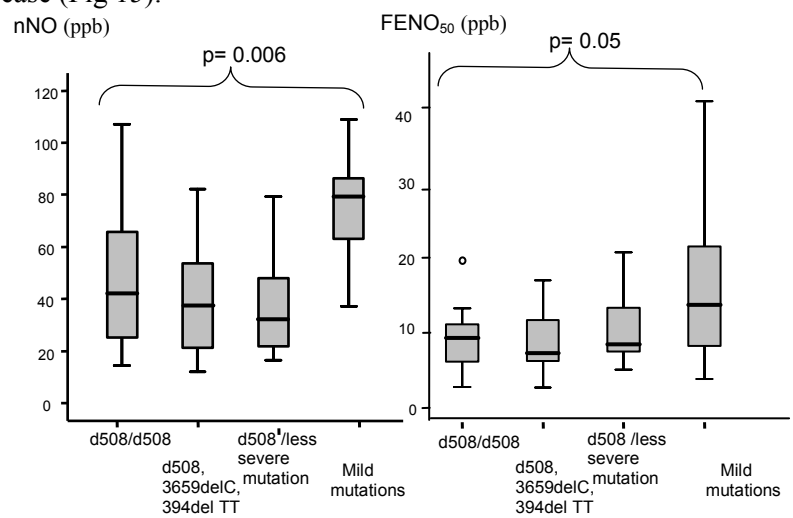


Figure 15

Nasal nitric oxide (nNO) (left) and exhaled nitric oxide ($FENO_{50}$) (right) in CF patients in relation to different cystic fibrosis transmembrane conductance regulator (CFTR) mutations. Box plot indicates median and 25th and 75th percentiles. Whiskers indicate 5th and 95th percentiles and outliers are indicated (○).

Similarly, there was a significant difference between pancreas insufficient and pancreas sufficient subjects (Fig 16).

NO and Pseudomonas aeruginosa

Chronic colonization with *Ps. aeruginosa* was associated with lower nNO and lower $FENO_{50}$ than non-colonization (Fig 16).

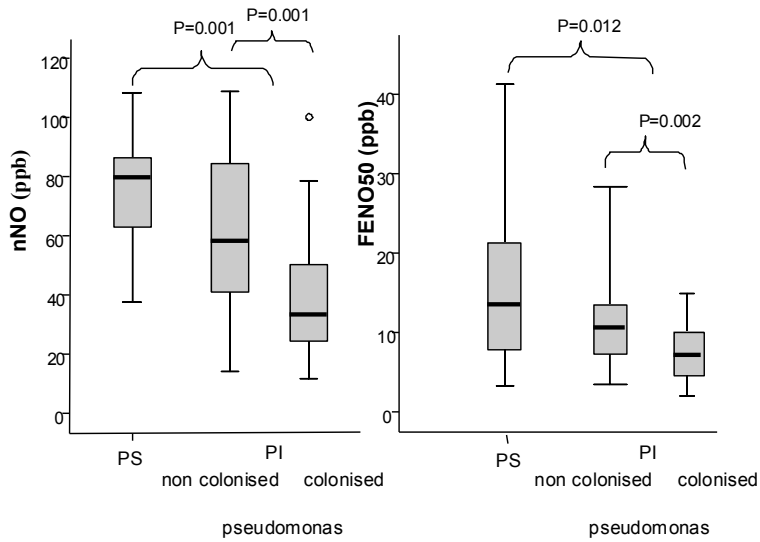
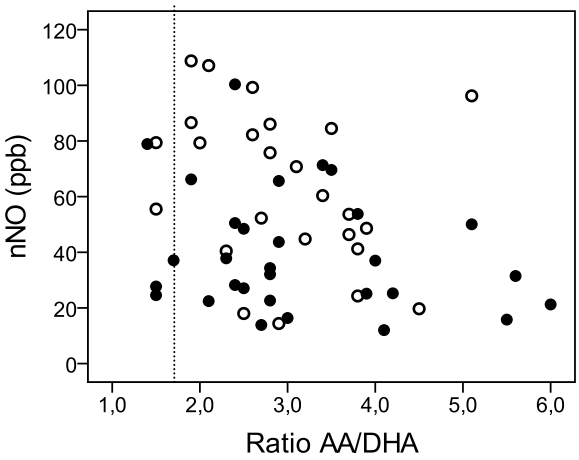


Figure 16

Nasal nitric oxide (nNO) (left) and $FENO_{50}$ (right) in relation to pancreatic function and chronic pseudomonas colonization in cystic fibrosis (CF) patients.
PS = pancreas sufficient; PI = pancreas insufficient.
Box plot indicates median and 25th and 75th percentiles. Whiskers indicate 5th and 95th percentiles and outliers are indicated (○).

NO and essential fatty acids in CF

There was a negative correlation between $FENO_{50}$ and nasal NO and the ratio arachidonic acid (20:4n-6) to docosahexaenoic acid (22:6n-3) in non colonised subjects (Fig 17). The ratio arachidonic acid to docosahexaenoic acid is often high in CF subjects and one of the characteristic lipid abnormalities seen in CF [204,206].



● = colonized with *Ps. aeruginosa*
r_s= -0.214, p= 0.27

○ = not colonized
r_s = -0.412, p= 0.032

Figure 17
Nasal nitric oxide (nNO) in CF patients in relation to the ratio between arachidonic acid (AA) and docosahexaenoic acid (DHA) in serum phospholipids. Line represents the ratio AA/DHA in reference population [269]

Paper II

Summary
Supplementation with different mixtures of dietary FA influences serum FA status and NO in the airways in patients with CF. Supplementation with n-3 FA attenuates the inflammatory response and n-6 supplemation aggravates the inflammatory response.

NO and n-3 and n-6 PUFA substitution

The serum phospholipid FA pattern changed significantly in all three groups, reflecting the intake of fatty acids.

Univariate and multivariate analysis showed that nNO decreased significantly in those subjects who received supplementation with n-6 fatty acids. There was no change in $FENO_{50}$ or nNO after intake of n-3 fatty acids.

The ratio of AA in serum phospholipids increased in 22 individuals (from group B and C) and in those subjects, there was a significant decrease in $FENO_{50}$ and nNO (Fig 18)

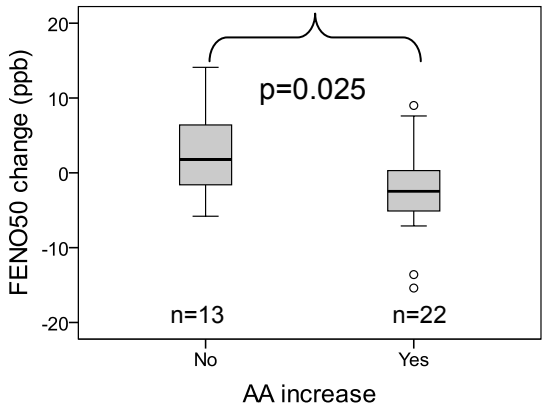


Figure 18
Change from baseline in $FENO_{50}$ in CF patients where serum concentration of arachidonic acid (=AA) increased and in those were there was no AA increase. Box plot indicates median and 25th and 75th percentiles. Whiskers indicate 5th and 95th percentiles and outliers are indicated (○).

N-3 and n-6 PUFA substitution and markers of systemic inflammation

Erythrocyte sedimentation rate (ESR) and IL-8, decreased after n-3 FA supplementation and ESR increased after n-6 FA supplementation (Table 21).

Table 21
Erythrocyte sedimentation rate (ESR) mm/h and IL-8 ng/L at baseline and after three months of FA supplementation. Values expressed as median (range).

	ESR			***IL 8***		
	baseline	*3 months*	*diff*	*baseline*	*3 months*	*diff*
Group A	7 (3–26)	6 * (3–25)	–1 (-11–3)	17.5 (0.8–25.0)	9.3 ** (0.8–22.0)	-11.1 (-26.4–8.0)
Group B	9 (3–13)	6 (2–27)	0 (-3–16)	19.5 (6.6–69.0)	21.5 (0.8–80.0)	-3.0 (-48.0–64)
Group C	7 (3–31)	9 * (3–32)	2 ††† (-1–7)	14.0 (6.0–88.0)	24.0 (4.7–61.0)	6.2 ††† (-32–29)

* p=0.04 within group, **p=0.003 within group
†††p=0.05 between group C and B

N-3 and n-6 PUFA substitution and urine metabolites

There were no changes in the marker for oxidative stress and no change in 15-keto-dihydro-PGF2a (a major metabolite of cyclooxygenase-catalyzed PGF2a) in urine.

Paper III

Summary
$FENO_{50}$ and bronchial NO flux are decreased in young subjects with CF while alveolar NO is no different compared to healthy controls. Children with CF have elevated LCI, indicating impaired small airway function. Low $FENO_{50}$ is associated with decreased airway function and low alveolar NO is associated with elevated markers of systemic inflammation and pseudomonas infection.

Exhaled NO in children with CF

$FENO_{50}$ and bronchial NO flux were lower in children with CF compared to healthy controls. There were no significant differences in alveolar NO, bronchial wall concentration and NO diffusion capacity between the groups (Table 22). Bronchial NO flux and alveolar NO were calculated with two different methods and the values differed significantly between the methods but they were highly correlated (Table 22). There were no differences according to gender in any of the NO variables in any of the groups.

Table 22
NO variables in CF and healthy children. Values are expressed as median (range). P-value is given for differences between groups.

	CF (n=45)	***Controls*** (n=35)	*p-value*
$FENO_{50}$ mL/s (ppb)	9.1 (2.7–38.8)	12.4 (5.2–40.1)	0.029
Alveolar NO (ppb)[1]	1.2 (0–3.1)	1.4 (0.3–3.6)	0.147
Bronchial NO flux (pL/s)[1]	378 (97–1772)	578 (123–1993)	0.036
Alveolar NO (ppb)[2]	1.03 (0.1–2.9)	1.3 (0.1–2.9)	0.490
Bronchial NO flux (pL/s)[2]	412 (23–2101.)	664. (127–2310)	0.024
Bronchial wall NO conc. (ppb)[2]	54.8 (2.8–8209.6)	43.4 (9.3–1420.2)	0.813
Bronchial NO diffusion capacity (nL/s)[2]	12.4 (0–97.2)	15.0 (1–51.9)	0.430

[1] Calculated according to (the linear method) Tsoukias
[2] Calculated according to (the non-linear method) Högman

Small airway function in children with CF

LCI was significantly elevated in the children with CF compared to the healthy controls, 8.33 (6.35–12.83) vs. 6.08 (5.05–6.96), p<0.001 (Fig 26). LCI was significantly correlated with FEV_1 but several CF subjects had increased LCI in spite of normal FEV_1, indicating that the children with CF had signs of small airway involvement in spite of near to normal FEV_1 (Fig 19).

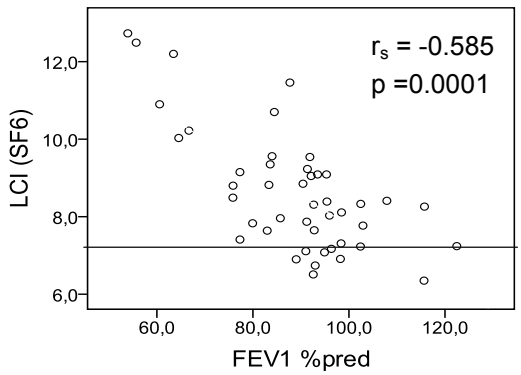


Figure 19
FEV_1 in relation to LCI in children with CF. Line indicates upper limit of normal for ref population.

Exhaled NO and ventilation inhomogeneity in children with CF

There was a significant negative correlation between $FENO_{50}$ and LCI (Fig 20). Alveolar NO also showed a negative correlation with LCI, r=-0.36, p=0.026.

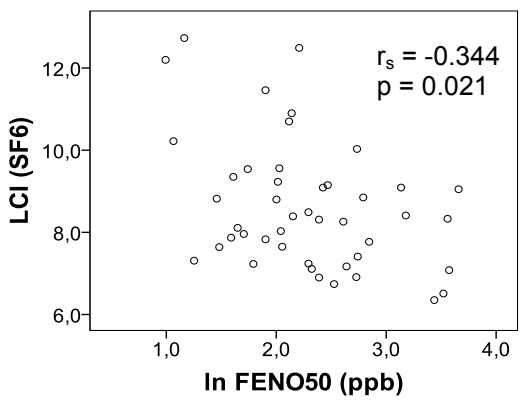
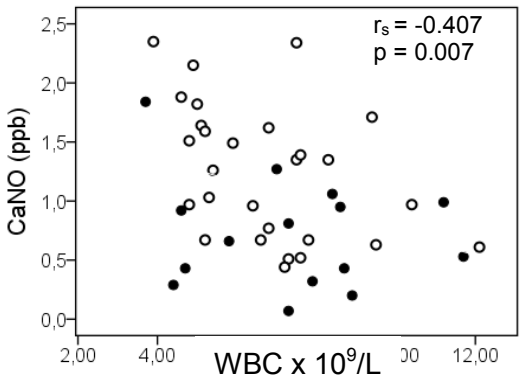


Figure 20
$FENO_{50}$ in relation to the lung clearance index (LCI) in children with CF.

Exhaled NO, inflammation and bacterial colonization

Alveolar NO was negatively associated with several markers of systemic inflammation (WBC, orosomucoid and IgG) (Fig 21). Subjects with chronic *Ps. aeruginosa* colonization had lower levels of alveolar NO and higher LCI than subjects who were not colonized (Table 23).



● = colonization with *Ps. Aeruginosa*
r_s = -0.286, p= 0.212

○ = no colonization
r_s = -0.482, p=0.009

Figure 21
Alveolar nitric oxide (Högman) concentration (ppb), in relation to white blood cell count (WBC x10^9/L).

Table 23
NO variables (non-linear method) and lung function in CF subjects with and without chronic colonization with Ps. aeruginosa. Values are median (range).

	Non-colonized (n=29)	***Colonized*** (n=16)	*p-value*
Age (years)	12.6 (6.2–17.8)	15.3 (7.8–18.3)	0.034
FEV_1 (% predicted)	92.3 (64.6–122.5)	84.2 (54.0–115.7)	0.017
LCI	7.8 (6.5–10.7)	9.4 (7.1–12.9)	0.0001
$FENO_{50}$ mL/s (ppb)	10.9 (3.5–35.6)	7.6 (2.7–38.8)	0.109
Alveolar NO (ppb)	1.3 (0.44–2.9)	0.6 (0–1.84)	0.002
Bronchial NO flux (pL/s)	421 (33–2101)	430 (23–1807)	0.864
Bronchial wall NO conc. (ppb)	53.1 (7.8–627.2)	28.6 (2.8–8210)	0.242
Bronchial NO diffusion capacity (nL/s)	12.4 (0.6–34)	23.2 (0.2–97.2)	0.150

Paper IV

Summary
Children with allergic asthma have dysfunction in the small conducting and acinar airways in spite of normal FEV_1. $FENO_{50}$ and bronchial NO flux are increased and there is an association between the increased levels of exhaled NO, the dysfunction in the small conducting airways and airway hyper-responsiveness in paediatric asthma. Alveolar NO is correlated with dysfunction in the small airways.

Exhaled NO in children with asthma

$FENO_{50}$ and bronchial NO flux were significantly increased but alveolar NO was no different in the asthmatic children compared to the healthy controls (Table 24).

Table 24
Atopy status and nitric oxide measurements reported as numbers (proportion) and median (range).

	Controls (n = 35)	***Asthma*** (n = 47)	*p-value*
Positive Phadiatop (n [%])	16 (43)	47 (100)	
$FENO_{50}$ mL/s (ppb)	12.4 (5.2–40.1)	22.3 (4.8–184)	0.010
Alveolar NO (ppb)[1]	1.4(0.1–3.6)	1.2 (-1–6.1)	0.361
Bronchial NO flux (pL/s)[1]	664 (127–2310)	1217 (155–12 139)	0.041
Bronchial wall NO concentration (ppb)[1]	43.4 (9.3–496)	84.0 (11.7–696.3)	0.034
Bronchial NO diffusion capacity (nL/s)[1]	15.0 (1.0–51.9)	23.4(0.6–254.4)	0.221

[1] *Non-linear method*

Small airway functionin children with asthma

The three variables derived from MBW (LCI, S_{cond} and S_{acin}) were increased in the asthmatic children (Table 25).

Table 25
Results of multiple breath inert gas washout presented as mean (SD).

	Controls (n = 36)	***Asthma*** (n = 47)	*p-value*	*95% CI for difference*
LCI (lung turnovers) *	6.07 (0.35)	6.44 (0.63)	<0.0001	-0.55 to -0.19
S_{cond} (Vt corrected)	0.017 (0.004)	0.032 (0.018)	<0.0001	-0.021 to -0.011
S_{acin} (Vt corrected)	0.071 (0.019)	0.095 (0.030)	0.001	-0.033 to -0.012

* n=74

LCI was increased in the children with asthma compared to the healthy controls, but it was S_{cond} that most clearly separated children with asthma from healthy controls (Fig 22).

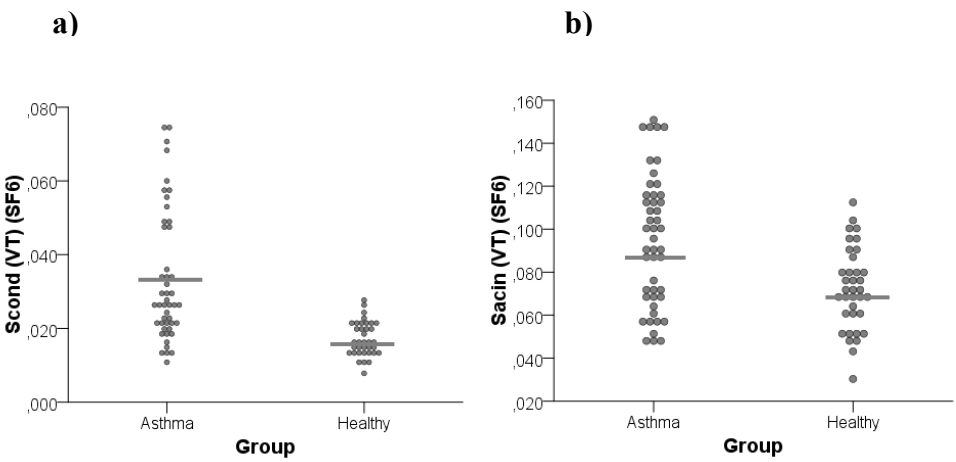


Figure 22
(a) S_{cond} (SF_6) corrected for tidal volume and **(b)** S_{acin} (SF_6) corrected for tidal volume in children with asthma and healthy controls. Marker corresponds to mean value.

Several asthmatic individuals had normal LCI but increased S_{cond} and S_{acin} (Fig 23).

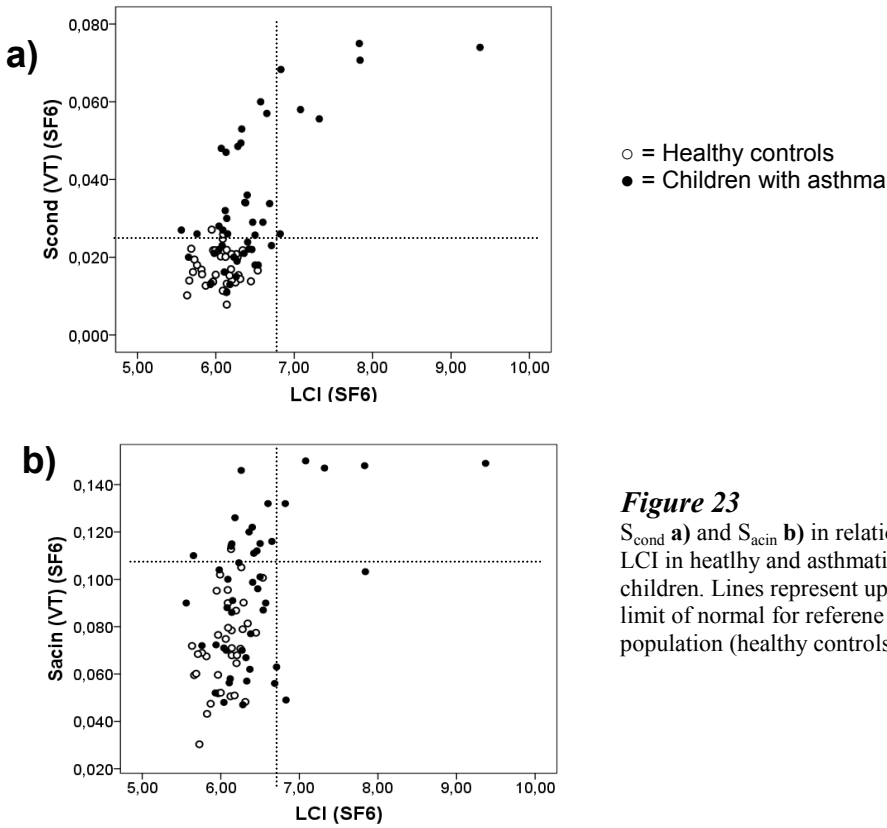


Figure 23
S_{cond} **a)** and S_{acin} **b)** in relation to LCI in heatlhy and asthmatic children. Lines represent upper limit of normal for referene population (healthy controls).

Exhaled NO, small airway function and airway hyper-responsiveness in children with asthma

$FENO_{50}$ was significantly correlated with S_{cond} (Fig 24a), airway hyper-responsiveness (Fig 24b) and bronchodilator response r_s= 0.354, p< 0.015 in the asthmatic children. S_{cond} was also significantly correlated with bronchodilator response to salbutamol (Fig 24c) and airway hyper-responsiveness r_s= 0.479, p= 0.001.

There were statistically significant correlations between alveolar NO and S_{cond} (r_s=0.402, p=0.011), alveolar NO and S_{acin} (r_s=0.402, p=0.015), alveolar NO and bronchodilator response (r_s=0.481, p=0.002), but there was no correlation between alveolar NO and AHR (r_s=0.212, p=0.194).

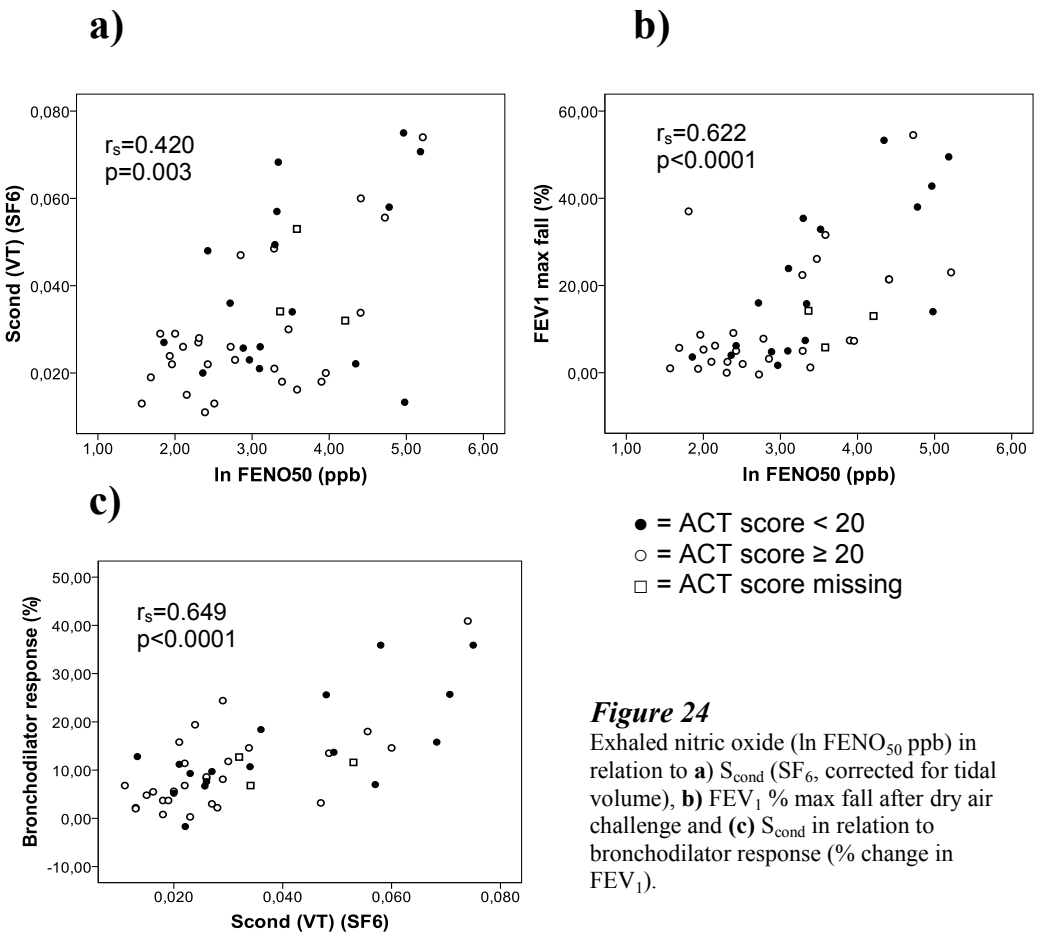


Figure 24
Exhaled nitric oxide (ln $FENO_{50}$ ppb) in relation to **a**) S_{cond} (SF_6, corrected for tidal volume), **b)** FEV_1 % max fall after dry air challenge and **(c)** S_{cond} in relation to bronchodilator response (% change in FEV_1).

Children with elevated S_{cond} had higher alveolar NO and than children with normal S_{cond} but the difference in alveolar NO between those who had elevated S_{acin} and those with normal S_{acin} did not reach significance (Table 26).

Symptoms

Subjects with well- and uncontrolled asthma (based on ACT score) are marked separately in the figures above. The symptom score is only partly associated with airway inflammation and lung function.

Table 26

Asthmatic children grouped according to MBW indices. Increased= > upper limit of normal for healthy controls. P-value and 95% CI are given for the differences between groups. Values are given as mean (SD) or median, range.

	Increased					
	LCI		***Scond***		***Sacin***	
Mean (SD) or median, range	***Yes*** n=7	***No*** n=40	***Yes*** n=28	***No*** n=19	***Yes*** n=17	***No*** n=30
FEV1 (Z.-score) p-value (95% CI)	-2.61 (0.7) p=0.010	-0.57 (1.1) (0.28 to 1.69)	-0.69 (1.1) p=0.799	-0.76 (1.0) (-0.72 to 0.56)	-1.01 (1.1) p=0.182	-0.56 (1.0) (-0.22 to 1.11)
FEV1 (%pred) p-value (95% CI)	85.0 (8.5) p=0.012	93.3 (12.4) (3.03 to 19.6)	92.2 (13.1) p=0.731	90.9 (11.9) (-8.70 to 6.15)	88.2 (12.7) p=0.163	93.6 (12.1) (-2.31 to 13.14)
FEV1/FVC (%pred) p-value (95% CI)	83.7 (8.5) p=0.035	92.5 (9.0) (0.78 to16.75)	89.7 (8.7) p=0.225	93.2 (10.1) (-2.26 to 9.27)	90.0 (10.5) p=0.448	92.0 (8.7) (-3.84 to 8.45)
MMEF (%pred) p-value (95% CI)	55.6 (17.7) 0.020	76.8 (21.9) (4.21 to 38.06)	71.0 (21.6) p=0.349	77.4 (23.6) (-7.30 to 20.17)	71.3 (26.7) p=0.632	74.9 (20.0) (-11.66 to 18.85)
$FENO_{50}$ (ppb) range p-value	118.8 22.3−183.8 p=0.002	17.6 4.8−145.3	27.3 6.1−183.8 p=0.162	16.1 4.8−145.3	26.8 5.4−178.3 p=0.842	22.1 4.8−183.8
$FENO_{300}$ (ppb) range p-value	29.3 6.9−39.7 p=0-001	5.1 1.3−34.0	6.5 2.5−39.7 p=0.041	4.0 1.3−3.4	6.4 1.6−39.7 p=0.789	5.1 1.3−37.7
Alveolar NO (ppb) range p-value	1.64 -1−6.1 p=0.531	1.14 -0.2−4.18	1.48 -1−6 p=0.007	0.6 0 -0.2−2.8	1.63 -1−6.1 p=0.311	1.17 -0.6−3.1
AHR n(%) p-value	6 (86) 0.037	14 (30)	17 (61) p=0.006	3 (16)	8 (47) 0.870	12 (40)
Low ACT score n(%) p-value	4 (57) p= 0.401	13 (35)	12 (48) p=0.113	5 (26)	8 (47) p=0.264	9 (33)

Exhaled NO and small airway function − comparing results in asthma and CF

Flow independent NO variables

Bronchial NO flux was closely associated to $FENO_{50}$ in all groups of children (healthy, CF and asthma) (Fig 25). Bronchial NO flux was correlated with the same lung function variables, as $FENO_{50}$ in children with asthma and CF. There was no correlation between alveolar NO and $FENO_{50}$ in any of the paediatric groups.

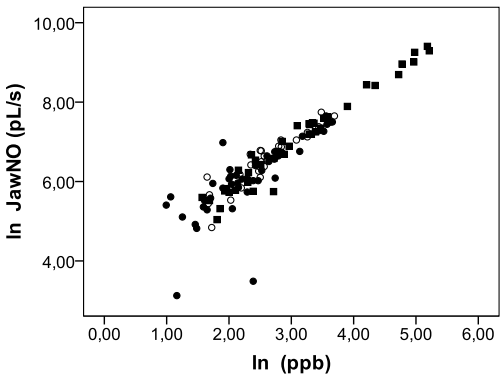


Figure 25
Bronchial NO flux in relation $FENO_{50}$ in children in study III-IV.

Bronchial wall NO concentration was increased in asthma and correlated to the same parameters as $FENO_{50}$ but also to FEV_1, r=0.463, r_s=0.002. Bronchial wall NO concentration was not different in CF compared to healthy controls.

Bronchial NO diffusion capacity was not statistically different in asthma or CF compared to healthy controls, however in CF, children with chronic colonization with *Ps. aeruginosa* had higher NO diffusion capacity than non-colonized children or healthy controls. There was a significant correlation between NO diffusion capacity and LCI in CF, r_s= 0.421, p=0.004

The two mathematical methods used, the linear and non-linear method, produced highly correlated results in all three groups, r=0.99, r=0.97 and r=0.98 for bronchial No flux and r=0.82, r=0.90 and r=0.89 for alveolar NO in the children with asthma, CF and healthy children respectively, p=0.0001 for all correlations above. Alveolar NO obtained by the linear method was higher and bronchial NO flux was lower compared to the levels obtained by the non-linear method in all groups.

Alveolar NO was corrected for axial back diffusion of NO to the alveolar region with two different mathematical corrections[103,272]. Both methods resulted in negative values for alveolar NO in a majority of the asthmatic children, in a third of the children with CF and in the same proportion in the healthy controls. Some children with high $FENO_{50}$ (n = 4) had negative alveolar NO according to the non-linear method and about 10% of the children were difficult to fit into the linear model (r^2 <0.75). For the non-linear method there was a good concordance between the estimated $FENO_{50}$ and the measured $FENO_{50}$ (± 10%).

Small airway function

LCI was significantly increased in children with asthma and CF but the increase in asthma was very small. S_{cond} could better differentiate between asthmatic children and healthy controls (Fig 26).

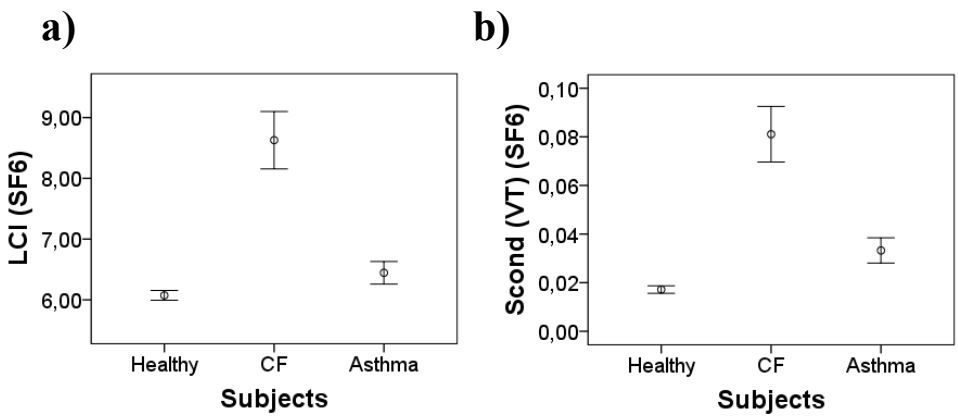


Figure 26
a) LCI in healthy children and children with CF and asthma
b) S_{cond} in healthy controls and in children with CF and asthma. Error bars show 95% of mean. p <0.0001 between the groups.

A majority of children with asthma and CF had elevated (> upper limit of normal) S_{cond}. S_{acin} was increased in more than half of the children with CF and one third of the children with asthma. There were significant differences in the proportions of subjects with asthma and CF who had increased levels of the MBW indices LCI, S_{cond} and S_{acin} (Table 27).

Table 27

Subjects with LCI, S_{cond} and S_{acin} above upper limit of normal (ULN; mean+1.96 SD for reference population, healthy controls) and those with reduced FEV1 (below lower limit of normal; LLN).

	CF (n=45)	***Asthma*** (n=47)	*p-value* (between groups)
LCI increased (>ULN) n (%)	42 (94)	7 (15)	<0.0001
S_{cond} increased (>ULN) n (%)	42 (94)	31 (66)	0.003
S_{acin} increased (>ULN) n (%)	33 (74)	18 (38)	0.002
FEV_1 reduced (<LLN) n (%)	9 (20)	7 (15)	0.511

Discussion

When analyzing the results in ***study I-IV*** there are five topics that raise new questions and warrant further discussion and possibly even future studies.

1) Exhaled NO and airway function in CF: Compared to healthy controls, subjects with CF have reduced levels of exhaled NO. Low $FENO_{50}$ is correlated with reduced airway function in spite of inflammation often being the cause of the decline in airway function seen in CF.
Is low $FENO_{50}$ a risk factor in CF or can reduced $FENO_{50}$ be used as an early marker of airway pathology?

2) Substitution with fatty acids in CF: Subjects with CF often have an impaired FA metabolism with an increased turnover of AA and low levels of DHA [204] and results from study II suggest that supplementation with n-3 FA can increase levels of n-3 FA in serum and attenuate systemic inflammation while AA supplementation leads to increased systemic inflammation and even more reduced levels of $FENO_{50}$.
Can FA supplementation prevent airway inflammation and maybe result in an increase of airway NO in CF?

3) Exhaled NO and small airway function in asthma: The results from study IV suggest that the small conducting airways are a major site for pathology in paediatric allergic asthma where the functional impairment is associated with airway inflammation and airway hyper-responsiveness.
Are $FENO_{50}$ and MBW useful methods to understand more about the different facets of paediatric asthma?

4) Alveolar NO: Alveolar NO is suggested as a marker of airway inflammation in the small acinar airways. Although alveolar NO is no different in children with asthma or CF compared to healthy controls, alveolar NO is correlated with acinar airway function in asthma and inversely correlated with systemic inflammation in CF. Children with *Ps. aeruginosa* have lower levels of alveolar NO indicating that alveolar NO is inversely correlated with inflammation in the airways in CF.
Is alveolar NO a useful marker of peripheral airway disease in asthma and CF?

5) Methodological questions about exhaled NO and MBW: *are these methods reliable and can they be used in the clinic?*

Exhaled NO and airway function in cystic fibrosis

Three studies in CF subjects are presented in this thesis and all three studies show that CF subjects, children as well as adults, have lower levels of exhaled and nasal NO than age matched healthy controls, supporting data from several other studies[28,143-146,148].

CF subjects homozygous for dF508 and other severe mutations associated with pancreas insufficiency have lower levels of $FENO_{50}$ and nNO compared to patients with mutations associated with mild disease. In the literature there is conflicting data regarding exhaled NO and genotype. Franklin *et al.* reported a non significant trend for exhaled NO being lower in young children homozygous for dF508 compared to other genotypes[273]. Thomas *et al*. reported no difference in FENO between subjects homozygous and heterozygous for dF508[144]. In study II all patients included were pancreas insufficient and there was no difference in nNO or $FENO_{50}$ between those homozygous and those heterozygous for dF508, indicating that it is not the genotype per se that cause the low airway NO. None of the above studies can provide evidence on whether low airway NO is caused by malabsorption due to pancreas insufficiency or another modifying gene or if is the result of the more severe clinical disease seen in subjects with severe mutations.

Clinical airway disease in CF is associated with chronic colonization with *Ps aeruginosa* and airway obstruction. Colonized CF patients have significantly lower NO levels than non-colonized patients. In the paediatric population (paper III), this difference did no reach significance but children colonized with *Ps. aeruginosa* had lower levels of alveolar NO than non-colonized subjects did. Other groups have studied the association between pseudomonas infection and exhaled NO with contradictory results. Some groups have found an association between pseudomonas and low levels of exhaled NO[151,274] , while others have reported no difference in exhaled NO between infected and non infected individuals[144,147,164].

Increased NO production is associated with host defense in both animal models and humans[43] and subjects with high airway NO may therefore be protected from chronic infection. Impairment in NO production could predispose for pseudomonas infection but low levels of NO could on the other hand be caused by consumption by the bacteria, for example *Ps. aeruginosa*[163]. The studies in this thesis cannot conclude whether the low airway NO is the cause or the result off bacterial colonization and there is support in the literature for both theories. Elphick *et al.* reported reduced NO in the airways in non-infected CF infants[145], suggesting that low airway NO comes before the infection. Franklin *et al.*

reported normal levels of exhaled NO in CF infants[273], thus suggesting that the reduced levels seen in CF is the result of bacterial colonization rather than the cause.

Forty-two out of the forty-five children with CF in study III had increased LCI, while only nine had pathological FEV_1. This supports other studies showing that MBW is more sensitive to early changes in the airways in CF[180, 234, 238]. Both S_{cond} and S_{acin} were affected providing evidence of small airway dysfunction early on in the CF airway disease.

Reduced levels of $FENO_{50}$ in children with CF are associated with impaired airway function and $FENO_{50}$ was not reduced in children with CF who had LCI within normal range (n=15), suggesting that the occurrence of small airway disease in young CF subjects is accompanied by reduced expiratory NO levels, also reported by Hubert *et al*.[147]. The present study showed that increased LCI was also correlated with increased bronchial NO diffusion capacity, speaking against NO being low due to mucus causing bad diffusion capacity.

Whichever come first, the low $FENO_{50}$ or CF airway disease? Our studies are exploratory and not mechanistic and whether the reduced levels of exhaled NO are the result or the cause of the airway pathology in CF is outside the scope of this thesis. The results in this thesis clearly show that the reduced levels of exhaled NO seen in subjects with CF are associated with a worse outcome and different interventions have been tried to increase exhaled NO in CF subjects. Grasemann *et al*. showed that inhaled arginine (the substrate for NO production) results in an increase in exhaled NO and FEV_1 shortly after the inhalation[161].

The most interesting clinical question if a reduction in $FENO_{50}$ is an indicator of airway disease remains unanswered. A large prospective study could provide further information whether reduced NO is an inborn risk factor of airway disease or a useful marker of reversible airway pathology. $FENO_{50}$ is not a good marker of airway inflammation in CF but atopic children with CF have significantly higher $FENO_{50}$ compared to non-atopic CF subjects. Many CF subjects report asthma like symptoms and some of them have higher levels of $FENO_{50}$, which subsequently are reduced by treatment with ICS. $FENO_{50}$ could therefore possibly indicate when young CF individuals might benefit from ICS, but this needs to be confirmed in a randomized study. The combined picture with low $FENO_{50}$ and increased LCI, which is common in CF, would also differentiate CF from asthma where $FENO_{50}$ often is elevated and LCI normal or just slightly increased.

Substitution with fatty acids in CF

There is a negative correlation between $FENO_{50}$ and nNO and the lipid abnormalities often seen in CF patients, an association which has not been reported elsewhere. In study II short term supplementation with dietary PUFA influences serum phospholipid FA pattern and to some extent the immune response, $FENO_{50}$ and nNO. Supplementation with n-6 FA results in aggravated inflammation and decreased levels of $FENO_{50}$ and nNO while the supplementation with n-3 FA leads to attenuated systemic inflammation and no change in airway NO. Exhaled and nNO are therefore not good markers of airway inflammation. The supplementation period is too short to expect any change in lung function and there are no differences in the number of positive sputum cultures before and after PUFA supplementation.

It has been suggested that AA, the substrate of proinflammatory eicosanoids, may play a role in the aggravated inflammation that is characteristic of CF patients. There is evidence that PUFA can have effects on NOS expression in macrophages. NO production has been shown to both increase and decrease under the influence of PUFA, possibly in a concentration dependent manner[212]. In a recent paper AA significantly reduced eNOS expression and NO production in human platelets[275]. AA has also been shown to reduce nNOS[276] .

Nuclear factor kappa B (NFκB) has been found to mediate the anti-inflammatory properties of PUFA[277]. iNOS and NO production are also activated by NFκB, suggesting a link between PUFA and NO[278].
Another possible link between airway NO and PUFA may be the profound effect essential fatty acids have on gene expression[279] suggesting that essential fatty acids might also influence the gene expression of NOS. Fatty acids interact with the genome through several mechanisms. They regulate nuclear receptor activity of several transcription factors or act indirectly through their effects on several specific enzyme mediated pathways or pathways involving changes in lipid membrane composition.

There are other intervention studies with n-3 PUFA in asthma and CF showing that n-3 PUFA inhibit the inflammatory response but also studies showing no such effect (for reviews see [211,214,215]). In spite of the short duration of study II, the study shows significant changes in inflammatory markers. The positive results with n-3 PUFA substitution regarding the systemic inflammatory response should encourage larger studies with PUFA supplementation.

Exhaled NO and small airway function in asthma

Study IV is the first paediatric study to show clear evidence of airway dysfunction in the small conducting airways in paediatric asthma. $FENO_{50}$ is significantly increased in the asthmatic children in study IV and correlates with the conducting airway function. Alveolar NO, although not increased, is also correlated with ventilation inhomogeneity in the small airways, both the small conducting airways and the acinar airways. These results provide evidence that airway inflammation is of importance in allergic paediatric asthma, not only in the large airways but also in the small airways, all the way down to the most peripheral acinar zone.

Based on our findings, it is important to monitor the peripheral airways in paediatric asthma. Anti asthmatic treatment should target the small airways.

FEV_1 is within normal limits in most subjects with asthma, clearly showing that this is not sensitive enough to detect early changes in the asthmatic airways in children.

The symptom score cannot differentiate between children with and without small airway dysfunction and inflammation, and symptom scores are not enough to support treatment decisions.

Study IV confirms the finding of residual small airway disease in asthmatic children reported by Macleod *et al*.[240] who reported increased LCI in children with asthma. We now provide clear evidence that the small conducting airways are the main site for disease process in asthma. Macleod *et al.* found no correlation between $FENO_{50}$ and LCI, but only just over half of the asthma subjects in the study were atopic, which could have influence the results

There are a few studies in adults assessing the association between small airway function and exhaled NO in asthma. Battaglia *et al.*[280] showed a correlation between exhaled NO and overall ventilation inhomogeneity. Verbanck *et al.* found signs of abnormal function in the conducting airways that was only poorly associated with bronchial NO flux[281]. Downie *et al.* showed that ventilation inhomogeneity arising in the small conducting airways is a major determinant of AHR in asthma, irrespective of airway inflammation measured as exhaled NO[241]. Our study is the first study in the paediatric age group showing clear associations between airway dysfunction in the small conducting airways, exhaled NO and AHR. This suggests that airway inflammation is a more important determinant of small airway dysfunction and AHR in children with allergic asthma than in adults where airway remodelling and other

structural changes could play a bigger role. However, it should be noted that there are individuals in the present study who have signs of small airway disease without increased levels of $FENO_{50}$, indicating that the functional changes can occur without ongoing inflammation. This could maybe be a sign of early airway remodeling since others have shown that the ventilation inhomogeneity is not fully reversible after acute treatment with β_2 agonists or long term treatment with inhaled corticosteroids[240,281]. Some subjects in the present study have AHR without increased $FENO_{50}$ indicating that AHR can also be present without concomitant allergic airway inflammation.

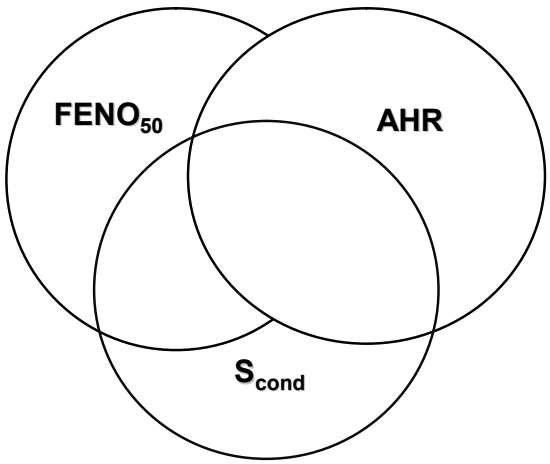


Figure 27
The correlation between $FENO_{50}$, AHR and S_{cond}

This absence of a strong correlation between S_{cond}, $FENO_{50}$ and AHR could represent different phenotypes that would benefit from different kinds of treatment.

Since the small conducting airways are a major site for disease process in paediatric asthma and spirometry is not sensitive enough to detect pathology in this region, it is important to add methods sensitive to small airway dysfunction and/or inflammation. LCI is increased in CF but not sensitive enough in the asthma population. S_{cond}, although not as simple to measure as LCI, is more sensitive to changes also in young asthmatic subjects. The findings in study IV suggest that adding $FENO_{50}$ and MBW to the current guidelines recommending symptoms and FEV_1 as a base for asthma monitoring could better characterize the asthma disease in children. Gaining more knowledge about asthma might change our view of treatment from "one drug fits all" to more personalized medicine where treatment is individualized. $FENO_{50}$ and MBW can add valuable information in those children who do not fully respond to the given treatment.

Alveolar NO in asthma and CF

Alveolar NO, presumably representing inflammation in the acinar airways, is correlated with acinar airway function and airway function in the small conducting airways in asthma. Low alveolar NO is correlated with increased markers of systemic inflammation and chronic bacterial airway colonization in the children with CF in study III. Alveolar NO was not different in children with asthma or CF on a group level compared to the healthy controls. However, some asthmatic children have high alveolar NO and increased S_{acin}, indicating that in a subpopulation within the asthmatic group dysfunction and inflammation in the distal airways could be of importance. The commonly used inhaled corticosteroids do not reach this airway zone.

Other studies have also shown "normal" levels of alveolar NO in mild to moderate asthma but increased levels in severe and nocturnal asthma[100,134-136]. Alveolar NO could be an important outcome in asthma studies evaluating the most peripheral airways and possibly as an outcome in treatment studies aiming at treating pathology in this region. However, there are still many methodological questions, discussed below, to solve before alveolar NO is used as an outcome in larger studies.

In CF, alveolar NO is significantly lower in children with chronic colonization with *Ps. Aeruginosa* than in non-colonized children. Suri *et al*. presented a study with somewhat contradictory results from the results presented here. They showed that alveolar NO was higher in children with CF compared to healthy controls and suggested that alveolar NO could be a marker of distal airway inflammation in CF[146]. It is difficult to explain why our results differ from the results reported by Suri *et al*. The present study has the same number of atopic individuals in both groups while Suri *et al.* included children with atopy only in the CF group and not in the healthy group, which could have affected the results. Data on alveolar NO in CF has also been presented by Shin *et al*. who reported somewhat lower levels in children with CF compared to healthy controls[101] and by Hofer *et al.* who presented data in adults[164] where the CF subjects had similar levels of alveolar NO compared to healthy controls. Based on these results one can argue against alveolar NO being a good marker of inflammation in the peripheral airways in subjects with CF.

Bronchial NO flux is significantly reduced in children with CF, and significantly increased in children with asthma. These results indicate that the divergent NO excretion/ production is located mainly in the conducting airway zone.

Methodological issues

$FENO_{50}$ is the method used in all studies in this thesis and just like others we find that $FENO_{50}$ is easy to measure and reproducible in healthy individuals from the age of six years as well as in subjects with asthma and CF [52,60,62]. All subjects in our studies were able to perform the NO measurements and perform three measurements for $FENO_{50}$ that were within ±10%. Nasal NO is also easy to measure, although the youngest children have difficulties with the airflow blowing into the one nostril.

Exhaled NO at multiple flows was assessed in all children however, the low flows (≤ 30mL/s) and high flows (≥ 250mL/s) are more difficult for the youngest children. Alveolar NO is derived from a mathematical model and small changes in the measured values results in large variations in the modelled variables. The calculated alveolar NO is negative in four of the subjects with $FENO_{50}$ above 100 ppb and subjects with high $FENO_{50}$ are more difficult to fit into the mathematical NO models. This has also been observed by Suresh and Shelley *et al.*[104,282], who suggested that patchy ventilation inhomogeneity could explain this. They proposed a new model based on several airway compartments as opposed to the two-compartment model used in this thesis. When modelling alveolar NO with this new model some individuals have much higher alveolar NO and this would suggest regional high NO production in the small airways. Three of the asthmatic individuals with negative alveolar NO in paper IV have increased LCI and S_{cond}. It would be interesting to introduce the multi compartment model into this group of asthmatic children.

The importance of retrograde axial diffusion on alveolar NO has been discussed by several authors who have suggested different mathematical equations to compensate for this[102, 103]. When applying two of these mathematical corrections to the alveolar NO levels in paper III and IV the corrected alveolar NO is negative in more than half of the asthmatic children and about one third of the children with CF and the same proportion in the healthy group. These corrections therefore are not applicable to this set of children.

Alveolar NO and bronchial NO flux derived from the linear and non-linear method are highly correlated even though the numbers differ significantly. Both methods can be used in healthy children, as also shown by Sepponen *el al.*[99], and in children with asthma and CF. When comparing results from different studies it is very important to know what method was used to calculate the NO variables.

Bronchial NO flux is closely correlated with $FENO_{50}$ in children with and without airway disease in paper III and IV and bronchial NO flux does not seem to provide more information compared to $FENO_{50}$. Alveolar NO is not correlated with $FENO_{50}$ in any of the groups and alveolar NO could therefore add information in conjunction with $FENO_{50}$; however, the methods used in this thesis are not robust enough.

Bronchial NO diffusion capacity and bronchial wall NO concentration show large inter and intra individual differences. Whether this is due to real differences or whether the models are not robust enough is still an open question. NO concentration in the airway wall is increased in children with asthma and it is correlated with S_{cond} and airway hyper-responsiveness. Bronchial wall NO diffusion capacity is not significantly increased in the asthmatic children and there is no correlation between bronchial wall NO diffusion capacity and airway function or airway hyper-responsiveness. Bronchial wall NO diffusion capacity is not significantly increased in the CF group compared to the healthy controls but in the CF group, bronchial wall diffusion capacity is associated with ventilation inhomogeneity. This shows that diffusion capacity increases while overall $FENO_{50}$ decrease with increased airway pathology indicating that NO diffusing capacity could be of importance in CF.

The multiple breath inert gas washout method is also a user-friendly method. All children in our studies perform MBW well enough to calculate LCI and others have shown that it is reproducible in most age groups[234]. S_{cond} and S_{acin} add information about the location of airway pathology, however these variables require specially trained personal to acquire measurements of good quality, especially difficult in healthy children since they are not used to the equipment and lab settings. Several of the healthy children in study IV have difficulties in fulfilling the quality criteria for evaluating S_{cond} and S_{acin}. Training could help this problem but it is time consuming. Evaluation of the Sn_{III} curve is done manually and therefore very dependent on the investigator. When presenting S_{cond} and S_{acin} it is recommended that they are adjusted for volume and breathing pattern, especially in children[246]. The equipment required for MBW is still very expensive and not commercially fully available which put limits to the usefulness of MBW in the clinic today.

Can exhaled NO be used as a biomarker in asthma and CF?

What are our and others findings in the case of the usefulness of exhaled NO as a biomarker in asthma and CF (Table 28)?

Table 28
Requirements for a biomarker and corresponding findings in regards ot exhaled NO in asthma and CF.

Requirement	*Exhaled NO in asthma*	*Exhaled NO in CF*
It should provide complimentary information- not the same information we already have.	FENO50 complimentary information to other markers such as AHR, LCI, ACT. Alveolar NO?	FENO50 could be a marker of airway disease? Alveolar NO??
It should respond to an intervention.	Treatment with ICS decrease FENO50. Alveolar NO can respond to po. steroid treatment.	FENO50 decreases after substitution with AA. Others have shown an increase after inhalation with arginine. No intervention study with alveolar NO
It should be relevant to the disease outcome.	FENO can predict steroid responsiveness.	?
It should be feasible.	FENO is very patient friendly. Small relatively inexpensive equipment is accessible . Alveolar NO patient friendly but equipment expensive.	See asthma

So yes - $FENO_{50}$ fulfils the requirements for a biomarker in asthma and possibly also in CF. Prospective longitudinal studies are needed to evaluate whether $FENO_{50}$ is relevant to disease outcome in CF. Others have suggested that $FENO_{50}$ could be used to predict the response to inhaled corticosteroids in asthma and to differentiate asthma from CF[11]. However, maybe $FENO_{50}$ can also be used in CF to diagnose asthma within the CF population and to predict a positive response to inhaled corticosteroids within this population.
Better methods for calculating alveolar NO are required before this variable is useful in the asthma clinic.

Conclusions and future studies

This thesis provides further evidence of early changes in the small airways in paediatric asthma and CF. The findings of dysfunction in the small airways associated with increased levels of NO in exhaled air in paediatric asthma are completely new. In CF, it is instead decreased levels of NO in exhaled air that is linked to declining lung function. The clinical significance of this is unknown.

Alveolar NO could provide additional information when assessing treatment directed against the smallest airways in patients with asthma, but the models used to calculate alveolar NO need to be standardised.

Treatment of inflammation is of paramount importance to the patient with CF and treatment with omega-3 fatty acids could lead to reduced inflammation.

Asthma and CF are like diamonds - multifaceted and expensive. They have a complex clinical picture and they constitute a major cost burden for both patients and society. The findings in this thesis provide new exciting insights into the pathology and pathophysiology of paediatric asthma and CF, which may allow for earlier and better targeted interventions.

Longitudinal data from prospective studies is required before implementing these findings in the clinic. Three such studies are proposed below.

$FENO_{50}$ longitudinally in CF patients

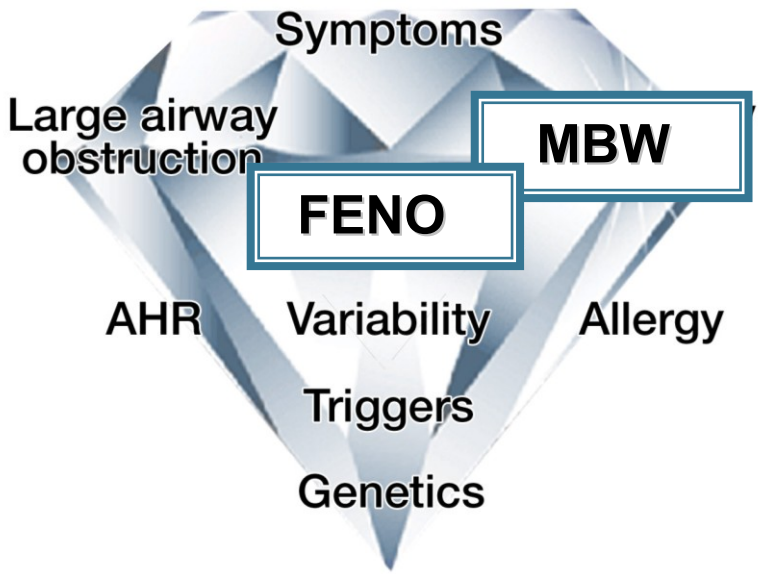
We have consistently found that CF patients with more severe disease have lower levels of exhaled NO than those with a less severe disease. It would therefore be of interest to see whether low levels of NO can predict further deterioration or if reduction of exhaled NO is an early result of infection, inflammation and damage in the airways and thereby maybe an early marker of airway disease. A future longitudinal study would be necessary to provide the answers to these questions.

Multi centre study with n-3 fatty acids in CF

Our data suggests that supplementation with n-3 fatty acids in CF can attenuate the inflammatory response. These findings need to be verified by larger multi centre randomized studies. In these studies, it would also be of interest to monitor the signs of pulmonary involvement. I would suggest the use of MBW in such a study since LCI has been shown to be a sensitive marker of airway disease in CF.

Multiple breath inert gas washout in asthma

We have shown that S_{cond} and, to a lesser extent, S_{acin} are increased in children with mild to moderate asthma as a sign of small airway involvement. It would be interesting to use this technique in combination with $FENO_{50}$ in the evaluation of new treatment targeting small airways in asthma to evaluate the effects on the peripheral airways. When methods for alveolar NO have been standardised this variable could also be included in evaluating anti-inflammatory treatment targeting the most peripheral airways.



Populärvetenskaplig sammanfattning

Utandat kväveoxid och funktion i små luftvägar vid astma och cystisk fibros

Bakgrund

Astma och cystisk fibros (CF) karaktäriseras av inflammation i luftvägarna som orsakar andningssvårigheter och hosta. Forskarna har under senare år insett att de riktigt små luftvägarna som ligger långt ut i lungorna, nära lungblåsorna, är av betydelse vid både astma och CF. Det är svårt att komma åt de små luftvägarna med dagens undersökningsmetoder och de har därför kallats för ”den tysta zonen”. Hos individer med CF startar inflammationen längst ut i lungorna redan tidigt i livet och denna inflammation leder sedan till icke reparerbara skador i lungorna. Astma är efter allergi den vanligaste kroniska sjukdomen hos barn i västvärlden. Stora studier har visat, att många barn med astma inte uppnår full astmakontroll trots behandling. Möjligen kan en bidragande orsak vara, att dagens behandling med inhalationsläkemedel inte når ut till de minsta luft-vägarna och därmed uppnår man inte önskad effekt fullt ut.

De små luftvägarna definieras hos vuxna som de luftvägar som har en diameter på mindre än 2 mm. Idag mäts lungfunktion med så kallad spirometri men denna metod är inte tillräckligt känslig för att påvisa förändringar i de små luftvägarna. Luftvägsinflammation kan mätas i lungsköljvätska och i vävnadsprov från lungorna, men dessa metoder är besvärliga och kräver narkos, då de ska utföras på barn. Det är därför av stor vikt att hitta andra metoder som kan påvisa försämrad funktion och/ eller inflammation i de allra minsta luftvägarna, och som är enkla att utföra.

Inertgasutsköljning är en metod för att mäta hur effektivt gasblandningen sker i lungorna. Ojämn gasblandning uppstår om olika luftvägsavsnitt ventileras ojämnt, dvs. om lungorna fungerar sämre. Denna metod är känslig för avvikelser i hela luftvägsträdet, inklusive de allra minsta luftvägarna. Metoden är lätt att utföra på barn i alla åldrar, dvs den kräver inte att barnet ska följa svåra instruktioner och den innebär ingen smärta eller obehag för patienten.

Koncentrationen av kväveoxid (NO) i utandningsluft är förhöjd hos individer med astma. Förhöjda NO nivåer har relaterats till markörer för allergisk inflammation i lungsköljvätska och vävnadsprover från luftvägarna. Utandat

NO avspeglar därför med stor sannolikhet allergisk inflammation i luftvägarna vid astma. Denna metod är lätt att använda på barn från cirka sex års ålder.

Syfte med avhandlingen

Syftet med studierna i denna avhandling är att undersöka om NO i utandningsluft och inert gasutsköljning kan bidra till ökad förståelse beträffande utbredning och svårighetsgrad av sjukdomsprocessen i luftvägarna vid astma och CF.

Studier i avhandlingen

De tre första artiklarna i avhandlingen är studier på individer med CF, där NO i utandningsluft karaktäriserades med hjälp av olika modeller för att avgöra från var i luftvägarna NO härstammar. NO i utandningsluft relaterades också till den genetiska defekten bakom CF, avsaknad av bukspottskörtel funktion, fettsyror i blodet och kronisk bakterieinfektion i lungorna. Lungfunktionen mättes med inert gas utsköljning och spirometri.

Resultaten av dessa studier visar att NO i utandningsluft är sänkt hos individer med CF trots att dessa har att pågående inflammation i luftvägarna. Orsaken är fortfarande oklar. CF individer med svårare genetisk defekt, icke fungerande bukspottskörtel och kronisk bakteriell infektion i lungorna har lägre halter av NO i utandningsluft än individer med något mindre allvarlig sjukdom utan kronisk infektion.

Majoriteten av de undersökta barnen med CF har en försämrad funktion i de små luftvägarna mätt med inertgasutsköljning medan endast ett fåtal av barnen har en försämrad spirometri. Inertgasutsköljning är därför en mer tillförlitlig metod för att påvisa påverkan på luftvägarna hos barn med CF än vad spirometri är. Ju sämre lungfunktion barnen med CF har desto lägre nivå av utandat NO.

Fettsyresammansättningen är påverkad hos individer med CF. Dessa har låga nivåer av omega-3 fettsyror och en ökad omsättning av omega-6 fettsyror. Omega-3 fettsyror har en antiinflammatorisk effekt. Den störda fettsyre sammansättningen kan därför ha betydelse för inflammationen i luftvägarna hos CF patienter. Mot bakgrund av detta fick en grupp CF patienter tillskott av olika fettsyror i kapselform under tre månader. Behandling med omega-3 fettsyror resulterade i sänkt inflammation medan behandling med omega-6 fettsyror förvärrade inflammationen och sänkte de redan låga NO nivåerna i utandningsluft även ytterligare. Kväveoxid har en antibakteriell effekt och de

låga nivåerna i utandningsluft hos CF patienter kan därför kanske utgöra en riskfaktor för kroniska infektioner i lungorna.

Den fjärde studien rör barn med allergisk astma, där NO från olika luftvägavsnitt mättes i förhållande till lungfunktion i samma luftvägsavsnitt.

NO nivåerna i utandningsluft är förhöjda hos många av barnen med astma trots pågående behandling, vilket är känt sedan tidigare. Vad som är dock är ny kunskap i denna avhandling är att 2/3 av barnen med astma i vår studie har påverkan på de små luftvägarna påvisad med inertgasutsköljning trots normal eller nära normal spirometri. NO nivåerna i utandningsluft ifrån de små luftvägarna korrelerar till dessa förändringar och talar för att inflammation är en viktig bakomliggande orsak till astma även ute i de perifera luftvägarna.

Betydelse

Resultaten i denna avhandling visar att genom att mäta NO i utandningsluft och undersöka lungfunktionen men hjälp av inertgasutsköljning kan man få en ökad förståelse för den bakomliggande luftvägssjukdomen vid astma och CF.

Fynden från studien på barn med astma är unika i det att denna studie för första gången visar, att de små luftvägarna är påverkade hos barn med allergisk astma med normal spirometri. Detta kan få konsekvenser på hur vi i framtiden behandlar barn och ungdomar med astma.

De låga NO nivåerna hos patienter med CF är av oklar betydelse men eventuellt skulle sänkta nivåer av utandat NO kunna vara en markör för tidig luftvägs-påverkan. Genom att följa NO nivåerna och se om de stiger igen efter be-handling skulle detta kunna utvärderas i studier där man följer individer med CF under en längre tid.

Fynden från studien med omega-3 fettsyror till individer med CF talar för att större studier bör göras för att undersöka huruvida dessa resultat kan upprepas och ligga till grund för en generell rekommendation om omega-3 tillskott till individer med CF.

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